AMP Deaminase
from *Saccharomyces cerevisiae*

A thesis submitted for the Degree of
Doctor of Philosophy at the
University of Glasgow

Jane MacKenzie

Institute of Genetics
University of Glasgow
Church Street
GLASGOW

December 1989

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Dedicated to Mrs Margaret MacKenzie (my mum)
for all her support, encouragement and love

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree

LIST OF CONTENTS

ABBREVIATIONS

Chemicals

ADP	- adenosine 5'-diphosphate
AMP	- adenosine 5'-monophosphate
ATP	- adenosine 5'-triphosphate
BSA	- bovine serum albumin
cDNA	- complementary DNA
dATP	- deoxyadenosine 5'-triphosphate
dGTP	- deoxyguanosine 5'-triphosphate
DEPC	- diethylpyrocarbonate
DNA	- deoxyribonucleic acid
DNP	- dinitrophenol
DTT	- dithiothreitol
EDTA	- ethylenediaminetetraacetic acid (disodium salt)
EtBr	- ethidium bromide
GTP	- guanosine 5'-triphosphate
GDP	- guanosine 5'-diphosphate
GMP	- guanosine 5'-monophosphate
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (sodium salt)
MOPS	- morphpropanesulfonic acid
mRNA	- messenger RNA
NTP	- nucleoside triphosphate
PMSF	- phenylmethylsulfonyl fluoride
RNA	- ribonucleic acid
SDS	- sodium dodecyl sulphate
TCA	- trichloroacetic acid
TEA	- triethanolamine
Xgal	- 5-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside
NAD	- nicotinamide adenine dinucleotide
NADP	- nicotinamide adenine dinucleotide phosphate
EHNA	- erythro-9-(2-hydroxy-3-nonyl) adenine
IMP	- inosine 5'-monophosphate
CF	- coformycin
dCF	- deoxycoformycin
Amp	- ampicillin

Growth supplements

ade	- adenine
his	- histidine
hpx	- hypoxanthine
leu	- leucine
met	- methionine
trp	- tryptophan
ura	- uracil

Measurements

V	- volts
kDa	- kilo Dalton
bp	- base pair
kb	- kilo base pair(s)
^{o}C	- degrees centigrade
x g	- gravitational force equal to gravitational acceleration
g	- gramme
mg	- milligramme (10^{-3}g)
µg	- microgramme (10^{-6}g)
ng	- nanogramme (10^{-9}g)
l	- litre
ml	- millilitre (10^{-3}l)
µl	- microlitre (10^{-6}l)
M	- molar (moles per litre)
mM	- millimolar (10^{-3}M)
M.W.	- molecular weight
Ci	- curie
µCi	- microcurie (10^{-6}Ci)

Miscellaneous

cpm	- counts per minute
fig.	- figure
log	- logarithm
UV	- ultraviolet light
PAGE	- polyacrylamide gel electrophoresis
MADD	- myoadenylate deaminase deficiency

ACKNOWLEDGEMENTS

I would like to thank Alistair Brown for all his help and supervision. I also wish to thank a number of members of the yeast group, past and present. Special thanks must go to Ian Purvis, for all his words of wisdom (often sardonic), to Gordon Lithgow for his endless enthusiasm and optimism and especially to Lynn Loughlin, who managed to keep the lab and the people in it going.

Thanks also to the many people at I.C.I. Billingham who helped me with the protein purification procedures when I was there, including my two supervisors Finn Wynne and Dave Byrom and all the Biochemistry department, especially Eleanor, John and Colin. Thanks also to Dr John Fitton of I.C.I., Pharmaceutical division for the amino acid sequence determinations. I also acknowledge the S.E.R.C. who have co-funded this project with I.C.I over the last few years.

Thanks also to Dr. R.L. Sabina for his gift of plasmid pBSAMPD, Dr. M. Black for his gift of deoxycoformycin, Dr Robin Wood for yeast strain, W1-2D and Professor V.L. Schramm for AMP deaminase sequence information and a pre-print of his paper.

I would especially like to thank the people who made the last few years (slightly) more pleasant. I especially thank Joyce, Arvind and Andrew for their good company, good advice and good taste. Last but not least, special thanks must go to Jim Pickett for drawing some of my diagrams (the good ones), but more importantly for his support, encouragement and friendship.

SUMMARY

Attempts were made to clone the yeast adenosine-5'-monophosphate deaminase (AMP deaminase) gene from S.cerevisiae using a number of strategies. Initially, it was hoped that overproduction of AMP deaminase could be detected in yeast, by selecting for resistance to the AMP deaminase inhibitor, deoxycoformycin (dCF). This involved the construction of ade, hpt1 yeast strains in which the AMP deaminase reaction is the major source of inosine-5'-monophosphate (IMP). Although yeast AMP deaminase is sensitive to dCF in vitro no effect was observed in vivo (possibly due to lack of uptake of the drug).

Alternative screening methods based on adaptations of histoenzymatic stains and colorimetric assays for AMP deaminase were then studied for their suitability for selection of the AMP deaminase gene. These were unsuccessful due to the production of unsuitably high background signals and the stains' relative insensitivity to yeast AMP deaminase activity.

AMP deaminase was then purified from S.cerevisiae using a new purification method based on standard FPLC techniques to allow the production of N-terminal amino acid sequence data. These data were used to design an oligonucleotide probe to screen a yeast genomic library. The N-terminal sequence of this purified protein proved to be "identical" to yeast heat shock protein 90 (hsp90), raising the question; are hsp90 and AMP deaminase the same protein?

Initial experiments were inconclusive since they revealed that AMP deaminase activity is maintained, but not markedly elevated during heat shock in yeast.

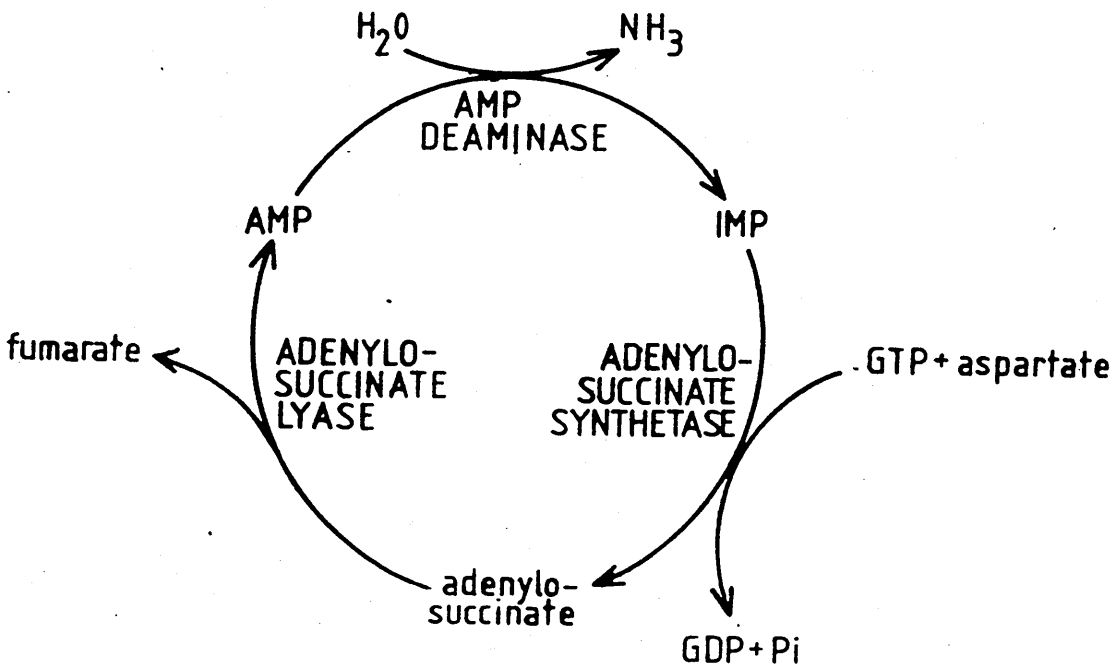
Since the yeast HSP90 gene was not available for this study (Farrelly and Finkelstein, 1984), the decision was made to construct a yeast integrating vector (YIp) to allow insertional inactivation of the HSP90 chromosomal locus. A YIp vector containing a hsp90:URA3 gene was constructed and was used to transform a suitable yeast strain. When this strain was compared under heat shock conditions with the host

strain and a strain carrying the entire HSP90 gene on a multicopy plasmid, no significant difference was observed in AMP deaminase activities. This strongly suggests that hsp90 and AMP deaminase are not the same protein and hence that hsp90 had copurified with AMP deaminase during all stages of the purification. Presumably, it was this contaminating protein that yielded the N-terminal amino acid sequence data.

CHAPTER 1

INTRODUCTION

FIGURE 1.1 THE PURINE NUCLEOTIDE CYCLE



1.1 THE ROLE OF AMP DEAMINASE IN THE PURINE NUCLEOTIDE CYCLE

AMP deaminase (AMP aminohydrolase E.C.3.5.4.6.) is one of the enzymes that comprise the purine nucleotide cycle in eukaryotes along with adenylosuccinate synthetase and adenylosuccinate lyase as shown in Figure 1.1. The overall reaction catalysed in the cycle is shown below:

$$\text{aspartate} + H_2O \text{ ----> } \text{fumarate} + NH_3$$

The functional significance of this cycling is obscure but it seems clear that the production of ammonia is of importance as discussed later (Lowenstein and Tornheim, 1971; Lowenstein, 1972).

In E.coli no adenine deaminase or AMP deaminase activities have been detected, only adenosine deaminase has been found (Leung and Schramm, 1980). Therefore in prokaryotes AMP catabolism occurs via dephosphorylation to adenosine by AMP nucleosidase. Eukaryotic cells also contain a dephosphorylating enzyme capable of using AMP as a substrate, called 5'-nucleotidase. This is a non-specific nucleotide-degrading enzyme which removes the phosphate group from mononucleotides. However, there is good evidence that in many eukaryotic systems the major purine degradation products are inosine and hypoxanthine rather than adenosine (Kather, 1988). Van den Berghe et al. (1980) reported that in isolated rat hepatocytes 5'-nucleotidase is inactive on AMP unless the nucleotide rises to unphysiological concentrations. This also appears to occur in human erythrocytes where AMP deaminase is the preferred route of AMP degradation (Paglia et al., 1986). Zoref-Shani et al. (1987), showed that a similar situation exists in primary rat muscle cultures. More recently the same group showed that while under normal circumstances rat cardiomyocytes produce and efflux inosine, hypoxanthine, xanthine and uric acid from the IMP produced by the AMP deaminase reaction, there is a greatly increased rate of AMP dephosphorylation by 5' nucleotidase during periods of rapid ATP degradation (i.e. the simultaneous inhibition of glycolysis and electron transport) (Zoref-Shani et al., 1988). Therefore it seems that the AMP deaminase reaction is the major route of AMP

catabolism under normal physiological conditions, while 5' nucleotidase acts at high AMP concentrations.

1.2 DISTRIBUTION OF AMP DEAMINASE

In a review by Zielke and Suelter (1971) it was reported that there is a virtual lack of adenine deaminase in animal tissues, although all eukaryotes contain both adenosine and AMP deaminases. As far as cellular localisation was concerned it was stated that in mammalian skeletal and heart muscle, AMP deaminase activity was found both in the cytoplasm and mitochondria, whereas in fish kidneys and gills the activity was located solely in the cytoplasm.

It is now clear that AMP deaminase activity is found at different levels in different tissues. In vertebrate systems it is found at the highest levels in skeletal muscle (Makarewicz and Stankiewicz, 1974; Koretz and Frieden, 1980). Fishbein (1985) has stated that skeletal muscle shows 10 to 50-fold higher AMP deaminase activity than any other tissue. The activity has been shown to be higher in type 2 fibres than type 1 (Higuchi et al., 1987). Human muscle however shows lower AMP deaminase activities compared with rat (Makarewicz and Stankiewicz, 1974).

High AMP deaminase activities are also found in erythrocytes (Askari and Rao, 1968) and in lymphoblasts, where higher activities are found in B rather than T lymphoblasts (Dornand et al., 1985).

There has been much less work done in invertebrate systems where AMP deaminase activities are generally very low, but again, highest in anaerobic muscle (Lazou, 1989). Far higher levels are found in the snail Helix lucorum's foot muscle than elsewhere in the molluscs and crustaceans surveyed. However, the general pattern of low AMP deaminase activity in invertebrates has led to the suggestion that there may be differences in the pattern of adenylate regulation in invertebrates relative to the better studied vertebrate systems (Lazou, 1989).

It the case of yeast, it was assumed for a long time that AMP deaminase was absent. It has now been established that the enzyme does exist in S.cerevisiae and it has been purified from that organism (Yoshino et al., 1979b). The yeast enzyme has been shown to be similar to other AMP deaminases as regards its regulatory properties. It was shown that AMP deaminase from yeast is specific, showing no activity with ATP, ADP, adenosine or adenine. It shows only 7% of its maximal activity with deoxyAMP as its substrate instead of AMP (Yoshino et al., 1979b).

1.3 DEVELOPMENTAL EXPRESSION OF AMP DEAMINASE

It is now clear that there are various isoforms of AMP deaminase in different vertebrate tissues (as indicated earlier, less is known about the invertebrate and lower eukaryote enzyme(s)). Ogasawara et al. (1972) identified 4 different isoenzymes from rat brain tissue which varied with respect to their molecular weights and kinetic properties. Ten years later it was shown that there are also four isozymes in human tissues (Ogasawara et al., 1982). These were designated E1 and E2 (the major erythrocyte forms), M form (major skeletal muscle form) and L (which is the major isozyme in liver and brain). In surveying other tissues it was shown that the heart, kidney and spleen contain a mixture of E1, E2 and L isoforms (Ogasawara et al., 1982). Again these different forms vary in molecular weights and at least in the case of E1, M and L, they are immunologically different (Ito et al., 1988). E1 and E2 are however very similar immunologically and kinetically (Ogasawara et al., 1982).

There is now good evidence that there is sequential expression of different isoforms of AMP deaminase during the development of the skeletal muscle of mammals. Chromatography on phosphocellulose revealed the presence of two well separable forms of AMP deaminase in skeletal muscle extracts of 11 and 16 week old human foetuses (Kaletha et al., 1987a; Kaletha and Novak, 1988). One form was identical to that found in adult muscle, and the second form (which is found in vestigial amounts in adult skeletal muscle) shows

different kinetic properties.

This situation has been studied more extensively in the rat. It has been shown that in drug induced myogenesis of myocytes in vitro there is an increase in AMP deaminase activity which parallels the appearance and accumulation of myosin (Sabina et al., 1984a). Similarily during the differentiation of myoblasts to myotubes there is a 10 to 100-fold increase in AMP deaminase activity. It is now clear that in the developing hind limbs of the rat, three different isoforms can be detected (Marquetant et al., 1987). Firstly, an embryonic form is seen 7 to 14 days through gestation. This isoform is not unique to skeletal muscle or to the embryo, but can be seen in many non-muscle tissues of the adult rat. There is also a perinatal form which is restricted to skeletal muscle and which is produced from 4 to 6 days before birth and is continued to be expressed for 2 to 3 weeks postnatally. The third or adult isoform appears at birth and reaches adult levels three weeks after birth (Marquetant et al., 1987). It was not clear whether these three isoforms represented the products of separate genes or the products of differential splicing of the same primary transcript.

Recently, Sabina et al. (1987) have succeeded in cloning a cDNA corresponding to the rat adult muscle AMP deaminase isoform mRNA and using this they have looked at the expression of AMP deaminase in the development of the muscles of the hind limb and during myocyte differentiation in vitro. Their conclusions are that in embryonic muscle and undifferentiated myoblasts a 3.4kb transcript encodes a 78kD peptide which cross-reacts with antisera raised against an isoform from adult kidneys. In perinatal muscle and myocytes at an intermediate stage of differentiation, a 2.5kb mRNA encoding a 77.5kD polypeptide is produced which cross reacts with antisera to the major adult heart muscle isozyme. And at birth, another 2.5kb transcript is produced representing the adult muscle isoform (Sabina et al., 1989). This group have now concluded that the two 2.5kb transcripts may be the products of the same gene, through differential splicing of a single primary transcript, but that the 3.4kb mRNA represents a functionally distinct gene.

A similar situation has been studied in the skeletal muscle of chickens, where there appears to be only two isoforms of AMP deaminase, an embryonic form (similar to the isozyme found in adult chicken brain) and an adult form (Sammons and Chilson, 1977 and 1978; Kaletha, 1983).

Spychala et al. (1985) have also studied the expression of AMP deaminase in the chicken liver. Unlike the development of rat skeletal muscle (Marquetant et al., 1987), and other chicken tissues, (for example; brain tissue where there is an increase in AMP deaminase activity throughout development (Spychala et al., 1985)), in the chicken liver there is no such continual increase. There are two distinct isoforms, type I and type II. Type I reaches 80% of the adult level within the first ten days of development. This may indicate that it plays a crucial function in the liver, possibly connected with the massive production of uric acid (Spychala et al., 1985). Koretz and Frieden (1980) showed that AMP deaminase binds strongly to subfragment 2 of myosin in vitro, and to the ends of the A bands of myofibrils in vivo. Cooper and Trinick (1984) showed that the interaction between AMP deaminase and myofibrils is due to the presence of specific binding sites for the enzyme (6 binding sites/thick filament). A similar situation seems to exist in invertebrate muscle where in general AMP deaminase activities are much lower. Raffin and Thebault (1987) have purified the tail muscle AMP deaminase from prawn. The enzyme was closely associated with contaminating contractile proteins, especially actin. There is also evidence of specific binding sites for AMP deaminase on the surface of hepatocytes (2.6×10^6/hepatocyte) which contribute to the rapid clearing of intravenously injected AMP deaminase from the circulation of chickens (Husic and Suelter, 1984).

Higuchi et al. have shown that in addition to its association with myosin, high AMP deaminase activity is found in rimmed vacuoles in muscles cells in certain myopathic conditions (Higuchi et al., 1986 and 1987). They suggest that AMP deaminase may be bound to membrane components as well as myosin in muscle cells.

TABLE 1.1 EFFECTORS OF AMP DEAMINASE

POSITIVE	NEGATIVE
ATP[1,2]	GTP[1,4]
dATP[1]	inorganic phosphate[1,3,13]
fatty acids[9]	fatty acids[9,11,12]
polyamines[8,21]	fatty acyl Co-As[6]
spermine[11,15]	polyamines[8]
monovalent cations[10,22]	2,3-diphosphoglycerate[23]
alkali metals[10]	polyphosphates[18]
alkaline earth metals[10,20]	transition metals[10,13]
polyphosphoinositols[19]	tetraiodofluorescein[7]
	blue dextran[5]
	anthraquinone compounds[17]
	napthosulfonate compounds[16]

1 Murakami (1979)
2 Cunningham and Lowenstein (1965)
3 Spychala and Marszalek (1987a)
4 Ashby et al. (1982)
5 Yoshino et al. (1974)
6 Yoshino et al. (1976)
7 Yoshino and Kawamura (1978a)
8 Yoshino and Murakami (1978b)
9 Yoshino et al. (1979a)
10 Yoshino and Murakami (1980)
11 Yoshino and Murakami (1981a)
12 Yoshino and Murakami (1981b)
13 Yoshino and Murakami (1981c)
14 Yoshino and Murakami (1981d)
15 Yoshino and Murakami (1982a)
16 Yoshino et al. (1984)
17 Yoshino and Kawamura (1986b)
18 Yoshino and Murakami (1988)
19 Spychala (1987b)
20 Almarez and Garcia-Sancho (1989)
21 Yoshino and Murakami (1987)
22 Raffin (1984)
23 Askari and Rao (1968)

1.4 REGULATION OF AMP DEAMINASE

The enzyme has been purified from a number of systems including rat liver (Smith and Kizer, 1969); rat skeletal muscle (Coffee and Kofke, 1975); S.cerevisiae (Yoshino et al., 1979b) and snail foot muscle (Stankiewicz, 1986). The purification of the yeast enzyme is discussed fully in Chapter 5.

Each of these enzymes has been found to be large, in the region of 240 and 360 kDaltons. And in each case AMP deaminase is tetrameric with subunits of identical size. The methods of purification have varied, although some have relied on the enzyme's affinity for ATP in either affinity adsorption or affinity elution chromatography. The purification of AMP deaminase from these sources has allowed detailed study of the regulation of the enzyme.

The regulation of AMP deaminase is highly complex. There are a host of cellular metabolites and synthetic compounds which allosterically activate or inhibit the enzyme. In some cases, the same effector will inhibit the enzyme from one tissue and activate it from another. These effectors are summarised in Table 1.1.

Most AMP deaminase isoenzymes are strongly activated by ATP, including the enzyme found in the brain (Cunningham and Lowenstein, 1965, Setlow et al., 1966), liver (Smith and Kizer, 1969), platelets (Ashby and Holmsen, 1983), small intestine (Spychala et al., 1986), kidney, lungs, pancreas, spleen and testes (Setlow et al., 1966). AMP deaminase from S.cerevisiae is also activated by ATP (Murakami, 1979). There are however, some forms of the enzyme which are insensitive to the effects of ATP including the heart muscle isoform (Skadanowski and Zydowa, 1988).

There is now evidence that $MgATP^{2-}$ is the true physiological activator, since most isozymes which are activated by ATP are maximally activated in the presence of MgATP (Ashby and Holmsen, 1983; Spychala et al., 1986). The presence of ATP has also been shown to largely prevent heat-inactivation of AMP deaminase from mammalian brains (Cunningham and

Lowenstein, 1965).

In general AMP deaminase from almost all sources is strongly inhibited by inorganic phosphate (Van den Berghe et al., 1977; Murakami, 1979; Ashby et al., 1982; Spychala et al., 1986). In contrast to ATP, another purine triphosphate nucleotide, GTP is a general and potent inhibitor of AMP deaminase from most sources (Setlow et al., 1966; Van den Berghe et al., 1977; Ashby et al., 1982). For some isoforms however, the effect of GTP is somewhat complex. The mammalian liver isoenzyme is activated by GTP when AMP is present at low concentrations, but inhibited by GTP at high substrate concentrations (Smith and Kizer, 1969).

It has been proposed that the regulation of AMP deaminase in any particular tissue, may be regulated at least in part by the endogenous concentrations of AMP, ATP and GTP (Smith and Kizer, 1969). This hypothesis may be an oversimplification because AMP deaminase activities respond to a wide range of allosteric effectors (Table 1.1).

Saturated fatty acids are inhibitors of purified yeast AMP deaminase (if there are more than ten carbon atoms in the hydrocarbon chain), but show little inhibition of the enzyme in the presence of ATP. Unsaturated fatty acids are more powerful inhibitors, whose inhibition is not significantly overcome by ATP (Yoshino and Murakami, 1981b). Similarly, spermine can release AMP deaminase in permeabilised yeast cells from fatty acid inhibition, whereas alkali metal ions cannot (Yoshino and Murakami, 1981a). Therefore, there appears to be a hierarchy in the regulation by effectors of this highly allosteric enzyme, which must contribute to the fine-control of its activity in the cell. Forms of the enzyme from different sources or tissues show different levels of inhibition or activation with respect to these effector molecules. For instance, brain AMP deaminase is more sensitive to inhibition by fatty acyl CoAs than the isozyme found in liver (Yoshino et al., 1976).

A great deal of information has been produced about the regulation of the yeast AMP deaminase either in its purified form or in situ in permeabilised cells. The results of such

work suggests that there are two types of cation binding sites on the enzyme. One class of binding site includes activating sites for alkali metals, polyamines, and free alkaline earth metals and the inhibition sites for transition metals. The other sites are the activating sites for ATP-metal complexes (Yoshino and Murakami, 1980).

Kinetic studies show that in the case of the yeast enzyme, there are two interaction sites for AMP and inorganic phosphate per molecule, and have led to the proposal that the enzyme (which is a tetramer) consists of two "protomer" units each of which consists of two polypeptide chains with identical molecular weights (Yoshino and Murakami, 1980).

There is strong evidence that at least in some systems, activation of AMP deaminase occurs, at least in part, by non-allosteric methods. Buniatian and Haroutunian (1971) showed that the addition of hexokinase (purified either from yeast or brain tissue) to mitochondrial fractions of rat brain causes a considerable increase in the formation of ammonia, due to the activation of AMP deaminase. Another isozyme of hexokinase isolated from the brain which was shown to be very similar to the skeletal muscle form showed no activating power (Haroutunian and Buniatian, 1974). Hexokinase is found in brain mitochondria where there is no glycolysis, and the function of this protein in this location is unknown (Buniatian and Haroutunian (1971). It is not yet clear how this form of regulation occurs since it appears that an active hexokinase is not required, since subunits of the yeast hexokinase (produced by urea treatment) also activate AMP deaminase.

Another interesting form of activation of AMP deaminase has come to light in two quite different tissues. In 1985, Ranieri-Raggi et al. demonstrated that limited proteolysis of rabbit skeletal muscle AMP deaminase with trypsin results in the conversion of the enzyme to a form which is no longer inhibited by ATP and exhibits hyperbolic kinetics even at low potassium concentrations and in the absence of ADP. The interaction with troponin T from white skeletal muscle or with the phosphorylated 42 amino acid N-terminal peptide of troponin T restores to the trypsin-treated AMP deaminase the

sensitivity to adenine nucleotides and increases the K_A for potassium activation of the enzyme from 1 to 12mM. Treatment of the N-terminal peptide of troponin T with alkaline phosphatase abolishes the modulating properties of the peptide suggesting that phosphorylation and dephosphorylation processes may be involved in the regulation of the enzyme.

Raffin (1986a) showed that trout gill AMP deaminase can be activated by an endogenous proteinase, in a manner similar to the alteration of the rabbit muscle enzyme described above. These alterations of the regulation of the enzyme include shifting the optimal activating concentration of sodium and potassium ions from 10 to 75mM resulting in a large increase of enzyme activity near the physiological concentrations of these ions. The modified enzyme is also much less sensitive to modifications in pH and inhibition by inorganic phosphate. In total the level of activation by this limited proteolysis was estimated to be approximately 40-fold in <u>in vivo</u> conditions (Raffin, 1986a). This may be a widespread form of regulation of this enzyme since Raffin also observed that in crude extracts of frog muscle, rabbit muscle and trout gill, the AMP deaminase activity increased with storage, suggesting that the activation may normally be mediated by an endogenous proteinase which can be mimicked by trypsin (Raffin, 1986a). He also showed that AMP deaminase purified from trout gills is insensitive to this proteolytic activation, suggesting that some modification has already occurred. Under environmental peturbations (starvation, pollution with pesticides, transfer to sea water, and reverse transfer to fresh water), the gills produce a greater proportion of modified enzyme (Raffin, 1986b). This proportion can vary from 8 to 100% of total depending on the nature of the stress and the length of time the fish is exposed to it. As a consequence of this modification during stress, increases in AMP deaminase activity of between 2 and 12-fold were observed. Raffin proposed that proteolysis occurs in the gill tissues to activate AMP deaminase under conditions of an increased energy demand (Raffin, 1986b). Raffin also suggests that AMP deaminase may be modified in this way in a wide range of systems under conditions of stress. Metabolic or cellular stress is often accompanied by and increase in the cellular inorganic phosphate concentration which will in

general inhibit the enzyme. Therefore by producing a modified form of the enzyme with a reduced sensitivity to inorganic phosphate a high specific activity may be maintained when it is most required (Raffin, 1986b and 1986c).

With reference to AMP deaminase from yeast, one group in particular have studied the regulation of this enzyme in depth. Yoshino and Murakami have been studying AMP deaminases since the early seventies and have carried out a detailed biochemical study of the yeast enzyme over the last ten years. They initially used purified AMP deaminase (Murakami, 1979; Yoshino and Murakami, 1979b) to study its regulation and then went on to study the regulation of the enzyme in situ in permeabilised yeast cells (Murakami et al., 1980).

The enzyme is similar in most respects to those found in other systems. It is activated by ATP, dATP, alkali metal ions, alkaline earth metals and polyamines (Murakami, 1979; Yoshino and Murakami, 1980). It is inhibited by GTP, inorganic phosphate, transition metals (Murakami, 1979; Yoshino and Murakami, 1980). In these respects it seems most similar to the mammalian liver isozyme (Murakami, 1979). The patterns of regulation are similar in situ and with the purified enzyme, with some minor modulations (Murakami, 1980).

There has been a great deal of interest in the effects of adenosine deaminase inhibitors including coformycin (CF), deoxycoformycin (dCF) and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) on AMP deaminase. This body of work on the effect of adenosine deaminase inhibitors has been produced, in part, because of the interest in adenosine deaminase as a suitable enzyme to be targetted for certain cancer treatments. CF and dCF especially are currently being tested for their effectiveness in the treatment of certain lymphoid malignancies (Bagnara and Hershfield, 1982). The drugs act by inhibiting adenosine deaminase resulting in an accumulation of adenosine, 2'deoxyadenosine and other metabolites which are toxic to the cell. Henderson et al. (1977) showed that high concentrations of EHNA and dCF

inhibit all pathways of purine ribonucleotide synthesis and also inhibit purified AMP deaminase from Ehrlich ascite tumour cells. Similarly, Agarwal and Parks (1977) showed that CF and dCF are potent inhibitors of rabbit muscle AMP deaminase. They appear to be behaving as transition state analogues (Frieden et al., 1980). Frieden et al. (1979) showed that the favoured inhibitor is likely to be the 5'-phosphate form of these drugs, which mimic more closely the structure of AMP.

A group headed by Debatisse, have used the analogue coformycin to select for Chinese hamster fibroblast lines with increased AMP deaminase activity due to amplification of the AMP deaminase gene (Debatisse et al., 1984). This is discussed fully in Chapter 3.

More recent work has been done to test the effectiveness of these analogues in conjunction with 2-halo-2',3'-dideoxyadenosine derivatives against the HIV-I virus. These halo-derivatives have in vitro activity against the HIV-I virus, but can be catabolised by human T cells even when the cell's adenosine deaminase is inhibited by CF (Haertle et al., 1988). The suggestion is that they are being deaminated by AMP deaminase which is not inhibited by the concentrations of CF being used (concentrations lower than those that produce cyto-toxicity in uninfected cells). The conclusion is that dideoxyadenosine derivatives are of limited value in preventing the spread of HIV-I infection, because of the high AMP deaminase activity found in lymphocytes (Carson et al., 1988).

Buchwald et al. (1981) have produced a murine T-cell lymphoma line which has altered AMP deaminase activity by selection in medium containing arabinosyl 2,6-diaminopurine. Selected cell lines were characterised and showed increased sensitivity to adenine and adenosine, in the presence of the adenosine deaminase inhibitor, EHNA. Adenine and adenosine cause inhibition of de novo purine biosynthesis and when used in conjunction with adenosine deaminase/AMP deaminase inhibitors they result in cytotoxicity caused by IMP and guanylate nucleotide starvation (Debatisse et al., 1981). This sensitivity was reduced by the addition of hypoxanthine,

guanosine or deoxyguanosine. The cell line also shows decreased sensitivity to thymidine, deoxyadenosine (plus EHNA), arabinosyl guanine and arabinosyl adenine (plus EHNA). They concluded that this phenotype was due to two separate mutations. This first was concluded to be an alteration of ribonucleotide reductase activity. The second was an AMP deaminase with altered kinetic properties which accounts for the increased sensitivity to growth inhibitors adenine or adenosine, and decreased flux of nucleotides from AMP to IMP. This altered AMP deaminase also showed increased sensitivity to inhibition by inorganic phosphate. Characterisation of such variants with altered AMP deaminase activity may give an insight into the true role of AMP deaminase in the cell.

1.5 THE ROLE OF AMP DEAMINASE IN HUMAN DISEASE

Two histoenzymatic stains have been developed to detect and localise AMP deaminase activity in skeletal muscle sections (which are discussed fully in Chapter 4) (Nakatsu, 1975; Fishbein, 1977a). However, the development of the more recent stain (Fishbein, 1977a) has led to in the discovery of a new disease of skeletal muscle, myoadenylate deaminase deficiency (MADD) (Fishbein, 1978). In applying the histoenzymatic stain to muscle biopsies it was found that 6 out of 300 biopsies surveyed had little or no AMP deaminase activity. This finding was confirmed by biochemically assaying for AMP deaminase (Fishbein et al., 1978). The biopsies were histologically normal, but ischemic forearm exercise followed by determination of plasma ammonia and lactate levels showed that MADD patients do not produce the expected increase in plasma ammonia relative to lactate (Fishbein et al., 1978). Patients with this deficiency suffer from mild to moderate cramping or fatique following exercise (Dimauro et al., 1980). The AMP deaminase activities from erythrocytes, leukocytes and cultured fibroblasts are all normal (Dimauro et al., 1980; Fishbein et al., 1981) which led to the suggestion of there being different genes involved in the different tissue-specific AMP deaminases. Intriguingly, primary muscle cultures from these patients also had normal AMP deaminase activities suggesting the persistant expression of a fetal isoenzyme

(Dimauro et al., 1980).

Fishbein et al. (1981) went on to show that some lymphoblast lines show an AMP deaminase which is more sensitive to CF, dCF and EHNA than their adenosine deaminases, again suggesting that immature lymphoblasts, too, may have a qualitatively distinct isozyme.

It now seems clear that MADD is an autosomal recessive disease (Sinkeler et al., 1988) which, it has been estimated, occurs at a frequency of 1-2% in the population. It is therefore the most common enzyme deficiency of skeletal muscle (Fishbein et al., 1985). It is clear that MADD disrupts purine metabolism which accounts for the muscle dysfunction observed (Sabina et al., 1984b; Valen et al., 1987). However, individuals with MADD were shown to be equally capable of maintaining a high adenylate energy charge value, as controls. This is probably due to the normal regulation between ATP consumption and regeneration (Sinkeler et al., 1987). However ATP, ADP and total adenylates (not AMP) are significantly elevated in MADD muscle cells (Sinkeler et al., 1987). The age of onset of the symptoms of the disease is variable as is the severity of the musculoskeletal symptoms. However the disease does appear to be progressive (Lally et al., 1985).

The discovery of this deficiency has attracted a great deal of interest, for a number of reasons. It has long been known that AMP deaminase activity can be reduced in different forms of muscular dystrophy (Pennington, 1961; Kar and Pearson, 1973; Young et al., 1984; Nagao et al., 1986). It is now clear that this deficiency of AMP deaminase is either coincidental or a secondary effect of the disease (Kar and Pearson, 1973). However, MADD has continued to be implicated in a wide range of neuromuscular disorders including primary hypokalaemic periodic paralysis (Engel et al., 1964), myalgic encephalomyelitis (Staines, 1985), McCardles disease (Sinkeler et al., 1986; Heller et al., 1987), Carnitine palmitoyl transferase deficiency (Reuschenbach and Zierz, 1988) and malignant hyperthermia susceptibility (Fishbein et al., 1985).

There is some evidence that MADD may be associated with malignant hyperthermia susceptibility, which is a susceptibility to anaesthesia induced fever, muscle rigidity and paralysis. This was believed to be inherited as a dominant trait with incomplete penetrance and variable expressivity (Fishbein et al., 1985). There is now evidence that this susceptibility requires a primary dominant gene defect and possibly a second recessive trait. It is suggested that this latter gene may encode AMP deaminase (Fishbein et al., 1985).

It seems likely that the implied association of AMP deaminase deficiency with most of the neuromuscular disorders listed above is coincidental because of the frequency of this gene disorder. Alternatively, it may be that some of these disorders result in a secondary deficiency in AMP deaminase (Fishbein et al., 1985). One suggestion is that such patients are heterozygous for AMP deaminase (which will be a common occurrence since MADD occurs in 1-2% of the population).

More recently a deficiency of AMP deaminase has been reported in erythrocytes, once again inherited as an autosomal recessive trait (Ogasawara et al., 1984). There appears to be no hematological disorder and the individuals with this deficiency are healthy. The deficiency is only seen in erythrocytes. Mononuclear cells and platelets have normal levels and the deficiency is only in one isozyme, one of the erythrocyte forms. Like the skeletal muscle disease, this deficiency is also seen at a high frequency, in the areas of Japan surveyed. It was estimated that approximately one in thirty individuals are heterozygotes. One similarity to MADD is that ATP in the deficient cells was approximately 50% higher than in control erythrocytes (Ogasawara et al., 1987) again emphasising the important role AMP deaminase plays in the regulation of cellular adenylates.

1.6 THE ROLE OF AMP DEAMINASE IN THE MAINTENANCE OF THE ADENYLATE ENERGY CHARGE

As previously stated, AMP deaminase is an important component

of the purine nucleotide cycle. However, the enzyme has been implicated in other important roles within the cell. In particular, it plays a part in the stabilisation of the adenylate energy charge. The adenine nucleotides stoichiometrically couple all the metabolic processes of a cell. The amount of metabolically available energy and thereby the cell's viability and growth capability that is momentarily stored in the adenylate system is known as the adenylate energy charge. The adenylate energy charge is measured by the following equation:

$$\frac{[(ATP) + 1/2(ADP)]}{[(ATP) + (ADP) + (AMP)]}$$

The energy charge must be maintained at a high value to maintain viability and growth. Values vary from organism to organism. In E.coli for instance the energy charge must be greater than 0.5 to prevent cell death, and must be maintained at or above 0.8 to allow growth (Chapman et al., 1971). Chapman and Atkinson (1973) showed that in liver cells, where the energy charge is normally 0.7 to 0.8 the rate of deamination of AMP increases sharply as the adenylate energy charge falls. Clearly the AMP deaminase reaction increases the energy charge by removal of AMP from the adenylate pool. However, the same group showed that this depletion of adenylates by the AMP deaminase reaction continues only until the adenylate pool drops to below a physiological range (Chapman and Atkinson, 1973), at which point AMP deaminase activity drops sharply. This may be a self-limiting response to prevent excessive depletion of the pool. This response has also been confirmed in muscle extracts (Tornheim and Lowenstein, 1974).

The pattern of stabilisation of the adenylate energy charge appears to be more complex in the yeast S.cerevisiae. When grown aerobically on ethanol or glucose and allowed to pass into stationary phase, yeast cells manage to maintain a normal energy charge value of 0.8-0.9 even during prolonged starvation in media depleted of nutrients. This is in contrast to yeast grown either anaerobically on glycogen or in the early stages of aerobic growth on glucose which when transferred to medium lacking an energy source, the energy

charge rapidly drops (Ball and Atkinson, 1975). The results suggest that functional mitochondria and/or the enzymes of the glyoxylate pathway are required for maintenance of a normal energy charge during starvation. Yeast are also unusual in that unlike E.coli they can maintain viability at energy charge values as low as 0.1 (Ball and Atkinson, 1975).

There is increasing evidence that cells respond more to the adenylate energy charge than the actual amount of cellular ATP. For instance, in platelets cell function is maintained even when two-thirds of metabolic ATP is lost, but becomes aberrant if the adenylate energy charge falls by only as much as 5% (Holmsen and Robkin, 1977). Again, the removal of AMP to stabilise the energy charge occurs primarily by the AMP deaminase reaction, although 5'-nucleotidase can respond to drops in the adenylate energy charge but only, as in the case of cultured lymphoblasts, when the AMP deaminase reaction alone has been unsuccessful and the energy charge has dropped below 0.6 (Matsumoto et al., 1979). Clearly, maintenance of a high adenylate energy charge is vital to normal cell function in many systems.

Recently, Ilzasa and Miyamoto (1987) have shown that the pattern of excretion from human lymphoblasts alters with the energy charge. When the energy charge is high, most of the excreted purines are intermediates of the de novo purine synthetic pathway, whereas when the energy charge drops, IMP produced by the AMP deaminase reaction, and its by-products are the major source of excreted purines.

It has been known for some time that the AMP deaminase reaction is the major source of NH_3 in blood (Conway and Cooke, 1938 and 1939). The AMP deaminase has also been shown to produce most of the ammonia which accumulates in working muscle (Lowenstein and Tornheim, 1971). In fact, it has been shown that in frog skeletal muscle, the amount of ammonia produced is proportional to the amount of work done by the muscle, although AMP deaminase is not directly involved in muscle contraction. (Lowenstein and Tornheim, 1971). There is now evidence that in the production of ammonia, the AMP deaminase reaction, in addition to its direct affect on the adenylate energy charge by the removal of AMP, may also

stimulates the regeneration of cellular ATP, thus indirectly enhancing stabilisation of the energy charge (Yoshino and Murakami, 1982b).

As already stated, AMP deaminase is activated when the energy charge falls and hence increases the production of ammonia. In so doing the AMP deaminase reaction has been proposed to stimulate at least two glycolytic enzymes. Ammonia is an allosteric activator of phosphofructokinase and pyruvate kinase. ATP inhibits these two enzymes (Weber et al., 1967), and hence when the energy charge is low, ATP concentrations are low and ammonia concentrations increase, allowing activation of these two enzymes. It has been proposed that this stimulation may result in an increase in glycolytic flux (Yoshino and Murakami, 1982c and 1983). Phosphofructokinase and pyruvate kinase along with hexokinase are often stated to be rate-limiting steps in glycolysis (Hess, 1971), i.e. have high control coefficients with respect to glycolytic flux (Kacser, 1983). However, there is some debate as to whether this is the case in yeast. One group has shown that overproduction of either phosphofructokinase or pyruvate kinase has little effect on glycolytic flux (Schaff et al., 1989). It now seems clear that these two enzymes have high control coefficients in many tissues although the situation in yeast awaits clarification (Bosca and Corredor, 1984; Fell, 1984). It may be that when the energy charge within cells is low (thus enhancing AMP deaminase activity), the production of allosteric activators of glycolytic enzymes leads to the enhanced regeneration of ATP suggested by Yoshino and Murakami (1982d).

This response to changes in the adenylate energy charge has been studied in great detail in yeast. Yoshino and Murakami (1983) showed, using permeabilised yeast cells, that both AMP deaminase and phosphofructokinase activities were activated by reductions in the adenylate energy charge. The optimal energy charge value for the response of phosphofructokinase being between 0.3 and 0.5. They also showed that the addition of NH_4^+ ions further stimulated phosphofructokinase activity. Spermine activation of AMP deaminase resulted in activation of phosphofructokinase, by the increase in ammonium ion concentration, (spermine has no direct affect on

phosphofructokinase). Similar effects were also noted for pyruvate kinase (Yoshino and Murakami, 1985a). The same group later showed that during aerobic growth where cellular citrate concentrations are high the AMP deaminase reaction can participate in the stabilisation of the energy charge only by the depletion of total adenylates and not by glycolytic stimulation (citrate is an inhibitor of phosphofructokinase) (Yoshino and Murakami, 1985c). Tornheim (1979) postulated that the purine nucleotide cycle and the glycolytic pathway oscillate to maintain a high ATP:AMP ratio. It was shown in skeletal muscle extracts that the control of phosphofructokinase activity by adenine nucleotides alone leads to the establishment of a steady state, but where appropriate, oscillations in the purine nucleotide cycle through the AMP deaminase reaction will also produce oscillations in the rate of glycolysis (Tornheim, 1979).

In platelets AMP deaminase is activated in response to changes brought about by thrombin stimulation, which leads to the loss of 30% of platelet metabolic adenine nucleotides (Ashby and Holmsen, 1983) and the suggestion has been made that, under these conditions, AMP deaminase may, instead of stimulating ATP production by NH_3 activation of glycolysis, simply cause displacement of the adenylate kinase reaction ($2ADP = ATP + AMP$) in favour of ATP production. E.coli AMP nucleosidase behaves in a qualitatively similar manner as platelet AMP deaminase with regard to its interactions with AMP, $MgATP^{2-}$ and inorganic phosphate and precisely the same mechanism has been proposed for the regulation of the bacterial enzyme (Ashby and Holmsen, 1983).

As previously stated, in muscle the ammonia produced by the AMP deaminase reaction is proportional to the work done by the muscle. Clearly work carried out by muscle depletes the energy stores of the muscle. Mommsen and Hockachka (1988) showed that during exhaustive exercise trout muscle ATP is depleted by more than 90%, resulting in a stoichiometric increase in IMP and ammonium ions. During exercise, of the three enzymes of the purine nucleotide cycle only AMP deaminase is active. Conversely, during slow recovery after exercise AMP deaminase is effectively inactive, while the

other two enzymes of the cycle, adenylosuccinate synthetase and adenylosuccinate lyase replenish the adenylate pool. Mommsen and Hockachka (1988) therefore propose that under certain conditions, the two halves of the purine nucleotide cycle, AMP deaminase and adenylosuccinate synthetase/adenylosuccinate lyase are functionally and temporally separated. They also proposed that AMP deaminase functions during exercise to supply allosteric effectors to aid recovery from whatever stress is applied. The situation in rat hepatocytes is somewhat different. In normally fed rats, ATP depletion can be induced in anoxic conditions, but the depletion is slow due to the presence of glycogen stores, allowing the generation of ATP by anaerobic glycolysis, whereas in hepatocytes from rats starved for 24 hours, the ATP almost completely disappears in the first five minutes of anoxic conditions, being replaced partly by the accumulation of AMP (Vincent et al., 1982). This can be explained by the fact that ATP (a major activator of AMP deaminase) disappears and is replaced by inorganic phosphate (a major inhibitor of AMP deaminase) and therefore AMP deaminase activity drops. Therefore, the AMP deaminase reaction can be shut down to prevent depletion of the adenylate pool under certain conditions.

Another central role in the metabolism of the cell has been suggested for the AMP deaminase reaction. It has been demonstrated that in rabbit reticulocyte lysates, AMP inhibits protein synthesis by inhibiting polypeptide chain elongation. This inhibition can be lifted by removing the AMP via the addition of AMP deaminase to the lysate (Mosca et al., 1983). Therefore, it has been proposed that under certain conditions the AMP deaminase reaction may play a role in controlling the rate of protein synthesis. In yeast the AMP deaminase reaction has also been implicated in the stimulation of threonine dehydratase activity by its production of ammonia, (Yoshino and Murakami, 1981d) and in the stimulation of fructose-1,6-bisphosphatase activity by the removal of AMP (Yoshino and Murakami, 1985b).

1.7 THE AIMS OF THIS STUDY

AMP deaminase has been shown to have a central role in many key reactions within the cell. At the outset of this project, although work was going on to elucidate the biochemical regulation of the enzyme itself, very little was known about the expression of the enzyme at the genetic level. There were no reported mutations of AMP deaminase in any system. Although in yeast, mutants for the other two members of the purine nucleotide cycle, adenylosuccinate synthetase and adenylosuccinate lyase had been isolated and named *ade12* and *ade13* respectively, little was known of the AMP deaminase gene (Dorfmann, 1968; Jones and Fink, 1982). Neither had the gene for AMP deaminase been cloned from any system. It was of interest to study the regulation of this enzyme as it related to metabolism in the cell. Clearly the enzyme is highly allosteric and thereby a proportion of the enzyme's activity would be controlled at that level. But it was of interest to study whether this enzyme is also regulated at the level of gene expression, and if so, just how that regulation was brought about. It was felt that a first step in achieving these objectives was to clone the gene from yeast. *S.cerevisiae* seemed an ideal system in which to study the expression of this enzyme because of the ease of growing the organism in a defined growth medium, studying the expression with respect to stages of growth and nutrient availability, and in the generation of specific mutations using well established "reverse genetics" techniques.

AMP deaminase was also of commercial interest. RNA is a biproduct of many biotechnological processes and as such it is of limited value. However, some of the degradative products of RNA are presently being used as flavour enhancers in the preparation of proprietary foods. In particular, two mononucleotides GMP and IMP can be used in this way and since IMP is one of the products of the AMP deaminase reaction this project has been partly funded by I.C.I.'s biological products division.

1.8 NOTES ON THE ORGANISATION OF THE THESIS

Not all the background information regarding this project has been covered in this chapter. Further information is included in the introductions to specific chapters. Therefore, further information regarding the use of adenosine analogues as inhibitors of AMP deaminase, and the use of one of these inhibitors to produce a variant mammalian cell line is discussed in Section 3.1. Similarly, the use of histoenzymatic stains for AMP deaminase are discussed in Chapter 4. At the beginning of Chapter 5 is an account of the previously described method for the purification of AMP deaminase from yeast. And finally, a brief review of the heat shock response especially with regard to hsp90 and AMP deaminase is given in Section 6.1.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Chemicals and enzymes used in this study and the sources from which they were obtained are listed below

Material	Source
General laboratory chemicals	Sigma, Formachem, BDHchemicals Koch-Light, Pharmacia, Biorad laboratories, May and Baker, BCL, Aldrich, Serva
Media	Difco, Oxoid
Biochemicals	Sigma, BCL
Antibiotics	Sigma
Agarose	Sigma, BRL
Radiochemicals	NEN
Nitrocellulose membrane	Schleicher and Schuell
Nylon membrane	Amersham
Amino acids	Sigma
DNA-modifying enzymes	BRL,
Lysozyme	Sigma
Novozyme	Novo Industries
Glucoronidase	Sigma
Zymolyase	Miles Laboratories
GIDH	BCL
X-ray film	Kodak
Gene Clean kits	Stratetech Scientific
Glass beads	BDH
phenol/acetone reagent	Fisons
hypochlorite	ICI

2.2 E.coli STRAINS

The E.coli strains used in this work are shown below with their genotypes.

Strain	Genotype	Source
C1400	supE, supF, hsd5, met$^-$, recA,	K. Murray
DS902	recA14, thr, leu, his, pro, str	David Sherratt

CB51	ara, thi, Δ(lac pro), rpsL, dam-3, str	Christopher Boyd

2.3 S.cerevisiae STRAINS

The S.cerevisiae strains used and produced in this work are shown below with the appropriate genotypes and sources

Strain	Phenotype	Source
DBY746	alpha, his3, ura3-52, leu2-3, leu2-112, trp1-289	David Botstein
DBY747	a, his3, ura3-52, leu2-3, leu2-112, trp1-289	David Botstein
DBY868	alpha, ade2-1, his4-619	David Botstein
W1-2D	a, hpt1.27	Woods et al., 1983
W1-2D rho°C	a, rho$^-$, hpt1.27	By mutation of W1-2D (Chapter3)
JMMDIP-1	a/alpha	By mating of DBY 747 and W1-2D (Chapter 3)
JMM1-1D	alpha, his4-619, ade2-1, hpt1.27	By sporulation of JMMDIP-1 (Chapter3)
JMM1-2C	a, ade2-1, hpt1.27	By sporulation of JMMDIP-1 (Chapter 3)
JMM1-5C	alpha, ade2-1, hpt1.27	By sporulation of JMMDIP-1(Chapter 3)
JMMDIP-2	a/alpha, his4	By mating of X4003-5B and JMM1-1D

JMM2-4C	a, ade2-1, hpt1-27, ura, his4, met2, leu2	By sporulation of JMMDIP-2 (Chapter 3)
JMM2-9D	a, ade2, hpt1.27, ura3, his4, met2, leu2	By sporulation of JMMDIP-2 (Chapter 3)
JMMDIP-3	a/alpha	By mating of X4003-5B JMM1-5C (Chapter 3)
JMM3-2A	a, ade1, hpt1.27, ura3, leu2, trp5	By sporulation of JMMDIP-3 (Chapter 3)
JMM3-7B	alpha, ade1, ade2, hpt1.27, ura3, met2, trp5	By sporulation of JMMDIP-3 (Chapter 3)
JMM3-8C	a, ade1, ade2, hpt1.27, ura3, met2, leu2, trp5	By sporulation of JMMDIP-3 (Chapter 3)
JMM3-9D	alpha, ade1, ade2, hpt1.27, ura3	By sporulation of JMMDIP-3 (Chapter 3)
X2928-3D-1A	a, ade1, gal1, trp1, ura3, his2, leu1, met14	
X2928-3D-1C	alpha, ade1, gal1, trp1, ura3, his2, leu1, met14	
X4003-5B	a, leu2, ade1, his4, met2, ura3, trp5, gal1	
X4003-22	a, leu2, ade1, his4, met2, trp5, gal1, hsp90-	By transforming X4003-5B with integrating vector pHSPUR22 (Chapter 6)

X4003-3.4	a, leu2, ade1, his4, met2, trp5, gal1,	By transforming X4003-5B with multi-copy plasmid p378-3.4 bearing the complete hsp90 gene (Chapter 6)

2.4 PLASMIDS

The plasmids used in this study, other than those whose construction, are described in this thesis are listed below.

Plasmid	Description	Source
pUC8	Cloning vector derived from pBR322	Pouwels et al., 1988
pSPUR2	1.1kb Hind III URA3-containing fragment from Yep24 cloned into pSP64	Alistair Brown
YEp24	E.coli-Yeast shuttle vector. Carries 2.2kb Eco RI fragment from 2um and 1.1kb URA3 - containing Hind III fragment	Carlson and Botstein, 1982
pBSAMPD	Blue script plasmid containing 2.5kb EcoRI fragment comprising rat muscle AMP deaminase cDNA	Sabina et al., 1987

2.5 E.coli CULTURE MEDIA AND GROWTH CONDITIONS

Media

L Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thymine, made up to 1 litre in distilled water and adjusted to pH7.0 with NaOH

L Agar: As above but without glucose and the addition of Bacto-agar to 2%

All growth media were sterilised by autoclaving at 120^{o}C for 15 minutes. Buffer solutions and nutrient additions, where

appropriate were autoclaved at 108°C for 10 minutes.

Antibiotics

In both liquid and plate selection, ampicillin was added to the medium at a final concentration of 100µg/ml. The drug was stored for several months at -20°C, as a stock solution of 100mg/ml in sterile, distilled H_2O.

Growth Conditions

Liquid cultures for transformation or plasmid DNA preparations were routinely grown in L broth at 37°C with vigorous shaking. Stationary phase overnight cultures were grown from small inocula in 2.5ml L broth with vigorous shaking,

Growth on plates was on L agar with antibiotics added as required. Plates contained 25ml agar solution and were incubated for approximately 16 hours at 37°C.

Strains were stored in the following way. The strain was grown in L broth at 37°C with vigorous shaking. 1.5ml of this culture was then added to 300µl of glycerol and after mixing the strain was stored at -70°C indefinitely. In order to use these stored strains, they were streaked on to L agar plates and incubated at 37°C overnight. These plates could then be stored at 4°C for some time.

2.6 S.cerevisiae CULTURE MEDIA AND GROWTH CONDITIONS

Media

Complete media (YPG)	2% glucose, 2% Bactopeptone, 1% yeast extract
YPG agar	As above with the addition of Bacto-agar to 2%
Minimal/Selective media (GYNB)	2% glucose, 0.65% yeast nitrogen base (amino acid free)

GYNB agar	As above with the addition of Bacto-agar to 2%
Presporulation media	0.8% Bacto yeast extract, 0.3% Bacto peptone, 10% glucose, 2% Bacto agar
Sporulation media	1% Potassium acetate, 0.1% Bacto peptone, 0.05% glucose, 2% Bacto agar
Transformation Top agar	2% glucose, 0.65% yeast nitrogen base (amino acid free), 2% Bacto agar, 1.2M Sorbitol
Transformation Bottom agar	2% glucose, 0.65% yeast nitrogen base (amino acid free), 3% Bacto agar, 1.2M Sorbitol

All growth media were sterilised by autoclaving at 120^{o}C for 15 minutes. Buffer solutions and nutrient additions, where appropriate were autoclaved at 108^{o}C for 10 minutes.

Nutrient additions

Amino acids and adenine were added to minimal media, where required for growth of auxotrophic mutants, to a final concentration of 50ug/ml unless otherwise stated. Stock solutions of these additions, at a concentration of 10mg/ml in distilled H_2O were sterilised by filtration or autoclaving and stored at 4^{o}C for up to several months.

Growth conditions

Strains were streaked onto YPG or GYNB agar with appropriate additions and grown for 3 days at 30^{o}C. Plates could be stored for several weeks at 4^{o}C.

Liquid cultures in either YPG or GYNB were normally innoculated with small volumes of a 2 day stationary culture and grown at 30^{o}C with vigorous shaking, overnight or until the appropriate phase of growth was attained. Growth was

monitored by absorbance at 600nm. Before use cultures were checked for bacterial contamination by light microscopy.

For storage purposes, 300µl of sterile glycerol was added to 1.5ml of a midlog culture grown in GYNB with the appropriate additions. This mixture was then stored at -70°C indefinitely.

2.7 BUFFERS AND SOLUTIONS

Buffer solutions, where appropriate were autoclaved at 108°C for 10 minutes.

Electrophoresis

10 x TBE Buffer, pH8.3	109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1 litre in distilled H_2O, the pH is 8.3
10 x TAE Buffer, pH8.2	48.4g Tris, 16.4g Na acetate, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH adjusted to 8.2 with acetic acid
Agarose Gel Loading Buffer	10% Ficoll, 0.5% SDS, 0.06% Bromophenol blue, 0.06 xylene cyanol
10 x Sequencing TBE	121.1g Tris, 51.35g Boric acid, 3.72g EDTA made up to 1 litre in distilled water, the pH is 8.3
SDS/PAGE Loading Buffer	80mM Tris, pH6.8, 10% glycerol, 0.0024% bromophenol blue, 2% SDS, 100mM DTT
5 x SDS/PAGE running buffer 5g SDS,	72.05g glycine, 15.15g Tris (base), made up to 1 litre with distilled water

10 x MOPS Buffer	0.2M 3-[N-Morpholino]-propane-sulphonic acid, 0.05M Na acetate, 0.01M Na_2EDTA
MMV solution	500µl formamide, 162µl of 37% formaldehyde, 100µl 10 x Mops buffer, made up to 1000µl with distilled H_2O
Northern gel loading buffer	50% glycerol, 0.4% bromophenol blue, 0.4% xylene cyanol, 1mM EDTA

Protein Phastgel staining solutions

Coomassie Blue stain	0.02% Phastgel Blue R (tablet form) dissolved in 30% methanol, 10% acetic acid
Destain	30% methanol, 10% acetic acid
Preserving solution	10% acetic acid, 5% glycerol
Wash 1	50% ethanol, 10% acetic acid
Wash 2	10% ethanol, 5% acetic acid
Sensitiser	8.3% glutaraldehyde
Silver nitrate	0.25% silver nitrate
Developer	0.04% formaldehyde in 2.5% sodium carbonate (prepared fresh each day)
Stop bath	5% acetic acid

DNA and RNA Preparation and Purification

Birnboim Doly I (BD-I)	50mM glucose, 25mM Tris-HCl ph 8.0, 10mM EDTA; add lysozyme to 1mg/ml immediately before use
Birnboim Doly II (BD-II)	0.2M NaOH, 1% SDS; used fresh

Birnboim Doly III (BD-III)	5M KAc, pH 4.8; mix equal volumes of 3M CH_3COOK and 2M CH_3COOH, pH should be 4.8
STET buffer	8% sucrose, 2% Triton X-100, 20mM EDTA, 50mM Tris, pH 8.0
Phenol	All phenol used in the purification of DNA was buffered against 0.1M Tris-HCl, pH 8.0
Phenol/chloroform	For most purposes a 1:1 mix of phenol (equilibrated as above) and chloroform
TEN solution	10mM Tris,HCl, pH 7.6, 1mM EDTA, 10mM NaCl
SB solution	0.2M Tris-HCl, pH 7.5, 1M sorbitol, 0.1M EDTA, 0.1M 2-mercaptoethanol
RNA extraction buffer	0.1M LiCl, 0.1M Tris, pH 7.5, 0.01M DTT (added fresh)

<u>DNA Manipulations</u>

Restriction Buffers	Restrictions were normally carried out using the appropriate BRL React™ buffer which were supplied with the enzyme used
10 x Ligation Buffer	250mM Tris, pH 7.5, 100mM $MgCl_2$, 100mM DTT, 4mM ATP
10 x Klenow Buffer	100mM Tris, pH 7.4, 100mM $MgCl_2$, 0.66% gelatin

DNA Hybridisation

Denhardt's Solution	2g BSA (Pentax fraction V), 2g Ficoll (MW 400000), 2g polyvinyl pyrrolidone (MW 360000) in 100ml distilled H_2O
20 x SSC	3M NaCl, 0.3M Na_3 citrate, pH7.0
20 x SSPE	3.6M NaCl, 200mM NaH_2PO_4, 20mM Na_2EDTA, pH7.4
Denaturation Buffer	1.5M NaCl, 0.5M NaOH
Neutralising Buffer	1.5M NaCl, 0.5M Tris.HCl, pH 7.2

Radiolabelling of DNA

10 x Kinase Buffer	0.5M Tris-HCl, pH 7.6, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA
Random Prime mix	200µl solution A, 500µl solution B and 300µl solution C (as below)
Random prime solution A	1ml solution O with 18µl 14.4M B-mercaptoethanol and 5µl each of triphosphate nucleotides; dA, dG and dT (stock solutions of 100µM)
Random prime solution O	1.25M Tris, pH8.0, 0.125M $MgCl_2$
Random prime solution B	2M Hepes, pH6.6
Random prime solution C	50 O.D. units of hexanucleotides $(p(dN)_6$ from Pharmacia in 556µl water

Transformation of E.coli and S.cerevisiae

SE solution	1M sorbitol, 25mM EDTA, pH8.0
SEN solution	1.2M sorbitol, 10mM EDTA, 100mM Na citrate, pH 5.8

SC solution	1.2M sorbitol, 10mM $CaCl_2$
CT solution	10mM $CaCl_2$, 10mM Tris, pH 7.5
SY solution	2 volumes of 1.8M sorbitol, 1 volume of 3 x YPG

Protein Analyses

Bradford Reagent	100mg Coomassie Brilliant Blue G-250 dissolved in 95% ethanol, to which 100mls 85% (w/v) phosphoric acid, is added, this was made up to 1 litre with distilled H_2O and was then filtered through Whatman filter paper into scrupulously clean glassware. This solution was stored in the dark for up to 6 months
Extraction Buffer	yeast proteins were normally extracted into 50mM potassium phosphate buffer, pH 7.5, 1mM PMSF
Oxoglutarate buffer	9.3g TEA.HCl, 95mg Na_2ADP, 670mg 2-oxoglutarate, pH to 8.0 with 5M NaOH and make up to 100mls with distilled water
NADH/$NaHCO_3$ solution	30mg Na_2 NADH, 60mg $NaHCO_3$ in 6ml distilled water
acetate buffer	13.61g sodium acetate trihydrate, 7mls conc. HCl made up to 1 litre with distilled water
Reagent 1 sodium	phenoxide/acetone solution (supplied by Fisons)

Reagent 2	0.5% hypochlorite; 50ml stock hypochlorite solution (5% free Cl^-, as supplied) made up to 500ml with acetate buffer
Reagent 3	0.01% sodium nitroprusside dissolved in acetate buffer

2.8 IN VIVO TECHNIQUES (E.coli)

Transformation of E.coli

A single colony of the appropriate recipient E.coli strain was innoculated into L broth from a fresh L agar plate which had been incubated overnight. The culture was then incubated at 37°C with vigorous shaking to a density of approximately 10^8 cells/ml. The cells were chilled rapidly in an ice-water bath and harvested by centrifugation at 3K for 5 minutes at 4°C and resuspended in 1/5 culture volume of ice cold 50mM $CaCl_2$. The cells were then incubated on ice for 30 minutes and then centrifuged at 1100 x g for 5 minutes at 4°C. The cells were then very gently resuspended in 1/100 culture volume of ice cold 50mM $CaCl_2$. This was then incubated on ice for 15 minutes. 50 - 200 ul aliquots were routinely used for each transformation. Plasmid DNA was added and gently mixed, and left on ice for at least 30 minutes. The cells were then heat shocked at 42°C for 2 minutes and returned to ice for at least a further 15 minutes. The cells were diluted to 1ml with L broth and incubated at 37°C for 30 minutes to allow ampicillin resistance to be expressed. The cells were then plated out on L agar + ampicillin. Unused cells were stored at -70°C in 20% glycerol.

2.9 IN VIVO TECHNIQUES (S.cerevisiae)

Transformation of S.cerevisiae

Transformation of S.cerevisiae was carried out by the method of Beggs (1978). A 100ml culture of the appropriate yeast strain grown in YPG to mid or late-logarithmic growth was used for each transformation. The cells were harvested by centrifugation at 1000 x g for 5 minutes at room temperature.

The cells were resuspended in 50ml SE solution containing 50mM DTT (added immediately before use and filter sterilised) and incubated at 30°C for 20 minutes with gentle shaking. The cells were reharvested by centrifugation at 100 x g for 5 minutes at room temperature. The cells were resuspended in 50ml SEN solution (containing 2-3% (v/v) B-glucoronidase added immediately before use and filter sterilised) and incubated at 30°C with gentle shaking until spheroplast formation had reached approximately 90% (as monitored by light microscopy). The spheroplasts were harvested by centrifugation at 1000 x g for 3 minutes at room temperature and washed gently twice with 1.2M sorbitol. They were then gently resuspended in 100µl SC solution to generate a thick suspension. DNA was added to 50ul of the spheroplasts in sterile eppendorf tubes and incubated at room temperature for 15 minutes. 500µl of 20% PEG in CT solution (made fresh and filter sterilised) was added and left for 45 seconds prior to centrifugation in a microfuge for 1 second. The PEG was immediately removed and the spheroplasts resuspended in 100µl SY solution. They were then incubated at 30°C for 30 minutes. The speroplasts were diluted as required with 1.2M sorbitol and aliquots were added to 10mls Top agar containing the appropriate supplements for growth of transformants (equilibrated at 48°C and immediately spread over Bottom agar containing the appropriate supplements. The plates were incubated at 30°C until the appearance of colonies (normally 3-7 days). Putative transformants were then streaked onto selective plates to confirm the predicted genotypes.

Standard mating of S.cerevisiae strains

This technique was used to produce strains with appropriate combinations of auxotrophic markers as described in this thesis (Chapter 3).

The parental strains were streaked onto YPG agar to produce single colonies. The 2 strains were then mixed on a fresh YPG agar plate with a sterile toothpick. This plate was incubated at 30°C overnight. At the end of this time mating had occurred and diploids had been produced. The cells were then streaked onto plates which will only support growth of the diploids. These plates were then incubated for 3-4 days. A single colony was then patched onto presporulation media,

grown overnight at 30°C, after which these cells were patched onto sporulation media, and then incubated at room temperature for 2-5 days. During this time sporulation was occasionally monitored by light microscopy, and once efficient ascus production was observed, the cells were subjected to tetrad dissection and analysis.

Tetrad dissection and analysis

Tetrad dissection and analysis was carried out by the method of Sherman et al. (1986) with the exception that B-glucuronidase was used instead of glusulase.

2.10 IN VITRO TECHNIQUES: NUCLEIC ACIDS

Birnboim Doly Plasmid DNA Preparations (Birnboim and Doly, 1979)

A 100-200ml L broth + Amp culture of the appropriate strain was harvested by centrifugation at 3800 x g for 10 minutes. The cell pellet was then resuspended in 2ml BD-I until an even cell suspension was achieved. To this 1ml of BD-I with lysozyme freshly added at 6mg/ml was added and thoroughly mixed. This was incubated at room temperature for approximately 5 minutes. To this 8mls of BD-II (freshly made) was added and mixed by inversion, followed by the addition of ice-cold BD-III which was again mixed by gentle inversion. This mixture was then incubated on ice for at least 5 minutes. Cell debris was then removed by centrifugation at 35000 x g for 20-30 minutes. The majority of the chromosomal DNA appears to be removed at this stage. The supernatant was removed to a fresh tube by straining through a sterile tissue and was precipitated by the addition of an equal volume of isopropanol for 15 minutes at room temperature. The precipitate was harvested by centrifugation at 35000 x g for 15 minutes at 20°C. The pellet was then washed with ice-cold 70% ethanol and drained.

The plasmid DNA was further purified on a CsCl/ethidium bromide gradient. The DNA was resuspended in 10mls TE and 500ul ethidium bromide (10mg/ml) and CsCl was added to a final density of 1.58g/ml. The gradients were then

centrifuged in a Beckman Ti70 rotor at 170000 x g at 20°C overnight. After this treatment, 2 bands were visible, the upper composed of chromosomal and relaxed plasmid DNA and the lower of supercoiled plasmid DNA which was removed using a 1ml syringe. The ethidium bromide was removed by repeated butanol extraction (with water saturated butanol) and the salts were removed by dialysis in 2 litres of TE (one change of buffer). The DNA was then ready to use.

STET DNA Preparations

This rapid method of plasmid preparation by the method of Holmes and Quigley (1981) and was used for the analysis of putative constructs where several plasmids were to be screened.

1.5ml of an overnight L broth + ampicillin culture containing the plasmid of interest was harvested by centrifugation in a 1.5ml eppendorf tube and resuspended in 35µl of STET buffer. 25µl of STET buffer containing lysozyme at a concentration of 10mg/ml (freshly made up) was added and the tube vortexed briefly. This mixture was boiled for 50 seconds and centrifuged in a microfuge for 15 minutes at room temperature. The pellet was discarded using a sterile toothpick. 40µl of 3M Na acetate and 400µl of isopropanol were added and mixed. This was incubated at room temperature for 15 minutes and then the nucleic acid was harvested by microcentrifugation for 10 minutes. The pellet was washed with 70% ethanol and dried briefly under vacuum. The pellet was then resuspended in 50µl sterile water. This DNA was suitable for restriction digestion and other *in vitro* manipulations.

Plasmid purification for sequencing

It was found that standard CsCl gradient purified plasmid was not always of sufficient quality to allow efficient dideoxy sequencing. When this was found to be the case the DNA was further purified in a adaptation of the method of Hattari and Sakaki, 1986.

1ml of normally purified DNA was first treated with RNAase A (preboiled for 10minutes) at a final concentration of 20µg/ml for 30 minutes at 37°C. The solution was then extracted once

with phenol/chloroform and once with chloroform. To the aqueous phase was added 0.6 volumes of 20% PEG, 2.5M NaCl (ice-cold). The DNA was precipitated on ice for at least 1 hour and then harvested by microcentrifugation for 10 minutes. The pellet was washed twice with ice-cold 70% ethanol and vacuum dried. The DNA was then resuspended in sterile, distilled water and its concentration estimated by agarose gel electrophoresis. The DNA was then suitable for plasmid DNA sequencing analysis.

Precipitation of DNA with Ethanol

Ethanol precipitation was routinely used for the concentration or harvesting of DNA where appropriate. The DNA solution was made 0.3M Na acetate and 2 volumes of ethanol was added. After mixing the DNA was precipitated by incubation at -20°C for 30 minutes or more. The DNA was then pelleted by centrifugation either in a microfuge, or at 27000 x g in a standard centrifuge for 15 minutes. The pellet was then washed in cold 70% ethanol and dried briefly under vacuum.

Restriction enzyme digestion of DNA

Restriction digests were performed with the DNA at a concentration no higher than 1µg/20µl. For small scale analyses the volume of the digest would normally be 20µl. The digest would be composed of 1 x the appropriate React™ buffer, the appropriate amount of DNA and at least 1 unit of enzyme/µg of DNA. The volume was made up with sterile, distilled water. For larger scale restrictions the volumes were scaled up accordingly. The reactions were allowed to proceed for 1 - 2 hours at the appropriate temperature. Reactions were stopped by the addition of gel loading buffer or by rapid heating to 70°C for 5 minutes followed by cooling on ice.

Ligation of DNA

The restriction fragments to be ligated were mixed such that the insert was in at least 3-fold excess over the vector (and at least 10-fold excess in the case of blunt-end ligations) and made up to 20µl by the addition of 2µl 10 x ligase buffer and sterile water. T4 DNA ligase was added at 0.5units/µg DNA for sticky end ligations and 2units/µg DNA in blunt end

ligations. The mix was incubated at 16°C overnight. Sticky end ligations could alternatively be incubated at room temperature for 3 hours. Aliquots of the ligation mix were diluted at least 5-fold with water prior to transformation of competent E.coli cells.

Calf Intestinal Phosphatase (CIP) treatment

To increase the cloning efficiency, CIP was used to remove the 5'terminal phosphate groups from the linearised vector to reduce recircularisation of the vector. This treatment is necessary if there is no direct selection method for plasmids containing inserts. CIP treatment was normally carried out in 1 x React™ 2 buffer immediately following restriction digestion. CIP was added at a concentration of 1-2units/ug DNA. Incubation was at 37°C for 20-30 minutes. The enzyme was heat-denatured and the vector gel purified prior to use to ensure removal of contaminating CIP.

Filling in 3' recessed ends with Klenow

This was carried out where a blunt-ended fragment was required to be produced from a fragment with 3' recessed ends. 0.1-1µg of DNA with 3' recessed ends were added to a 20µl mix made up of 2µl 10 x Klenow buffer, and containing all four dNTPs at a final concentration of 50uM each. 1 unit of Klenow fragment was added and reaction carried out for 15 minutes at 23°C.

Gel purification of DNA fragments: Gene Clean

The kit for this method was obtained from Stratatech Scientific. This method was routinely used where the fragment to be purified was less than 3kb in size. Fragments larger than this were normally purified by electroelution. Restriction digests were run on TAE agarose gels until the band to be purified is well resolved. The band of interest was then cut out and trimmed with a fresh scalpel blade, placed in an eppendorf tube, and dissolved in and 3 volumes of NaI solution (as supplied) at 60°C. Glass milk suspension (as supplied) was then added (5µl solutions containing less than 5µg DNA and an additional 1µl for each additional 1µg DNA). This was mixed thoroughly by pipetting and incubated on ice for 10 minutes. The glass beads were then pelleted in a microfuge for 5 seconds and the supernatant removed. The

glass beads were then washed three times with 500µl ice-cold NEW (as supplied) keeping the mixture ice-cold at all times. The glass beads were then pelleted and all the NEW removed. They were then resuspended in 50µl water and heated to 65°C for 5-10 minutes. The tube was then microfuged for 30 seconds and the supernatant removed to a fresh tube. DNA purified in this way was used directly in ligations and labelling procedures.

Gel purification of DNA fragments: Electroelution

This method was used routinely to purify fragments greater than 3kb in size. The DNA band was cut and trimmed as described above. The gel fragment was placed at one edge in a wide piece of dialysis tubing, the bag was then filled with TE until the gel fragment was covered and then the bag was sealed. The bag was then placed in a normal horizontal gel electrophoresis tank and 1 x TAE was added to just cover the bag. The edge of the bag with the gel fragment was placed farthest from the positive terminal in the tank. The bag was then subjected to a current of 10-20 mAmps for up to 1 hour depending on the size of the fragment. During this time the DNA would run out of the gel fragment and into the buffer along the other edge of the bag. This could be monitored with a hand held UV lamp since the fragment will remain complexed with ethidium bromide. Once the fragment has been successfully electroeluted the terminals were reversed and a low current run in the opposite direction for approximately 1 minute. The TE in the bag was then removed and the DNA precipitated with ethanol in the standard manner. This DNA could be used directly in ligations and labelling procedures.

Isolation of high molecular weight DNA from S.cerevisiae

The method used was an adaptation of the method of Lautenberger and Chen (1987). This adaptation was communicated by Stewart Finlayson and produces 1-2mg of DNA/litre culture of a size greater than 40kb.

A 1 litre stationary culture (more than 2 x 10^8 cell/m)l in YPG was harvested by centrifugation at 15000 x g for 10 minutes at 4°C. The cell pellet was resuspended in 10ml TEN, centrifuged at 15000 x g for 10 minutes and the resulting pellet weighed. The pellet was then resuspended in SB buffer

at a concentration of 0.25g cells/ml. 5mg Zymolyase 20000 (or 1.5mg Zymolyase 60000) was mixed in and the cell suspension incubated at 30°C with occasional gentle mixing for approximately 1 hour or until more than 80% of the cells are spheroplasted as determined by light microscopy. After this stage it is of the utmost importance that the mixing steps be by very gentle inversion and pipetting be carried out with wide-bore cut tip pipettes. The spheroplasts were harvested by centrifugation at 2500 x g for 5 minutes and then gently resuspended in 20ml SB. To this was added 40ml TEN, 5ml 10% SDS and 5mg RNAase A. This mixture was incubated at 37°C for 2 hours. After this 2mg Proteinase K was added and mixed by inversion. The mixture was incubated at 37°C for a further 2 hours, then heated to 65°C for 30 minutes and then cooled rapidly to room temperature. To this was added an equal volume of phenol/chloroform and mixing was continued for 15 minutes by gentle inversion. The phases were separated by centrifugation at 15000 x g for 10 minutes, after which the aqueous phase was removed and similarly extracted with an equal volume of chloroform. The aqueous phase was removed to a fresh tube and to it was added 1/25 volume of 5M NaCl and 2 volumes of ethanol (chilled to -20°C). This was then mixed by gentle inversion until the DNA was clumped together. The DNA was spooled out to a fresh tube and the excess ethanol removed by aspiration. The DNA was resuspended in 50ml TEN. THis step was carried out at 37°C to allow the DNA to completely dissolve. 1mg of RNAase A was added and the solution was incubated at 37°C for 1 hour. The DNA was then subjected to one phenol/chloroform and one chloroform extraction reprecipitated as described above. The DNA was spooled out again and resuspended in approximately 10mls TE. Once dissolved the DNA solution was dialysed against TE at 4°C overnight. This DNA was suitable for restriction analysis.

Isolation of RNA from S.cerevisiae

RNA was isolated according to the procedure of Lindquist (1981). Cultures in mid-logarithmic growth were harvested by centrifugation at 960 x g for 5 minutes at 4°C. The pellet was rapidly resuspended in 5ml lysis buffer and the resulting cell suspension was transferred to tubes containing 14g glass beads (40 mesh), 1ml 10%SDS, 10ml phenol/chloroform. This

mixture was vortexed continuously for 5 minutes and the centrifuged at 12000 x g for 5 minutes at 4°C. The aqueous phase was removed and added to 10ml phenol/chloroform and then vortexed for 1 minute and centrifuged at 12000g for 5 minutes at 4°C. The aqueous phase was made to 0.3M Na acetate, and 2.5 volumes of ethanol were added. This was incubated at -20°C for at least 1 hour. The RNA was then harvested by centrifugation at 15Krpm for 15 minutes and resuspended in 2ml water. The RNA was further purified by centrifuging through a CsCl cushion according to the method of Chirgwin et al. (1979).

2.11 IN VITRO TECHNIQUES; PROTEINS

Extraction of native soluble proteins from S.cerevisiae

10 mls or more of a yeast culture was harvested by centrifugation at 3500 x g for 5 minutes. The pellet was washed 1-2 times with water and resuspended in at least 1ml of extraction buffer (the choice of extraction buffer is detailed where appropriate in the text of this thesis) to give an even cell suspension. To this glass beads (40 mesh) were added to the meniscus. This mixture was then chilled on ice and then vortexed for 30 seconds, 3-4 times with 1 minutes on ice between each 30 second vortex. To this an appropriate volume of extraction buffer was added and vortexed briefly. The supernatant was removed to a fresh tube. Cell debris was removed by centrifugation at 12000 x g for 10 minutes.

Bradford assay for protein concentration

Bradford assays for protein concentration were carried out by the method of Bradford (1978). For any batch of measurements a set of BSA standard assays were carried out to allow the production of a calibration curve.

AMP deaminase reaction mixes

The AMP deaminase activity was determined by carrying out a reaction for a known period of time followed by determination of the ammonia produced by either the GIDH assay or the phenol-hypochlorite assay, as detailed below.

The standard reaction mix was as follows, unless otherwise stated:

20mM cacodylic acid, pH7.1, 100mM KCl, 10mM NaCl, 10mM AMP, 1mM ATP, 10mM $MgCl_2$, 0.02% BSA

In addition, for every sample assayed for AMP deaminase activty a substrate blank was carried out to allow the endogenous NH_3 in protein extracts and solutions to be taken into account. The reactions were stopped either by the addition of 10% TCA in the case of the GIDH assay (see below) or the sodium phenoxide/acetone reagent in the case of the phenol/hypochlorite assay.

Glutamate dehydrogenase (GIDH) ammonia assay

Initially it was decided to assay the ammonia produced from the AMP deaminase reaction by the sensitive enzymatic assay system decribed by Bergmeyer and Beutler (1982). This relies upon the use of NH_3 by glutamate dehydrogenase (GIDH) in the reduction of NADH to NAD with its concomitant decrease in absorbance at 339nm. The principle of this assay is shown below.

$$\text{2-oxoglutarate} + \text{NADH} + NH_4 \xrightarrow{\text{GIDH}} \text{L-glutamate} + NAD^+ + H_2O$$

This assay was used for the determination of NH_3 produced by the AMP deaminase reaction as detailed above. Into a 4ml cuvette the following were pipetted successively:

1ml oxoglutarate buffer
1.5 - 2.0ml water to bring volume up to 3.2ml
0.1ml NADH/$NaHCO_3$ solution
0.1 - 0.6ml sample of deproteinised reaction mix

The absorbance at 339nm was measured and noted and 0.02ml of the GIDH solution was added and stirred. Reactions were then incubated at room temperature for 20-30 minutes, by which time the reaction was complete and the absorbance at 339nm was again noted. The amount of NH_3 in the determination is then calculated thus

ΔA_{339}nm x 5.111 = molarity of a 0.1ml sample in a 3.2ml mix.

Samples containing proteins required to be deproteinised prior to the assay. This was carried out by adding one half volume of ice-cold 10% TCA to the sample or reaction mix to be assayed. The sample was then incubated on ice for 5-10 minutes and then microfuged for 5 minutes to remove any particulate material. The supernatant was removed and neutralised by the addition of one half volume of 2M $KHCO_3$. When CO_2 production had ceased the mixture was subjected to normal NH_3 determination in the GIDH assay.
This method was used in all analyses detailed in Chapter 3 and the sensitivity and accuracy of this assay is further discussed in appendix I. Subsequent analyses used the phenol-hypochlorite ammonia assay.

Phenol/hypochlorite ammonia assay

This method is an adaptation of Guisti's method (1974) as communicated by John Fitton. No deproteinisation step is required in this determination since the sodium phenoxide/acetone reagent brings about immediate denaturation of all proteins in the reaction mix.

Normally a 0.1ml sample was added to 1ml of sodium phenoxide/acetone and mixed. To this was added successively 1.5ml 0.5% hypochlorite and 1.5ml 0.01% sodium nitroprusside. The mixture was incubated at room temperature for 30 minutes (by which time colour production is complete) and the absorbance at 595nm was measured. Colour production was found to be stable for up to 24 hours. With each patch of determinations a set of NH_4Cl standards were also assayed to allow the production of a calibration curve. The method was adapted to increase its sensitivity. Again, in each case a suitable set of standard determinations were carried out. The adaptation was simply to reduce the volumes of the reagents used. Thus a 0.1 or 0.2ml sample was added to 0.33ml sodium phenoxide/acetone reagents followed by the addition of 0.5ml each of the hypochlorite and nitroprusside solutions. This allowed spectrophotometric measurements to be made in standard 1ml cuvettes.

Precipitation of proteins with ammonium sulphate or acetone

The following method was adopted for the analytical precipitation of proteins with ammonium sulphate or acetone.

At all stages of the analysis the extract was kept at between 0 and 4°C. A known volume of crude protein extract was cooled to 4°C and placed in a glass beaker on a magnetic stirrer. To this was added, slowly with stirring the appropriate volume of acetone or amount of solid ammonium sulphate (see Figure 2.12.1). This mixture was then stirred continuously and slowly for 15 - 30 minutes. At the end of this time the precipitated protein was harvested by centrifugation at 10K for 10 minutes, and the supernatants subjected to further precipitations as required (and detailed in Chapter 5). The precipitate was then resuspended in the smallest convenient volume of extraction buffer, and in the case of ammonium sulphate precipitations dialysed against 2 litres of extraction buffer at 4°C, with one change of buffer. These precipitated fractions were then subjected to AMP deaminase and protein analysis.

Liquid chromatography using an FPLC system

All chromatography with the exception of Cibacron blue sepharose analyses was carried out on a Pharmacia FPLC™ system. All methods were carried out according to each specific column's data sheet (supplied with column) and are detailed in Chapter 5. Extract and semi-purified material was filtered through 0.22μm filters before application to the column. In the case of Phenyl Superose and Mono Q columns the sample was always applied in the start buffer (the buffer with which the column had been pre-equilibrated). Once the sample was applied the column was washed with this start buffer until no appreciable protein elution was observed on a trace of absorbance at 280nm. After this linear gradients were applied to the column as detailed in Chapter 5. Fractions were collected in small test tubes and removed to ice as soon after elution as possible. Fractions were then assayed for AMP deaminase activity and aliquots of the fractions of interest were retained for protein concentration and SDS gel electrophoretic analyses. The fractions used for further analysis were pooled as indicated in Chapter 5.

2.12 GEL ELECTROPHORESIS

Standard agarose gel electrophoresis of DNA

Agarose gels of 0.6 - 1.5% (normally 0.8 or 1.0%) were used in this work for the separation of DNA molecules on the basis of length (MW). DNA was visualised by ultraviolet fluorescence after staining with EtBr. The EtBr was either added to the agarose at a final concentration of 0.2ug/ml, or after running, the gel was soaked in 1 x running buffer plus 0.6µg/ml EtBr. Gels were photographed with 260nm UV trans-illumination using a Polaroid camera loaded with 4x5 Land Film (no.57). The camera was fitted with a Kodak Wratten Filter No.6 (red).

Formaldehyde gel electrophoresis of RNA: Northern analysis

These gels were used for the electrophoresis of RNA under denaturing condition, such as in the case of Northern blot analysis. The were run under RNase-free conditions according to the modified procedures of Maniatis et al. (1982). 1.5g of RNase-free agarose was melted in 73mls of RNase-free water and cooled to 60°C. 10ml of 10 x MOPS buffer and 16.2ml 37%(v/v) formaldehyde were added, mixed well and poured. Well formers (made RNase-free) were inserted into the molten agarose and removed after the agarose was set. The RNA sample for analysis was mixed with 8 volumes of MMF and the mixture was incubated for 15 minutes at 65°C to denature the RNA. The denatured RNA samples were mixed with formaldehyde gel loading buffer, loaded on to the gel and run at 100V with 1 x MOPS as running buffer with constant circulation of the buffer from anode to cathode to maintain a constant pH. This gel was then suitable for Northern blot analysis.

Standard SDS polyacrylamide gel electrophoresis of proteins

Standard 7 or 9% SDS polyacrylamide gel electrophoresis was used both in some analyses during purification (Chapter 5) and for the confirmation of heat shock (Chapter 6) and was carried out by the standard method of Laemmli (1970). In the case of heat shock gels, after electophoresis the gels were dried down and autoradiographed.

Phastgel SDS polyacrylamide gel electrophoresis of proteins

Most electrophoretic analyses during purification were

TABLE 2.12.1 COOMASSIE BLUE STAINING USING THE PHASTSYSTEM

Reagent	Temperature	Time(minutes)
Stain	50°C	8
Destain	50°C	5
Destain	50°C	8
Destain	50°C	8
Preserving solution	50°C	5

TABLE 2.12.2 SILVER STAINING USING THE PHASTSYSTEM

Reagent	Temperature	Time(minutes)
Wash 1	50°C	2
Wash 2	50°C	2
Wash 2	50°C	4
Sensitiser	50°C	6
Wash 2	50°C	3
Wash 2	50°C	5
Water	50°C	2
Water	50°C	2
Silver nitrate	40°C	13
Water	30°C	0.5
Water	30°C	0.5
Developer	30°C	0.5
Developer	30°C	4
Stop bath	50°C	2
Preserving solution	50°C	3

carried out using the Pharmacia Phastsystem™. This system uses small pre-cast gels and solid buffer strips and the electrophoretic conditions are controlled automatically. Standardly used in this study were 10-15% gradient gels and SDS buffer strips. 1µl samples in SDS loading buffer were loaded and the electrophoretic conditions used were as follows. The sample was applied for 1Vh (250V/10mA), followed by electrophoresis for 60Vh (250V/10mA). Separation takes approximately 30 minutes and in carried out at 15°C. After this the gel was either Coomassie-blue or silver stained, again these processes were automated by the Phastsystem. The details of staining are shown in Tables 2.12.1 and 2.12.2.

Sequencing gels

8% denaturing polyacrylamide wedge gels were used for all sequence analysis and were made from the following stocks. 40% acrylamide (acrylamide:bisacrylamide, 19:1), urea and 10 x sequencing TBE buffer. The gel was prepared thus (for an 8% gel). 42g of urea, 20ml 40% acrylamide stock and 10ml 10 x TBE were made up to 100mls (once the urea had completely dissolved) and filtered through a 0.45µm membrane filter prior to the addition of 0.8ml 10% ammonium persulphate and 40ul TEMED. The gel was poured into pre-cleaned and sealed plates and allowed to polymerise for at least 2 hours. A shark's tooth comb was used for the application of samples. The gel was pre-electrophoresised for 30 - 60 minutes at a constant power 60W. Prior to loading the samples were heated at 100°C for 5 minutes and cooled on ice. After loading the gels were electrophoresised for 2-3 hours. The gel was then soaked in 10% acetic acid, 10% methanol, prior to drying onto filter paper (Whatman 3MM) and autoradiography.

2.13 DNA AND RNA HYBRIDISATION TECHNIQUES

Southern Blots

DNA was transferred from agarose gels to nitrocellulose or nylon membrane using the procedure of Southern (1975), and those modifications proposed by the manufacturers of the membranes. In the case of genomic digests the DNA was partially depurinated by soaking the gel in 0.25M HCl for 15-

20 minutes. In all cases the gel was soaked in denaturing solution for 30 minutes with gentle agitation, after which the solution was replaced with neutralising solution and agitated for a further 30-60 minutes. A glass plate spanning two reservoirs of 20 x SSC was covered with 2 or 3 sheets of filter paper (Whatman 3MM), the filter paper being submerged in the 20 x SSC at both sides. The gel was placed on the paper/glass support and a pre-cut sheet of membrane was placed over the gel excluding any bubbles. Several sheets of filter paper were placed on top of the membrane, which was then covered with a stack of paper towels and a glass plate. This sandwich was weighted down and transfer was allowed to continue for 12-16 hours. The membrane was then removed and fixed either by baking at 80°C for 1-2 hours, in the case of nitrocellulose membrane and by UV fixing on a transilluminator for 2-3 minutes in the case of nylon membranes.

Colony Hybridisations

For the screening of the YEp24 library (described in Chapter 6), standard colony hybridisation techniques were employed. Nitrocellulose membrane was cut to the appropriate size and laid gently onto the surface of an agar plate bearing E.coli colonies stabbed in an array. Using a sterile needle marks were made through the membrane into the agar to allow orientation of the filters with the plate. The membrane was removed after 1 minute and placed colony side up on chloramphenicol (250µg/ml) plates. The plates were then incubated at 37°C for 8 hours to allow amplification of the plasmid. The filters were then removed and placed, colony side up, on a pad of filter paper soaked in 10% SDS for 4 minutes. This acts as a prewash to remove debris. The filters were then placed on a pad of filter paper soaked in denaturing solution for 10 minutes or until the colony appeared smeary. This lyses the bacteria and denatures the DNA. The filter is then placed on a pad soaked in neutralising solution for 10 minutes and finally on a pad of 2 x SSC for 5 minutes. The filters were then baked at 80°C for 1 hour. Because of the amount of cell debris the filters were washed in 2 x SSC. The cell debris was gently brushed off with the gloved hand prior to prehybridisation.

Northern Blots

RNA was transferred from formaldehyde agarose gels to nylon membrane to facilitate Northern blot analysis. The technique used was the same as that for Southern blot analysis with the exception that the gel does not need to be treated with denaturing and neutralising solution because the RNA has been denatured by the formaldehyde gel.

Hybridisation Conditions

Filters with nucleic acid fixed as described in the sections above were sealed in plastic bags. To the bag was added enough prehybridisation solution to allow adequate coverage of the filter. All air was excluded and the bag was sealed. Filters were generally prehybridised at 37°C and the concentration of formamide in the prehybridisation solution was altered to vary the stringency. Standardly 20% formamide was used (unless otherwise stated) and the normal prehybridisation solution consists of the following (for 20mls)

5mls 20 x SSC
1ml 0.4M Na PO_4, pH7.0
1ml 100 x Denhardt's solution
0.8ml 0.25M EDTA, pH8.0
1ml 10% SDS
make up total volume to 20ml with distilled water

Filters were prehybridised for 2-24 hours prior to the addition of the probe. The probe was then added to the bag and hybridisation continued at 37°C for at least 3-4 hours (in the case of oligonucleotide probes, hybridisation was continued for 12-24 hours). Filters were then washed. Oligonucleotide probes were standardly washed off in 6 x SSPE, 0.1% SDS at room temperature and then 42°C or as required. Filters hybridised with random primed probes were generally washed in 2 x SSPE, 0.1% SDS at room temperature and 42°C as required. After washing the filters were again sealed in plastic bags and autoradiographed.

Radioactive probe production: end labelling oligonucleotides

Mixes containing 100-200ng of oligonucleotide DNA, 1 x kinase buffer, 50μCi [^{32}P]-dATP, and 1 unit of T4 kinase were

incubated at 37°C for 30 minutes. The probe was then purified on a Sephadex G-50 column. A portion of the purified probe was counted in a scintillation counter and the specific activity in cpm/µg DNA was calculated. On average oligonucleotide probes had a specific activity of between 10^8-10^9 cpm/µg. Probe was then added to prehybridised filters at approximately 10^6 cpm/ml of prehybridisation solution.

Radioactive probe production: random priming (Feinberg and Vogelstein, 1983)

Between 10 and 100ng of a DNA fragment was incubated at 100°C for 3 minutes. It was then cooled at 37°C for 10-15 minutes. To the DNA was added the following; 10µl random prime mix, 50µCi [^{32}P]-dCTP, 2µl (DNase free) BSA, and water to make up the volume to 54µl. 1 unit of Klenow fragment was then added and this mix was incubated overnight at room temperature or for 1 hour at 37°C. The probe was then purified on a Sephadex G-50 column and the specific activity of the probe was calculated as for oligonucleotide probes. Again the specific activity was routinely between 10^8-10^9 cpm/µg. Prior to the addition of the probe to the hybridisation solution the probe was boiled for 10 minutes.

2.14 PLASMID DNA SEQUENCING

Plasmid DNA sequencing was carried out using the Pharmacia T7 polymerase sequencing kit. The method was based upon the dideoxynucleotide sequencing method of Sanger et al. (1977) and was carried out as detailed in instructions supplied with the kit. Gel electrophoresis conditions and method of plasmid purification have been detailed previously.

CHAPTER 3

ATTEMPTED CLONING OF THE AMP DEAMINASE GENE BY RESISTANCE TO DEOXYCOFORMYCIN

3.1 INTRODUCTION

The initial aim of this project was to isolate and clone the AMP deaminase gene from Saccharomyces cerevisiae. Theoretically a number of strategies could be used to achieve this aim.

At the outset of this project there were no AMP deaminase genes cloned from any organism. Therefore, it was not possible to consider a strategy whereby sequences are selected by homologous hybridisation techniques. Had there been a readily available source of anti-AMP deaminase antibodies, or a source of the purified protein (to allow the manufacture of such antibodies) it might have been possible to isolate clones of the yeast AMP deaminase cDNA from a library in a suitable E.coli expression vector such as lambda gt11. This however was not the case. The only alternative was therefore to use a selection strategy which makes use of the expression of the active enzyme. Unfortunately, there had been no identified mutants in the gene for AMP deaminase in a suitable system to allow selection by complementation. There was also no obvious method by which a S.cerevisiae strain defective in AMP deaminase could be produced other than by random mutagenesis and assaying all resulting mutants biochemically until a strain lacking AMP deaminase activity was found. Initially it was anticipated that this was unlikely to succeed since AMP deaminase might be an essential enzyme given its prominant role in the regulation of the adenylate energy charge (see Chapter 1).

The only alternative was to find some strategy to detect and select for elevated intracellular levels of AMP deaminase in yeast. There are certain classical strategies to allow selection of a cloned gene in this way. All such strategies require a library which must be representative of all sequences in the organism's genome. The library must be made in a suitable vector depending on the requirements of the screening strategy and whether the DNA fragments borne on the vector are to be genomic fragments or cDNAs. To this end it was judged advisable to use a genomic library cloned into a multicopy episomal vector, which should allow elevated levels of the product of most genes borne on that vector when

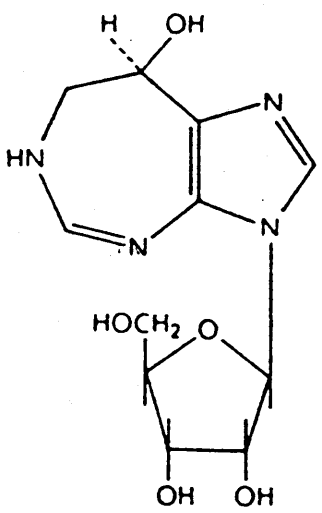
transformed into S.cerevisiae. It was also advisable to choose a vector which can also be transformed and stably maintained in E.coli. This facilitates easy amplification, purification and manipulation of the vector. Therefore, the ideal choice was a genomic library based upon an episomal S.cerevisiae-E.coli shuttle vector. The genomic DNA fragments must be of a great enough size to allow a large proportion of them to contain entire coding sequences and flanking regulatory sequences which make up a specific gene. At the outset of this project a suitable genomic library was available from David Botstein. This is a size selected (average insert size is reported as 10kb) S.cerevisiae genomic library cloned into the YEp24 shuttle vector which is maintained at multiple copies in the yeast cell (Carlson and Botstein, 1982)

Using the choice of this multicopy, episomal library, a method was required to allow selection of overproduction of AMP deaminase in clones containing the S.cerevisiae AMP deaminase structural gene. This might have been achieved by simply assaying transformants for an increased AMP deaminase specific activity, but this was adjudged laborious. It would have required the growth of transformants to a similar cell density, followed by protein extraction and finally assaying the specific activity of AMP deaminase in individual transformants. It was calculated that several thousand transformants would have to be screened to allow a high probability of finding any one gene using the genomic library described above. The formula used for the calculation of this number is shown below:

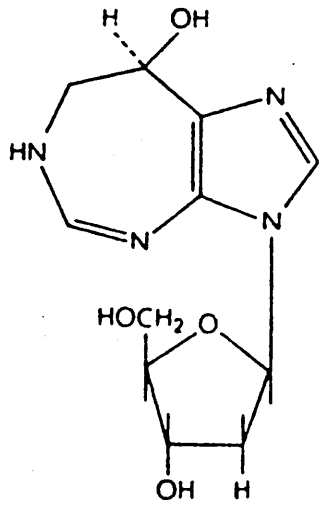
$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where N = the number of clones which must be screened,
P = the probability of finding a particular sequence
f = the fractional proportion of the genome in a single recombinant which is in this case $1 \times 10^4 / 1.4 \times 10^7$ since the average insert size of this library is 10kb and the haploid yeast genome is 1.4×10^7

FIGURE 3.1.1 THE STRUCTURES OF COFORMYCIN AND DEOXYCOFORMYCIN



COFORMYCIN



DEOXYCOFORMYCIN

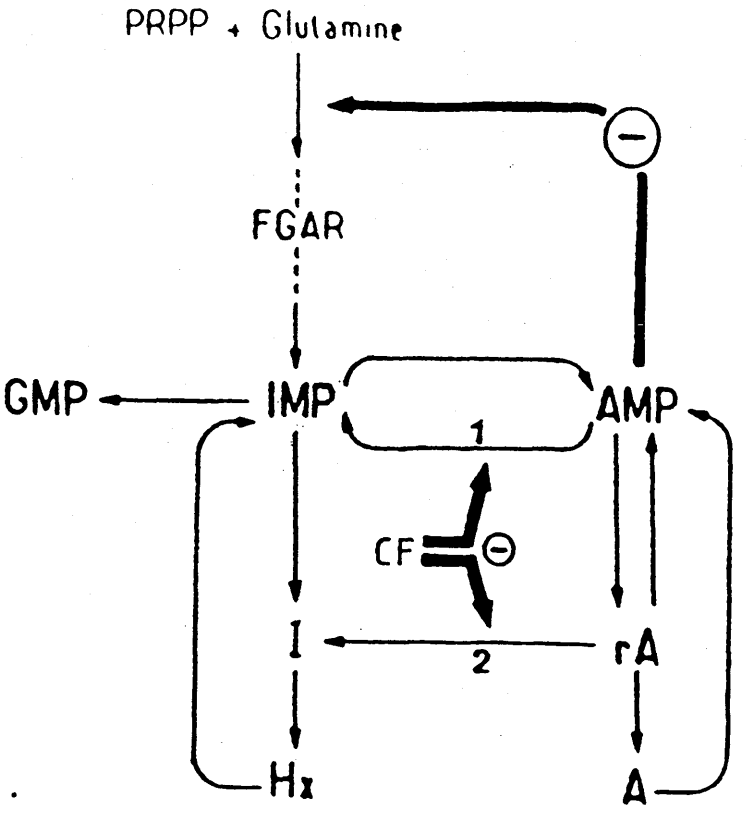
The answer to this calculation is 6450 recombinants which must be screened to give a 99% probability of finding any particular sequence and therefore this strategy was rejected.

An alternative strategy was to use the classical method of looking for increased resistance to a drug which inhibits the enzyme in question and thus select for increased enzyme synthesis. Coformycin and deoxycoformycin are two adenosine analogues currently being used effectively in the treatment of some lymphoid malignancies (Bagnara and Hershfield, 1982). The structure of these two compounds is shown in Figure 3.1.1. This toxicity to tumour cells is due to the drug's ability to inhibit adenosine deaminase, resulting in an accumulation of adenosine, 2'deoxyadenosine and other metabolites toxic to the cells. Coformycin (CF) and deoxycoformycin (dCF) are also powerful non-competitive inhibitors of AMP deaminase in all mammalian cell lines so far studied (Henderson et al., 1977; Agarwal and Parks, 1977).

CF and dCF have been studied fairly extensively in in vitro studies with purified rabbit muscle AMP deaminase. Under the conditions used (Agarwal and Parks, 1977) AMP deaminase was inhibited 45% and 66% by 1.3μM and 2.8μM of dCF respectively whereas, CF inhibited the enzyme 67% and 99.5% with 55nM and 1.3μM respectively. In other words, CF is the more powerful inhibitor and the level of inhibition was much greater when the enzyme was preincubated with the inhibitor prior to addition of the substrate to initiate the assay. This preincubation effect was less marked in the case of dCF.

Freiden et al. (1979) chemically synthesised deoxycofomycin 5'-phosphate which proved to be a much more potent inhibitor of AMP deaminase than deoxycoformycin. This is to be expected since dCF is an analogue of deoxyadenosine while deoxycoformycin 5'-phosphate is more similar to deoxy-adenylate (dAMP) which, though not the natural substrate for AMP deaminase can be used as a substrate, though at a lesser rate (Yoshino et al., 1979b). There is good evidence that deoxycoformycin 5'-phosphate binds to the active site of the enzyme of which there appears to be four per molecule (i.e. one per subunit; AMP deaminase is a tetramer) (Freiden et

FIGURE 3.1.2 PRINCIPLE OF COFORMYCIN TOXICITY



The method of "IMP starvation" arising from simultaneous shutoff of the de novo biosynthetic pathway (by adenine) and the two adenylic purine deamination pathways (Debatisse et al., 1982)

(-) inhibitor action
CF high concentration of coformycin (0.5ug/ml)
rA adenosine
I inosine
A adenine
Hx hypoxanthine
FGAR the last intermediate in the purine biosynthetic pathway
1. AMP deaminase reaction
2. adenosine deaminase reaction

al., 1979). They also showed that inhibition is time dependent which may reflect that deoxycoformycin 5'-phosphate induces conformational changes in the enzyme. The inhibitor binds extremely tightly, only being released from the enzyme-inhibitor complex after protein denaturing treatments. The inhibitor is then still active and is able to inhibit freshly added enzyme (Freiden et al., 1979).

These inhibitors have also been used in studies involving intact cells as well as in in vitro studies. Debatisse et al. (1982) found that although AMP deaminase was not strongly inhibited by 0.5µg/ml CF in cell free extracts of Chinese hamster fibroblasts, AMP deaminase activity is reduced to 15 - 20% of its normal specific activity in intact cells cultured in medium containing the same concentration of CF for 48 hours. They also showed that this concentration of CF is toxic to Chinese hamster fibroblasts when they are cultured in medium containing adenine. This toxicity is thought to be due to starvation of IMP and resulting guanylic derivatives. Evidence for this is that the concentration of adenine required for this toxic effect is the same as the concentration required to inhibit and block de novo biosynthesis of IMP (Debatisse and Buttin, 1977). The above cells can be rescued from these conditions by the addition of hypoxanthine as long as they contain hypoxanthine-guanine phosphoribosyl-transferase activity. They hypothesised that this toxicity requires both the inhibition of de novo biosynthesis of IMP and the inhibition of adenosine deaminase and AMP deaminase by CF as shown in Figure 3.1.2. (Debatisse et al., 1981).

This treatment resulted in the isolation of CF-resistant lines which fall into two categories: stable and unstable (Debatisse et al., 1982). The stable lines showed altered de novo IMP biosynthesis, that is they are no longer inhibited in the production of IMP by the presence of adenine. The second, unstable class showed a 6 to 10-fold increase in their AMP deaminase specific activity. They went on to study this second class of variants and found that their AMP deaminase was biochemically unaltered with regard to its Km, its cofactor requirements or its chromatographic properties.

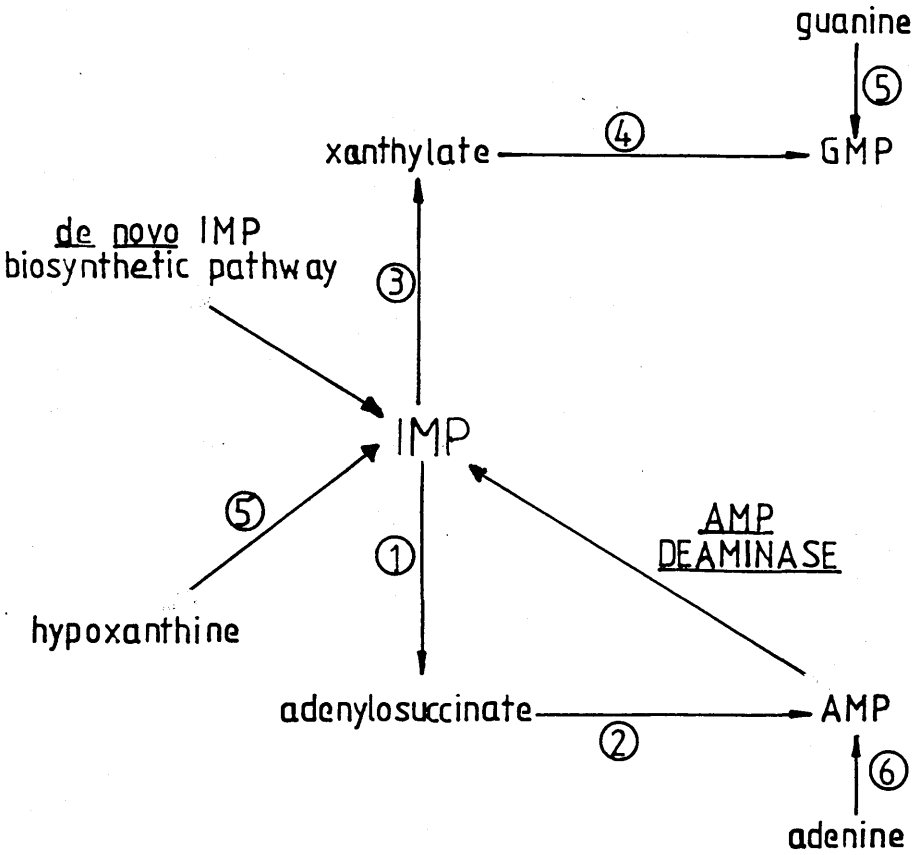
By stepwise selection using increasing concentrations of CF, Debatisse et al. (1982) isolated variants with up to 150-fold increased AMP deaminase activity. From SDS polyacrylamide electrophoresis it was clear that this increased AMP deaminase activity correlated with an accumulation of the protein. Several other, unidentified polypeptides also accumulated (Debatisse et al., 1982 and 1984) which led to the hypothesis that gene amplification was responsible for this overexpression of AMP deaminase. By isolating RNA from amplified variants they succeeded both in showing in vitro translation of four of the accumulating polypeptides, though not of AMP deaminase itself. They also isolated the cDNAs of the same four polypeptides but again, not of AMP deaminase (Debatisse et al., 1984). They suggested that this failure to clone AMP deaminase cDNA by this method may be due to a failure to isolate its mRNA, possibly because of some inherent instability in this system, or that there is something about this isolated mRNA which makes it a poor substrate for reverse transcription in their hands.

The four isolated cDNA clones were used as probes to show that there are differential degrees of amplification in each cell line, and that although the four open reading frames corresponding to these four polypeptides are linked, the end points of the amplification units vary in their positions in different amplified isolates (Debatisse et al., 1986). They have recently managed to clone the open reading frame assumed to code for AMP deaminase by chromosome walking using these cloned cDNAs as starting points (Debatise et al., 1988).

Our strategy was to select a plasmid containing the AMP deaminase structural gene by its increased resistance to dCF. In mammalian systems these drugs are only effective in the presence of adenine which inhibits de novo IMP synthesis. The toxic effects observed result from the cell being blocked in its production of IMP from both the de novo biosynthetic route and the AMP deaminase reaction, and thereby being unable to produce guanine containing nucleotides.

Our plan was to mimic this effect by producing strains whose only route of production of IMP was via the deamination of

FIGURE 3.1.3 THE BIOSYNTHESIS OF PURINE NUCLEOTIDES
(Genetic blocks are indicated below)



	REACTION	GENETIC BLOCK
1	adenylosuccinate synthetase	ade12 (Dorfman, 1969)
2	adenylosuccinate lyase	ade13 (Dorfman, 1969)
3	IMP dehydrogenase	pur5? (Burridge et al., 1978)
4	GMP synthetase	gua1 (Reichert and Winter, 1975)
5	hypoxanthine-guanine phosphoribosyl transferase	hpt1 (Woods et al., 1983)
6	adenine phosphoribosyl transferase	

facing page 60

AMP by the AMP deaminase reaction. The central position of IMP in purine metabolism is shown in the Figure 3.1.3. It was assumed that this strain would have a basal sensitivity to dCF which could be overcome by the presence of higher levels of AMP deaminase brought about by the presence of multiple copies of a plasmid bearing the AMP deaminase gene. In this manner clones containing this gene would hopefully be selected. To mimic the inhibitory effect of adenine on de novo synthesis of IMP in mammalian systems the decision was made that our constructed yeast strain would be mutated in one of the steps of de novo IMP biosynthesis, for instance ade1 or ade2. Another obvious route to IMP is from hypoxanthine via the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity encoded by the HPT1 gene (Woods et al., 1983). Fortuitously this enzyme has a second activity in the phosphorylation of guanine to GMP. A strain defective in this enzyme therefore cannot produce GMP via this route and must rely on metabolism of IMP. This would also further our ultimate aim in producing a dCF-sensitive strain.

Other factors in the design of our sensitive strain were also taken into consideration. It was thought possible that the enzymes adenylosuccinate synthetase (ade12) and adenylosuccinate lyase (ade13) may, under certain conditions, catalyse the reverse reactions. There is evidence however, that in yeast these reverse reactions do not occur (Jones and Fink, 1982) and initially it was considered that the strain constructed would not have to contain one of these mutations.

Another possible problem is that one of the intermediary bi-products of the histidine biosynthetic pathway is an intermediate in the IMP biosynthetic pathway. This intermediate is phospho-ribosylamino-imadazole-carboxamide (AICAR). Hence the introduction of a mutation making the strain defective in the early stages of histidine biosynthesis may have proved necessary.

It is necessary finally that the strain has a suitable auxotrophic mutation to allow selection of cells receiving plasmids bearing a suitable auxotrophic marker gene. Yeast vectors routinely carry marker genes such as LEU2, HIS3 and

URA3. The marker in the chosen YEp24 library is URA3. The constructed strain should therefore carry a mutation in the URA3 gene.

Thus a suitable and new yeast strain was to be constructed that theoretically relies largely on AMP deaminase for the production of IMP and thereby guanylate nucleotides. It was hoped that this strain would have an increased sensitivity to the AMP deaminase inhibitor dCF, and therefore that clones resulting from transforming such a strain with the chosen genomic library would show relatively high resistance to this drug because they had increased levels of AMP deaminase due to the presence of multiple copies of the AMP deaminase gene.

3.2. PRODUCING A DEOXYCOFORMYCIN SENSITIVE STRAIN

A list of the strains used and produced in this project can be found in chapter 2.

3.2.1. Confirming the hpt1$^-$ phenotype of W1-2D

A strain W1-2D carrying a mutation in the hypoxanthine-guanine phosphoribosyl transferase gene, hpt1 was kindly donated by Robin Woods (Woods et al., 1983). This strain was to be used in the production of our dCF sensitive strain. According to Woods et al. (1983), strain W1-2D was isolated by selecting for resistance to the base analogue 8-azaguanine, at a concentration of 200µg/ml. 8-azaguanuine is toxic by virtue of it being incorporated into nucleic acids via the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) reaction. Hence W1-2D should be resistant to azaG because it lacks HGPRT activity.

Attempts to confirm the hpt1$^-$ genotype by its resistance to this analogue over other strains have been wholly unsuccessful. W1-2D shows resistance up to 400µg/ml azaguanine, but DBY868 which was presumed to be HPT1$^+$ shows a similar if not greater level of resistance to the analogue. The basis of DBY868's resistance has not been characterised, and use of the analogue as a selective agent in the yeast

matings was abandoned.

A hpt1$^-$ mutant by definition is unable to utilise hypoxanthine to produce IMP. Therefore an ade2$^-$, hpt1$^-$ strain according to Woods et al. cannot grow on hypoxanthine (hpx) in the absense of adenine (ade) (Woods et al., 1983). The ability of an ade2$^-$ strain, DBY868, to grow on hypoxanthine containing medium in the absense of adenine was confirmed, but since the inability of an ade2$^-$, hpt1$^-$ mutant to grow on this same medium could not be confirmed prior to mating it was assumed to be the case, and subsequently used successfully in the selection of mating progeny, (see section 3.2.3).

3.2.2 Production of a rho$^-$ W1-2D variant strain

The strain W1-2D carried no chromosomal markers apart from hpt1$^-$. It was therefore necessary to have some way to select for diploids made by crossing W1-2D with DBY868 (the strain used to donate the ade2$^-$ marker) since the strain W1-2D can grow on minimal medium (GYNB).

It was decided that the simplest way to make a variant of W1-2D was to make it rho$^-$, that is, defective in its mitochondrial-based respiration. Rho$^-$ mutants cannot therefore grow on non-fermentable carbon sources such as glycerol. Rho$^-$ mutants in yeast are also known as petites, since they produce small colonies in comparison to wildtype strains on the appropriate media (Monnolou et al., 1966; Nagley and Linnane, 1970). To check that both DBY868 and W1-2D are capable of growth on glycerol, they were both plated on glycerolYNB and GYNB with the necessary supplements and incubated at 30^{o}C. Most yeast strains will grow well on glucose and will produce reasonably sized colonies after 2-3 days. However, both DBY868 and W1-2D grew much more slowly on glycerol containing medium and colonies attained a reasonable size only after approximately seven days.

Rho$^-$ mutants were isolated by mutagenesis using ethidium bromide, and was carried out in the following manner. (Brian Cox, personal communication. A single W1-2D colony was

picked into 1ml of sterile water in an eppendorf tube. This was serially diluted on to YPG plates, in duplicate, to give a density of approximately 100 colonies per plate. A single ethidium bromide crystal is placed in the centre of one of the duplicate plates and then the plates are incubated, non-inverted for 3 days. At the end of this time, a clear zone of inhibition of growth was seen on the plate due to diffusion of the ethidium bromide from the crystal, and immediately outside this was a zone where only small colonies grew. Finally, colony size increased towards the outside of the plate. Several of the small colonies were stabbed in duplicate onto glycerolYNB and GYNB and allowed to grow at 30°C for ten days. At the end of this time everything had grown on GYNB, and only some on glycerolYNB. Eight of those strains incapable of growth on glycerol were picked and streaked on to YPG. Of these, six gave clearly small colonies and the two remaining gave rise to a mixture of small and larger colonies. These latter two were discarded and the remaining six (named W1-2D rho$^-$ A,B,C,D,F and G) were retained for future use.

3.2.3 The production of an ade2$^-$, hpt1$^-$ mutant strain

DBY868 and W1-2D rho$^-$C were mated and sporulated using standard methods (see Chapter 2). In addition, control crosses of DBY868 with X1056-1C, and of W1-2D rho$^-$C with DBY747 were carried out to confirm the mating types of DBY868 and W1-2D rho$^-$C. Diploids were selected by streaking on to glycerol YNB, which only supports the growth of these diploids. The diploids were then presporulated and sporulated in the standard manner. The resulting asci were dissected and the resulting tetrads were grown and their phenotypes analysed. The phenotypes of ten such tetrads and the analysis of this cross are shown in appendix 1.

Differential growth on minimal medium +hpx, -ade (ie containing hypoxanthine but excluding adenine) was observed for ade2$^-$, hpt1$^+$ and ade2$^-$, hpt1$^-$ strains. Ade2$^-$, hpt1$^-$ strains were not completely inhibited in their growth on this medium but their rate of growth was considerably less than ade2$^-$, hpt1$^+$ strains. It was this much slower growth rate on

minimal medium +hpx -ade which allowed assignment of the ade2, hpt1 genotype.

From the ten tetrads analysed, only 3 spores had the required genotype of ade2$^-$, hpt1$^-$. These are spores 1D, 2C and 5C. The mating types of these 3 haploids were analysed thus.

1D, 2C AND 5C were each mixed with X2928-3D-1A and X2928-3D-1C in the standard method for yeast matings. Diploids of these reciprocal matings were selected on GYNB. In the case of 1D and 5C, diploids were only yielded in crosses with X2928-3D-1A, and hence 1D and 5C are therefore mating type alpha. In contrast, 2C yielded diploids with X2928-3D-1C, and is therefore mating type a.

Therefore the 3 constructed strains have the following genotypes.

JMM1-1D alpha, ade2$^-$, hpt1$^-$, his4$^-$
JMM1-2C a, ade2$^-$, hpt1$^-$
JMM1-5C alpha, ade2$^-$, hpt1$^-$

3.2.4. Making the ade2$^-$, hpt1$^-$ strain into a suitable transformation strain

Before using these strains as hosts for a transformation with a genomic library, a suitable auxotrophic mutation must be introduced to allow for selection and stable maintenance of the vector. In this instance, the decision was made to use ura3 as the selectable marker since this was the marker gene carried by the YEp24 library discussed in the introduction. Therefore an ade2$^-$, hpt1$^-$ strain was also made ura3$^-$. This was done by classical yeast genetic mating. The decision was made to use the strain X4003-5B as a source of the mutated ura3 allele. This strain was also chosen because of its phenotype of giving high transformation efficiencies. It was hoped that some of the resulting haploids of this mating would also exhibit this phenotype.

Therefore the following two matings were set up in the standard manner

Cross 2: JMM1-1D x X4003-5B
Cross 3: JMM1-5C x X4003-5B

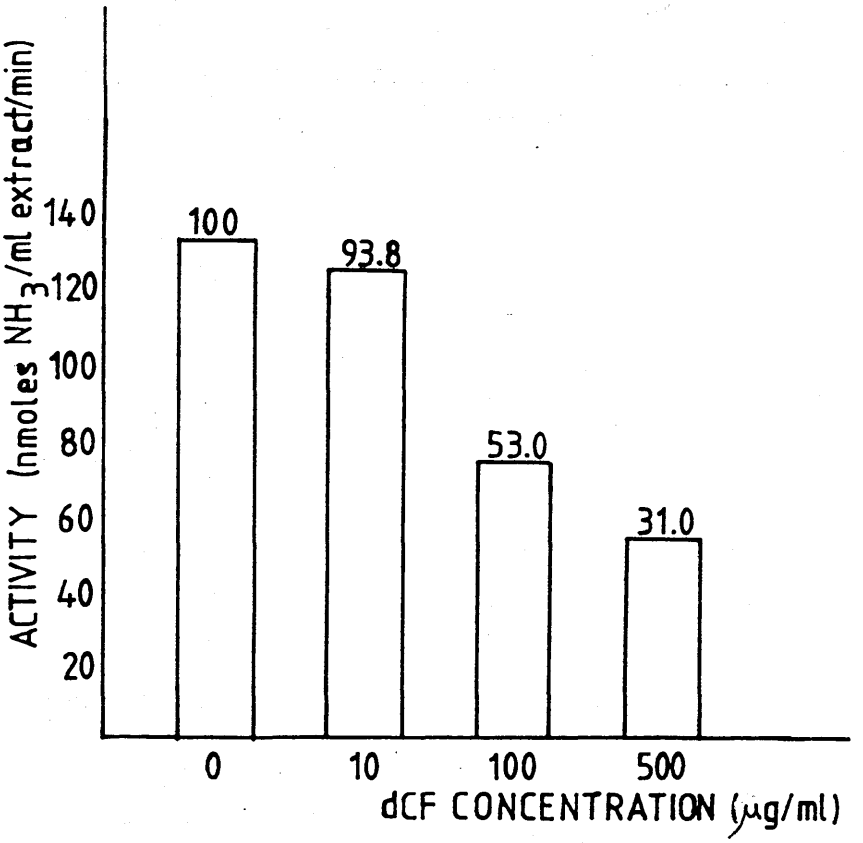
The diploids of these crosses were selected by streaking onto GYNB with histidine and GYNB, respectively. This allows selection of diploids only. The diploids thus produced were induced to sporulate in the standard way. The resulting asci were dissected and the phenotypes of the spores and partial genetic analysis of these crosses are presented in appendix 2. As expected, all progeny of cross 2 are his$^-$ (with the exception of spore 2-3B). The two alleles of his4 in the two parent strains, X4003-5B and DBY868 are not the same. Therefore, this his4$^+$ spore may have resulted from a rare recombination event between the two closely linked mutations or it may have been mis-assigned. Analysis with respect to the hpt1 mutation was only analysed in strains which proved to be ade$^-$, ura3$^-$, as listed below

Cross 2 1A; 2C; 4C; 6A; 8B; 9D; 11C; 14B; 15C; 16C; 17C; 18C

Cross 3 2A; 6D; 8C; 9D

It was necessary to characterise these strains for their full ade genotype. By the nature of the crosses carried out they could fall into three categories, either ade1$^-$, ade2$^-$ or the double mutant ade1$^-$/ade2$^-$. This characterisation was carried out by mating each strain in the standard way in the crosses described below to produce diploids. The resulting diploids were then streaked out on media which would either support growth of the diploid in the absence or presence of adenine. Those crosses which produced diploids which were able to grow on medium in the absence of adenine did so because complementation of two separate mutations had occurred allowing further classification of the haploid strains. X2928-3D-1A and X2928-3D-1C are isogenic strains, differing only in mating type, carrying the ade1$^-$ mutation and therefore able to complement ade2$^-$ mutants in the diploid but

FIGURE 3.3.1 INHIBITION OF AMP DEAMINASE BY DEOXYCOFORMYCIN IN CELL FREE EXTRACTS



The percentage of maximal activity is given in figures on top of each bar in the histogram.

These results were obtained using identical cell free extract from yeast strain X4003-5B in standard 100ul reactions (see Chapter 2), incubated at 37^{o}C for 10 minutes.

All determinations of AMP deaminase activity were carried out in triplicate and the results represent the average of these data.

not the other two categories. The haploids were therefore initially crossed with strains X2928-3D-1A and X2928-3D-1C. These crosses could distinguish between ade2$^-$ mutants and ade1$^-$ or ade1$^-$/ade2$^-$ mutants Those mutants which were not complemented in the X2928-3D-1A/1C crosses were then crossed with either JMM1-1D or -2C which as previously stated are isogenic except at the mating locus and carry the ade2$^-$ mutation. These crosses could distinguish between ade1$^-$ mutants and ade2$^-$ or ade1$^-$/ade2$^-$ mutants. Those strains which did not complement for ade requirement in either of the test crosses were therefore ade1$^-$/ade2$^-$ double mutants. These test crosses also allowed classification of mating type for each strain of interest. A total of six strains were retained for future use and the full genotypes of these strains are shown below.

Cross 2: JMM2-4C - a, ade2$^-$, hpt1$^-$, his4$^-$, ura3$^-$, met2$^-$, leu2$^-$
JMM2-9D - a, ade2$^-$, hpt1$^-$, his4$^-$, ura3$^-$, .lm23 met2$^-$, leu2$^-$, trp5$^-$

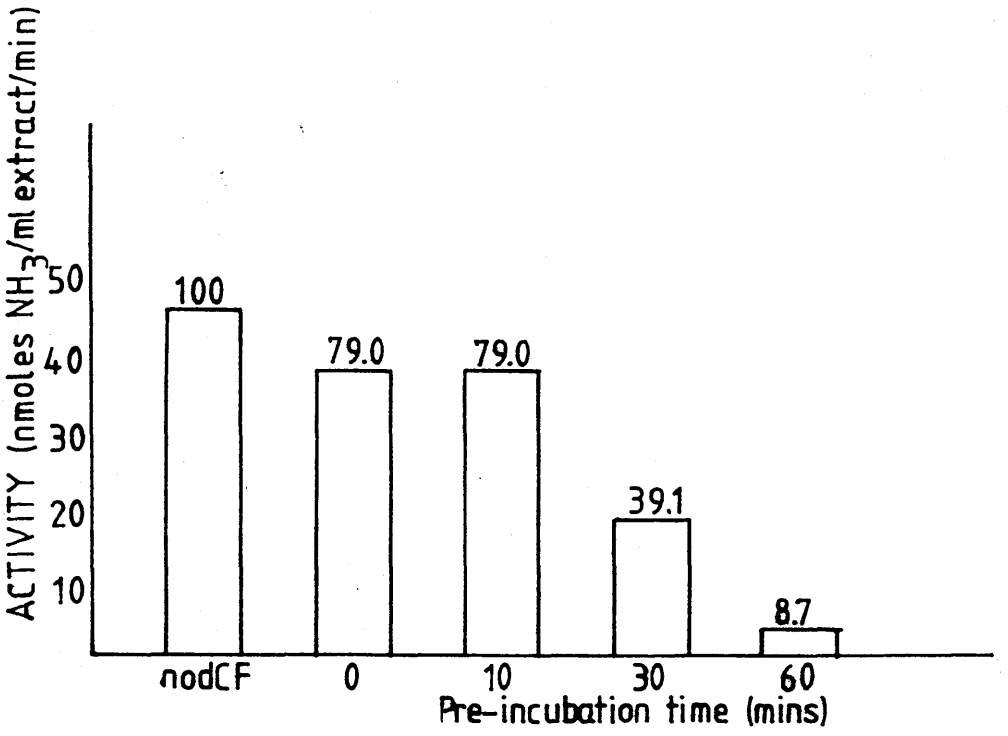
Cross 3: JMM3-2A - a, ade1$^-$,ade2$^-$, hpt1$^-$, ura3$^-$, leu2$^-$, trp5$^-$
JMM3-7B - alpha, ade1$^-$, hpt1$^-$, ura3$^-$, met2$^-$, trp5$^-$
JMM3-8D - alpha, ade1$^-$, ade2$^-$, hpt1$^-$, his4$^-$, ura3$^-$, met2$^-$, leu2$^-$
JMM3-9D - alpha, ade2$^-$, hpt1$^-$, ura3$^-$

3.3. IN VITRO EXPERIMENTS WITH DEOXYCOFORMYCIN

Because of the value of CF and dCF as therapeutic chemicals, and because they are only produced in a few laboratories throughout the world they are not widely available. We were fortunate, however to be given 100mg of dCF. This was the generous gift of Martin L. Black, Warner Lambert.

The first priority was to show that in our hands yeast AMP deaminase is inhibited by dCF. To this end, AMP deaminase reactions were set up using yeast cell free extracts containing a variety of concentrations of deoxycoformycin. The results of this experiment are shown in figure 3.3.1.

FIGURE 3.3.2 THE EFFECT OF PREINCUBATION OF CELL FREE EXTRACT WITH DEOXYCOFORMYCIN PRIOR TO DETERMINING AMP DEAMINASE ACTIVITY



The percentage of maximal activity is given in figures on top of each bar in the histogram.

Reaction mixes were set up with identical X4003-5B cell free extract and were incubated at 37°C in the presence (or absence, where indicated) of 100ug/ml deoxycoformycin prior to the addition of 10mM AMP to start a standard AMP deaminase assay (see Chapter 2). The reactions mixes were in a total volume of 100ul containing 10ul extract and the AMP deaminase assay was carried out for a total of ten minutes.

All determinations of AMP deaminase activity were carried out in triplicate and the results represent the average of these data.

Note: All reaction mixes were incubated at 37°C for a total of 60 minutes prior to the addition of substrate
All reactions were carried out in triplicate

Note: All the AMP deaminase determinations described in this chapter were carried out using the glutamate dehydrogenase ammonia assay method (Bergmeyer and Buetler, 1982) (as detailed in Chapter 2). All the activities are the average of three identical reactions and a single, parallel substrate blank reaction. Errors (not shown) are in the range of not greater than +/- 4%.

From these results it is clear that yeast AMP deaminase is inhibited by concentrations of 100µg/ml dCF although the inhibition is not complete even at 500µg/ml under these conditions. Since deoxycoformycin is an adenosine analogue, it has been postulated that it is a more effective inhibitor when it is phosphorylated to form deoxycoformycin 5'-phosphate (Freiden et al., 1979). There is also strong evidence that at least in the case of CF, pre-incubation of AMP deaminase with the inhibitor prior to the assay increases the inhibitory power of the drug. To test if this was also the case for dCF with respect to yeast AMP deaminase, reaction mixes were incubated in the presence or absence of 100µg/ml dCF, at 37°C for a variety of times, prior to the start of the reaction, by the addition of AMP. The results of this experiment are presented in figure 3.3.2.

It is clear that the level of inhibition increases markedly with pre-incubation time of the enzyme with the inhibitor, showing almost complete inhibition after 60 minutes. It is also clear from these results that the AMP deaminase is differentially inhibited from one cell-free extract to another, ie the extract used in the experiment described in figure 3.3.1 is inhibited 47% by the addition of 100ug/ml dCF with no pre-incubation whereas the extract used in the production of the results in figure 3.3.2 is inhibited only 21% under the same conditions. The differential sensitivity of these two extracts may be explained in a number of ways. For example, minor differences in the extraction process may yield differential recovery of the enzyme(s) responsible for the phosphorylation of dCF which is thought to be required for maximum inhibition of the enzyme.

Therefore, although there may be some experimental variability in the level of inhibition by dCF, there is

TABLE 3.4.1 THE EFFECT OF DEOXYCOFORMYCIN ON RECOVERING PROTOPLASTS

Recovery Time (mins)	THE NUMBER OF COLONY FORMING UNITS/ML x 10^6 Concentrations of dCF (μg/ml)				
	0	1	10	100	1000
0	328	238	232	286	279
30	330	314	291	387	478
60	291	393	226	259	279
120	205	193	87	162	149

The number of colony forming units produced following the adapted transformation technique (detailed in section 3.4) in the presence (and absence) of various concentrations of deoxycoformycin in the recovery media.

Protoplasts were made in the in the manner of normal transformation with DNA (Beggs, 1978) with the exception that after PEG treatment, identical aliquots of cells were allowed to recover in YPG media containing various concentrations of deoxycoformycin (as indicated). The protoplasts were then plated onto standard transformation media with the appropriate supplements and incubated at 30°C. The number of colony forming units produced in each case was then scored.

obviously an observable level of inhibition of the yeast AMP deaminase by dCF.

3.4 IN VIVO STUDIES WITH DEOXYCOFORMYCIN

The next priority was to show that dCF could inhibit AMP deaminase *in vivo* in yeast cells and would thereby be toxic to the cells under prescribed conditions. This was a prerequisite for our cloning strategy.

The intent was to find a concentration of dCF which is toxic to normal, untransformed cells of our specially constructed strain, whose sensitivity would theoretically be reduced by the presence of multiple copies of the complete AMP deaminase gene. Initial investigations of the toxicity of dCF on laboratory strains of *S.cerevisiae* were carried out prior to the production of our constructed strains. The following strains were patched ond later plated in dilution series on GYNB with necessary additions and 0, 50 and 100ug/ml dCF. No appreciable difference was seen either in the rate of growth or the number of surviving colonies produced in the presence or absence of dCF. These experiments were later carried out with the *ade2*$^-$, *hpt1*$^-$ strains, JMM1-1D, JMM1-2C AND JMM1-5C described in section 3.2.3. These strains too, showed no detectable sensitivity.

It has been shown that yeast AMP deaminase is sensitive to inhibition by dCF *in vitro*. It was hypothesised that if the dCF was entering the cells one would expect to see some effect, at least on the rate of growth of the *ade2*$^-$, *hpt1*$^-$ strains in comparison to other laboratory strains. Therefore, it was argued that either the concentrations of dCF we were using were too low to have a toxic effect, or that the cells were either metabolising the drug or failing to take it up.

The experiments were also carried out on glycerol containing medium since it has been suggested that glycerol may increase the uptake of certain molecules by yeast cells (Alistair Brown, personal communication). This too, had no appreciable effect on the growth of cells in the presence of dCF.

The decision was made to see whether cells undergoing normal yeast transformation treatments would show sensitivity to dCF. One technique frequently used for the transformation of S.cerevisiae involves protoplasting cells in an osmotically stabilized buffer, ie partially removing their cell walls by enzymatic treatment. The cells are then treated with polyethylene glycol (PEG) to bring about cell fusion which allows uptake of DNA. It was hoped that during this treatment cells would also be able to take up dCF. To this end a number of experiments were carried out.

Protoplasts were produced from strain JMM1-2C in the standard manner (see chapter 2). After PEG treatment the protoplasts were allowed to recover normally in 100µl YPG at 30 C for 30 minutes. The protoplasts were then plated in overlays containing 0, 1, 10 and 100µg/ml dCF. No difference could be seen in the resulting number of colonies (data not shown).

In the next experiment, identical aliquots of protoplasts were recovered in YPG containing 0, 1, 10, 100 and 1000µg/ml dCF following PEG treatment. Aliquots from each tube were then plated at various times and at various dilutions in agar overlays without dCF. The results are shown in table 3.4.1. There appears to be no significant difference between those cells recovered in the presence or absence of dCF. It does show however that a 30 minute recovery period is the most efficient for the regeneration of protoplasts under these conditions, and that longer recovery results in a loss of viability. In subsequent experiments these results were borne out, and viability is effectively wiped out after 8 hours (results not shown).

Although dCF was shown to be a potent inhibitor of yeast AMP deaminase in vitro, all attempts to make dCF toxic to yeast cells failed. Since the use of larger concentrations of dCF could not be contemplated due to the unavailability of this drug, our strategy to select for the AMP deaminase gene using this drug was abandoned.

3.5 DISCUSSION

As previously stated the initial aim in this study was to design a suitable screening system to clone the AMP deaminase gene.

The initial strategy was to demonstrate sensitivity of yeast AMP deaminase to an AMP deaminase inhibitor, dCF which would then be overcome by the presence of increased levels of AMP deaminase. This method relied on the overproduction of AMP deaminase due to the presence of multiple copies of the gene.

The strategy involved the production of a specially constructed strain where in theory only the AMP deaminase reaction could supply the cell with the necessary IMP and guanylic derivatives thereof. Suitable strains were produced but at no stage could any sensitivity to the AMP deaminase inhibitor be shown in these intact cells. Attempts were made to promote uptake of the drug dCF, but these were unsuccessful. Attempts using spheroplasting treatments were unlikely to succeed since the cell membrane is the more likely barrier to dCF. Other methods of finding sensitivity were considered. These methods included using certain permeabilising treatments such as toluene or toluene and heat treatments (Weitsman and Hewson, 1973; Murakami et al., 1980) but no way of incorporating such intrinsically damaging treatments into a screening system could be conceived.

Another alternative which was considered was to screen for mutants which are sensitive to dCF in liquid culture, by selecting for non growing cells by one of the so called suicide methods (Snow, 1966; Littlewood, 1972; Littlewood and Davies, 1973; Ferenczy et al., 1975; Henry and Horowitz, 1975; Henry et al., 1975; Young et al., 1976). This strategy however required that the uptake of dCF and the inhibition of AMP deaminase within the cell merely prevented the cell from actively growing, but was not lethal; there is no evidence to this effect. Such strategies were however discarded because of the scarcity of dCF. In fact at all stages of the study involving dCF the availability of the drug proved to be a critical factor, because sufficient quantities of dCF or CF, for detailed studies could not be obtained. Therefore the

plan to use dCF as a means of selecting for the AMP deaminase gene was abandoned.

CHAPTER 4

MODIFICATION OF HISTOENZYMATIC STAINS AS A SELECTION SYSTEM FOR THE ISOLATION OF THE YEAST AMP DEAMINASE GENE

4.1 INTRODUCTION

Since the approach described in chapter 3 to isolate the yeast AMP deaminase gene had failed, it was hoped that some quick and convenient biochemical method of screening a large number of transformants could be found. Using our chosen genomic library which has an average insert size of 10kb (Carlson and Botstein, 1982), the number of transformants that would have to be screened to give a 99% probability of selecting a single copy gene has been calculated to be approximately six and a half thousand (see section 3.1).

Therefore screening the library by carrying out assays on extract produced from this number of transformants, to detect individual transformants with increased AMP deaminase activity, was deemed too time-consuming and it was hoped that a more efficient method might be found. For example, small liquid cultures could be grown in microtitre wells, the cells lysed by the addition of wall degrading enzymes and detergents, and then a sensitive assay for AMP deaminase employed to detect over-production of the enzyme.

Two histochemical for stains for AMP deaminase in muscle sections have been reported. Both rely for their specificity on aspects of the AMP deaminase reaction.

The first stain makes use of the fact that spontaneous reduction of nitro blue tetrazolium by a thiol to produce a coloured formazan will occur when the pH in a weakly buffered solution is raised from 6.1 to 6.4 (Fishbein, 1977b). This increase in pH occurs by the production from NH_3 from the AMP deaminase reaction (see figure 4.1.1).

The second method makes use of an AMP analogue, 6-chloropurine ribonucleotide, in which the NH_3 group at position 6 of AMP is replaced by a Chlorine atom. This analogue can be used as a substrate by AMP deaminase at approximately 9% the efficiency of AMP (Nakatsu, 1975). The Cl^- ions produced are trapped by Ag^+ ions to produce AgCl which precipitates from the solution and which is visible in the form of black deposits by visible light.

FIGURE 4.1.1 PRINCIPLE OF THE NITROBLUE TETRAZOLIUM HISTOENYMATIC STAIN FOR AMP DEAMINASE

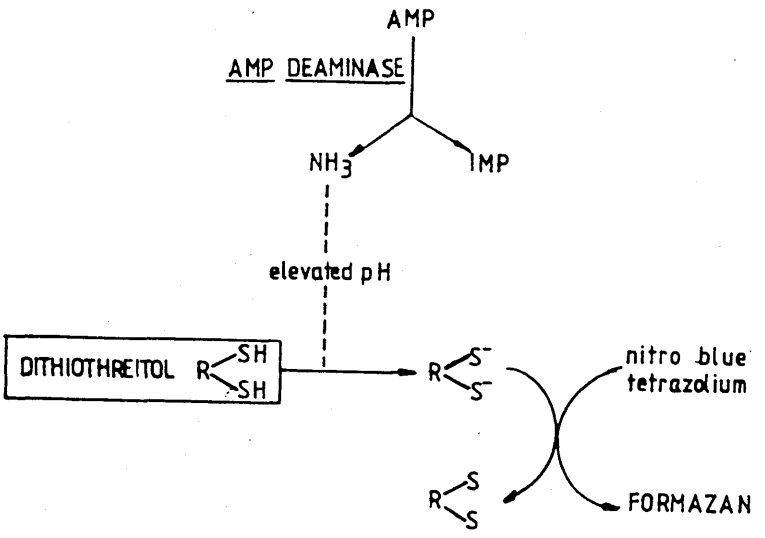
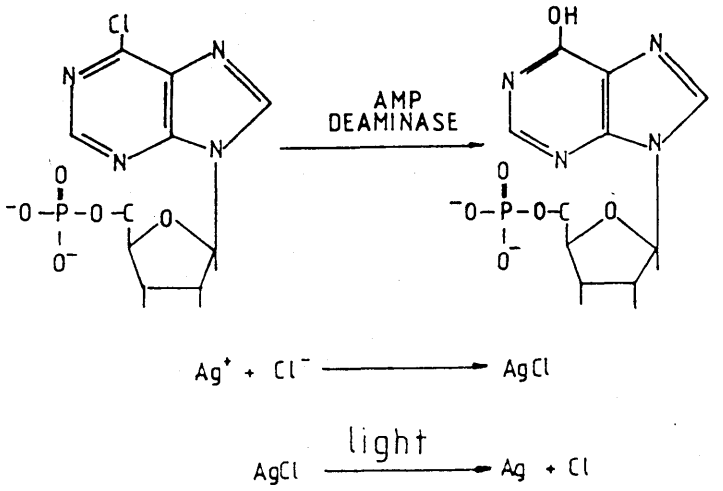


FIGURE 4.1.2 PRINCIPLE OF THE 6-CHLOROPURINE RIBONUCLEOTIDE HISTOENZYMATIC STAIN FOR AMP DEAMINASE



The structure of the analogue and the principal of the stain is shown in figure 4.1.2.

It was hoped that one of these stains could be modified to screen a yeast genomic library for transformants with excess AMP deaminase, produced by multiple copies of the AMP deaminase gene.

4.2 RESULTS

Attempts were made to modify both stains for use as a microtitre assay. Although both methods gave reasonable results when used with varying amounts of NH_4^--containing standard solutions, the assaying of either cell free extract or lysed cells as described above proved more difficult.

The tetrazolium stain requires a pH change from 6.1 to 6.4, however the pH optimum of yeast AMP deaminase has been shown to be pH 7.1 (Yoshino et al., 1979b). It became even more difficult when attempting to lyse cells using either glucoronidase or novozyme in conjunction with detergents, since both the enzyme preparations contained extremely high levels of NH_3 which interfered greatly with the stain. Since the specific activity of AMP deaminase is extremely low in unpurified cell-free extracts the significant signals could not be obtained above background levels even in concentrated extracts (see chapter 2).

The 6-chloropurine ribonucleotide stain also had its problems, but for quite different reasons. For this method to be successful, it was vital that there should be insignificant levels of chloride ions in buffers and solutions, since they interfere with the staining reaction. The removal of chloride ions proved extremely difficult. In addition, many other anions such as acetate, will associate with Ag^+ ions and generate precipitates of silver metal with light, and this also caused problems. Even when such problems were overcome by pHing buffers with nitric acid, it was still not possible to detect AMP deaminase activity from cells lysed in microtitre-scale assays. All cell wall degrading enzyme preparations commercially available are

fairly crude and resulted in immediate precipitation of silver salts on mixing with the stain. This in conjunction with the low specific activity of the cellular AMP deaminase made assaying in this manner impossible.

A final colorimetric assay was tested for its usefulness in this type of screening system. This was the ammonia assay using a phenol and hypochlorite mixture, communicated by John Hinton, of I.C.I. (Bergmeyer and Beutler, 1982). This assay again gave reasonable results both with gradients of NH_4Cl and also with cell-free extracts, but no activity could be detected in cells lysed in microtitre wells. This was again attributed to the high NH_3 concentrations in cell wall degrading enzyme preparations and to the low AMP deaminase specific activity.

Having attempted to modify these published AMP deaminase assays, further attempts at finding a microtite-scale visible assay were abandoned.

The phenol/hypochlorite method of NH_3 determination however proved much more rapid and convenient than the glutamate dehydrogenase method previously used and hence it was subsequently adopted as the assay of choice (see Chapter 2)

4.3 DISCUSSION

As previously stated the initial aim in this study was to clone the yeast AMP deaminase gene. In this chapter possible screening strategies based upon biochemical assays for AMP deaminase are described.

Attempts to modify the two histoenzymatic stains described, to microtitre scale screens were unsuccessful.

All attempts to find a suitable screening system making use of the putative overproduction of the AMP deaminase protein in cells carrying multiple copies of a vector with a cloned genomic fragment carrying the AMP deaminase gene proved unsuccessful (see also chapter 3).

As previously mentioned no cloned genes or purified protein were available to allow gene selection by heterologous probing or antibody production and screening. Unfortunately at this time, there were no known null mutants for AMP deaminase in any suitable organism to allow cloning by complementation. Attempting to mutagenise and screen for AMP deaminase mutants would have proved extremely laborious. Therefore, the decision was made to purify AMP deaminase from S.cerevisiae and to obtain N-terminal amino acid sequence data to allow oligonucleotide production for use as hybridisation probes (see chapter 5).

CHAPTER 5

PURIFICATION AND N-TERMINAL SEQUENCING OF AMP DEAMINASE

TABLE 5.1.1 SUMMARY OF AMP DEAMINASE PURIFICATION FROM S.cerevisiae (Yoshino et al, 1979b)

	Specific activity (μmoles NH_3/min/mg protein)	Total Protein (mgs)	Purification factor	%Recovery
Crude extract	0.08	26 700	1	100
Column I	2.0	600	25	56.1
Column II	16.1	48	201	36.2
Column III	343.0	0.3	4290	4.8

The steps in the purification method of AMP deaminase adopted by Yoshino et al., 1979b are discussed in section 5.1.

5.1 INTRODUCTION

AMP deaminase from S.cerevisiae has been previously purified by Yoshino et al. (1979b). Their purification procedure uses phosphocellulose adsorption chromatography and affinity elution of AMP deaminase with ATP. The method they used is outlined below.

Phosphocellulose column I

The crude extract, made in 50mM potassium phosphate,pH7.5 was loaded on to a phosphocellulose column equilibrated with the same buffer. The enzyme-containing fraction was then eluted with 0.2M potassium phosphate buffer.

Phosphocellulose column II

The enzyme-containing fraction from the first column was diluted two-fold and loaded on to a second phosphocellulose column equilibrated with 0.1M potassium phosphate, pH7.5. The active fraction is then eluted with 5mM ATP.

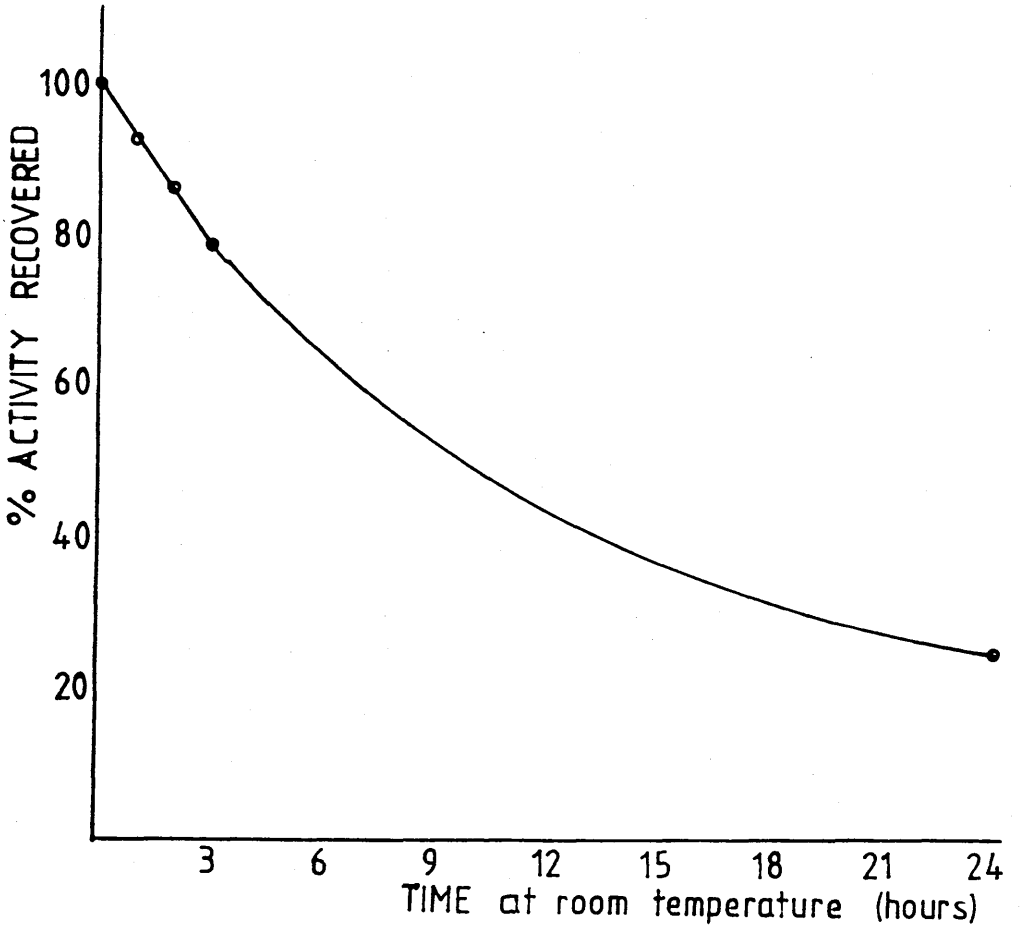
Phosphocellulose column III

The active fraction from column II is diluted two-fold and loaded on to a third phosphocellulose column equilibrated with 50mM potassium phosphate,pH7.5. The column was then washed with 0.1M potassium phosphate, 0.15M NaCl. The column was then subjected to a linear ATP gradient from 0 - 0.6mM and the active fraction was then collected. This fraction was electrophoretically homogeneous. The degree of purification at each stage is shown in table 5.1.1.

As can be seen from this table this purification resulted in a purification of over 4000-fold. This was an extremely large scale purification with starting material of over 27g of protein. However the entire purification only yielded 300 ug of protein, and only 4.8% of the original activity.

It was hoped that a rapid, efficient and smaller scale purification of AMP deaminase from S.cerevisiae could be developed which would yield material of sufficient purity to allow the N-terminal amino acid sequence of AMP deaminase to be determined. With this information an oligonucleotide would be designed to allow cloning of the AMP deaminase gene.

FIGURE 5.2.1.1 THE STABILITY AT ROOM TEMPERATURE OF AMP DEAMINASE IN CRUDE YEAST EXTRACT



Aliquots of a single preparation of crude extract were incubated at room temperature for various times as indicated prior to assaying for AMP deaminase activity in the standard manner. All reactions were carried out in triplicate.

5.2 STUDIES TO FACILITATE AMP DEAMINASE PURIFICATION

5.2.1 The Stability of AMP deaminase

Most stages of an enzyme purification should be carried out at low temperatures, assuming the enzyme of interest is not cold-labile. This reduces the activity of contaminating proteases, and is less likely to lead to chemical denaturation. However, it is not always practicable for all stages to be carried out in a cold room, so prior to the purification it is desirable to know something of the enzyme's stability at room temperature.

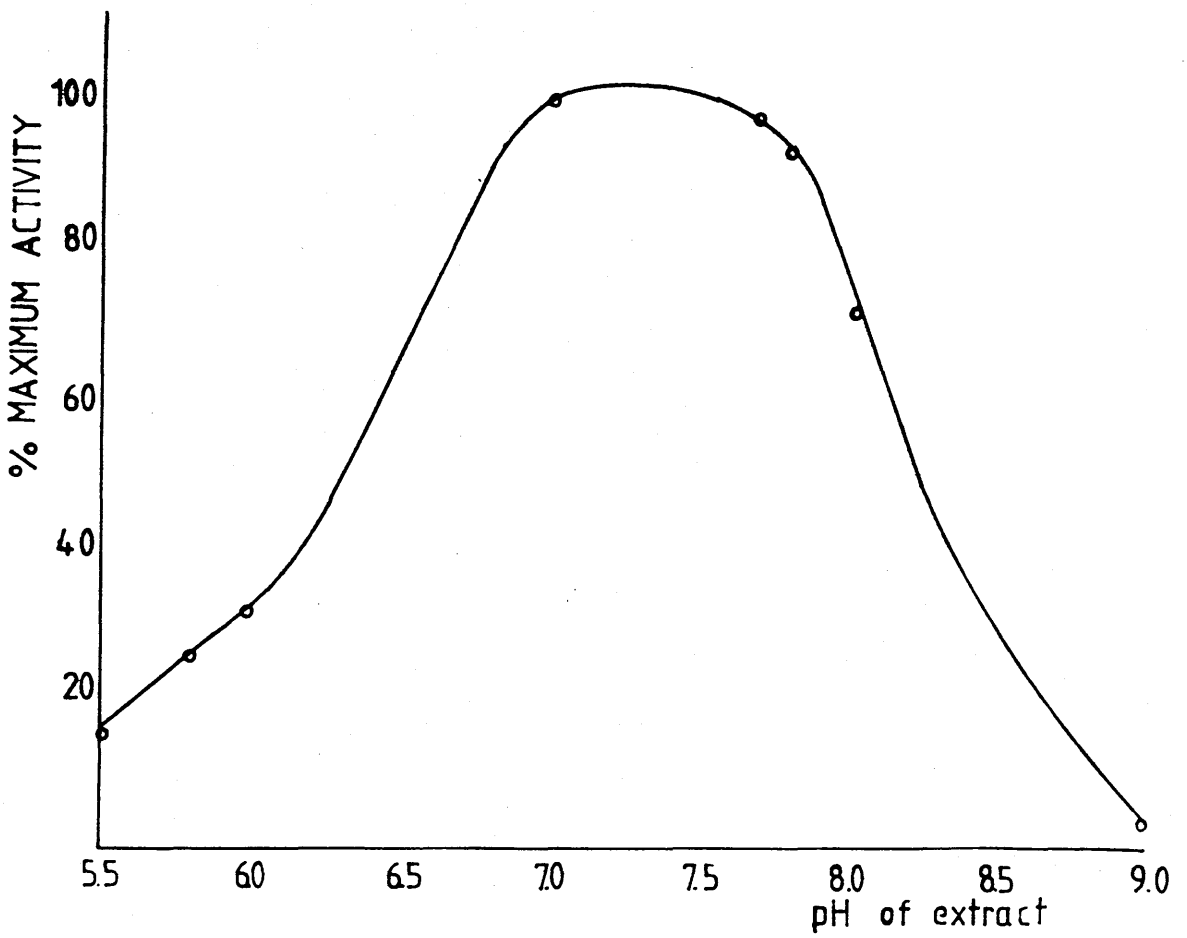
When yeast cell-free extract was made it was stored at -20^{o}C and rapidly thawed prior to use when required. An experiment was carried out whereby extract was assayed for AMP deaminase activity at various times after this thawing process. The results of this experiment are shown in Figure 5.2.1.1. It is clear that under these conditions the enzyme is reasonably stable at room temperature for one to two hours.

As in Chapter 3, AMP deaminase activities quoted in this chapter are the average of three reactions (unless otherwise stated) with a single substrate blank to account for endogenous NH_3 in reagents and extracts. For all determinations quoted from now on the phenol-hypochlorite ammonia assay was employed (see Chapter 2). Errors for this assay are in the range of +/- 4%.

It is also of interest to know under what buffering and ionic conditions the enzyme remains active. Clearly the buffer should also not interfere with the AMP deaminase assay. To this end a range of buffers were tested for their effect on the assay system. It was found that several buffers, including Tris, are unsuitable for use with this assay system and thus they were not used during purification procedures. Suitable buffers included cacodylate (the buffer used in AMP deaminase determinations), phosphate buffers and TEA, all of which had little effect on the assay system.

It was also desirable to know what extremes of pH the enzyme would withstand while maintaining activity. To this end an

FIGURE 5.2.1.2 THE EFFECT OF pH ON YEAST AMP DEAMINASE ACTIVITY



Specific AMP deaminase activities were measured when soluble proteins were extracted in 50mM potassium phosphate buffer at various pHs, prior to assaying in the standard manner (section 2.11). All reactions were carried out in triplicate.

experiment was set up extracting total yeast proteins into 50mM phosphate buffer at a range of pHs. The actual pH of these extracts was then measured. These extracts were stored at -20°C, overnight and then assayed in the standard reaction mix. Therefore this experiment does not test the optimum pH for AMP deaminase activity (which has been shown to be 7.1 by Yoshino et al, 1979b), but the optimum pH for storage and maintenance of activity. The results of this experiment are shown in figure 5.2.1.2. Clearly, under these conditions the pH optimum for maintenance of AMP deaminase is between pH 7.0 and 7.5 and activity is greatly reduced wher the extract's pH drops below pH6 or rises above pH8 suggesting that extremes beyond these limits should be avoided. It has been shown previously that yeast AMP deaminase's pH optimum is 7.1 and that cacodylic acid is the most suitable assay buffer. Therefore samples are assayed in 20mM cacodylic acid, pH7.1 (see Chapter 2 for full assay details) and any purification steps were carried out using 20mM cacodylic acid or TEA, pH7.1 - 7.5 unless otherwise stated.

The cytoplasm contains a number of compounds which help maintain a high reducing potential. Many enzymes lose activity rapidly during extraction due to covalent modifications which occur spontaneously under oxidising conditions. This can often be alleviated by maintaining the reducing conditions by the addition of such compounds as DTT, or B-mercaptoethanol to all buffers. Both these compounds were tested for their effect on AMP deaminase activity. DTT or B-mercaptoethanol at a concentration of only 1mM reduce the AMP deaminase activity (results not shown) as well as affecting the efficiency of the AMP deaminase assay, therefore the use of these compounds was not investigated further.

Many proteins are also very sensitive to the effects of contaminating heavy metal ions in buffers, and to prevent this happening a chelating agent such as EDTA is frequently used. To check the affect of this chelating agent on AMP deaminase activity a series of reactions were set up with varying concentrations of EDTA in the standard AMP deaminase reaction mix. The results of this experiment are shown in table 5.2.1.1. As can be seen from these results low

TABLE 5.2.1.1 THE EFFECT OF EDTA ON AMP DEAMINASE ACTIVITY

CONCENTRATION OF EDTA IN REACTION MIX (mM)	AMP DEAMINASE ACTIVITY (nmoles NH_3/ml/min)
0	420
1	397
5	396
10	380
50	52

EDTA was added at various concentrations to standard assay mixes containing the same amount of identical extract. All the reactions were carried out in triplicate with a single substrate blank reaction. Specific activities were not examined in this case.

TABLE 5.2.1.2 THE EFFECT OF EDTA ON YEAST AMP DEAMINASE ACTIVITY AFTER OVERNIGHT STORAGE AND THE EFFECT OF STORAGE TEMPERATURE

	AMP DEAMINASE ACTIVITY (nmoles NH_3/ml/min)	
EDTA IN EXTRACT (mM)	STORAGE AT 4°C	STORAGE AT -20°C
0	181	393
1	52	207
10	69	206

EDTA was added at various concentrations to identical aliquots of crude extract which was then store overnight at the stated temperatures. These extracts were then assayed for AMP deaminase activity in the standard manner. All the reactions were carried out in triplicate with a single substrate blank reaction. Specific activities were not examined in this case.

concentrations of EDTA have apparently little effect on the enzymes activity, although 50mM virtually abolishes activity.

To further test the effects of EDTA, and also to discover the best method of storage, enzyme extracts were stored overnight at either 4°C or -20°C with various concentrations of EDTA. The results of this experiment are shown in Table 5.2.1.2. It is clear from these results firstly that -20°C is the more suitable temperature of storage of the enzyme at least in its crude form, and secondly that the addition of even 1mM EDTA greatly reduces the activity of AMP deaminase when it is used in a buffer for storage. The nature of this delayed effect of inhibition is unclear and was not studied further. EDTA was not adopted in any future analyses.

Since a number of purification steps rely on elution in differential salt concentrations an experiment was carried out to see what effect varying concentrations of NaCl has on the activity of AMP deaminase. Cell free extracts were prepared into buffer containing varying concentrations of NaCl, up to 5M and the AMP deaminase activity was assayed under normal conditions by diluting enzyme ten-fold with reaction mix, see Chapter 2). These results are shown in Table 5.2.1.3. It is clear that NaCl does not reduce AMP deaminase activity, but appears to increase it. Therefore NaCl could be used in ion exchange chromatography as the chosen eluant.

5.2.2. The effect of ammonia on AMP deaminase activity

Many enzymes are inhibited allosterically by one or more of the products of their reaction. It was not inconceivable that AMP deaminase fell into this category, although ammonia has not been reported as an allosteric effector of the enzyme. Since ammonium sulphate precipitations were one of the methods of purification to be tested it was necessary to know whether the presence of small amounts of NH_3 remaining after dialysis would affect the activity of AMP deaminase. It is of course essential to always account for small contaminating amounts of NH_3 left over after precipitation since the AMP deaminase activity is assayed by the production

TABLE 5.2.1.3 THE EFFECT OF NaCl ON AMP DEAMINASE ACTIVITY IN YEAST EXTRACT

Concentration of NaCl in extract (M).	Final Concentration NaCl in mix (mM)	Specific Activity (nmoles NH_3/mg/min)
0	0	30.3
1	100	51.1
5	500	64.1

The effect of extracting yeast proteins into standard 50mM potassium phosphate, pH7.5 buffer in the presence of absence of various concentrations of NaCl. All reactions carried out in triplicate with a single parallel substrate blank reaction.

of NH_3. This possible contamination is accounted for by carrying out substrate blank reactions, along with every actual assay reaction. To measure the effect of NH_3 on AMP deaminase activity, reactions were set up containing various concentrations of NH_4Cl in parallel with substrate blank reactions. The results of this experiment are shown in Table 5.2.2.1. It is clear from these results that small concentrations of NH_3 in reaction mixes have no effect on AMP deaminase activity.

5.3 PURIFICATION BY DIFFERENTIAL PRECIPITATION: AMMONIUM SULPHATE PRECIPITATIONS

5.3.1 Introduction

There are a number of methods of protein purification which rely on the differential solubility of proteins under different salt or ionic conditions. This process is often known as salting out. A protein solution is taken stepwise from low ionic strength to high, by the addition of a salt or in some cases an organic solvent (the effect of the latter class is somewhat different and is discussed in section 5.4.1).

Salting out largely depends on the hydrophobicity of the protein in question. Typically a protein in solution has hydrophobic regions on its surface (normally due to the side chains of Phenylalanine, Tyrosine, Tryptophan, Leucine, Isoleucine, Methionine and Valine). Forcing these patches into contact with the aqueous solvent causes an ordering of water molecules, effectively "freezing" them around the side chains. This ordering is thermodynamically unstable since it represents a large decrease in entropy compared with the unsolvated proteins and free water molecules. As salt is added and the salt ions become solvated, there is a greater tendency for the ordered water structures around the hydrophobic regions to be disrupted thus allowing these patches to interact with one another leading to precipitation of the proteins.

TABLE 5.2.2.1 THE EFFECT OF NH_4Cl ON AMP DEAMINASE ACTIVITY

Concentration of NH_4Cl	Activity (nmoles NH_3/ml/min)
0	37.7
0.01μM	36.4
0.1μM	35.5
1μM	39.6
10μM	34.3
100μM	32.5
1mM	37.4

Each assay (carried out in triplicate) was accompanied by an appropriate substrate blank (i.e. contained the same concentration of NH_4Cl).

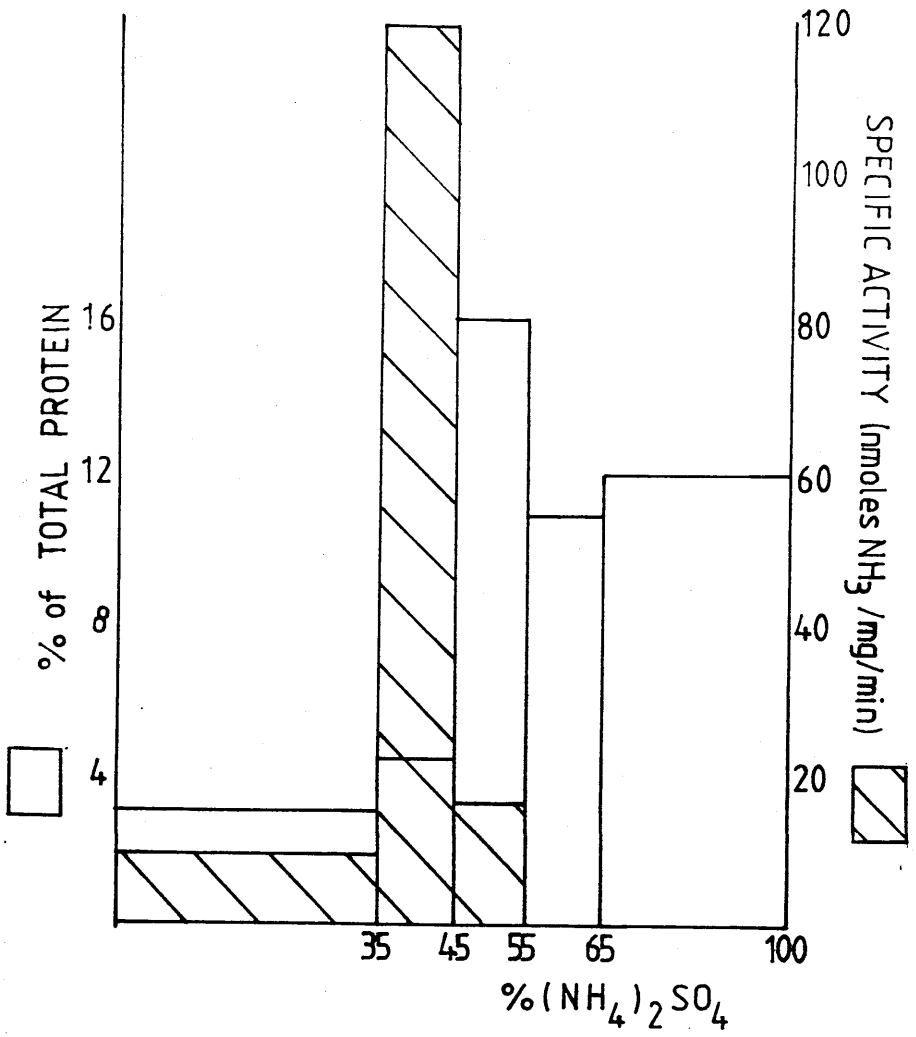
In a crude mixture coaggregation is great and a proportion of many proteins, which in isolation would precipitate at a much higher salt concentration, will be aggregated with very hydrophobic fractions, at relatively low salt concentrations. However, the major portion of most proteins will precipitate in a sufficiently narrow range of salt concentration to make ammonium sulphate precipitations an effective method of enrichment. Other factors are involved in this salting out process, including pH and temperature.

Many different salts can be used for this method of purification the most commonly used is ammonium sulphate There are a number of factors in favour of its use. Sulphate is the most effective anion for salting out purposes because of its multiple charge, and NH_4^+ is a good choice of cation because it is monovalent. In practice the choice of anion is the more important. Another factor to be considered is the density of a concentrated solution of the chosen salt, since centrifuging a precipitate depends on the difference in density between the aggregate and the solvent. A saturated solution of ammonium sulphate has a sufficiently low density at $1.235 g/cm^3$ (Skopes, 1987). Also the solubility of ammonium sulphate varies little between 0 and 30^oC. It is also available in a sufficiently pure state to be used economically in large quantities. One major advantage of ammonium sulphate is that it stabilises most proteins, allowing them to be stored in the form of a precipitate, while the high salt also inhibits proteolysis and bacterial action. For the above reasons ammonium sulphate has been the traditional salt of choice for most salting out procedures.

5.3.2 Results

Various experiments were carried out using small amounts of crude cell-free extract (10 - 30 mls). Briefly, following the addition of $(NH_4)_2SO_4$ to either crude extract or the remaining supernatant after a precipitation step, the mixture is stirred slowly for 15 - 30 minutes at 4^oC. The mixture is then centrifuged at 10000 x g for 15 minutes. The supernatant is then treated appropriately. The pellet after each precipitation step is resuspended in the smallest

FIGURE 5.3.2.1 THE PATTERN OF PRECIPITATION OF AMP DEAMINASE WITH AMMONIUM SULPHATE



Specific activity (nmoles NH_3/mg/min)	% of original activity	% $(NH4)_2SO_4$ of cut	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)
66.4	(100)	Crude extract	666.4	10.0
9.5	0.42	0 - 35	99.9	10.5
118.8	8.0	35 - 45	1366.1	11.5
15.8	4.0	45 - 55	299.9	19.0
0.0	0.0	55 - 65	0.0	15.0
0.0	0.0	65 - 100	0.0	13.0

facing page 84

suitable volume, and then dialysed, in a 1000-fold excess of buffer, 20mM TEA, with a single change of buffer. Each fraction was assayed for protein concentration and AMP deaminase activity. By varying the percentages of the cuts it was found that most of the AMP deaminase activity was recovered in the fraction precipitated between 35-45% ammonium sulphate. One such experiment demonstrating this is summarised in figure 5.3.2.1. In this experiment only 12.4% of the original activity was recovered. In all experiments the recovery was in this range, the average recovery being 14.7%. The maximum purification in any experiment was only 2.1 fold.

5.3.3. Discussion

As stated above, both the recovery and purification of AMP deaminase using ammonium sulphate precipitation were low. Many proteins are very stable in ammonium sulphate and can be stored in the precipitated form for some time. However, it is obviously essential when assaying for an ammonia producing enzyme activity such as AMP deaminase to remove the salt in some way. It is possible that the low recoveries were due to loss of activity during dialysis. This commonly occurs due to the loss of an essential low molecular weight co-factor. Dialysis in the presence of 1mM MgCl, 10mM NaCl or 100mM KCl (all known activators of AMP deaminase) had no effect on the amount of activity recovered. Another possibility is that AMP deaminase is extremely sensitive to some of the denaturing effects of raising the concentration of ammonium sulphate (see section 5.3.1.). The recoveries and low level of purification made ammonium sulphate precipitations an unsuitable step in a purification strategy, and further analysis was abandoned.

5.4 PURIFICATION BY DIFFERENTIAL PRECIPITATION: ACETONE PRECIPITATIONS

5.4.1 Introduction

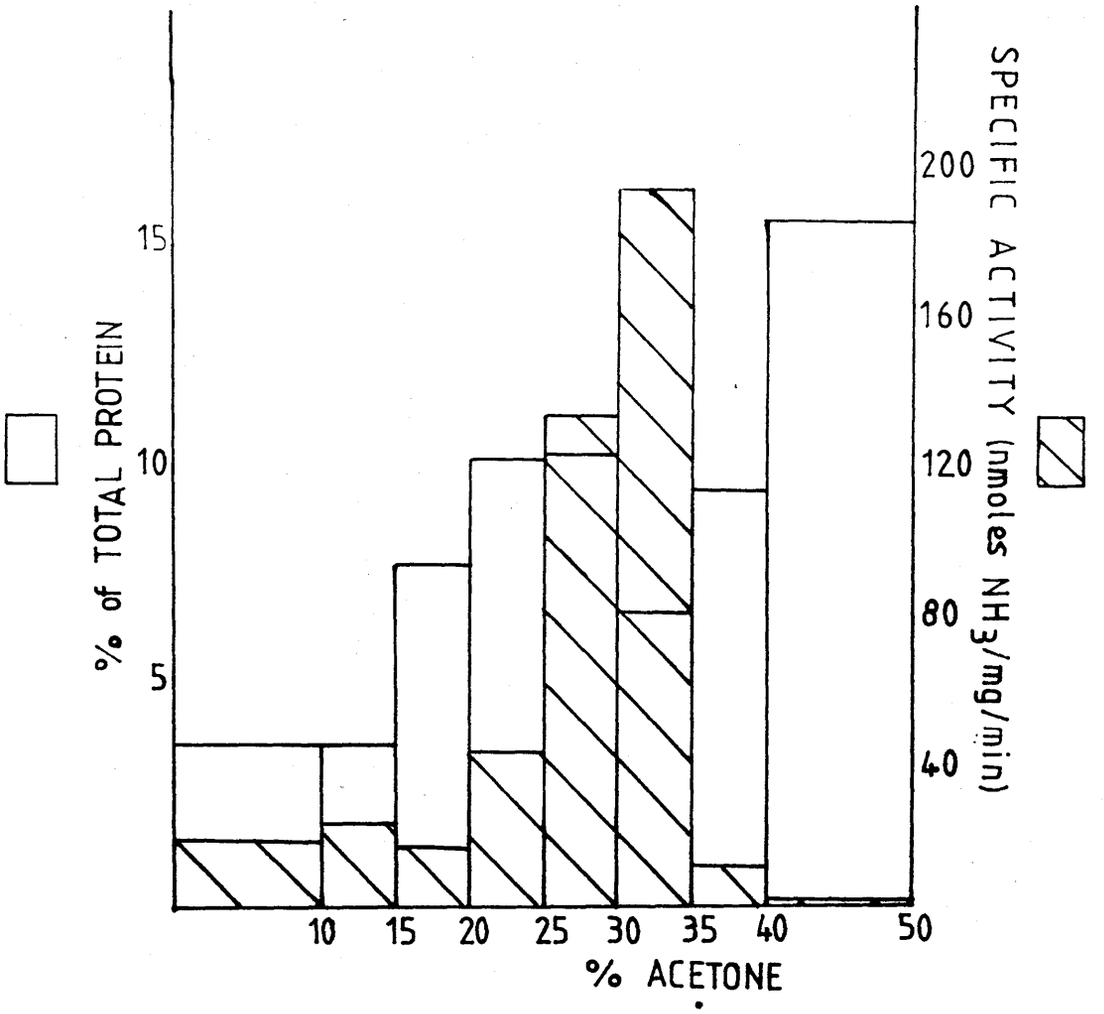
Addition of a solvent such as ethanol or acetone to an

aqueous extract containing proteins has a variety of effects which lead to protein precipitation. The principles causing precipitation are different to those in salt precipitations, and precipitation with organic solvents can be an additional rather than an alternative step in purification. The principal effect of the addition of an organic solvent is the reduction of water activity. The solvating power of water for a charged hydrophobic enzyme is decreased as the concentration of the solvent increases. Hydrophobic attractions are less involved in the formation of aggregates because of the solubilizing influence of the solvent in these areas. Aggregation tends to occur rather by interactions between oppositely charged areas on the protein surface. Another feature affecting organic solvent precipitation is the size of the molecule. All else being equal, the larger the molecule the lower the concentration of solvent required to precipitate it. Thus, if one could compare a range of proteins with different molecular weights but similar hydrophobicity and isoelectric points the precipitation order would be the reverse of the order of size.

There are a number of factors involved in the choice of solvent. It must be completely water miscible, unreactive with proteins, and have good precipitating properties. The most commonly used solvents are ethanol and acetone. Other possible solvents are methanol, n-propanol, i-propanol and various other exotic alcohols, ethers and ketones.

One advantage of organic solvent fractionation is that it can be carried out at sub-zero temperatures since the aqueous-solvent mixtures freeze well below $0^{o}C$. This is advantageous since at about $10^{o}C$ denaturation effects become significant. At low temperatures a lack of conformational flexibility means that organic solvent molecules are unlikely to penetrate the internal structure and cause destabilisation. Long chain alcohols are more denaturing than short ones. Although ethanol is standardly used for plasma protein fractionations (Curling, 1980), acetone has a lesser tendency to cause denaturation than ethanol partly because lower concentrations are needed to cause comparable amounts of precipitation at low temperatures.

FIGURE 5.4.2.1 THE PATTERN OF PRECIPITATION OF AMP DEAMINASE WITH ACETONE



% Acetone of cut	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific activity (nmoles NH_3/mg/min)	% of original activity
Crude extract	499.8	8.5	58.6	(100)
0 - 10	206.7	11.4	18.1	1.1
10 - 15	266.7	11.7	22.7	1.4
15 - 20	300.0	18.0	16.7	2.2
20 - 25	901.8	21.2	42.5	7.2
25 - 30	1483.3	11.3	131.3	22.5
30 - 35	1492.5	7.8	191.3	21.5
35 - 40	99.9	8.8	11.4	1.8
40 - 50	16.7	14.6	1.1	0.3

facing page 86

Ideally acetone precipitations should be carried out on a solution with low salt concentrations, otherwise electrostatic aggregation is impaired and higher levels of organic solvent are needed and denaturation is more likely. Addition of solvent to water causes heat evolution, consequently slow addition with efficient cooling is desirable.

5.4.2 Results

All steps were carried out as close to 0°C as possible. The method used is detailed in chapter 2.

A number of pilot experiments were set up, typically precipitating 10mls of cell-free extract (at approximately 5mg/ml protein). Recovery values were reasonable, averaging 50 - 60% of original activity. The best purifications were 3.3-fold. Most of the AMP deaminase activity was found to precipitate with between 25 and 40% acetone. It was found that the most efficient way of obtaining reasonable amounts of sufficiently purified material was to take the sample in multiple steps to the correct concentration of acetone. If a single 0 - 25 % acetone cut was carried out, a significant proportion of the AMP deaminase activity was precipitated in this crude first step, presumably due to coaggregation effects (data not shown). A typical acetone precipitation is detailed in figure 5.4.2.1. In this experiment a total of 58% of the original activity was recovered and of this 44% is located in the 25-30 and 30-35% cuts. The 30-35% cut material represents a 3.3-fold purification.

5.4.3 Discussion

Acetone precipitation appeared to represent a reasonable first crude purification step. It removed a large proportion of the cell protein, and allowed a small level of purification while maintaining a large proportion of the AMP deaminase activity. The precipitated material was simply resuspended in a small volume of 20mM TEA, pH 7.1 buffer. Attempts to remove the acetone by lyophilisation resulted in

large losses of activity. However, when this material was filtered or loaded on to a PD-10 buffer exchange column, for use in further purification, more than 90% of the activity was lost. This appeared to be due to the incomplete resolubilisation of the protein after acetone precipitation. The protein mixture cannot be redissolved effectively but remains particulate. The AMP deaminase activity is still assayable, however. Attempts to completely dissolve this particulate material were unsuccessful either in a larger buffer volume or by changing the buffer conditions. At all stages the AMP deaminase activity remained associated with this particulate material thoughout all attempts to clear the protein solution. For this reason the use of acetone precipitation as a purification step was abandoned.

5.5 AFFINITY CHROMATOGRAPHY WITH CIBACRON BLUE SEPHAROSE

5.5.1 Introduction

A standard method of enzyme purification is affinity adsorption chromatography. Here, the protein of interest is adsorbed to a medium by a mechanism normally related to the ligand binding properties of the protein. There are two basic classes of affinity adsorbents. Firstly, there are the true affinity adsorbents which specifically bind proteins that have binding sites for the immobilised ligand. The second class contains the more general adsorbents, sometimes called pseudo-affinity adsorbents which include the dye adsorbents.

Many ready-made adsorbents are available commercially. Because of the low capacity of these adsorbents the method is most useful for purifying proteins that make up a small proportion of the total protein present, in which case it can be used as the first step in the purification as was hoped in this case.

Falling into the class of true affinity adsorbents are adsorbents with AMP as the protein binding ligand. AMP agarose media are commercially available and would seem the ideal choice in the purification of AMP deaminase. However,

the commercially available chromatographic media have their AMP ligand bound to the media matrix by the N-6 group of AMP. Since the N-6 group is that which undergoes catalysis during the AMP deaminase reaction it was judged that this media would be unsuitable for the purification of AMP deaminase (Ohlsson et al., 1972).

The second class of more general affinity adsorbents include the dye adsorbents. Often the dye used as a protein binding ligand bears little obvious resemblance to the true ligand, hence the term pseudo affinity adsorbents. One such dye is Cibacron blue F3GA, which behaves as an analogue of ADP-ribose and has been shown to bind to most enzymes that bind a purine nucleotide including AMP, ATP, IMP, NAD, NADP and GTP (Biellmann et al., 1979). This type of media has been used successfully in the purification of yeast pyruvate kinase (Haeckel et al., 1968). Fortuitously, these media have a good protein binding capacity of 20-30mg/cm^3. Hence a media with Cibacron Blue as its ligand such as cibacron blue sepharose seemed like an extremely good choice of adsorbent in the purification of AMP deaminase.

5.5.2 Results and Discussion

Small scale columns were set up with a bed volume of approximately 1.0 ml. These columns were equilibrated with 20mM cacodylic acid, pH7.1. Varying amounts of yeast cell free extract were then loaded and the column washed with several volumes of the above buffer. When a small amount of cell free extract (less than 5mg total protein) was applied to this column the AMP deaminase activity appeared to be retained on the column. However, if the column was loaded with a greater amount of protein a proportion of the AMP deaminase activity was eluted immediately from the column. This unbound material showed no purification in terms of specific activity and protein concentration.

Step gradients were run on the columns described above with the following eluants;

1) 5, 10, 20, 50 and 100mM AMP in Start buffer
2) 0.1, 1, 10, 50 and 100mM ATP in Start buffer
3) 0.1, 1, 2, and 3M NaCl in Start buffer
4) 10, 20 and 100mM $MgSO_4$ in Start buffer
5) 10, 20, 50 and 100mM AMP in Start buffer + 0.1 or 1M NaCl
6) 20mM ATP and 20mM AMP in Start buffer

In each of the cases described above no AMP deaminase activity was detected in the eluted fractions.

It seems likely that yeast AMP deaminase is bound by the Cibacron blue dye ligand rather than somehow becoming inactivated during the course of the chromatography. This view is supported by the fact that when a column is overloaded the unbound AMP deaminase maintains its activity. It has also been reported that Cibacron blue binding is very often tighter than the true substrate with a K_i in the uMolar range (Skopes, 1987). It is possible that since the enzyme is tetrameric it can become complexed with more than one ligand molecule thus being more stably bound than an enzyme with only one ligand binding site per molecule. It is possible that the conditions for elution described above are not strong enough to overcome this binding, or alternatively, the inherent structure of the enzyme itself may have been disrupted during adsorption and/or elution. Another possibility is that although adsorption generally weakens with increasing ionic strength, the adsorption of a given protein may be due to hydrophobic attraction of regions of the protein rather than to ionic attractions. Therefore, increasing the ionic strength can, under certain circumstances, strengthen adsorption. This was not examined further and Cibacron blue sepharose medium was abandoned as a method of purification.

5.6. ION EXCHANGE CHROMATOGRAPHY USING FPLC MONO Q MEDIA

5.6.1. Introduction

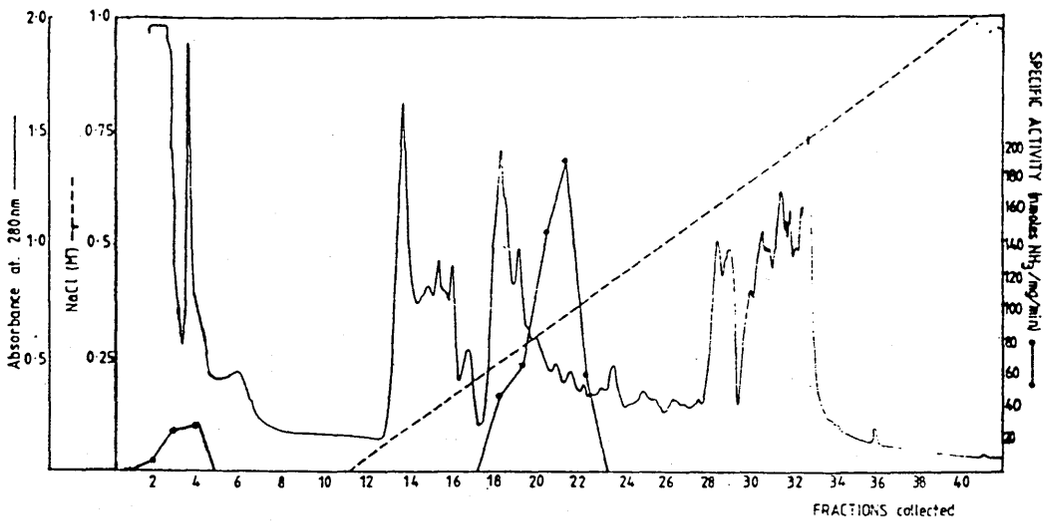
Proteins bind to ion exchangers mainly by electrostatic forces between its surface charges and the dense clusters of charged groups on the exchangers. The substitution level of a typical DEAE-cellulose or CM-cellulose may be as much as 0.5mmol/cm^3, ie 0.5M of charged groups. The charges are balanced by counter-ions such as metal, chloride or buffer ions. A protein must displace the counter-ions and become attached.

Protein molecules in solution are neutralised by counter-ions to become effectively neutral, i.e. a protein with a net negative charge is pre-equilibrated with say Tris.Cl buffer and therefore the counter-ion associated with the protein is H^+Tris. An anion exchange column is equilibrated with Cl- counter-ions. The protein then displaces the Cl^-, and Tris.Cl is discharged. This Tris.Cl is the acid salt of Tris and if displacement is rapid it could lead to a lowering of the effective pH in the column ahead of the band of adsorbed protein. Also the pulse of Tris.Cl represents an increase in the ionic strength of the buffer flowing through the column. Typically for every 1mg/ml protein that is adsorbed, it is replaced by approximately 1mM of extra buffer salts. The pH of non-adsorbed fractions can be shown to be lower than the preceding buffer wash (or higher in cation exchangers) if the protein concentration of the applied sample is too high. To avoid pH and ionic aberrations, the applied protein concentration should not be too high, especially if a large proportion of it is expected to bind. In addition, inadequate buffering could allow extremes of pH to develop causing denaturation. Generally 10mM of a buffer within 0.3 of its pKa is sufficient. Protein being adsorbed should be no more than 5mg/ml (non-adsorbing proteins are less important but should not be more than 10mg/ml) (Skopes, 1987). Capacity of an ion exchanger is protein size dependent. Large molecules can only bind to the surface of the ion exchange particles and therefore capacity for these is low. Overloading of columns may result in the emergence of the larger molecular weight protein first, although there

FIGURE 5.6.2.1 ELUTION OF AMP DEAMINASE FROM A MONO Q 5/5 COLUMN LOADED WITH CRUDE EXTRACT

Column volume: 1ml
Flow rate: 1ml/min
Fraction size: 1ml
Buffer: 20mM potassium phosphate, pH 7.5

Sample: 1.5ml crude extract (see below)



Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
extract	941.0	10.3	90.9
2	26.7	2.8	9.5
3	16.7	0.52	31.8
4	11.6	0.3	38.8
18	36.6	0.82	44.8
19	43.4	0.68	63.8
20	56.6	0.39	145.0
21	58.3	0.30	191.0
22	15.0	0.26	57.7

may be sufficient capacity to adsorb all the smaller molecules.

All chromatographic studies and purification steps discussed from now on were carried out using a Pharmacia FPLC system. Most of this work was carried out at the Biological products division of ICI in Billingham. All ion exchange was carried out using pre-packed Mono Q columns. Mono Q is a strong anion exchanger based on a beaded hydrophilic resin with one of the narrowest particle size distributions available. The beads have a particle size of 10um. Since there are no small particles with these beads the packed columns have void volumes of 40% and therefore a very low back pressure. The charged group on the gel is $-CH_2-N-(CH_3)_3$. The ionic capacity of the gel is 0.27-0.37 mmoles/ml and the exclusion limit of the beads is 10^7 daltons.

Note: all subsequent analyses of AMP deaminase in this chapter consisted of only a single reaction mix and a parallel substrate blank. Reactions were no longer carried out in triplicate, to reduce losses of purified material and to aid the speed of analysis. As previously stated, errors using this assay are in the range of +/- 4%.

5.6.2. Pilot experiments with FPLC Mono Q media

Using a 1ml Mono Q 5/5 column, pilot experiments were carried out using yeast cell-free extract made in 20mM TEA, pH7.5. The protein was found to bind under these conditions and was eluted when a 0 - 1M NaCl linear gradient was applied, the AMP deaminase activity peaking at around 0.32M NaCl. Recovery was in the range of 20-37%, and the purification was on average 3.4-fold under these conditions. A typical small scale Mono Q run is detailed in Figure 5.6.2.1. Fraction 21 represents a 2.1-fold purification and 22.3% of the activity which was loaded on the column was recovered in fractions 18 to 22. The run detailed here gave a particularly low purification factor, but this value never exceeded 5 or 6-fold.

As can be seen a small but significant proportion of the activity does not bind to the column but appears to be eluted immediately. Data sheets regarding capacity of Mono Q beads state it as 25mg/ml. As can be seen in the case of this run only 15.5mg of protein was loaded, and therefore, it would appear that this is not simply a case of protein overloading. This problem is discussed further in section 5.6.3. More worrying than the loss of this material, is the low recovery of activity, and the low purification factor.

The situation improved somewhat when the same conditions were scaled up for use on large columns. On Mono Q 16/10 columns (20ml bed volume) the recovery of activity was lower, averaging 15% of that loaded. But the level of purification increased to 9.6-fold on average, and on occasion was considerably higher. However, the low recoveries were unacceptable and the steps taken to improve these results are discussed in section 5.6.3.

5.6.3. Improving the performance of Mono Q anion exchange

Data provided with FPLC suggest that recoveries for chromatography of most proteins should approach 100%. This clearly was not the case for AMP deaminase under the conditions discussed in the previous section.

All chromatography was carried out at room temperature. However, no analysis would require samples to be at room temperature for more than two hours. Results shown in figure 5.2.1.1 show that AMP deaminase activity might be expected to fall at most 20%, therefore losses of up to 85% can not be explained by mere heat inactivation.

Many proteins require the presence of reducing conditions as provided by DTT or B-mercaptoethanol for their activity to be maintained. However, this is not the case for AMP deaminase as shown in section 5.2. Nor does the addition of EDTA help maintain AMP deaminase activity as it does for many enzymes.

Many proteins lose stability when diluted. Enzymes exist in a very concentrated solution within the cell, but when

TABLE 5.6.3.1 FRACTIONS CONTAINING AMP DEAMINASE ACTIVITY FROM MONO Q 16/10 WHEN CRUDE EXTRACT IS APPLIED AT HIGH CONCENTRATIONS

Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
extract	156.6	12.0	13.0
5	49.6	1.3	39.0
6	72.9	5.3	13.8
7	250.0	6.1	41.0
8	335.0	3.1	108.0
9	305.0	5.6	54.5
10	302.0	7.9	38.3
11	308.0	7.1	43.4
12	318.0	8.2	38.7
13	315.0	7.5	42.0
14	270.0	5.4	50.1
15	20.3	1.5	13.5
30	10.3	0.54	19.1
31	21.0	0.72	29.2
32	14.3	0.99	14.5
33	7.3	1.09	6.7

Elution of AMP deaminase activity from a Mono Q 16/10 column run under standard conditions with the exception that the sample of 48mls crude extract is applied at a concentration of 12mg/ml (see figure 5.9.2.1).

Fractions 5-15 represent AMP deaminase activity which does not adsorb to the Mono Q media (i.e. is eluting prior to the application of the NaCl gradient).

Fractions 30-33 represent adsorbed AMP deaminase activity which is eluted at around 0.3M NaCl on application of the NaCl gradient.

released from the cell the environment radically changes. AMP deaminase appears to be reasonably stable in the form of a crude cell extract, especially when stored at -20°C. It is possible that dilution during purification was causing destabilisation. Alternatively, purification may lead to the loss of an unidentified but essential co-factor.

The first of these problems is sometimes overcome by adding a known protein such as BSA to storage solutions. (This is why BSA is included in the AMP deaminase reaction mix). This seemed an undesirable step and was not considered at this stage since protein concentrations were still reasonably high. Nothing could be done to overcome the loss of a co-factor without first identifying the putative co-factor and including it in all chromatography buffers.

In fact this low recovery or instability problem was drastically improved by carrying out all purification steps in potassium phosphate buffer, pH7.5. Many enzymes are stabilised by this buffer, but the reason for this is somewhat obscure. This one change, from 20mM TEA to 20mM potassium phosphate for Mono Q 5/5 columns, vastly improved average recoveries from 30% to 114% with a new average purification factor of 10.7-fold. In the case of the preparative scale columns (8 or 20ml volumes) the recovery rose from 15% to 114%, with an average purification of 13-fold.

Unfortunately, the second problem regarding the proportion of AMP deaminase activity not adsorbed to the column was not improved by the use of phosphate buffer. An extreme example of this unbound activity is detailed here in table 5.6.3.1. 97.9% of the recovered activity is found in fractions 5 to 15, with only 2.1% being adsorbed to the column and then eluted as normal at around 0.32M. This phenomenon is not simply due to overloading the column. Here, 480mg of protein was loaded, the column has a stated capacity of 500mg. Also as can be seen from the specific activities of fractions 7 to 15, there appears to be a low level of purification. It would appear that some cellular proteins are being bound preferentially over AMP deaminase hence the increase in specific activity of these non-adsorbed fractions.

The capacity of an ion exchanger is protein size dependent. Large molecules can only bind to the surface of the ion exchange particles and therefore capacity for them is low. Overloading of columns may result in the emergence of the larger molecular weight proteins first, although there may be sufficient capacity to adsorb all the smaller molecules. AMP deaminase, at a reported molecular weight of 320 kDa (it is a tetramer) (Murakami, 1979), could easily be included in the former category.

There can also be an effect by loading a protein mixture at too high a concentration, as discussed in section 5.6.1. If too high a concentration is applied to the column, and a high proportion of this is adsorbed, a pulse of buffer, at a more acid pH (in the case of anion exchange) and higher ionic strength may be released.

To check whether this concentration effect may be a contributing factor, two parallel experiments were set up, using identical conditions with the exception that one column was loaded with 0.5ml cell free extract at a protein concentration of 9.30mg/ml and the other with 2mls of the same extract diluted 4-fold at a protein concentration of 2.35mg/ml. In the case of the first column only 40.6% of the activity recovered was bound, the remaining 59.4% running straight through. In the case of the column loaded with dilute extract 85.7% of recovered activity was bound and then eluted with 0.3M NaCl. This clearly shows that the concentration of extract loaded on to the column is of importance as well as the actual amount. In subsequent experiments extracts were diluted to a final concentration of 1.5-3mg/ml prior to loading, and the stated capacity of 25mg/ml was not adhered to, the amount loaded never exceeded 16mg/ml. In this way all further Mono Q columns were found to adsorb a high percentage of the AMP deaminase activity loaded.

Therefore, ion exchange chromatography for the actual purification was carried out with the following modifications to that detailed in section 5.6.2.

Low protein concentration:	1.5-3.0mg/ml
Low protein loading:	less than 70% of the manufacturer's stated capacity
Buffer conditions:	changed from 20mM TEA to 20mM potassium phosphate

Another point to note is that in some cases the activity recovered exceeds that which was loaded. There are a number of possible reasons for this. The activity of some proteins is actually increased by an increase in temperature during chromatography. AMP deaminase does not fall into this category of thermo-activation, as shown in section 5.2.

Another possibility is that during purification the enzyme may have been separated from some inhibitory component. Alternatively the increase in ionic strength of the eluted fraction may in some way be stabilising or activating AMP deaminase. This was not studied further.

5.7. HYDROPHOBIC INTERACTION CHROMATOGRAPHY WITH FPLC PHENYL-SUPEROSE MEDIA

5.7.1. Introduction

During the development of affinity chromatographic support mediums a number of control experiments were carried out looking at the behaviour of matrices containing spacer arms but no ligand. In a few cases, enzymes were found to bind strongly to hexamethylene arms even without charged ends (Er-el et al., 1972). These findings were used in the development of hydrophobic interaction chromatography, so called because it involves the interaction between the aliphatic chains on the adsorbent and hydrophobic regions on the surface of the proteins (Hofstee and Otillio, 1978; Ochoa, 1978).

Relatively few proteins bind to short immobilised aliphatic chains at low ionic concentrations, but hydrophobic interaction chromatography can be extended to cover all proteins since the hydrophobic interactions increase with increasing salt concentration. The reasons for hydrophobic

interactions are similar to those involved in the precipitation of proteins with ammonium sulphate as discussed in section 5.3.1. Consequently at high salt concentrations most proteins can be adsorbed to hydrophobic groups attached to inert matrices. Typical hydrophobic adsorbents include C4, C6, C8 and C10 linear aliphatic chains, and the same chains containing a terminal amino group. Phenyl-based matrices are also available (Gopalakrishna and Anderson, 1982), such as the one used here, Phenyl Superose produced by Pharmacia in the form of pre-packed columns to be compatible with their FPLC systems. Phenyl Superose is a derivative of the rigid, cross-linked agarose-based Superose 12 and contains covalently bonded phenyl groups. The degree of substitution is approximately 50umoles/ml of gel bed. The average particle size is 10um. Phenyl Superose has a negligible amount of charged groups meaning that true hydrophobic interaction chromatography is possible.

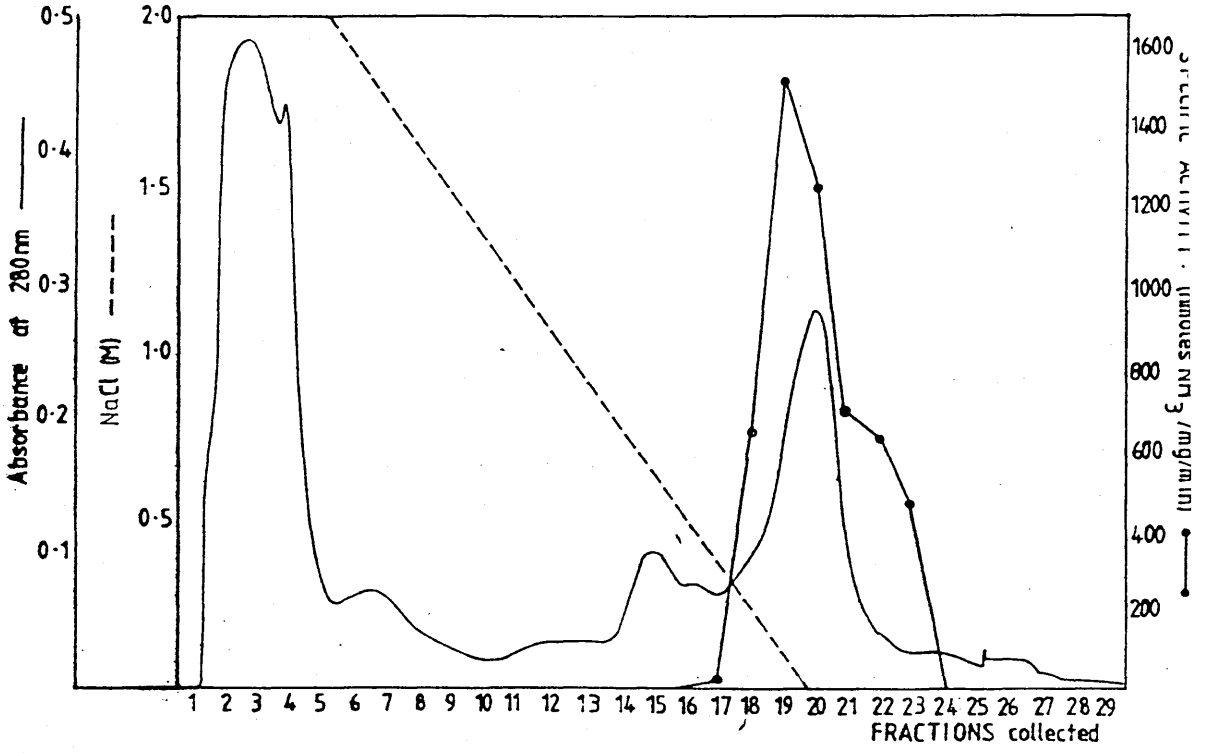
Hydrophobic interaction adsorbents provide a complementary purification medium to ion exchange. In practice, hydrophobic interaction chromatography does not usually achieve very high resolution. This is because binding behaviour changes only slowly with altering conditions and therefore there can be a relatively large degree of overlap between components. However, the protein capacity of this type of medium is very high (10-100mg/cm3). It also does not require any buffer exchange, since samples are loaded in high salt buffer and salt can simply be added to the sample to achieve this.

For the above reasons, hydrophobic interaction chromatography was considered a suitable second step in the purification of AMP deaminase. This is a suitable point to consider it in this purification strategy for a number of reasons. Firstly, precipitation methods, based upon protein hydrophobicity had not been previously used, and secondly a method with a reasonably high protein capacity is required at this stage since fairly large amounts of protein are still to be dealt with. Therefore experiments using the FPLC phenyl-superose pre-packed column were carried out.

FIGURE 5.7.2.1 ELUTION OF AMP DEAMINASE FROM A PHENYL-SUPEROSE 5/5 COLUMN LOADED WITH CRUDE EXTRACT

Column volume: 1ml
Flow rate: 0.5ml/min
Fraction size: 1ml
Buffer: 20mM potassium phosphate, pH 7.5

Sample: 2.0ml crude extract (see below)



Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
extract	399.0	4.05	98.8
17	3.32	0.12	27.7
18	83.3	0.13	641.0
19	173.0	0.115	1510.0
20	200.0	0.16	1250.0
21	123.0	0.175	704.0
22	56.7	0.09	637.0
23	33.3	0.07	477.0

facing page 97

5.7.2. Pilot experiments with FPLC phenyl-superose media: Results and Discussion

Pilot experiments were carried out using crude extract loaded onto a Phenyl Superose 5/5 column (1ml bed volume). Using cell-free and partially purified material from an ion exchange column were not expected to give comparable results since the differing components in each mixture would compete differently in binding. However, experiments with cell-free extract give an indication of the suitability of this method of purification.

NaCl was chosen as the salt to be used in these analyses since its effect on AMP deaminase at high concentrations had already been studied (Section 5.2.2). Initially the material was loaded in 4M NaCl but since AMP deaminase was found to elute at a low salt concentration when subjected to a linear gradient from high to low salt, the material was subsequently loaded in 2M NaCl, to reduce the competitive binding of less hydrophobic components. These pilot experiments were encouraging, giving high returns of activity in the range of 70-85% of the material loaded. The level of purification, as expected, was not particularly high ranging from 5 to 15-fold. One such experiment is shown in figure 5.7.2.1. AMP deaminase activity peaks at approximately 0.24M NaCl, and fraction 19 represents a 15.3- fold purification. 84% of the loaded activity was recovered. Therefore, Phenyl Superose chromatography appears to represent a reasonable second step in the purification of AMP deaminase. Hence, similar experiments were carried out using fractions purified from an ion exchange column.

Those fractions with the highest AMP deaminase specific activity after Mono Q chromatography were pooled, salt was then added to make the sample up to 2M NaCl. The column was then run as described above. In general, the recoveries from such columns loaded with partially purified material were much lower than those from columns loaded with crude extract. The average recovery was only 20.8% ranging from as low as 4.9% to 44.4%. Related to this was the poor level of purification, averaging only a 2.9-fold increase. It was also found that the AMP deaminase from this partially

purified material was generally eluted at a higher salt concentration than that when using crude extract. The activity normally peaked at around 0.7M NaCl, as opposed to 0.2M NaCl using crude material. These differences are discussed below. The Phenyl Superose chromatographic step in the final purification is detailed in section 5.9.

This reduced recovery is not too surprising when coupled with the fact that Mono Q semi-purified fractions lost AMP deaminase activity rapidly when stored. It was found that activity could decrease dramatically overnight, and all activity could be lost when the fractions are stored at -20^{o}C for 2 to 3 days (data not shown). Storage at 4^{o}C did not improve matters. As nothing was known about the nature of this activity decay, the final purification procedures were carried out rapidly without storing fractions for longer than necessary. This was accomplished by carrying out the entire fractionation in one day. In this way at least some AMP deaminase activity was maintained and assayable.

The reason for the different elution patterns seen with crude and partly-purified material is somewhat obscure. It may be that partially purified AMP deaminase is competing for binding with a population of relatively more hydrophobic proteins than that encountered in the crude extract.

5.8 GEL FILTRATION WITH FPLC SUPEROSE 6

5.8.1 Introduction

The previous methods of purification have involved phase changes, i.e. the proteins pass from a liquid phase (when in solution) to a solid phase (when adsorbed or precipitated) and back again. Not all proteins can withstand these stresses, and as has been discussed previously, AMP deaminase appears to lose a high proportion of its activity during all attempts to purify it.

Gel filtration is a relatively gentle technique, where the proteins remain in solution at all times. It was hoped that this technique coupled with the two adsorptions processes

TABLE 5.8.1.1 PROPERTIES OF PHARMACIA SUPEROSE GEL FILTRATION MEDIA

PROPERTIES	SUPEROSE 6	SUPEROSE 12
Exclusion limit for globular proteins (MW)	approx 4×10^7	approx 2×10^6
Optimal separation range (MW)	$5000 - 5 \times 10^6$	$1000 - 3 \times 10^5$
Average particle size (μm)	13 +/- 2	10 +/- 2
Yields and activity recovered (%)	80 - 100	80 - 100

Information quoted from data sheets regarding Superose 6 and 12 supplied with prepacked columns from Pharmacia.

described in sections 5.6 and 5.7, would allow enough AMP deaminase activity to be maintained to sufficiently purify the enzyme for N-terminal amino acid sequencing.

The basic principles of gel filtration are simple. The gel matrix consists of an open cross-linked 3-dimensional molecular network, cast in bead form. The pores within the beads are of such sizes that they are not accessible to the largest molecules, and the smallest molecules can penetrate all pores. Within these two extremes are molecules which can penetrate many or few of the pores depending on the size of the molecules. In this way large molecules are excluded from most of the pores, and therefore, move more rapidly through the column. They are eluted ahead of smaller molecules which are impeded in their passage by having to pass through pores in the beads. In this way gel filtration medium can separate molecules largely on the basis of size.

Again all purifications were carried out using the Pharmacia FPLC system. There are two gel filtration media available with this system, Superose 6 and Superose 12.

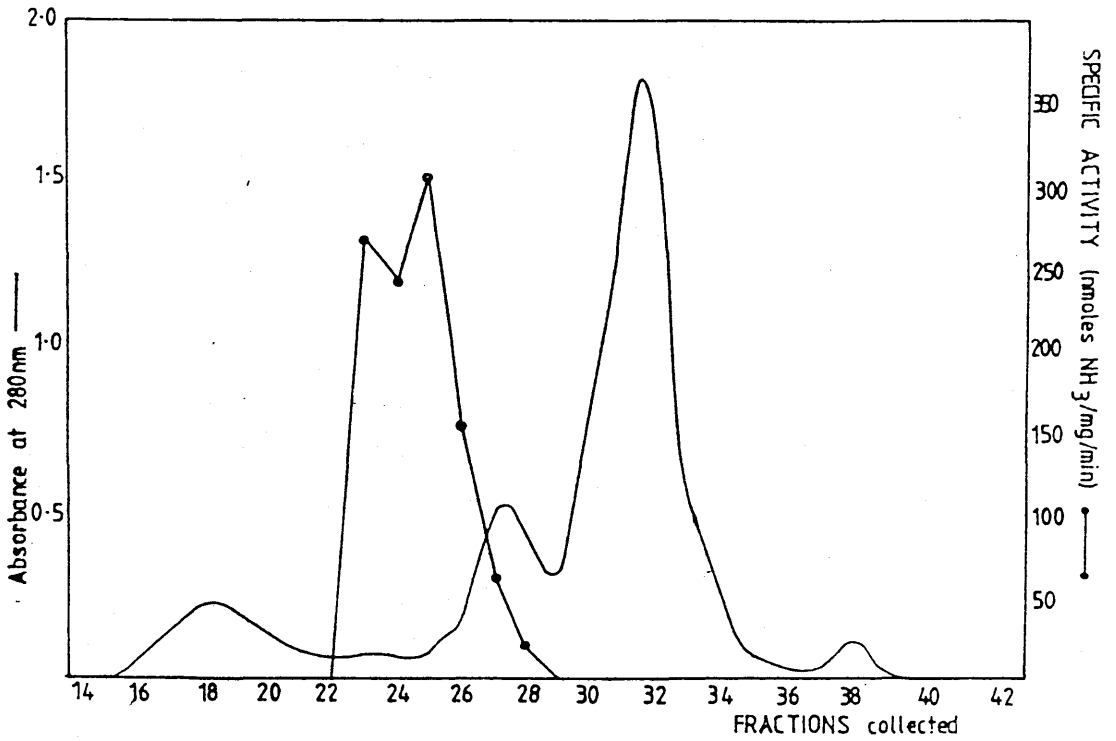
Superose is a cross-linked agarose based medium which exhibits negligible ionic interactions at ionic strengths of 0.05M and above. However, some hydrophobic interactions have been shown, and therefore some proteins may be eluted somewhat later than predicted. The properties of the two media are shown in table 5.8.1.1. Since the molecular weight of AMP deaminase from S. cerevisiae is reported to be 320 kDa, Superose 6 was chosen for use in the purification of this enzyme because of its greater exclusion limit. The column has a bed volume of 20mls.

Gel filtration requires that a sample be applied in a small volume (commonly not greater than 1-2% of the column volume) because of its ability to seperate molecules on the basis of their size. Therefore, since the column used has a volume of 20mls, no more than 400µl of a protein solution could be loaded. The actual amount of protein is of less importance, but obviously a protein solution which is too concentrated results in precipitation of proteins. This curtails the actual capacity of the column, and although pilot experiments

FIGURE 5.8.2.1 ELUTION OF AMP DEAMINASE FROM A SUPEROSE 6 COLUMN LOADED WITH CRUDE EXTRACT

Column volume: 20ml
Flow rate: 1ml/min
Fraction size: 0.5ml
Buffer: 50mM potassium phosphate, pH 7.5

Sample: 200µl crude extract (see below)



Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
extract	403.0	14.2	28.4
23	11.7	0.044	265.0
24	26.7	0.112	238.0
25	33.3	0.1	333.0
26	30.0	0.195	154.0
27	25.0	0.405	61.7
28	13.3	0.68	19.7
21	58.3	0.30	191.0

were carried out using semi-pure fractions from ion exchange it soon became apparent that another purification procedure would be advisable prior to gel filtration. Hence the use of hydrophobic interaction chromatography, which although not giving a great overall purification does considerably reduce the amount of protein to be dealt with.

Gel filtration requires no buffer exchange of the sample, but does normally require a concentration step, to reduce the volume. Protein concentration was carried out using Amicon filter concentrators, which remove water from a solution by blotting it through a membrane (see chapter 2). This was found to be a satisfactory method of concentration, which could be carried out rapidly and at low temperatures.

5.8.2 Pilot experiments with FPLC Superose 6 media: Results and Discussion

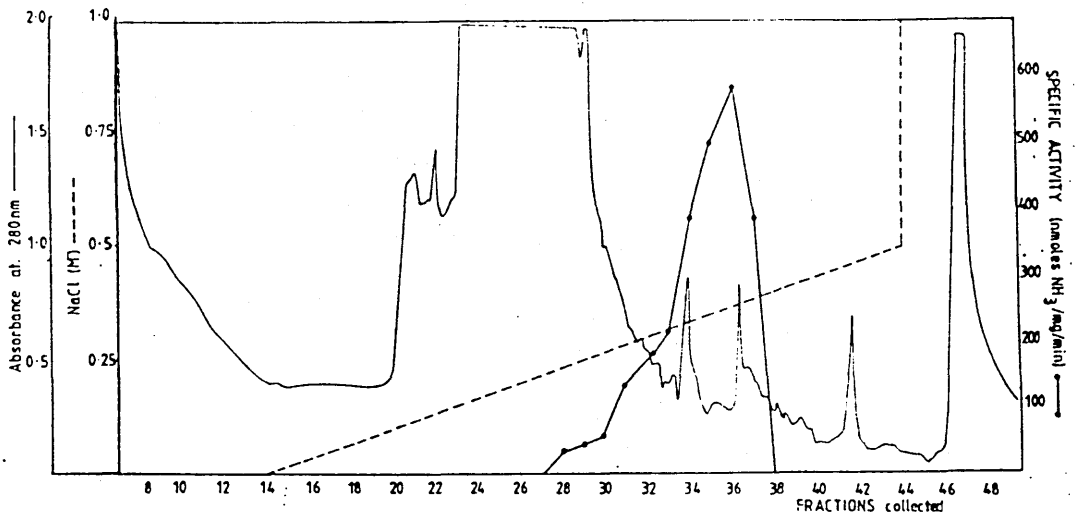
Pilot experiments were carried out using crude extract to determine the elution characteristics of AMP deaminase from Superose 6. AMP deaminase was routinely eluted between 12 and 14mls after loading. Recovery of this activity was normally high, approaching 100%. A typical Superose 6 run is detailed in figure 5.8.2.1. In this case, the activity peaks at 12.5mls, and fraction 25 represents a 11.7-fold purification. 87% of the loaded activity was recovered. However, as previously discussed, partially purified AMP deaminase rapidly loses activity, and purification by Superose 6 chromatography using partially purified material was less efficient (see section 5.9.2).

As at every stage, all attempts to stabilise the enzyme had failed. Also the AMP deaminase assay was the only method of monitoring the enzyme during the purification. Hence, it was felt that the entire purification procedure would have to be carried out as quickly as possible, preferably in a single day.

FIGURE 5.9.2.1 ELUTION OF AMP DEAMINASE FROM A MONO Q 16/10 COLUMN LOADED WITH CRUDE EXTRACT

Column volume: 20ml
Flow rate: 8ml/min
Fraction size: 8ml
Buffer: 20mM potassium phosphate, pH 7.5

Sample: 100ml crude extract (see below)



Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
extract	128.0	3.1	41.3
28	23.3	0.67	34.8
29	39.9	0.89	44.8
30	61.6	1.10	55.7
31	125.0	0.98	127.5
32	192.0	1.07	179.0
33	239.0	1.05	227.0
34	356.0	0.92	387.0
35	476.0	0.96	496.3
36	512.0	0.88	581.2
37	290.0	0.75	387.0

5.9 THE PURIFICATION OF YEAST AMP DEAMINASE

5.9.1 Introduction

What follows is a detailed description of the purification of AMP deaminase from crude extract to the point where activity recovered in a fraction correlates with the major band on an SDS polyacrylamide gel. This material was used for N-terminal sequencing.

The entire procedure was carried out without long term storage (i.e. overnight) of semi-purified material. Although all chromatography was carried out at room temperature, wherever possible fractions were collected and immediately stored on ice, prior to assaying for AMP deaminase activity. Small samples were taken at each stage for AMP deaminase assays, protein determination, and for analysis by SDS polyacrylamide electrophoresis.

The phenol-hypochlorite method for ammonia determination as described in chapter 2 was used in all AMP deaminase assays throughout the purification. However, the assay was increased in its sensitivity by determining the ammonia produced by a 100ul reaction mix by the addition of 0.33ml reagent 1, followed by the addition of 0.5ml of both reagent 2 and 3 (as opposed to the normal additions of 1ml, 1.5ml and 1.5ml respectively). This increased the lower limit of detection of the assay to 5nmoles of NH_3. For every AMP deaminase assay, a substrate blank reaction was set up to account for endogenous ammonia in the protein sample and reagents. A set of NH_4Cl standards was included in every batch of ammonia determinations carried out.

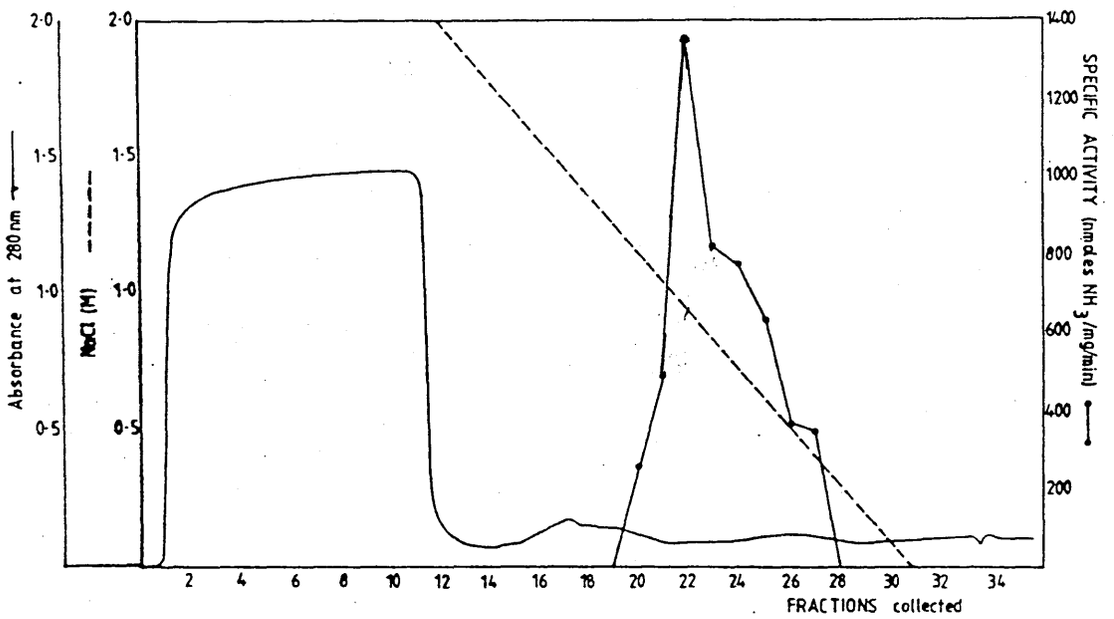
5.9.2 Results

The first step of the purification was carried out using crude extract at a protein concentration of 3.1mg/ml, with an AMP deaminase specific activity of 41.3nmoles/min/mg. 100mls of this extract in 20mM potassium phosphate buffer, pH7.5 was loaded on to the Mono Q 16/10 (20ml) column equilibrated with the same buffer. The column was then washed with the

FIGURE 5.9.2.2 ELUTION OF AMP DEAMINASE FROM A PHENYL-SUPEROSE 5/5 COLUMN WHEN LOADED WITH PARTIALLY PURIFIED MONO Q 16/10 FRACTIONS

Column volume: 1ml
Flow rate: 0.5ml/min
Fraction size: 1ml
Buffer: 20mM potassium phosphate, pH 7.5

Sample: 10.5ml of pooled Mono Q fractions 35 and 36 (see figure 5.9.2.1) in 2M NaCl (see below)



Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
sample	323.0	0.87	372.0
20	18.7	0.072	260.0
21	46.3	0.095	487.4
22	67.5	0.05	1350.0
23	130.0	0.158	823.0
24	132.0	0.172	770.3
25	108.0	0.170	637.0
26	60.0	0.162	370.0
27	26.3	0.075	351.0

facing page 102

starting buffer until there was no further protein elution as determined by absorbance at 280nm. A linear gradient of 0-0.5M NaCl in 20mM potassium phosphate was then applied to the column. 8ml fractions were collected and assayed for AMP deaminase activity.

A trace of the elution of proteins from this column is shown in figure 5.9.2.1. Activity was found in fractions 28 through to 37, the activity peaking at around 0.34M NaCl. 144.6% of the activity loaded was recovered in these fractions, and fraction 36 represents a 14-fold purification.

Fractions 35 and 36 were pooled and made up to 2M NaCl. This pooled material had a protein concentration of 0.87mg/ml and an AMP deaminase specific activity of 371.5 nmoles/min/mg. 10.5mls of this material was then loaded on to a Phenyl-superose 5/5 (1ml) column, equilibrated with 2M NaCl in 20mM potassium phosphate, pH 7.5. The column was then washed with this buffer until no more protein was seen to elute. A linear gradient of 2M-0M NaCl in 20mM potassium phosphate, pH7.5 was then applied to the column and 1ml fractions were collected. These fractions were then assayed for AMP deaminase activity. The elution pattern is shown in figure 5.9.2.2. Fractions 20 through to 27 showed AMP deaminase activity. However only 15.8% of the loaded activity was recovered in these fractions, with fraction 22 representing a 3.6-fold purification. The elution of AMP deaminase peaked at around 1M NaCl. Fractions 21 through to 26 were pooled and concentrated in an Amicon concentrator.

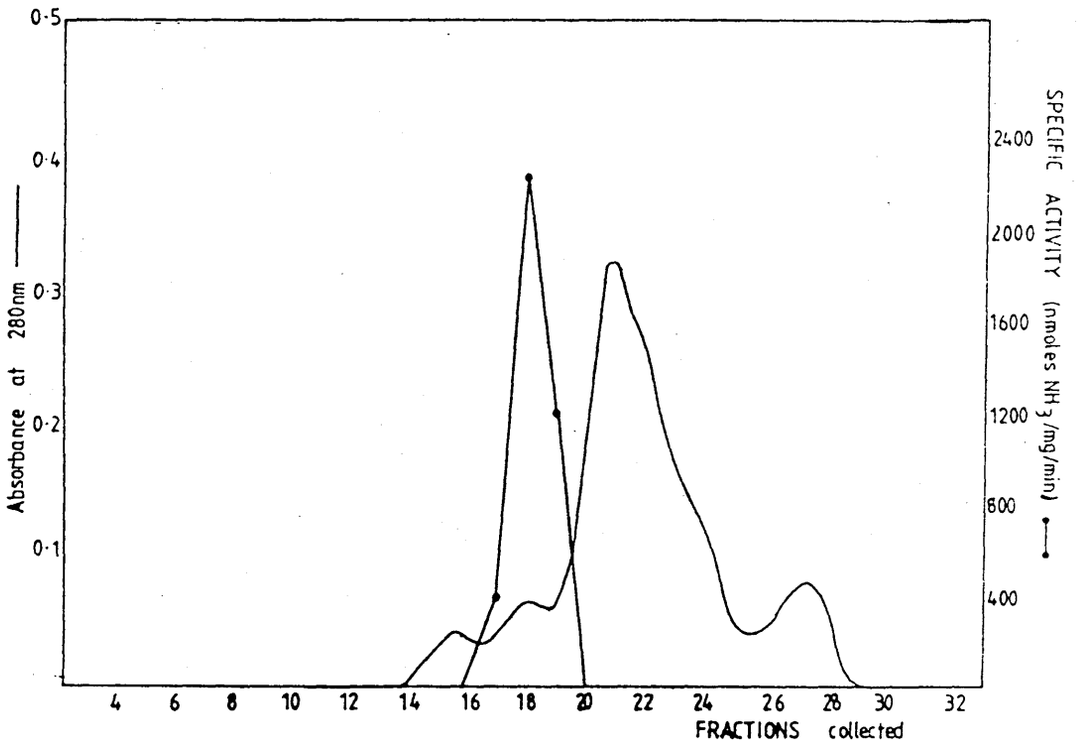
The material was concentrated approximately 10-fold, and 200µl of this material was loaded on a Superose 6 column equilibrated with 50mM potassium phosphate, pH 7.5. This loaded material has an AMP deaminase specific activity of 432 nmoles/min/mg and a protein concentration of 3.5 mg/ml. All protein was then eluted with this buffer and 0.67ml fractions were collected and assayed for AMP deaminase activity. A trace of absorbance at 280nm is shown for this run in figure 5.9.2.3.

Activity was found in fractions 17, 18 and 19. Of the loaded activity 102% was recovered in these three fractions and

FIGURE 5.9.2.3 ELUTION OF AMP DEAMINASE FROM A SUPEROSE 6 COLUMN WHEN LOADED WITH PARTIALLY PURIFIED PHENYL SUPEROSE FRACTIONS

Column volume: 20ml
Flow rate: 0.333ml/min
Fraction size: 0.67ml
Buffer: 20mM potassium phosphate, pH 7.5

Sample: 200µl of concentrated Phenyl superose fractions 21-26 (see figure 5.9.2.2 and below)



Fraction	Activity (nmoles NH_3/ml/min)	Protein (mg/ml)	Specific Activity (nmoles NH_3/mg/min)
sample	1510.0	3.52	429.0
17	117.0	0.3	389.0
18	226.0	0.1	2260.0
19	120.0	0.1	1200.0

fraction 18 represents a 5.2-fold purification. Samples from each stage of the purification were subjected to SDS/PAGE and this gel is shown in Figure 5.9.2.4. All protein electrophoresis was carried out using the Pharmacia Phast Gel system (Chapter 2).

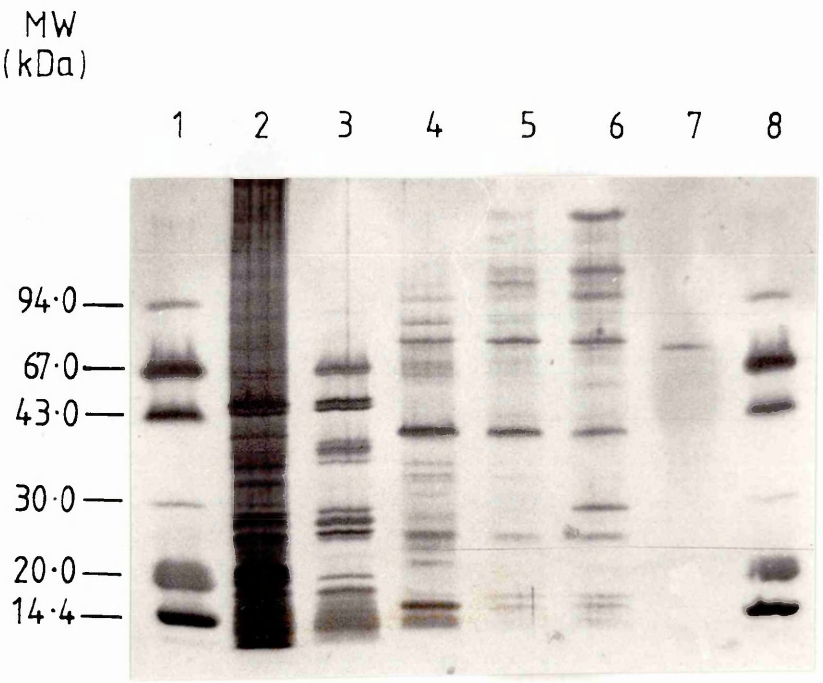
5.9.3 Discussion

The entire purification is detailed in table 5.9.3.1. If these results are compared with those in table 5.1.1 the purification of yeast AMP deaminase by Yoshino et al. (1979b), it can be seen that this purification results in a sample of much lower specific activity, i.e. the purified material described here has a specific activity of 2.26μmoles/min/mg in comparison with that described by Yoshino et al.; 343.3μmoles/min/mg. Both purifications started with extracts of similar specific activity i.e. 0.041 (here) and 0.08μmoles/min/mg (Yoshino et al., 1979b). This low specific activity is also accompanied with a low level of recovery after the three rounds of purification and with a low level of purification (only 54.7-fold purification in the method described here as opposed to 4291-fold purification in the Yoshino method).

It is not clear why the figures from this purification should be so low. It has been seen at each stage of the purification that activity is lost. The reasons for these losses are unknown. It seems unlikely to be due to protease contamination for a number of reasons. Firstly, there was no apparent differences in the activities recovered during purifications which included the protease inhibitor, PMSF and those that did not (data not shown). PMSF was routinely added at all steps of the purification at 1mM.

Alternatively, the enzyme may be undergoing some form of denaturation or inactivation of the catalytic site. Denaturation is normally brought about by extremes of either pH or temperature or by the presence of denaturants. All of these conditions have been avoided as far as possible.

FIGURE 5.9.2.4 AN ELECTROPHORETIC SUMMARY OF THE PURIFICATION OF AMP DEAMINASE



LANE

1 Protein molecular weight standards
2 Crude extract
3 Mono Q fractions 36 (see figure 5.9.2.1)
4 Phenyl-superose fraction 22 (see figure 5.9.2.2)
5 Phenyl-superose fraction 23 (see figure 5.9.2.2)
6 Pooled and concentrated Phenyl-superose sample (see figure 5.9.2.3)
7 Superose 6 fraction 18 (see figure 5.9.2.3)
8 Protein molecular weight standards

Catalytic site inactivation is more complex. It may be brought about by the loss of an essential co-factor, as suggested initially in section 5.3.3 when dialysis appeared to greatly reduce AMP deaminase activity. The loss of this putative co-factor may contribute to a destabilisation of the enzyme, possibly allowing breakdown of its tertiary structure (i.e. the tetrameric enzyme may be breaking down into its monomeric form which is either completely inactive, unstable or is lost at some stage of the purification). This type of inactivation could be prevented if the co-factor was known, which is not the case here.

Another form of active site inactivation is when there is a covalent modification of active site. Cysteine residues at the active site are most susceptible to modification. The sulphydryl group may be in the ionised form and therefore very prone to oxidation. Sulphydryls can undergo a number of modifications, including disulphide bond formation and either partial oxidation to sulphinic acid or irreversible oxidation to sulphonic acid. These modifications are normally avoided by the addition in buffers of a chelating agent, such as EDTA to remove trace divalent cations which activate oxygen molecules and complex with sulphydryls and the addition of a sulphydryl containing compound such as DTT or B-mercaptoethanol to maintain reducing conditions. As discussed in section 5.2.1 all of these precautions are deleterious both to the assay system employed and to the AMP deaminase activity itself.

Another possibility is that AMP deaminase is a fragile enzyme and that during the phase change methods of chromatography the enzyme cannot maintain its structural integrity. AMP deaminase is a large multimeric protein (a tetramer of apparently four identical subunits (Yoshino et al., 1979b) and it is possible that the methods of purification which involve adsorption to a matrix from solution and back again are too stressful to allow the protein to be maintained efficiently in its active form. The final gel filtration, which does not involve a phase change, allows 102% of loaded activity to be maintained suggesting that this more gentle type of purification is more suited to AMP deaminase.

TABLE 5.9.3.1 SUMMARY OF AMP DEAMINASE PURIFICATION FROM S.cerevisiae AS PRESENTED IN THIS CHAPTER

	Specific activity (nmoles NH_3/min/mg protein)	Total Protein (mgs)	Purification factor	% Recovery
Crude extract	41.3	310.0	1	100
Applied to P-sup	372.0	9.14	9.0	26.5
Applied to Sup 6	199.0	0.70	10.5	2.36
Sup 6-Fraction 18	2260.0	0.07	54.7	1.17

Finally, it is known that very dilute enzyme solutions lose activity quickly. This is often prevented by the addition of another protein such as BSA. Extreme dilution of a protein may lead to dissociation of subunits which whether active or not may be unstable and denature quickly. The addition of BSA was not considered appropriate in this purification although with hindsight some trial experiments with it might have proved advantageous. Other stabilising compounds might have been added such as glycerol. The non aqueous components of a cell equal 10-15%, most of which is protein. This high non aqueous component reduces the water activity. Glycerol and some sugars like sucrose or glucose appear to mimic this by forming strong hydrogen bonds with water, slowing down the motion of water molecules and therefore reducing water activity (Gekko and Timasheff, 1981; Smith, et al., 1978). 20-30% glycerol has apparently little effect on ion exchange (Skopes, 1987) although salting out methods and hydrophobic interaction chromatography may well be affected.

Regardless of the reasons for the loss of activity, enough activity was maintained up until this point in the purification to allow the correlation of the activity with a major band on an SDS polyacrylamide gel (data not shown). The size of the AMP deaminase subunit has been reported to be approximately 83kDa (Murakami, 1979). This single major band in fraction 18 (see figure 5.9.2.4), representing approximately 80% of the total protein in this sample, has an apparent molecular weight of approximately 83kDa.

Fraction 18 was freeze dried and sent to ICI Pharmaceutical Division for N terminal amino acid determination.

5.10 N-TERMINAL SEQUENCE OF THE PURIFIED PROTEIN

5.10.1 Introduction

The analyses described in this section were carried out by John Fitton, of ICI Pharmaceutical Division. The protein in fraction 18 was run on a 15% SDS polyacrylamide gel, and the gel was then electroblotted. Protein on the membrane was then detected by Coomassie blue staining and

FIGURE 5.10.1 N-TERMINAL AMINO ACID SEQUENCE OF THE MAJOR POLYPEPTIDE BAND IN FRACTION 18

RESIDUE:	1	2	3
AMP deaminase[1]	?	ALANINE/GLUTAMINE/ LYSINE	SERINE/GLYCINE
hsp90[2]	METHIONINE	ALANINE	SERINE

RESIDUE:	4	5	6
AMP deaminase	GLUTAMIC ACID	?	PHENYLALANINE
hsp90	GLUTAMIC ACID	THREONINE	PHENYLALANINE

RESIDUE:	7	8	9
AMP deaminase	GLUTAMIC ACID	PHENYLALANINE	GLUTAMIC ACID
hsp90	GLUTAMIC ACID	PHENYLALANINE	GLUTAMIC ACID

RESIDUE:	10
AMP deaminase	ALANINE
hsp90	ALANINE

1 This study
2 Farrelly and Finkelstein, 1984

facing page 106

the portion of the membrane corresponding to the 83kDa protein was cut out. This material was then applied directly to an Applied Biosystems Gas Phase Sequencer.

5.10.2 Results

The first ten amino acids of the N terminal of this pure protein were determined, with the assumption that the first residue was methionine since this residue was lost either during purification or the sequencing process. Some of the remaining residues showed ambiguity and the fifth residue could not be determined. The sequence is shown in Figure 5.10.2.1.

This sequence data was compared with those in the protein database and homology was found to the S.cerevisiae heat shock protein 90 (hsp90)(Farrelly and Finkelstein, 1984). The first ten amino acids of this protein are also shown in Figure 5.10.2.1. All the residues which could be determined from the pure protein in fraction 18 match with the reported sequence of hsp90 (Farrelly and Finkelstein, 1984). At residue 5 in the hsp90 sequence corresponds to a threonine. This residue could not be determined in the putative AMP deaminase sequence, but threonine is especially susceptible to degradation in this sequencing process (John Fitton, personal communication).

5.10.3 Discussion

The protein was purified on the basis of AMP deaminase activity and was found to have a subunit size which correlates exactly with that previously reported for AMP deaminase. The N-terminal sequence of this protein is homologous to yeast hsp90. Clearly this raises some interesting questions.

Is it possible that this homology has simply occurred by chance? The chances of two proteins having this level of similarity in the N terminal region is $(1/20)^6 \times 2/20 \times 3/20$ which equals 2.34×10^{-10}. It therefore seems very unlikely

that the sequence homology is a chance occurrence, suggesting that the 80kDa band on SDS gels does correspond to hsp90. Hence, it would appear that AMP deaminase and hsp90 may be one and the same protein. This matter is addressed in the next chapter.

CHAPTER 6

THE RELATIONSHIP BETWEEN AMP DEAMINASE AND HSP90

The Heat Shock Response

In 1962 Ritossa noted that a sudden increase in temperature resulted in the production of novel chromosome puffs in Drosophila cells. Chromosome puffs were known to indicate sites of active transcription. This was accompanied by the concomitant subsidence of previously active puffs (Ritossa, 1962). This finding received little attention until other workers noted that this treatment also resulted in the production of a new subset of proteins (Tissieres et al., 1974). This phenomenon is known as the heat shock response and the particular set of proteins synthesised during heat shock are known as heat shock proteins (hsps).

It is now clear that this phenomenon is not unique to Drosophila but is common to all organisms so far studied. It represents one of the most highly conserved genetic regulatory systems known. Subsequently it became apparent that this reponse was not a function of temperature but can be brought about by a number of stimuli, including amino acid analogues and ethanol (for review of stimuli, see Ashburner and Bonner, 1979). The cellular trigger for this response is unknown but it seems likely that the heat shock proteins function to repair damage caused by different types of stress (for reviews of response see Ashburner and Bonner, 1979; Pelham, 1985; Lindquist, 1986).

Of the heat shock proteins produced, there is great conservation between different organisms. All organisms produce an hsp of between 80 and 90 kDa, one or more of approximately 70 kDa and one or more of between 15 and 30 kDa. The proteins in each class are highly conserved in different organisms suggesting that they have similar functions in the different cell types.

It is worth noting, at this point, that the hsps have been named by their apparent sizes on SDS polyacrylamide gels. Fortunately, in most organisms, hsp70 does appear to be very close to this molecular weight. The case is not as simple

for hsp90. As its name suggests yeast hsp90 had an apparent molecular weight of 90 kDa on the gel systems used in the first reports of this protein (Finkelstein et al., 1982). Subsequent work has shown that the derived molecular weight of this protein from DNA sequence data is approximately 82-83kDa (Farrely and Finkelstein, 1984). It is now frequently known as yeast hsp83, and the homologous proteins in different organisms can be called, variously, hsp82, hsp83 and hsp89. For the purposes of this report I have elected to maintain the original name of hsp90 for the yeast protein, and where the homologous protein from other organisms is named otherwise, it will be accompanied by hsp90 in brackets.

There are differences in the response depending on the organism and the degree/type of stress. In some organisms the response to a temperature rise is transient when the shift is to a temperature at which normal growth can occur. In some organisms heat shock is not transient and this pattern of protein synthesis maintained while the high temperature is maintained. When the temperature is decreased, normal transcription and translation resumes (Lindquist, 1986). Where heat shock is to a non-growth permissive temperature or induced by a lethal stimulus the cell maintains the production of hsps and the response continues until cell death occurs. Even under circumstances where the response is transient there is some evidence that even after normal synthesis is resumed the accumulated hsps are maintained at higher levels than prior to the heat shock for some time (Neidhardt et al., 1984). The response is also modulated by the metabolic state of the cell. Fermenting yeast cells when heat shocked to 36^{o}C show a transient heat shock response, whereas at 40^{o}C the response is maintained. Respiring yeast cells in contrast show the transient response at 34^{o}C but maintain it at 36^{o}C (Lindquist, 1986).

Different organisms and cell types have different mechanisms to facilitate expression of hsps. In almost all cases, immediately upon heat shock, transcription of normal cellular mRNAs ceases, polysomes are seen to disappear for several minutes (Ashburner, 1982) and then polysomes representing heat shock specific mRNAs appear. In Drosophila, the organism in which heat shock was first widely studied, there

appears to be a specific translational control mechanism in addition to transcriptional specificity. Pre-existing mRNAs remain in the cytoplasm but are no longer translated while heat shock lasts. Instead, heat shock messages are specifically translated, and since these messages are induced up to 1000-fold the major hsps rapidly become highly abundant. In Drosophila after prolonged heat shock, hsp70 is more abundant than actin (Pelham, 1985). Once heat shock is over and the cell starts to revert to its normal pattern of gene expression, pre-existing messages are at this stage again translated, indicating that these normal mRNAs are not modified or inactivated in any way, but that upon heat shock the cells translational machinery must be able to specifically recognise and translate heat shock messages (Storti et al., 1980). Therefore, there must be selective alterations in the translation components of heat shocked cells causing selective translation of heat shock mRNAs, and hence there must be information in the heat shock mRNAs to allow their specific translation.

Although this is the classic example of the heat shock response, other organisms respond differently. In yeast, for example, again polysomes break down and normal mRNAs cease to be transcribed as the heat shock mRNAs become actively transcribed to a fairly high level. There appears, however, to be no translational specificity. All cellular messages are translated, including pre-existing ones (Lindquist,1984). Therefore the gradual reduction in the normal translational products are due simply to the depletion of normal cellular mRNAs. This depletion appears to be a function of the specific messages inherent stability. Even so, the hsps soon become major products of protein synthesis. Hsp100 in S.cerevisiae represents 2.5% of total soluble protein 60 minutes after heat shock (McAlister et al., 1979). These differences between Drosophila and yeast account for the more dramatic changes seen on gels of labelled proteins for Drosophila in comparison to yeast (Lindquist, 1984).

It is thought that these differences in the evolution of mechanisms are at least partly due to the inherent differences in mRNA stability in the different organisms. Drosophila mRNAs have average half lives in the region of 8

hours and therefore to allow the remaining cellular mRNAs to compete for the translational machinery of the cell would mean a long delay in the accumulation of the required hsps. Yeast on the other hand, has mRNAs with stabilities in the region of 5 to 100 mins (Santiago et al., 1986). Therefore most cellular messages are quickly degraded allowing the hsp-specific mRNA to quickly become the major substrate of translation (McAlister and Finkelstein, 1980b). In support of this theory is the differences in the mechanism of heat shock response seen in Xenopus somatic cells and oocytes. Xenopus somatic cells react to heat shock in a way very similar to Drosophila with regulation at both the transcriptional and translational level. Oocytes, conversely show no transcriptional effects on heat shock. It appears that hsp mRNAs are in existence at high levels within the oocyte, prior to heat shock and upon heat shock they become the sole substrates for translation, hence the hsps rapidly accumulate (Lindquist, 1984). It is thought that the reason for this novel response to heat shock is due to the unusually large size of the oocyte. If the accumulation of hsps required depended on newly transcribed messages from a single genome, it is estimated that for the hsps to reach the necessary concentration within this large cell would take several days.

Hsps are generally defined as those whose synthesis is sharply and dramatically induced at high temperatures. It is presumed that these proteins are required for the homeostatic regulation of the cell to a rapid but physiological environmental change. However there are a number of reports of minor proteins which are also induced by heat. These analyses normally involve the use of very large 2-dimensional protein gels. Some estimates for the number of these minor induced proteins are as high as 70 (Anderson et al., 1982; Reiter and Penman, 1983; Maytin et al., 1985).

As well as the induction of a specific subset of proteins, other physiological effects of heat shock have been reported. In particular it has been shown in yeast that a heat shock to a moderate temperature or treatment with ethanol can save the cells from thermal killing at a non physiological temperature as well as effects of some mutagens, including ionising

radiation (Plesset et al., 1982; Mitchel and Morrison, 1983 and 1986). There appears to be a good correlation under these conditions between the levels of hsps and the level of protection, but subsequent work has shown that protein synthesis is not required to induce this thermotolerance (Hall, 1983; Watson et al., 1984; Cavicchioli and Watson, 1986). It seems likely that thermotolerance is induced either by the activation of a pre-existing cellular component activated at the higher temperature or by the inactivation of a component at the increased temperature which in some way interferes with thermotolerance.

Heat shock has a number of other effects. It has been shown in two species of Tetrahymena that within 3 minutes of heat shock, the cellular ATP content is reduced by 50% (Findley et al., 1983). There is no accompanying increase in ADP or AMP, but there is an increase in Pi, suggesting that ATP is hydrolysed to the free nucleoside.

It has also been shown that heat shock or other stimuli induce a significant drop in internal pH (Weitzel et al., 1985 and 1987). Other effects reported are the accumulation of trehalose in yeast (Attfield, 1987; Hottinger et al., 1987) and an increast in the level of phosphotyrosine in proteins in most cultured cells (Maher and Pasquale, 1989).

Another interesting effect of heat shock is that cells appear to be transiently arrested in G1 phase (Shin et al., 1987). Also it was shown that yeast cells which are in G0 show qualitative differences to actively growing cells. They show different RNAase activity, poly A content and specifically synthesise about 20 proteins, 5 of which are hsps (Lindquist, 1984; Boucherie, 1985). This finding has subsequently been found in chick embryo fibroblasts, mouse lymphocytes and Drosophila cells (Iida and Yahara, 1984a). Along with this is the finding that stationary cells are more resistant to thermal killing than exponentially growing cells.

Hsps in development

As previously stated several hsps are also present in non heat shocked cells, suggesting that they fulfil necessary functions in the unstressed cell. Interestingly a specific subset of hsps are induced during normal development in a number of organisms. Hsps 28, 26 and 83 (hsp90) are abundantly transcribed during oogenesis in Drosophila ovarium nurse cells (Lindquist, 1984). They then pass into the oocyte at stages 10 - 11. Curiously, pre-blastoderm embryos fail to accumulate these hsps even if heat shocked (Zimmerman et al., 1983; Kurtz, 1986). Hsp70 is not induced at this time. Similarily yeast hsp26 and 90 are strongly induced as the cells approach stationary phase and during spore formation (Kurtz and Lindquist, 1984; Lindquist, 1986). This differential accumulation indicates the existence of differential and possibly multiple controls of heat shock gene expression and belies the original view of the heat shock response as the classical example of coordinate regulation of a family of genes.

The expression of Heat Shock proteins

The question of the regulation of the hsps expression has attracted a great deal of attention. A number of workers have examined the chromatin structure of heat shock genes. In Drosophila the 5' ends of heat shock genes are hypersensitive to treatment with DNAse I, as though they are prepared for immediate transcription (Wu, 1980). Fine mapping of this region in 5 Drosophila heat shock genes shows that there are at least 2 hypersensitive regions, one representing the TATA box, the other is a region found approximately 115bp upstream of the transcriptional start site. This region has been widely studied and is now known as the heat shock element (HSE) (Holmgren et al., 1981; Costlow, 1984). There is evidence that this is a protein binding site (Sorger and Pelham, 1987). The element appears to be a hyphenated dyad, and the consensus in Drosophila is given thus

C__GAA__TTC__G

Although only a 5\8 match is sufficient for the 10-fold induction of one hsp (Craig, 1985), this element is highly conserved throughout evolution, and the Drosophila HSE has been shown, when present in two or more copies, to be sufficient for heat shock specific induction of genes in yeast (Wei et al., 1986; deBanzie et al., 1986) and monkey COS cells (Pelham, 1985), suggesting great conservation not only of the hsps themselves but also of the regulatory mechanisms associated with their expression.

Using these consensus sequences as substrates for affinity chromatography a heat shock binding or transcription factor (HSBF or HSTF) has been purified from both yeast and Drosophila (Wiederrecht et al., 1987; Sorger and Pelham, 1987 and 1988). Both are 70kDa polypeptides, and the Drosophila HSTF has been show to stimulate HSE-dependent transcription in yeast.

A recent report has suggested that the HSE is better described as a dimer of the following 10bp consensus (Xiao and Lis, 1988)

NTTCNNGAAN

and this has led to the suggestion of the following yeast specific consensus (Tuite et al., 1988)

nnTTCTAGAAnn

Of the heat shock genes studied very few have introns. In fact only the hsp83 (hsp90) of Drosophila, chick and human (Lindquist, 1986; Vourch et al., 1989; Hickey et al., 1989) and a small hsp of nematodes (Lindquist, 1986) fall into this category. There may be a very good reason for this. In Drosophila, hsp83 (hsp90) shows a different pattern of expression to other hsps. It is produced at moderately high temperatures but not very high temperatures. There appears to be a block in the processing of the RNA at very high temperatures (Lindquist, 1986), hence it makes sense that few HSP genes contain introns. Interestingly, the first intron in all these hsp90 genes are all adjacent to the ATG translational start codon.

Prior to heat shock hsp90 RNA is found solely in the poly $(A)^-$ fraction, but after heat shock it is dispersed between the poly $(A)^+$ and $(A)^-$ fractions (Craig, 1985; Storti et al., 1980). In the case of Hela cells at least, this distribution between both fractions is also seen for hsp100, 70 and 37 (Burdon and Cutmore, 1982).

The function of the hsps

Although a great deal is known about the expression of hsps and the physiological effects of stress, very little is known about the specific functions of hsps. Most progress has been made in this field where known gene products have been inadvertantly shown to be heat inducible. Included in this category are several yeast glycolytic genes including glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (Piper et al., 1986). In addition, in yeast an isozyme of enolase has been shown to be hsp48 (Iida and Yahara, 1985) but this was not confirmed by another report (Uemara et al., 1986). One interesting addition to this category is ubiquitin (Finley et al., 1984; Bond and Schlesinger, 1985; Munro and Pelham, 1985). This small polypeptide has a role in directing the attack of proteinases (Finley et al., 1984). It has been suggested that ubiquitin-linked protein degradation has a key role in the regulation of the heat shock response. One of the physiological effects of heat shock is to cause the accumulation of denatured or aberrant proteins, which may require to be removed from the cell before the cell can recover (Pelham, 1986).

As yet, none of the major hsps have been assigned a proven function. To help elucidate their functions a lot of effort has been put into localising the proteins within the cell. It is now apparent that hsp70, is a cytoplasmic protein which moves to the nucleus, and particularily the nucleolus on heat shock (Welch and Feramisco, 1985). Hsp90 appears to be an abundant cytoplasmic protein. The low molecular weight hsps in mammals appears to form aggregates of unknown function both in the cytoplasm and the nucleus (Lindquist, 1986). Hsp100 in yeast and hsp110 in mammals also appear to be associated with the nucleolus, whereas mammalian hsp100 seems

to be a golgi protein. However in mouse, two proteins related to hsp70 and hsp90 have been found in the lumen of the endoplasmic reticulum (Pelham, 1986; Craig et al., 1987). In most eukaryotes there are several members of the hsp70 family, some of which are heat shock induced, some not. It is possible that each member is fulfilling similar functions but in different cellular compartments both prior to and during heat shock.

There is an ever increasing number of fragments of information regarding the function of the hsps. Hsp26 in Drosophila has been shown to be an RNA-binding protein (Kurtz and Lindquist, 1984). Hsp70 as the most abundant and conserved hsp has attracted the greatest effort, and its function is becoming apparent. It was shown in 1985 to be an ATP binding protein (Welch and Feramisco, 1985; Lewis and Pelham, 1985) and Chirico et al. (1988) recently demonstrated that yeast cytoplasm contains two distinct activities that stimulate protein translocation. Both activities are constitutively expressed hsp70-related proteins. It is now clear that at least some of the hsp70 family are involved in post-translational import of precursor polypeptides into the mitochondria and endoplasmic reticulum lumen (Deshaies et al., 1988). This has led to the classification of hsp70 as a molecular chaperone or chaperonin (Ellis and Hemmingsen, 1989) along with such proteins as BiP which has been implicated in the assembly of the oligomeric enzyme ribulosebisphosphate carboxylase oxygenase in chloroplasts (Hemmingsen et al., 1988). It is proposed that during heat shock, nuclear components are damaged and that binding to, being released from and rebinding to hsp70 can reassemble these denatured or aggregated proteins (Pelham, 1988).

Heat Shock Protein 90

Hsp90 has, as yet, been assigned no clear function. It is the second most highly conserved hsp; the Drosophila and E.coli proteins are 36% homologous, with some regions showing 90% homology at the amino acid level (Lindquist,1986). All organisms so far studied contain an hsp in the region of 80 - 90 kDa and the protein always has a fairly acidic pI at

between 5.1 and 5.4. In eukaryotes it is a major cytoplasmic protein under non-stressed conditions, and in vertebrates it is methylated at lysine and arginine residues, and phosphorylated. It has also been shown to be glucose regulated. Under glucose starvation its synthesis is repressed (Kasambalides and Lanks, 1983).

It has also been shown in Hela cells, rabbit reticulocytes, Xenopus and Arbacia egg extracts that double stranded DNA induces the phosphorylation of several proteins including hsp90. This double stranded DNA-dependent phosphorylation is not seen in mouse cell extracts (Walker et al., 1985). The implication of this form of regulation of hsp90 phosphorylation is unclear.

Recently a tissue specific transplantation antigen has been purified to homogeneity from a methylcholanthrene-induced tumour in mice. The antigen consists of two similar polypeptides of 84 and 86 kDa both of which are highly homologous to Drosophila and yeast hsp90. The antigen is a highly abundant cytosolic protein but it is also present at the cell surface and is hence accessible to the host's immune system (Ullrich et al., 1986).

Most cellular hsp90 is found in a monomeric form (Lindquist, 1986) but it has also been reported that chick hsp89 (hsp90) can be isolated as an aggregate with the predominate form having a molecular weight of 560 kDa (Schlesinger et al., 1982). Very recently, Radanyi et al. (1989) presented evidence that in chick oviduct cells, hsp90 exists as a dimer.

There is a great deal of evidence to show that hsp90 is to be found associated with a wide number of cellular components, including viral transforming proteins and steroid hormone receptors.

In 1981, Brugge et al. used sera from rabbits bearing tumours induced by Rous Sarcoma virus to purify the transforming protein $pp60^{src}$. The precipitated protein consisted not only of the $pp60^{src}$ but two other transformation specific phosphoproteins named pp50 and pp90. These proteins were not

precipitated with sera from uninfected cells or transformation defective-virus infected cells (Brugge et al., 1981). Sedimentation analysis revealed that $pp60^{src}$ is found within the cells in two forms. 95% are monomeric and the remaining 5% is associated with the other two proteins in a complex of approximately 200 kDa. pp50 is as yet unidentified and pp90 is now known to be hsp90. $pp60^{src}$ functions by phosphorylating IgG, but only the monomeric form is capable of this. $pp60^{src}$ in the monomeric form has both phophotyrosine and phosphoserine residues, in its complexed form it has been shown to contain much less phosphotyrosine.

Other work has shown that these associations occur in many vertebrate sources (Oppermann et al., 1981) and also in cells transformed by Fujinami and Yamaguchi Sarcoma viruses (Lipsich et al., 1982). The complexed form is mostly cytosolic, the monomer is membrane associated (Yonemoto et al., 1982), In 1983, Brugge et al. went on to show that only a discrete population of $pp60^{src}$ complexes with pp90 and pp50 and that this population represents newly synthesised $pp60^{src}$. The complex is short lived with a half life of approximately 15 minutes, and it has been found under a number of lysis conditions, even in the presence of detergents (Brugge et al., 1983). The complex has not yet been reconstructed in vitro.

Shortly after the finding that hsp90 associates with the $pp60^{src}$ transforming protein, came a report that hsp90 is also found associated with immunoprecipitated chick oviduct progesterone receptor (Catelli et al., 1983; Gasc et al., 1984). Later it was shown that this association was not confined to progesterone receptors, but was also apparent for at least four other steroid hormone receptors (Joab et al., 1984). In fact the antibody raised for the immunoprecipitation of these receptors was directed against hsp90. However, it was also reported by Sanchez et al. (1985) that in the case of the glucocorticoid receptor, the association was only seen in the presence of molybdate and that if the complex was incubated at 25°C, dissociation of subunits occurs.

Non-transformed hormone receptors found in the cytosol of target cells have a sedimentation coefficient of approximately 8S and a molecular weight of 250 - 300 kDa. The receptor alone has a sedimentation coefficient of 4S, while the receptor-hsp90 complex has a sedimentation coefficient of 8S. The 8S complex is incapable of binding further hsp90 (Catelli et al., 1985). There is evidence that two molecules of hsp90 and one hormone receptor make up this 8S complex (Radanyi et al., 1989).

In 1985, Binart et al. reported that the amino acid sequence of chick hsp90 reveals a "DNA-like" structure which may be the potential site of interaction with steroid receptors (Binart et al., 1985). They proposed that this region, in the absence of hormone, can interact with and "cap" the positively charged binding domain of steroid receptors. Recently the hormone receptor-hsp90 complex has been demonstrated in vitro (Denis and Gustafsson, 1989), suggesting that the complex is not merely an artefact of homogenisation.

There have been isolated reports of hsp90 associating with other cellular proteins including actin (Koyasu et al., 1986), the halogenated aromatic hydrocarbon receptor (Ah receptor) (Perdew, 1988), tubulin (Sanchez et al., 1988) and the heme-sensitive eukaryotic initiation factor-2 kinase (Rose et al., 1989). It has been proposed that hsp90 functions by binding to newly synthesised proteins and serves to move them about within the cell to the membrane or other cellular location, possibly holding them in an inactive form (Lindquist, 1986). However hsp90 is an abundant protein and at least in the chick oviduct cytoplasm only about 1% of it is associated in the 8S hormone receptor complex. This begs the question, is there a hundred-fold excess to ensure that all target proteins will be successfully complexed or does hsp90 have another function?

Yeast hsp90

In 1982, Finkelstein et al. showed that the pattern of poly $(A)^+$ RNA dramatically changes on heat shock. Using in vitro

translation of heat shock RNA they demonstrated that a 2.9kb RNA codes for hsp90. By differential plaque hybridisation using heat shock and unshocked RNA, heat shock responsive genes (including the gene for hsp90) were isolated. The same group later went on to study the effect of an increased gene dosage of HSP90 as supplied on a multicopy plasmid. This apparently had no affect other than increasing the level of hsp90 on heat shock (Finkelstein and Strausberg, 1983; Finkelstein, 1983).

In 1983 the same group (Farrely et al., 1983) isolated the HSC90 gene (heat shock cognate), whose product is 90% homologous to HSP90. They showed that hsc90 is an abundant protein under non-stress conditions whose expression is only affected slightly by heat shock. Hsp90 from yeast has a predicted molecular weight of 81418 Daltons. It is an acidic protein, composed of 13.4% glutamate which contains no cysteines. It has a novel region of 21 charged amino acids with the structure $(acid_3\ lys_2)_3\ acid_4\ lys_2$. The most significant difference between hsp90 and its cognate is that hsc90 lacks one of the charged pentapeptide units (Farrely et al., 1983).

In 1984 the entire DNA sequence of the HSP90 gene was published (Farrely and Finkelstein, 1984). All the sequences for the heat shock regulated expression reside no more than 273 residues 5' of the transcription origin. By transcript mapping it was shown that the hsp90 mRNA carries a 59 nucleotide 5' untranslated region, a coding sequence of 2130 bases and a 3' untranslated region of 128 nucleotides. The 5' end of this gene shows only limited homology with the Drosophila HSP83 (HSP90) gene, and the codon useage is not random, diagnostic in yeast of an efficiently expressed gene (Sharp et al., 1988)

Is hsp90 actually AMP deaminase?

As stated at the end of the last chapter, when purified AMP deaminase-containing fractions were subjected to N terminal amino acid analysis, it was found that those of the first 10 residues which could be determined were identical to the N

terminal of yeast hsp90 (Farrely and Finkelstein, 1984). This finding begs the question; are AMP deaminase and hsp90 the same protein? There are several lines of evidence both for and against this hypothesis.

The major fact in favour of the above hypothesis is that AMP deaminase activity co-purifies with the hsp90 protein in samples apparently containing only a single protein species of the appropriate size. Is it likely that AMP deaminase would function during heat shock? AMP deaminase (as discussed in Chapter 1) has been strongly implicated in the stabilisation of the cell's adenylate energy charge. The cell must maintain the ratio of ATP to ADP to AMP at a high level to remain viable. It seems likely that during heat shock the maintenance of the energy charge would play a major role in the cells ability to recover, since most cellular metabolism must at the very least be perturbed by this form of induced stress. Pelham (1985) has suggested, that a major physiological effect of heat shock, and possibly the actual trigger for the response is the accumulation of denatured and aberrant protein. He also suggests that this protein aggregate is renatured and reassembled by the action of hsp70 via the hydrolysis of ATP. If this is case the cellular requirement for ATP will be greatly increased. Along with this, goes the finding by Findley et al. (1983), that at least in two species of Tetrahymena the cellular ATP concentration is reduced by 50% within the first 3 minutes of heat shock. It seems possible that the cell, in its attempt to recover from this stress, would evolve methods of maintaining a high energy charge, both by synthesising extra ATP and possibly by removing cellular AMP by the action of AMP deaminase as described in Chapter 1. There have been several reports of glycolytic genes which are induced during heat shock, at least in yeast (Piper et al., 1986), presumably this induction is to allow continued ATP production via glycolysis during heat shock. Therefore, it is not unlikely that an enzyme which maintains a high energy charge might also be induced during heat shock. In addition, the AMP deaminase reaction may be useful to the cell in a different way during heat shock. There have been a number of reports that the pH of the cell is reduced significantly during heat shock (Weitzel et al., 1985 and 1987). This

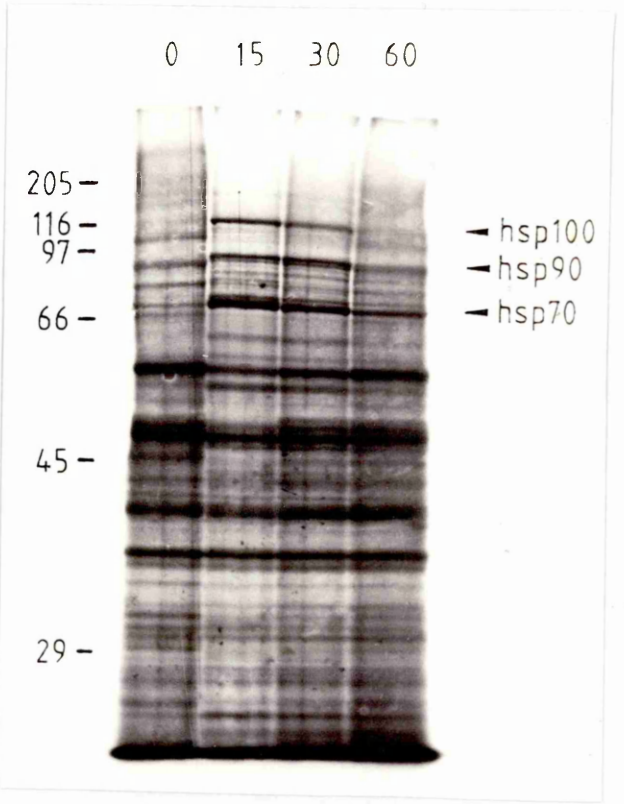
major physiological alteration must be restored in some way and there is evidence in a number of tissue types that the AMP deaminase reaction is one of the major sources of cellular ammonia. This increase in the production of ammonia may also help the cell to restore its pH to normal.

Another feature which supports the hypothesis that AMP deaminase and hsp90 are the same protein is their respective molecular weights. Hsp90 from yeast has a calculated molecular weight of 81412 (Farrelly and Finkelstein, 1984), which is in very good agreement with the apparent molecular weight of yeast AMP deaminase at 82000 (Yoshino et al., 1979b). Conversely, this close agreement in size might explain why only a single band is resolved on SDS gels in the case that the two proteins have simply co-purified.

There is however some strong evidence against the proposed hypothesis. There is strong evidence to indicate that a homologue of eukaryotic hsp90 exists in E.coli. It seems likely that the E.coli heat inducible dnaK gene product is functionally homologous to hsp90 in eukaryotes. AMP deaminase activity has never been shown in any prokaryotic species, and it is widely held that the role AMP deaminase plays in the eukaryotic purine nucleotide cycle is played, at least in part, by the enzyme AMP nucleosidase (Leung and Schramm, 1980). DnaK and the Drosophila hsp83 (hsp90) are only 36% homologous, although some regions are up to90% homologous. Is it possible that both are AMP metabolising enzymes which do not fulfil the same function. This would account for regions of homology.

Stronger evidence against the hypothesis exists in the fact that hsp90 has been shown to be a glucose regulated protein in mammalian cells (Lanks and Kasambalides, 1979; Kasambalides and Lanks, 1983; Welch et al., 1983; Craig, 1985). Its synthesis is strongly repressed upon glucose starvation. This does not fit in with the theory that hsp90 is AMP deaminase. During glucose starvation one would expect that the importance of a balanced energy charge, as aided by AMP deaminase activity, would be more essential than under good growth conditions.

FIGURE 6.2.1 IN VIVO ^{35}S LABELLED YEAST PROTEINS BEFORE AND AFTER HEAT SHOCK



Aliquots of a culture were labelled for 10 minutes with [^{35}S]-methionine in GYNB and the proteins were extracted. Equal portions of radioactivity were loaded onto each lane of a standard 9% SDS polyacrylamide gel. The gel was dried and the resulting autoradiograph is shown. Note that the heat shock response is confirmed by the production of diagnostic heat shock proteins.

Lane designations - the times at which samples were taken for AMP deaminase assays following transfer from 23^{o}C to 37^{o}C.

Note: Sample denoted 15 was labelled from T_{10} to T_{20}, sample denoted 30 was labelled from T_{25} to T_{35} etc., as explained in section 6.2.

Numbers on the left of the gel are the sizes of molecular weight standards in kDaltons and the positions of hsps 70, 90 and 100 are noted at the right of the diagram.

facing page 124

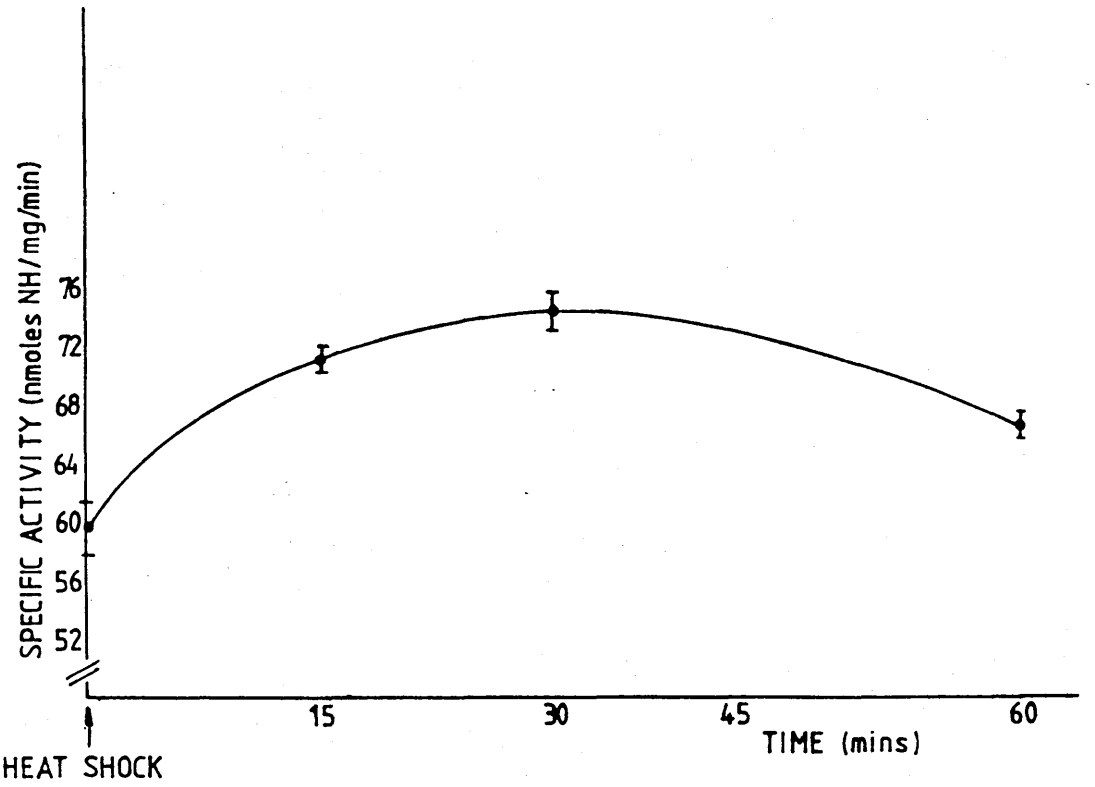
The largest body of information about hsp90 is with respect to its apparent association in the cell with a large and diverse number of proteins and cellular components, notably with steroid hormone receptors and sarcoma virus transforming proteins in vertebrate cells. These associations have led to the proposal that hsp90 functions by associating with these proteins and shuttling them to different cellular compartments. Pelham has proposed that on heat shock the cells accumulate aggregates of denatured proteins both in the nucleus and elsewhere and that hsp90 is induced to aid the disassembly and transport of these complexes (Pelham, 1985 and 1986).

Intriguingly, there is some conflicting information about the form in which hsp90 normally exists within the cell. Apparently most exists as a monomer (Lindquist, 1986). However two separate reports have indicated that in chick oviduct cells the major form has a molecular weight of approximately 560 kDa (Schlesinger et al., 1982) and a very recent report puts the major form as a dimer of molecular weight of 180 kDa (Radanyi et al., 1989). This conflicting information may give an indication of the nature of hsp90. A lot of the complexes studied have been shown to be short lived and few have been reconstructed in vitro. Could it be that at least some of the reports are simply of artefacts of the extraction processes used, and that in actual fact hsp90 is simply a highly hydrophobic protein which during extraction may not maintain its normal physiological form but complex, preferentially with other hydrophobic proteins. AMP deaminase is apparently a tetramer protein (Murakami, 1979), and so presumably there are hydrophobic regions on the surface of each subunit, which might account for its possible association with other proteins.

6.2 THE EFFECT OF HEAT SHOCK ON AMP DEAMINASE ACTIVITY

Is hsp90 AMP deaminase? In an attempt to answer this question, the effect of heat shock on AMP deaminase activity in yeast was investigated. To this end mid log cultures of strain DBY 747 were heat shocked as described in Chapter 2 and small portions of the culture were in vivo labelled with

FIGURE 6.2.2 AMP DEAMINASE ACTIVITY BEFORE AND AFTER HEAT SHOCK (23°C - 37°C)



TIME AFTER HEAT SHOCK (mins)	SPECIFIC ACTIVITY (nmoles NH_3/mg protein/min)
0	59 +/-2.5
15	71 +/-1.0
30	74 +/-2.0
60	66 +/-1.0

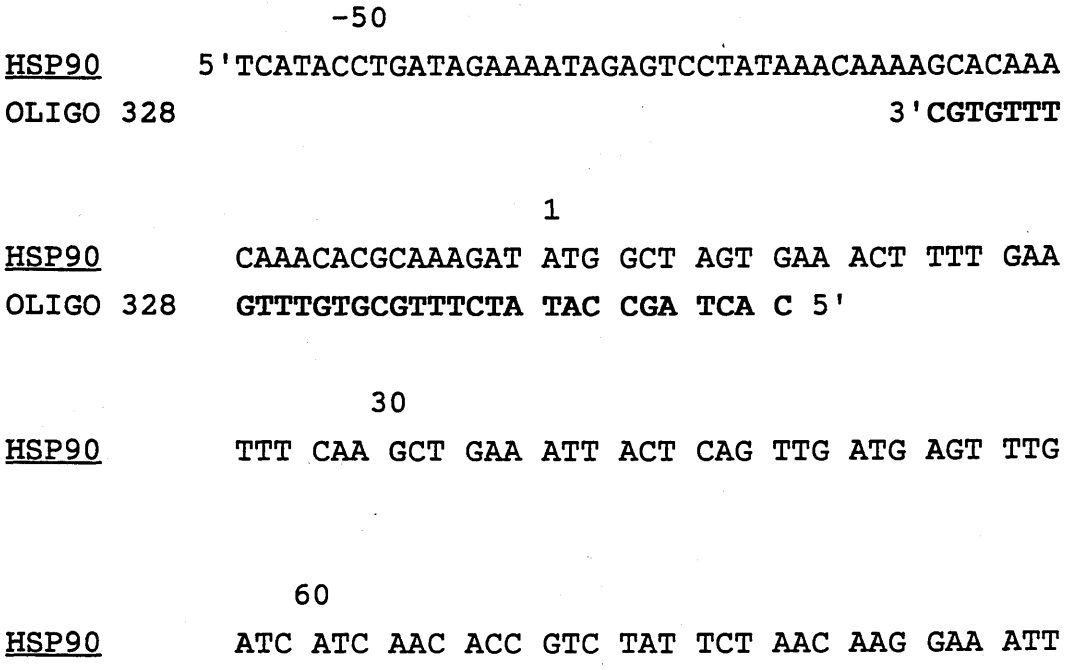
AMP deaminase determinations were carried out in triplicate with a single substrate blank and the figures given are an average of these determinations

[^{35}S]-methionine at different times during heat shock to confirm that heat shock had occurred. The [^{35}S]-labelled protein samples taken at times, T_0, T_{15}, T_{30} and T_{60} minutes were extracted, into SDS loading buffer (a detailed account of this method is given in section 6.5). Portions of these labelled samples were counted in a scintillation counter and equal amounts of radioactivity were applied to a 9% SDS polyacrylamide gel. An autoradiograph of this gel is shown in Figure 6.2.1. As indicated hsps 100, 90 and 70 are clearly induced in T_{15} and T_{30} samples, as well as a number of minor species. Most of these changes have reverted by the T_{60} time point as might be expected from a fairly mild heat shock to 37^{o}C (Piper et al., 1988). From this gel it is clear that the culture had been successfully heat shocked. Protein extracts were prepared from larger portions of the culture taken at the same time points and these extracts were assayed for AMP deaminase specific activity. The results of this experiment are shown in Figure 6.2.2.

As in chapter 3 all AMP deaminase determinations in this chapter are the average of three identical AMP deaminase assays with a single substrate blank. In this chapter ranges are shown and are in agreement with those previously quoted (+/- 4%).

As can be seen from these results there is a slight increase in the AMP deaminase specific activity for the first 30 minutes which then recedes by 60 minutes. Hsp90 has been reported to be induced ten-fold in heat shocked cultures, which correlates well with the result shown in Figure 6.2.1. However these findings do not correlate the increase in the synthesis of hsp90 with an increase in AMP deaminase activity. However, the synthesis of most yeast proteins is greatly reduced during heat shock since their mRNAs are no longer being transcribed, and this does not seem to be the case for AMP deaminase, since its activity is at least maintained through heat shock. This discrepancy does not rule out the possibility that hsp90 is AMP deaminase. In most reported heat shock experiments only changes in protein synthesis are examined, not the actual abundances of a particular protein species. As discussed in Section 6.1 yeast, as well as containing a gene for HSP90 which is highly

FIGURE 6.3.1.1 THE DESIGN OF AN HSP90 GENE SPECIFIC OLIGONUCLEOTIDE PROBE



The nucleotide sequence presented is the sense strand of the HSP90 gene. The nucleotide marked +1 is the putative translation origin of hsp90. The nucleotide sequence of oligonucleotide 328, which is shown in bold, is complimentary to the plus strand, and therefore is also complimentary to the mRNA.

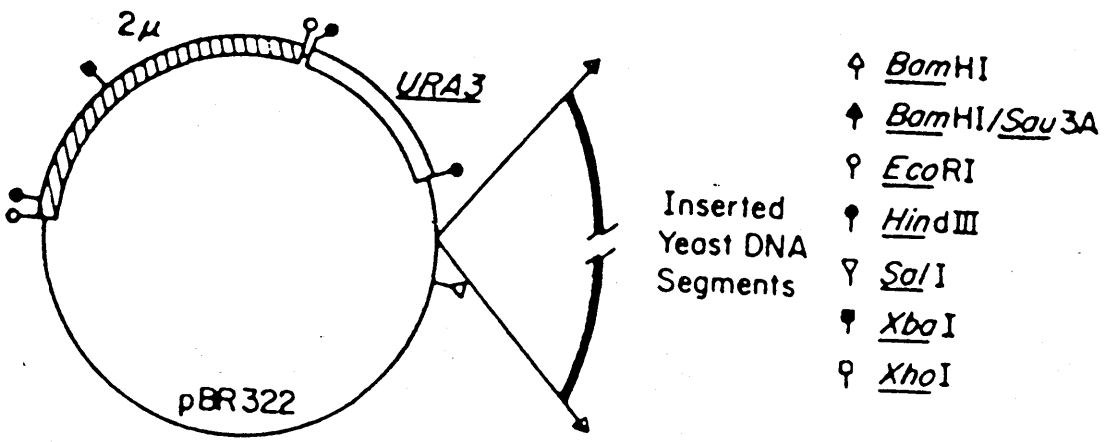
inducible under heat shock, contains the heat shock 90 cognate gene HSC90, which is only mildly affected by heat shock (Farrelly et al., 1983). The two genes are 90% homologous and are therefore probably fulfilling similar functions. Together, hsp90 and hsc90 constitute a major cytoplasmic protein under normal conditions. Therefore, although the rate of hsp90 synthesis may be induced ten-fold, this may not reflect the actual increase in abundance of the protein. This experiment was inconclusive in determining whether hsp90 and AMP deaminase are one and the same and hence the decision was made to study the effects of insertionally inactivating the HSP90 gene and studying the effect on AMP deaminase activity, before and during heat shock. The steps to facillitate this, and the results of this experiment are discussed in the rest of this chapter.

6.3 ISOLATING THE HSP90 GENE FROM A YEAST GENOMIC LIBRARY

6.3.1 Designing an oligonucleotide to facilitate HSP90 gene isolation

The first step in constructing a strain which is insertionally inactivated for HSP90 by the integration of a yeast integrating vector is the isolation of the HSP90 gene. As stated earlier, the HSP90 gene of S.cerevisiae has been cloned and sequenced by Farrelly, Finkelstein and coworkers (Finkelstein et al., 1982; Finkelstein and Strausberg, 1983; Farrelly and Finkelstein, 1984). Although a request for this gene was made, it was not sent. Figure 6.3.1.1. shows the nucleotide sequence of the HSP90 gene as determined by these workers from -60bp upstream of the coding region and including the first 30bp of the coding region. Not shown, are the transcriptional start and the heat shock consensus element (Tuite et al., 1988) The sequence of the oligonucleotide synthesised for the purposes of this experiment is shown in this figure. The oligonucleotide is a 32mer spanning the beginning of the coding region and some of the 5'untranslated region of the mRNA. The two genes HSP90 and HSC90 are highly homologous (Farrelly and Finkelstein, 1984). The sequence of the HSC90 gene has not been published but it was assumed that because of the differences in expression of these two genes

FIGURE 6.3.2.1 STRUCTURE OF THE YEp24 LIBRARY (Carlson and Botstein, 1982)



Yeast genomic DNA was partially digested with Sau 3A and fragments of greater and equal to 10kb were selected and cloned in the the unique Bam HI site of vector YEp24.

the 5' regions was likely to be different. Therefore, this part of the sequence was used for the construction of the oligonucleotide. It was thought that an oligo hybridising to this part of the HSP90 gene would be less likely to select inadvertantly for the HSC90 gene. In addition if the HSC90 gene were selected it could easily be distinguished from the HSP90 gene by sequence analysis of clones using this oligo as primer. The oligo was also synthesised to be complementary to the RNA sequence as well as the DNA, to allow it to be used in RNA analyses, as well as in the isolation of the HSP90 gene. Therefore the sequence of the synthesised oligonucleotide, named oligo 328 is

5' CACTAGCCATATCTTTGCGTGTTTGTTTGTGC 3'

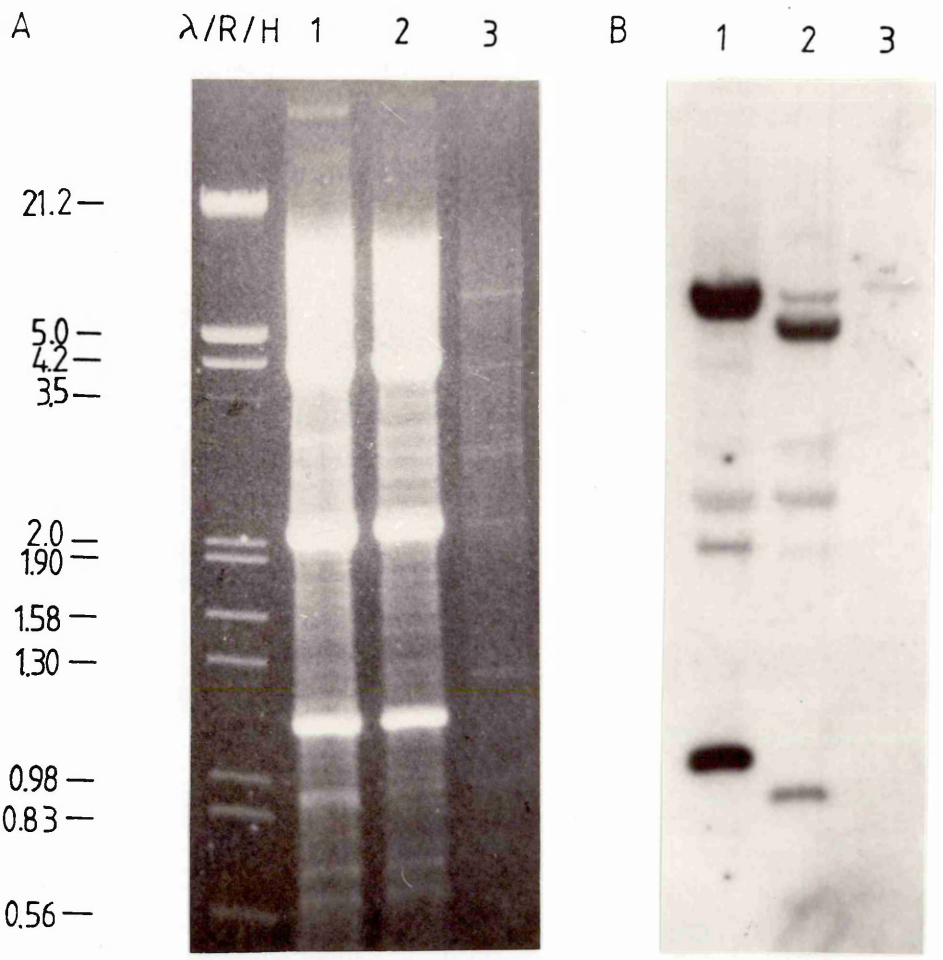
Oligo 328 was synthesised by Dr. V. Math of the Biochemistry Department, Glasgow University.

6.3.2 Screening the YEp24 libraries with Oligo 328

Available in this laboratory were aliquots of David Botsteins YEp24 yeast genomic library (Carlson and Botstein, 1982) transformed into E.coli. This library was made by cloning partial Sau3A yeast genomic fragments into the BamHI site of the yeast-E.coli shuttle vector YEp24, see Figure 6.3.2.1. The inserted fragments were size selected to 10kb and greater. The aliquots available represented two separate libraries made in parallel experiments (David Botstein, personal communication) and are named 378 and 380. Aliquots of these libraries were plated on to LB agar + ampicillin. In total approximately 100,000 colonies were plated per library. These plates were grown overnight at 37^{o}C. The cells were then scraped off into LB and this cell suspension was treated in the usual way to Birnboim-Doly plasmid DNA preparation followed by Caesium Chloride density gradient centrifugation (see Chapter 2).

Equal amounts of DNA from both libraries were subjected to restriction endonuclease digestion with Hind III and run on a 0.8% agarose gel. Figure 6.3.2.2.A shows this gel. In lane 3 is X4003-5B genomic DNA also digested with Hind III, to

FIGURE 6.3.2.2 SOUTHERN BLOT ANALYSIS OF PLASMID LIBRARIES 378 AND 280 AND X4003-5B GENOMIC DNA



A LANE 1 library 378
2 library 380
3 X4003-5B

B LANE 1 library 378
2 library 380
3 X4003-5B

A Agarose gel (stained with ethidium bromide) of DNA prepared from YEp24 plasmid libraries 378 and 380 and X4003-5B genomic DNA, all digested with Hind III (see section 6.3.2)

B This represents an autoradiograph of the above gel Southern blotted and probed with oligonucleotide 328 (See section 6.3.2.2)

λ/R/H here and in future figures refers to lambda DNA which has been digested with Eco RI and Hind III to yield fragments of the sizes shown on the left of this diagram

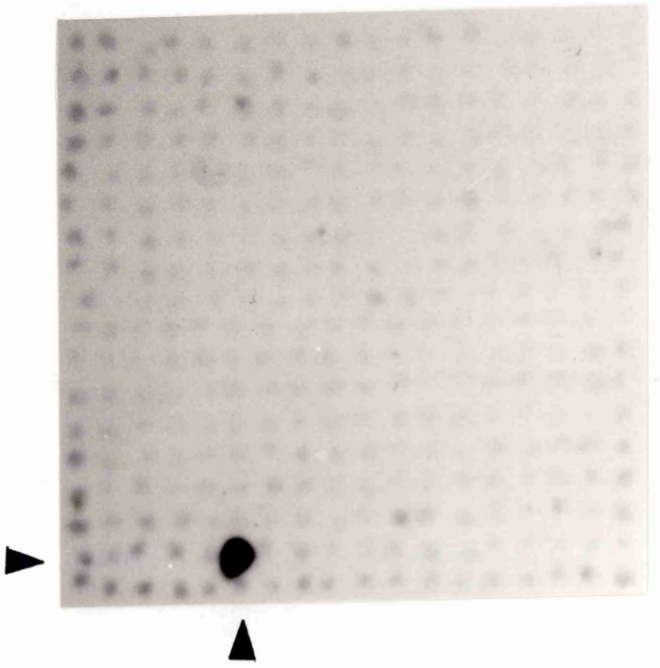
facing page 128

compare the representation of DNA sequences homologous to the oligo 328. This gel was subjected to Southern blot analysis (see Chapter 2). The radioactive probe used was oligo 328 which was end labelled with [^{32}P]-dATP with T4 DNA kinase (see Chapter 2). The result from this analysis is shown in Figure 6.3.2.3.B. There are approximately equivalent amounts of DNA on this gel representing the two libraries, 378 and 380. It is clear from the Southern analysis that the 378 library has better representation of the sequences homologous to oligo 328. In lane 3 the Hind III digested yeast genomic DNA, although not equivalent in amount to the two libraries clearly shows that the two libraries have an efficient representation of oligo 328 homologous sequences. There is in fact 2 to 5-fold less yeast DNA in lane 3 than in lanes 1 and 2, but it is clear that sequences homologous to oligo 328 are better represented in lanes 1 and 2, even taking into account the lower loading. This lends merit to the use of these libraries for screening with oligo 328 to isolate the HSP90 gene.

Also worthy of note is the fact that the major bands in the Southern analysis for libraries 378 and 380 are not the same in the two libraries. In fact both of the two major bands in library 378 are substantially larger than the two major bands in library 380. The reason for this is unclear. There are a number of bands which show up on this autoradiographic exposure which are common to the two libraries. Presumably although the libraries were made in parallel experiments the Sau3A digestions have resulted in the preferential production of the strong bands indicated. It is possible that the two libraries were made from separate genomic DNA preparations which have led to this differential digestion.

For this reason, both libraries were screened. It was calculated that 6400 colonies would have to be screened to allow a 99% chance of selecting any one sequence, as discussed in Section 3.1. To this end plasmid DNA prepared from both libraries as described previously was transformed into E.coli strain C1400, by the standard method and plated on to LB agar + ampicillin. Of these colonies approximately 3200 from each library were picked randomly in arrays on to LB agar + ampicillin in 10cm square petri dishes. These

FIGURE 6.3.2.3 IDENTIFICATION OF PUTATIVE HSP90 CLONES BY COLONY HYBRIDISATION



Legend: E.coli colonies transformed with library 378 plated in an array. These colonies were then transferred to a nitrocellulose filter, the colonies were lysed (see Chapter 2) and the filter was probed using oligo 328. A positive clone, is clearly visible.

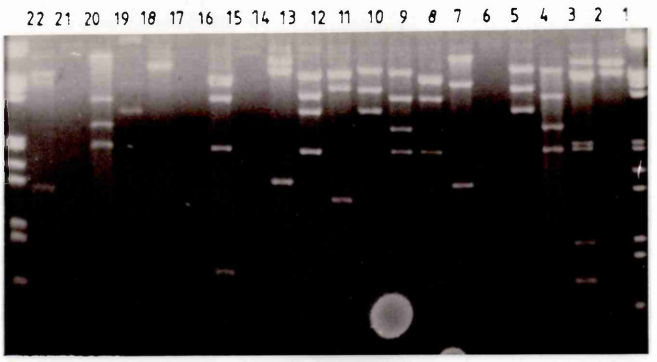
arrays were grown overnight and the colonies were lifted on to nitrocellulose filters. These filters were transferred colony side up to chloramphenicol plates and were incubated at 37°C for 8 hours. The filters were then removed and the colonies lysed and the DNA fixed as described in Chapter 2. After fixing, the filters were wet in 2 x SSC and gently rubbed with a gloved hand to remove most of the remaining cell debris. The filters were then prehybridised in 20% formamide at 37°C, as described in Chapter 2 and end labelled oligo 328 was added and allowed to hybridise for approximately 24 hours at 37°C. The filters were then washed and autoradiographed. A typical autoradiograph of one of these filters is shown in Figure 6.3.2.3. Colonies giving a positive reaction with the oligo 328 probe were picked and stabbed on to a second array, along with several negative colonies, including non-insert containing YEp24 transformants. These colonies were screened in exactly the same way as the first screening and 21 positive colonies were retained for further analysis. 15 of these clones originated from library 378 and 6 from 380. Of the 21 clones, eight had given much stronger signals in both rounds of the screening. These 21 colonies were grown in small liquid cultures which were then subjected to STET plasmid DNA preparation. These DNA samples were then subjected to 0.6% agarose gel electrophoresis alongside a YEp24 standard. All but one of the 20 selected plasmids were of a greater molecular weight than YEp 24 and this exception, YEp373.2.3, was discarded from further analyses.

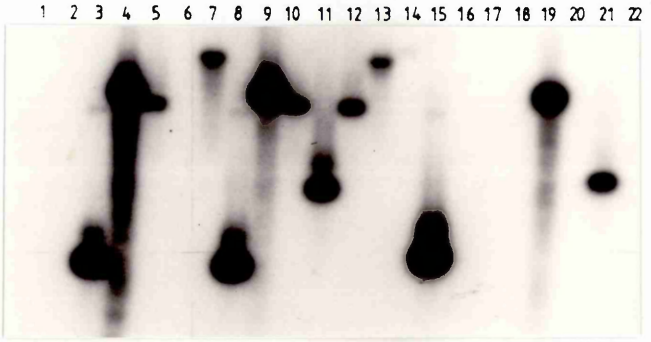
6.3.3. Confirmation that selected plasmids contain HSP90 sequences

To further confirm that the twenty remaining selected clones do in fact react positively with the oligo 328, the STET prepared DNA was digested to completion with Xba I and subjected to 1% agarose gel electrophoresis. This gel is shown in Figure 6.3.3.1.A. Xba I was chosen to digest the clones since there is an Xba I fragment of approximately 700bp that spans the region of the HSP90 gene, to which the oligo 328 corresponds, as shown in the map in Figure 6.3.4.1. Note that this 700bp Xba I fragment is frequently used as a

FIGURE 6.3.3.1 SOUTHERN BLOT ANALYSIS OF PLASMIDS SELECTED DURING SCREENING OF LIBRARIES 378 AND 380

LANE	SAMPLE
1	λ/R/H
2	YEp24
3	378.2.1
4	378.2.2
5	378.2.4
6	378.3.1
7	378.3.3
8	378.3.4
9	378.4.1
10	378.4.2
11	378.5.1
12	378.5.2
13	378.5.3
14	378.5.5
15	378.7.3
16	378.8.1
17	380.1.5
18	380.2.5
19	380.3.1
20	380.4.3
21	380.4.4
22	λ/R/H

A 

B 

A Agarose gel of mini-preps of plasmids selected during screening of libraries 378 and 380, digested with Xba I

B The gel shown in A, subjected to Southern blotting and probed with oligonucleotide 328, the autoradiograph of this blot is shown here

Note: λ/R/H are molecular weight standards comprising lambda DNA digested with EcoRI and Hind III Sizes of standards f top to bottom of gel (in kbs) are 21.2, 5.0 (doublet), 4.2, 3.5, 2.0, 1.9, 1.58, 1.3, 0.98, 0.83 and 0.56.

probe in other analyses described in this chapter. Therefore clones containing this region should produce a 700bp band on this gel to which the oligo 328 will hybridise. This gel was blotted on to nitrocellulose and probed with oligo 328 as previously described. The autoradiograph of this Southern blot is shown in Figure 6.3.3.1.B. It is clear from this autoradiograph that there are several different families of clones within the twenty selected plasmids. These families are shown below

Family A : yields a strong, approx 700bp band on this Southern analysis. Members are 378.2.1; 378.3.4 and 378.7.3

Family B : yields a strong approx 3.5kb band on this Southern analysis. Members are 378.2.1; 378.4.1; 378.5.5 and 380.3.1

Family C : yields a strong, approx 1.2kb band on this Southern analysis. Members are 378.5.1; 380.4.4 and 380.7.1

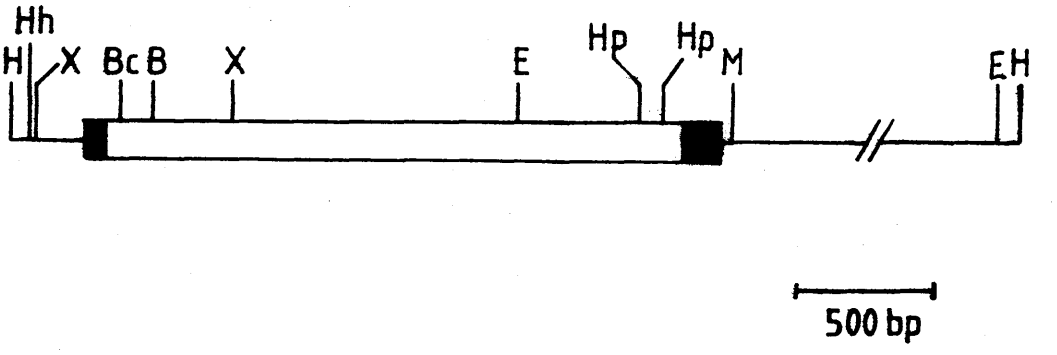
Family D : yields a weak, high molecular weight band on this Southern analysis. Members are378.3.3 and 378.5.3

Family E : yields a weak, approx 3kb band on this Southern analysis. Members are 378.2.4; 378.4.2 and 378.5.2

The remaining five plasmids were discarded because they produced little or no signal when hybridised with oligo 328 on this Southern analysis. Several of the family members appeared to be identical, in this and future analyses, for instance 378.2.2 and 378.4.1. In fact only nine different plasmids remained at this stage. They are listed below in their family groups.

A - 378.2.1 and 378.3.4
B - 378.2.2 and 380.3.1
C - 378.5.1
D - 378.3.3 and 378.5.3
E - 378.2.4 and 378.5.2

FIGURE 6.3.4.1 PARTIAL RESTRICTION MAP OF THE 5.5kb Hind III FRAGMENT ON WHICH THE HSP90 GENE RESIDES



B - Bgl II
Bc - Bcl I
E - Eco RI
H - Hind III
Hh - Hha I
Hp - Hpa II
X - Xba I

The open bar indicates the location of the HSP90 coding region. The filled bars denote sequence encoding the 5' and 3' untranslated region of the mature HSP90 mRNA. Data taken from Farrelly and Finkelstein, (1984)

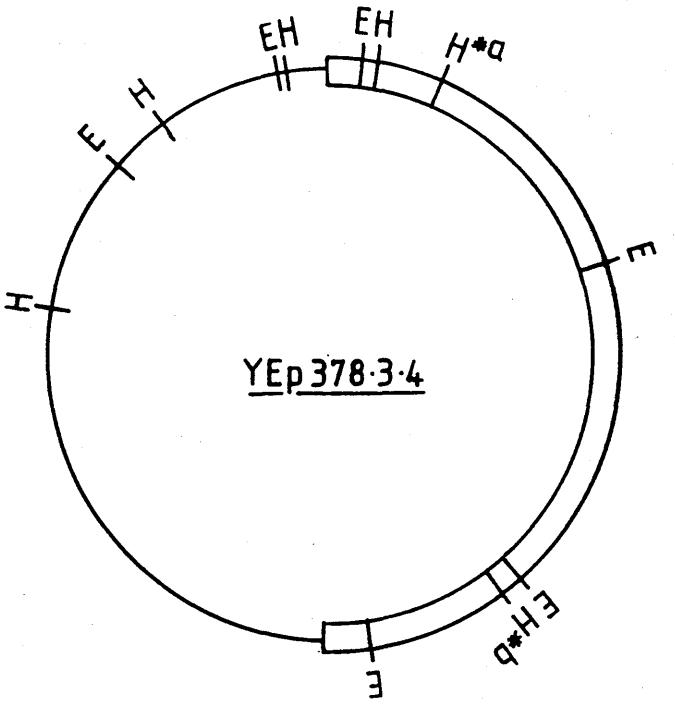
It still remained to be confirmed that oligo 328 in this diagnostic blot and in the library screening, was actually hybridising to the predicted sequence within the HSP90 gene, as shown in Figure 6.3.1.1. Clearly family A, which produce a 700bp band to which oligo 328 hybridises on digestion with Xba I would appear to be the correct species from the previously published sequence (Farrelly and Finkelstein, 1984). However, the decision was made to obtain some DNA sequence data from a member of each family to confirm that oligo 328 is hybridising to the expected sequence and which if any of the selected clones contain HSP90 specific sequence. To this end, a member of each family was subjected to dideoxy plasmid sequencing using T7 polymerase (see Chapter 2), using oligo 328 as the sequencing primer. The plasmids to be sequenced were large, averaging 15kb, and the oligo used as primer, at 32 residues was considerably longer than 17 residues which has been shown to be optimal for use as a sequencing primer (Hattari and Sakaki, 1986). It became apparent that the quality and purity of the plasmid DNA was of prime importance to allow any sequence to be determined. The method of plasmid purification used is described in Chapter 2.

From these analyses it was clear that those families to which oligo 328 hybridised most strongly, namely A,B and C contained sequence identical to the published sequence of HSP90 (data not shown). No sequence of members of family D could be determined using oligo 328 as primer, despite repeated attempts and this analysis yielded unrelated sequences from family E. Therefore members of families D and E were discarded, leaving only five HSP90 plasmids for future analyses.

6.3.4 Partial mapping of HSP90 clones

Since the object of selecting the HSP90 gene was to construct a plasmid vector capable of integrating at the HSP90 chromosomal locus, and thereby disrupting the HSP90 gene, it was essential that at least a large portion of the HSP90 gene was available for use in the vector construction. Since the sequence of the entire HSP90 coding and 5' and 3' coding

FIGURE 6.3.4.2 PARTIAL RESTRICTION MAP OF PLASMID YEp378.3.4



H - Hind III
E - Eco RI

The figure shows a partial restriction map of YEp378.3.4. This plasmid is approximately 16kb, about 8.3kb of which represents yeast genomic DNA (shown as boxed region). Within this, the entire HSP90 coding and flanking regulatory sequences are located (between asterixed Hind III sites (Farrelly and Finkelstein, 1984))

a - according to the map of Finkelstein and Strausberg (1983) there are three Hind III sites closely situated in this region

b - according to the map of Finkelstein and Strausberg (1983) there are two Hind III sites closely situated in this region

regions was known, a restriction map could be produced. Figure 6.3.4.1 shows some of the restriction sites within the HSP90 gene (Farrelly and Finkelstein, 1984).

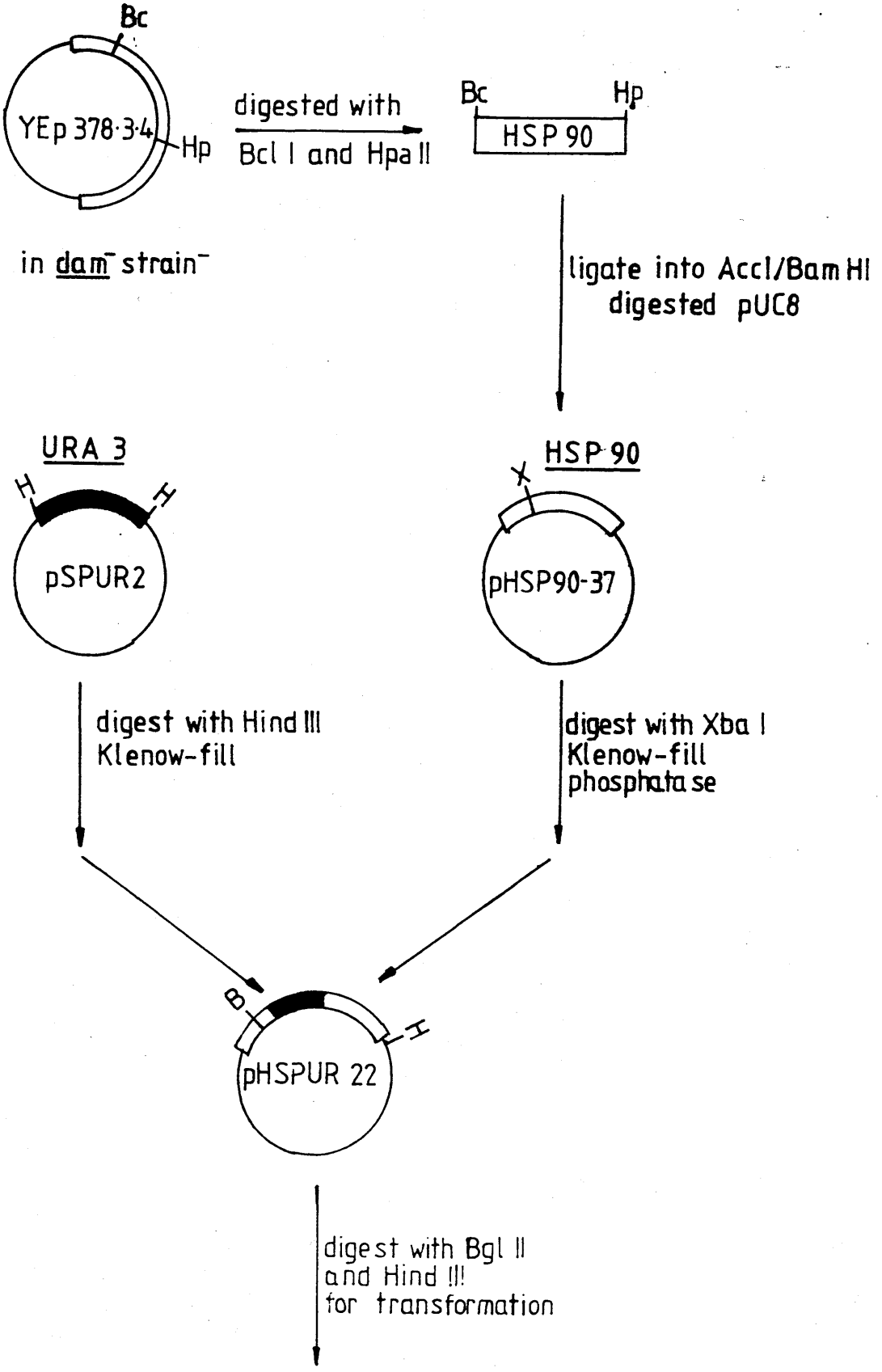
To test whether any of the remaining clones contained a sizeable part of this sequence, including the entire coding region, the plasmids were digested with Hha I and Mlu I. This should yield a fragment of 2488bp if the entire region is present, to which the oligo 328 will hybridise. These digested samples were therefore subjected to Southern blot analysis as previously described. In this analysis, only plasmid YEp378.3.4 yielded a band of approximately the correct size (data not shown). To confirm that this fragment was the expected one, plasmid 378.3.4 was digested, again with MluI and HhaI followed by digestion with several enzymes. These digests were subjected to 1% agarose gel electrophoresis. The gel was then blotted and probed as before. The sizes of fragments, to which oligo 328 hybridised agreed in each case with what is expected, confirming that this 2.5kb Hha I/Mlu I fragment is the expected one (data not shown). Clearly plasmid YEp378.3.4. does indeed contain at least this region of the HSP90 gene, including the entire coding region. The estimated size of this plasmid is approximately 16kb of which approximately 8.3kb is yeast genomic DNA (YEp 24 is approximately 7.7kb). Therefore as well as containing the 2.5kb fragment described plasmid 378.3.4 also contains a further 5.8kb of yeast genomic DNA. A partial map of YEp378.3.4 is shown in figure 6.3.4.2.

6.4 CONSTRUCTION OF A YEAST INTEGRATING VECTOR TARGETED AT HSP90

6.4.1 Introduction

Since the demonstration by Hinnen et al. (1978) that S.cerevisiae can be transformed with DNA via homologous recombination between the transforming DNA and the yeast chromosome, this method has been used in a number of analyses. In most cases these integrations occur only at the chromosomal position represented in the integrating vectors.

FIGURE 6.4.2.1 STEPS IN THE PRODUCTION OF A YIp VECTOR TO INSERTIONALLY INACTIVATE THE HSP90 GENE



Not to scale

Bc - Bcl I H - Hind III B - Bgl II

Hp - Hpa II X - Xba I

facing page 133

Exceptions to this, where the vector integrates at a low frequency at other chromosomal locations can be explained by the presence of repeated sequences on the plasmid used (Kingsman et al., 1979).

The frequency of integration at a particular site is at least somewhat proportional to the length of the homologous sequence so that manipulation of sequence lengths can give some direction to the integration process. Increased direction of integration can also be achieved by cleavage of the vector in the sequence homologous to the target site. The free ends that are generated are increased in their recombinogenic potential (Orr-Weaver et al., 1981).

6.4.2 Subcloning the HSP90 gene into pUC 8

To facilitate the production of a YIp vector the sequence which is desired to target the integration must be isolated. To allow the construction of the intended integrating vector, the first step was to subclone HSP90 sequence into a suitable vector. The E.coli multicopy cloning vector pUC8 was chosen for this purpose. The vector allows colorimetric selection for recombinant plasmids (via Lac Z) and rapid plasmid amplification and purification.

The cloning strategy used, was designed to use two different restriction sites at opposite ends of the HSP90 insert which could be cloned into complementary sites in pUC 8. The sites chosen, within the HSP90 gene were Bcl I at position 385 and Hpa II which cuts twice, at positions 2293 and 2415. Therefore the subcloned HSP90 fragment would span 385 to 2293 which was cloned into the Bam HI and Acc I sites in pUC8, as shown in Figure 6.4.2.1.

The enzyme Bcl I is sensitive to dam methylation, that is where adenosine residues are methylated by the E.coli enzyme dam methylase. DNA, purified from E.coli strains which have an active dam methylase, containing the target sequence for the Bcl I enzyme cannot be digested by the enzyme. Therefore to allow digestion of 378.3.4 with this enzyme, the plasmid had first to be transformed into and reisolated from an

E.coli strain which is mutant for the dam gene. YEp378.3.4 was transformed into the dam⁻ strain, CB51 (see Chapter 2). Transformation efficiencies were extremely low for this plasmid into this strain. The reason for this is not clear. The plasmid was re-isolated by standard Birnboim-Doly plasmid preparation.

The pUC 8 vector was prepared by digestion with Acc I and Bam HI, followed by gel purification by the gene clean method (see Chapter 2). This was done primarily to remove the small fragment cleaved from the polylinker by digestion with these two enzymes, to prevent its reinsertion during ligation. The HSP90 insert was prepared by firstly digesting plasmid 378.3.4 with Bcl I and Hpa II. This digested DNA was applied to agarose gel electrophoresis, and the 1.9kb band was cut out of the gel and also purified by gene cleaning (see Chapter 2). Digestion of 378.3.4 with Bcl I and Hpa II results in the production of at least 6 fragments of varying sizes, the largest of which is approximately 1.9kb and the next largest between 1.5 and 1.6kb.

This purified material was mixed with prepared pUC 8 vector and ligated in the standard manner at 16°C overnight (See Chapter2). This material was then transformed into E.coli strain, DS902. Forty white transformants were subjected to STET DNA preparation to allow identification of plasmids containing the correct insert by means of several diagnostic restriction digestions. In this way a plasmid was isolated which contained the correct HSP90 sequences. This plasmid was named pHSP90.37.

6.4.3 Inserting the URA3 gene into the coding region of the HSP90 gene

Yeast insertional vectors target the insertion of the vector at a chromosomal region homologous to that within the vector, as discussed in Section 6.4.1. In this case, the vector under construction was to be targeted to the yeast HSP90 gene. For two reasons this vector, which was intended to insertionally inactivate the chromosomal locus of HSP90, must also contain a yeast auxotrophic marker. Firstly the marker

can allow for selection of transformants over untransformed yeast cells by growth on selective medium, following the transformation process. Secondly, since the intention of these experiments is to produce a yeast strain with an inactive HSP90 gene brought about by the insertion of the vector at the targeted chromosomal locus by homologous recombination between the vector and the targeted chromosomal locus, the vector must contain a homologous gene which has been made functionally inactive. The most simple way of constructing such a vector is to insert a fragment of DNA into the coding region of the gene used for targeting. It is therefore convenient that this piece of DNA used to mutate the gene of interest should also contain the required autosomal marker.

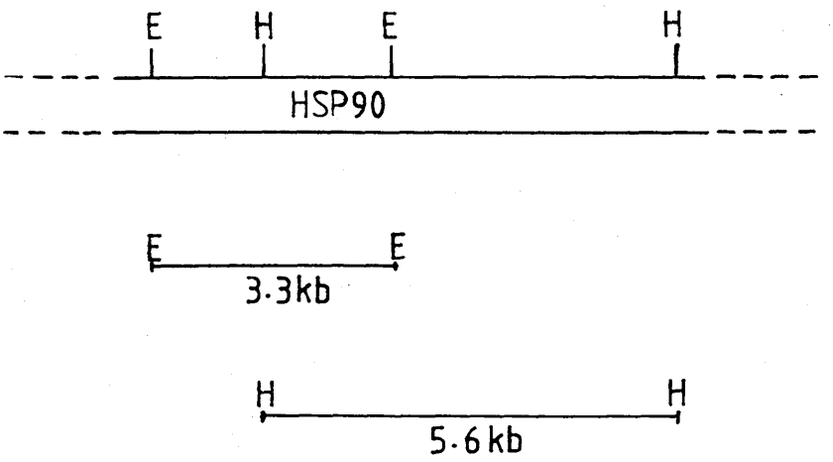
It was intended that the yeast strain to be used in these analyses should be the same one from which the AMP deaminase enzyme was purified, X4003-5B. This strain has a number of auxotrophic mutations, which allowed a choice of marker genes. The gene chosen was URA3. URA3 can be isolated on a 1.1kb HindIII fragment from a number of plasmids available in this laboratory (Rose et al., 1984). This fragment was to be subcloned within the coding region of the HSP90 gene located in plasmid pHSP90.37. Unfortunately there is no suitable restriction site within the HSP90 coding sequence to allow a forced cloning of the Hind III fragment. Instead, the URA3 bearing fragment was blunt ended and cloned into a unique site in pHSP90.37. This strategy is shown in Figure 6.4.2.1.

To this end, the plasmid pHSP90.37 was digested with Xba I. Since a small piece of the polylinker of pUC 8, carrying the Xba I site was lost during the production of pHSP90.37, Xba I no longer cuts within the vector sequences of pHSP90.37. It does however cut within the coding region of HSP90 at position 799 and this is the only Xba I site in pHSP90.37. The ends of the Xba I digested pHSP90.37 were then filled with Klenow and phosphatased using Calf Intestinal Phosphatase (See Chapter 2).

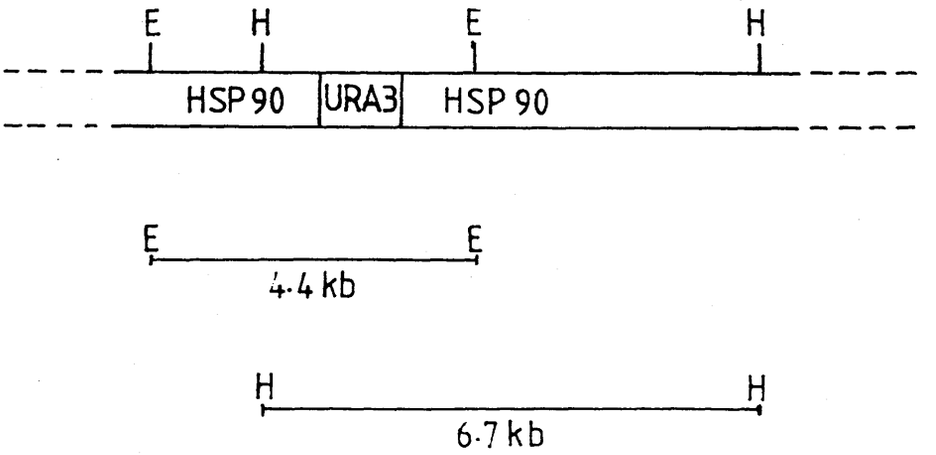
The URA3 gene to be inserted was isolated from pSPUR2, see Figure 6.4.2.1. pSPUR2 was digested with Hind III and then Klenow filled to yield blunt ended molecules and the 1.1kb

FIGURE 6.4.5.1 FRAGMENTS EXPECTED IN GENOMIC SOUTHERN BLOT ANALYSES OF X4003-5B AND X4003-22 GENOMIC DNA

HSP90 CHROMOSOMAL LOCUS IN STRAIN X4003-5B



HSP90 CHROMOSOMAL LOCUS IN STRAIN X4003-22



Fragments that will be yielded in genomic Southern blot analysis using an HSP90-specific probe, if insertion of Ylp vector has occurred as predicted.

E = Eco RI
H = Hind III

fragment was gel purified. This DNA was then ligated in the standard reaction with the end-filled and phosphatased pHSP90.37 at room temperature for five hours. The ligation mix was then transformed into E.coli strain C1400. Since the pUC8 LacZ gene was already inactivated by the insertion of the HSP90 sequences, a blue/white selection on Xgal medium was not possible. Forty transformants were subjected to STET DNA preparation followed by restriction analysis, and in this way a plasmid with the URA3 gene correctly inserted was isolated. This plasmid was named pHSPUR22. It was not known in which orientation the URA3 Hind III fragment had inserted, but this was not examined since the 1.1kb Hind III fragment used to provide the URA3 gene contains all flanking sequences required for expression of the gene, and therefore orientation of the fragment should not affect its expression.

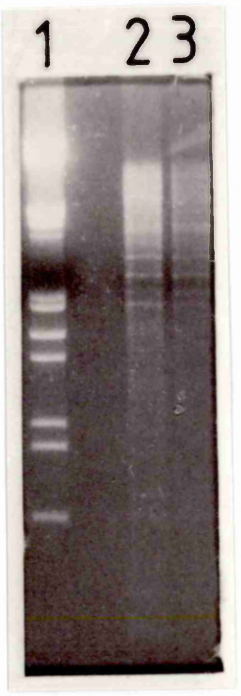
6.4.4 Transformation of yeast strain, X4003-5B with integrating vector pHSPUR22

Integrative transformation into yeast is much less efficient than transformation with an episomal vector. Whereas one might expect to get in the region of 10^3 transformants/ug DNA with an episomal vector, this figure is reduced to about 1 - 10 transformants/ug DNA with an integrating vector. Therefore, a 50ul aliquot of X4003-5B spheroplasts produced in the standard method of yeast transformation (see Chapter 2) was mixed with 10ug of pHSPUR22 digested to completion with Bgl II and Hind III (Figure 6.4.2.1.). These mixture was then treated and plated in the normal manner on to soft agar medium containing the necessary amino acid supplements (in this case adenine, leucine, histidine, methionine and tryptophan each at a concentration of 100ug/ml).

The selectable marker for pHSPUR22 is URA3, hence anything growing on these selective plates should be transformants. The transformation plates were incubated at 30°C for 4 days. At the end of this time, a number of transformants were streaked on to YPG plates to obtain single colonies. A single colony was then patched on to selective plates to confirm that the correct genotype had been maintained. The transformation efficiency was in the region of those

FIGURE 6.4.5.2 AGAROSE GELS OF GENOMIC DNA OF STRAINS X4003-5B AND X4003-22 DIGESTED WITH Eco RI OR Hind III

A



B

A	LANE		B	LANE	
	1	λ/R/H		1	X4003-5B/Eco RI
	2	X4003-5B/Hind III		2	X4003-22/Eco RI
	3	X4003-22/Hind III			

λ/R/H molecular weight sizes are as shown in Figure 6.3.2.2.

expected. A single transformant with the correct genotype was retained for future use and this strain was named X4003-22.

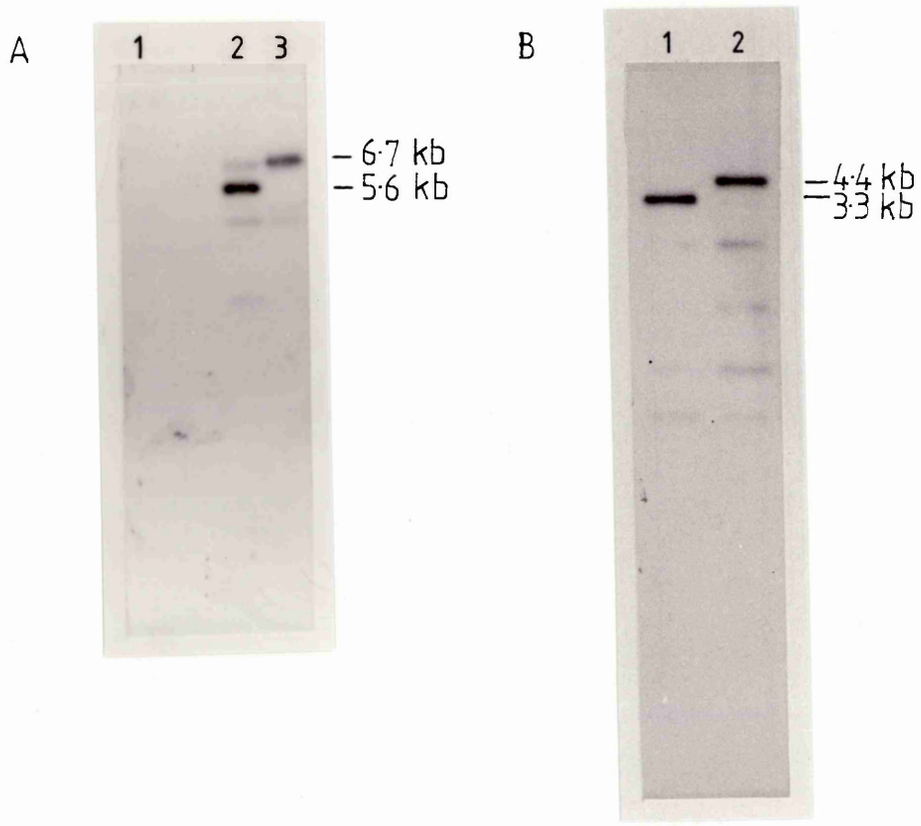
6.4.5 Confirmation of the integration of pHSPUR22

It was necessary at this stage to confirm that insertion of the integrating vector had occurred as predicted. The classical way to confirm that an introduced, auxotrophic marker such as URA3 has integrated at a novel and predicted position in the yeast genome is to study its behaviour in genetic crosses with respect to other marker genes. That is, the auxotrophic marker should now be linked to genes which are linked under normal conditions with the site of integration. This would have been difficult to do in this instance since the chromosomal location of HSP90 is not known. To confirm the position of integration it was decided to use Southern blot analysis of the genomic DNA from the control strain and X4003-22. The strategy behind this analysis is shown in Figure 6.4.5.1.

A restriction enzyme which cuts outwith the gene of interest will produce a single band of a specific size on an autoradiograph of a Southern blot analysis of genomic DNA digested with the enzyme in question, when a probe specific the the gene in question is used. If an integration event has occurred within this gene (in this case, the URA3 gene borne on pHSPUR22), the same restriction enzyme will produce in the same Southern blot analysis, a band which is bigger than that seen for the control strain (assuming the enzyme in question does not cut within the auxotrophic marker gene). The increase in size in this case will be 1.1kb (the size of the URA3 gene-bearing fragment).

Thus, to confirm the integration of pHSPUR22 at the correct chromosomal position, genomic DNA was prepared from X4003-5B and X4003-22. This DNA was then digested both with Eco RI and Hind III in separate digestions and then subjected to 0.8% agarose gel electrophoresis. The gels of these digests are shown in Figure 6.4.5.2. These gels were blotted on to nylon membranes and fixed and prehybridised in 20% formamide

FIGURE 6.4.5.3 SOUTHERN BLOT ANALYSIS OF GENOMIC DNA FROM STRAINS X4003-5B AND X4003-22



A Lane 1 λ/R/H
Lane 2 X4003-5B/Hind III
Lane 3 X4004-22/Hind III

B Lane 1 X4003-5B/Eco RI
Lane 2 X4003-22/Eco RI

The calculated sizes of the major band in each lane is given to the right of each picture.

A Southern blot of gel shown in figure 6.4.5.2A probed with a 700bp Xba I fragment from the HSP90 gene

B Southern blot of gel shown in figure 6.4.5.2B probed as above

In the two Southern blot analyses shown the major band detected, using a HSP90 -specific probe was approximately 1.1kb larger in strain X4003-22 than X4003-5B as was expected if the integration of pHSPUR22 had occurred as predicted (see figure 6.4.5.1). The probe used was a random primed 700bp Xba I fragment from the HSP90 gene (see figure 6.3.4.1). Other bands were also detected in these analyses, using this probe, but in most cases these bands were common to both strains and their nature was not investigated further

at 37°C. The probe used was a gel purified 700bp Xba I fragment derived from YEp378.3.4, which was random primed to a specific activity of approximately 10^9cpm/ug DNA (see Chapter 2). Probing with end labelled oligo 328 failed to give clear results, since there was a high degree of non-specific binding (data not shown).

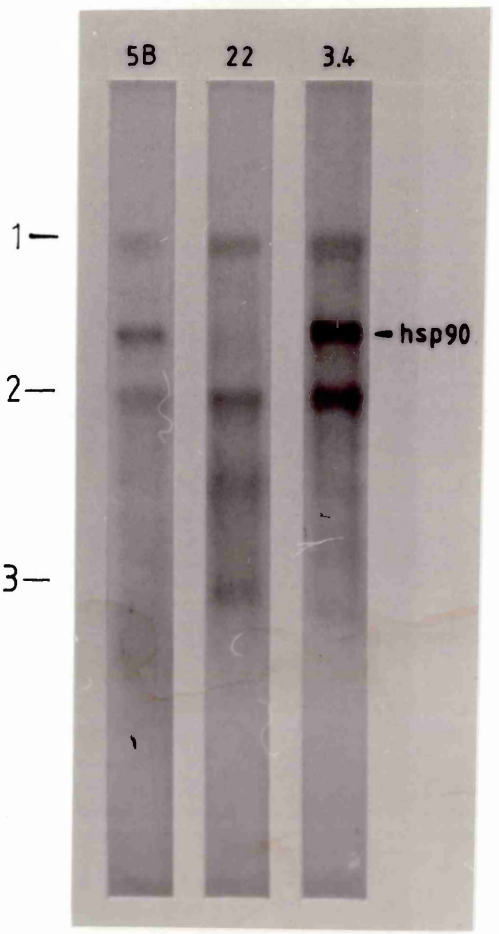
Eco RI cuts the HSP90 gene at position 1543 and at -1820bp upstream of the AUG (Szent-Gyorgi et al., 1987). Therefore when genomic DNA is digested with Eco RI, a probe specific to the 5' end of the HSP90 gene (such as the Xba I fragment, described above) should hybridise to a fragment of 3363bp. Eco RI does not cleave within the 1.1kb URA3 fragment, therefore if insertion has occurred as expected DNA from strain X4003-22 treated in the same way, should yield a fragment of 4463bp.

Similarly, Hind III cuts outwith the entire HSP90 gene (Farrelly and Finkelstein, 1984). Using this enzyme the entire gene and its necessary flanking regions can be isolated on a 5.6kb fragment. Neither does this enzyme cut within the URA3 fragment, therefore an HSP90 specific probe should hybridise to a 5.6kb and 6.7kb band on Southern blot analysis of strains X4003-5B and X4003-22 respectively using the restriction enzyme, Hind III.

These Southern blots are shown in Figure 6.4.5.3. Clearly, these results are in complete agreement with the predictions in the case of integration of pHSPUR22 at the HSP90 locus.

To further confirm that the insertional inactivation of HSP90 has occurred, RNA was prepared from mid-log cultures of X4003-5B and X4003-22 as described in Chapter 2. To allow analysis of the overproduction of hsp90 on AMP deaminase activity, the control strain, X4003-5B was transformed with pYEp378.3.4. This is a multi-copy vector bearing the entire HSP90 gene (see section 6.3). Therefore, RNA was also made from this strain named X4003-3.4. The RNA was purified by spinning through a caesium chloride cushion, and the resulting pellet was resuspended in water. The absorbance at 260nm was measured, and equivalent amounts of RNA were applied to a formaldehyde/MOPS agarose gel (Chapter 2). This

FIGURE 6.4.5.4 NORTHERN BLOT ANALYSIS OF RNA FROM STRAINS X4003-5B, X4003-22 AND X4003-3.4



Equal amounts of RNA were applied to each lane of a denaturing formaldehyde gel and blotted onto a nylon membrane which was then probed with a random primed 700bp Xba I fragment from the HSP90 gene (see figure 6.3.4.1).

From this analysis it was apparent that strain X4003-22 was not producing a mRNA corresponding to HSP90. It would also appear that strain X4003-3.4 has a higher amount of this same mRNA, confirming X4003-22 as a strain lacking a functional HSP90 gene and X4003-3.4 as a strain containing an increased number of copies of the HSP90 gene.

Other bands are apparent in this Northern blot analysis. Attempts at more stringent washing of the filter failed to remove them. It was thought likely that the bands indicated on the left of the picture as 1 and 2 represent the 25S and 18S ribosomal RNAs, respectively. These two bands are common to all three strains in this analysis. It is possible that the band indicated as 3 may represent a truncated mRNA produced by the HSP90/URA3 integrated gene visible only in the lane representing X4003-22 RNA. This was not investigated further.

gel was blotted on to nylon membrane and probed with random primed Xba I fragment of p378.3.4., as described elsewhere. The resulting autoradiograph of this Northern blot is shown in Figure 6.4.5.4. Clearly, there is a high degree of non-specific binding in this analysis which could not be removed by prolonged washing. The nature of this hybridisation was not examined. It is however clear from this analysis, that X4003-22 no longer produces a functional hsp90 mRNA and that X4003-3.4 appears to produce a higher basal level of hsp90 mRNA. From this evidence it was clear that X4003-22 and X4003-3.4 represent strains with no functional hsp90 and an increased level of hsp90, respectively.

6.5 THE EFFECT OF LOSS AND OVERPRODUCTION OF HSP90 ON AMP DEAMINASE ACTIVITY

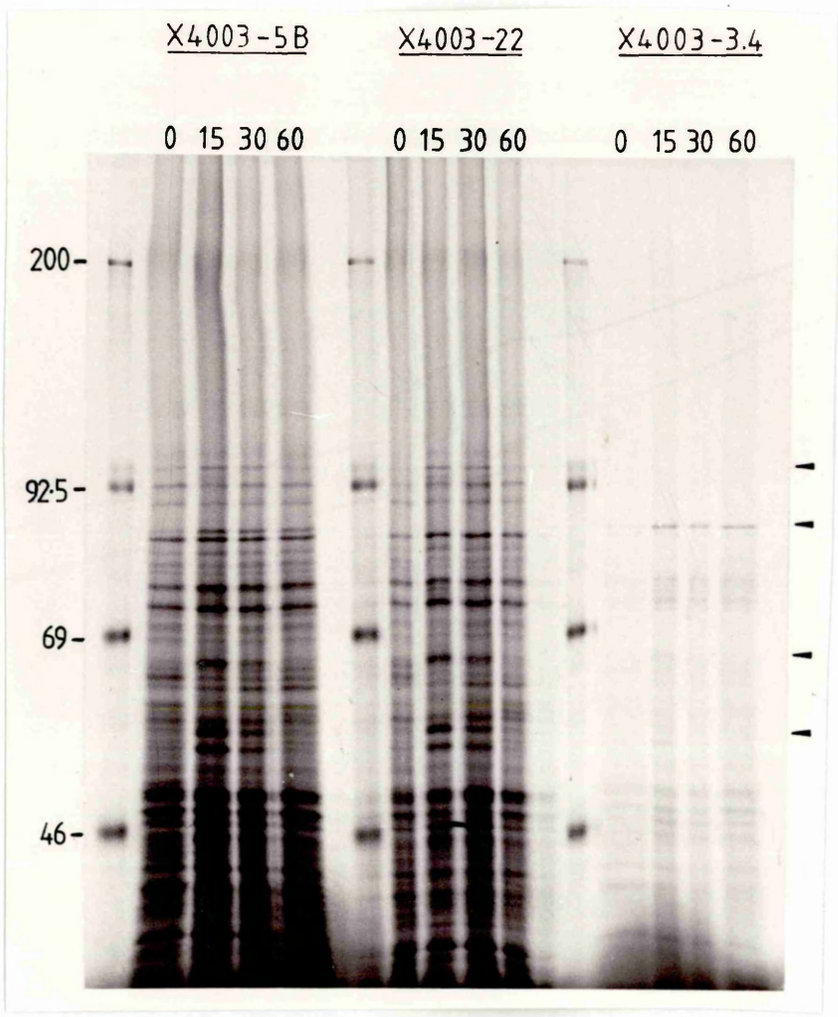
If hsp90 and AMP deaminase are the same protein then by inactivating the HSP90 gene, one would expect to see a reduction in AMP deaminase activity. This statement is complicated by the fact that in yeast there is also the heat shock cognate 90, HSC90 gene, which is highly homologous to HSP90, and that presumably their proteins are fulfilling similar functions. Hsc90 is abundantly expressed in normal cells, and little affected by heat shock (Farrelly et al., 1983). Hsp90 on the other hand is strongly induced by heat shock. The two genes are fulfilling an essential function in the cell, since strains with mutations in both genes are not viable. Mutations in one or other gene have no apparent effect on the viability of the cell (Lindquist, 1986). We have shown in the previous section that yeast strain, X4003-22 has been insertionally inactivated at the HSP90 locus, but it is known, because of the lethal nature of the double mutation, that the HSC90 gene must be intact and normal. If we assume that hsp90 and AMP deaminase are the same, then hsc90 should also produce AMP deaminase activity. Presumably at normal temperatures hsc90 accounts for the largest proportion of the activity, with hsp90 accounting for only a small proportion. If this is the case, a strain which is mutated in the HSP90 gene would be expected to have similar AMP deaminase specific activities at the normal temperature, but upon heat shock, the mutated strain cannot induce hsp90

synthesis, and therefore will have less AMP deaminase activity than the control strain. Similarly, a strain overproducing hsp90 (due to multiple copies of the gene), should have a slightly greater basal level of AMP deaminase activity, which will become much greater than the control strain upon heat shock.

To test whether this hypothesis is correct, strains X4003-22 and X4003-3.4 have been constructed and an experiment was set up to measure the AMP deaminase specific activity of these strains, along with the control strain X4003-5B,before and after heat shock.

To this end, cultures of X4003-5B, X4003-22 and X4003-3.4 grown in GYNB with the necessary growth supplements were set up and grown at 23^{o}C to mid-log and an absorbance at 600nm of 0.5-0.55. To confirm that heat shock has occurred in each case, small samples of each culture were <u>in vivo</u> labelled with ^{35}S-methionine to allow the production of the diagnostic heat shock gels. These denaturing protein gels are autoradiographed and the labelled bands which are exposed represent newly synthesised proteins. If heat shock has been successful, samples taken after heat shock should show the synthesis of novel proteins, the hsps, relative to the control sample. Yeast heat shock gels are far less dramatic than those obtained from <u>Drosophila</u> cells, since normal cellular proteins are still being synthesised as explained in Section 6.1. The experiment was carried out thus. A 100ml portion of the culture was removed, and 100mls of GYNB and the necessary growth supplements at 23^{o}C were added. This dilution step was carried out because the rapid heat shock to 37^{o}C was achieved by the addition to the culture of an equal volume of GYNB and the necessary growth supplements at 51^{o}C. Therefore the control sample must be treated in the same way with the exception that the added media was at 23^{o}C. 1.0ml of this dilution was removed to a plastic microfuge, it was then spun briefly in a microfuge at room temperature, the supernatant was removed and the cell pellet resuspended in 1ml of GYNB and the necessary growth supplements (without methionine). In this 1ml of media was 25μCi of ^{35}S methionine. This mixture was then incubated for 10 minutes at 23^{o}C. At the end of this incubation, 100μl of methionine

FIGURE 6.5.1.A IN VIVO ^{35}S LABELLED PROTEINS FROM STRAINS X4003-5B, X4003-22 AND X4003-3.4 BEFORE AND AFTER HEAT SHOCK



Aliquots of a culture were labelled for 10 minutes with [^{35}S]-methionine in GYNB and the proteins were extracted. Equal portions of radioactivity were loaded onto each lane of a standard 9% SDS polyacrylamide gel. The gel was dried and the resulting autoradiograph is shown. Note that the heat shock response is confirmed by the production of diagnostic heat shock proteins.
Note: Samples denoted 15 were labelled from T_{10} to T_{20}, samples denoted 30 were labelled from T_{25} to T_{35} etc., as explained in section 6.5.

Lane designations - the times at which samples were taken for AMP deaminase assays following transfer from 23°C to 37°C.

Numbers on the left of the gel are the sizes of molecular weight standards in kDaltons and the positions of several heat shock proteins, including hsp90 are noted at the right of the diagram.

Note: hsp90 is induced upon heat shock of X4003-5B, and over-induced (with respect to other cellular proteins) in X4003-3.4. However, hsp90 does not appear upon heat shock of X4003-22.

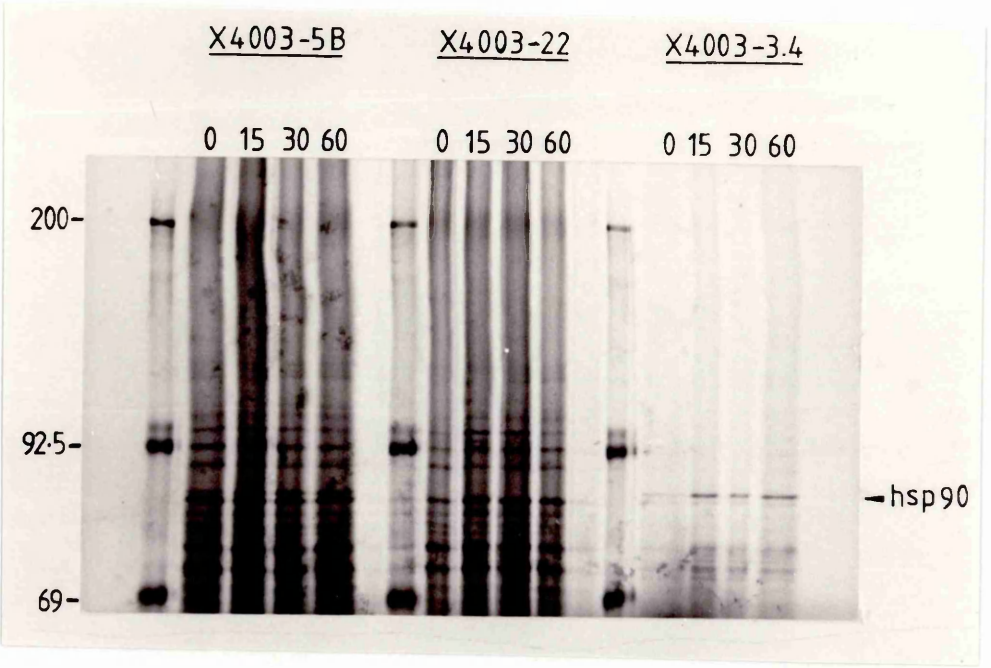
facing page 141

(10mg/ml) was added and the mixture was placed on ice prior to extraction. This treatment of spinning and resuspending the cells in media lacking methionine was necessary to allow the incorporation of ^{35}S methionine into newly synthesised proteins. All three strains used in this experiment are methionine auxotrophs, and therefore require media supplemented with methionine for growth. Under these conditions ^{35}S methionine labelling is extremely inefficient (data not shown) and to increase the efficiency, the methionine-containing media has to be removed and replaced with media containing no added methionine as described here. The harvesting of yeast cells by centrifugation and resuspension has been shown, in this and other laboratories, not to induce the production of stress proteins (data not shown; Sorger et al., 1987b). The remainder of the 200ml sample was centrifuged at 10000 x g for 5 minutes and then the supernatant was removed. The cell pellet was then chilled on liquid nitrogen and stored at -20°C till required.

The rest of the culture had an equal volume of GYNB and the necessary growth supplements (pre-equilibrated at 51°C) added rapidly and from then the culture was incubated at 37°C. The temperature of the culture was thus changed to 37°C immediately on addition of the hot media. At 15, 30 and 60 minutes after this heat shock 200ml samples were taken for protein extraction. The samples were harvested and frozen as described for the T_0 sample. 1ml samples of the heat shocked culture were removed and labelled as described before with the exceptions that the ^{35}S methionine containing media had been pre-equilibrated at 37°C and the ten minute incubations were carried out at 37°C. These samples were taken and incubated at times, T_{10-20}, T_{25-35} and T_{55-65} respectively.

The labelled protein extracts were then treated thus. The samples were spun for 2 minutes in a microfuge, the cell pellet was washed once with distilled water and resuspended in 200µl SDS sample buffer. Glass beads were added till no fluid remained, and the samples were vortexed for 30 seconds, three times, resting on ice between each vortex. 200µl SDS sample buffer was added, the mix was briefly vortexed and the supernatant was removed. This material was spun in a microfuge for two minutes to remove cell debris, and the

FIGURE 6.5.1.B



This photograph represents a longer exposure of the same gel detailed in figure 6.5.1.A. All the details pertaining to this figure have been previously indicated.

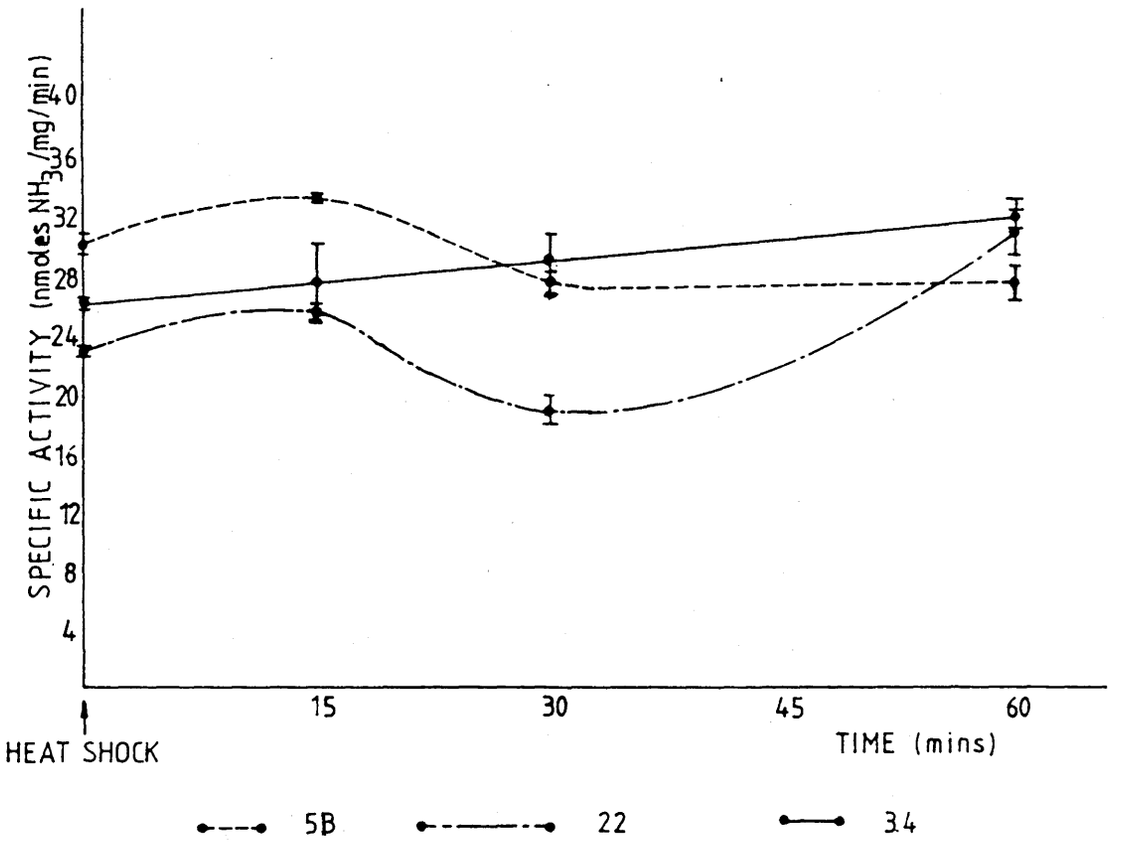
Note: there is clearly a massive over-production of hsp90 (relative to other cellular proteins) in strain X4003-3.4.

supernatant was removed to a fresh tube. Aliquots of these samples were counted for radioactivity in a scintillation counter, and then the samples were heated to 100°C for 5 minutes, and then spun for 2 minutes. Equal counts for each sample were then applied to a standard 7.5% SDS polyacrylamide gel along with ^{14}C protein standards. The gel was dried and autoradiographed. This autoradiograph is shown in Figure 6.5.1.A. Clearly for each of the three strains heat shock has occurred. Time points T_{15} and T_{30} clearly show the synthesis of new proteins. Most of these changes have reverted to normal by T_{60}. This is in line with the finding of Sorger et al., (1987), who showed that a transient heat shock in yeast lasts no longer than one hour.

In this gel, the band corresponding to hsp90 is clearly recognisable. In Figure 6.5.1.B this region of the gel is shown in greater detail. Hsp90 is quite clearly induced in the control strain, X4003-5B across the time of the heat shock. In stark contrast is the protein profile for X4003-22 where the band corresponding to hsp90 is apparently completely absent, again confirming successful inactivation of the hsp90 gene. X4003-3.4 shows clear induction of hsp90 to a greater level than with the control strain. This is to be expected in a strain carrying multiple copies of the gene.

Clearly, heat shock has occurred in all three strains. The protein extract pellets were therefore extracted thus. The frozen pellets were thawed into ice cold 50mM potassium phosphate buffer, pH7.5, the cells were re-harvested and resuspended in 1ml extraction buffer. Glass beads were added to this cell suspension till no fluid remained. This mix was then vortexed three times, for 30 seconds each time, with incubation of ice between each vortex. Another 1ml of extraction buffer was added and the mix vortexed briefly and the supernatant removed. The supernatant was then spun in a microfuge for 5 minutes to remove cell debris and the supernatant was removed to a fresh tube. 100ul of this extract was then added to standard 200ul AMP deaminase reaction mixes and incubation at 37°C was carried out for 30 minutes. At the end of this time a 100ul sample was removed from this reaction mix and assayed for ammonia production in the standard way (addition of 0.33ml reagent 1, followed by

FIGURE 6.5.2 AMP DEAMINASE ACTIVITIES IN STRAINS X4003-5B, X4003-22 AND X4003-3.4 BEFORE AND AFTER HEAT SHOCK



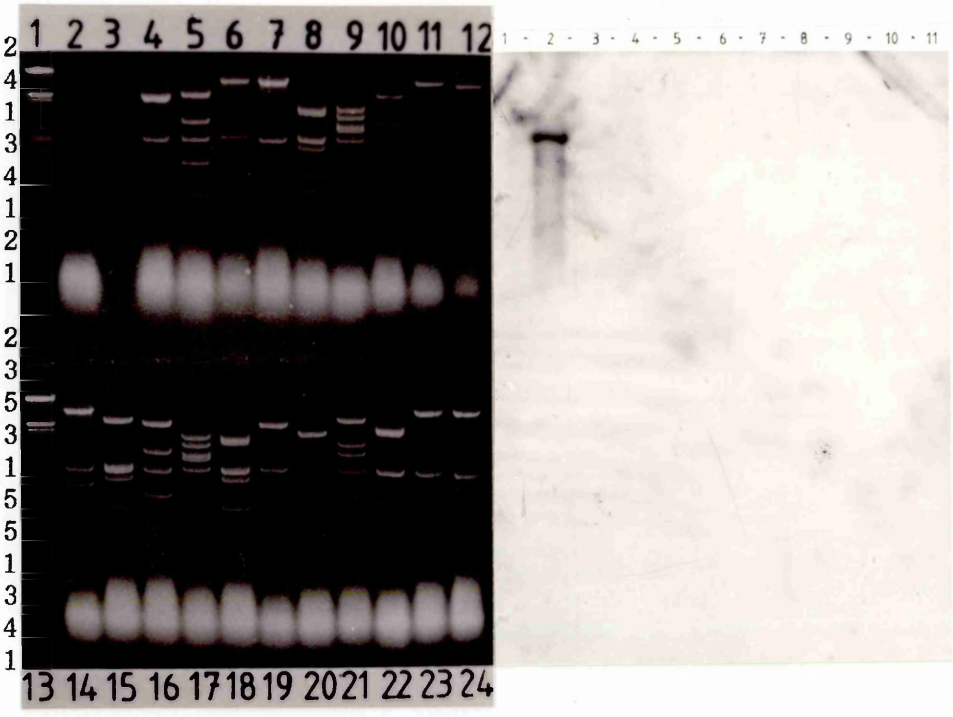
AMP deaminase determinations were carried out in triplicate and the figures given are an average of these determinations. Also shown in this figure are the errors produced by the assay system.

0.5ml each of reagents 2 and 3). Each sample was assayed in triplicate in this way. Included with each set of assays was a substrate blank reaction. The same protein extracts were also subjected to standard Bradford protein assays and the AMP deaminase specific activity was calculated. The results of this experiment is shown in figure 6.5.2.

From these results it is clear that a similar pattern of AMP deaminase activity is seen in strain X4003-5B here, as was seen in Figure 6.2.2. However the pattern of AMP deaminase activity in strains X4003-22 and X4003-3.4 during heat shock do not agree with those predicted above if hsp90 and AMP deaminase are the same. It was hypothesised that if AMP deaminase and hsp90 are the same, the mutation of the HSP90 gene would result in little difference in AMP deaminase activity prior to heat shock, but that the inactivated strain would have greatly reduced AMP deaminase activity after being heat shocked. Clearly strain X4003-22 does have a slightly lower basal level of AMP deaminase specific activity than X4003-5B but after heat shock the pattern of AMP deaminase activity is very similar (i.e. there is no dropping off of AMP deaminase activity in X4003-22 as predicted if AMP deaminase is hsp90). Similarly, one would predict that if AMP deaminase is hsp90 a strain bearing multiple copies of the HSP90 gene would have a slightly elevated AMP deaminase activity prior to heat shock and considerably more AMP deaminase activity after heat shock. Clearly this is not the case in this experiment. The strain bearing multiple copies of the HSP90 strain has been shown to synthesise elevated levels of hsp90 protein during heat shock (Figure 6.5.1) but there is not a concomitant increase in AMP deaminase activity. Small differences in specific activities can be explained by slightly different growth conditions in the three different cultures used. However, the overall changes in the pattern of AMP deaminase activity predicted in these strains during heat shock are clearly not seen. It therefore seems clear that hsp90 and AMP deaminase are not the same protein.

FIGURE 6.6.1 SOUTHERN BLOT ANALYSIS OF PLASMIDS SELECTED FROM COLONY SCREENING WITH OLIGONUCLEOTIDE 328 PROBED WITH A RAT AMP DEAMINASE cDNA

LANE	SAMPLE
1	λR/H
2	pBSAMPD
3	
4	YEp24
5	378.2.2
6	378.2.4
7	378.3.1
8	378.3.3
9	378.3.4
10	378.4.1
11	378.4.2
12	378.5.1
13	λR/H
14	378.5.2
15	378.5.3
16	378.5.5
17	378.7.3
18	378.8.1
19	380.1.5
20	380.2.5
21	380.3.1
22	380.4.3
23	380.4.4
24	380.7.1



A agarose gel of pBSAMPD and plasmids which had been selected by colony hybridisation as described in section 6.3 (i.e during screening for HSP90-containing plasmids). All are digested with Eco RI (see section 6.6)

Note: only approximately 20ng of plasmid pBSAMPD digested with Eco RI was loaded in lane 2, not visible on photograph

B Southern blot analysis of gel shown in A probed with a 2.5kb Eco RI fragment comprising a rat skeletal muscle AMP deaminase cDNA (from pBSAMPD). This fragment was gel purified twice and [^{32}P]-labelled by random priming.

Note: Hybridisation was carried out at low stringency (37^{o}C with hybridisation solution containing 20% formamide).

facing page 144

6.6 PROBING OF HSP90 SELECTED PLASMID WITH A RAT AMP DEAMINASE cDNA CLONE

Towards the end of 1987, there was a report of the cloning of a rat myoadenylate deaminase cDNA (Sabina et al., 1987). Dr. Sabina kindly sent us this clone, to help in our analyses. The clone was in plasmid bluescribe, and the AMP deaminase insert could be removed on a 2.5kb Eco RI fragment. This plasmid is called pBSAMPD. Sabina et al. had used this clone to probe genomic digests from a wide variety of sources including yeast. They had shown that at low stringencies a single Bam HI fragment of yeast genomic DNA, to which the rat cDNA probe hybridised to, was visible on a Southern blot. This band disappeared at higher stringencies. When we recieved this clone, the selection of HSP90 clones was already in progress, and the decision was made to use this cDNA as a probe against the selected YEp plasmids discussed in Section 6.3. To this end, the YEp clones were digested with Eco RI and run on a 1% agarose gel, along with Eco RI digested pBSAMPD. This gel is shown in Figure 6.6.1.A. The gel was blotted and prehybridised with 20% formamide at 37°C. The probe used was the 2.5kb Eco RI cDNA fragment from pBSAMPD which had been random primed to a specific activity of approximately 10^9cpm/µg DNA. This insert had been twice gel purified to eliminate any contaminating plasmid based DNA. The blot was washed at very low stringency, 6 x SSPE, 0.1% SDS at room temperature. The filter was then autoradiographed. This autoradiograph is shown in Figure 6.6.1.B. Clearly the only thing which the probe hybridises to is itself. Even after prolonged exposure no other hybridisation was visible.

6.7 CONCLUSIONS

Described in this chapter is the work entailed in answering the question asked at the end of the last chapter. Are AMP deaminase and hsp90 the same protein?

The first experiment carried out was the heat shocking of yeast and the assaying of the resulting AMP deaminase activity. As indicated in Section 6.2 the result of this

experiment was inconclusive. AMP deaminase activity was maintained and in fact induced slightly for at least the first 30 minutes of heat shock (See Figure 6.2.2).

The decision was made to insertionally inactivate the HSP90 gene by transforming a yeast strain with an integrating yeast vector targeted to the HSP90 chromosomal locus. The stages in the production of this vector are described and integration was achieved. This was confirmed by Southern and Northern blot analyses and later on by heat shock protein gels.

This constructed strain along with a control strain and the control strain transformed with a multicopy yeast plasmid carrying the HSP90 gene and all the sequences necessary for its heat shock-specific expression were heat shocked and their AMP deaminase activities were studied. The results show that inactivation or overproduction of hsp90 have little effect on AMP deaminase activity either prior to or during heat shock. This is conclusive evidence that hsp90 is not AMP deaminase.

This finding begs the question of why in AMP deaminase purified protein samples does there appear to be only one protein species of the appropriate size. Hsp90 and AMP deaminase from yeast are of very similar sizes (Farrelly and Finkelstein, 1984; Murakami, 1979). If the two proteins have coincidentally co-purified, they may have run as a single band.

But why, if this is the case, was there no indication of two proteins during amino acid sequence analysis. The determined sequence was compatible with only one polypeptide being present (John Fitton, personal communication). One answer to this is that AMP deaminase is N terminally blocked in some way. This is the case with rat myoadenylate deaminase (Sabina et al., 1987). This and other possible reasons for the lack of sequence from AMP deaminase is discussed in the following chapter.

CHAPTER 7

CONCLUDING REMARKS

As discussed in the individual chapters several different strategies have been adopted during the course of this study to facilitate the cloning of the AMP deaminase gene from S.cerevisiae.

In Chapters 3 and 4 methods have been described which relied on the overproduction of AMP deaminase when the gene is introduced into yeast on a multicopy plasmid. During the course of these studies there was no evidence that such an increase in activity would be observed. It was felt that because of the role the enzyme plays in central energy metabolism and because of its exquisite control by allosteric effectors the cell might be extremely disadvantaged by increased amounts of the enzyme. Therefore, when the two different types of methods of screening described in Chapters 3 and 4 failed, a time-consuming screen of the library (by simply assaying several thousand individual transformants) was not attempted. However, there is now some evidence that a strategy like this might have worked as will be discussed later.

The decision was therefore made to attempt to purify AMP deaminase, generate N-terminal amino acid sequence data and hence design and make a suitable oligonucleotide for use as a probe to screen a genomic library. The decision was also made to design a new and more rapid purification method than that previously reported (Yoshino et al., 1979b).

Attempts to use precipitation techniques as an initial purification step resulted in rather mixed success but were finally rejected (as discussed in Chapter 5). Similarly affinity chromatography was not used in the final purification as the types of affinity media studied were not appropriate for the purification of this enzyme. Finally, all purification steps were carried out using the Pharmacia FPLC system. At all stages of this purification problems were encountered in maintaining the enzyme's activity and all attempts to alleviate this situation proved fruitless. Finally, all the steps in the purification were carried out in a single day to maintain enough activity for the identification of an active fraction. Another problem was encountered in that AMP deaminase [at least from mammalian

sources (Fishbein, personal communication) and apparently from yeast] does not enter non-denaturing polyacrylamide gels under normal running conditions and hence attempts to adapt a suitable activity gel were unsuccessful. Therefore, once purified material was produced it was subjected to SDS/PAGE and the presence of a single band of an apparent M.W. of approximately 83kDa (the reported size of yeast AMP deaminase (Yoshino and Murakami, 1979b) was observed. This was deemed to be evidence of sufficient purity to allow amino acid determination to be carried out. In fact there was some minor contamination of lower molecular weight material, but as the 83kDa band was further purified by electroblotting this would not lead to contamination during sequencing.

Sequence analysis, as discussed in Chapter 5 led to the hypothesis that AMP deaminase was identical to heat shock protein 90. The only possible alternative to explain sequence results was that hsp90 had copurified with AMP deaminase.

An initial attempt to distinguish between these two possibilities was to heat shock a culture of yeast and extract the cells and assay for AMP deaminase activity before and after heat shock. The reasoning behind this was that if AMP deaminase activity decreases sharply on heat shock it would be extremely unlikely that it is a heat shock protein. This experiment proved inconclusive since AMP deaminase activity appeared to be maintained, and possibly elevated slightly during heat shock. This could be due to a number of reasons (as will be discussed later) but did not rule out the possibility that hsp90 and AMP deaminase are the same protein.

Therefore, the chromosomal HSP90 gene was insertionally inactivated via the production of a YIp vector. The AMP deaminase activity upon heat shock was then compared in this hsp90 mutated strain and a control strain. The results indicated that AMP deaminase is not hsp90. This is strongly suggestive that hsp90 was copurified with AMP deaminase and that this contaminant yielded the amino acid data.

In 1985, Meyer and Schramm reported that they had cloned the AMP deaminase from yeast. A lambda gt11 library containing randomly sheared S.cerevisiae genomic DNA (to which Eco RI linkers were ligated) was screened using rabbit anti-yeast AMP deaminase antiserum. From this study the same recombinant phage was selected on three separate occasions using this antiserum. Western blot analysis revealed that the phage was producing a hybrid protein of 180kDa comprising 114kDa of B-galactosidase and approximately 66kDa of the cloned gene product (Meyer and Schramm, 1985).

This clone was subjected to DNA sequence analysis and the sequence was compared to amino acid sequence data available from purified yeast AMP deaminase. This comparison revealed that the selected lambda clone did not contain even part of the AMP deaminase gene (V.L. Schramm, personal communication). Therefore this screening system had been unsuccessful in selecting for the AMP deaminase gene, and had yielded only false positives.

As the work described in the body of this thesis was nearing completion, a personal communication from Professor V.L. Schramm indicated that the same group had succeeded in cloning and sequencing the yeast AMP deaminase gene. This work has now been published (Meyer et al., 1989) and the method used by this group to clone the gene is summarised here.

Firstly, a yeast strain deficient in AMP deaminase was isolated by direct enzymatic assay of colonies surviving treatment with nitrosoguanidine. Western blot analysis confirmed that the strain was deficient in AMP deaminase (i.e. amd). Purified AMP deaminase was subjected to tryptic digestion and small peptides were HPLC purified. The N-terminal sequence of some of these peptides along with the amino acid sequence of the intact protein were determined. Amino acid data from one of these internal tryptic peptides was used to design two synthetic oligonucleotides of 15 and 20 residues respectively. These oligonucleotides were used to screen a lambda gt11 yeast genomic library which allowed selection of a clone containing part of the AMP deaminase gene. A fragment from this clone was then used to screen a

yeast genomic library in the yeast-E.coli shuttle vector YCp50. Selected plasmids were then transformed in to the amd strain and one clone restored AMP deaminase activity to the deficient strain, and restored the band corresponding to AMP deaminase on Western blots.

Some of the findings of this group are pertinent to the study presented here. Firstly, they found that an amd$^-$ strain (lacks AMP deaminase activity) is viable. They also found that when introduced into the cell on a multi-copy vector, multiple copies of the AMP deaminase gene resulted in a 15-fold increase in AMP deaminase specific activity, and this had little effect on the strain's growth (Meyer et al., 1989). These findings suggest that production of an AMP deaminase mutant would have been an alternative strategy to adopt. We had reasoned that, because of the enzymes central role in energy metabolism the AMP deaminase gene may be essential.

Western blot analysis performed by this same group revealed that extracts prepared from actively growing yeast indicates a major band at approximately 96kDaltons (which is in close agreement with their predicted molecular weight of 93,286 Daltons). However, in such extracts several bands at lower molecular weight, including 83kDaltons are detected by the same antisera. This 83kDa polypeptide is the major form found in purified preparations of AMP deaminase, as previously reported by Yoshino et al (1979b). It appears that during purification, and possibly to a certain extent within the cell, limited proteolysis occurs which results in a truncated, but active form of AMP deaminase. This is highly reminiscent of the findings discussed in Chapter 1 where AMP deaminase of rabbit muscle, frog muscle, trout gill and muscle is subjected to limited proteolysis when stored (Raffin, 1986a and 1986b; Ranieri-Raggi et al., 1985). Raffin found that in the case of trout muscle AMP deaminase this situation could be mimicked trypsin treatment. These cases of limited proteolysis resulted in the enzyme having altered kinetics. This situation remains to be seen in the case of yeast AMP deaminase.

This does not however answer the question, why did purified AMP deaminase material yield no amino acid sequence data, but instead hsp90 was successfully sequenced. There was no evidence during sequencing that more than one protein was present in the fraction purified on the basis of AMP deaminase activity. That is, the quality of the sequence was in agreement with there being only one protein present (John Fitton, personal communication). However, it is possible that AMP deaminase was present in the purified material in such low quantities as to yield not even evidence of there being more than one polypeptide species present. As stated at the end of the previous chapter this seems unlikely. Unfortunately, the purification procedure adopted in this study was inefficient in terms of recovery. It did not yield sufficient material to allow protease treatment to allow N-terminal sequencing of internal peptides to be carried out. Meyer and Schramm have found no evidence that the 83kDa truncated protein was N-terminally blocked (V.L. Schramm, personal communication). However the question of what they term a "ragged" end (i.e. a number of protein species of various molecular weights can be detected) to AMP deaminase is intriguing. They found that in actively growing cultures the predominant species is the 96kDa one, and throughout purification, protease contamination lowers the yield of this high molecular weight form in favour of the lower molecular weight form. Might it be that in cells which are not actively growing (i.e. stationary cells, such as those that used to produce protein extracts) the proteolysed forms are predominant. It may even be that under certain conditions a further proteolysis to yield a slightly smaller polypeptide can occur and this intermediate may be blocked to Edman degradation of the N-terminal. A range of protease inhibitors were tested, but with apparently no effect. Hence, only 1mM PMSF was added to buffers in the final purification. Clearly, further study is required to answer these questions.

Using the sequence data produced by Meyer et al. (1989), the AMP deaminase gene has been selected and cloned in this laboratory, as descibed briefly here. Two oligonucleotides were designed and produced which are complementary to 5' and 3' regions of the gene respectively. Using these oligonucleotides a lambda gt11 genomic library was screened

and lambda clones representing the 5' and 3' sections of the gene were selected. The inserts of these lambda clones were subcloned into pUC18 and subjected to double-stranded sequence analysis to confirm that they are indeed AMP deaminase gene-specific sequences.

Work will continue to study some aspects of AMP deaminase expression. Interestingly, within the AMP deaminase gene promoter there is a 7 out of 8 match to the consensus described by Tuite et al. (1988), to best represent the heat shock responsive element in yeast. This consensus sequence and its positioning in the 5' regions of certain heat shock regulated genes is shown below.

Consensus		nnTTCTAGAAnn	
hsp26	-357	GGTTCTAGAAAG	-346
hsp70 A	-356	CATTCTAGAAAG	-345
hsp70 B	-203	TTTTCCAGAAAG	-192
hsp90	-237	TTTTCTAGAACG	-226
PGK1	-366	GGTTCTGGAAAG	-355
AMPD	-271	AGTTCTAGTAGC	-263

As can be seen from this data, other genes known to be regulated by heat shock, namely HSP70B and PGK1 (the gene for phosphoglycerate kinase) have only a 7 out of 8 match to this consensus sequence. Therefore, one of the prime objectives is to study the effect of heat shock (and other related stresses) on the expression of AMP deaminase at the level of RNA. Clearly the results indicated in chapter 6 have strongly suggested that AMP deaminase activity is maintained during heat shock. This now awaits confirmation by Northern blot analysis.

APPENDICES

APPENDIX 1:

Genotypes of progeny of DBY868/W1-2D rho$^-$C cross

1.	A ade2$^+$, hpt1$^+$, his4$^+$	2. A ade2$^+$, hpt1$^+$, his4$^+$
	B ade2$^+$, hpt1$^-$, his4$^+$	B ade2$^-$, hpt1$^+$, his4$^-$
	C ade2$^-$, hpt1$^+$, his4$^-$	C ade2$^-$, hpt1$^-$, his4$^+$
	D ade2$^-$, hpt1$^-$, his4$^-$	D ade2$^+$, hpt1$^-$, his4$^-$
3.	A ade2$^-$, hpt1$^+$, his4$^-$	4. A ade2$^+$, hpt1$^-$, his4$^-$
	B ade2$^-$, hpt1$^+$, his4$^+$	B ade2$^+$, hpt1$^-$, his4$^+$
	C ade2$^+$, hpt1$^-$, his4$^-$	C ade2$^-$, hpt1$^+$, his4$^+$
	D ade2$^+$, hpt1$^-$, his4$^+$	D ade2$^-$, hpt1$^+$, his4$^-$
5.	A ade2$^+$, hpt1$^-$, his4$^+$	6. A ade2$^-$, hpt1$^+$, his4$^+$
	B ade2$^-$, hpt1$^+$, his4$^-$	B ade2$^-$, hpt1$^+$, his4$^-$
	C ade2$^-$, hpt1$^-$, his4$^+$	C ade2$^+$, hpt1$^-$, his4$^-$
	D ade2$^+$, hpt1$^+$, his4$^-$	D ade2$^+$, hpt1$^-$, his4$^+$
7.	A ade2$^-$, hpt1$^+$, his4$^-$	8. A ade2$^+$, hpt1$^-$, his4$^+$
	B ade2$^-$, hpt1$^+$, his4$^-$	B ade2$^-$, hpt1$^+$, his4$^-$
	C ade2$^+$, hpt1$^-$, his4$^+$	C ade2$^+$, hpt1$^-$, his4$^+$
	D ade2$^+$, hpt1$^-$, his4$^+$	D ade2$^-$, hpt1$^+$, his4$^-$
9.	A ade2$^+$, hpt1$^-$, his4$^+$	10. A ade2$^+$, hpt1$^-$, his4$^+$
	B ade2$^-$, hpt1$^+$, his4$^+$	B ade2$^-$, hpt1$^+$, his4$^-$
	C ade2$^-$, hpt1$^+$, his4$^-$	C ade2$^+$, hpt1$^-$, his4$^-$
	D ade2$^+$, hpt1$^-$, his4$^-$	D ade2$^-$, hpt1$^+$, his4$^+$

Genetic Analysis of Cross 1

The recombination frequencies presented here have been calculated thus (Fincham, 1983)

$$R = \frac{\text{Tetratypes} + (2 \times \text{Non-parental ditypes})}{2 \times \text{Total asci}}$$

Note: The status of the HPT1 locus could not be determined in ade2$^-$ strains. However, probable HPT1 status has been assigned wherever possible, i.e. where two spores from the same tetrad are identical at the ADE2 and HIS4 loci, and one must be Hpt1$^-$ and one Hpt$^+$.

ADE2/HPT1 recombination analysis

PDT 7

NPDT

TT 3

The recombination frequency between ADE2 and HPT1 markers is calculated as 15% within the limits of this experiment. The ADE2 marker is known to reside on chromosome XV (Mortimer and Schild, 1985). As yet HPT1 has not been assigned to a chromosome. A recombination frequency between these markers of 15% suggests that HPT1 may also resides on chromosome XV. Further evidence that these two loci are linked is the excess of parental ditype asci compared to non-parental ditype asci (i.e. 7 to 0).

ADE2/HIS4 recombination analysis

PDT 3

NPDT

TT 7

The recombination frequency between ADE2 and HIS4 is calculated to be 35% in the limits of this experiment. This is considered as not a significant deviation from the expected frequency of 50%. This result is not surprising since as previously stated ADE2 resides on chromosome XV and HIS4 has been reported as residing on chromosome III (Mortimer and Schild, 1985).

HPT1/HIS4 recombination analysis

PDT 2

NPDT

TT 8

The recombination frequency between HPT1 and HIS4 is calculated as 48% within the limits of this experiment. This is considered to be 50% which is as expected since as previously stated HIS4 has been assigned to chromosome III, and we have hypothesised here that HPT1 resides on chromosome XV which would mean that these two genes are linked.

APPENDIX 2:

Genotypes of the progeny of JMM1-1D/X4003-5B cross

1.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	−	+	−	−	−	−
B	−	+	−	+	−	−	+
C	+	−	−	−	−	+	
D	+	+	+	+	−	+	

2.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	+	−	+	−	−	
B	−	−	+	−	−	+	
C	−	+	+	−	−	−	−
D	+	−	−	+	−	−	

3.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	−	−	−	−	+	
B	−	+	−	−	+	−	+
C	+	−	+	+	−	−	
D	+	+	+	+	−	−	

4.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	−	−	−	−	+	
B	+	+	+	+	−	−	
C	−	−	+	−	−	−	−
D	+	+	−	+	−	−	

5.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	−	+	+	−	−	
B	−	−	−	−	−	−	+
C	+	+	+	−	−	+	
D	−	+	−	+	−	−	+

6.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	+	−	−	−	+	
B	+	+	+	+	−	−	
C	−	−	−	−	−	−	−
D	+	−	+	+	−	−	

7.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	+	+	−	−	−	+
B	−	−	+	−	−	−	+
C	+	−	−	+	−	−	
D	+	+	−	+	−	+	

8.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	+	+	+	−	−	+
B	−	−	+	+	−	−	−
C	+	−	−	−	−	+	
D	+	+	−	−	−	−	

9.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	+	−	−	−	+	
B	+	−	+	+	−	−	
C	+	+	+	+	−	−	
D	−	−	−	−	−	−	−

10.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	+	+	+	−	−	
B	−	−	−	−	−	−	+
C	−	+	+	+	−	+	
D	+	−	−	−	−	−	

11.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	−	−	+	−	+	
B	−	−	+	−	−	−	+
C	−	+	+	−	−	−	−
D	+	+	−	+	−	−	

12.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	+	+	+	−	−	+
B	+	−	−	−	−	+	
C	+	+	−	+	−	−	
D	−	−	+	−	−	−	+

13.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	−	+	−	−	−	−	+
B	+	−	−	+	−	−	
C	−	−	+	−	−	+	
D	+	+	+	+	−	−	

14.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	-	+	+	-	-	
B	-	+	-	-	-	-	-
C	+	-	+	+	-	-	
D	-	+	-	-	-	-	+

15.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	+	-	-	-	-	+
B	+	-	+	+	-	+	
C	-	-	-	-	-	-	-
D	+	+	+	+	-	-	

16.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	-	+	-	+	-	
B	-	-	+	+	-	+	
C	-	+	-	+	-	-	-
D	+	+	-	-	+	+	

17.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	+	+	+	-	-	
B	-	-	-	-	-	+	
C	-	+	-	-	-	-	-
D	+	-	+	+	-	-	

18.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	-	+	+	-	-	
B	-	+	-	-	-	+	
C	+	-	+	+	-	-	
D	-	+	-	-	-	-	-

Genotypes of the progeny of JMM1-5C/X4003-5B cross

1.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	-	-	+	+	-	
B	+	+	+	+	-	-	
C	-	+	-	-	-	-	+
D	-	-	+	-	+	-	+

2.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	+	-	-	+	-	-
B	-	-	-	-	-	-	+
C	+	+	+	+	+	-	
D	+	-	+	+	-	-	

3.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	-	+	-	+	+	
B	-	+	-	+	-	-	+
C	-	+	-	+	+	+	
D	+	-	+	-	-	-	

4.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	+	+	+	-	-	
B	-	-	-	-	-	+	
C	-	-	-	+	+	-	+
D	+	+	+	-	+	-	

5.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	+	-	+	+	+	-	
B	-	+	-	-	-	+	
C	+	+	+	+	+	-	
D	-	-	-	-	-	-	+

6.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	-	+	-	+	-	+
B	+	+	-	+	+	-	
C	+	-	-	+	-	+	
D	-	+	+	-	-	-	-

7.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	-	+	+	+	-	+
B	-	-	-	+	+	-	+
C	+	+	+	-	-	-	
D	+	+	-	-	-	+	

8.

	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	+	-	+	+	+	
B	+	+	-	+	+	-	
C	-	-	+	-	-	-	-
D	+	-	+	-	-	+	

9.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	-	+	+	+	+	
B	+	-	-	-	+	-	
C	+	+	-	-	-	-	
D	-	+	+	+	-	-	-

10.	ura3	met2	trp5	leu2	his4	ade$^-$	hpt1
A	-	+	+	+	+	+	
B	+	-	+	-	-	-	
C	-	+	-	-	+	-	+
D	+	-	-	+	-	-	

Genetic Analysis of Crosses 2 and 3

The recombination frequencies presented here have been calculated thus (Fincham, 1983)

$$R = \frac{\text{Tetratypes} + (2 \times \text{Non-parental ditypes})}{2 \times \text{Total asci}}$$

Note: In all instances with the exception of HIS4, data from both crosses can be analysed together. Because both parents of cross 3 are his4$^-$, no assortment can be seen in this cross for this marker therefore data relating to HIS4, is taken only from cross 2.

All reference to chromosomal location is from Mortimer and Schild, 1985.

URA3/MET2 recombination analysis

PDT	3
NPDT	4
TT	21

The recombination frequency between URA3 and MET2 is calculated as 52%. Therefore the two genes are unlinked. Ura3 resides on chromosome V and MET2 on chromosome XIV.

URA3/TRP5 recombination analysis

PDT	12
NPDT	7
TT	9

The recombination frequency between URA3 and TRP5 is calculated as 41% which does not deviate significantly from 50%. The URA3 gene has been assigned to chromosome V and is therefore not linked to TRP5 which has been assigned to chromosome VII.

URA3/LEU2 recombination analysis

PDT	16
NPDT	5
TT	7

The recombination frequency between URA3 and LEU2 is calculated as 28.2% which does not deviate significantly from 50%. The URA3 gene has been assigned to chromosome V and is therefore not linked to LEU2 which has been assigned to chromosome III.

MET2/TRP5 recombination analysis

PDT	2
NPDT	5
TT	21

The recombination frequency between MET2 and TRP5 is calculated as 55% which does not deviate significantly from 50%. The MET2 gene has been assigned to chromosome XIV and is therefore not linked to TRP5 which has been assigned to chromosome VII.

MET2/LEU2 recombination analysis

PDT	6
NPDT	3
TT	19

The recombination frequency between MET2 and LEU2 is calculated as 44% which does not deviate significantly from 50%. The MET2 gene has been assigned to chromosome XIV and is therefore not linked to LEU2 which has been assigned to chromosome III.

TRP5/LEU2 recombination analysis

PDT	12
NPDT	6
TT	10

The recombination frequency between TRP5 and Leu2 calculated as 39% which does not deviate significantly from 50%. The TRP5 gene has been assigned to chromosome VII and is therefore not linked to LEU2 which has been assigned to chromosome III.

HIS4/URA3 recombination analysis

PDT	2
NPDT	1
TT	7

The recombination frequency between HIS4 and URA3 calculated as 45% which does not deviate significantly from 50%. The HIS4 gene has been assigned to chromosome III and is therefore not linked to URA3 which has been assigned to chromosome V.

HIS4/MET2 recombination analysis

PDT	3
NPDT	3
TT	4

The recombination frequency between HIS4 and MET2 is calculated as 50%. The HIS4 gene has been assigned to chromosome III and is therefore not linked to MET2 which has been assigned to chromosome XIV.

HIS4/TRP5 recombination analysis

PDT	1
NPDT	1
TT	8

The recombination frequency between HIS4 and TRP5 is calculated as 50%. The HIS4 gene has been assigned to chromosome III and is therefore not linked to TRP5 which has been assigned to chromosome VII.

HIS4/LEU2 recombination analysis

PDT	3
NPDT	
TT	7

The recombination frequency between HIS4 and LEU2 is calculated as 35% which does not deviate significantly from 50%. However both HIS4 and LEU2 have been mapped to chromosome IIIL and the 35% recombination frequency may represent some level of linkage between these two loci.

REFERENCES

Agarwal, R.P. and Parks, R.E.,Jnr.(1977).
Potent inhibition of muscle AMP deaminase by the nucleoside antibiotics coformycin and deoxycoformycin
Biochemical Pharmacol 26: 663-666.

Almarez, L. and Garcia-Sancho, J. (1989).
Activation by calcium of AMP deaminase from the human red cell
FEBS Lett 244: 417-420.

Anderson, N.L., Giomettim, C.S., Gemmell, M.A., Nance, S.L. and Anderson, N.G. (1982).
A two-dimensional analysis of the heat shock-induced proteins of human cells
Clin Chem 28: 1084-1092.

Ashburner, M. (1982).
Effects of heat shock and other stress on gene activity: an introduction
In Heat shock: from Bacteria to Man, Schlesinger, M.J., Ashburner, M. and Tissieres, A. New York: Cold Spring Harbour.

Ashburner, M. and Bonner, J.J. (1979).
The induction of gene activity in Drosophila by heat shock
Cell 17: 241-254.

Ashby, B. and Holmsen, H. (1983).
Platelet AMP deaminase: Regulation by Mg-ATP and inorganic phosphate and inhibition by the transition state analogue coformycin
J. Biol. Chem. 258: 3668-3672.

Ashby, B., Wernick, E. and Holmson, H. (1982).
Coformycin inhibition of AMP deaminase in broken and intact platelets
Fed Proc 41: 701.

Askari, A. and Rao, S.N. (1968).
Regulation of AMP deaminase by 2,3-diphosphoglyceric acid: A possible mechanism for the control of adenine nucleotide metabolism in human erythrocytes
Biochim Biophys Acta 151: 198-203.

Attfield, P.V. (1987).
Trehalose accumulates in yuck during exposure to agents that induce the heat shock response
FEBS Lett 225: 259-263.

Bagnara, A.S. and Hershfield, M.S. (1982).
Mechanism of deoxyadenosine induced catabolism of adenine ribonucleotides in adenosine deaminase inhibited human T lymphoblastoid cells
Proc. Natl. Acad. Sci. USA 79: 2673-2677.

Ball, W.J.,Jnr. and Atkinson, D.E. (1975).
Adenylate energy charge in yuck during starvation
J. Bact. 121: 975-982.

Beggs, J.D. (1978).
Transformation of yeast by a replicating hybrid plasmid
Nature 275: 104-109.

Bergmeyer, H.U. and Beutler, H-O. (1982)
Ammonia in Methods in Enzymatic Analysis (3rd Ed), pp454-462
New York: Verlag Chemie

Biellmann, J.F., Samama, J.P., Branden, C.I. and Eklund, H. (1979).
X-ray studies on the binding of Cibacron Blue F3G-A to liver alcohol dehydrogenase
Eur J Biochem 102: 107-110.

Binart, N., Chambraud, B., Dumas, B., Rowlands, D.A., Bigogne, R., Levin, J.M., Garnier, J., Baulieu, E-E. and Catelli, M-G. (1985).
The cDNA derived amino acid sequence of chick HSP Mr 90000 (hsp90) reveals a "DNA-like" structure: potential site of interaction with steroid receptors
Biochem Biophys Res Commun 159: 140-147.

Birnboim, H.C. and Doly, J. (1979).
A rapid alkaline extraction procedure for screening recombinant plasmid DNA
Nucleic Acids Res. 7: 1513-1523.

Bond, U. and Schlesinger, M.J. (1985).
Ubiquitin is a heat shock protein in chick embryo fibroblasts
Mol Cell Biol 5: 949-956.

Bosca, L. and Corredor, C. (1984)
Is phosphofructokinase the rate-limiting step of glycolysis?
Trends in Biochem Sci 9: 372-374

Boucherie, H. (1985).
Protein synthesis during transition and stationary phase under glucose limitation in S.cerevisiae
J. Bact 161: 385-392.

Bradford, M.M. (1978).
A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding
Anal Biochem 72: 248-254.

Brugge, J.S., Eriksson, E. and Eriksson, R.L. (1981).
The specific interaction of the Rous Sarcoma Virus transforming protein, pp60src, with two cellular proteins
Cell 25: 363-372.

Brugge, J.S., Yonemoto, W. and Darrow, D. (1983).
Interaction between the Rous Sarcoma Virus transforming protein and two cellular phosphoproteins: Analysis of the turnover and distribution of this complex
Mol Cell Biol 3: 9-19.

Buchwald, M., Ullman, B., Martin, D.W. and Jnr, (1981).
Biochemical and genetic analysis of AMP deficiency in cultured mammalian cells
J. Biol. Chem. 256: 10346-10353.

Buniatian, H.C.H. and Haroutunian, A.V. (1971).
Potentiation of AMP aminohydrolase activity of brain mitochondria by hexokinase
J. Neurochem 18: 859-867.

Burdon, R.H. and Cutmore, C.M.M. (1982).
Human heat shock gene expression and the modulation of plasma membrane Na, K -ATPase activity
FEBS Lett 140: 45-48.

Carlson, M. and Botstein, D. (1982).
Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase
Cell 28: 145-154.

Carson, D.A., Haertle, T., Wasson, D.B. and Richman, D.D. (1988).
Biochemical genetic analysis of 2',3'-deoxyadenosine metabolism in human T lymphocytes
Biochem Biophys Res Commun 151: 788-793.

Catelli, M.G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E.E., Feramisco, J.R. and Welch, W.J. (1985).
The common 90kD protein component of non-transformed 8S steroid receptors is a heat shock protein
EMBO J. 4: 3131-3135.

Catelli, M-G., Joab, I., Renoir, J-M., Mester, J., Binart, N., Radanyi, C., Buchou, R., Zoorob, R. and Baulieu, E-E. (1983).
Immunological studies of the chick oviduct progesterone receptor
2nd International Congress on Hormones in Cance J Ster Biochem 19: Abstr 402.

Cavicchioli, R. and Watson, K. (1986).
Loss of heat shock acquisition of thermotolerance in yeast is not correlated with loss of heat shock proteins
Febs Lett 207: 149-152.

Chapman, A.F. and Atkinson, D.E. (1973).
Stabilisation of the adenylate energy charge by the adenylate deaminase reaction
J. Biol. Chem. 248: 8309-8312.

Chapman, A.G., Fall, L. and Atkinson, D.E. (1971).
Adenylate energe charge in E.coli during growth and starvation
J Bact 108: 1072-1086.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979).
Isolation of biologically active RNA from sources enriched in ribonuclease
Biochem. 24: 5294-5299.

Chirico, W.J., Waters, M.G. and Blobel, G. (1988).
70K heat shock-related proteins stimulate protein translocation into microsomes
Nature 332: 805-810.

Coffee, C.J. and Kofke, W.A. (1975).
Rat muscle 5' Adenylic acid Aminohydrolase I: Purification and subunit structure
J. Biol. Chem. 250: 6653-6658.

Conway, E.J. and Cooke, R. (1938).
Blood Ammonia and the deaminases of adenosine and adenylic acid
Nature 142: 720.

Conway, E.J. and Cooke, R. (1939).
The deaminases of adenosine and adenylic acid in blood and tissues
Biochem J. 33: 479.

Cooper, J. and Trinick, J. (1984)
Binding and localisation of AMP deaminase in rabbit psoas muscle myofibrils.
J. Mol. Biol. 177: 137-152

Costlow, N. and Lis, J.T.
High resolution mapping of DNaseI-hypersensitive sites of Drosophila heat shock gene in D.melanogaster and S.cerevisiae
Mol. Cell. Biol. 4: 1853-1863

Craig, E.A. (1985).
The Heat Shock Response
Crit Rev Biochem 18: 239-280.

Craig, E.A., Kramer, J. and Kosk-Smithers, J. (1987).
SSC1, a member of the 70kDa heat shock protein multigene family of S.cerevisiae is essential for growth
Proc. Natl. Acad. Sci. USA 84: 4156-4160.

Cunningham, B. and Lowenstein, J.M. (1965).
Regulation of AMP deaminase by ATP
Biochim Biophys Acta 96: 535-537.

Curling, J.M. (ed) (1980).
Methods of plasma protein fractionation
New York : Academic Press.

DeBanzie, J.S., Sinclair, L. and Lis, J.T. (1986).
Expression of a major heat shock gene of D.melanogaster in S.cerevisiae
Nucleic Acids Res. 14: 3587-3601.

Debatisse, M., Berry, M. and Buttin, G. (1981).
The potentiation of adenine toxicity to Chinese Hamster cells by coformycin: suppression in mutants with altered regulation of purine biosynthesis or increased AMP deaminase activity
J Cell Physiol 106: 1-11.

Debatisse, M., Berry, M. and Buttin, G. (1982).
Stepwise isolation and properties of unstable Chinese Hamster cell variants that over produce adenylate deaminase
Mol Cell Biol 2: 1346-1353.

Debatisse, M. and Buttin, G. (1977).
The control of cell proliferation by preformed purines: A genetic study I. Isolation and preliminary characterisation

of Chinese hamster lines with single or multiple defects in the purine "salvage" pathways
Somat Cell Genet 3: 497-511.

Debatisse, M., De Saint Vincent, B.R. and Buttin, G. (1984).
Expression of several amplified genes in an adenylate deaminase overproducing variant of Chinese hamster fibroblasts
EMBO J. 3: 3123-3128.

Debatisse, M., Hyrien, O., Petit-Koskas, E., De Saint Vincente, B.R. and Buttin, G. (1986).
Segregation and rearrangement of coamplified genes in different lineages of mutant cells that overproduce adenylate deaminase
Mol Cell Biol 6: 1776-1781.

Debatisse, M., Satto, I., Buttin, G. and Stark, G.R. (1988).
Preferential amplification of rearranged sequences near amplified adenylate deaminase genes
Mol Cell Biol 8: 17-24.

Denis, M. and Gustafsson, J.A. (1989).
Translation of glucocorticoid receptor mRNA in vitro yields a non-activated protein
J. Biol. Chem. 264: 6005-6008.

Deshaies, R.J., Koch, B.D., Werner-Washburne, D., Craig, E.A. and Schekman, R. (1988).
A subfamily of stress proteins facilitate translocation of secretory and mitochondrial precursor polypeptides
Nature 332: 800-810.

DiMauro, S., Gihanda, A.F., Hays, A.P. and Franck, W.A. (1980).
Myoadenylate deaminase deficiency, muscle biopsy and muscle culture in a patient with gout
J Neurol Sci 47: 191-202.

Dorfman, B-Z. (1968).
The isolation of adenylosuccinate synthetase mutants in yeast by selection for constitutive behaviour in pigmented strains
Genetics 61: 377-389.

Dornand, J., Clofent, G., Bonnafous, J-C., Favero, J. and Mani, J.C. (1985).
Purine metabolising enzymes of lymphocyte cell populations: correlation between AMP deaminase activity and dATP accumulation in murine lymphocytes
Proc Soc Exp Biol Med 179: 448-455.

Ellis, R.J. and Hemmingsen, S.M. (1989).
Molecular chaperones: proteins essential for the biogenesis of some macro-molecular structures
Trends Biochem Sci 14: 339-342.

Engel, A.G., Potter, C.S. and Rosevear, J.W. (1964).
Nucleotides and adenosine monophosphate deaminase activity of muscle in primary hypokalaemic periodic paralysis
Nature 202: 670.

Er-el, Z., Zaidenzaig, Y. and Shaltiel, S. (1972).
Hydrocarbon coated Sepharoses: Use in the purification of glycogen phosphorylase
Biochem Biophys Res Comm 49: 383-390.

Farrelly, F.W. and Finkelstein, D.B. (1984).
Complete sequence of the heat shock inducible hsp90 gene of S. cerevisiae
J Biol Chem 259: 5745-5751.

Farrelly, F.W., Davis, A.H. and Finkelstein, D.B. (1983).
The yeast hsp90 gene family
J. Cell Biol 97: abstract 496.

Feinberg, A.P. and Vogelstein, B. (1983).
A technique for radiolabelling DNA restriction fragments to high specific activity
Anal Biochem 132: 6-13.

Fell, D.A. (1984)
Phosphofructokinase and glycolytic flux
Trends in Biochem Sci 9: 515-516

Ferenczy, L., Sipiczki, M. and Szegedi, M. (1975).
Enrichment of fungal mutants by selective cell wall lysis
Nature 253: 46-47.

Findly, R.C., Gillies, R.J. and Shulman, R.G. (1983).
In vivo Phosphorus-31 nuclear magnetic resonance reveals lowered ATP during heat shock of Tetrahymena
Science 219: 1223.

Finkelstein, D.B. (1983).
Heat shock regulation production of E.coli B-galactosidase in S.cerevisiae
Mol Cell Biol 3: 1625-1633.

Finkelstein, D.B. and Strausberg, S. (1983).
Identification and expression of a cloned yeast heat shock gene
J. Biol. Chem. 258: 1908-1913.

Finkelstein, D.B., Strausberg, S. and McAlister, L. (1982).
Alterations of transcription during heat shock of Saccharomyces cerevisaie
J. Biol. Chem. 257: 8405-8411.

Finley, D., Ciechanover, A. and Varshavsky, A. (1984).
Thermolability of Ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85
Cell 37: 42-55.

Fishbein, W.N. (1977a).
Identification of adenosine deaminase and AMP deaminase in gels, frozen sections and blood smears by direct tetrazolium reduction
Fed Proc 36: 1061.

Fishbein, W.N. (1977b).
Direct catalytic staining methods for histochemical and gel localisation of adenosine deaminase and AMP deaminase
Am J Pathol 86: 79a-80a.

Fishbein, W.N. (1978).
Myoadenylate deaminase deficiency: a new disease of muscle
Science 200: 545-548.

Fishbein, W.N. (1985).
Myoadenylate deaminase deficiency: inherited and acquired forms
Biochem Med 33: 158-169.

Fishbein, W.N., Davis, J.I., Winkert, J.W. and Strong, D.M. (1981).
Levels of adenosine deaminase, AMP deaminase and adenylate kinase in cultured human lymphoblast lines: exquisite sensitive of AMP deaminase to adenosine deaminase inhibitors
Biochem Med 26: 377-386.

Fishbein, W.N., Griffin, J.L. and Armbrustmacher, V.W. (1978).
Application of a new histochemical stain for adenylate deaminase to skeletal muscle biopsies and the discovery of a new disease
J Neuropathol Exp Neurol 104: 613.

Fishbein, W.N., Muldoon, S.M., Deuster, P.A. and Armbrustmacher, V.W. (1985).
Myoadenylate deaminase deficiency and malignant hyperthermia susceptibility: is there a relationship?
Biochem Med 34: 344-354.

Freiden, C., Kurz, L.C. and Gilbert, H.R. (1980).
Adenosine deaminase and adenylate deaminase: comparative kinetic studies with transition state and ground state analogue inhibitors
Biochemistry 19: 5303-5309.

Frieden, C., Gilbert, H.R., Miller, W.H. and Miller, R.L. (1979).
Adenylate deaminase: potent inhibition by 2'-deoxycoformycin-5'-phosphate
Biochem Biophys Res Commun 91: 278-283.

Gasc, J-M., Renoir, J-M., Radanyi, C., Joab, I., Tuohimaa, P. and Baulieu, E-E.(1984).
Progesterone receptor in the chick oviduct: an immuno-histochemical study with antibodies to distinct receptor components
J Cell Biol 99: 1193-1201.

Gekko, K. and Timasheff, S.N. (1981).
Thermodynamic and kinetic examination of protein stabilisation by glycerol
Biochemistry 20: 4677-4686.

Gopalakrishna, R. and Anderson, B. (1982).
Ca2+-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-superose affinity chromatography
Biochem Biophys Res Commun 104: 830-836.

Guisti, G. (1974)
Determination of ammonia
In Methods of Enzymatic Analysis Vol 4, pp314-323, Bergmeyer, H.V. (ed) Basel: Verlagchemie.

Haeckel, R., Hess, B., Lauterborn, W. and Wurster, K. (1968).
Purification and allosteric properties of yeast pyruvate kinase
Hoppe-Seyler's Z Physiol Chem 349: 699-714.

Haertle, T., Carrera, C.J., Wasson, D.B., Sowers, L.C., Richman, D.D. and Carson, D.A. (1988).
Metabolism and anti-human immunodeficiency virus-1 activity of 2-halo-2',3'-dideoxyadenosine derivatives
J. Biol. Chem. 263: 5870-5875.

Hall, B.G. (1983).
Yeast thermotolerance does not require protein synthesis
J Bact 156: 1363-1365.

Haroutunian, A.V. and Buniatian, H.C.H. (1974).
On the mechanism of stimulation of AMP aminohydrolase activity by hexokinase in brain tissue
J Neurochem 23: 297-302.

Hattari, M. and Sakaki, Y. (1986)
Dideoxy sequencing method using denatured plasmid templates
Anal Biochem 152: 232-238

Heller, S.L., Kaiser, K.K., Planer, G.J., Hagberg, J.M. and Brooke, M.H. (1987).
McArdle's disease with myoadenylate deaminase deficiency: observations in a combined enzyme deficiency
Neurology 37: 1039-1042.

Hemmingsen, S.M., Woolford, C., Van, , der, , Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W. and Ellis, R.J. (1988).
Homologous plant and bacterial proteins chaperone oligomeric protein assembly
Nature 333: 330-334.

Henderson, J.F., Brox, L., Zombor, G., Hunting, D. and Lomax, C.A. (1977).
Specificity of adenosine deaminase inhibitors
Biochem Pharmacol 26: 1967-1972.

Henry, S.A., Donahue, T.F. and Culbertson, M.R. (1975).
Selection of spontaneous mutants by inositol starvation in yeast
Molec Gen Genet 143: 5-11.

Henry, S.A. and Horowitz, B. (1975).
A new method for mutant selection in S.cerevisiae
Genetics 79: 175-186.

Hess, B. (1973)
Organization of glycolysis: Oscillatory and stationary control
SEB Symp 27: 105

Hickey, E., Brandon, S.E., Smale, G., Lloyd, D. and Weber, L.A. (1989).
Sequence and Regulation of a gene encoding a human 89kD heat shock protein
Mol Cell Biol 9: 2615-2626.

Higuchi, I., Ishiura, S., Nonaka, I. and Sugita, H. (1987).
Immunohistochemical localisation of AMP deaminase in rimmed vacuoles in human skeletal muscle
Muscle Nerve 10: 790-800.

Higuchi, I., Nonaka, I., Ishiura, S., Ishihara, T. and Sugita, H. (1986).
High AMP deaminase activity in rimmed vacuoles of skeletal muscle
J Neurol Sci 75: 329-342.

Hinnen, A., Hicks, J.B. and Fink, G.R. (1978).
Transformation of Yeast
Proc. Natl. Acad. Sci. USA 75: 1229-1333.

Hofstee, BHJ. and Otillio, N.F. (1978).
Modifying factors in hydrophobic protein binding by substituted agaroses
J Chromatogr 161: 153-163.

Holmes, D.S. and Quigley, M. (1981).
A rapid boiling method for the preparation of bacterial plasmids
Anal Biochem 114: 193-197.

Holmgren, R., Corces, V., Morimoto, R., Blackman, R. and Meselson, M. (1981).
Sequence homologies in the 5' regions of 4 Drosophila heat shock genes
Proc. Natl. Acad. Sci. USA 78: 3775-3778.

Holmsen, H. and Robkin, L. (1977).
Hydrogen peroxide lowers ATP levels in platelets without altering adenylate energy charge and platelet function
J. Biol. Chem. 252: 1752-1757.

Hottinger, T., Boller, T. and Wiemken, A. (1987).
Rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in S.cerevisiae cells subjected to temperature shifts
FEBS Lett 220: 113-115.

Husic, H.D. and Suelter, C.H. (1984).
Binding internalisation and degradation of AMP aminohydrolase by avian hepatocyte monolayers
J. Biol. Chem. 259: 4365-4371.

Iida, H. and Yahara, I. (1984).
Heat shock proteins in G0 cells of yeast and other eukaryotes
J Cell Biol 99: 199-207.

Iida, H. and Yahara, I. (1985).
Yeast heat shock protein of Mr 48000 is an isoprotein of enolase
Nature 315: 688-690.

Ilzasa, T. and Miyamoto, T. (1987).
Major determinants of purine excretion from human lymphoblasts
Biochem Med Metab Biol 38: 292-299.

Ito. K., Yamamoto, H. and Mizugaki, M. (1988)
Purification and general properties of AMP deaminase from sheep brain
J. Biochem. (Tokyo) 103: 259-262

Joab, I., Radanyi, C., Renoir, J.M., Buchou, T., Catelli, M.G., Binart, N., Mester, J. and Baulieu, E.E. (1984).
Common non-hormone binding component in non-transformed chick oviduct receptors for 4 steroid hormones
Nature 308: 850-853.

Jones, E.W. and Fink, G.R. (1982).
Regulation of amino acid and nucleic acid biosynthesis in yeast
In The Molecular Biology of the Yeast Saccharomyces: Metabolism and gene expression, Strathern, J.N., Jones, E.W., Broach, J.R. and Eds, New York: Cold Spring Harbour

Kacser, H. (1983)
The control of enzyme systems *in vivo*: Elasticity analysis of the steady state
Biochem. Soc. Transactions 11: 35-40

Kaletha, K. (1983)
Regulatory properties of 14-day embryo and adult hen skeletal muscle AMP deaminase. The influence of pH on enzyme activity
Biochim. Biophys. Acta 784: 90-92

Kaletha, K. and Nowak, G. (1988).
Developmental forms of human skeletal muscle AMP deaminase. The kinetic and regulatory properties of the enzyme
Biochem J 249: 255-261.

Kaletha, K., Spychala, J. and Nowak, G. (1987).
Developmental forms of human skeletal muscle AMP deaminase
Experientia 43: 440-443.

Kar, N.C. and Pearson, C.M. (1973).
Muscle adenylic acid deaminase activity: selective decrease in early-onset Duchenne Muscular dystrophy
Neurology 23: 478-482.

Kasambalides, E.J. and Lanks, K.W. (1983).
Dexamethasone can modulate glucose-regulated and heat shock protein synthesis
J Cell Physiol 114: 93-98.

Kather, H. (1988).
Purine accumulation in human fat cell suspensions. Evidence that human adipocytes release inosine and hypoxanthine rather than adenosine
J. Biol. Chem. 263: 8303-8309.

Kingsman, A.J., Clarke, L., Mortimer, R.K. and Carbon, J. (1979).
Replication in *S.cerevisiae* of plasmid pBR313 carrying DNA from the yeast trp1 region
Gene 7: 141-152.

Koretz, J.F. and Freiden, C. (1980).
Adenylate deaminase binding to synthetic thick filaments of myosin
Proc. Natl. Acad. Sci. USA 77: 7186-7188.

Koyasu, S., Nishida, F., Kadowaki, T., Matsuzaki, F., Iida, K., Karada, F., Kasagu, M., Sakai, H. and Yahara, I. (1986).
Two mammalian heat shock proteins, hsp90 and hsp100 are actin-binding proteins
Proc. Natl. Acad. Sci. USA 83: 8054-8058.

Kurtz, S. (1986)
Heat shock proteins induced in sporulation and oogenesis
Science 231: 1154-1157

Kurtz, S. and Lindquist, S. (1984).
Changing patterns of gene expression during sporulation in yeast
Proc. Natl. Acad. Sci. USA 81: 7323-7327.

Laemmli, U.K. (1970).
Cleavage of structural proteins during the assembly of the head of bacteriophage T4
Nature 227: 680-685.

Lai, B-T., Chin, N.W., Staner, A.E., Keh, W. and Lanks, K.W. (1984).
Quantitation and intracellular localisation of the 85K heat shock protein by using monoclonal and polyclonal antibodies
Mol Cell Biol 4: 2802-2810.

Lally, E.V., Friedman, J.H. and Kaplan, S.R. (1985).
Progressive myalgias and polyarthralgias in a patient with myoadenylate deaminase deficiency
Arthritis Rheum 28: 1298-1302.

Lanks, K.W. and Kasambalides, E.J. (1979).
Purification and characterisation of a major component from the cytoplasmic matrix of cultured murine cells
Biochim Biophys Acta 578: 1-12.

Lautenberger, J.A. and Chen, Z-Q. (1987).
A method for the preparation of high molecular weight yeast DNA
Gene. Anal. Techn. 4: 87-88.

Lazou, A. (1989).
Adenylate metabolising enzymes in invertebrate tissues
Comp Biochem Physiol 92B: 175-180.

Leung, H.B. and Schramm, V.L. (1980).
Adenylate degradation in E.coli The role of AMP nucleosidase and properties of the purified enzyme
J. Biol. Chem. 255: 10867-10874.

Lewis, M.J. and Pelham, H.R.B. (1985).
Involvement of ATP in the nuclear and nucleolar functions of the 70K heat shock protein
EMBO J. 4: 3137-3143.

Lindquist, S. (1981)
Regulation of protein synthesis during heat shock
Nature 293: 311-314

Lindquist, S. (1984).
Heat shock: Comparison of Drosophila and Yeast
J Embryol Exp Morphol 83 Supplement: 147-161.

Lindquist, S. (1986).
The heat shock response
Ann Rev Biochem 55: 1151-1191.

Lipsich, L.A., Cutt, J.R. and Brugge, J.S. (1982).
Association of the transforming proteins of Rous, Fujinami and Y73 Avian Sarcoma viruses with the same two cellular proteins
Mol Cell Biol 2: 875-880.

Littlewood, B.S. (1972).
A method of obtaining antibiotic sensitive mutants in S.cerevisiae
Genetics 71: 305-308.

Littlewood, B.S. and Davies, J.E. (1973).
Enrichment for temperature sensitive and auxotrophic mutants in S.cerevisiae by tritium suicide
Mutation Res 17: 315-322.

Lowenstein, J.M. (1972).
Ammonia production in muscle and other tissues: the purine nucleotide cycle
Physiol Rev 52: 382-414.

Lowenstein, J.M. and Tornheim, K. (1971).
Ammonia production in muscle; The purine nucleotide cycle
Science 171: 397-400.

Maher, P.A. and Pasquale, E.B. (1989).
Heat shock induces tyrosine phosphorylation in cultured cells
J Cell Biol 108: 2029-2035.

Makarewicz, W. and Stankiewicz, A. (1974).
AMP aminohydrolase of human skeletal muscle: partial purification and properties
Biochem-Med 10: 180-197.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982).
Molecular Cloning, A Laboratory Manual
New York : Cold Spring Harbour Laboratories.

Marquetant, R., Desai, N.M., Sabina, R.L. and Holmes, E.W. (1987).
Evidence for sequential expression of multiple AMP deaminase isoforms during skeletal muscle development
Proc. Natl. Acad. Sci. USA 84: 2345-2349.

Matsumoto, S.S., Raivio, K.O. and Seegmiller, E. (1979).
Adenine nucleotide degradation during energy depletion in human lymphoblasts: adenosine accumulation and adenylate energy charge correlation
J. Biol. Chem. 254: 8956-8962.

Maytin, E.V., Colbert, R.A. and Young, D.A. (1985).
Early heat shock proteins in primary thymocytes: Evidence for transcriptional and translational regulation
J. Biol. Chem. 260: 2384-2392.

McAlister, L. and Finkelstein, D.B. (1980).
Alterations in translatable RNA after heat shock in S.cerevisiae
J. Bact 143: 603-612.

McAlister, L., Strausberg, S., Kuluga, A. and Finkelstein, D.B. (1979).
Altered patterns of protein synthesis induced by heat shock of yeast
Curr Genet 1: 63-74.

Meyer, S.L. and Schramm, V.L. (1985).
Cloning and Sequencing of yeast AMP deaminase
Fed Proc : 1599.

Meyer, S.L., Kvalnes-Krick, K.L. and Schramm, V.L. (1989)
Characterization of AMD, the AMP deaminase gene in yeast. Production of amd strain, cloning, nucleotide sequence and properties of the protein
Biochemistry 28: 8734-8773

Mitchel, R.E.J. and Morrison, D.P. (1983).
Heat shock induction of UV light resistance in S.cerevisiae
Radiat Res 96: 95-99.

Mitchel, , REJ, and Morrison, D.P. (1986).
Inducible error prone repair in yeast in suppressed by heat shock
Mutat Res 159: 31-39.

Mommsen, T.P. and Hochachka, P.W. (1988).
The purine nucleotide cycle as two temporally separated metabolic units: a study on trout muscle
Metabolism 37: 552-556.

Monnolou, J.C., Jakob, H. and Slonimiski, P.P. (1966).
Mitochondrial DNA from yeast "petite" mutants: specific changes in buoyant density corresponding to different cytoplasmic mutations
Biochem Biophys Res Commun 24: 218-224.

Mortimer, R.K. and Schild, D. (1985)
Genetic map os S.cerevisiae, Edition 9
Microbiol. Rev. 49: 181-212

Mosca, J.D., Wu, J.M. and Suhadolnik, R.J. (1983).
Restoration of protein synthesis in lysed rabbit reticulocytes by the enzymatic removal of 5'AMP with either AMP deaminase or AMP nucleosidase
Biochemistry 22: 346-354.

Munro, S. and Pelham, H.R.B. (1985).
What turns on heat shock genes?
Nature 317: 477-478.

Murakami, K. (1979).
AMP deaminase from Baker's yeast
J. Biol. Chem. 86: 1331-1336.

Murakami, K., Nagura, H. and Yoshino, M. (1980).
Permeabilization of yeast cells: Application to study on the regulation of AMP deaminase activity in situ
Anal Biochem 105: 407-413.

Nagao, H., Habara, S., Morimoto, T., Sano, N., Takahashi, M., Kida, K. and Matsuda, H. (1986).
AMP deaminase activity of skeletal muscle in neuromuscular disorders in childhood Histochemical and biochemical studies
Neuropediatrics 17: 193-8.

Nagley, P. and Linnane, A.W. (1970).
Mitochondrial DNA deficient petite mutants of yeast
Biochem Biophys Res Commun 39: 989-996.

Nakatsu, K. (1975).
Adenylate deaminase: A method for its localisation in skeletal muscle
J Histochem Cytochem 23: 194-199.

Neidhardt, F.C., Vanbogelen, R.A. and Vaughn, V. (1984).
The genetics and regulation of the heat shock proteins
Ann Rev Genetics 18: 295-329.

Ochoa, J.L. (1978).
Hydrophobic (Interaction) Chromatography
Biochimie 60: 1-15.

Ogasawara, N., Goto, H., Yamada, Y., Nishigaki, I., Itoh, T., Hasegawa, I. and Park, K.S. (1987).
Deficiency of AMP deaminase in erythrocytes
Human Genet 75: 15-18.

Ogasawara, N., Goto, H., Yamada, Y., Nishigaki, I., Itoh, Y. and Hasegawa, I. (1984).
Complete deficiency of AMP deaminase in human erythrocytes
Biochem Biophys Res Commun 122: 1344.

Ogasawara, N., Goto, H., Yamada, Y., Watanabe, T. and Asano, T. (1982).
AMP deaminase isozymes in human tissues
Biochim Biophys Acta 714: 298-306.

Ogasawara, N., Yoshino, M. and Kawamura, Y. (1972).
Multiple forms of AMP deaminase from rat brain
Biochim Biophys Acta 258: 680-684.

Ohlsson, R., Brodelius, P. and Mosbach, K. (1972).
Affinity chromatography of enzymes on an AMP analogue: specific elution of dehydrogenases from a general ligand
FEBS Lett 25: 234-238.

Oppermann, H., Levinson, W. and Bishop, J.M. (1981).
A cellular protein that associates with the transforming protein of Rous Sarcoma virus is also a heat shock protein
Proc. Natl. Acad. Sci. USA 78: 1067-1071.

Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J. (1981).
Yeast transformation: A model system for the study of recombination
Proc. Natl. Acad. Sci. USA 78: 6354.

Paglia, D.E., Valentine, W.N., Nakatani, M. and Brockway, R.A. (1986).
Mechanisms of adenosine 5'-monophosphate catabolism in human erythrocytes
Blood 67: 988-992.

Pelham, H.R.B. (1985).
Activation of heat shock genes in eukaryotes
Trends in Genetics : 31-34.

Pelham, H.R.B. (1986).
Speculations on the function of the major heat shock and glucose-regulated proteins
Cell 46: 959-961.

Pelham, H.R.B. (1988).
Heat Shock Proteins; Coming in from the Cold
Nature 280: 776-777.

Pennington, R.J. (1961).
5'-adenylic acid deaminase in dystrophic mouse muscle
Nature 192: 884.

Perdew, G.H. (1988).
Association of the Ah receptor with the 90kD heat shock protein
J. Biol. Chem. 263: 13802-13805.

Piper, P.W., Curran, B., Davies, M.W., Hirst, K., Lockheart, A. and Seward, K. (1988).
Catabolite control of the elevation of phosphoglycerate kinase mRNA levels by heat shock in S.cerevisiae
Molecular Micro 2: 353-361.

Piper, P.W., Curran, B., Davies, M.W., Lockheart, A. and Reid, G. (1986).
Transcription of the phosphoglycerate kinase gene of S.cerevisiae increases when fermentative cultures are stressed by heat shock
Eur J Biochem 161: 525-531.

Plesset, J., Palm, C. and McLaughlin, C.S. (1982).
Induction of heat shock proteins and thermotolerance by ethanol in S.cerevisiae
Biochem. Biophys. Res. Commun. 108: 1340-1345.

Pouwels, P.H., Enger-Valk, B.E. and Brammar, W.J. (1988)
Cloning Vectors
New York: Elsevier

Radanyi, C., Renoir, J-M., Sabbah, M. and Baulieu, E-E. (1989).
Chick heat shock protein of Mr = 90000, free or released from progesterone receptor is in a dimeric form
J. Biol. Chem. 264: 2568-2573.

Raffin, J.P. (1984).
AMP deaminase from the gill of Salmo gairdnerii Richardson: effects of anions, cations and buffers
Comp. Biochem. Physiol. 79B: 499-504.

Raffin, J.P. (1986a).
Activation of trout gill AMP deaminase by and endogenous proteinase: I Effects on the regulatory properties of the enzyme
Comp Biochem Physiol 85B: 157-162.

Raffin, J.P. (1986b).
Activation of trout gill AMP deaminase by an endogenous proteinase: II Modification of the properties of the enzyme during starvation, pollution and salinity changes
Comp. Biochem. Physiol. 85B: 163-171.

Raffin, J.P. (1986c).
Activation of trout gill AMP deaminase by an endogenous proteinase: III Comparative studies on the gill AMP deaminase from different fresh water and sea water teleosts
Comp. Biochem. Physiol. 85B: 173-182.

Raffin, J.P. and Thebault, M.T. (1987).
Purification and partial characterisation of an AMP deaminase from the marine invertebrate Palaemon serratus
Comp. Biochem. Physiol. 88B: 1071-1076.

Ranieri-Raggi, M., Moir, A.J. and Raggi, A. (1985).
Interaction with troponin T from white skeletal muscle restores in white skeletal muscle AMP deaminase those allosteric properties removed by limited proteolysis
Biochim. Biophys. Acta. 827: 93-100.

Reiter, T. and Penman, S. (1983).
"Prompt" heat shock proteins: Translationally regulated synthesis of new proteins associated with the nuclear matrix - intermediate filaments as an early response to heat shock
Proc. Natl. Acad. Sci. USA 80: 4737-4741.

Reuschenbach, C. and Zierz, S. (1988).
Mutant carnitine palmitoyltransferase associated with myoadenylate deaminase deficiency in skeletal muscle
J. Pediatr 112: 600-603.

Ritossa, F. (1962).
A new puffing pattern induced by temperature shock and DNP in Drosophila
Experentia 18: 571-573.

Rose, D.W., Welch, W.J., Kramer, G. and Hardesty, B. (1989).
90K heat shock protein: Regulation of proteins synthesis
J. Biol. Chem. 264: 6239-6244.

Rose, M., Grisafi, P. and Botstein, D. (1984).
Structure and function of the yeast URA3 gene: expression in E.coli
Gene 29: 113-124.

Sabina, R.L., Desai, N., Marquetant, R. and Holmes, E.W. (1984a).
5-azacytidine and 5-asadeoxycytidine potentiation of myocyte differention and the expression of AMP deaminase in L6 myoblasts
Circulation Supplement 2: 197.

Sabina, R.L., Marquetant, R., Desai, N.M., Kaletha, K. and Holmes, E.W. (1987).
Cloning and sequence of the rat myoadenylate deaminase cDNA. Evidence for tissue-specific and developmental regulation
J. Biol. Chem. 262: 12397-12400.

Sabina, R.L., Ogasawara, N. and Holmes, E.W. (1989).
Expression of three stage specific transcripts of AMP deaminase during myogenesis
Mol. Cell. Biol. 9: 2244-2246.

Sabina, R.L., Swain, J.L., Olanow, C.W., Bradley, W.G., Fishbein, W.N., Dimauro, S. and Holmes, E.W. (1984b).
Myoadenylate deaminase deficiency: Functional and metabolic abnormalities associated with disruption of the purine nucleotide cycle
J Clin Invest 73: 720-730.

Sammons, D.W. and Chilson, O.P. (1977).
Stage specific isozymes of AMP deaminase in chick muscle
Fed Proc 36: 725.

Sammons, D.W. and Chilson, O.P. (1978).
AMP deaminase: Stage-specific isozymes in differentiating chick muscle
Arch Biochem 191: 561-570.

Sanchez, E.R., Redmont, T., Scherrer, L.C., Bresnick, E.H., Welsh, M.J. and Pratt, W.B. (1988).
Evidence that the 90kD heat shock protein is associated with tubulin-containing complexes in L cell cytosol and intact PtK cells
Mol Endocrinology 2: 756-760.

Sanchez, E.R., Toft, D.O., Schlesinger, M.J. and Pratt, W.B. (1985).
Evidence that the 90K phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein
J. Biol. Chem. 260: 12398-12401.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977).
DNA sequencing with chain terminating inhibitors
Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Santiago, T.C., Purvis, I.J., Bettany, A.J.E. and Brown, A.J.P.B. (1986).
The relationship between mRNA stability and length in S.cerevisiae
Nucleic Acids Res. 14: 8347-8360.

Schaff, I. Heinisch, J. and Zimmerman F.K. (1989)
Overproduction of glycolytic enzymes in yeast
Yeast 5: 285-290

Schlesinger, M.J., Aliperti, G. and Kelley, P.M. (1982).
The response of cells to heat shock
Trends Biochem Sci 7: 222-225.

Setlow, B., Burger, R. and Lowenstein, J.M. (1966).
Adenylate deaminase: The effects of adenosine and guanosine triphosphates on activity and the organ distribution of the regulated enzyme
J. Biol. Chem. 241: 1244-1245.

Sharp, P.M., Cowe, E., Higgins, D.G., Shields, D.C., Wolfe, K.H. and Wright, F. (1988).
Codon usage patterns in E.coli, B.subtilis, S.cerevisiae, Schizosaccharomyces pombe, D.melanogaster and H.sapiens; A review of the considerable within-species diversity
Nucleic Acids Res. 16: 8207-8211

Sherman, F., Fink, G.R. and Hicks, J.B. (1986).
Meiotic mapping
In Laboratory Course manual for methods in yeast genetics, New York: Cold Spring Harbour.

Shin, D.Y., Matsumoto, K., Iida, H., Uno, I. and Ishikawa, T. (1987).
Heat shock response of S.cerevisiae mutants altered in cAMP-dependent protein phosphorylation
Mol. Cell. Biol. 7: 244-250.

Sinkeler, S.P., Binkhorst, R.A., Joosten, E.M., Wevers, R.A., Coerwinkei, M.M. and Oei, T.L. (1987).
AMP deaminase deficiency: study of the human skeletal muscle purine metabolism during ischaemic isometric exercise
Clin Sci 72: 475-482.

Sinkeler, S.P., Joosten, E.M., Wevers, R.A., Binkhorst, R.A., Oerlemans, F.T., Van Bennekom, C.A., Coerwinkel, M.M. and Oei, T.L. (1986).
Ischaemic exercise test in myoadenylate deaminase deficiency and McCardles disease: measurement of plasma adenosine, inosine and hypoxanthine
Clin Sci 70: 399-401.

Sinkeler, S.P., Joosten, E.M., Wevers, R.A., Oei, T.L., Jacobs, A.E., Veerkamp, J.H. and Hamel, B.C. (1988).
Myoadenylate deaminase deficiency: a clinical, genetic and biochemical study in 9 families
Muscle Nerve 11: 312-317.

Skadanowski, A.C. and Zydowa, M.M. (1988).
Two forms of AMP deaminase in bovine heart
Acta Biochim Pol 35: 29-37.

Skopes, R.K. (1987).
Protein Purification : Principles and Practice
Springer Verlag : New York.

Smith, L.D. and Kizer, D.E. (1969). Purification and properties of rat liver AMP deaminase
Biochim. Biophys. Acta. 191: 415-424.

Smith, M.B., Oakenfall, D.G. and Back, J.F. (1978).
Thermal stabilisation of protein by sugars and polyols
Proc Aust Biochem Soc 11: 4.

Snow, R. (1966).
An enrichment method of auxotrophic yeast mutants using the antibiotic nystatin
Nature 211: 206-207.

Sorger, P.K., Lewis, M.J. and Pelham, H.R.B. (1987).
Heat shock factor is regulated differently in yeast and HeLa cells
Nature 329: 81-84.

Sorger, P.K. and Pelham, H.R.B. (1987).
Purification and characterisation of a heat shock element binding protein from yeast
EMBO J. 6: 3035-3041.

Sorger, P.K. and Pelham, H.R.B. (1988).
Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation
Cell 54: 855-864.

Southern, E. (1975).
Detection of specific sequences among DNA fragments separated by gel electrophoresis
J Mol Biol 98: 503-533.

Spychala, J. (1987).
The interaction of polyphosphoinositols with AMP deaminase
Biochem. Biophys. Res. Commun. 148: 106-111.

Spychala, J., Kaletha, K. and Makarewicz, W. (1985).
Developmental changes of chicken liver AMP deaminase
Biochem J 231: 329-333.

Spychala, J. and Marszalek, J. (1987).
The role of GTP in the regulation of two forms of AMP deaminase from chicken kidney
Comp. Biochem. Physiol. 88: 1077-1082.

Spychala, J., Marszalek, J. and Kucharczyke, E. (1986).
AMP deaminases of rat small intestine
Biochim. Biophys. Acta. 880: 123-130.

Staines, D. (1985).
Myalgic encephalomyelitis hypothesis
Med J Aust 143: 91.

Stankiewicz, A.J. (1986).
Nonspecific snail muscle adenylate deaminase: simplified purification, characterisation and use for the preparation of deamino derivatives of NAD, NADH and AMP-P(NH)P
Enzyme 36: 187-96.

Storti, R.V., Scott, M.P., Rich, A. and Pardue, M.L. (1980).
Translational control of protein synthesis in response to heat shock in D.melanogaster cells
Cell 22: 825-834.

Szent-Gyorgyi, C., Finkelstein, D.B. and Garrard, W.T. (1987).
Sharp boundaries demarcate the chromatin structure of a yeast heat shock gene
J Mol Biol 193: 71-80.

Tissieres, A., Mitchell, H.K. and Tracy, U.M. (1974).
Protein synthesis in salivary glands of D.melanogaster: relation to chromosome puffs
J Mol Biol 84: 389-398.

Tornheim, K. (1979).
Oscillations of the glycolytic pathway and the purine nucleotide cycle
J Theor Biol 79: 491-541.

Tornheim, K. and Lowenstein, J.M. (1974).
The purine nucleotide cycle: IV Interactions with oscillations of the glycolytic pathway in muscle extracts
J. Biol. Chem. 249: 3241-3247.

Tuite, M.F., Bossier, P. and Fitch, I.T. (1988).
A highly conserved sequence in yeast heat shock promoters
Nucleic Acids Res. 16: 11845.

Uemara, J., Shiba, T., Paterson, M., Jigami, Y. and Tanaka, H. (1986).
Identification of a sequence containing the positive regulatory region of S.cerevisiae gene ENO1
Gene 45: 67-75.

Ullrich, S.J., Robinson, E.A., Law, L.W., Willingham, M. and Appella, E. (1986).
A mouse tumour-specific transplantation antigen is a heat shock-related protein
Proc. Natl. Acad. Sci. USA 83: 3121-3125.

Valen, P.A., Nakayama, D.A., Veum, J., Sulaiman, A.R. and Wortmann, R.L. (1987).
Myoadenylate deaminase deficiency and forearm ischemic exercise testing
Arthritis Rheum 30: 661-668.

Van den Berghe, G., Bontemps, F. and Hers, H.G. (1980)
Purine catabolism in isolated rat hepatocytes: influence of coformycin
Biochem. J. 188: 913-920

Van den Berghe, G., Brontman, M., Vanneste, R. and Hers, H.G. (1977).
Purine catabolism in isolated rat hepatocytes: influence of coformycin: A kinetic study of liver adenylate deaminase
Biochem J 162: 601-609.

Vincent, M.F., Van den Berghe, G. and Hers, H.G. (1982).
Pathway of adenine nucleotide catabolism and its control in isolated rat hepatocytes subjected to anoxia
Biochem J 202: 117-124.

Vourch, C., Binart, N., Chambraud, B., David, J.P., Jerome, V., Baulieu, E.E. and Catelli, M.G. (1989).
Isolation and functional analysis of chicken 90kD heat shock protein gene promoter
Nucleic Acids Res. 17: 5259-5272.

Walker, A.I., Hunt, T., Jackson, R.J. and Anderson, C.W. (1985).
Double stranded DNA induces the phosphorylation of several proteins including the 90K MW heat shock protein in animal cell extracts
EMBO J. 4: 139-145.

Watson, K., Dunlop, G. and Cavicchioli, R. (1984).
Mitochondrial and cytoplasmic protein synthesis is not required for heat shock acquisition of ethanol and heat shock tolerance in yeast
FEBS Lett 172: 299-302.

Weber, G., Lea, M.A., Hirdconvery, H.J. and Stamm, N.B. (1967).
Regulation of gluconeogenesis and glycolysis: studies of mechanisms controlling enzyme activity
Adv Enzyme Regul 5: 257-300.

Wei, R., Wilkinson, H., Pfeifer, K., Schneider, C., Young, R. and Guarente, L. (1986).
Two or more copies of Drosophila heat shock consensus sequence serve to activate transcription in yeast
Nucleic Acids Res. 14: 8183-8188.

Weitzel, G., Pilatus, U. and Rensing, L. (1985).
Similar dose response of heat shock protein synthesis and intracellular pH change in yeast
Exp Cell Res 159: 252-256.

Weitzel, G., Pilatus, U. and Rensing, L. (1987).
The cytoplasmic pH, ATP content and total protein synthesis rate during heat shock protein inducing treatment in yeast
Exp Cell Res 170: 64-79.

Weitzman, P.D.J. and Hewson, J.K. (1973).
In situ regulation of yeast citrate synthase. Absense of ATP inhibition observed in vitro
FEBS Lett 36: 227-231.

Welch, W.J. and Feramisco, J.R. (1985).
Rapid purification of mammalian 70 000 Dalton Stress proteins: Affinity of the proteins for nucleotides
Mol. Cell. Biol. 5: 1229-1237.

Welch, W.J., Garrels, J.I., Thomas, G.P., Lin, JJ-C. and Feramisco, J.R. (1983).
Biochemical characterisation of the mammalian stress proteins and identification of two stress proteins as glucose and Ca2+-ionophore-regulated proteins
J. Biol. Chem. 258: 7102-7111.

Wiederrecht, G., Shuey, D.J., Kibbe, W.A. and Parker, C.S. (1987).
The Saccharomyces and Drosophila heat shock transcription factors are identical in size and DNA binding properties
Cell 48: 507-515.

Woods, R.A., Roberts, D.G., Friedman, T., Jolly, D. and Filipula, D. (1983).
Hypoxanthine:guanine phosphoribosyl transferase mutants in S.cerevisiae
Mol Gen Genet 191: 407-412.

Wu, C. (1980).
The 5' ends of Drosophila heat shock genes in chromatin are hypersensitive to DNase I
Nature 286: 854-860.

Xiao, H. and Lis, J.T. (1988).
Germline transformation used to define key features of heat shock response elements
Science 239: 1139-1142.

Yonemoto, W., Lipsich, L.A., Darrow, D. and Brugge, J. (1982).
An analysis of the interaction of the Rous Sarcoma virus transforming protein pp60src, with a major heat shock protein
In Heat shock: Bacteria to Man, Schlesinger, M.J., Ashburner, M. and Tissieres, A. New York: Cold Spring Harbour.

Yoshino, M. and Kawamura, Y. (1978).
Inhibition of chicken erythrocyte AMP deaminase by tetraiodofluorescein compounds
Biochim. Biophys. Acta. 526: 640-646.

Yoshino, M. and Kawamura, Y. (1986).
Kinetics of the interactions of the chicken erythrocyte AMP deaminase with anthraquinone compounds
Int J Biochem 18: 241-244.

Yoshino, M., Kawamura, Y. and Ogasawara, N. (1974).
Inhibition of chicken erythrocyte AMP deaminase by blue dextran 2000
J Biochem 75: 1391-1393.

Yoshino, M., Miyajima, E. and Tsushima, K. (1976).
Inhibition of AMP deaminase of bovine brain and liver by fatty acyl-CoA derivatives
FEBS Lett 72: 143-146.

Yoshino, M., Miyajima, E. and Tsushima, K. (1979a).
Kinetics of interactions of AMP deaminase with fatty acids
J. Biol. Chem. 254: 1521-1525.

Yoshino, M. and Murakami, K. (1978).
The role of polyamines in the regulation of AMP deaminase isozymes
Biochim. Biophys. Acta. 542: 177-179.

Yoshino, M. and Murakami, K. (1980).
Role of cations in the regulation of Baker's yeast AMP deaminase
Biochim. Biophys. Acta. 616: 82-88.

Yoshino, M. and Murakami, K. (1981a).
Effect of spermine on the inhibition by fatty acid of AMP deaminase reaction as a control system of the adenylate energy charge in yeast
Biochem. Biophys. Res. Commun. 102: 905-910.

Yoshino, M. and Murakami, K. (1981b).
In vitro and in situ studies on the inhibition of yeast AMP deaminase by fatty acids
Biochim. Biophys. Acta. 660: 199-203.

Yoshino, M. and Murakami, K. (1981c).
In situ studies of AMP deaminase as a control system of the adenylate energy charge in yeast
Biochim. Biophys. Acta. 672: 16-20.

Yoshino, M. and Murakami, K. (1981d).
The effects of spermine on the interaction of AMP deaminase with Threonine dehydratase activity in yeast
Biochim. Biophys. Acta. 672: 313-316.

Yoshino, M. and Murakami, K. (1982a).
Interaction of the AMP deaminase-NH_4^+ system with glutamate dehydrogenase in yeast
Biochim. Biophys. Acta. 706: 111-116.

Yoshino, M. and Murakami, K. (1982b).
The regulatory role of spermine and fatty acid in the interaction of AMP deaminase with phosphofructokinase
Biochim. Biophys. Acta. 719: 474-479.

Yoshino, M. and Murakami, K. (1982c).
AMP deaminase reaction as a control system of glycolysis in yeast : Activation of phosphofructokinase and pyruvate kinase by the AMP deaminase-ammonia system
J. Biol. Chem. 257: 2822-2828.

Yoshino, M. and Murakami, K. (1982d).
AMP deaminase as a control system of glycolysis in yeast: mechanism of the inhibition of glycolysis by fatty acid and citrate
J. Biol. Chem. 257: 10644-10649.

Yoshino, M. and Murakami, K. (1983).
Role of the AMP deaminase reaction in the response of phosphofructokinase to the adenylate energy charge
Biochem. Biophys. Res. Commun. 112: 96-101.

Yoshino, M. and Murakami, K. (1985a).
AMP deaminase reaction as a control system of glycolysis in yeast: Role on ammonium ion in the interaction of Phosphofructokinase and Pyruvate kinase activity with the adenylate energy charge
J. Biol. Chem. 260: 4729-4732.

Yoshino, M. and Murakami, K. (1985b).
Role of AMP deaminase reaction in the control of Fructose-1,6-bisphosphatase activity in yeast
Biochem. Biophys. Res. Commun. 128: 1020-1024.

Yoshino, M. and Murakami, K. (1985c).
Stabilisation of the adenylate energy charge by the depletion of adenylates without glycolytic stimulation
Biochem. Biophys. Res. Commun. 129: 287-292.

Yoshino, M. and Murakami, K. (1987).
Quantitative structure activity relationship studies on the activation of yeast AMP deaminase by polyamines
Int. J. Biochem. 19: 209-211.

Yoshino, M. and Murakami, K. (1988).
A kinetic study of the inhibition of yeast AMP deaminase by polyphosphate
Biochim. Biophys. Acta. 954: 271-276.

Yoshino, M., Murakami, K. and Kawamura, Y. (1984).
Kinetics of the inhibition by naptholsulfonate compounds of AMP deaminase from chicken erythrocytes
Biochim. Biophys. Acta. 791: 364-369.

Yoshino, M., Murakami, K. and Tsushima, K. (1979b).
AMP deaminase from Bakers's yeast: Purification and some regulatory properties
Biochim. Biophys. Acta. 570: 157-166.

Young, J.D., Gorman, J.W. and Gorman, J.A. (1976).
Indirect selection for auxotrophic mutants in S.cerevisiae using the antibiotic netropsin
Mutat Res 35: 423-428.

Young, R.B., Hopkins, L.H. and Fleming, G.D. (1984).
Elevated ammonia release from dystrophic chicken muscle cell and fibroblast cultures
Muscle Nerve 7: 626-628.

Zielke, C.L. and Suelter, C.H. (1971).
Purine, purine nucleoside and purine nucleotide aminohydrolases
In The Enzymes, Boyer, P.D. and Ed, New York: Academic Press.

Zimmerman, L.J., Petri, W. and Meselson, M. (1983).
Accumulation of a specific subset of D.melanogaster heat shock mRNAs in normal development without heat shock
Cell 32: 1161-1170.

Zoref-Shani, E., Kessler-Icekson, G. and Sperling, O. (1988).
Pathways of adenine nucleotide catabolism in primary rat cardiomyocyte cultures
J Mol Cell Cardiol 20: 23-33.

Zoref-Shani, E., Shainberg, A. and Sperling, O. (1987)
Pathways of adenine nucleotide catabolism in primary rat muscle cultures
Biochim. Biophys. Acta. 926: 287-295