ASPECTS OF THE STRUCTURE AND CATALYTIC FUNCTION OF THE *arom* MULTIFUNCTIONAL ENZYME FROM *Neurospora crassa*

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Thesis submitted to the University of Glasgow

for the degree of Doctor of Philosophy

Department of Biochemistry

November, 1983

FOR MY PARENTS

Acknowledgements

I would like to thank my friends and colleagues on D-floor, Amanda, Ann, Andy, Barbara, Betty, Brian, David M., David M.M., Drumond, Elizabeth and Ian (for the perspex ruler), James, Jill, John G., John L., Ken, Mansi, Murray and Suven for all their help. I also thank Professor C.A.Fewson and Nigel Allison for their advice. Dr.Stuart Ridley of I.C.I. and his colleagues kindly made available analytical grade glyphosate and certain structural analogues. My especial thanks go to John Lambert, who collaborated in the development of the 3rd generation purification procedure and to Hugh Nimmo, who read the draft of parts of chapter 6 and gave me a great deal of sound advice during the course of this study. I am also very grateful to Mrs.S.Brown, who has typed and printed this thesis and executed the tables and figure legends with great skill and patience. My thanks to the Science and Engineering Research Council, for financial support, and to Professor R.M.S.Smellie, for providing the facilities in this department.

Finally I would like to thank my supervisor, John Coggins, for giving me his enthusiastic support throughout my three years in Glasgow.

ABBREVIATIONS

The abbreviations used are those recommended in the Biochemical Journal "Instructions to Authors, 1981", with the following additions:

arom	the *arom* multifunctional enzyme of *N.crassa*
As_i	inorganic arsenate
bis-tris	bis(2-hydroxyethyl)imino-tris-(hydroxymethyl) methane
bis-tris-propane	1,3-bis[tris(hydroxymethyl)methylamino]propane
DAHP	3-deoxy-D-*arabino*-heptulosonate 7-phosphate
DCPIP	2,6-dichloroindophenol
DEAE	diethyl aminoethyl
DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
DTNB	5,5'-dithio-bis(2-nitrobenzoate)
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetate
enz	enzyme
EPSP	5-enolpyruvylshikimate 3-phosphate
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HPGFC	high performance gel filtration chromatography
KP_i	potassium phosphate
MTT	thiazolyl blue tetrazolium
βME	β-mercaptoethanol
NBT	nitro blue tetrazolium
NEM	N-ethyl maleimide
P_i	inorganic phosphate
PEP	phosphoenolpyruvate
PAGE	polyacrylamide gel electrophoresis
PMS	phenazine methosulphate
PMSF	phenylmethyl sulphonyl fluoride
pZn	$-\log[Zn^{2+}]$
Shik3P	Shikimate 3-phosphate
SDS	sodium dodecyl sulphate
SDS PAGE	gel electrophoresis in the presence of SDS
UDPGlcNAc	uridine 5'-diphospho-N-acetyl-2-amino-2-deoxyglucose

CONTENTS

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SUMMARY

1. The established methods for the isolation of the *arom* multifunctional enzyme from *N.crassa* have been modified to take into account the instability of two of the component activities. The copurification in constant ratio of the five activities of the enzyme complex has been demonstrated.

2. The homogeneous multifunctional enzyme isolated by the modified procedures contains approximately 1 atom/subunit of tightly bound zinc, which is essential for DHQ synthase (E1) activity. DHQ synthase activity is very rapidly reconstituted on addition of the inactive EDTA-treated enzyme to zinc ion "buffering" systems.

3. Chorismate synthase and anthranilate synthase suitable for use as coupling enzymes in steady state kinetics experiments have been isolated from *N.crassa*. Chorismate synthase has been purified to electrophoretic homogeneity. The catalytic properties of the purified enzyme are qualitatively different from those of the highly purified chorismate synthase preparation of Welch *et al* (1974).

4. The catalytic properties of chorismate synthase isolated from *N.crassa* strongly suggest that the enzyme is bifunctional, and that reduced FMN participates in the conversion of EPSP to chorismate.

5. A detailed study of the steady state kinetic properties of the EPSP synthase (E5) activity of the *arom* enzyme complex has been undertaken. The results support an ordered sequential mechanism in which shikimate 3-phosphate is the first substrate to bind to the enzyme in the forward reaction; EPSP and P_i bind to the enzyme in a random order in the reverse reaction.

6. Arsenate can substitute for phosphate in the reverse reaction of EPSP synthase. Kinetic experiments using arsenate as a pseudo-substrate indicate that the release of enzyme-bound EPSP is the rate-limiting step in the forward reaction.

7. The herbicide glyphosate (N-phosphonomethyl glycine) is a potent reversible inhibitor of the EPSP synthase activity of the arom enzyme complex. Glyphosate interacts uniquely with an enzyme • shikimate 3-phosphate kinetic complex, and excludes productive binding of PEP. The steady state kinetic properties of the arom EPSP synthase strongly suggest that glyphosate mimics the enzyme-bound conformation of the natural substrate, PEP.

8. The equilibrium constant of the EPSP synthase reaction, re-estimated at pH 7.0 under the conditions used in the steady state kinetics experiments, is 116.

9. The structural organisation of the arom multifunctional enzyme has been investigated by limited proteolysis, and catalytically active fragments have been resolved by chromatography under nondenaturing conditions. In particular, the products of limited trypsin/chymotrypsin digestion of the enzyme complex have been separated into active fragments carrying E5 activity and E2/E3 activities. These fragments have been isolated and characterised, and are derived from non-overlapping sub-regions of the arom polypeptide.

10. The limited proteolysis results are best interpreted in terms of a "gene fusion" model for the origin of the arom multifunctional enzyme.

CHAPTER 1 GENERAL INTRODUCTION

1.1 The arom multifunctional enzyme and the shikimate pathway

In plants, fungi, protozoa and bacteria the three aromatic amino acids phenylalanine, tyrosine and tryptophan, and a number of other aromatic compounds are derived from a common precursor, chorismic acid (see figures 1.1 and 1.2); the field has been reviewed by Haslam (1974). The biosynthesis of chorismate from phosphoenolpyruvate (PEP) and erythrose 4-phosphate is a seven-step process. One of the key metabolic intermediates, shikimic acid, has given its name to the entire pathway (see figure 1.1).

The seven enzyme-catalysed reactions that make up the "early common pathway" of chorismate biosynthesis are the same in all organisms, but the structural organisation of the enzymes in the pathway is varied (see figues 1.1 and 1.4). In *E.coli* seven discrete enzymes are required for chorismate biosynthesis (Berlyn and Giles, 1969). In the filamentous fungus *Neurospora crassa* five reactions of the common pathway are catalysed by the *arom* enzyme complex (Giles *et al*, 1967a). This enzyme complex consists of two identical 165kDa polypeptide chains and is a catalyst for the five independent reactions numbered 1 to 5 in figure 1.1 (Lumsden and Coggins, 1978). The *arom* complex is thus a multifunctional enzyme consisting of two identical pentafunctional polypeptides.

1.2 Organisation of material in this thesis

The present study is concerned with aspects of the structural organisation and catalytic function of the *arom* multifunctional enzyme and in particular the mechanism of EPSP synthase, the fifth activity of the enzyme complex. Progress in these directions necessitated the

development of new methods for the isolation of the arom multifunctional enzyme and the next enzyme in the shikimate pathway, chorismate synthase. The experiments thus fall into four broad areas:

1. The purification and characterisation of the arom multifunctional enzyme (chapter 4)
2. The purification and characterisation of chorismate synthase (chapter 5)
3. Kinetic and mechanistic experiments with EPSP synthase (chapter 6)
4. An investigation of the structure of the arom complex by limited proteolysis (chapter 7)

A detailed introduction to previous work in each area is given in the appropriate chapter, where it is presented in the context of the research objectives. The structure of the arom multifunctional enzyme and the genetics or the arom locus are therefore discussed in detail in chapter 7, and the enzymology of EPSP synthases is reviewed in chapter 6.

The remainder of this general introduction is devoted to an overview of the chemistry and enzymology of the shikimate pathway, while chapter two provides an introduction to multifunctional enzymes. Many of the ideas presented in chapter two form an essential background to the discussion of the structure of the arom complex which follows in chapter seven. Chapters 4, 5 and 6 are, by contrast, relatively self-contained.

1.3 Nomenclature

Throughout this thesis the full names for the five component enzyme activities of the arom multifunctional enzyme and the shorthand forms E1, E2 etc. are used interchangeably, as defined in figure 1.1. It should be noted that in this convention arom E1 is the second enzyme of the common pathway. The terms "arom enzyme complex" and "arom multifunctional enzyme" are synonymous.

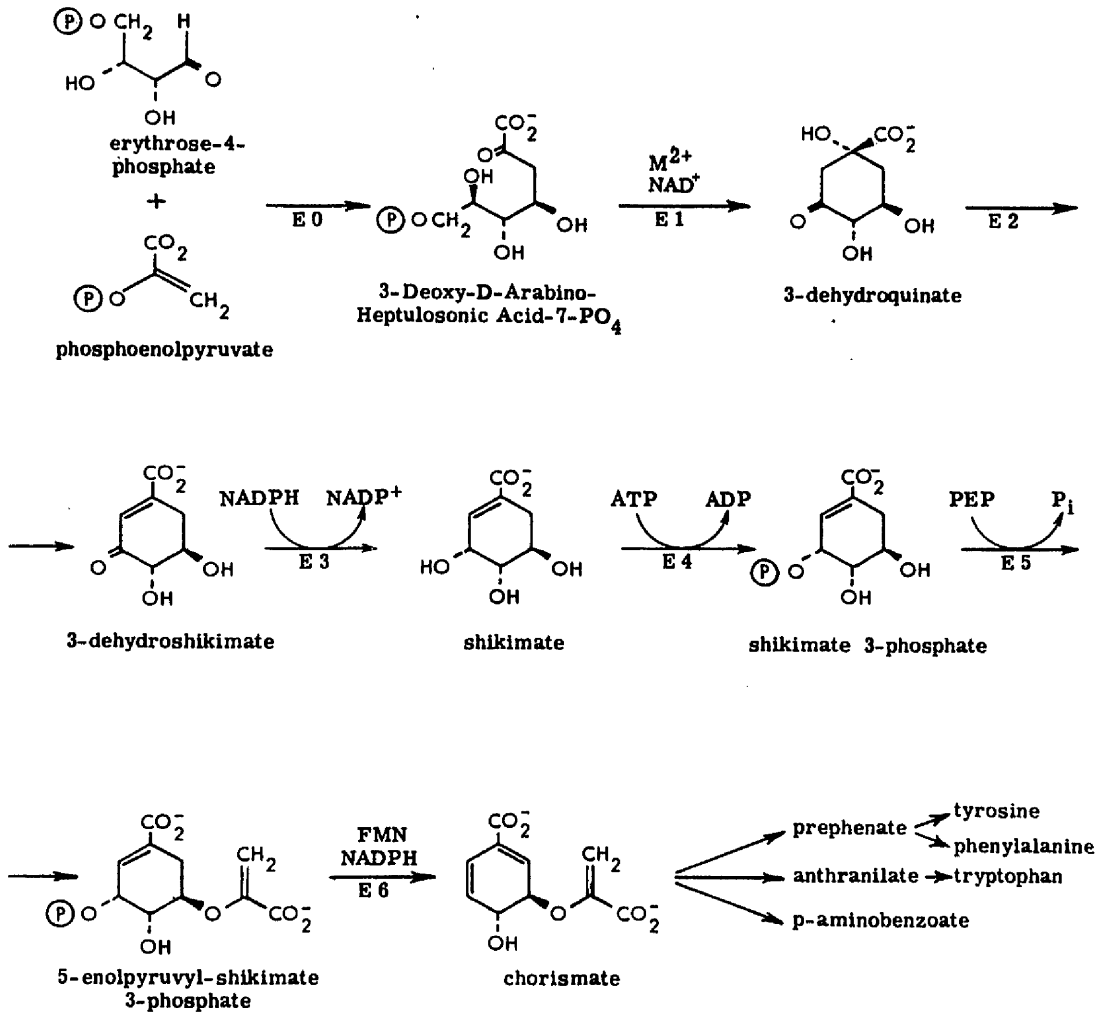


Figure 1.1 The shikimate pathway

abbreviations:

EO DAHP synthase

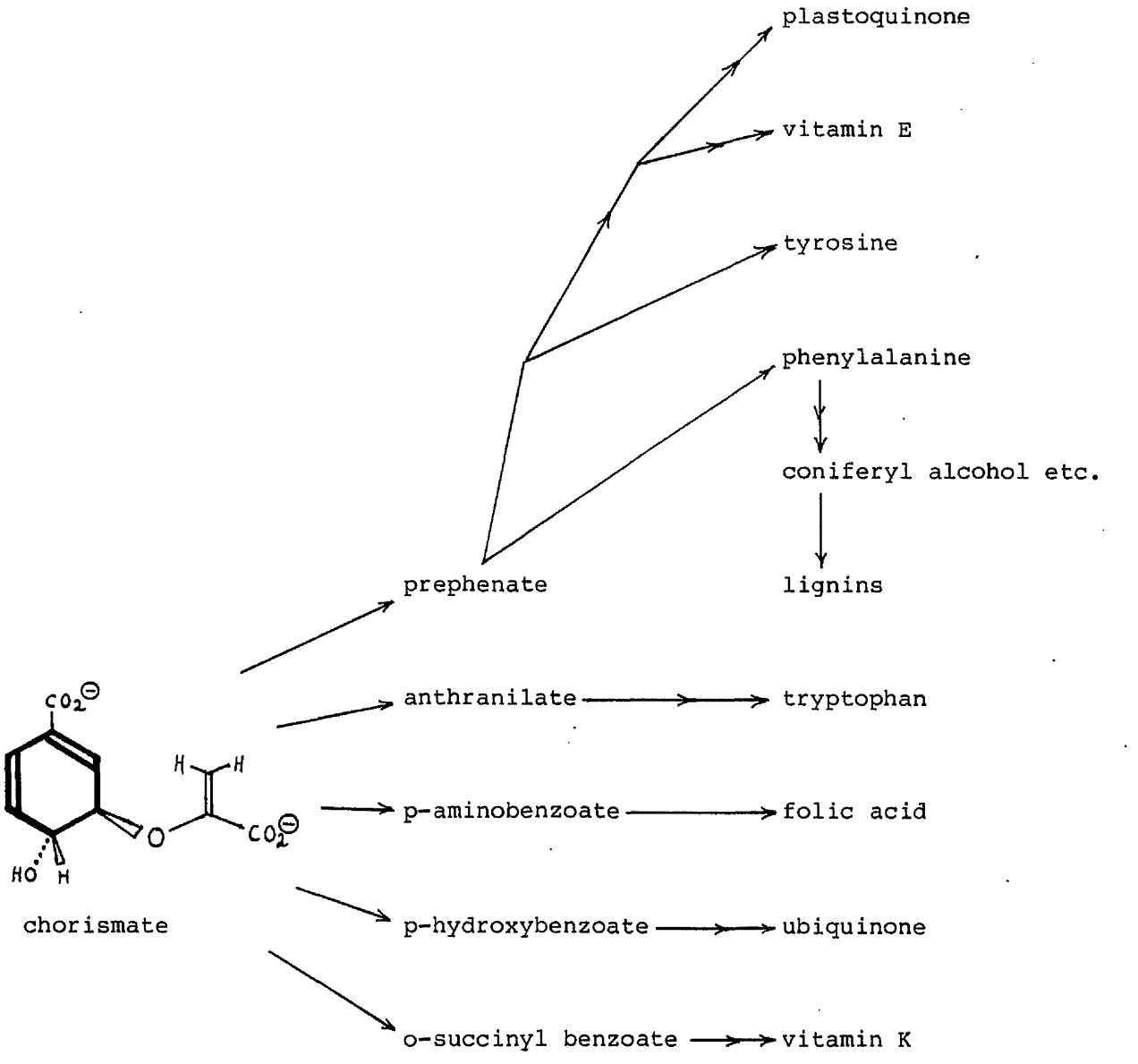
E1	DHQ synthase
E2	dehydroquinase
E3	shikimate dehydrogenase
E4	shikimate kinase
E5	EPSP synthase

enzyme activities of the arom multifunctional enzyme of N.crassa

E6 chorismate synthase

DAHP 3-deoxy-D-arabino-heptulosonic acid 7-phosphate
DHQ 3-dehydroquinate
DHS 3-dehydroshikimate
Shik3P shikimate 3-phosphate
EPSP 5-enolpyruvyl-shikimate 3-phosphate

Figure 1.2 Utilisation of chorismate (after Haslam, 1974)



A number of abbreviations are used for the names of metabolic intermediates, and these are also defined in Figure 1.1. and on page (ii).

1.4 The shikimate pathway and the biosynthesis of aromatic compounds

In a series of classic experiments Bernard Davis and his coworkers isolated for the first time auxotrophic strains of E.coli and Salmonella that were deficient in aromatic amino acid biosynthesis, and identified shikimic acid as a common precursor of phenylalanine, tyrosine and tryptophan (see figure 1.1; Davis, 1951). The crucial importance of certain unusual chemical properties of chorismic acid was first recognised by R.B.Woodward, and it was then possible to complete the jigsaw-puzzle of aromatic amino acid biosynthesis (R.B.Woodward, 1954: cited by Levin and Sprinson, 1963).

In aqueous solutions chorismate is spontaneously converted into prephenate in an extraordinary electrocyclic reaction analogous to a Claisen rearrangement. The enzyme chorismate mutase catalyses the same reaction and is perhaps unique in catalysing a symmetry-allowed reaction of this type (Heyde, 1979; Andrews and Haddon, 1979). The product of the reaction, prephenate, can be converted into either tyrosine or phenylalanine in two simple steps (see figure 1.3).

Chorismate is one of the most versatile intermediates in primary metabolism. It is converted into tryptophan in a complex six-step sequence of enzyme-catalysed reactions (figure 1.3). Chorismate is also a precursor for the growth factors and coenzymes ubiquinone, plastoquinone, tocopherol (vitamin E), folic acid and vitamin K. In plants phenylalanine is a precursor of coniferyl alcohol, the monomeric unit required for the biosynthesis of lignins, the major structural polymers of woody tissues (figure 1.2).

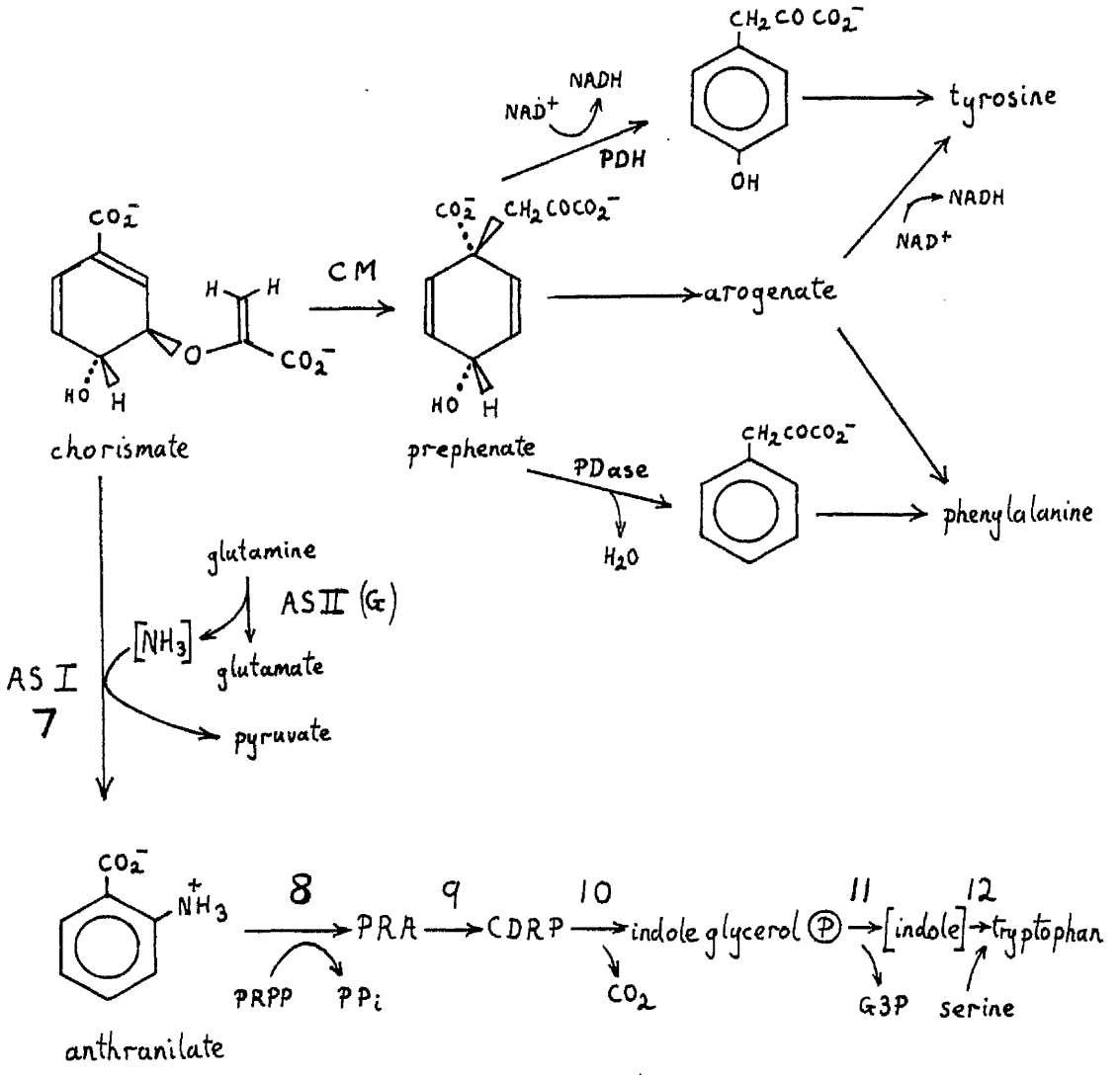


Figure 1.3 Biosynthesis of tyrosine, phenylalanine and tryptophan from chorismate

enzymes	CM	chorismate mutase
	PDH	prephenate dehydrogenase
	PDase	prephenate dehydratase
	ASI(7)	anthranilate synthase catalytic subunit
	ASII(G)	anthranilate synthase glutaminase subunit
	8	anthranilate phosphoribosyl transferase
	9	phosphoribosyl anthranilate isomerase
	10	indole glycerol phosphate synthase
	11	tryptophan synthase step A
	12	tryptophan synthase step B
intermediates	PRA	phosphoribosyl anthranilate
	CDRP	(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate
	PRPP	phosphoribosyl pyrophosphate
	G3P	glyceraldehyde 3-phosphate

In all organisms that do not rely on an external supply of "essential" aromatic amino acids the "common pathway" of chorismate biosynthesis is a major synthetic route leading to homocyclic aromatic compounds. The carbon skeleton of chorismate is derived from one molecule of erythrose 4-phosphate, an intermediate in the pentose phosphate pathway, and two molecules of phosphoenolpyruvate (see figure 1.1). The first committed step in the pathway is a condensation reaction between erythrose 4-phosphate and PEP, which yields the seven-carbon sugar derivative DAHP. The second enzyme in the sequence closes the six-carbon ring that eventually forms the aromatic nucleus. The second PEP unit is added in the EPSP synthase reaction; this three-carbon unit subsequently migrates around the ring in the chorismate mutase reaction to form the side chain of phenylalanine and tyrosine (figures 1.1 and 1.3).

1.5 Enzymology of the early common aromatic pathway

The enzymes of the early common aromatic pathway of chorismate biosynthesis have been well characterised in N.crassa and E.coli, and to a lesser extent in plants. Figure 1.4 illustrates the diversity in the degree of structural organisation of the enzymes of the common pathway and the tryptophan pathway, and lists the subunit structures of the enzymes in several organisms.

The seven enzymes of the common pathway are structurally independent in many species of bacteria, including E.coli, and the corresponding genes are expressed from independent loci (Gollub et al, 1967; Berlyn and Giles, 1969). There is no evidence that the enzymes are aggregated within the cell.

In a wide range of plants the common pathway enzymes also appear to be structurally independent, with the exception of dehydroquinase and

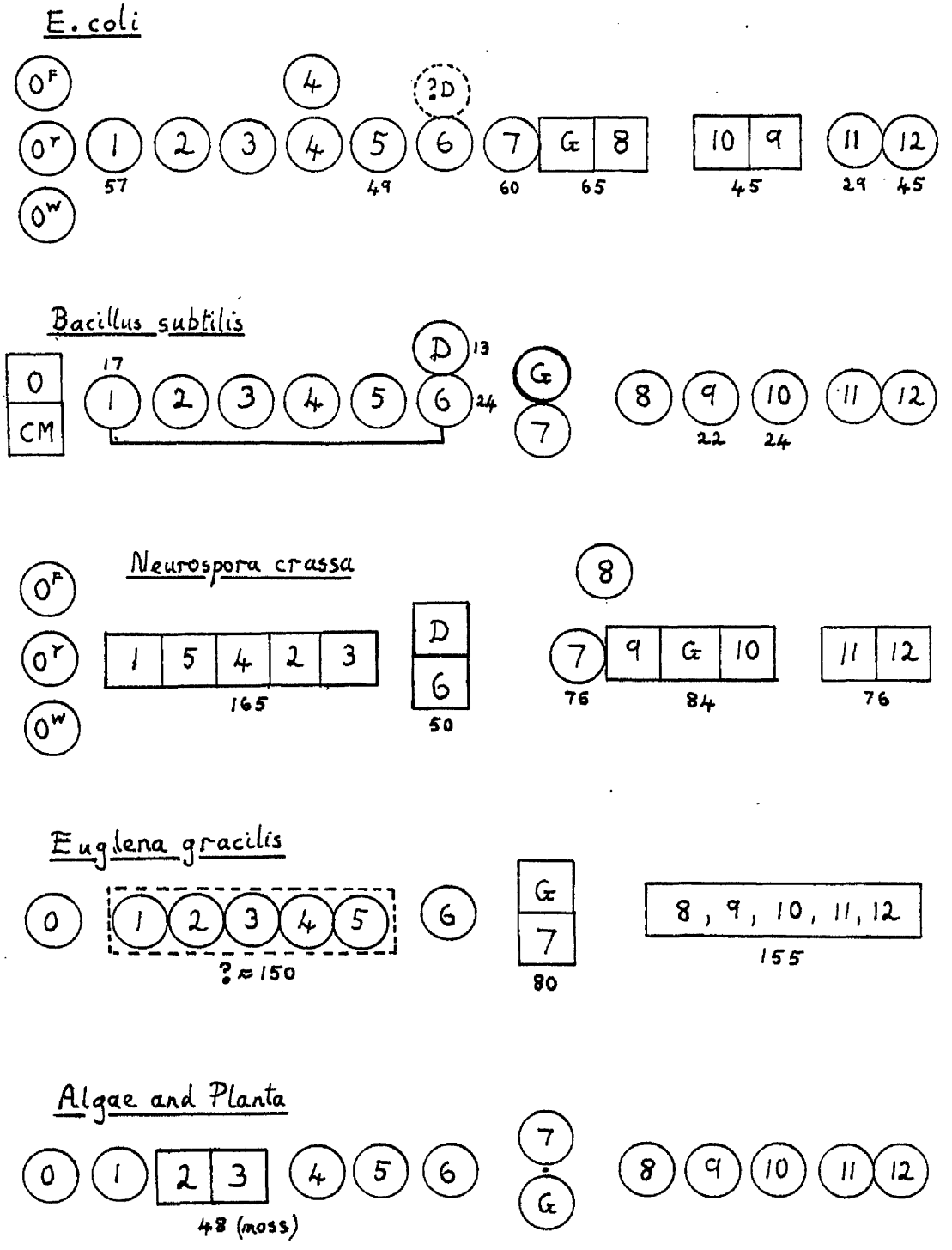


Figure 1.4 Structural organisation of enzymes in the common aromatic pathways and tryptophan pathways of a range of organisms

The enzymes are numbered as in figures 1.1 and 1.3. Rectangles represent multifunctional polypeptides; circles represent monofunctional polypeptides. Joined circles represent multienzyme complexes. Polypeptide sizes are in kDa. After Welch and De Moss (1978) and Crawford (1980).

shikimate dehydrogenase (steps 2 and 3), which are tightly associated in many species. At least in the moss Physcomitrella patens, dehydroquinase and shikimate dehydrogenase activities are carried by a bifunctional polypeptide (Berlyn et al, 1970; Koshiba, 1979a; Boudet and Lēcussan, 1974; Polley, 1978).

In filamentous fungi, yeasts and the green flagellate Euglena gracilis the enzymes catalysing steps 1 to 5 of the common pathway are associated in an arom enzyme complex (Ahmed and Giles, 1969). The arom complex of N.crassa consists of two identical pentafunctional polypeptides (Coggins and Lumsden, 1978). Genetic evidence suggests that at least in the yeasts saccharomyces cerevisiae and schizosaccharomyces pombe the arom enzyme complexes may be organised in the same way, but these enzymes have not yet been purified to homogeneity (de Leeuw, 1967; Strauss, 1979).

1.5.1 DAHP synthases and the metabolic regulation of the common pathway

DAHP synthase (EO) catalyses a condensation reaction between erythose 4-phosphate and PEP; this is the first committed step in the common aromatic pathway. N.crassa, E.coli and a number of other organisms possess three DAHP synthase isoenzymes, each sensitive to feedback inhibition (or repression) by one of the three aromatic amino acids phenylalanine, tyrosine and tryptophan (Nimmo and Coggins, 1981a, Doy and Brown, 1965). Feedback regulation at the level of DAHP synthase gene expression appears to be particularly important in prokaryotes (McCray and Herrmann, 1976). In E.coli and N.crassa other common pathway enzymes are expressed constitutively; in E.coli only the genes coding for enzymes in the separate branches of the pathway from chorismate onwards are organised into operons that are regulated by the end-products of the reaction sequences (Umbarger, 1978). There are thus strong

indications that DAHP synthases play an important part in metabolic regulation of the common pathway of chorismate biosynthesis.

The tryptophan-sensitive DAHP synthase of Neurospora crassa has been purified to homogeneity, and is perhaps the best-characterised of these enzymes. It probably requires a transition metal ion for activity, and has a rapid equilibrium ordered mechanism in which PEP is the first substrate to bind to the enzyme (Nimmo and Coggins, 1981 a,b). The tyrosine-sensitive DAHP synthase of E.coli is an iron-containing enzyme; the N-terminal sequence is strongly homologous to the iron-binding region of the oxygen-carrying protein hemerythrin of marine worms (Herrmann et al, 1980).

1.5.2 Dehydroquinate synthase (E1)

Monofunctional DHQ synthases from sorghum, mung bean, E.coli and B.subtilis have been characterised (Saijo & Kosuge, 1978; Yamamato, 1980; Maitra and Sprinson, 1978; Hasan and Nester, 1978c). All these enzymes require catalytic amounts of NAD^+ and a divalent transition metal cation for activity. The two bacterial enzymes and the mung bean enzyme have been purified to homogeneity. The DHQ synthase of E.coli is monomeric, while the B.subtilis enzyme is associated with two different functional subunits of chorismate synthase in a multienzyme complex.

Transition metal requirements for DHQ synthase action are discussed in chapter 4.

1.5.3 Dehydroquinase (E2)

No monofunctional biosynthetic dehydroquinase has yet been purified to homogeneity. Experiments in our laboratory have indicated that the dehydroquinase of E.coli may be a dimeric enzyme with subunits of 30kDa (J.M.Lambert, A.A.Coia, S.Choudhury, unpublished work).

A tight association between the biosynthetic dehydroquinase and shikimate dehydrogenase (step 3) appears to be universal in higher plants (Boudet and Lēcussan, 1974). The associated dehydroquinase and shikimate dehydrogenase activities from mung beans, and from pea seedlings, have been very extensively purified (Koshiba, 1978; M.S.Campbell, unpublished work). A bifunctional dehydroquinase/shikimate dehydrogenase has been purified to homogeneity from the moss Physcomitrella patens (Polley, 1978). It is a monomeric enzyme of 48kDa.

1.5.4 Catabolic dehydroquinases and the quinate pathway

In N.crassa the dehydroquinase reaction is a component of two independent metabolic pathways, the biosynthetic shikimate pathway, and a catabolic pathway that allows the fungi to grow on the plant product quinate as sole carbon source (see figure 1.5). In addition to the "biosynthetic" dehydroquinase, the second activity of the arom multifunctional enzyme, there is an inducible "catabolic" dehydroquinase, the product of the qa-2 structural gene (Giles et al, 1967b). Three independent enzymes of the inducible quinate pathway are coordinately expressed from the qa region, which has now been cloned in E.coli. The qa system is an important model for the regulation of gene expression in eukaryotes (Schweizer et al, 1981).

The catabolic dehydroquinase of N.crassa has been purified to homogeneity (Hautala et al, 1975). Results from our own laboratory indicate that the subunit size is 20kDa, (S.Choudhury and J.R.Coggins, in preparation)) as predicted from the DNA sequence, (Giles et al, unpublished work), and that the enzyme is probably a dodecamer.

The properties of the catabolic dehydroquinase of N.crassa are in a number of respects rather different from those of the arom

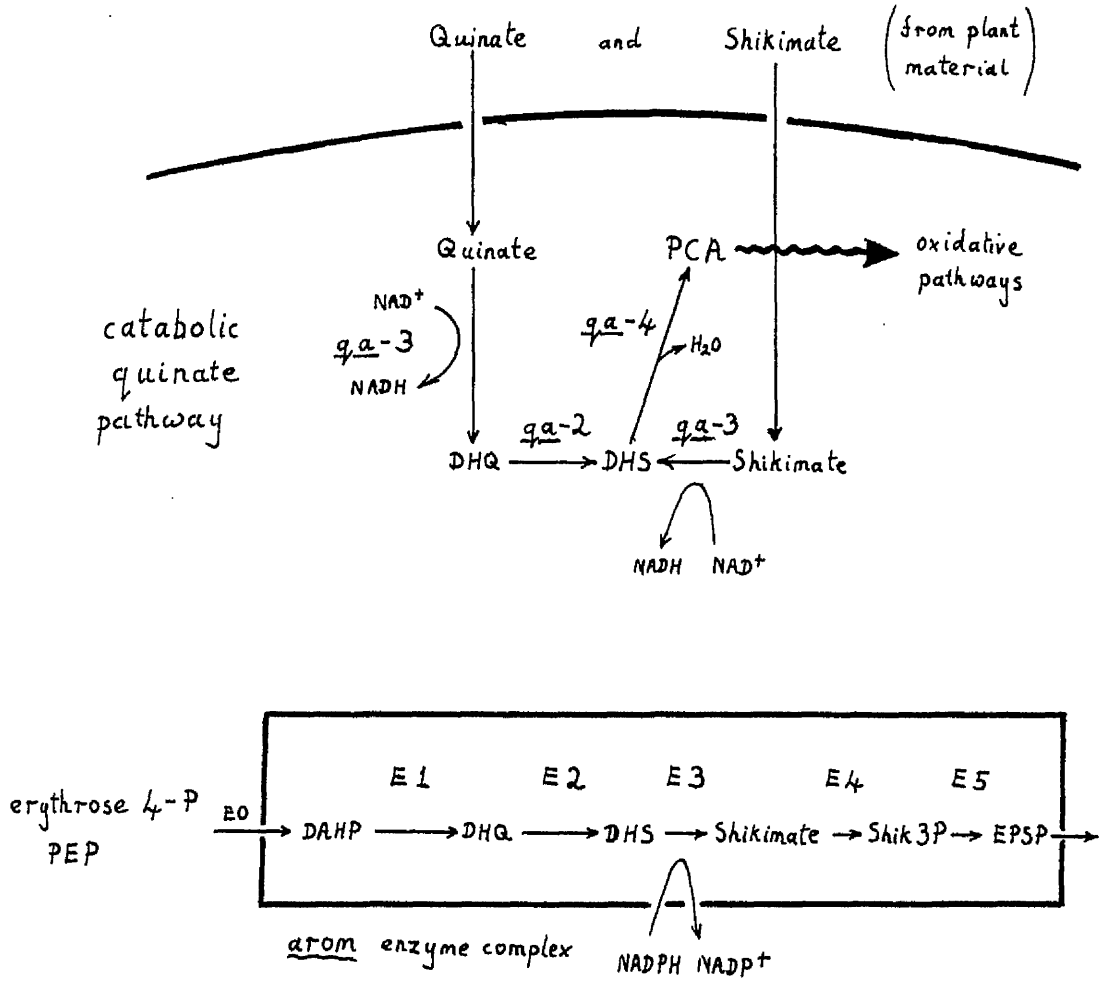


Figure 1.5 The catabolic quinate pathway and the biosynthetic shikimate pathway in Neurospora crassa

intermediates:	DHQ	dehydroquinate
	DHS	dehydroshikimate
	PCA	protocatechuic acid
enzymes:	qa-2	catabolic dehydroquinase
	qa-3	catabolic quinate dehydrogenase/shikimate dehydrogenase
	qa-4	dehydroshikimate dehydratase

dehydroquinase (E2), although the reaction mechanism appears to be the same (Giles et al, 1967b; Choudhury and Coggins, 1982; see section 1.6.3). It is thus of enormous interest to examine the evolutionary relationships between the catabolic dehydroquinase and the catabolic quinate/shikimate dehydrogenase (Lopez-Barea and Giles, 1978), and the biosynthetic dehydroquinase and shikimate dehydrogenase activities of the arom multifunctional enzyme.

Giles (1967a) has suggested that one function of the arom enzyme complex is to ensure that the metabolic intermediate dehydroshikimate (DHS) is "channelled" into the DHS reductase (E3) reaction of the biosynthetic pathway, and is not siphoned off into the catabolic pathway by the enzyme DHS dehydratase (Stronan et al, 1978; see figure 1.5). Mutants that lack the biosynthetic arom dehydroquinase (E2) are able to use the "catabolic" activity to grow on minimal medium, but mutants lacking the catabolic dehydroquinase cannot grow on quinate as sole carbon source (Case et al, 1972; N.H.Giles and M.E.Case, personal communication). These observations may be consistent with some form of intracellular compartmentation of DHS.

It has been reported that in many monocotyledenous plants there are two dehydroquinases, one associated with a quinate dehydrogenase activity and the other with the biosynthetic shikimate dehydrogenase (Graziana et al, 1980). It is conceivable that both of those enzymes are bifunctional.

1.5.5 Shikimate dehydrogenase (E3)

No monofunctional biosynthetic shikimate dehydrogenase has yet been purified to homogeneity from any source. The biosynthetic shikimate dehydrogenase (E3) activities of E.coli, plants and N.crassa have a strong preference for $NADP^+$ over NAD^+ as cofactor (Yaniv and

Gilvarg, 1955; Boudet and Lēcussan, 1974). Shikimate dehydrogenase of E.coli is likely to be a small monomeric enzyme of approximately 30kDa (I.A.Anton, A.Lewendon, A.A.Coia, unpublished work; Berlyn and Giles, 1969).

The catabolic "quinate" dehydrogenase of N.crassa has been purified to homogeneity; its activities as a shikimate dehydrogenase and as a quinate dehydrogenase are of comparable magnitude (Lopez-Barea and Giles, 1978). The catabolic dehydrogenase is a monomeric enzyme of 40kDa, and unlike the biosynthetic dehydrogenase (E3) it has a strong preference for NAD^+ as cofactor. This enzyme is presumably used by N.crassa in the catabolic assimilation of the widely distributed plant products quinate and shikimate (see figure 1.5).

A shikimate dehydrogenase activity from peas has been subjected to detailed kinetic studies. It appears to have an ordered sequential mechanism in which $NADP^+$ or NADPH is the first substrate to bind to the enzyme (Balinsky et al, 1971).

1.5.6 Shikimate kinase (E4)

No monofunctional shikimate kinase has yet been fully characterised. E.coli possesses two shikimate kinase isoenzymes that are expressed from independent genetic loci. Synthesis of one of the isoenzymes, the aro L gene product, is under the control of the tyrosine repressor (Ely and Pittard, 1979). There are thus strong indications that metabolic regulation of the common pathway is exercised at this point.

The shikimate kinase activity of B.subtilis appears to be associated with a 10kDa polypeptide that is catalytically inactive except when it aggregates with the bifunctional DAHP synthase/chorismate mutase complex (see figure 1.4; Huang et al, 1975). It is difficult to assess

the importance of the proposed regulatory functions of this multienzyme complex, since it has recently been shown that the bifunctional DAHP synthase/chorismate synthase has arisen "artificially" in certain laboratory strains of B.subtilis (Llewellyn et al, 1980).

The shikimate kinases of mung beans and E.coli, like that of B.subtilis, are rather small enzymes with estimated molecular weights of 29kDa and 20kDa respectively (Koshiba, 1979b; Ely and Pittard, 1979).

1.5.7 EPSP synthase (E5)

Monofunctional EPSP synthases have recently been purified to homogeneity from E.coli and from pea seedlings (Lewendon and Coggins, 1983; Mousdale and Coggins, 1983). They are monomeric enzymes of 49kDa. The enzymology of EPSP synthases is discussed in some detail in the introduction to chapter 6.

EPSP synthase is probably the unique cellular target for the herbicide glyphosate (see chapter 6).

1.5.8 Chorismate synthase (E6)

Chorismate synthases of E.coli, N.crassa and B.subtilis are stimulated by a "flavin reductase" or "diaphorase" (D) activity; under aerobic conditions the biosynthetic reaction is totally dependent on the availability of flavin and NAD(P)H as cofactors (Morell et al, 1967). The B.subtilis enzyme has been purified to homogeneity and consists of three different polypeptides, which specify the chorismate synthase and associated diaphorase functions and also DHQ synthase (E1) (Hasan and Nester, 1978b). Chorismate synthase of N.crassa has been highly purified, and is reported to consist of two 55kDa sununits (Welch et al, 1974). It is therefore possible that the N.crassa chorismate synthase is a bifunctional enzyme. The purification and characterisation of this enzyme is considered in detail in chapter 5.

The function of the "diaphorase" activity in chorismate synthase catalysis is not known. It has recently been reported that an enzyme identical to the diaphorase subunit of the B.subtilis chorismate synthase is involved in the reductive assimilation of iron (III) compounds (Gaines et al, 1981; see Section 1.6.6 and chapter five).

1.6 Enzyme chemistry of the common pathway

The biosynthetic pathway that leads from carbohydrates to simple aromatic compounds has long intrigued chemists and enzymologists alike (reviewed by Ganem, 1978). The DAHP, DHQ, EPSP and chorismate synthase reactions (steps 0, 1, 5 and 6 in figure 1.1) are by any standards unusual and exotic chemical processes, and they may even tax the ingenuity of enzyme catalysis.

Most of the mechanistic experiments on shikimate pathway enzymes have been carried out on partially purified preparations from bacteria. There is generally no direct evidence that the component activities of the arom multifunctional enzyme follow the same chemical mechanism, but this would be a reasonable working hypothesis.

1.6.1 DAHP synthase (EO)

There are two enzymes in the common pathway that catalyse a condensation reaction involving phosphoenolpyruvate - DAHP synthase and EPSP synthase. Since many of the experiments described in this study relate to the mechanism of EPSP synthase a comparison of the modes of PEP utilisation by the two enzymes is of some interest.

Unlike EPSP synthase, DAHP synthase does not scramble the positions of the two protons that originate from the C3 methylene group of PEP, and a freely rotating methyl group therefore cannot be formed at this position during the course of the reaction. In both the DAHP synthase reaction and the EPSP synthase reaction the entire phosphate

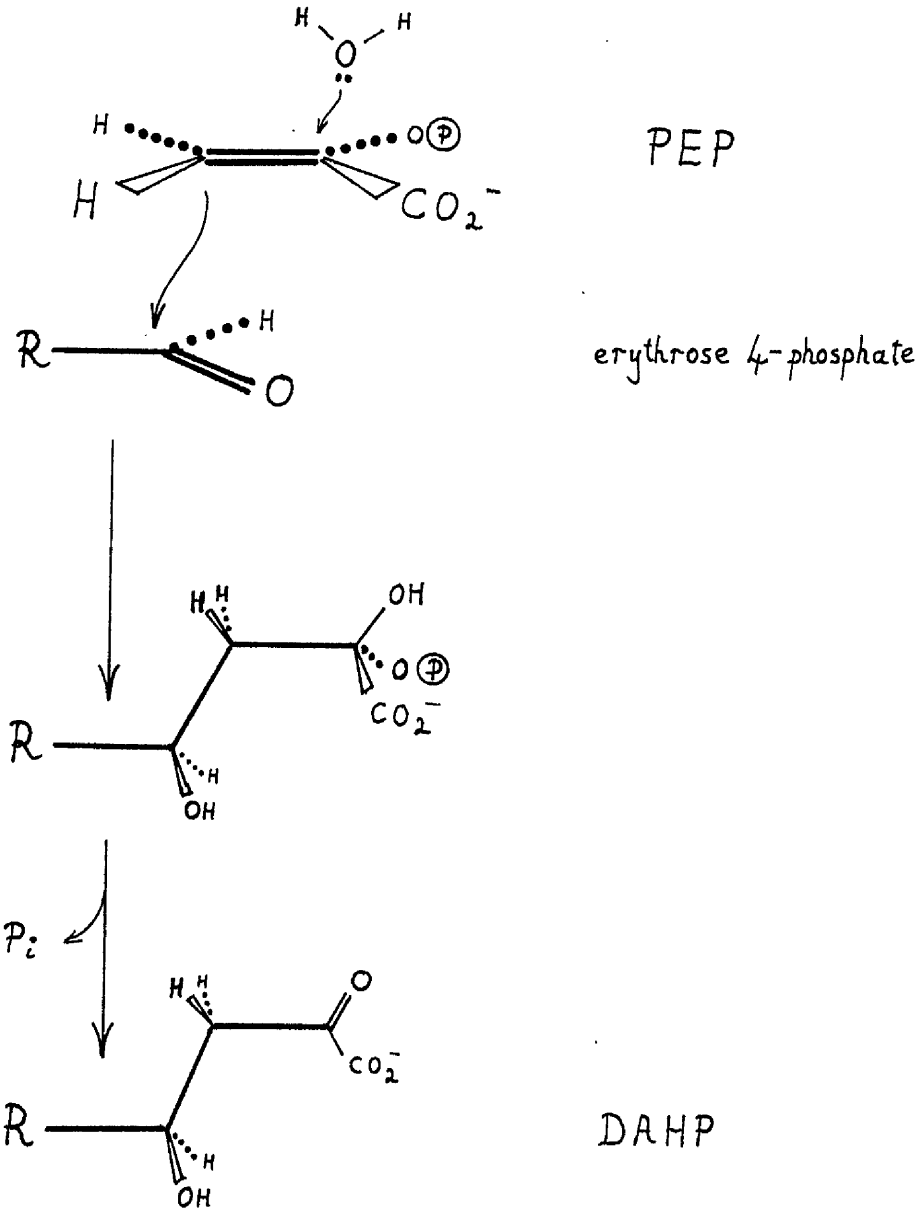


Figure 1.6 A catalytic mechanism for DAHP synthase proposed by de Leo et al (1973)

($-OPO_3^{2-}$) group from PEP is eliminated intact. In this respect both reactions are quite different from more conventional enzymatic reactions of PEP in which only the phospho ($-PO_3^{2-}$) group is transferred (Floss et al, 1972). The implication is that in both of the synthase reactions a nucleophile must add to the C2 position of PEP before the phosphate group can be eliminated.

A plausible mechanism for DAHP synthase that is consistent with all the isotope tracer results is shown in figure 1.6. The key feature of this proposed mechanism is the concerted addition of the elements of erythose 4-phosphate and water across the double bond of PEP (de Leo et al, 1973). EPSP synthase may catalyse an analogous concerted addition of an electrophile (H^+) and a nucleophile across the PEP double bond (Grimshaw et al, 1982; see section 6.1). De Leo et al (1973) were not able to rule out more complex reaction schemes for DAHP synthase in which a nucleophilic group in the enzyme initiates the attack on the double bond of PEP; Ganem (1978) has suggested that the nucleophile is likely to be an enzyme thiol group, rather than a carboxylate anion.

1.6.2 DHQ synthase (E1)

DHQ synthase catalyses the crucial ring-closing reaction in the common aromatic pathway. The reaction is essentially irreversible.

All DHQ synthases require catalytic quantities of NAD^+ for activity. There is good evidence that the hydroxyl group at the C5 position of DAHP is transiently oxidised by NAD^+ during the course of the reaction (Rotenberg and Sprinson, 1978; see figure 1.7). Oxidation at the C5 position (step 1) apparently precedes elimination of phosphate from the C7 position (step 2). The C5 position can then be re-reduced to give the enol intermediate III (step 3) which immediately participates in the ring-closing reaction (step 4) without tautomerising to the

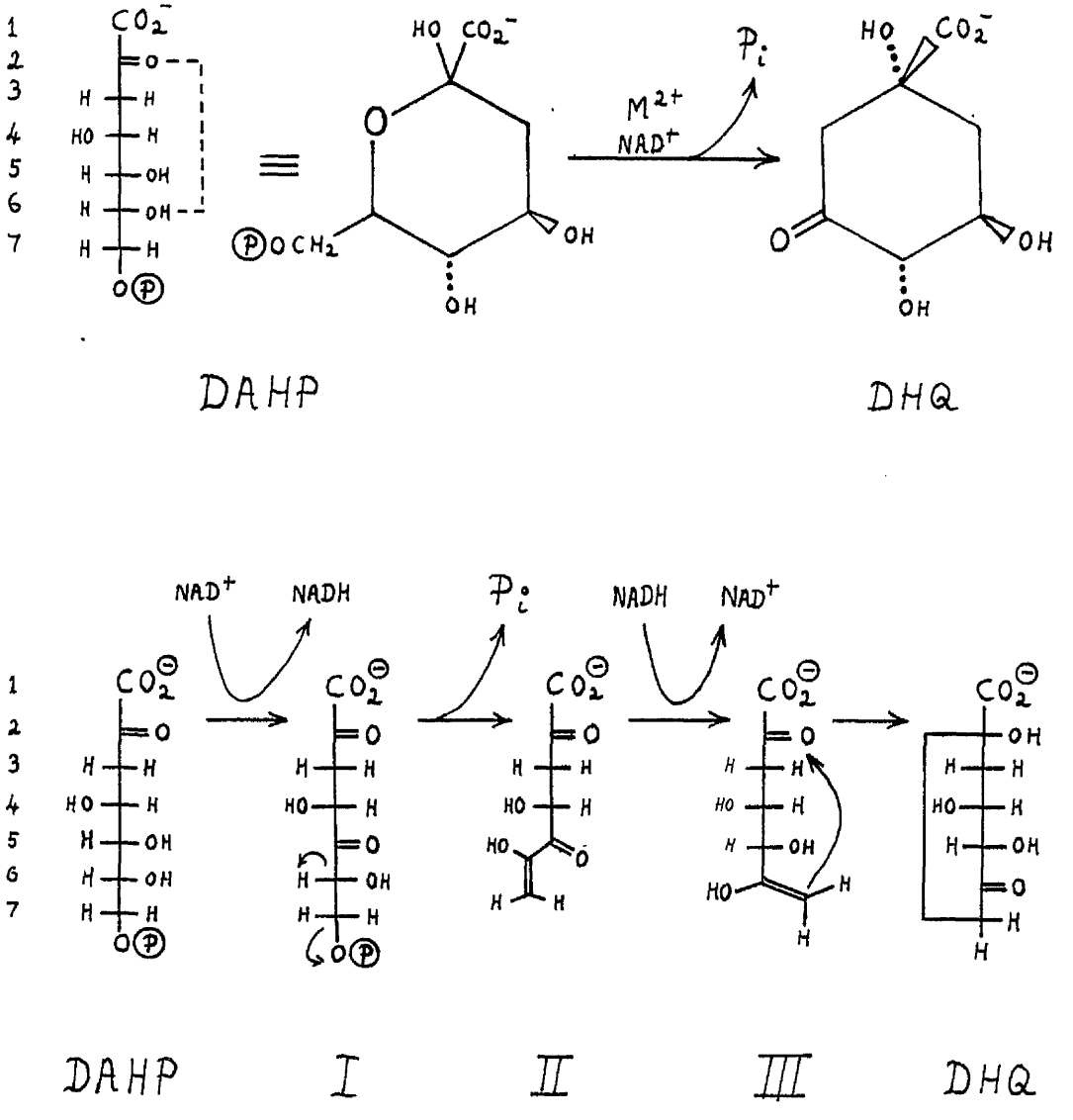


Figure 1.7 The DHQ synthase (E1) reaction and the catalytic mechanism proposed by Maitra & Sprinson (1978)

corresponding diketone. This proposed mechanism provides the most satisfactory explanation of the results of many elegant experiments that have been designed to probe the stereochemistry of the reaction and trap the catalytic intermediates (Maitra and Sprinson, 1978).

In aqueous solution a cyclic pyranose tautomer of DAHP may be the majority species; the protons at C3 of DAHP do not rapidly exchange with the solvent (Floss et al, 1972), and the fractional concentration of available keto groups is very low (J.M.Lambert, M.R.Boocock and J.R.Coggins, in preparation; see figure 1.7). It has been suggested that a cyclic pyranose tautomer of DAHP may be the true substrate of the synthase reaction. This would be consistent with the finding that the methyl glycoside of DAHP and various other closed-ring analogs of DAHP are potent inhibitors (Le Marechal and Azerad, 1976; Maitra and Sprinson, 1978; Le Marechal et al, 1981). It is not known at what point in the mechanism the glycosidic ring of DAHP is opened.

The role of transition metal cations in DHQ synthase catalysis remains obscure (see chapter 4).

1.6.3 Dehydroquinase (E2)

Dehydroquinase catalyses the dehydration of DHQ to give DHS. The reaction is freely reversible with $K_{eq}' \approx 15$ (Mitsuhashi and Davis, 1954).

The dehydration of DHQ proceeds by an unusual syn elimination of the elements of water. The orientation of the leaving groups is thus opposite to that seen in most comparable enzymatic reactions; in all straightforward concerted elimination reactions of this type an anti orientation of the leaving groups is expected (Hanson and Rose, 1963). Butler et al (1974) therefore proposed a completely different reaction pathway for dehydroquinase (see figure 1.8). DHQ first reacts with a

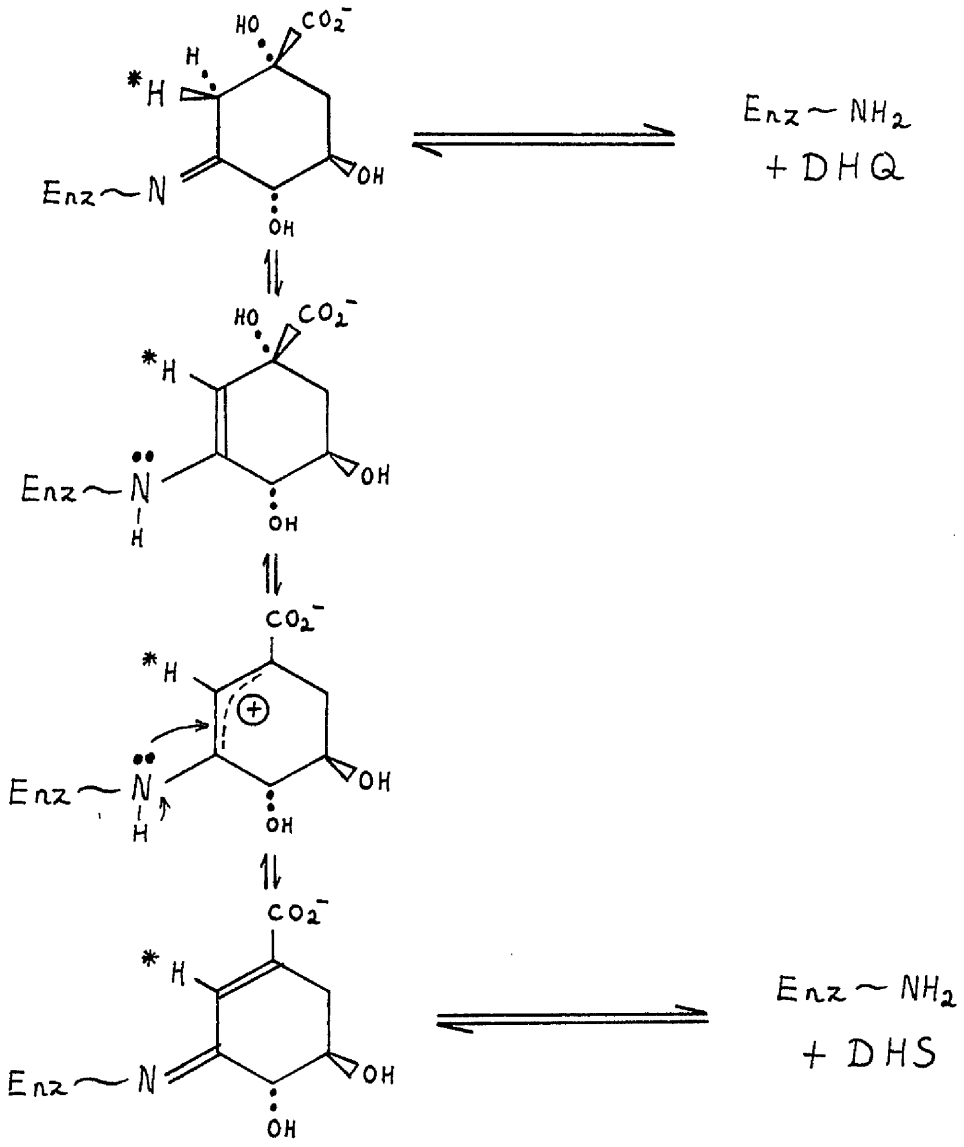


Figure 1.8 A proposed catalytic mechanism for dehydroquinase (E2); Butler et al (1974)

lysine side chain at the active site of the enzyme to form a Schiff base, and the elements of water are then eliminated in two separate stages. Crude bacterial dehydroquinase preparations are inactivated by borohydride only in the presence of DHQ, as predicted by this model (Butler et al, 1974). The biosynthetic (arom E2) and catabolic (qa-2) dehydroquinases of N.crassa can be specifically labelled by tritiated borohydride in the presence of DHQ, and are thus likely to follow the same mechanism (Choudhury and Coggins, 1982; Smith and Coggins, 1982).

1.6.4 Shikimate dehydrogenase (E3)

Shikimate dehydrogenase (dehydroshikimate reductase, E3) catalyses the reduction of DHS by NADPH (see figure 1.1). The reaction is freely reversible with $Keq' \approx 28$ (Balinsky et al, 1971; Yaniv and Gilvarg, 1955). The magnitude of the equilibrium constant is relatively small for a dehydrogenase reaction, presumably because the keto group of DHS is part of a conjugated system.

Shikimate dehydrogenases from pea seedlings and from E.coli transfer the hydrogen atom from the A side of the reduced nicotinamide ring to C3 of DHS (Davies et al, 1972; Dansette and Azerad, 1974).

1.6.5 Shikimate kinase and EPSP synthase (E4 and E5)

Shikimate kinase catalyses a straightforward phosphoryl transfer from ATP to shikimate. Reversal of the kinase reaction can be observed when the arom complex is incubated with shikimate 3-phosphate and ADP in the presence of glucose and hexokinase, but at equilibrium shikimate 3-phosphate and ADP predominate (G.A.Nimmo, unpublished work).

The chemistry of the EPSP synthase reaction is considered in section 6.1.

1.6.6 Chorismate synthase, E6

Chorismate synthase catalyses a 1,4 elimination of a phosphate group and a proton from EPSP. The two leaving groups are in an *anti* orientation. The observed stereoselectivity of chorismate synthase, like that of dehydroquinase, is thus at odds with the steroselectivity predicted on the basis of simple model reactions and orbital symmetry theory (Floss *et al*, 1972; Hill and Newkome, 1969; Hill and Bock, 1978; Ganem, 1978).

The sterochemistry of the chorismate synthase reaction can be rationalised if a nucleophilic group in the enzyme initiates the reaction sequence by attacking the double bond of EPSP; the phosphate group and the proton can then be eliminated in two separate stages. A plausible mechanism is shown in figure 1.9 (Floss *et al*, 1972).

The activity of all known chorismate synthases is dependent on a "flavin reductase" activity (Morell *et al*, 1967; Welch and Gaertner, 1974; Hasan and Nester, 1978a). Since there is no obvious role for redox processes in the chorismate synthase reaction the requirement for flavin and a reducing agent, normally NAD(P)H, is a total surprise. However, early studies on chorismate synthase from *E.coli* yielded evidence that an iron (II) centre is essential for catalysis. It was proposed that the function of the "flavin reductase" activity is to reduce an iron (III) centre in the inactive enzyme to iron (II) (Morell *et al*, 1967). Recently it has been reported that the "flavin reductase" (diaphorase) subunit of the *B.subtilis* chorismate synthase complex has a second physiological function in the assimilative reduction of iron (III) chelate complexes (Gaines *et al*, 1981). There is, however, no evidence that the activated *B.subtilis* chorismate synthase enzyme complex contains iron (II).

The "flavin reductase" activity associated with a highly purified chorismate synthase from *N.crassa* has been investigated in some detail by Welch *et al*, (1974).

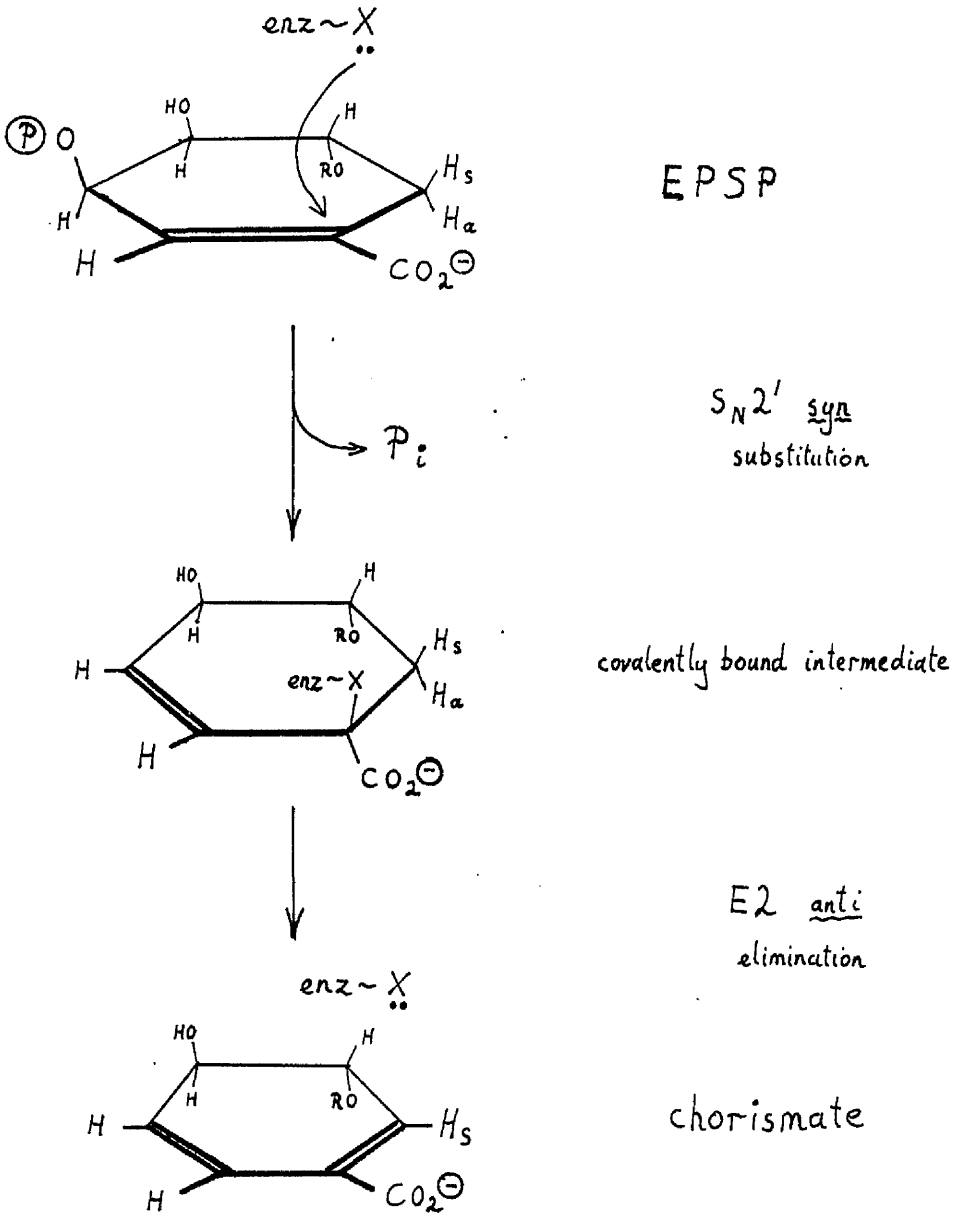


Figure 1.9 The catalytic mechanism for chorismate synthase proposed by Floss et al (1972)

"X" is an unidentified nucleophilic group in the enzyme.

These workers concluded that NADPH and FMN do not participate directly in the conversion of EPSP to chorismate, but are required for the reduction of an unidentified oxygen-sensitive functional group in the enzyme. Welch et al (1974) did not favour the possibility that the oxygen-sensitive functional group is an iron (II) centre, although they obtained no conclusive evidence on this point. Instead they suggested that the function of the "flavin reductase" activity is to reduce a disulphide bond at the catalytic site of the enzyme. One of the resulting free thiol groups could then act as the nucleophile "X" in a mechanism of the type proposed by Floss et al (1972). This hypothesis builds on the idea that the "flavin reductase" activity of chorismate synthase may be analogous to the flavin-dependent dihydrolipoate dehydrogenase activity of the pyruvate dehydrogenase complex (Reed, 1974).

In the light of the discovery that the flavin reductase component of the B.subtilis chorismate synthase complex is an iron (III) reductase it would probably be premature to dismiss the possibility that the activated N.crassa chorismate synthase contains an iron (II) centre. How such an iron (II) centre might participate in chorismate synthase action remains wholly mysterious.

1.7 The shikimate pathway: outstanding problems

A satisfactory description of the catalytic action of several shikimate pathway enzymes is not yet available (see section 1.6). At present the arom multifunctional enzyme is the only homogeneous preparation of three of the common pathway enzymes; it is thus a system of choice for the detailed study of individual steps in the pathway.

The evolutionary relationships of enzymes in the shikimate pathway are not clear. Dehydroquinase, for instance, the second activity of the arom multifunc tional enzyme, exists in at least four other distinct structural guises in various organisms (see figures 1.4

and 1.5). There are numerous indications that the diversity of enzymes that catalyse a given shikimate pathway reaction share many common features, including cofactor specificity and catalytic mechanism. Detailed characterisation of the enzymes at the protein and DNA levels may establish clear structural and functional homologies and contribute to our understanding of the dynamics of molecular evolution.

It is not known why the structural and genetic organisation of the enzymes of the shikimate pathway is so varied (see figure 1.4). It is our hope that a critical comparison of the shikimate pathway genes and enzymes of N.crassa, E.coli and pea seedlings will lead to a deeper understanding of the evolution of this pathway as an integrated unit of function.

CHAPTER 2 MULTIFUNCTIONAL ENZYMES

Multifunctional enzymes have been defined as the class of enzymes that contain polypeptide subunits which catalyse more than one distinct biochemical reaction (Kirschner and Bisswanger, 1976). Enzymes like trypsin that have broad substrate specificities are excluded from the definition, as are conventional multienzyme complexes such as pyruvate dehydrogenase. The fatty acyl synthase of yeast is classified both as a multienzyme complex - because it contains more than one type of polypeptide and catalyses more than one reaction - and as a multifunctional enzyme - because only two types of subunit are required for at least seven distinct biochemical reactions. Multifunctional enzymes are thus a group of biological catalysts for which the classical unitary relationship between gene, enzyme and polypeptide is invalid.

2.1 Ubiquity of multifunctional enzymes

A large number of multifunctional enzymes have now been characterised. Multifunctional enzymes are found in all classes of organisms, including bacteria, higher plants and mammals, but they appear to be particularly prevalent in the amino acid biosynthetic pathways of prokaryotes and fungi (Kirschner and Bisswanger, 1976; Schminke-Ott and Bisswanger, 1980).

A number of generalisations can be made about the reactions catalysed by multifunctional enzymes. Many multifunctional enzymes catalyse two or more consecutive reactions in a biosynthetic pathway. In some cases the pathway intermediates are covalently bound to the enzyme system - as in fatty acyl synthases - but more frequently the products of the individual reactions are free to diffuse away from the enzyme surface. The arom multifunctional enzyme is a good example of the latter type. Where the metabolic intermediates are non-covalently

bound there is sometimes evidence that they are "channelled" into the next reaction of the sequence before they can dissociate from the surface of the enzyme. Multifunctional enzymes that do not catalyse consecutive reactions generally carry enzyme activities from a single metabolic pathway. There are no known examples of naturally occurring multifunctional enzymes that catalyse reactions that are not functionally related, although a large number of artificial "chimaeras" have now been created (Kania and Müller-Hill, 1980).

Multifunctional enzymes are involved in a diversity of important cellular processes. Enzymes such as DNA polymerase I and the rec A protein of E.coli, and acetyl CoA carboxylase, fatty acyl synthase, carbamoyl phosphate synthase and methenyltetrahydrofolate dehydrogenase of mammalian tissues are all multifunctional (Kirschner and Bisswanger, 1976).

Groups of enzyme activities that are encountered in a multifunctional polypeptide in one species are often organised in a different way in unrelated species; the same enzyme activities may be carried by independent polypeptides, or a number of distinct subunits may associate to form a multienzyme complex. The pattern of occurrence of multifunctional enzymes is, however, far from random; there are some interesting phylogenetic relationships in the structural organisation of enzyme activities such as those of the shikimate pathway (see figure 1.4).

2.2 The structure of multifunctional enzymes

There have been many conjectures about the structure and function of multifunctional enzymes. In this section and the subsequent section (2.3) I have attempted to summarise the more important concepts and experimental approaches. Many of the ideas are illustrated in section 2.5, which briefly reviews some properties of a representative

group of well-characterised multifunctional enzymes, and in the introduction to chapter 7, which deals with the structure of the arom multifunctional enzyme.

2.2.1 Structural domains and multifunctional enzymes

Structural "domains" were first recognised in immunoglobulin molecules, and they are an almost universal feature of the structural organisation of enzymes and other proteins. Rossmann and Argos (1981) have listed a number of features that are characteristic of domains and that may aid in their delineation:

1. Similar structures may be recognised elsewhere, either within the same protein (as in immunoglobulins) or in another protein (as in dehydrogenases)
2. Domains are spatially distinct, or at least form a compact folded "glob"
3. Domains often have a specific function
4. Active sites often lie between domains

Two further characteristics of domains are frequently cited:

5. Domains are made up of one, or a small number of, continuous stretches of the polypeptide backbone of the protein
6. Limited proteolytic cleavage will often separate the component domains of a protein.

In regard to limited proteolysis Rossmann and Argos (1981) noted that "whether excised domains in general retain their fold and function is subject to dispute.....excision may expose large hydrophobic patches".

Kirschner and Bisswanger (1976) have proposed that the component enzyme activities of a multifunctional enzymes correspond to discrete structural "domains" within the folded polypeptide. This proposal can be viewed as an extension of the hypothesis that domains are the

evolutionary building-blocks of all proteins. If the component activities of a multifunctional enzyme do not reside on independent domains it follows that the enzyme must carry components of more than one catalytic site on a single structural domain, or that it must catalyse more than one independent reaction at a single active site. In practice there is generally some evidence that the catalytic sites associated with the component enzyme activities are autonomous, although chorismate mutase/prephenate dehydrogenase may be one example of a multifunctional enzyme that can carry out chemically unrelated reactions at a single substrate binding site (Heyde, 1979). We are thus principally concerned with the questions of whether or not a single structural domain can contribute to more than one catalytic site.

2.2.2 Indications of the existence of stuctural domains in multifunctional enzymes

The structural domains within a protein are readily distinguished if the 3-dimensional structure has been determined by x-ray crystallography. Unfortunately high resolution diffraction data are not yet available for any multifunctional enzyme. If one wishes to investigate the possibility that the component activities of a multifunctional enzyme are associated with independent structural domains it is thus necessary to seek alternative methods for recognising structural domains.

In many cases the component enzyme activities of a multifunctional enzyme can be found in another organism as independent enzymes, or as distinct subunits of a multienzyme complex. This finding could be consistent with onecriterion for recognising a domain - that a similar structure be present in another protein (Rossmann and Argos, 1981; see above). There is, however, at present only limited evidence

of structural homologies between regions of multifunctional enzymes and monofunctional enzymes that share a common activity (e.g. Miozzari and Yanofsky, 1979).

Fine structure mapping of the genes for multifunctional enzymes frequently reveals that discrete non-overlapping regions of the genetic map are associated with each of the catalytic activities. This could be consistent with the general observation that structural domains within a protein are folded from continuous segments of the polypeptide chain.

Limited proteolytic digestion of a number of multifunctional enzymes has allowed the component enzyme activities to be resolved and assigned to discrete regions of the polypeptide chain. The resolution of a multifunctional enzyme into independent "active fragments" is generally taken as good evidence that the enzyme activities reside on different structural domains. There are two reasons why such a conclusion is likely to be valid.

Firstly, it is known that regions of the polypeptide chain that link different structural domains are often particularly susceptible to limited proteolysis under native conditions; cleavage in these vulnerable regions may thus yield fragments that correspond to the structural domains of the native enzyme (e.g. Ghelis et al, 1978). Secondly, it is implicit in the definition of a domain that any two internal subfragments will lack the structural integrity necessary to retain a native folded conformation and catalytic activity.

In several cases active polypeptide fragments isolated after limited proteolytic digestion of multifunctional enzymes have turned out to be stable globular proteins. Sometimes they display a capacity to recover their active folded conformation after treatment with denaturants (e.g. Cohen and Dautry-Varsat, 1980). There is no doubt that such fragments amply satisfy the criteria for designation as component structural domains of the original multifunctional enzyme.

In the absence of x-ray diffraction data limited proteolysis appears to be the most promising technique for demonstrating the existence of structural domains corresponding to the component activities of a multifunctional enzyme.

2.3 Catalytic properties of multifunctional enzymes

Discussion of the catalytic properties of multifunctional enzymes has often been dominated by flights of speculation. This section summarises some of the more useful concepts; concrete examples are given in section 2.5.

By definition, a multifunctional enzyme is able to catalyse a number of different reactions. Frequently these are consecutive reactions in a biosynthetic pathway, as with the arom multifunctional enzyme. In such cases one may ask whether or not the component activities interact with each other in a way that would be impossible for structurally independent enzymes. In enzymes such as the multifunctional fatty acyl synthases catalytic coupling between the component activities is clearly inevitable, as the metabolic intermediates are covalently bound and cannot be released from the enzyme surface until the reaction sequence has been completed. However, in the majority of multifunctional enzymes the common intermediates are not covalently bound. In general the component activities can be assayed independently, as is the case with the arom multifunctional enzyme, and the products of the individual reactions readily dissociate from the enzyme surface. One might therefore expect the component enzymes to act independently, the rate of each reaction being set simply by the concentrations of substrates, products and effectors present in the solvent. In this case the behaviour of the multifunctional system would be indistinguishable from that of a mixture of independent monofunctional

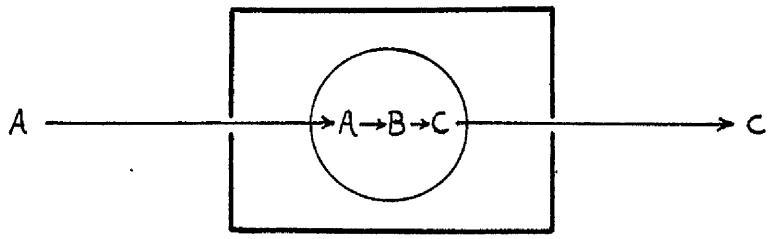
enzymes (McClure, 1969). There is no theoretical basis for the common assumption that the occurence within a single polypeptide chain of two consecutive enzyme activities from a metabolic pathway nesessarily leads to catalytic interactions between the two enzymes. There are indeed a number of natural and artifically created multifunctional enzymes in which the component activities appear to act independently (e.g. Bisswanger et al, 1979).

A number of authors have proposed that a principal characteristic of many multifunctional enzymes is their ability to "channel" the products of a given enzymatic reaction into consecutivereactions of the same metabolic pathway (Giles et al, 1967a; Stark, 1977; Schminke-Ott and Bisswanger, 1980). This could happen in a number of different ways (see figure 2.1).

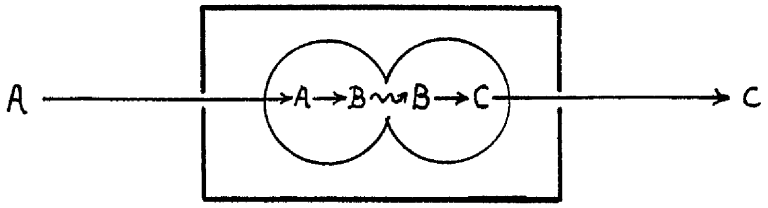
If two consecutive reactions are catalysed at a single substrate binding site the enzyme-bound product of the first reaction is immediately available for utilisation in the second reaction (Heyde, 1979). Very few enzymes, if any, appear to fit this model, which requires that a single substrate binding site should stabilise the transition states for two different reactions (Jencks, 1975).

Where there are two spatially distinct catalytic sites coupling is possible if the common intermediate can migrate between them. Migration of the common intermediate could be facilitated by a close juxtaposition of the binding sites in a "composite active site" as suggested for tryptophan synthase (DeMoss, 1962; Creighton, 1970), or by a general tendency of the intermediate to diffuse over the surface of the enzyme before it goes into free solution, as proposed by Davis (1967). The "local concentration" of the reaction product in the "free" solvent in the vicinity of one active site could conceivably influence the rate of reaction in a nearby site, although the magnitude of "local

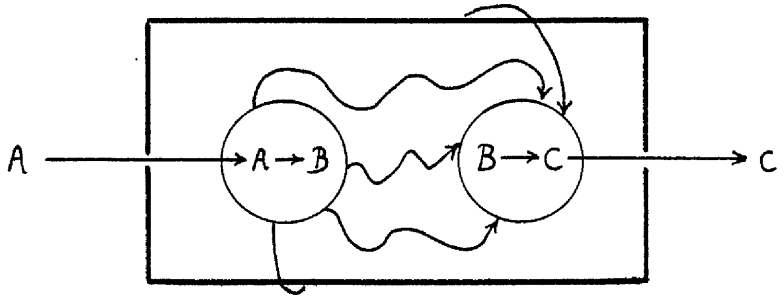
Figure 2.1 Models for catalytic facilitation in a bifunctional enzyme



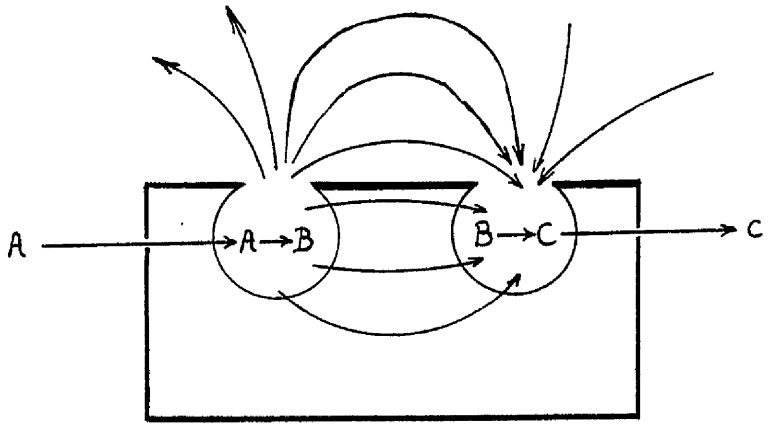
single site model
Heyde (1979)



composite active site
De Moss (1962)
Creigton (1970)



surface diffusion model Davis (1965)



free diffusion model

concentration" effects in "free" aqueous solution is expected to be very slight because of the rapidity of diffusion (Atkins, 1978).

All of these possible mechanisms for facilitated transfer of intermediates between catalytic sites lead to a situation in which the reaction velocity of an enzyme is controlled, in part, by the turnover rate of other enzymes in the sequence, and is not simply a function of the concentrations of intermediates in the bulk solvent. For this reason it is frequently proposed that interactions between the catalytic sites of a multifunctional enzyme allow the metabolic intermediates to be "channelled" and that "channelling" is of some adaptive significance in cellular metabolism. Welch (1977), and others, have speculated that interactions of the same type are prevalent in most functioning cellular pathways, and that the concept of a bulk solvent concentration of a metabolic intermediate is of generally limited value. Welch (1977) has even suggested that the very existence of multifunctional enzymes implies that higher levels of structural organisation within the cell serve to facilitate catalysis in metabolic pathways.

The problems surrounding the structure and action of multifunctional enzymes are sometimes confused with the wider question of the validity of the reductionist view of the cell as a "bag of enzymes" that are not organised into higher order structures (Welch, 1977). This has tended to obscure the fact that there is at present little evidence that "catalytic facilitation" or "substrate channelling" is a general property of multifunctional enzymes. There is nevertheless an interesting parallel between speculations about the spatial organisation of metabolic pathways and their associated flows of intermediates, and proposals for "localised coupling" between electron transport and ATP synthesis in mitochondria and chloroplasts (Kell, 1979; Williams, 1978). Both proposals amount to a claim that the products of

enzyme-catalysed reactions do not diffuse freely within membrane-bound compartments, but are "channelled" from one active site to the next.

2.4 Catalytic properties of the arom multifunctional enzyme

The arom enzyme complex is often cited as an example of a multifunctional enzyme with an established function in the integration of shikimate pathway metabolism in N.crassa (e.g. Stark, 1977; Gaertner, 1978a; Schminke-Ott and Bisswanger, 1980). There have been a number of reports of exotic kinetic properties of the complex. It has been claimed that the five activities are "coordinately activated" by the substrate for E1, DAHP, that the overall reaction reaches a steady state faster than is consistent with the "independent sites" model, and that the overall reaction is faster than some of the component steps (Gaertner et al, 1970; Welch and Gaertner, 1975, 1976). It has also been acknowledged that the experiments should be repeated, using homogeneous and proteolytically intact arom complex (Gaertner, 1978b). Preliminary experiments carried out in our own laboratory have failed to demonstrate any catalytic interactions between the five enzymes of the complex (G.A.Nimmo, J.M.Lambert, M.R.Boocock, unpublished work). It may thus be premature to draw any conclusions about the catalytic integration of shikimate pathway functions in N.crassa, despite the extraordinary level of structural organisation of the enzymes (see figure 1.4).

2.5 Examples of multifunctional enzymes in the shikimate pathway

2.5.1 Tryptophan synthase of N.crassa

Tryptophan synthase of N.crassa is a multifunctional enzyme with two identical subunits of 76kDa. It catalyses the final two steps in the tryptophan pathway (Matchett and DeMoss, 1975). In E.coli and many

other organisms the same reactions are catalysed by two different subunits of a multienzyme complex (see figures 1.3 and 1.4; Yanofsky and Crawford, 1972).

Fine structure genetic mapping in N.crassa has indicated that there are two distinct regions of the tryptophan synthase polypeptide that are each mainly concerned with a single enzyme activity. A third, central, region of the polypeptide appears to be essential for both activities. These observations lend support to the proposal that the Neurospora tryptophan synthase arose through fusion of two independent genes, and that the two component activities retain some structural individuality in the multifunctional enzyme (Bonner et al, 1965).

Although the two component activities can be assayed independently, the overall tryptophan synthase reaction is 50-fold faster than the saturated velocity of the first component. The kinetic behaviour of the enzyme has been rationalised in terms of facile "channelling" of indole from the active site of the first component enzyme to a spatially distinct indole binding-site in the second catalytic centre. Indole synthesised by the first component enzyme appears to be 200 times more likely to "channel" to the second catalyic site than to dissociate from the enzyme (Matchett, 1973). The impressive extent of active site coupling in the N.crassa tryptophan synthase is, however, not a unique attribute of the multifunctional enzyme; the tryptophan synthase multienzyme complex of E.coli has very similar catalytic properties (Creighton, 1970).

2.5.2 Indole glycerol phosphate synthase/phosphoribosyl anthranilate isomerase of E.coli

In E.coli the third and fourth steps of the tryptophan pathway are catalysed by the bifunctional enzyme indole glycerol phosphate

synthase/phosphoribosyl anthranilate isomerase (InGPS/PRAI) (Creighton and Yanofsky, 1966). These two enzyme activities are carried by independent polypeptides in many organisms (steps 9 and 10 in figures 1.3 and 1.4).

Distinct regions of the trpC gene of E.coli are associated with the InGPS and PRAI activities (Smith, 1967). The bifunctional enzyme is refractory to limited proteolysis under native conditions, but large fragments derived from opposite ends of the polypeptide by proteolysis under denaturing conditions have been reconstituted and assigned to the two component activities (Kirschner et al, 1980). The region of the E.coli enzyme associated with PRAI activity is highly homologous to the corresponding monofunctional PRAI from yeast (Christie and Platt, 1980). These results indicate that the bifunctional enzyme consists of independent folded regions or "domains" that correspond to the two component activities, and strongly suggest that it arose through fusion of adjacent genes in the trp operon.

PRAI/InGPS is a particularly good example of a multifunctional enzyme with non-interacting catalytic sites. A close scrutiny of the enzyme has revealed no sign of cooperation or communication between the two component activities, nor of "channelling" of their common intermediate, CDRP (Creighton, 1970; Bisswanger et al (1979); Cohn et al (1979)).

2.5.3 Chorismate mutase/prephenate dehydrogenase of A.aerogenes

In A.aerogenes and E.coli the first two steps of the tyrosine pathway are catalysed by the bifunctional enzyme chorismate mutase/prephenate dehydrogenase (Koch et al, 1971). A rather similar bifunctional enzyme, chorismate mutase/prephenate dehydratase, catalyses the first two steps of the phenylalanine pathway in these organisms (see figure 1.3).

The two component activities of chorismate mutase/prephenate dehydrogenase can be assayed independently, but there is evidence that prephenate synthesised by chorismate mutase can be utilised in the prephenate dehydrogenase reaction before it equilibrates with prephenate in free solution (Koch et al, 1972). The kinetic properties of the enzyme are consistent with the possibility that only a single binding-site for prephenate is required for both component activities. It has therefore been suggested that the chorismate mutase and prephenate dehydrogenase reactions take place at a single catalytic site (Heyde and Morrison, 1978; Heyde, 1979). Chorismate mutase/prephenate dehydrogenase is thus a possible counterexample to the proposal that the component activities of all multifunctional enzymes correspond to structurally independent polypeptide domains (Kirschner and Bisswanger, 1976).

Chorismate mutase catalysis presumably depends on stabilisation of the transition state of the electrocyclic reaction; it is possible to draw a plausible scheme for catalysis of the prephenate dehydrogenase reaction at the same substrate binding-site (Andrews and Haddon, 1979; Andrews and Heyde, 1979). Conclusive evidence of the existence of a truly bifunctional active site of this type is not available for any enzyme. The unconventional chemistry of the chorismate mutase reaction would make it relatively easy to rationalise the existence of a bifunctional active site in chorismate mutase/prephenate dehydrogenase.

The limited extent of "coupling" of the two enzyme reactions in chorismate mutase/prephenate dehydrogenase suggests that "channelling" of prephenate may not be an important function of the enzyme in vivo (Heyde, 1979).

2.6 Evolution of multifunctional enzymes

The evolution of a complex structure such as a multifunctional enzyme can only be understood if one has a clear picture of its function and adaptive significance. This presents a problem, since appropriate functional criteria for assessing the selective value of multifunctional enzymes have not yet been identified.

Many ideas have been put forward to explain why an organism with multifunctional enzymes may have a selective advantage over an organism with equivalent structurally independent enzymes (Stark, 1977; Kirschner and Bisswanger, 1976; Giles et al, 1967a). It is possible that unique catalytic and regulatory properties of multifunctional enzymes are important in cellular metabolism. Multifunctional enzymes may simply be more efficient catalysts, and their ability to "channel" intermediates may allow compartmentation of competing pathways. Alternatively the advantages of multifunctional enzymes could lie in their economical demands on the machinery of gene expression; the component activities of a multifunctional enzyme are coordinately expressed in fixed proportions, and there is no need for recognition and assembly of unlike subunits as in a multienzyme complex. A multifunctional enzyme could conceivably require a shorter polypeptide than equivalent monofunctional enzymes.

One final possibility should be borne in mind; some multifunctional enzymes may have no particular functional advantage and may be retained only because their genes are difficult to eliminate once they have become fixed in the population.

2.6.1 Evolutionary precursors of multifunctional enzymes

It is generally assumed that multifunctional enzymes have evolved from monofunctional enzymes. Three plausible mechanisms for such a process can be listed:

1. adaptation of an existing monofunctional enzyme to catalyse a second reaction (Llewellyn et al, 1980).
2. internal duplication within the gene for a monofunctional enzyme and adaptation of one active site for catalysis of a second reaction (Schminke-Ott and Bisswanger, 1980).
3. fusion of two existing genes (Bonner et al, 1965)

Processes of the second and third types would be expected to yield a multifunctional enzyme with discrete structural domains that correspond to the component enzyme activities.

It has been proposed that the bifunctional enzyme chorismate mutase/DAHP synthase of B.subtilis arose through adaptation of a regulatory prephenate binding-site in an existing DAHP synthase (Llewellyn et al, 1980). This isolated example of the recent "evolution" of a multifunctional enzyme from a monofunctional enzyme is unlikely to be typical, because the nature of the chorismate mutase reaction is such that a catalytic site need be no more than a binding site for a particular conformation of the substrate (Andrews and Haddon, 1979).

If a multifunctional enzyme is to arise through gene fusion it is necessary for two existing genes to be brought together in the genome, and for the fusion event to allow the production of a single mRNA transcript and a single hybrid polypeptide. This could happen relatively easily within a bacterial operon; deletion of nonsense codons and "read-through" of protein synthesis from a polycistronic message would yield a chimaeric protein. There is very suggestive evidence that such a process has occurred within the trp and thr operons of E.coli (Miozzari and Yanofsky, 1979; Katinka et al, 1980).

Plausible intermediates in eukaryotic gene fusion events are less readily identified. Genes of related function are generally not

clustered on the chromosomes of eukaryotes, and there are no known examples of structurally independent enzymes that share a polycistronic message. Gene duplication has therefore been put forward as an attractive mechanism for the evolution of multifunctional enzymes. The duplication and subsequent adaptation of an existing catalytic site could accommodate the requirement that sequential enzyme activities from a metabolic pathway that are carried by a multifunctional enzyme must recognise structurally similar intermediates (e.g. Schminke-Ott and Bisswanger, 1980). There are strong indications that internal gene duplications have played an important part in the evolution of multifunctional proteins such as the immunoglobulins, and of the tandemly repeated segments of enzymes such as the transacetylase component of the pyruvate dehydrogenase complex (Guest et al, 1983).

2.6.2 Multifunctional enzymes and evolutionary adaptation

In the "gene fusion" model for the evolution of multifunctional enzymes the second component function evolves independently before the fusion event; in the "gene duplication" model it arises only after the duplication event, as the two homologous regions adapt for separate functions. This difference raises the vexed question of whether or not it is necessary that each step in the evolution of a complex structure like a multifunctional enzyme need be associated with a selective advantage of the new structure over its evolutionary precursor (Gould and Lewontin, 1979). It has been suggested that the difficulty in attaining an immediate functional advantage through fusion of two existing genes may bar this route for the evolution of multifunctional enzymes (Schminke-Ott and Bisswanger, 1980). On the other hand it is possible that many "neutral" and slightly deleterious mutations do achieve fixation, and that many evolutionary innovations, including multifunctional enzymes, only later prove to be adaptive (Gould, 1980).

The problems associated with the evolution of multifunctional enzymes will only be resolved when the structure and function of these enzymes is better understood. A number of features of multifunctional enzymes may then betray their evolutionary origins. Strong homologies between different component activities of a multifunctional enzyme may indicate that gene duplication has taken place, while strong homologies between the component activities and the corresponding monofunctional enzymes from other organisms would imply that gene fusion events have occurred. Homologies may be noticeable in the sequence and structure of the genes, and in the tertiary structure and catalytic function of the enzymes.

2.6.3 Evolution of the arom multifunctional enzyme

A longterm objective of work in our laboratory, of which this study forms a part, is to make a comparison of the shikimate pathway genes and enzymes of N.crassa, E.coli and pea seedlings. Since the structural organisation of the enzymes is so different in these three organisms (see figure 1.4) we hope that the comparison will yield some insights into the evolution of multifunctional enzymes, and the evolution of the shikimate pathway as an integrated unit of function.

The interplay of structure, function and evolutionary adaptation in the arom multifunctional enzyme is of particular interest. It is perhaps worthwhile to list a number of interesting hypotheses that are open to experimental investigation:

1. the arom multifunctional enzyme is made up of structural "domains"
2. the structural domains correspond to particular enzyme activities

3. the structural domains are derived from the domains of one or more monofunctional enzymes

4. the precise disposition of the domains in the multifunctional enzyme is essential for certain catalytic properties of the complex.

5. some of these catalytic properties are important for normal functioning of the shikimate pathway *in vivo*.

These ideas will be taken up in chapter 7.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1 Chemicals and Biochemicals

Bis-tris, bis-tris-propane, monocyclohexylammonium ADP, DCPIP and FMN were obtained from Sigma (London) Chemical Co. Poole, Dorset. PEP, pyridine nucleotides ATP and ADP, DTT, PMSF, triethanolamine • HCl and tris base were from Boehringer Corp. (London), Lewes, Sussex. Benzamidine • HCl and shikimic acid were from Aldrich Chemical Co., Gillingham, Dorset. Zinc acetate, βME, disodium EDTA, glutamine, anthranilic acid, sodium arsenate, NBT and PMS, all reagents for gel electrophoresis and many inorganic salts were obtained from BDH Chemicals, Poole, Dorset. Analytical grade glyphosate was a kind gift from Dr.S.Ridley of ICI Plant Protection Division, Jeallot's Hill, Berkshire, as were many structural analogues of glyphosate; other analogues were obtained from Calbiochem Corp., Los Angeles, U.S.A. Triton X-100 (scintillation grade) was from Packard Instruments Co.Inc., Warrenville, Illinois, U.S.A.

All other chemicals were of analytical reagent grade or of the highest available purity.

3.1.2 Chromatographic Media

"Dowex" resins were from B.D.H., Poole, Dorset, England. Sephadex gel filtration media and DEAE-Sephacel were from Pharmacia (GB) Ltd., London W5. Phosphocellulose and DEAE-cellulose (DE52) were from Whatman Biochemicals, Maidstone, Kent. Trypsin inhibitor immobilised on agarose was from Miles-Yeda Ltd., Slough, Berkshire. Blue Dextran Sepharose was prepared as described by Ryan and Vestling (1974) and was a gift from Dr.P.A.Lowe.

3.1.3 Enzymes and Proteins

The following commercial enzyme preparations were obtained from the Boehringer Corporation (London, Lewes, Sussex, England:

aldolase (EC 4.1.2.13) from rabbit muscle

carbonic anhydrase (EC 4.2.1.1) from bovine erythrocytes

catalase (EC 1.11.1.6) from beef liver

β-galactosid ase (EC 3.2.1.23) from E.coli

glucose 6-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle

hexokinase (EC 2.7.1.1.) from yeast (HK)

lactate dehydrogenase (EC 1.1.1.27) from pig heart (LDH)

malate dehydrogenase (EC 1.1.1.37) from pig heart mitochondria (MDH)

phosphoglycerate kinase (EC 2.7.2.3) from yeast (PGK)

pyruvate kinase (EC 2.7.1.40) from rabbit muscle (PK)

The following enzymes were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.:

α-chymotrypsin (EC 3.4.4.5) from bovine pancreas

TPCK-treated trypsin (EC 3.4.21.4) from bovine pancreas

The following enzymes and proteins were obtained from the Sigma Chemical Company (London), Fancy Road, Poole, Dorset, England:

bovine serum albumin

cytochrome C from horse heart

trypsin inhibitor from soybean

Trypsin/chymotrypsin inhibitor from lima bean was a gift from Dr.J.Kay, Department of Biochemistry, University College Cardiff. Pyruvate dehydrogenase complex from E.coli was a gift from Dr.J.R.Coggins. Glutamine synthetase from E.coli was a gift from Dr.C.Oliver, NIH, Bethesda, Maryland, U.S.A.

Dehydroquinase was partially purified from E.coli to a specific activity of approximately 0.5U/mg (J.M.Lambert, M.R.Boocock & J.R.Coggins, in preparation) and was a gift from A.A.Coia.

3.1.4 N.Crassa

N.crassa strain 74-OR23-1A (F.G.S.C. No.987, obtained from the Fungal Genetics Stock Centre, Humboldt State University Foundation, Arcata, CA 95521, U.S.A.) was grown, harvested and stored at -20°C as freeze-dried powdered cells, as described by Lumsden and Coggins (1977). The cells were grown by B.A.Brodie, A.A.Coia and J.Greene.

3.2 General Methods

pH measurements were made with a Radiometer pH probe, calibrated at room temperature.

Conductivity measurements were made with a Radiometer conductivity meter type CDM2e (Radiometer, Copenhagen, Denmark).

Protein estimations were by the method of Bradford (1976) and used bovine serum albumin as a standard. The protein concentration of highly purified arom preparations was calculated from E_{280} measurements, taking $E^{1cm}_{1\%} = 11.0$ (Lumsden & Coggins, 1978).

3.3. Polyacrylamide Gel Electrophoresis

Electrophoresis of proteins in polyacrylamide gels was carried out in the discontinuous buffering systems of Davis (1964) and Laemmli (1970). Gels were polymerised from solutions consisting of 375mM tris/HCl pH 8.8 and the stated concentration of acrylamide (at an acrylamide:bis-acrylamide ratio of 30:0.8), 0.03% v/v, N,N,N'N', tetramethylene diamine and 0.05% w/v ammonium persulphate, supplemented with 0.1% SDS ("Laemmli" gels) or 10μM zinc acetate (nondenaturing gels). Laemmli gels were poured with a stacking gel in a slab format (20cm x 15cm x 1.2mm: Raven Scientific Ltd., Haverhill, Suffolk) while nondenaturing gels were poured in siliconised tubes. Samples for SDS PAGE were supplemented with 1% SDS, 1% β ME, bromophenol blue and glycerol and immediately raised to 100°C for 2 minutes in the presence of 1% SDS and 1% β ME. Samples for nondenaturing PAGE were treated with 1mM DTT at 0°C. SDS PAGE was carried out at ambient temperatures while

nondenaturing gels were run in a cold room at 4°C. The well buffer stock solution is 30g/l tris base, 144g/l glycine, diluted 10-fold and supplemented with 0.1% SDS (SDS PAGE) or diluted 25-fold and supplemented with 0.4mM DTT and 10μM zinc acetate (nondenaturing PAGE).

Gels were stained for protein with 0.1% coomassie brilliant blue G250 in 10% v/v glacial acetic acid, 50% v/v methanol, and destained at room temperature in 10% acetic acid, 10% methanol. For certain experiments the silver staining method of Wray et al (1981) was employed.

Non-denaturing gels were stained for arom E1 and arom E5 activity by the calcium phosphate precipitation method of Nimmo and Nimmo (1981). Gels were stained for arom E3 activity by the triazolium dye-linked method, as adapted by Lumsden and Coggins (1977). The staining mixture consisted of 0.5mM $NADP^+$, 1mM shikimic acid and 0.75mg/ml nitro-blue tetrazolium, in 200mM tris/HCl pH 8.8. Nondenaturing gels were first soaked in 200mM tris/HCl for 20 min to remove excess DTT. SDS polyacrylamide gels to be stained for arom E3 activity were run as above except that gel solutions contained 1mM EDTA and the well buffer contained 0.1mM EDTA and 0.01%βME. The gels were prepared for activity staining by a modification of a method developed by I.A.Anton:

1. soak the gel with gentle agitation at room temperature in 50mM sodium phosphate pH 7.0, 0.1% Triton X-100, 0.4mM DTT, 0.1mM EDTA: use a total of 1 l in several changes over 7-12 hours.
2. continue for approximately 12 hours using a further 1 l of the same buffer without Triton X-100
3. soak in 200mM tris/HCl pH 8.8 (30 min)

Nondenaturing gels were stained for "diaphorase" activity in a cocktail consisting of 0.25mM NADPH, 0.01mM FMN, 60μM DCPIP and 1mM MTT in 100mM tris/HCl pH 8.8.

The molecular weight markers in most SDS PAGE experiments were E.coli pyruvate dehydrogenase (100kDa, 84kDa, 56.5kDa), carbonic anhydrase (29kDa), the arom enzyme complex (165kDa), and its two primary proteolytic fragments (110kDa and 68kDa); see Smith (1980).

3.4 Preparation and Standardisation of Enzyme Substrates

DAHP was isolated as the lithium salt from culture supernatants of E.coli strain AB 2847a and was a gift from J.M.Lambert; stock solutions were standardised by conversion to shikimate using the first three activities of the arom complex (Lambert, Boocock & Coggins; in preparation). Ammonium dehydroquinate, prepared chemically from quinic acid (Grewe & Haendler, 1968), was a gift from S.Chaudhuri, and was standardised by conversion to shikimate. Dehydroshikimic acid, prepared chemically from dehydroquinate was also a gift from S.Chaudhuri; the stock solution was standardised by absorbance measurements at 234nm (ε=12,000). Shikimic acid was obtained from Aldrich Chemical Co., Gillingham, Dorset and was standardised by conversion to DHS using arom E3. Shikimate 3-phosphate was isolated as the ammonium salt from culture supernatants of an A.aerogenes aromatic auxotroph and was a gift from G.A.Nimmo. It was standardised by conversion to chorismate, taking ε_{275nm}= 2,630 (Gibson, 1970). Phosphoenolpyruvate (potassium salt) was obtained from Sigma Chemical Co., Poole, Dorset, and was standardised by conversion to lactate. EPSP was prepared enzymatically from PEP and shikimate 3-phosphate using the fifth activity of the arom enzyme complex, and was isolated as the barium salt essentially as described by Knowles et al (1970). The barium salt was added to 5mM K_2SO_4 to give a 1mM stock solution of the potassium form of EPSP, which was standardised by enzymatic conversion to lactate. Potassium phosphate for use as a substrate for the reverse reaction of EPSP synthase was prepared from anhydrous KH_2PO_4 (BDH "Analar" reagent) as a 200mM solution and

neutralised with KOH. Chorismic acid was isolated from culture supernatants of a multiple aromatic auxotroph (Gibson, 1970) and was a gift from J.R.Coggins.

3.5 Enzyme Assays

Continuous spectrophotometric and fluorimetric enzyme assays were conducted at 25°C in masked semi-micro quartz cuvettes (1cm path, 1ml) and a set of matched quartz fluorimeter cells (1cm x 1cm, 3ml). The instruments used throughout were Gilford-Unicam model 252 spectrophotometers with slave recorder attachments and a Hitachi/Perkin Elmer model MPF-2A spectrofluorimeter. One unit of activity is defined as the amount of enzyme required to catalyse the conversin of 1μmole of substrate/minute under the defined conditions.

Many of the enzyme assays were conducted in one of the following buffers, which were prepared as two-fold concentrated stock solutions.

100mM KP_i pH 7.0	100mM KH_2PO_4/KOH pH 7.0
buffer A	50mM KCl, 2.5mM $MgCl_2$, 50mM bistris/HCl pH 7.0
buffer B	50mM KCl, 2.5mM $MgCl_2$, 50mM triethanolamine.HCl/KOH pH 7.0
buffer C	50mM bistris propane/HCl pH 7.0

DAHP synthase was assayed spectrophotometrically at 232nm in 50mM bistrispropane/HCl pH 7.0 as described by Nimmo and Coggins (1981a).

DHQ synthase (arom E1) was assayed spectrophotometrically at 234nm by coupling DHQ release to the dehydoquinase reaction. Assays contained 400μM DAHP, 50μM NAD^+ and 10mU/ml E.coli dehydroquinase in 100mM KP_i pH 7.0.

Dehydroquinase (arom E2) was assayed spectrophotometrically at 234nm, taking ε=12,000 for DHQ (Mitsuhashi & Davis, 1954). Assays

contained 100μM DHQ in 100mM KP_i pH 7.0.

Shikimate dehydrogenase (dehydroshikimate reductase, arom E3) was routinely assayed in the reverse of the biosynthetic reaction at pH 10.6 (Lumsden & Coggins, 1977). Assays contained 4mM shikimic acid and 2mM $NADP^+$ in 100mM sodium carbonate/bicarbonate pH 10.6. The enzyme was also assayed at pH 7.0 in 100mM KP_i; assays contained 150μM DHS and 170μM NADPH (forward reaction) or 4mM shikimic acid and 2mM $NADP^+$ (reverse reaction).

Shikimate kinase (arom E4) was assayed spectrophotometrically at 340nm by coupling the release of ADP to the pyruvate kinase and lactate dehydrogenase reactions (G.A.Nimmo, unpublished work). Assays contained 1mM shikimic acid, 2.5mM ATP (neutralised), 1mM PEP (neutralised), 100μM NADH, pyruvate kinase (3U/ml) and lactate dehydrogenase (2.5U/ml) in buffer B, pH 7.0.

EPSP synthase (arom E5) was assayed by several methods. For routine purposes and for the determination of activity ratios the reverse reaction was assayed spectrophotometrically at 340nm by coupling the release of PEP to the pyruvate kinase and lactate dehydrogenase reactions. Assays contained 50μM EPSP, 2.5mM ADP/KOH, 2.5mM $MgCl_2$, 100μM NADH, pyruvate kinase (3U/ml) and lactate dehydrogenase (2.5U/ml) in 100mM KP_i pH 7.0. The forward reaction was routinely assayed spectrophotometrically at 275nm by coupling the release of EPSP to the chorismate synthase reaction, taking ε=2,630 for chorismate (Gibson, 1970). For the activity ratio experiment summarised in Table 4.13 the assays contained 300μM shikimate 3-phosphate, 300μM PEP, 10μM NADPH, 10μM FMN and 12mU/ml chorismate synthase in buffer B, pH 7.0.

Different assay systems were employed in the kinetic studies (see Section 3.6).

Chorismate synthase was assayed spectrophotometrically at 275nm, taking ε=2,630 for chorismate (Gibson, 1970). Assays contained 50μM

EPSP, 20μM NADPH and 10μM FMN in buffer B, pH 7.0. Chorismate synthase is somewhat less active in phosphate buffers and in the bistris/KCl-based buffer A used in kinetics experiments, and behaves very poorly in bistrispropane-based buffers e.g. "buffer C". Anoxic chorismate synthase assays were conducted in buffer B as described above, except that NADPH was omitted and 5mg of solid sodium dithionite were gently stirred into 1ml of assay cocktail in a semi-micro cell. Oxygen is efficiently removed by autoxidation of reduced (bleached) FMN.

Diaphorase activities of chorismate synthase were assayed spectrophotometrically by following the reduction of the artificial electron acceptors DCPIP and cytochrome c. All assays were conducted in buffer B, pH 7.00; the concentrations of other components are listed in Chapter 5. DCPIP reductase assays were conducted at 600nm taking ε=21,000 for oxidised DCPIP (cf. Welch et al, 1974); blank rates of nonenzymatic DCPIP reduction were subtracted. Cytochrome c reductase assays were conducted at the extinction maximum of the α band of the reduced cytochrome (approximately 548nm) using the narrowest monochromator bandwidth available and assuming $\Delta\varepsilon$ =20,000 (e.g. Metzler, 1977).

Anthranilate synthase was assayed fluorimetrically using excitation/emission wavelengths of 315nm/400nm (De Moss, 1974). The assays contained 100μM chorismic acid, 10mM glutamine and 2.5mM $MgCl_2$ in 100mM KP_i pH 7.0. Anthranilate synthase from N.crassa has comparable activity in buffer A, but cannot be used as a coupling enzyme in buffer B.

Determination of activity ratios In the experiments described in Chapters 4 and 7 the following standard protocol was followed for the determination of the ratios of the five activities of the arom multifunctional enzyme:

1. Prepare stock assay cocktails for the forward reactions of E1, E2 and E4 and the reverse reactions of E3 and E5 by mixing stock solutions of the components listed above; use 0.2M KP_i pH 7.0, 2-fold buffer B concentrate and 0.5M sodium carbonate/bicarbonate pH 10.6, and make no further adjustment to the pH. Omit NADH and coupling enzymes and for work with crude extracts omit shikimate and EPSP. Store cocktails at -20°C.

2. Warm completed assay cocktails to 25°C; dispense into cuvettes as required (and add dehydroquinase for E1 assays).

3. If necessary dilute enzyme stock solution with 100mM KP_i pH 7.0; keep on ice.

4. With highly purified arom preparations initiate the assays by adding enzyme. In purification experiments check for blank rates and initiate the assays with the appropriate pathway intermediate. Always use exactly the same volumes of enzyme solution dispensed from a glass syringe for all five assays.

5. The assays are designed to show good linearity over an absorbance full scale of 0.1A; do not exceed rates of 0.04A/min. Avoid excessive absorbance at 234nm in E1 and E2 assays and use standardised monochromator settings on the same instrument for any individual experiment.

6. If two or more samples are to be compared obtain at least two assay traces for each enzyme activity.

7. Using a transparent ruler, draw lines parallel to all assay traces. Then calculate activity ratios, taking $\Delta\varepsilon$ =12,000 for dehydroquinase-linked assays and $\Delta\varepsilon$=6,200 for dehydrogenase-linked assays.

3.6 Steady-state Kinetic Studies on EPSP Synthase

For the steady state kinetic experiments described in Chapter 6 the continuous assay systems listed below were employed. The assays were conducted at 25°C in buffer A pH 7.0, except in two experiments

with EPSP as a product inhibitor of the forward reaction, where the u.v.-transparent buffer C, pH 7.0 was used instead. Instrumentation was as described above; for most spectrophotometric experiments a chart recorder full scale of 0.05A and fixed chartspeed of 2cm/min were employed. The common components of the assay were combined and maintained at 25°C; for selected experiments the cocktails were passed through a millipore filter. Coupling enzymes were either dialysed against the assay buffer (and stored at -20°C) or added as concentrated solutions in tris/HCl-buffered 50% glycerol (chorismate synthase and anthranilate synthase; the final glycerol concentration in the fluorimetric assays of the forward reaction was approximately 0.5% v/v). Substrate and inhibitor stock solutions were neutralised with KOH or HCl and standardised as in Section 3.4. The arom enzyme complex was diluted into buffer A containing 1mg/ml BSA and 1mM DTT and kept on ice to ensure stability during the experiments. Assays were generally initiated by adding enzyme; significant "blank" rates were not seen when the assays were initiated with substrates. Enzymes and substrates were dispensed from glass syringes; for assays at the lowest substrate concentrations an integral multiple of standard aliquots of arom was added to keep the measured initial velocities of reaction within a narrow range. Chart-recorder and monochromator settings were not altered during the course of individual experiments.

A transparent ruler was used to draw initial velocity tangents to the reaction traces. The linearity of the assay traces was very good except at the lowest of the substrate concentrations used in certain experiments. Particular care was taken to check the linearity of the response of the two fluorimetric coupled assay systems, as discussed by McClure (1969).

All calculated data points were plotted individually on the double reciprocal primary plots illustrated in Chapter 6. Lines were fitted by eye, and secondary replots were constructed as appropriate from

the resulting data. Analytical line-fitting procedures were avoided as these generally make unwarranted assumptions about the error distribution in kinetic experiments (e.g. Cornish-Bowden & Eisenthal, 1973).

1. Fluorimetric assay of forward reaction EPSP release is coupled to the chorismate synthase and anthranilate synthase reactions (Gaertner & De Moss, 1970) and anthranilate fluorescence is monitored at excitation/emission wavelengths of 315/380nm with a chart recorder full scale deflection routinely equivalent to 250nM anthranilate. Assays contained 10μM FMN, 50μM NADPH, 10mM glutamine, 8mU/ml chorismate synthase, 5mU/ml anthranilate synthase, substrates and inhibitors in buffer A and were normally initiated by the addition of PEP.

2. Coupled spectrophotometric assay of forward reaction Assays contained 10μM FMN, 20μM NADPH, 10mU/ml chorismate synthase, substrates and inhibitors in buffer A. Chorismate production was followed at 275nm.

3. Direct spectrophotometric assay of forward reaction Assays contained substrates and inhibitors in buffer C. The progress of the reations was followed by monitoring the change in absorbance at 240nm (Grimshaw et al, 1982). For the two product inhibition experiments a very bright stabilised u.v. light source was used in conjunction with fixed monochromator settings and a chart full-scale deflection of 0.04A (at 1cm/min), and an electronic "backoff" facility (up to 0.8A) was essential.

4. Fluorimetric assay of reverse reaction The release of PEP was coupled to the pyruvate kinase, hexokinase and glucose 6-phosphate dehydrogenase reactions. NADPH fluoresence was monitored using excitation/emission wavelength of 340/460nm and a chart full-scale deflection equivalent to 270nM NADPH. The assay cocktail was filtered and degassed before use and contained 2mM glucose, and 40μM $NADP^+$ in buffer A. It was supplemented with 50μM monocyclohexyammonium ADP,

2U/ml pyruvate kinase, 17U/ml hexokinase, 3U/ml glucose 6-phosphate dehydrogenase, and EPSP synthase substrates and inhibitors.

5. Spectrophtometric assay of the reverse action Assays contained 1mM ADP/KOH, 100μM NADH, 3U/ml pyruvate kinase, 2.5U/ml lactate dehydrogenase, and EPSP substrates and cofactors in buffer A. The release of PEP is coupled to the lactate dehydrogenase reaction, which is followed at 340nm.

6. Spectrophotometric assay of the arsenate-dependent activities of EPSP synthase Assays contained 100μM NADH and 2.5U/ml lactate dehydrogenase and the appropriate concentrations of sodium arsenate, EPSP, shikimate 3-phosphate, PEP and chorismate synthase in buffer A. The arsenate stock solution was 100mM Na_2HAsO_4 (BDH, Analar) neutralised with HCl. The release of pyruvate is coupled to the lactate dehydrogenase reaction, which is followed at 340nm.

3.7 Purification of the arom multifunctional enzyme

In Chapter 4 a number of variants of the basic purification scheme devised by Lumsden and Coggins (1977) are mentioned. The "2nd generation" protocol incorporates the modifications made by Smith (1980). The development of the third and fourth generation procedures is discussed in Chapter 4. Decribed here in detail are the procedures followed in second, third and fourth generation experiments, and in the modified third generation experiment A21.

Stock solutions and preparation of buffers

Prepare chromatography buffers by mixing the listed components: add βME, solid DTT, solid benzamidine, zinc salts and PMSF (as an ethanolic solution; final 1.2mM PMSF, 3.3% ethanol). Do not make any

further adjustment to the pH.

1M KCl, 2M KCl

1M tris/HCl pH 7.5: titrate to pH 7.5 at room temperature

100mM zinc acetate in 100mM acetic acid

200mM sodium/potassium phosphate pH 7.0 or pH 7.5

glass distilled water

Chromatography

Precycle and de-fine Whatman DE52 ion exchanger before use. Recycle and wash a 2-3ml column of Blue Dextran Sepharose with the following solutions:

2nd generation:	1.	25ml 2M KCl, 100mM tris/HCl pH 7.5
	2.	25ml 100mM tris/HCl pH 7.5
3rd generation:	1.	5ml 5M guanidine • HCl, 50mM EDTA/NaOH, 50mM tris/HCl pH 7.5
	2.	70ml 1.9M KCl, 5mM EDTA/NaOH, 100mM tris/HCl pH 7.5
	3.	50ml 30mM KCl, 50mM tris/HCl, 1.4mM βME
4th generation:	1.	20ml 6M guanidine • HCl, 0.1% Triton X-100, 1% βME, 5mM EDTA, 50mM tris/HCl pH 7.5
	2.	40ml 1.9M KCl, 1mM EDTA, 50mM tris/HCl pH 7.5
	3.	60ml 30mM KCl 50mM tris/HCl pH 7.5

Dialysis membranes

2nd generation:	Soak the membrane in 1mM EDTA at 100°C for 10 minutes
3rd & 4th generation:	Agitate the membrane in 1mM EDTA/NaOH pH 7.5 at 100°C (15 mins). Rinse in H_20, soak in 1mM zinc acetate (15 mins) and finally rinse in H_20.

Detailed procedures for the purification of the arom enzyme complex

Carry out all steps at 0-4°C.

1. Mycelial extraction Thaw 100g of powdered, freeze-dried N.crassa mycelia and stir gently into 1500ml of extraction buffer. Continue stirring for 1 hour, then centrifuge the extract at 10,000g for 30 min. Filter the supernatant through several layers of cheesecloth; discard the pelleted cell debris. The mycelial extraction buffer is 100mM tris/HCl pH 7.5, 1.2mM PMSF supplemented with:

2nd generation; 0.4mM DTT, 5mM Na_2 EDTA; titrate to pH 7.5 at room temperature with concentrated NaOH.

3rd generation; 1.4mM β-mercaptoethanol. Cool to 4°C.

4th generation; As for 2nd generation but titrate to pH 7.6, then cool to 4°C.

2. "Negative" chromatography on DEAE-cellulose Adjust the conductivity of the crude extract to 4.0mmho by adding 1M KCl to a final concentration of approximately 15mM. Pump the extract at 400ml/h through a column of DE52 (2nd generation: 15cm x $32cm^2$; 3rd & 4th generation: 10cm x $32cm^2$) pre-equilibrated with 75mM KCl,50mM tris/HCl pH 7.5,1.2mM PMSF supplemented with 2nd and 4th generation: 0.4mM DTT/3rd generation: 1.4mM βME. Continue elution of the column with the equilibration buffer; pool all fractions with significant E2 activity.

3. 37°C step

2nd generation: incubate the extract for 1-2 hours at 37°C; centrifuge for 45 min at 10,000g and discard the pelleted proteins.

3rd & 4th generation: omit this step altogether.

4. Ammonium sulphate fractionation Add benzamidine to a final concentration of 1mM. Add 242g/litre (initial volume) finely powdered ammonium sulphate, in small portions, stirring gently, over approximately 15 minutes. Stir gently for a further 20 minutes after all the ammonium

sulphate has dissolved. Centrifuge at 10,000g for 30 minutes to pellet the precipitated proteins. Add 63g/litre (initial volume) powdered ammonium sulphate, as before, stir gently for 20 minutes then centrifuge for 30 minutes at 10,000g. Carefully remove all of the supernatant fraction from the pelleted proteins, then resuspend the proteins in approximately 20ml of 100mM tris/HCl pH 7.5,1.2mM PMSF supplemented with:

2nd generation: 0.4mM DTT

3rd generation: 1.4mM β-mercaptoethanol, 10μM $ZnSO_4$

4th generation: 0.4mM DTT, 20μM zinc acetate, 1mM benzamidine

5. Dialysis I Dialyse the resuspended proteins overnight versus 2 l of 50mM tris/HCl pH7.5, 1.2mM PMSF supplemented with the protective agents listed above. 3rd & 4th generation: Centrifuge the dialysed enzyme sample briefly at 15,000g and discard the pellet of precipitated proteins.

6. Ion exchange chromatography Equilibrate the ion exchange column with 50mM tris/HCl pH 7.5,1.2mM PMSF supplemented with the protective reagents listed.

2nd generation: 12cm x $5cm^2$ column of DE52; 0.4mM DTT

3nd generation: 12cm x $5cm^2$ column of DE52; 1.4mM βME, 10μM $ZnSO_4$

4th generation: 12cm x $5cm^2$ column of DEAE Sephacel; 0.4mM DTT, 20μM zinc acetate.

Load the enzyme sample onto the column, and briefly continue pumping at 100ml/h with the equilibration buffer. Apply a step of equilibration buffer supplemented with 30mM KCl (2nd & 3rd generation) or 40mM KCl (4th generation), pumping at 200ml/h until the E_{280} of the effluent falls below 0.06. Then apply a linear gradient of total volume 1000ml consisting of equilibrium buffer supplemented with:

2nd & 3rd generation: 30mM - 300mM KCl

4th generation: 40mM - 400mM KCl,

at a flow rate of 100 - 150ml/h. Pool the fractions of highest E2 specific activity.

7. Dialysis II

2nd generation: omit this step

3rd generation: dialyse the pooled enzyme fractions for at least 5 hours versus ten volumes of 30mM KCl, 50mM tris/HCl pH 7.5, 1.2mM PMSF, 1.4mM βME without any zinc supplement.

4th generation: dialyse the pooled fractions versus twenty volumes of 30mM KCl, 50mM tris/HCl pH 7.5, 1.2mM PMSF, 0.4mM DTT, 1mM benzamidine without any zinc supplement.

8. Blue Dextran Sepharose chromatography Load the enzyme pool onto a 2-3ml column of Blue Dextran Sepharose, equilibrated with the dialysis buffer, pumping overnight at 5-10ml/h. Wash the column briefly with equilibration buffer (5ml) then apply a step of 500mM KCl 50mM tris/HCl pH 7.5 1.2mM PMSP supplemented with:

2nd generation: 0.4mM DTT

3rd & 4th generation: 1.2mM βME and no zinc salts,

at a flow rate of 10ml/h. A large amount of protein should elute from the column in a sharp peak; in 3rd and 4th generation experiments approximately 10% of the E2 activity applied to the column may also elute at this point. When the E_{280} of the effluent has dropped to approximately 0.03 elute the arom enzyme complex with a step of 1.5M KCl 50mM tris/HCl pH 7.5 1.2mM PMSF supplemented with 2nd generation: 0.4mM DTT/3rd & 4th generation: 1.4mM βME and 20μM zinc acetate or sulphate, at a flow rate of 10ml/h, collecting 6 minute fractions. In 3rd and 4th generation experiments rinse the fraction tubes with H_2O and add 20μl of 0.8mM $ZnSO_4$ 50mM tris/HCl pH 7.5 to each tube before use. Pool all fractions with an E2 specific activity equivalent to that of the peak fraction and determine the specific activity of the pool.

9. Final dialysis As soon as possible dialyse the enzyme pool against 500ml of the appropriate final dialysis buffer, precooled to 4°C.

2nd generation: 50mM sodium phosphate pH 7.5, 50% w/v glycerol, 1mM benzamide, 0.4mM DTT.

3rd & 4th generation: 50mM potassium phosphate pH 7.0, 50% w/v glycerol 1mM benzamidine, 0.4mM DTT, 40μM zinc acetate.

In experiment A7 the enzyme pool was first dialysed versus 500ml of 50mM potassium phosphate pH 7.0, 50% w/v glycerol, 1mM benzamidine, 1.4mM βME, 1.2mM PMSF with no added zinc salts. The enzyme was later dialysed against the standard 3rd generation final dialysis buffer (see Chapter 4).

10. Storage Divide the final pool into several parts and store at -20°C in individual stoppered 2ml glass vials. With uninterrupted storage under these conditions the five activities of 3rd generation arom enzyme complex are essentially stable for over 12 months.

Purification experiment A21

The results of this experiment are discussed in Chapters 4 & 7. The procedure followed was almost identical to the 4th generation scheme described above except that a less stringent anti-protease strategy was employed. The points of departure from the 4th generation protocol were:

1. The mycelial extraction buffer was supplemented with 10μM zinc acetate and contained no EDTA.
2. The DE52 column for the first ion exchange step was equilibrated with buffers containing 20μM zinc acetate.
3. The conductivity of the crude extract was somewhat lower than 4.0mmho when it was pumped through the DE52 bed.
4. Benzamidine was omitted from dialysis buffers I and II; PMSF was omitted from dialysis buffer II.
5. 24 hours were allowed for dialysis II (normally 5-6 hours).
6. Zinc was omitted from the 1.5M KCl buffer in the Blue Dextran Sepharose step.

Evaluation of arom purification procedures

In the experiments described in Chapter 4 small samples were taken from each pool and stored on ice in eppendorf tubes; these samples were used for E2 assays, activity ratio measurements, protein determinations and analytical SDS PAGE. In routine purification experiments the E2 specific activity and the pattern of polypeptides seen in SDS PAGE was recorded at all important stages. In practice the minimal criteria for the homogeneity of the final product are:

1. E2 specific activity
2. polypeptide homogeneity by SDS PAGE
3. E1:E2:E5 activity ratios

3.8 Purification of chorismate synthase and anthranilate synthase

Stock solutions and preparation of buffers

Prepare stock solutions and buffers as for arom purification experiments, with the following additions:

1M potassium phosphate pH 6.5 (room temperature)

0.5M potassium phosphate pH 7.0

Chromatography

Prepare anion exchange columns and a Blue Dextran-Sepharose column as in the 2nd and 3rd generation arom purification procedures, respectively. For phosphocellulose columns use precycled, de-fined Whatman P11 material.

Purification procedure (carry out all steps at 0-4°C)

This is based in part on purification steps derived from the procedures of Wegman and De Moss (1965), Cole and Gaertner (1975) Lumsden and Coggins (1977) and A.A. Coia and J.R. Coggins (unpublished work).

1. Mycelial extraction As for arom purification procedure, except that the extraction buffer is 100mM potassium phosphate (pH 7.0), 5mM EDTA; bring to pH 7.0 at room temperature with concentrated NaOH, cool to 4°C, then supplement with 0.4mM DTT and 1.2mM PMSF.

2. "Negative" chromatography on DEAE-cellulose As for 2nd generation arom purification experiments, except that 100mM potassium phosphate pH 7.0 replaces the tris/HCl, KCl buffer. Pool all fractions with significant anthranilate synthase activity.

3. Ammonium sulphate fractionation As for arom purification experiments, except that the proteins precipitating between 40% and 50% saturation in ammonium sulphate are resuspended in 100mM KP_i pH 7.0, 0.4mM DTT, 1.2mM PMSF (40ml). Note that the use of phosphate buffer throughout is critical for the quantitative recovery of anthranilate synthase activity in these steps.

4. Dialysis I As for 2nd generation arom purification experiments. Centrifuge briefly to remove precipitated proteins.

5. Ion exchange chromatography on DEAE-cellulose As for step 6 of 2nd generation arom purification procedure. Arom E2, anthranilate synthase and chorismate synthase elute in the same region; it is probably quickest to pool all fractions with high arom E2 and anthranilate synthase specific activities.

6. "Negative" chromatography on Blue Dextran Sepharose Pump the pool from step 5 through a 2-3ml column of Blue Dextran-Sepharose, as in the 2nd generation arom purification procedure. Assay the flow-through fractions for arom E2 and anthranilate synthase activities; all arom E2 activity should remain bound to the column, while anthranilate synthase and chorismate synthase are not retarded. Pool these fractions.

7. Dialysis II Dialyse the pool from step 6 versus 2 x 2 l 10mM potassium phosphate pH 6.5, 0.2mM DTT, 0.6mM PMSF (3 hours + 5 hours). Check the conductivity of the sample after recovery from dialysis.

8. Phosphocellulose chromatography I Meanwhile prepare a long column (25cm x $1cm^2$) of phosphocellulose, and very carefully equilibrate with 10mM potassium pH 6.5. Finally equilibrate the column with the second

dialysis buffer of step 7. Load the sample from step 7 overnight, pumping at 16ml/h. Pool all flow-through fractions with significant anthranilate synthase activity.

Elute the phosphocellulose column with two linear gradients of potassium phosphate supplemented with 0.4mM DTT. Use an LKB "Ultrograd" gradient manager.

Gradient I 12-100mM KP_i pH 6.5; 8 hours at 30ml/h

Gradient II 100-400mM KP_i pH 6.5; 16 hours at 12ml/h

Pool the fractions of highest chorismate synthase specific activity.

9. Concentration of anthranilate synthase Pump the pooled fractions from step 8 through a 1-2ml column of DEAE-cellulose pre-equilibrated with 10mM potassium phosphate pH 6.5. Pump through several column volumes of a low ionic strength tris buffer to remove phosphate, then elute the proteins with 300mM KCl, 50mM tris/HCl pH 7.5, 0.4mM DTT, pumping at 10ml/h and collecting 1ml fractions. Do not pool the fractions, but to each add an equal volume of glycerol, vortex gently to mix, and store immediately at $-20^{o}C$ in stoppered glass vials.

10. Phosphocellulose chromatography II Dialyse the chorismate synthase pool from step 8 versus 1 l. of 40mM potassium phosphate pH 6.5, 0.2mM DTT and equilibrate a 2cm x $1cm^2$ column of phosphocellulose with the same buffer. Pump the dialysed pool to the column at 10ml/min. Continue pumping with equilibration buffer, collecting 6 min fractions, for approximately 1 hour. Then apply "substrate elution" steps (5ml each) in which the equilibration buffer is sequentially supplemented with 50μM NADPH, 20μM FMN and 100μM EPSP. Do not interrupt the flow of buffer at any point. Finally elute the column with 250mM potassium phosphate pH 6.5, 0.2mM DTT. Assay fractions for chorismate synthase activity and record the E_{280}, pH and conductivity as appropriate. Dialyse fractions of high chorismate synthase activity versus 500ml of 50mM

tris/HCl pH 7.5, 50% v/v glycerol, 0.4mM DTT. Store chorismate synthase in tightly stoppered glass vials at -20°C.

3.9 Atomic Absorbtion Sectroscopy

The purified arom enzyme complex was dialysed for 9 days at 4°C against four changes of 10mM sodium phosphate, 3mM NaCl pH 7.0 which had been extracted with 0.01% diphenylthiocarbazone in CCl_4 (Thiers, 1957). All glassware was washed with HNO_3, then with diphenylthiocarbazone solution, and finally with distilled water. The dialysed enzyme samples were assayed for protein and for arom E1 and E2 activity. Zinc analyses were kindly performed by Dr. John Farmer of the Department of Forensic Medicine and Science, Glasgow University. Duplicate samples were analysed in the air-acetylene flame of an 1L151 atomic absorbtion spectrophotometer equipped with a deuterium continuum lamp for background corrections. The detection limit was 0.005μg/ml zinc.

3.10 Zinc Buffering Systems

The pH-independent zinc buffers employed in the experiment summarised in Table 5.4 were constructed according to the general method of Perrin and Dempsey (1974). The parameter pZn is analagous to pH:

$$pZn = -\log_{10}\left([Zn^{2+}] \text{ /mole } l^{-1}\right)$$

Calculations of pZn were based on values of pKd for EDTA complexes cited by Perrin and Dempsey (1974).

Zinc buffer concentrates contain 100mM $CaCl_2$, 40mM EDTA, 30mM acetate and the following concentrations of zinc (acetate salt) 10mM (pZn=8.8), 2mM (9.6), 0.5mM (10.2) and 0mm ("no zinc" blank control). Make up all solutions in deionised water, titrate immediately to pH 7.5 with 10M NaOH and store at 4°C.

Zinc buffered E1 assay cocktails for the reconstitution experiment contained 100μM DAHP, 50μM NAD^+, 50mM tris/HCl pH 7.5,

supplemented with 0.03u/ml phosphate-free E.coli dehydroquinase, 2.5% glycerol and a tenfold dilution of the appropriate zinc buffer concentrate.

Enzyme samples for this experiment contained 40mM tris/HCl and:

1. "EDTA treated" 20% v/v A7 arom in storage buffer and 1mM EDTA
2. "control" 20% v/v A7 arom in storage buffer
3. "blank" 20% v/v storage buffer without arom

Samples were incubated at 0°C for 60 hours. Assays were initiated by adding 2.5μlitre of the appropriate sample to 1ml of the appropriate zinc-buffered assay cocktail; the progress of the reaction was followed at 234nm.

3.11 Determination of the Equilibrium Constant for the EPSP Synthase Reaction

Incubation mixtures initially contained approximately 90 nanomoles EPSP, either≈10mM or≈20mM potassium phosphate pH 7.0, 4mU arom EPSP synthase (phosphate free) in buffer A, 0.5mM DTT, pH 7.0. The stock solutions were prepared and standardised as in kinetics experiments. Incubation mixtures were kept at 25°C for 15 hours in sealed eppendorf tubes. The contents were then vortexed vigorously, and samples were raised to 100°C for 2 minutes to inactivate the enzyme. Duplicate aliquots from each sample were then assayed sequentially for pyruvate, PEP and EPSP by conversion to lactate, taking ε_{340nm} = 6,220 for NADH. In all experimental samples ≤2% of the total revealed pyruvate was present as free pyruvate. In control samples containing

1. EPSP, arom and no P_i
2. EPSP alone

≤4% of the total revealed pyruvate was present as free pyruvate. The results indicate that the components of the incubation mixtures were essentially free of adventitious P_i and of EPSP-degrading

activities. For each pair of measured final concentrations of PEP and EPSP the final concentrations of P_i and Shik3P, and hence the apparent equilibrium constant, were calculated on the assumption of stoicheiometric conversion of EPSP and P_i to Shik3P and PEP. The results are summarised in Table 6.2.

3.12 Limited Proteolysis of the arom Multifunctional Enzyme

Limited proteolysis of the arom enzyme complex was conducted under standardised conditions at 25°C. The stock enzyme concentrate was diluted approximately fourfold into 50mM potassium phosphate pH 7.00 to give a final protein concentration of approximately 100µg/ml. Trypsin and chymotrypsin were stored at -20°C as 1mg/ml stock solutions in 1mM HCl, and were added to the proteolysis incubation at concentrations up to 100µg/ml. Samples for analytical procedures were removed at intervals; proteolysis was terminated with a threefold weight excess of lima bean trypsin/ chymotrypsin inhibitor. These samples were kept at 25°C for the duration of the experiment, then removed to ice.

In larger-scale proteolysis experiments the digestion products were passed through a 1ml column of immobilised trypsin inhibitor to terminate proteolysis, and were then subjected to chromatography on 1ml columns of chromatographic materials poured in 1ml disposable syringes. All chromatographic steps were carried out at 4°C.

3.13 High Performance Liquid Chromatography (HPLC)

Gel permeation chromatography was carried out at room temperature on a 60cm x 0.75cm TSK G3000 SW column (LKB Ltd., South Croydon, London) with 100mM potassium phosphate, 0.4mM DTT pH 7.0 as mobile phase, at a flow rate of 0.4ml/min, using a model 303 pump (Gilson France, S.A., Villiers-le-Bel, France). The column eluate was monitored at 215nm with an LC-UV detector (Pye Unicam, Cambridge) fitted with an 8µl flow cell. Molecular weight calibration proteins were selected from those listed in Section 3.1.2.

CHAPTER 4 COPURIFICATION OF THE FIVE ACTIVITIES OF THE AROM MULTIFUNCTIONAL ENZYME AND CHARACTERISATION OF E1 AS A ZINC ENZYME

4.1 Previous work on the purification and characterisation of the arom multifunctional enzyme

4.1.1 The arom gene cluster

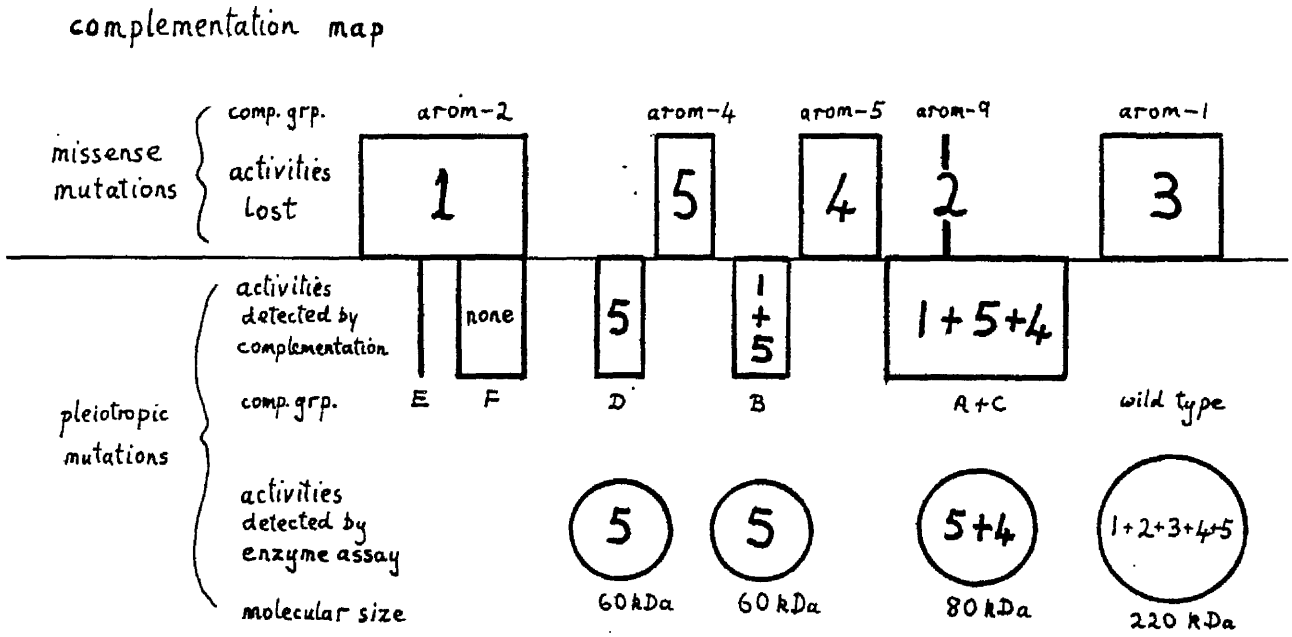
Auxotrophic strains of N.crassa that are functionally deficient in the early common aromatic pathway are known as arom mutants. Many arom mutations map within a complex gene cluster on linkage group II of N.crassa, the arom locus. Five distinct complementation groups of arom mutations are associated with defects in the five enzyme activities (E1, E2, E3, E4, E5) required for the biosynthesis of EPSP from DAHP; these mutations are located within five discrete segments of the linkage map of the arom region (Rines et al, 1969; see Figures 4.1 and 1.1). A more detailed discussion of the genetic mapping experiments follows in chapter 7.

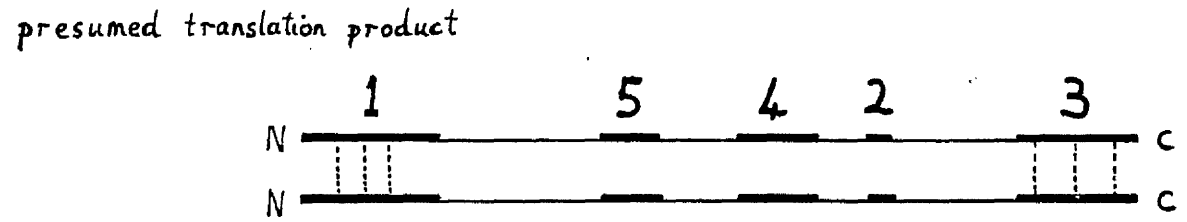
4.1.2 Evidence for an arom multienzyme complex

Early work revealed that the five enzyme activities defined by the arom gene cluster exist in the cell as a single structural aggregate, the arom enzyme complex (Giles et al, 1967a). Although the five enzyme activities of the complex remain tightly associated during protein fractionation the isolation of the arom enzyme complex and the definition of its subunit structure proved to be a far from trivial problem.

Burgoyne et al (1969) obtained a preparation of the arom complex that appeared to be essentially homogeneous and carried all five enzyme activities. A single major species of 100,000kDa was seen in SDS PAGE. Determined efforts to disrupt the complex into smaller

Figure 4.1 Genetic map of the arom locus of N.crassa (Giles et al, 1967a; Rines et al, 1969; Case & Giles, 1971)





constituent subunits did not meet with success. At this point in the investigation the accumulated biochemical and genetic evidence was open to interpretation in terms of an arom complex with two identical multifunctional subunits of 100,000kDa (Burgoyne et al, 1969). Further work produced evidence for smaller arom "subcomplexes", and it was proposed that the intact arom complex consists of at least four types of polypeptide subunit (Jacobson et al, 1972, Gaertner, 1972).

4.1.3 The subunit structure of the arom enzyme complex

A new scheme for the purification of the arom complex was devised by Lumsden and Coggins (1977); several features of this scheme were expressly designed to minimise problems arising from the action of endogenous proteases. The purified complex was judged homogeneous by several criteria; SDS PAGE revealed a single size of polypeptide subunit of 165,000kDa. The molecular weight of the native arom complex was estimated to be 270kDa by density gradient centrifugation. The products of enzyme cross-linking reactions were consistent with a homodimeric structure for the enzyme complex, and "mapping" of the cysteine- and methionine-containing peptides provided no evidence for more than one type of subunit. All five arom activities were present in the homogeneous enzyme. It was concluded that the intact arom complex is a dimer of identical pentafunctional polypeptides of 165,000kDa (Lumsden and Coggins, 1978). A subunit size of approximately 150,000 daltons for the "intact" complex has been reported by Gaertner and Cole (1977).

The purified arom complex of Lumsden and Coggins (1977) differed from earlier preparations of the enzyme in its stability, homogeneity and high specific activity. The copurification in constant ratio of three of the activities, E2, E3 and E4, was documented for a substantial part of the purification scheme. Assay of E1 and E5 activities in partially

purified enzyme fractions proved to be technically difficult but there was no reason to suppose that E1 and E5 did not copurify with the other three activities.

A "second generation" protocol for purification of the arom complex was developed in our laboratory by Smith and Coggins (1983). The only substantial change was the introduction of Blue Dextran-Sepharose as an "affinity matrix" for the final stage of chromatography. The new scheme proved to be more convenient, gave reliably high yields of homogeneous arom enzyme complex, and allowed the coupling enzymes anthranilate synthase and chorismate synthase to be separated as byproducts (A.A.Coia and J.R.Coggins, unpublished results). In other respects the final products of the "first" and "second" generation procedures were very similar. The "second generation" procedure is outlined in table 4.1, as it is the basis of most of the purification methods described here.

4.1.4 Obstacles to the purification of the arom multifunc tional enzyme

4.1.4.1 Endogenous proteases

The success of the arom purification method of Lumsden and Coggins (1977) was attributed to the steps that were taken to remove and inactivate the suite of active and latent proteases that are present in N.crassa mycelial extracts (Yu et al, 1973). It is likely that these proteases were responsible for the confused picture of the subunit composition of the arom complex that emerged from the earlier work. Endogenous proteases have presented a serious obstacle to the purification of a number of multienzyme complexes from fungi. The difficulties experienced with such enzymes as the multifunctional anthranilate synthase complex of N.crassa (Keesey et al, 1981) provide case histories similar to that of the arom complex.

Table 4.1 Outline of 2nd, 3rd and 4th generation purification procedures

	Stage	Protective reagents in 2nd generation experiments
1.	Mycelial extraction	EPD
2.	Passage of extract through column of DE52	EPD
3.	37°C incubation (omitted in 3rd and 4th generation schemes)	D
4.	Ammonium sulphate fractionation (40-50% saturation)	DB
5.	Dialysis I	PD
6.	Ion exchange chromatography	PD
7.	Dialysis II (omitted in 2nd generation scheme)	-
8.	Chromatography on Blue Dextran Sepharose	PD
9.	Final dialysis into buffer containing 50% v/v glycerol	PDB
10.	Storage at -20°C	DB

The protective reagents in the "second generation" protocol of Smith (1980) are:

E 5mM EDTA

P 1.2mM PMSF

D 0.4mM DTT

B 1mM benzamidine

The key elements in the anti-protease strategy of Lumsden and Coggins (1977) are the "negative" chromatography step that precedes all the other fractionation procedures, and the extensive use of the protease inhibitors EDTA, benzamidine, and particularly PMSF. There is considerable evidence that proteolytically degraded arom complex is generally not removed by procedures that fractionate the intact complex from all other cell components (Smith and Coggins, 1983). It is therefore reasonable to conclude that the anti-protease strategy of Lumsden and Coggins (1977), incorporated in the "second generation" purification procedure of Smith and Coggins (1983), is wholly successful in eliminating proteolytic damage to the complex at all stages in its isolation.

4.1.4.2 Oxidation

In some early studies it was found that the arom multienzyme complex was highly sensitive to oxidation, and fractionation procedures were carried out under nitrogen in buffers containing thiol reducing agents (Burgoyne et al, 1969). Lumsden and Coggins (1977) simply employed 0.4mM DTT as a thiol reducing agent throughout purification, and no problems arising from oxidation were recognised.

4.1.5 Homogeneity of the purified arom multifunctional enzyme

The final products of the "first" and "second" generation arom purification procedures developed in our laboratory have been judged homogeneous by SDS PAGE, and by gel electrophoresis under native conditions - and the coincidence of single bands staining for protein and for E1, E3 and E5 activity (Lumsden and Coggins, 1977; Nimmo and Nimmo, 1982; Smith and Coggins, 1983). SDS PAGE has routinely been used for "quality control" of the purified enzyme.

4.1.6 The measurement of activity ratios

A successful enzyme purification procedure should normally yield a homogeneous product with a well-defined specific activity. When the final product is a homogeneous pentafunctional enzyme five well-defined specific activities are required. If the five enzymes activities are only present in the cell as components of a single species of multifunctional enzyme it follows that they will copurify in constant ratio unless their catalytic activities are perturbed by the fractionation procedures.

Four of the arom activities are uniquely expressed from the four corresponding complementation regions of the arom gene cluster, but there are two genes for dehydroquinase (E2) in N.crassa. In addition to the biosynthetic arom dehydroquinase there is a second, inducible, enzyme that is expressed from the qa-1 gene (Giles et al, 1967b; Rines et al, 1969). The qa gene products are not induced unless high levels of quinate or related compounds are present in the cell, and they are essentially absent in wild type cells grown on minimal medium, as in this study. Copurification of the five arom activities in constant ratio is therefore to be expected unless the fractionation procedures alter the enzyme activities of the native arom complex.

The criterion of constant "activity ratios" is of proven utility in the purification of multienzyme complexes, and is also useful when different aspects of the same catalytic activity have differential sensitivities to covalent modification (for example see Nimmo and Tipton, 1975; Meek, 1982). Interpreting changes in the activity ratios may not be straightforward, since in principle it is impossible to distinguish between activation of one subset of enzymes and inactivation of the complementary subset. In this study the activities of the arom complex are always normalised to give a value of 100 for the

dehydroquinase (E2) activity. Specific activities of 1, 2, 3, 2, 1 units/mg for enzymes, 1, 2, 3, 4, 5, respectively, are thus expressed as an activity ratio 50:100:150:100:50. This choice of notation requires some explanation. E2 is chosen as the reference activity because it can be assayed simply and reliably in crude mycelial extracts. E3 shares this advantage, but the assay is much more sensitive to pH and temperature changes and a "blank rate" must frequently be subtracted. Fortunately E2 activity is the most useful reference point for interpreting the activity ratios, as it is often retained quantitatively under conditions where other activities are selectively destroyed. It should, however, be emphasised that the use of E2 as a reference activity never implies any assumptions about the absolute stability of this component of the arom complex.

4.1.7 Objectives of the present study

In the course of this study it became clear that the "second generation" arom purification protocol has certain shortcomings. This development fortuitously coincided with the finding by J.M.Lambert that E1 is a metalloenzyme. It then became an important objective to develop, in collaboration with J.M.Lambert, a "third generation" protocol, and to demonstrate the copurification, in constant ratio, of all five activities of the arom multifunctional enzyme. This was seen as a crucial test of the homogeneity of the purified enzyme complex, and as a prerequisite for any detailed study of its catalytic properties. Progress in the enzyme purification work was assisted by further studies on aspects of the DHQ synthase (E1) activity of the arom enzyme complex that relate to its function as a metalloenzyme.

4.2 Development of a method for copurification of the five arom activities

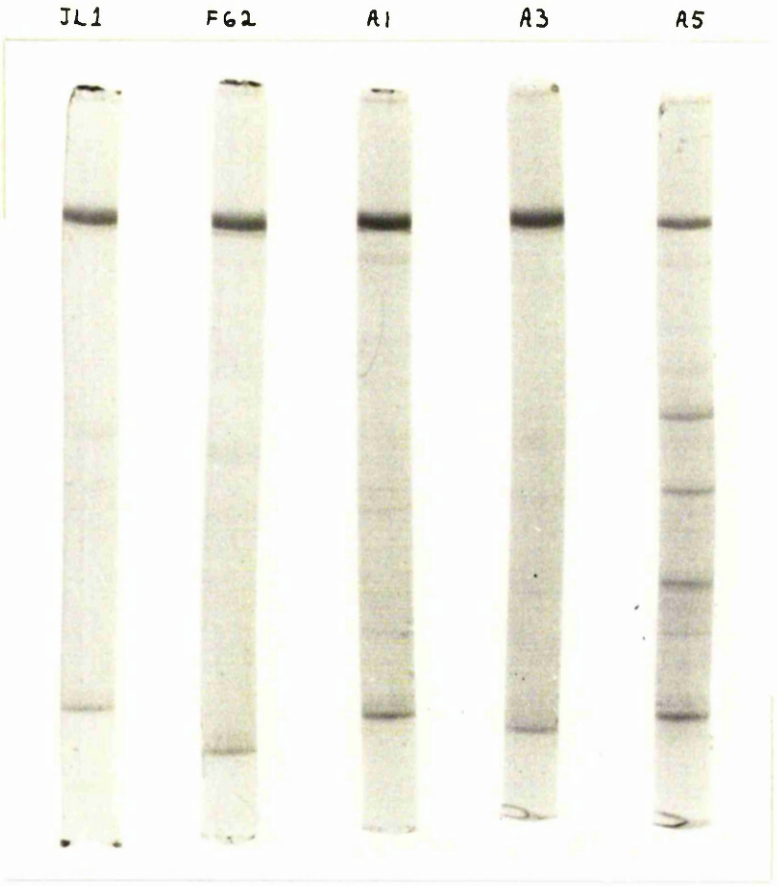
4.2.1 Evidence of selective inactivation of E1 and E5

The activity ratios estimated for three "second generation" preparations of the arom multifunctional enzyme, judged homogeneous by SDS PAGE (figure 4.2), are summarised in table 4.2. In all three samples E2, E3 and E4 were present in essentially the same ratio. This result is in line with the earlier observations that E2, E3 and E4 copurify in the later stages of the "first generation" protocol, that the final product has a well-defined E3 specific activity, and that E2, E3 and E4 are stable on storage (Lumsden and Coggins, 1977).

In contrast the relative activities of E1 and E5 in the three samples varied independently over a wide range; the E5:E2 ratios ranged from 4.8:100 to 66.4:100. Similar variations were seen in other samples of purified arom. The simplest explanation of these results is that E1 and E5 are relatively labile during purification or storage, and that the differences between the activity ratios are due to independent inactivation of E1 and E5.

There were minor differences between the three samples A1, F62 and JL1 in the details of the purification procedures and storage conditions. Attention was initially focused on the difference between the low E5:E2 ratio of sample A1 (stored for periods at the higher temperature of 0°C) and the high E5:E2 ratio of sample JL1 (stored exclusively at -20°C and not subjected to the 37°C heat step during purification). A progressive decline in the E5 activity of sample A1 was monitored on continued storage, while E2 activity was retained. These observations lent support to the hypothesis that the E5 and E1 activities of arom are relatively labile. We therefore hoped that

Figure 4.2 SDS PAGE of five preparations of the arom enzyme complex



Samples of the five numbered preparations of the arom enzyme complex were subjected to SDS PAGE in 8% gels, which were stained for protein. The activity ratios of these five samples of the arom enzyme complex are recorded in table 4.2.

TABLE 4.2 Activity ratios of five samples of the arom complex

Sample	Age (mths)	changes to 2nd generation purification protocol	Activity Ratios E1	E2	E3	E4	E5
FG2	12	none	21.8	100	230	66.4	9.8
A1	6	none	34.3	100	223	61.2	4.8
JL1	9	omit heat step	7.9	100	216	66.2	61.3
A3	0	omit heat step omit EDTA	10.4	100	228	77.2	64.4
A5	0	omit heat step, EDTA, DTT add Zn^{2+}/βME/NAD+	88.0	100	244	82.5	71.1

Activity ratios were calculated from triplicate assay traces (F62, A1, JL1 and A3), or quadruplicate assay traces (A5 and all E2 assays).

identifying the factors responsible for the relative instability of E1 and E5 would help us to isolate the homogeneous arom complex with its full complement of enzyme activities.

4.2.2 Evidence that E1 is a metalloenzyme

DHQ synthase (E1) activity has normally been assayed in our laboratory in cocktails lacking a transition metal ion supplement (see section 3.5). The addition of powerful transition-metal chelating agents to the assay cocktail has no significant effect on the rate of DHQ synthesis (G.A.Nimmo, unpublished results). Only when the arom multifuncational enzyme is preincubated with a chelating agent before addition of the substrate, DAHP, is there any indication that a metal cation may be essential for E1 action (J.M.Lambert, unpublished results). The DHQ synthase activity of the arom multifunctional enzyme is rapidly and specifically inhibited by pretreatment with EDTA, and it is also sensitive to 1,10-phenanthroline and to high concentrations of DTT (J.M.Lambert, unpublished results). The susceptibility of E1 to inactivation by these three different metal chelating agents is taken as good evidence that a metal cation is essential for catalysis.

It is at first sight surprising that EDTA fails to block E1 activity when the arom complex is added directly to complete assay cocktails containing the chelating agent. The result indicates that the metal cofactor remains bound to the enzyme when the substrates are absent, and that there is a kinetic barrier to its removal once the reaction has been initiated. The result also suggests that the essential metal cation must remain bound to the enzyme throughout the purification procedures. This immediately raises the possibility that there is a danger of E1 inactivation during the first stage of the "second generation" purification procedure, where the cells are extracted

into a buffer that contains EDTA - a precaution against metal-dependent proteases. We therefore speculated that the variable E1:E2 ratios of "second generation" arom samples reflected variations in the proportion of the initial E1 activity that was lost in the first stage of purification.

4.2.3 Evidence that E1 is selectively inactivated during chromatography

In the light of the evidence that E1 is a metallo enzyme it was important to examine the effect of omitting EDTA from the first stage of the "second generation" purification procedure. The resulting arom sample, A3, appeared homogeneous by SDS PAGE, and the final activity ratios were compared with those of the earlier preparations of arom (see table 4.2 and fig. 4.2). The final E2:E3:E4 ratios and specific activities were similar to those of the other samples, and the E5:E2 ratio was at the upper end of the range. The final E1:E2 ratio did not show the expected dramatic improvement over the earlier preparations, and fell within the range of values seen with the three samples of arom derived from cells extracted into buffers containing EDTA.

Estimates of the activity ratios at various stages in the purification procedures suggested an explanation for these results (see table 4.3). It was found that the E2:E3:E4:E5 ratios remained essentially the same at all stages where they could be determined reliably. In contrast the E1:E2 ratio in the earliest stages of purification was threefold higher than had previously been seen in any purified arom sample, but the ratio dropped precipitately across the two chromatography steps - ion exchange on DEAE cellulose and affinity chromatography with Blue Dextran-Sepharose.

On this evidence it was concluded that EDTA does not constitute an indispensible element in the anti-protease strategy, and that the most

TABLE 4.3 Activity ratios during modified "second generation" purification experiment A3

Stage	E2 activity (units)	Activity Ratios E1	E2	E3	E4	E5
DEAE cellulose I	27.8	80.2	100	(309)*	nd	nd
$(NH_4)_2SO_4$ fractionation	17.3	103	100	276	57.0	81.4
dialysis I	19.4	91.8	100	260	nd	73.2
DEAE cellulose II	16.6	53.5	100	249	48.3	63.0
Blue-dextran-sepharose	9.6	14.3	100	235	81.6	74.0
final dialysis	nd	10.4	100	228	77.2	64.4

NOTES:

1. Purification was by modified "second generation" method, omitting EDTA and 37°C step; 50 g cells were extracted into 750 ml buffer.
2. Activity ratios were calculated from duplicate assay traces.

* blank rate not determined

serious cause of variable E1:E2 activity ratios is the instability of E1 activity to chromatography. There was little doubt that preferential inactivation of E1 underlay the observed changes in the activity ratios, since the evidence for copurification of the other four activities and for the invariance of the specific E2 activity of the final product was satisfactory. Nevertheless the inactivation of E1 during chromatography was puzzling, as there was no obvious mechanism for removal of an essential metal cation. It was considered unlikely that passive dissociation of the metal cofactor was responsible for losses of E1 activity, because the E1:E2 ratio was not substantially affected by dialysis or by ammonium sulphate fractionation (see table 4.3).

4.2.4 Evidence that E1 is zinc-dependent

In its sensitivity to metal-chelating agents and its strict dependence on NAD^+ for catalysis the DHQ synthase of the arom complex (E1) has two properties in common with the DHQ synthases characterised in plants and bacteria. After treatment with chelating agents the activities of the plant and bacterial enzymes can be wholly or partially restored on addition of excess divalent transition metal cations. Divalent cobalt stimulates the largest number of these DHQ synthases, while Cu^{++} and Mn^{2+} are also effective in some cases (Hasan and Nester, 1978c; Maitra and Sprinson, 1978; Saijo and Kosuge, 1978; Yamamato, 1980). This type of result is often cited as conclusive evidence that catalysis is dependent on the metal cation that appears to reconstitute the activity. One criticism of this approach is that the affinity of some enzymes for their metal cofactors is so high that activity may be restored at transition metal concentrations that are commonly present as contaminants in dilute solutions of analytical grade magnesium chloride. A further criticism is that the chelating agent may block catalysis by binding reversibly to an enzyme-bound metal centre, rather than by removing it from the enzyme. In either of these cases

the results of simple reconstitution experiments must be treated with some caution.

A more satisfactory approach is be to use metal ion "buffering" systems in which very low free metal cation concentrations may be specified and interference from adventitious metals can be eliminated (Perrin and Dempsey, 1974).

When EDTA-treated arom was diluted into solutions containing an excess of a divalent metal cation, E1 activity was only reconstituted quantitatively when the metal cation was Zn^{2+}. Co^{2+}, Mn^{2+} and Ca^{2+} were not effective, neither were a range of other D block dications (J.M.Lambert, unpublished results). These results were taken as preliminary evidence that E1 is a zinc-dependent enzyme.

When the arom sample A3 was treated with zinc no increase in its E1 activity could be detected, despite the apparent loss of over 80% of the initial E1 activity during the final two stages of purification (see table 4.3). It was found that the E1:E2 ratio of a number of "second generation" arom samples returned to approximately its initial value, but did not increase, when the samples were treated first with EDTA, and then with excess zinc. The latent E1 activity of arom incubated with EDTA was essentially stable for many days at $0^{o}C$. It therefore had to be concluded that if losses of a metal cofactor are the primary cause of changes in the E1:E2 ratio during purification they are not readily reversible on subsequent addition of the presumed cofactor, zinc. This paradox has not been resolved, although it is possible to speculate that removal of the metal cofactor is followed by an irreversible secondary modification of E1, perhaps through binding of adventitious heavy metals. Such a sequence of events would be consistent with the stability of the latent E1 activity in the presence of excess EDTA.

There are some precedents for the removal of catalytically essential zinc from enzymes during purification. The behaviour of the

zinc-dependent enzyme phosphomannose isomerase, in particular its sensitivity to chromatography on DEAE cellulose, provides an interesting parallel to the results obtained with arom E1 (Gracy and Noltmann, 1968). Phosphomannose isomerase is stabilised during chromatography by the addition of low concentrations of Zn^{2+} to the buffers. We were therefore encouraged to seek means for stabilising arom E1 activity during chromatography.

4.2.5 Experiments with zinc buffers

Experiments on the reconstitution of arom E1 activity in metal ion buffers at very low defined zinc concentrations were initiated with two immediate objectives: firstly to evaluate critically the link between zinc and E1 catalysis, and secondly to define conditions necessary for the rapid reconstitution of E1 activity after removal of the presumed cofactor. The results of one experiment are summarised in table 4.4. Central to the design of this experiment are modified E1 assay cocktails which allow the kinetics of the recovery of E1 activity to be observed directly at specified concentrations of free zinc ions. Free zinc concentrations as low as 10^{-10}M (pZn=10) are readily obtained in cocktails where $[Ca^{2+}] > [EDTA] > [Zn^{2+}]$, by virtue of competition between Ca^{2+} and Zn^{2+} for available EDTA (Perrin and Dempsey, 1974, see section 3.10).

When untreated arom was assayed for E1 activity in the zinc buffering system the increase in absorbance at 234nm was linear with time after the initial "lag" period associated with the conversion of DHQ to DHS by the coupling enzyme, E.coli dehydroquinase. With untreated arom identical reaction traces were obtained in cocktails at calculated pZn 8.8, 9.6 and 10.0, and in the equivalent cocktails containing only the Ca^{2+} and EDTA components of the "zinc buffer" and lacking added zinc.

Table 4.4 Reconstitution of E1 activity of EDTA-treated arom in assay cocktails containing subnanomolar concentrations of zinc

calculated pZn	Total concentrations of components of assay cocktails (mM)			E1 activity ($\Delta E_{234} min^{-1}$) at stated time after addition of arom sample to assay cocktail; apparent lag-time, τ, of coupled enzyme assay	
	Ca^{2+}	EDTA	Zn^{2+}	control untreated arom	EDTA-treated arom
"no zinc"	10	4	0	0.020 (5 min) τ = 0.7 minutes	$\leq$ 0.001 (5 min) –
10.2	10	4	0.05	0.0175 (5 min) τ = 0.75 minutes	0.010 (5 min) $\tau \gg$ 5 minutes
9.6	10	4	0.2	0.018 (5 min) $\tau <$ 1.0 minute	0.018 (10 min) τ = 3 minutes
8.8	10	4	1.0	nd	0.018 (10 min) $\tau \leq$ 1.5 minutes

Reconstitution of E1 activity was monitored by coupling the release of DHQ to the dehydroquinase reaction (see Section 3.10)

However, when the arom complex was treated with EDTA before assay in these systems the results were quite different. Little or no activity was detectable on addition of EDTA-treated arom to the cocktails lacking added zinc, although the activity was rapidly recovered on addition of small amounts of the pZn 8.8 "buffer concentrate". EDTA-treated arom recovered its full E1 activity within two minutes of addition to the cocktail at pZn 8.8, while at pZn 9.6 the kinetics of reactivation were clearly slower, although the full activity was recovered within 10 minutes. At pZn 10.0 EDTA-treated arom only gradually recovered E1 activity over 10 minutes, reaching about half the initial activity in this time.

These results can be interpreted in a fairly straightforward manner. Untreated arom presumably retains its full initial E1 activity in all the cocktails, with or without added zinc, because there is a kinetic or thermodynamic barrier to the removal of the metal ion cofactor in the presence of the substrate and cofactor DAHP and NAD^+. This result is consistent with the earlier finding that E1 gives linear reaction traces even in the presence of high concentrations of free EDTA. The failure of the cocktail with $[Ca^{2+}]>[EDTA]$, and no added zinc, to reactivate EDTA-treated arom E1 is good evidence that the inhibition is not simply a consequence of reversible binding of EDTA to a site in the enzyme. A similar cocktail containing a total concentration of 200μM Zn^{2+} and a calculated free zinc concentration of 2.5×10^{-10}M (pZn=9.6) allowed full recovery of the initial E1 activity within 10 minutes; this is persuasive evidence that the essential component that is removed from arom on EDTA treatment can be replaced by exogenously supplied Zn^{2+}.

The slower kinetics of the recovery of E1 activity at lower zinc concentrations (pZn=10) suggest that the progress of the bimolecular association reaction

$$\underline{arom} + Zn^{2+} \longrightarrow \underline{arom} \bullet Zn^{2+}$$

determines the rate of reconsitution of E1 activity. Two other possible rate-determining steps must be considered. Exchange of zinc between EDTA and the metal-binding site in the enzyme

$$EDTA \bullet Zn^{2+} + \underline{arom} \longleftrightarrow \underline{arom} \bullet Zn^{2+} + EDTA$$

can be ruled out, since it is known that the activity of EDTA-treated arom is not reconstituted at a measurable rate in complete cocktails containing significant concentrations of zinc and a higher concentration of EDTA, despite the fact that the E1 activity of untreated arom is readily assayed under these conditions. It must therefore be concluded that it is "free" hydrated Zn^{2+}, rather than any metal chelate, that confers reconstituting activity on the zinc buffer cocktails. A more likely alternative rate-limiting step in the reconstitution of arom E1 activity is the slow re-equilibration of the metal ion buffer in the step

$$EDTA \bullet Zn^{2+} \longrightarrow EDTA + Zn^{2+}$$

since the concentration of arom in the assays ($\approx 10^{-9}$M) was somewhat higher than the free zinc concentration. This explanation is plausible, as the dissociation of $Zn^{2+} \bullet$ EDTA in the pZn=10 buffer cannot proceed much faster than 10^{-11} M s^{-1}, even if the reaction is diffusion controlled. In any case it is clear that under the conditions of the assay the reconstitution of E1 activity, presumably through reassociation of the cofactor zinc, goes essentially to completion at 10^{-10} M Zn^{2+}. It appears that E1 activity is reconstituted very quickly once the zinc ion has bound to the enzyme. The rapidity of the reassociation reaction is remarkable, and on the preliminary evidence of the experiment shown in table 4.4 it must proceed with a second order rate constant $k_{on} \geqslant 10^{7}$ M^{-1} s^{-1}.

A more ambitious series of experiments using metal ion buffers has since been conducted by J.M.Lambert. Zn^{2+} was the only metal from the group Zn^{2+}, Co^{2+} and Mn^{2+} that, at submicromolar concentrations, allowed significant reconstitution of the E1 activity of EDTA-treated arom.

It should be stressed that the results described above do not demonstrate that zinc is the cofactor for DHQ synthase catalysis in vivo; they do, however, support the hypothesis that zinc is the metal that is removed from the purified arom multifunctional eyzyme by EDTA treatment.

4.2.6 The need for a third-generation purification protocol

The serious losses of E1 activity sustained in the two chromatography steps of the "second generation" procedure indicated that it would be necessary to modify the purification scheme to stabilise E1 activity. Inclusion of zinc salts in the chromatography buffers was the most promising option, the rationale being that permanent losses of E1 activity might be avoided if any zinc dissociating from the arom complex were immediately replaced from an external supply. Unfortunately the "second generation" scheme used DTT throughout as a thiol reducing agent. DTT is known to be a strong zinc chelating agent, with estimated pK_d=10.4 (see table 4.5); it inactivates zinc-dependent enzymes such as phosphmannose isomerase and histidase (Gracy and Noltmann, 1968; Cornell and Crivaro, 1972) and at very high concentrations it inactivates arom E1 relatively quickly. DTT was therefore abandoned as a thiol reducing agent in favour of βME - a weaker zinc chelating agent with pK_d=5.9 - and appropriate buffers were supplemented with zinc salts.

Table 4.5 Affinities of some common ligands for Zn^{2+}

ligand	pK_d
β-mercaptoethanol (βME)	5.9
dithiothreitol (DTT)	10.3
EDTA	16.4
glycine	5.4
cysteine	9.9

Sources: Cornell and Crivaro (1972)

Perrin and Dempsey (1974)

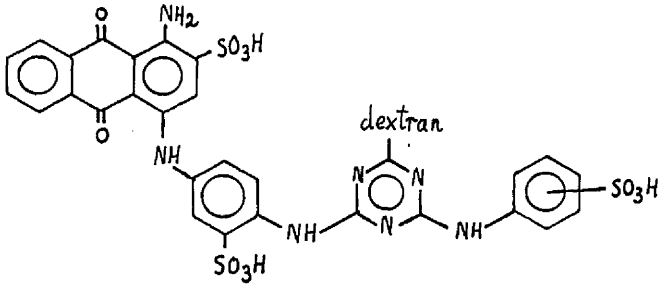


Figure 4.3 Structure of Cibacron Blue F3G-A ~ dextran

4.2.7 Copurification of E1 and E2

In the first attempt to design a "third generation" purification protocol all dialysis and chromatography buffers of the second generation scheme (from the resuspension of the protein after ammonium sulphate fractionation onward) were supplemented with 10μM NAD^+, 100μM $ZnSO_4$ and 1mMβME. The concentrations were chosen to guarantee a calculated free zinc concentration of 10^{-7} M, a hundred-fold higher than was necessary for rapid recovery of E1 activity in the reconstitution experiments. The E1:E2 ratio remained essentially the same at all stages of purification, and it was concluded that supplementing the buffers with zinc salts and βME has the intended effect of stabilising arom E1 during chromatography. The final E1:E2 ratio was therefore 3-fold higher than had been recorded for any "second generation" arom sample. The final E2:E3:E4:E5 activity ratios in this experiment, A5, were comparable to those of the "second generation" arom samples with the highest relative E5 activities (see Table 4.2).

It appeared likely that the activity ratios of the final product in this purification experiment (A5) reflected the full intrinsic activities of all five enzymes of the complex. After the normal fractionation procedures had been completed the arom was not homogeneous by the criterion of SDS PAGE, and the overall yield was only 1%. A major setback was the anomalous behaviour of the Blue Dextran-Sepharose matrix in this experiment. Subsequent work showed that the affinity of Blue Dextran-Sepharose for arom is greatly reduced in the presence of zinc. It is likely that chelation of zinc by the immobilised blue dye alters the properties of the affinity matrix. This idea suggests a possible rationale for the selective losses of E1 activity previously observed during chromatography on Blue Dextran Sepharose; the metal cofactor may be removed from the enzyme by the dye and its binding site

may then be "poisoned" by adventitious metals that accumulate on the affinity matrix.

4.2.8 Copurification of the five arom activities

The final "third generation" protocol for the purification of the arom multifunctional enzyme was designed and executed in collaboration with J.M.Lambert. The major preoccupations were

1. to avoid the stronger zinc-chelating agents (EDTA and DTT)
2. to supplement the buffers with βME and, where necessary, with zinc salts.

A competing demand was that the total amount of Zn^{2+} loaded onto the Blue Dextran-Sepharose column should be minimised. A number of additional measures were taken to remove adventitious metal ions from dialysis membranes, and from the affinity matrix. All these precautions were designed to eliminate losses of E1 activity, and they are summarised in detail in table 4.6, and in section 3.7.

The purification of the arom multifunctional enzyme by the "third generation" procedure is documented in tables 4.7 and 4.8. In one experiment, A7, the enzyme complex was purified to electrophoretic homogeneity in 42.9% overall yield. The activity ratios of the complex were estimated, where possible, at all stages in the purification procedure (table 4.8). The final two chromatographic steps achieved a 70-fold copurification of the five activities of the arom complex in essentially constant ratio. There was no evidence of any substantial change in the activity ratios in the earlier stages of purification, although E4 activity could not be determined, and the E1 and E5 assays were technically difficult. The results in table 4.8 constitute clear evidence of the copurification in constant ratio of the five arom activities, from crude mycelial extracts to electrophoretically

Table 4.6 Summary of alterations made to second generation purification protocol in the development of the third generation protocol

Change	Rationale
DTT (0.4mM) replaced by βME (1.2mM) throughout	DTT chelates zinc, inactivates E1
EDTA omitted from mycelial extraction buffer	unnecessary
37°C step omitted	unnecessary, inactivates E5?
10μM $ZnSO_4$ added to buffers for dialysis I and for ion exchange chromatography	stabilise E1 during chromatography
Dialysis tubing treated 1. with EDTA	remove adentitious metals
2. with dH_2O	remove EDTA
3. with zinc salts	saturate metal-binding sites
Blue Dextran Sepharose Chromatography	
A. Column treated with 5M guanidine . HCl and 50mM EDTA before loading sample	remove adventitious metals and denatured proteins
B. Sample dialysed versus zinc-free buffer	avoid saturating column with zinc
C. 20μM $ZnSO_4$ added to final elution buffer	stabilise E1
D. $ZnSO_4$ added to fraction tubes	zinc may remain bound to column
Final dialysis buffer	
A. Supplemented with 40μM Zn^{2+}, 0.4mM DTT (1μM Zn^{2+}, 1mM βME in experiment A7)	stabilise E 1, E5 (ill-considered)
B. Sodium phosphate replaced by potassium phosphate	convenience

TABLE 4.7 Purification table for "third generation" experiment A7

Stage	Volume (ml)	Protein (mg)	E2 (units)	E2 specific activity (u/mg)	E2 pfn factor (fold)	E2 net yield (%)
mycelial extract	1170	7020	67.7	0.0096	1.0	100
DEAE cellulose I	1340	3819	68.7	0.0180	1.87	101.5
$(NH_4)_2SO_4$ fractionation	39.5	742.6	61.3	0.083	8.56	90.6
dialysis I	54.5	654.0	64.3	0.098	10.1	95.0
DEAE cellulose II	100.0	118.0	43.0	0.364	37.8	63.6
Blue-dextran-sepharose	9.8	*4.13	29.0	*7.04	730	42.9

*The protein concentration of the final pool was estimated from E_{280} measurements, taking $E^{1\%}_{280}$=11.0 (Lumsden & Coggins, 1978). Other protein estimations were by the method of Bradford (1978). The table was compiled by J.M.Lambert, who executed all the fractionation procedures in this experiment.

TABLE 4.8 Activity ratios during "third generation" purification experiment A7

Stage	E2 pfn. factor (fold)	E2 yield (%)	Activity Ratios E1	E2	E3	E4	E5
mycelial extract	1.0	100	-	100	248	-	(48.8)
DEAE cellulose I	1.87	101.5	(94.0)	100	259	-	(55.8)
$(NH_4)_2SO_4$ fractionation	8.56	90.6	78.4	100	257	-	80.7
dialysis I	10.1	95.0	81.9	100	265	83.9	74.7
DEAE cellulose II	37.8	63.6	97.4	100	263	82.1	76.3
blue-dextran-sepharose	730	42.9	94.4	100	270	87.5	68.9
final dialysis I Zn^{2+}/β ME	-	-	87.7	100	268	86.2	51.8
final dialysis II Zn^{2+}/DTT	-	-	84.2	100	235	87.3	67.3

Activity ratios were calculated from quadruplicate assay traces, except those (in parentheses).

homogeneous enzyme. The final activity ratios were essentially the same in independent "third generation" experiments, and were comparable to those of the earlier copurification experiment A5 which was described in section 4.2.7. The final E2 specific activity was identical to that obtained in an independent "third generation" experiment, and within the narrow range of specific activities estimated for electrophetically homogeneous "second generation" arom.

Taken together these results indicate that the electrophoretically homogeneous "third generation" arom multifunctional enzyme has very similar catalytic properties to the enzyme present in crude cell extracts, and that it is isolated with all five enzyme activities essentially intact. The results also demonstrate that the variation in the final E1:E2:E5 ratios of "second generation" arom samples is due to selective inactivation of E1 and E5, as we had originally supposed.

4.3 Further developments in the purification and characterisation of the arom multifunctional enzyme

4.3.1 Characterisation of arom E1 as a zinc enzyme

A number of samples of the purified arom complex were dialysed exhaustively against "metal-free" buffer. The E1:E2 ratios and protein concentrations of the dialysed samples were estimated, and the total concentrations of zinc present were determined by atomic absorbtion spectroscopy (see section 3.9). The assay results are collected in table 4.9.

Exhaustive dialysis of the arom samples against the "metal-free" buffer led only to a small reduction in the E1:E2 ratio and E2 specific activity of the samples (tables 4.9 and 4.8). This finding is

Table 4.9 Zinc content of three samples of arom complex

Sample	E2 specific activity (units/mg)	E1:E2 activity ratio	[arom] (μM in subunits)	[zinc] (μM)	Zinc content (mole Zn/ mole arom subunits)	E1:E2/ maximal E1:E2
JL 1	6.32	14.0	2.85	0.58	0.20	0.15
E 80	4.79	41.1	2.36	1.18	0.50	0.44
A 7	6.49	68.0	2.36	1.57	0.67	0.72
dialysis buffer	- -	-	-	$\leq$ 0.08	-	-

NOTES:

1. Samples were dialysed exhaustively against "metal-free" buffer, and were then assayed for E2 activity, relative E1 activity, protein and zinc.

2. The concentration of the arom complex was estimated from E_{280nm} measurements [$E^{1\%}_{280}$ = 11.0 (Lumsden and Coggins, 1978)], taking M_r = 165,000 for arom subunits (Lumsden and Coggins, 1977).

3. Zinc concentrations were estimated by atomic absorbtion spectroscopy. The analyses were performed by Dr.J.Farmer.

4. The "maximal" E1:E2 ratio of the arom complex is taken to be 94.4%.

5. Full details of the experiment are given in section 3.9.

6. This table compiled by John Lambert and myself.

consistent with the earlier evidence that the metal cofactor of arom E1 dissociates very slowly in free solution. There was a strong correlation between the final E1:E2 ratio of the samples and the zinc content, calculated as g.atom zinc per mole of arom subunits. The "third generation" arom sample A7, with an E1:E2 ratio of 68:100 after dialysis, contained 0.67 atom/subunit of zinc. On the assumption that the specific E1 activity is proportional to the zinc content it can be calculated that the E1:E2 ratio of 94.4:100 estimated for this arom preparation immediately after purification would correspond to a zinc content of 0.93 atom/subunit.

The specific E1 activities of different samples of the arom complex, as determined from the E1:E2 activity ratios, thus appear to be directly related to the zinc content of the enzyme. The specific E1 activities recorded after the "third generation" purification procedures correspond very closely to a zinc content of 1 atom/subunit. There is therefore little doubt that the zinc component of the arom complex isolated by the second and third generation procedures is essential for E1 catalysis. If the E1 activity of the "third generation" arom represents the full potential Zn^{2+}-dependent E1 activity of the complex - as is likely - it follows that a single zinc ion per subunit is needed for full catalytic activity. This conclusion does not preclude the possibility that both subunits of the arom complex (and two zinc ions) are required for DHQ synthase (E1) activity.

4.3.2 Zinc and E1 action in the cell

The zinc analysis results for the isolated arom complex do not allow one to specify the metal cofactor utilised for E1 action in the cell, as it can always be argued that the authentic cofactor is exchanged for zinc during purification. The zinc content of sample JL1, purified

without any deliberate supplementation of the buffers with zinc, is not so readily discounted, but it could still be explained by exchange of the endogenous cofactor for adventitious zinc.

There are good reasons for believing that the cellular cofactor is indeed zinc. Significant dissociation of the cofactor is not expected before the ammonium sulphate fractionation step in the arom purification procedure, since the available evidence indicates that in free solution dissociation of the cofactor from the enzyme is very slow. If this is the case replacement of the endogenous cofactor - presumably by zinc - during the subsequent stages of the "third generation" purification procedures cannot greatly alter the relative E1 activity, since the E1:E2 ratio remains constant. Detailed investigations by J.M.Lambert have failed to identify any metal other than zinc that allows full reconstitution of E1 activity after EDTA treatment. Zinc is therefore the only metal likely to be bound to arom at the outset of purification, and is probably the only metal important for DHQ synthase (E1) catalysis in N.crassa. A direct means to test this idea would be to grow the cells in medium supplemented with ^{65}Zn, and to purify the labelled enzyme.

4.3.3 Arom E1 and other DHQ synthases

The differences in apparent metal cofactor specificity between arom E1 and the DHQ synthases of other organisms require some comment. Potential shortcomings in the assignment of the cofactor specificity of the other enzymes have already been mentioned, (section 4.2.4) and in the light of the very high affinity of arom E1 for zinc, should be taken seriously. Nevertheless the mung bean, sorghum, B.subtilis and E.coli enzymes all appear to differ from arom E1 in their specificity for Co^{2+}, Mn^{2+} or Cu^{2+}, and in their inability to utilise Zn^{2+} (Saijo

and Kosuge, 1978; Yamamato, 1980; Hasan and Nester, 1978c; Maitra and Sprinson, 1978). All five DHQ synthases share a number of common features, including a strict requirement for NAD^+ and a transition metal cation in catalysis. The E.coli and N.crassa enzymes are both inhibited by NADH and are unstable to purification. The only other purified enzyme to be analysed chemically - that from mung bean - contained 0.2 g atom/mole of copper. The DHQ synthase activity of crude E.coli and B.subtilis extracts is normally stimulated by exogenously supplied cobalt or manganese ions, which suggests that the enzymes do not bind their metallic cofactors as tightly as does arom E1.

One may reasonably speculate that all known DHQ synthases are catalytically and structurally homologous. The enormous chemical diversity of the metallic cofactors that have now been identified, (Mn^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}) probably indicates that subtle transition metal chemistry is not important for catalysis in this extraordinary condensation reaction.

4.3.4 Evidence that arom E5 is sensitive to oxidation

When the product of the successful "third generation" purification experiment A7 was dialysed at $4^{o}C$ into a storage buffer containing 50% v/v glycerol, βME and a trace of zinc, an alarming decline in the E5:E2 ratio was recorded (see table 4.8). No such change in the E5:E2 ratio had been seen when the products of modified "second generation" purification procedures were dialysed into storage buffer supplemented with DTT. At this stage in the investigation a convincing rationale for the variation in the E5:E2 ratios of "second generation" arom samples was still lacking, and it became an urgent priority to establish the reasons for the instability of E5.

A further deterioration in the E5:E2 ratio of "third generation" arom was recorded when a sample was incubated without supplementary thiol

reagents under conditions comparable to those in the final dialysis step. There was a slight recovery in the E5:E2 ratio when a similar sample was incubated with DTT. The E5 activity of the arom complex was found to be highly sensitive to inactivation by N-ethyl-maleimide (NEM), while the other four activities were relatively stable. When the "second generation" arom sample A1, which appeared to have lost almost all E5 activity (see table 4.2) was treated with a high concentration of DTT (50mM), a 37-fold increase in E5 activity was monitored over two days. When the same sample was pretreated with NEM (2mM) to inactivate the residual E5 activity, and then incubated with DTT, a 22-fold increase over the initial E5 activity was still recorded.

The results listed above suggest that arom E5 activity is particularly sensitive to the thiol redox potential, and that modification of thiol groups by NEM or by oxidation can selectively inactivate the enzyme. The evidence is also consistent with the possibility that the progressive oxidation of thiol groups in the enzyme underlies the enormous range in the E5:E2 ratios of "second generation" arom samples. The failure of NEM pretreatment to abolish the DTT-dependent reactivation of latent E5 activity in these samples suggests that any thiol groups essential for E5 action are masked when E5 is inactivated. Formation of an intramolecular disulfide bridge (or of a mixed disulfide) is therefore a likely mechanism for the inactivation of the enzyme. In any case a substantial proportion of the lost activity is reconstituted on treatment with DTT.

4.3.5 Conflicting requirements for E1 and E5 stability

The documented instability of E5 activity posed a serious practical problem. An important stratagem adopted to improve the stability of E1 in third generation purification experiments was the

replacement of DTT by the combination of Zn^{2+} and βME; this change in the protective reagents was now directly implicated in contributing to the instability of E5. There was clear evidence that arom E5 activity declines progressively in buffers containing Zn^{2+} and substantial concentrations of unoxidised βME, and that the activity is stable in solutions containing fresh DTT. It should, however, be emphasised that in at least two "third generation" purification experiments a serious deterioration in the E5:E2 ratio was not experienced until the final dialysis step.

In the light of these findings stocks of "third generation" arom were dialysed into a storage buffer containing 0.4mM DTT and 0.04mM Zn^{2+}; there was a significant recovery in the E5:E2 ratio and no substantial loss of E1 activity (see table 5.8). All five arom activities were stable over many months at $-20^{o}C$ in the new storage buffer (see table 4.13).

4.3.6 Stability of arom E5

Despite its outstanding success on a number of occasions - and its reliable performance in the copurification of E1, E2, E3 and E4 - the "third generation" purification scheme did not always yield homogeneous arom with a high E5:E2 ratio. The evidence presently available indicates that there are probably three independent factors to be considered in the selective inactivation of E5.

E5 losses on storage of "second generation" arom probably occurred only after oxidative deterioration of the protective agent DTT, and may be associated with oxidation of arom E5 (see section 4.2.12). This is likely to be the explanation for the large variation in the E5:E2 ratio of "second generation" arom samples. The problem has not arisen when the purified enzyme complex is carefully stored in narrow glass tubes at $-20^{o}C$.

E5 losses sustained during purification by the "third generation" protocol may - in part - be due to the relative instability of E5 activity in buffers supplemented with zinc and βME. The evidence that latent E5 activity can be reconstituted in buffers containing DTT and traces of zinc implies that the presence of zinc is not the chief problem. It is possible that relatively low thiol redox potentials, obtainable only with DTT - and not with βME - are necessary to ensure the thermodynamic stability of E5 activity against oxidation. Zinc may catalyse the oxidation reactions, but the protective reagents themselves are not destroyed rapidly under the conditions of a purification experiment.

No evidence has been obtained in this study to substantiate a claim that PMSF can inactivate arom E5 (Gaertner et al, 1979).

4.3.7 Evidence of residual protease activity

Further modifications to the "third generation" purification protocol were investigated in an attempt to ensure quantitative recovery of E5 activity (see section 3.7). In one experiment, A21, there was a serious decline in the E5:E2 ratio during the dialysis step that precedes chromatography on Blue Dextran-Sepharose. There was no reason to suppose that oxidation was responsible for inactivation of E5, as there was direct evidence from DTNB assays that the protective thiol reagent (DTT) was intact (see table 4.10). SDS PAGE of the final product showed two large polypeptides in approximately equal abundance, the 165kDa arom polypeptide and a smaller polypeptide of approximately 125kDa (fig. 4.4). Both polypeptides stained for E3 activity after SDS PAGE. Samples taken before and after the critical dialysis step showed that it was associated with a decline in the relative abundance of the 165 kDa polypeptide with respect to the 125kDa polypeptide. All the evidence

TABLE 4.10 Purification table for modified "third generation" experiment A21

Stage	Vol. (ml)	Protein (mg)	E2 (units)	E2 specific activity (u/mg)	E2 pfn. factor (fold)	E2 net yield (%)	E5:E2 activity ratio
mycelial extract	1020	3670	42.5	0.012	1.0	100	(44.0)
DEAE cellulose I	1020	2350	36.6	0.016	1.33	86.1	(45.0)
$(NH_4)_2SO_4$ fractionation	23.3	350	28.1	0.081	6.75	66.1	62.3
dialysis I	31.5	213	28.1	0.132	11.0	66.0	58.9
DEAE-sephacel	115	55.2	22.8	0.412	34.3	53.6	55.2
dialysis II	127	81.3	23.7	0.292	24.3	55.8	38.2
Blue-dextran--sepharose	7.5	2.42	16.5	6.81	568	38.8	33.2

NOTES:
1. All protein concentrations were estimated by the method of Bradford (1976)
2. E5:E2 activity ratios were calcuated from triplicate assay traces, except those (in parentheses), which were calculated from duplicate assays on high speed supernatants of the crude extracts

pointed to a direct link between the loss of E5 activity and a proteolytic cleavage of the arom polypeptide that left the other four activities intact and did not affect the chromatographic properties of the enzyme complex. Re-examination of the results of earlier purification experiments suggested that this was not an isolated instance of proteolytic damage to the enzyme complex during the later stages of purification; the 125kDa polypeptide had been a persistently troublesome "contaminant" in third generation purification experiments. The action of an endogenous protease therefore provides a rationale for the failure of E5 to copurify with the other activities in these experiments.

4.3.8 Development of a fourth generation purification protocol

The conflicting interests of E5 and E1 stability were finally reconciled through the development of a "fourth generation" purification scheme. There were three major departures from the "third generation" protocol:

1. DTT (0.4mM) was reinstated as the protective thiol reagent, in conjunction with 20 μM zinc acetate, on the basis that this combination was equally as effective as βME/Zn^{2+} in protecting E1 from inactivation during chromatography on DEAE-sephacel, and was also able to stabilise E5 activity. The estimated free zinc concentration of this cocktail is 2.5×10^{-12}M.
2. EDTA was reinstated as a component of the antiprotease strategy, on the basis that it does not inactivate E1 when added to the cell extraction buffer. This is presumably because total divalent cations are in excess of EDTA during mycelial extraction. If so the extraction medium in the "fourth generation" scheme may constitute a "metal ion buffer" since $[M^{2+}] > [EDTA] > [Zn^{2+}]$. At least one

major N.crassa protease is metal-dependent (Siepen et al, 1975) and there are grounds for suspecting that the protease problem resurfaces intermittently in third generation procedures precisely because steps are taken to avoid removing transition metal cations. Benzamidine was added to all dialysis buffers, in addition to PMSF, as an additional precaution against residual protease activities.

3. DEAE-cellulose was replaced by DEAE-Sephacel in the second chromatography step. The latter material gave more efficient and reproducible separations.

Further details of the "fourth generation" purification scheme are given in table 4.11 and in section 3.7.

4.3.9 Comparison of third and fourth generation purification procedures

Table 4.12 summarises the results of experiment A23, in which the arom complex was purified to electrophoretic homogeneity in almost 50% overall yield by the "fourth generation" procedure. The final E2 specific activity was comparable to that of homogeneous "third generation" samples. The final activity ratios were compared to those of the "third generation" sample A7 - which had been stored at $-20^{\circ}C$ for 18 months - and to those of the proteolytically damaged sample A21 (table 4.13).

It should be clear that the catalytic activities of the "fourth generation" product A23 were essentially the same as those of the third generation product A7, and that the subunit structure and all five catalytic activities of the A7 product were essentially intact after 18 months storage at $-20^{\circ}C$ (compare tables 4.13 and 4.8 and see fig. 4.4).

It is interesting to note that earlier workers have experienced unpredictable behaviour of E5 during purification. Jacobson et al

Table 4.11 Summary of alterations made to third generation purification protocol in the development of the fourth generation protocol

Change; new feature	Rationale
DTT is preferred to β ME (except in elution of Blue column)	β ME does not stabilise E5 against oxidation
Buffers supplemented with zinc contain 0.4mM DTT, 20μM zinc acetate	stabilise both E1 and E5
Myocelial extraction buffer contains EDTA (5mM)	inactivate metal-dependent proteases
DEAE-Sephacel replaces DEAE-cellulose in "positive" chromatography	improved yield and reproducibility
Benzamidine (1mM) included in all dialysis buffers	inhibit trypsin-like proteases
Blue Dextran Sepharose chromatography A. New washing/recycling procedure, incorporating nonionic detergent	remove denatured proteins, zinc and adventitious metals from previous runs
B. Sample dialysed against 20 volumes of zinc-free buffer containing 0.4mM DTT before loading onto column	reduce zinc concentration and ionic strength; stabilise E5 against oxidation

TABLE 4.12 Purification table for "fourth generation" experiment A23

Stage	Vol. (ml)	Protein (mg)	E2 (units)	E2 specific activity (u/mg)	E2 pfn. factor (fold)	E2 net yield (%)	E5:E2 activity ratio (%)
mycelial extract	830	2822	40.2	0.0141	1.0	100	nd
DEAE cellulose	850	2890	41.6	0.0144	1.02	103	nd
$(NH_4)_2SO_4$ fractionation	18	450	39.3	0.087	6.17	97.8	nd
dialysate I	28.5	399	36.4	0.091	6.45	90.5	65.9
DEAE-sephacel	105	84.0	30.9	0.368	26.1	76.9	64.8
dialysate II	102	77.5	29.2	0.376	26.6	72.6	67.4
Blue Dextran Sepharose	9.75	*2.06	19.8	*9.63	683	49.3	59.4
final dialysate	-	-	-	-	-	-	69.1

NOTES: 1. Protein concentrations were estimated by the method of Bradford (1976) except those marked *; the concentration of the homogeneous arom complex was estimated by taking $E^{1\%}_{280} = 11.0$ (Lumsden & Coggins 1978)

2. E5:E2 activity ratios were calculated from duplicate assay traces

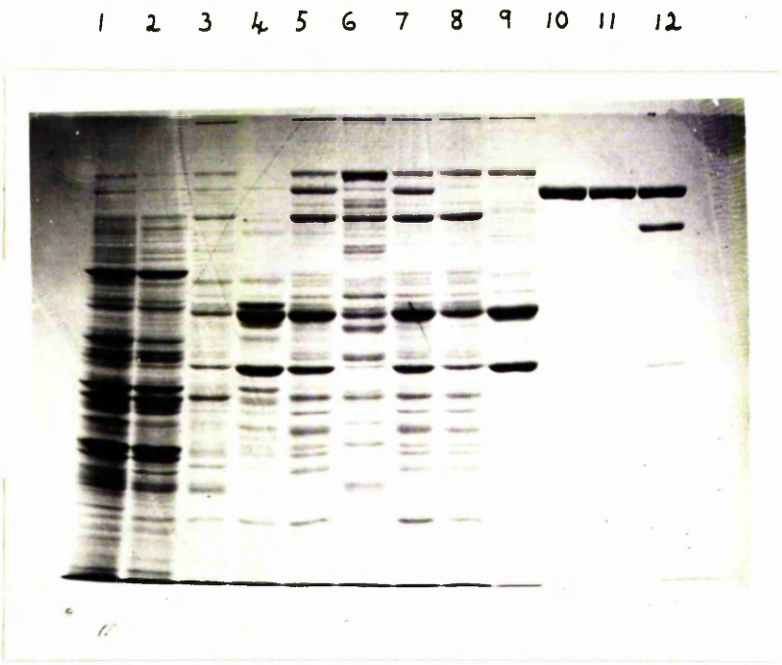
Table 4.13 Activity of "third" and fourth" generation arom samples

arom sample	age (mths)	activity ratios					activity ratios		
		$\overrightarrow{E1}$	$\overrightarrow{E2}$	$\overleftarrow{E3}$ pH10.6	$\overrightarrow{E4}$	$\overleftarrow{E5}$	$\overrightarrow{E3}$ pH7.0	$\overleftarrow{E3}$ pH7.0	$\overrightarrow{E5}$
A 7	20	89.3	100	262.6	89.3	63.7	269.4	38.3	180.5
A 21	1	96.1	100	267.1	98.0	43.3	243.2	35.4	105.3
A 23	0	98.0	100	266.3	94.4	69.1	263.9	36.5	173.5

NOTES: 1. Standard activity ratios were calculated from triplicate assay traces.

2. Other activity ratios were calculated from duplicate assays (A 23) or single assays (A 21 and A 7).

Figure 4.4 Purification of the arom multifunctional enzyme



Samples from pools at different stages in the 4th generation purification experiment A23 were subjected to SDS PAGE through a 10% gel, which was stained for protein:

1. crude extract
2. DEAE cellulose pool
3. 40-50% ammonium sulphate fraction (dialysate I)
5. DEAE Sephacel pool
7. dialysis II
8. material not bound to Blue column
9. material eluted from Blue column at 0.5M KCl
10. A23 arom final pool after dialysis
11. A7 arom (used in kinetics experiments)
12. A21 arom (modified 3rd generation experiment)

(1972) reported that E5 activity occasionally vanished during purification, and speculated that this was due to dissociation of an E5 "subunit". It is possible that the endogenous protease activity associated with E5 losses in "third generation" procedures is not always expressed to the same extent under routine growth conditions for N.crassa.

No evidence of the residual E5-specific protease has ever been encountered in "second generation" purification procedures. The same suite of protective reagents, EDTA, PMSF, benzamidine and DTT, is employed in the "fourth generation" purification scheme. It is therefore hoped that the "fourth generation" protocol will prove to be more reliable than the original "third generation" method for the copurification of the five arom activities.

4.4 Discussion: The development of methods for the purification of the arom multifunctional enzyme

The isolation of an "essentially homogeneous" arom multienzyme complex was first reported by Burgoyne et al (1968). These workers used gel electrophoresis under native conditions and analytical ultracentrifugation as their main criteria of homogeneity. A new purification scheme was developed by Lumsden and Coggins (1977) who isolated a multifunctional arom enzyme complex that was homogeneous by the criterion of polypeptide size, as judged by SDS PAGE. Subsequent work demonstrated conclusively that the arom multifunctional enzyme consists of two identical polypeptide subunits of 165,000 Da (Lumsden and Coggins, 1978). The earlier reports of a more complex subunit structure for the arom complex (Jacobson et al, 1972; Gaertner, 1972) were rationalised in terms of the proven susceptibility of the intact arom polypeptide to damage by endogenous proteases (Lumsden and Coggins, 1977; Gaertner and Cole, 1976).

In the present study the copurification in constant ratio of the five activities of the arom complex has been introduced as an additional criterion for evaluating the homogeneity of the purified enzyme. This condition follows from the general requirement that a homogeneous enzyme should have well-defined specific activities and should not be contaminated by species that lack one or more catalytic activities. In the case of a multifunctional enzyme such as the arom complex it is particularly important to demonstrate that the enzyme can be isolated with all of its catalytic activities essentially intact. This is because the study of a family of species that are partially deficient in particular enzyme activities will lead to serious errors in the evaluation of the overall catalytic properties of the complex.

During the course of the present study it has been shown that the established procedures for the purification of the arom multifunctional enzyme, including the "second generation" procedure developed in our own laboratory (Smith and Coggins, 1983), do not fulfill the stringent "copurification" criterion for the homogeneity of the final product. The only scheme developed elsewhere that has been reported to yield a preparation of the arom multifunctional enzyme that is homogeneous with respect to polypeptide size is that of Gaertner and Cole (1977). No particular precautions were taken to stabilise the activities of E1 and E5, and the specific activities of the purified enzyme were not reported. An earlier report from the same laboratory (Welch and Gaertner, 1976) quotes relative E1 and E2 activities of 5 and 100 respectively, for a sample of the arom complex that was partially purified by the general method of Gaertner (1972), which yields proteolytically degraded enzyme (Lumsden and Coggins, 1977). A comparison of this activity ratio with the E1:E2 ratio of the arom complex purified by the "third generation" protocol (table 4.8) reveals

that about 95% of the E1 activity had probably been lost. Welch and Gaertner (1975, 1976) nevertheless proceeded to use an arom preparation of this quality in a series of experiments that is frequently cited as a definitive demonstration that the arom multifunctional enzyme has "novel" kinetic and regulatory properties (for example Price and Stevens, 1980). It may be advisable to interpret the results of these experiments with some caution.

Two methods that allow the copurification in constant ratio of the five activities of the arom complex have now been developed. The evolution of the new procedures from the original protocol of Lumsden and Coggins (1977) proceeded in a series of discrete steps, as the strengths and weaknesses of the earlier procedures were evaluated. It was found that different aspects of the stability of the enzyme complex could not be treated independently. The stability of E1 was greatly improved by supplementing the chromatography buffers with zinc, but in making this change it was necessary to take into account the stability of E5 and the choice of thiol reducing-agent, the behaviour of the Blue Dextran-Sepharose affinity matrix, and the action of endogenous proteases.

It should be noted that the shortcomings of the established arom purification methods were only recognised when anomalies in the activity ratios of the electrophoretically homogeneous enzyme were encountered. Much of the subsequent work on the development of modified purification schemes was guided by careful observations of changes in the activity ratios. The "copurification" criterion adopted in this study therefore proved to be a crucial additional test of the homogeneity of the end-product of the fractionation procedures. Our experience with the arom multifunctional enzyme indicates that if one wishes to isolate the undamaged enzyme it is not sufficient to show that all five activities remain tightly associated during purification. Similar

considerations may apply quite generally to the purification of multifunctional enzymes and multienzyme complexes.

The arom multifunctional enzyme isolated by the new purification procedures is considered to be essentially homogeneous with respect to subunit structure and catalytic activity. There is no evidence that it is significantly different from the enzyme present in the living cell. This preparation is therefore suitable for detailed studies on the overall catalytic properties of the complex.

Attention is drawn to the relative velocities of the "forward" reactions of the five enzyme activities of the arom complex, assayed at saturating substrate concentrations and at pH 7.00. The activity ratios for the forward reactions previously estimated for a partially purified and proteolytically damaged sample of arom were 5:100:20:10:25 (Welch and Gaertner, 1976). The activity ratios estimated here under comparable conditions (except E5) for the intact arom complex are 98:100:264:94.4:173.5 (table 4.13). The significance of these activity ratios is discussed in section 8.1.

CHAPTER 5 PURIFICATION AND PRELIMINARY CHARACTERISATION OF CHORISMATE SYNTHASE FROM N.CRASSA

5.1 Introduction

Chorismate synthase is probably the most unusual and least well-understood enzyme in the shikimate pathway. For the purposes of this project it also assumes enormous practical importance as a coupling enzyme in continuous fluorimetric and spectrophotometric assays of the forward reaction of arom E5, EPSP synthase (see Chapter 6).

Two particular features of chorismate synthase action, the cofactor requirement for NADPH and the time-dependent "activation" of the enzyme, can complicate the design of continuous coupled assay systems. The modification of the sensitive fluorimetric assay system of Gaertner and De Moss (1970) for use in steady state kinetics experiments on EPSP synthase called for relatively large amounts of chorismate synthase and anthranilate synthase that were essentially free of interfering activities such as NADPH oxidase and EPSP synthase. It was therefore necessary to undertake further work on the purification and characterisation of chorismate synthase from N.crassa. The subsequent isolation of chorismate synthase of very high specific activity provided an attractive opportunity for a preliminary examination of outstanding problems relating to the structure and catalytic mechanism of this enzyme.

5.2 Previous work on chorismate synthase

The chemistry of chorismate synthase catalysis and the significance of the associated "flavin reductase" and diaphorase activities is very poorly understood; the subject was discussed in section 1.6.6.

5.2.1 Purification of chorismate synthases

Chorismate synthase from E.coli has not been purified substantially. The enzyme is somewhat inconvenient to handle, as it is most readily assayed under hydrogen (Morell et al, 1967).

The chorismate synthase/dehydroquinate synthase complex of B.subtilis has been purified to homogeneity (Hasan and Nester, 1978 a,b,c). It consists of three types of polypeptide subunit, which have been assigned enzyme activities as follows:-

13 kDa	"flavin reductase" (diaphorase)	required for chorismate synthase activity
24 kDa	chorismate synthase	
17 kDa	dehydroquinate synthase	

The subunit stoicheiometry of the intact complex was not reported.

Chorismate synthase has been very extensively purified from N.crassa. After ammonium sulphate fractionation of the crude mycelial extract, a further 70-fold purification yielded material of "$\geqslant$90% homogeneity" with respect to polypeptide size, in less than 3% overall yield (Welch et al, 1974). Chorismate synthase activity appeared to be associated with a polypeptide of approximately 55 kDa. A native molecular weight of 110 kDa had earlier been estimated by density gradient centrifugation (Gaertner and Cole, 1973) but "at least two multimeric states" were observed in the later study (Welch et al, 1974). The most highly purified enzyme fractions exhibited an NADPH-dependent DCPIP reductase (diaphorase) activity which was stimulated by EPSP. It was proposed that the diaphorase function is essential for the "activation" of chorismate synthase.

On the strength of the results summarised above Welch et al (1974) concluded that the native chorismate synthase enzyme is a dimer of identical polypeptides which catalyses the conversion of EPSP to chorismate and also the associated diaphorase reaction. In view of the evidence that there are numerous distinct diaphorase activities in

N.crassa extracts (Welch et al, 1974), the insubstantial evidence presented for the polypeptide homogeneity of the purified enzyme, and the serious losses of chorismate synthase activity sustained at several stages during enzyme fractionation, this conclusion may be considered a little premature.

A method for the separation of chorismate synthase, anthranilate synthase, and the arom multifunctional enzyme from N.crassa has been developed in our own laboratory (J.R.Coggins, A.A.Coia, unpublished results). This method exploits an earlier observation that all three enzyme complexes behave in a rather similar way during ammonium sulphate fractionation and chromatography on DEAE cellulose, and that only the arom multifunctional enzyme and chorismate synthase readily bind to phosphocellulose (Cole and Gaertner, 1975). In the positive DEAE-cellulose chromatography step of the "second generation" arom purification scheme chorismate synthase and anthranilate synthase elute in peaks that closely overlap the peak of arom E3 activity. When these fractions are pooled and pumped through a Blue Dextran Sepharose column only the arom E3 activity is bound to the column. Chorismate synthase and anthranilate synthase acitivites are subsequently separated by chromatography on phosphocellulose, but are far from homogeneous (J.R.Coggins, and A.A.Coia, unpublished).

5.2.2 Enzyme activities of purified chorismate synthases

The "flavin reductase" (diaphorase) subunit of the B.subtilis chorismate synthase complex has been isolated (Hasan and Nester, 1978a). It catalyses the NADPH-dependent reduction of the synthetic electron-acceptor DCPIP. The other subunits of the enzyme complex apparently confer flavin dependence on this reaction. The isolated "flavin reductase" also catalyses the NADPH-dependent reduction of the 2,3-dihydroxybenzoate chelate of iron (III), an important step in the

reductive assimilation of iron(III) compounds by B.subtilis (Gaines et al, 1981).

Like the isolated B.subtilis "flavin reductase", the highly purified chorismate synthase from N.crassa has also been reported to catalyse the flavin-independent reduction of DCPIP by NADPH. Welch et al (1974) reported that the DCPIP reductase and chorismate synthase activities of their enzyme preparation were of comparable magnitude, and that the DCPIP reductase activity was stimulated by EPSP.

Under aerobic conditions both NADPH and FMN are required for activation of the N.crassa and B.subtilis chorismate synthases. Full activation of the N.crassa enzyme is not instantaneous, and typically takes 3-4 minutes (Welch et al, 1974; Hasan and Nester, 1978b). This is an important consideration, since the success of a coupled assay system involving chorismate synthase is dependent on the rapid response of the coupling enzymes to their respective substrates (McClure 1969).

There is some evidence that the chorismate synthases of E.coli and N.crassa can be activated without the intervention of a flavin nucleotide (Morell et al, 1967; Welch et al, 1974). Under a hydrogen atmosphere both enzymes are activated by the powerful reducing agent dithionite, but the evidence that this process is flavin-independent is not conclusive. The partially purified E.coli enzyme is reported to be activated by hydrogen alone, in the presence of a platinum catalyst, after it has been carefully "scrubbed" of flavin.

Two possible roles for flavin reductase activity in chorismate synthase catalysis were discussed in section 1.6.6:

1. flavin reductase confers catalytic activity on chorismate synthase by reducing an iron (III) centre in the inactive enzyme to an oxygen-sensitive iron (II) centre (Morell et al, 1967).

2. flavin reductase confers catalytic activity on chorismate synthase by reducing a functional disulphide bond at the active site (Welch et al, 1974).

5.3 Objectives of the present study

N.crassa was considered to be the most promising source of the two coupling enzymes required for fluorimetric EPSP synthase assays. Preliminary work revealed that chorismate synthase and anthranilate synthase isolated as byproducts of the "second generation" arom purification procedure, as described in section 5.2.1, are not suitable for use in coupled fluorimetric assays. The partially purified chorismate synthase is contaminated by EPSP synthase activity - probably a minor proteolytic fragment of the arom complex - while the quantities of anthranilate synthase activity available are wholly insufficient for use in coupled assays. Further purification of chorismate synthase yielded material that was very far from homogeneous but exhibited a higher specific activity than the "$\geqslant$90% homogeneous" material of Welch et al (1974). This result cast some doubt on the validity of the subunit structure assigned to chorismate synthase in the earlier study.

The development of new strategies for the purification of chorismate synthase from N.crassa was undertaken with two immediate objectives:

1. to ensure supplies of chorismate synthase suitable for use as a coupling enzyme.
2. to establish optimal conditions for the use of this enzyme in fluorimetric EPSP synthase assays.

5.4 Results and Discussion

5.4.1 Purification of chorismate synthase to electrophoretic homogenity

The purification of chorismate synthase from N.crassa to electrophoretic homogeneity is documented in Table 5.1 and in Figure 5.1. The purification procedure is described in section 3.8. It consists of a succession of steps modified from earlier procedures (Wegman and De Moss, 1965; Cole and Gaertner, 1975; Lumsden and Coggins, 1977; A.A.Coia and J.R.Coggins, unpublished work), followed by a final affinity chromatography step.

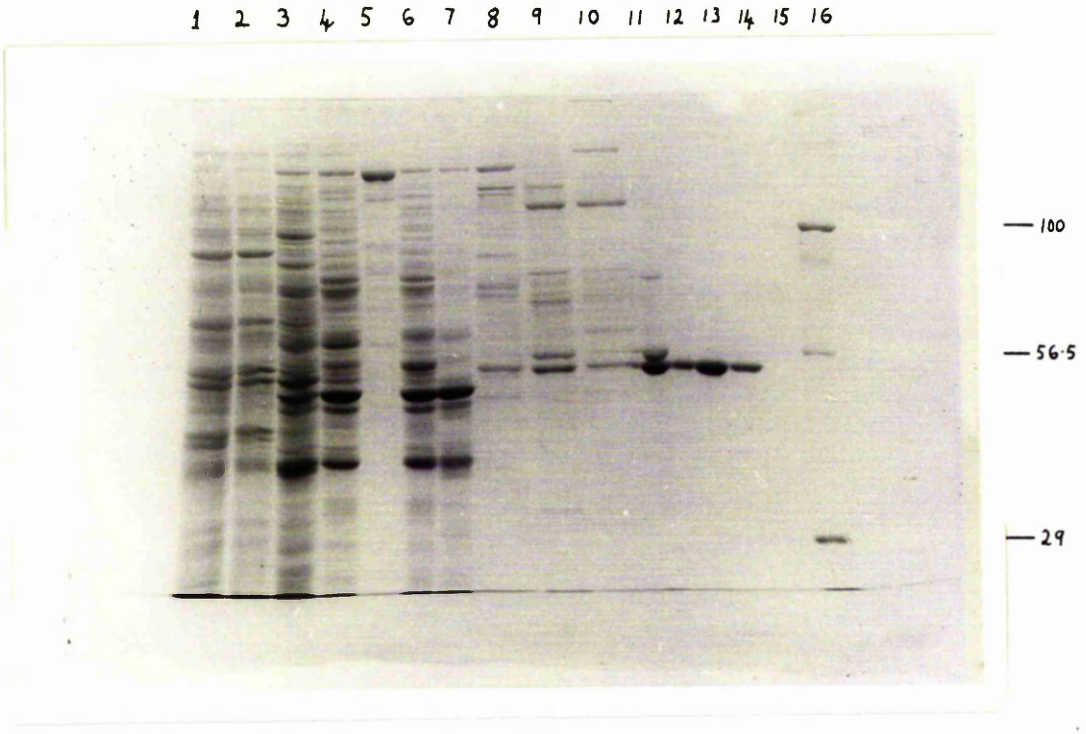
The material that failed to bind to the first phosphocellulose column in the new purification procedure contained valuable amounts of anthranilate synthase activity. After concentration the partially purified (15-fold) anthranilate synthase was suitable for use in coupled fluorimetric assays, although it was not completely free of a troublesome NADPH oxidase activity. Chorismate synthase was eluted from the first phosphocellulose column with a concentration gradient of potassium phosphate. The pooled material had a specific activity of 5.86 U/mg, higher than that reported by Welch et al (1974) for the "homogeneous" enzyme, and it was suitable for use as a coupling enzyme in fluorimetric assays.

Chorismate synthase was further purified in a second stage of chromatgraphy utilising phosphocellulose. The enzyme was bound to the column after equilibration with 40 mM potassium phosphate, 0.2 mM DTT, pH 6.5, and was eluted from the column with the same buffer supplemented with the substrate and cofactors EPSP, NADPH and FMN, at the same pH and conductivity. Approximately 25% of the activity applied to the column was recovered by "substrate elution". The remainder was eluted with a salt step. Results from a number of experiments indicate that there is no specific displacement of chorismate synthase from phosphocellulose at low phosphate concentrations unless both FMN and (valuable) EPSP are

Table 5.1 Purification of chorismate synthase

Stage	V (ml)	Protein (mg)	Chorismate synthase activity (units)	Chorismate synthase sp. act. (u/mg)	Chorismate synthase pfn. (fold)	Anthranilate synthase activity (units)	Anthranilate synthase sp. act. (u/mg)	Anthranilate synthase pfn. (fold)
Mycelial extract	1240	6700	nd	-	-	9.94	.0015	1.0
DE52 I	1180	4720	nd	-	-	9.25	.00195	1.3
40-50% $(NH_4)SO_4$ (dialysate)	58	812	46.7	.058	1.0	7.12	.0088	5.9
DE52 II	144	190	38.9	.205	3.5	4.21	.022	14.7
Blue Dextran Sepharose	150	174	36.5	.207	3.6	3.70	.021	14.2
Phosphocellulose I flow-through	155	121	0.0	-	-	3.22	.027	17.8
phosphate gradient	29.5	4.5	26.4	5.86	101.0	0.0	-	-
Phosphocellulose II substrate elution	17.0	nd	6.7	nd	-	-	-	-
salt step	4.6	3.24	11.9	3.67	63.2	-	-	-

Figure 5.1 Purification of chorismate synthase



Samples taken from pools at different stages in the chorismate synthase purification experiment documented in table 5.1 were subjected to SDS PAGE in a 10% Laemmli gel:

1. mycelial extract
2. DEAE cellulose I
3. 40-50% ammonium sulphate fraction
4. DEAE cellulose II
6. Blue Dextran Sepharose flow-through
7. phosphocellulose I : anthranilate synthase pool
9. phosphocellulose I : chorismate synthase pool
12, 13, 14. phosphocellulose II : substrate elution pools
16. E.coli pyruvate dehydrogenase, carbonic anhydrase.

included in the eluting buffers. It should be noted that these are the two components required in the "slow" phase of chorismate synthase activation (Section 5.4.3).

SDS PAGE showed that the enzyme pool prior to the final chromatography step contained at least eight major polypeptide species. The material eluted from the phosphocellulose column with substrates and cofactors was judged homogeneous by the criterion of SDS PAGE (see Figure 5.1). A reliable estimate of the final specific activity was not obtained because of the low protein concentration and the difficulty of removing flavin from the enzyme pool. However the SDS PAGE results and the quantitative recovery of enzyme activity in the final chromatography step indicate that at least a further threefold purification of the enzyme was achieved, giving material with a final specific activity of approximately 15-20 Units/mg. This implies that the homogeneous chorismate synthase is purified approximately 1500-fold over crude mycelial extracts.

The final two stages of chromatography on phosphocellulose purify chorismate synthase approximately 100-fold. The key step in the purification of the tryptophan sensitive DAHP synthase (EO) of N.crassa was also substrate elution from phosphocellulose (Nimmo and Coggins, 1981[a]); the EPSP synthases (E5) of E.coli and pea seedlings have recently been purified in a similar way (Lewendon and Coggins, 1983; Mousdale and Coggins, 1983). All of these enzymes are relatively tightly bound to anion exhangers such as DEAE cellulose at neutral pH; it is therefore unlikely that their high affinity for phosphocellulose is due simply to electrostatic effects. Cole and Gaertner (1975) have suggested that phosphocellulose acts as a specific "affinity absorbent" for a number of shikimate pathway enzymes which have phosphate-containing substrates. The results described above are fully consistent with this proposal.

5.4.2 Specificity of chorismate synthase for flavins and reducing agents

Under aerobic conditions the activity of the highly purified chorismate synthase obtained as described above is absolutely dependent on the two cofactors NADPH and FMN. The enzyme is saturated by very low concentrations ($\ll 10\mu M$) of the substrate EPSP. In agreement with the results of Welch et al (1974) and Gaertner and Cole (1973), NADH cannot substitute for NADPH, and FAD gives only partial stimulation. (It is possible that the latter effect is due to contaminating FMN in commercial preparations of FAD). A number of other reducing agents, in conjunction with FMN, failed to elicit significant chorismate synthase activity (see Table 5.2).

The powerful one-electron reducing agent dithionite was also ineffective in stimulating chorismate synthase when supplied alone, or in conjunction with the reduced form of the electron carrier PMS, or in an oxygen-free environment with reduced FAD and dithiothreitol. Reduced FMN and dithionite together gave rapid stimulation of the enzyme; the rate of chorismate synthesis was almost identical to that seen in the "aerobic" assay with FMN and NADPH (see Table 5.2). These results differ from those of Welch et al (1974) who observed stimulation of chorismate synthase activity by dithionite under anoxic conditions in the absence of exogenously supplied FMN. In the presence of other powerful reducing agents (dithiothreitol, reduced FAD and reduced PMS), and in the absence of molecular oxygen, the highly purified chorismate synthase obtained in the present study is essentially inactive (Table 5.2). It is therefore proposed that reduced FMN has a specific function in the activation of chorismate synthase and that it cannot be replaced by other reducing agents. Under aerobic conditions the requirement for reduced flavin is presumably met by the NADPH-dependent reduction of FMN.

Table 5.2 Activation of chorismate synthase by various reducing agents and specificity of the enzyme for FMN

Concentrations of cofactors and reducing agents etc. (all assays contain 50 μM EPSP)	Relative chorismate synthase activity
No reducing agent	≤1.0
+ 10 μM FMN	≤1.0
20 μM NADPH	≤1.0
+ 10 μM FMN	100
+ 10 μM FMN + 5 mM EDTA	84
+ 10 μM FMN + 10 μM (oxidised) DCPIP	≤2.5
+ 10 μM FAD	10 (after 4 mins)
50 μM NADH + 10 μM FMN	≤1.0
100 μM ferrocyanide + 10 μM FMN	≤1.0
1 mM DTT + 10 μM FMN	≤1.0
5 mg/ml sodium dithionite	≤2.5
+ 10 μM PMS (reduced)	≤1.0
+ 10 μM FAD (reduced)	≤2.0
+ 10 μM FAD (reduced) + 1 mM DTT	≤2.0
+ 10 μM FAD + 10 μM FMN (reduced)	60
+ 10 μM FMN (reduced)	96
+ 10 μM FMN + 10 μM DCPIP (reduced)	98

Material with a specific chorismate synthase activity of 5 U/mg was assayed as described in Chapter 3.

Welch et al (1974) have speculated that FMN is an intermediate electron carrier in the NADPH-dependent reduction of an unidentified oxygen-sensitive group at the active centre of chorismate synthase, although they presented no evidence to support this hypothesis. The results of Welch et al (1974) and of the present study are fully consistent with the alternative proposal that reduced FMN itself is a necessary component of the catalytically active form of chorismate synthase; it then becomes unnecessary to postulate the existence of an additional, unidentified, oxygen-sensitive centre in the enzyme.

5.4.3 Time-dependence of chorismate synthase activation

Unlike most conventional enzymes chorismate synthase does not immediately attain full activity when presented with the appropriate substrate and cofactors. When the chorismate synthase reaction is initiated by adding enzyme to the standard "aerobic" assay cocktail the reaction gradually accelerates over several minutes until a final constant rate of reaction is achieved. In this study no evidence was obtained to support the contention of Welch et al (1974) that the rapidity of chorismate synthase activation depends on the purity of the enzyme. As noted in the earlier study the activation of the enzyme is substantially slower at lower FMN concentrations, although the final rate of reaction is little altered (Table 5.3). This is the one established property of the enzyme that appears to favour the view that FMN is simply a cofactor in chorismate synthase "activation" and does not participate directly in the conversion of EPSP to chorismate.

The time required for full activation of chorismate synthase after initiation of the reaction is substantially reduced if the enzyme is preincubated with EPSP and FMN together. Preincubation with NADPH and FMN, or NADPH and EPSP, is ineffective. The reaction "lag" cannot be eliminated completely in this way, as was claimed by Welch et al 1974 (see Table 5.3).

Table 5.3 Time-dependence of chorismate synthase activation

Components of assay pre-incubated for 4 mins at 25°C	Component added to initiate assay	Activation time (mins)	Final relative velocity of chorismate synthase
Chorismate synthase			
+ 20μM NADPH + 1μM FMN	50μM EPSP	0.8	93
+ 20μM NADPH + 50μM EPSP	1μM FMN	0.8	90
+ 50μM EPSP + 1μM FMN	20μM NADPH	0.2	98
50μM EPSP + 20μM NADPH			
+ 1μM FMN	enzyme	0.8	94
+ 10μM FMN	enzyme	0.4	100
50μM EPSP + 5 mg/ml sodium dithionite			
+ 1μM FMN (reduced)	enzyme	$\leqslant$ 0.4	61
+ 10μM FMN (reduced)	enzyme	$\leqslant$ 0.2	96

NOTES:

1. All assays were conducted in buffer B; highly purified chorismate synthase (5 U/mg) was diluted into buffer B, 10mU were added to each assay (final volume 1ml).

2. The progress of the chorismate synthase reaction was followed directly at 275nm.

3. The "activation time" of the assay is a measure of the length of the characteristic "lag" period that precedes the linear phase of the reaction; it was estimated by the method of McClure (1968).

The results in Table 5.3 indicate that EPSP and FMN are both required in a relatively slow process ($\tau \approx 0.8$ mins) that "primes" chorismate synthase for catalytic activity. The fully active state is probably attained in a somewhat faster process ($\tau \approx 0.2$ mins) when EPSP, FMN and NADPH are all present. These events are very much slower than the turnover rate of the enzyme, which is estimated to be approximately 200 s^{-1}. Since the reducing agent NADPH is not required for the slow phase of enzyme activation it is unlikely that redox processes are involved. The slow phase of chorismate synthase activation is most easily rationalised if the reversible binding of FMN to chorismate in the presence of EPSP is followed by a slow conformational change which locks FMN into its binding site. The "activated" form of the enzyme would then contain tightly bound FMN that would dissociate only relatively slowly. This explanation of events is compatible with all the established properties of the enzyme, and with the proposal that FMN is not only required in the "activation" process, but also participates in the conversion of EPSP to chorismate.

When chorismate synthase is assayed in an anoxic environment with reduced FMN and dithionite the activation of the enzyme is very rapid. The extent of activation is more strongly influenced by the total flavin concentration than in comparable "aerobic" assays with FMN and NADPH (Table 5.3). This result may be interpreted in terms of activation of the enzyme through reversible binding of the reduced form of FMN.

The practical consequence of the time-dependence of chorismate synthase activation is that considerable caution must be exercised in the use of this enzyme in continuous coupled fluorimetric assays of EPSP synthase activity. However, if sufficiently large amounts ($\approx$10 mU/ml) of chorismate synthase are employed the conversion of EPSP to chorismate reaches a steady state within one minute, and the steady-state

concentration of EPSP in the coupled assay (estimated by the general method of McClure, 1968) is vanishingly low ($\leq$ 5 nM) under routine assay conditions. This implies that the active and inactive forms of chorismate synthase both have a very high affinity for EPSP.

5.4.4 Utilisation of NADPH by chorismate synthase

The specific requirement for NADPH in chorismate synthase activation raises the question of whether there is stoicheiometric oxidation of NADPH to $NADP^+$ when the reaction is conducted in an aerobic environment, as has been assumed by Hasan and Nester (1978b). Welch et al (1974) reported that under anoxic conditions NADPH is not consumed stoicheiometrically by the N.crassa enzyme. The results obtained in the present study using highly purified material indicate that in air-saturated buffer NADPH is oxidised at less than 0.2% of the rate of EPSP conversion to chorismate. The enzyme also exhibits insignificant NADPH oxidase activity in the absence of EPSP (Table 5.4).

When highly purified chorismate synthase is supplied with an artificial electron acceptor the enzyme-catalysed oxidation of NADPH is readily demonstrated (Table 5.4). The NADPH-dependent DCPIP reductase or "diaphorase" activity of chorismate synthase is totally dependent on FMN and is 95% inhibited by 25μM EPSP. Electrophoretically homogeneous material and enzyme purified up to the first phosphocellulose chromatography step have essentially the same relative diaphorase and chorismate synthase activities (Table 5.5). After non-denaturing gel electrophoresis of the most highly purified chorismate synthase (containing FMN) an identical multiplet of very closely spaced bands was seen when the gels were stained for DCPIP reductase activity and for protein (not shown). These results are taken as clear evidence that the DCPIP reductase is a component activity of the isolated chorismate synthase enzyme complex.

Table 5.4 "Diaphorase" activities of highly purified chorismate synthase

Enzyme activity assayed: concentrations of substrates and cofactors	Relative diaphorase activity (aerobic chorismate synthase = 100)
DCPIP reductase	
50μM DCPIP + 50μM NADPH	≤ 0.1
+ 25μM EPSP	≤ 0.1
+ 10μM FMN	10.9
+ 10μM FMN + 5 mM EDTA	13.3
10μMDCPIP + 50μM NADPH	
+ 10μM FMN	10.7
+ 10μM FMN + 25μM EPSP	0.6
+ 10μM FMN + 250μM chorismate	7.8
+ 1μM FMN	2.3
+ 10μM FAD	≤ 0.25
10μM DCPIP + 270μM NADH + 10μM FMN	≤0.25
Cytochrome c reductase	
0.5 mg/ml cytc + 50μM NADPH	≤ 0.1
+ 10μM FMN	12.5
+ 10μM FMN + 25μM EPSP	0.9
NADPH oxidase (0_2 as acceptor)	
50μM NADPH	≤ 0.20
+ 10μM FMN + 50μM EPSP	≤ 0.20
+ 50μM EPSP	≤ 0.20
+ 10μM FMN	≤ 0.40

Material with a specific chorismate synthase activity of 5U/mg was assayed as described in Chapter 3.

Highly purified chorismate synthase can also catalyse the NADPH and FMN-dependent and EPSP-sensitive reduction of horse cytochrome C (Table 5.4) and of ferricyanide (not shown). The DCPIP reductase activity is absolutely specific for NADPH and FMN, and will not utilise NADH or FAD (Table 5.4). The DCPIP reductase therefore has the same cofactor specificity as the aerobic chorismate synthase reaction. In the presence of only 10μM oxidised DCPIP, but not of 10μM reduced DCPIP, the conversion of EPSP to chorismate is completely abolished (Table 5.2).

These findings are fully consistent with the idea that the isolated N.crassa chorismate synthase is able to catalyse the NADPH-dependent reduction of FMN, as was originally proposed by Welch et al (1974). The results also clearly indicate that the two component enzyme activities are carried by polypeptide chains that are identical or of very similar size. The failure of chorismate synthase to exhibit significant NADPH oxidase activity (molecular oxygen as final electron acceptor) indicates that if reduced FMN does indeed participate in the NADPH-dependent reduction of DCPIP and in the conversion of EPSP to chorismate it must normally remain bound to the enzyme throughout the catalytic cycle.

5.4.5 Reduced flavins and chorismate synthase catalysis

It has been suggested that there is an oxygen-sensitive iron(II) centre or a reduced disulphide bridge at the catalytic site of chorismate synthase that attains its reduced state during "activation" of the enzyme, by accepting reducing equivalents derived from NADPH (Morell et al, 1967; Welch et al, 1974).No evidence to support the existence of a redox centre of either type has been obtained in the present study. In the presence or absence of the cofactor/substrate cocktail FMN(10μM),

NADPH(20μM) and EPSP(50μM) chorismate synthase is not highly sensitive to inactivation by powerful metal-chelating agents (EDTA, dithizone, 1,10-phenanthroline, 8-hydroxyquinoline; 30 mins at 1 mM chelator in 40 mM potassium phosphate, 5% v/v ethanol pH 7.00, 25°C) and is only partially inhibited by the sulphydryl-directed reagent NEM (20 mins at 1 mM NEM. pH 7.00, 25°C). It is therefore suggested that:

1. the properties of the electrophoretically homogeneous N.crassa chorismate synthase are most simply explained if FMN is the final enzyme-bound electron acceptor in the diaphorase reactions and in the "activation" of the enzyme.
2. reduced FMN may be an essential cofactor in the catalytic conversion of EPSP to chorismate.

5.4.6 Comparison of different preparations of chorismate synthase

Welch et al (1974) have reported the isolation of "$\geq$ 90% homogeneous" chorismate synthase from N.crassa. There are serious discrepancies between the properties of their enzyme preparation and the properties of the electrophoretically homogeneous enzyme isolated in the present study (Table 5.5). The specific chorismate synthase activity of the enzyme obtained in the present study is at least seven-fold higher than the most highly purified material of Welch et al (1974). The latter material was purified only 70-fold over a 40-50% saturation ammonium sulphate fraction of mycelial extracts, while the material obtained in the present study was purified approximately 300-fold over a similar ammonium sulphate fraction. In addition the DCPIP reductase activities of the two enzyme preparations are completely different. The DCPIP reductase activity of chorismate synthase as obtained in the present study is totally dependent on FMN and is 95% inhibited by EPSP. In contrast, Welch et al (1974) reported that the DCPIP reductase activity of chorismate synthase was stimulated 5-fold by EPSP, and they

Table 5.5 Comparison of different preparations of chorismate synthase

Source of data	Enzyme preparation	Specific chorismate synthase activity Units/E_{280nm}	Relative activities of:			
			Chorismate synthase (=100)	DCPIP reductase +NADPH	+NADPH +FMN	+NADPH +FMN +EPSP
This study	Partially purified chorismate synthase	5.06	100	≤ 0.1	11.0	0.6
This study	Electrophoretically homogeneous enzyme	15.0 (estimated)	100	nd	9.9	≤ 1.0
Welch et al (1974)	" ≥90% homogeneous" chorismate synthase	0.69 (2.07)	100 (100)	47.3 (15.8)	47.3 (15.8)	247.0 (82.3)

Assay systems: Welch et al (1974) assayed chorismate synthase in 100 mM potassium phosphate pH 7.0 (25°C) and the DCPIP reductase activities in 100 mM potassium phosphate pH 8.0 (25°C). In the present study all enzyme activities were assayed in buffer B, pH 7.0, 25°C. Chorismate synthase is threefold more active in buffer B than in 100 mM KP_i pH 7.0; the DCPIP reductase activities are essentially the same in both buffers. The figures in parentheses in the final line of the Table are corrected for the differences between assay conditions in the two studies.

observed no flavin-dependence of the DCPIP reductase activity in the absence of EPSP. In the present study flavin-independent DCPIP reductase activity has only been observed in less highly purified chorismate synthase fractions. The purified enzyme ran as a single band in SDS PAGE, corresponding to a polypeptide size of approximately 50 kDa; Welch et al (1974) reported a polypeptide size of approximately 55 kDa for the predominant species in their "$\geqslant$90% homogeneous" material. It should be noted that in the purification scheme described here a number of contaminant proteins of comparable polypeptide size are only removed in the final substrate elution chromatography step (Figure 5.1), and that a vigorous anti-protease regime (EDTA, benzamidine, PMSF) was imposed; no protease inhibitors (other than EDTA) were employed in the purification protocol of Welch et al (1974). For these reasons, and in the light of our earlier experiences with the purification of enzyme complexes from N.crassa, (chapter 4; Lumsden and Coggins, 1977) it is considered that the enzyme characterised in the present study is more likely to resemble the intact cellular catalyst than is the material isolated by Welch et al (1974).

5.4.7 Preliminary evidence that chorismate synthase is a bifunctional enzyme

The results presented above clearly demonstrate that chorismate synthase activity and an NADPH and FMN-dependent DCPIP reductase activity are associated with a single protein aggregate which consists only of 50 kDa polypeptide chains. Since the corresponding enzyme activities in B.subtilis are carried by independent and separable polypeptides of 24kDa (chorismate synthase) and 17 kDa (DCPIP reductase) the question arises of whether chorismate synthase of N.crassa is a bifunctional enzyme. Genetic studies have identified only a single complementation group of arom-3 (chorismate synthase) mutations, which exhibit extensive allelic

complementation, as might be expected if there is a single bifunctional chorismate synthase gene product (Rines et al, 1969). Further protein chemical evidence will be needed to clarify the situation.

It is interesting to note that the DCPIP reductase activities of the N.crassa chorismate synthase more closely resemble those of the intact B.subtilis chorismate synthase multienzyme complex than those of the isolated B.subtilis DCPIP reductase subunit. The isolated B.subtilis reductase subunit exhibits flavin-independent NADPH-dependent DCPIP reductase activity, while the intact enzyme complex, like the N.crassa enzyme, only oxidises NADPH in the presence of FMN. These results are consistent with the possibility that the flavin binding site of the B.subtilis enzyme is within the catalytic site of the 24 kDa "chorismate synthase" subunit and receives reducing equivalents from a spatially distinct NADPH binding-site in the small 13 kDa "reductase" sub unit. A similar arrangement of catalytic sites in the N.crassa enzyme could perhaps underly the requirement for FMN and EPSP, but not NADPH, in the "slow" phase of enzyme activation.

5.4.8 The catalytic mechanism of chorismate synthase

The properties of chorismate synthase summarised in the preceding sections are consistent with a catalytic mechanism in which reduced FMN participates directly in the conversion of EPSP to chorismate. If chorismate synthase has a catalytic mechanism of the type proposed by Floss et al (1972; section 1.6, figure 1.9) the nucleophile "X" could perhaps arise by transfer of reducing equivalents from FMN to an unidentified functional group at the active site of the enzyme. It is, however, tempting to speculate that there is neither an iron(II) centre (Morell et al, 1967) nor a reduced disulphide group (Welch et al, 1974) at the active site, and that reduced FMN itself acts as the nucleophile "X". Since reducing equivalents are consumed at a

negligible rate during aerobic chorismate synthesis, it is proposed that enzyme-bound reduced FMN is reconstituted at the end of each catalytic cycle, and that it only infrequently reacts with molecular oxygen or dissociates from the enzyme before the next cycle is initiated.

5.5 Future prospects

The new purification scheme for the N.crassa chorismate synthase and the preliminary results described above open the way for a more detailed investigation of this extraordinary enzyme. It should now be relatively easy to define the subunit structure, and to study the "activation" of the enzyme by sophisticated spectroscopic techniques.

CHAPTER 6 CATALYTIC PROPERTIES OF THE EPSP SYNTHASE ACTIVITY OF THE AROM MULTIENZYME COMPLEX

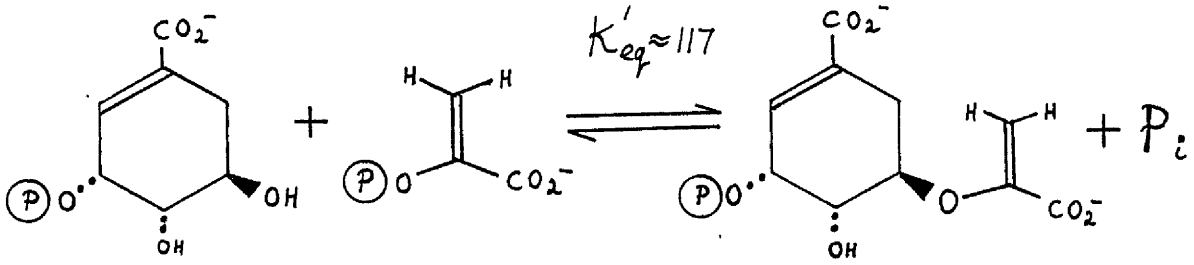
6.1 Introduction : enzymology of EPSP synthases

EPSP synthase catalyses the penultimate step in the biosynthetic pathway that leads to chorismic acid, the common precursor of the three aromatic amino-acids phenylalanine, tyrosine and tryptophan. (Fig. 1.1). The EPSP synthase reaction entails a condensation between shikimate 3-phosphate and the carbon skeleton of phosphoenolpyruvate, and results in the formation of an unusual enol ether linkage. The products are EPSP and inorganic phosphate. (Fig. 6.1). An equilibrium constant $Keq'' = 15$ has been estimated for the EPSP synthase reaction at pH 6.1. (Levin & Sprinson, 1964).

6.1.1 Structural organisation of EPSP synthases

EPSP synthase activity has been detected in bacteria, fungi and plants. No cofactor requirements have been established. (Levin & Sprinson, 1964). In bacteria and higher plants EPSP synthases appear to be monofunctional enzymes with a single subunit of 49 kDa (Lewendon & Coggins, 1983; Mousdale & Coggins, 1983; Berlyn *et al*, 1970; Berlyn & Giles, 1969). In *N.crassa* EPSP synthase is the fifth activity of the pentafunctional *arom* enzyme complex. (Lumsden & Coggins, 1977). An analagous pentafunctional *arom* complex may be present in many other fungi, and in the green flagellate *Euglena gracilis*. (Ahmed & Giles, 1969; Patel & Giles, 1979).

The location of EPSP synthase within a multifunctional *arom* complex in *N.crassa* raises two interesting problems. The first concerns the evolutionary origin of the EPSP synthase catalytic site in the multifunctional *arom* polypeptide. If the multifunctional enzyme arose

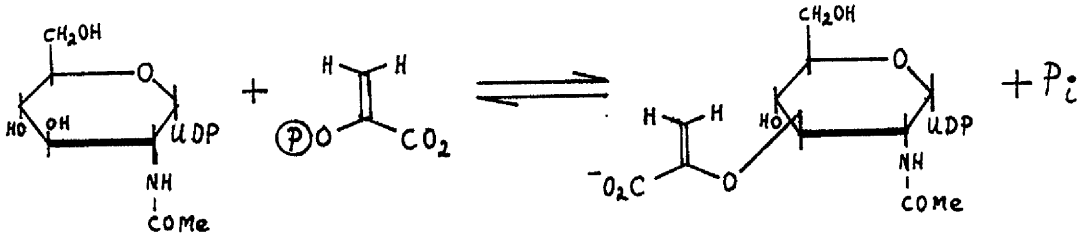


Shik3P — shikimate 3-phosphate

PEP — phosphoenolpyruvate

EPSP — 5-enolpyruvylshikimate 3-phosphate

P_i



UDPGlcNAc

PEP

EPU

P_i

Figure 6.1 The reactions catalysed by EPSP synthase and UDPGlcNAc pyruvyl transferase

through fusion of genes coding for monofunctional enzymes, structural and mechanistic homologies with present-day monofunctional EPSP synthases may be evident.

A second problem concerns the source of shikimate 3-phosphate consumed by the EPSP synthase activity of the arom complex. It has been proposed that there is intramolecular transfer of shikimate 3-phosphate between the shikimate kinase and EPSP synthase sites of the multifunctional enzyme,but no conclusive evidence has been presented on this point (Welch & Gaertner, 1975, 1976; see Section 4.4) The extent of any catalytic interaction between the skikimate kinase and EPSP synthase activities of the arom complex is amenable to detailed investigation, as sensitive fluorimetric and radioactive tracer assays are available for the intermediates shikimate 3-phosphate and EPSP (see "Methods").

6.1.2 EPSP synthase and metabolic regulation in the skikimate pathway

A regulatory function has not been proposed for any EPSP synthase. The EPSP synthase reaction is, however, flanked by two steps in the shikimate pathway that are essentially irreversible. Potential regulatory properties have been reported for enzymes that catalyse both of these steps. Thus in E.coli one of the two shikimate kinase isoenzymes is repressible by tyrosine, while the activity of N.crassa and B.subtilis chorismate synthases can be modulated in vitro by the NADP/NADPH ratio (Ely & Pittard, 1979; Welch et al, 1974; Hasan & Nester, 1978[a]).

It is not known whether the close association of five common pathway enzymes in the arom multienzyme complex has any bearing on the function of the pathway. Conjecture has centred around the possibility that integration of the five catalytic activities of the enzyme complex

may restrict the free diffusion of shikimate pathway intermediates within the cell (Giles et al, 1967). A detailed knowledge of the catalytic properties of the individual enzymes will be a prerequisite for any experimental investigation of the catalytic function of the arom complex as a whole.

6.1.3 Mechanistic studies on bacterial EPSP synthases

6.1.3.1 The reaction pathway

EPSP synthase catalyses a highly unusual chemical transformation, and the elucidation of the elementary steps in the reaction is of considerable interest. (Ganem, 1978).

Tracer studies have shown that the ester oxygen attached to carbon 2 of PEP is recovered quantitatively in one product of the synthase reaction, inorganic phosphate, and not in EPSP. (Bondinell et al, 1971). Many PEP-utilising enzymes exploit the reactive species related to enol-pyruvate that are formed as the P-O bond in the enol ester link is broken. EPSP synthase cannot conform to this pattern, as it is the C-O bond of PEP that is broken instead. (see Fig. 6.2; Davies, 1979). The synthase reaction is therefore best imagined as a replacement of the phosphate ($-OPO_3$) group of PEP by the alkoxy (-OR) group from shikimate phosphate. One other enzymatic reaction of this type is known, UDPGlc-NAc pyruvyl transferase, which substitutes the UDPGlc-Nac unit for the phosphate group of PEP. (Strominger, 1958) (see Fig. 6.1).

When the EPSP synthase reaction is conducted in tritiated water the two enolic hydrogens of the product EPSP become labelled to equal extents. (see Fig. 6.1). The only simple explanation of this result is that a freely-rotating methyl group is formed at the PEP C3 position during the course of the reaction. (Bondinell et al, 1971). There are

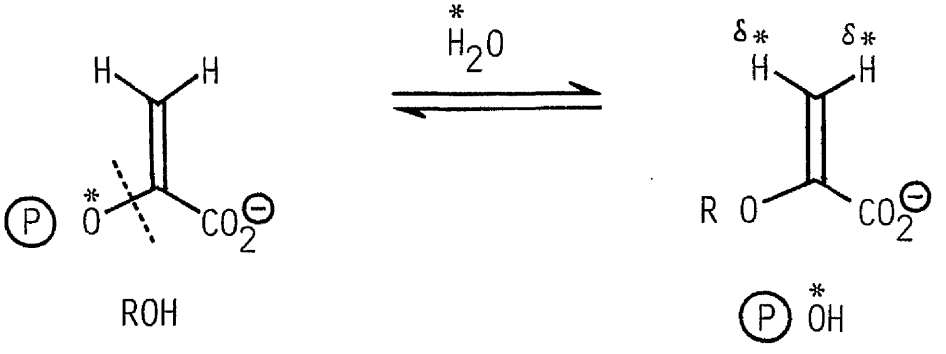
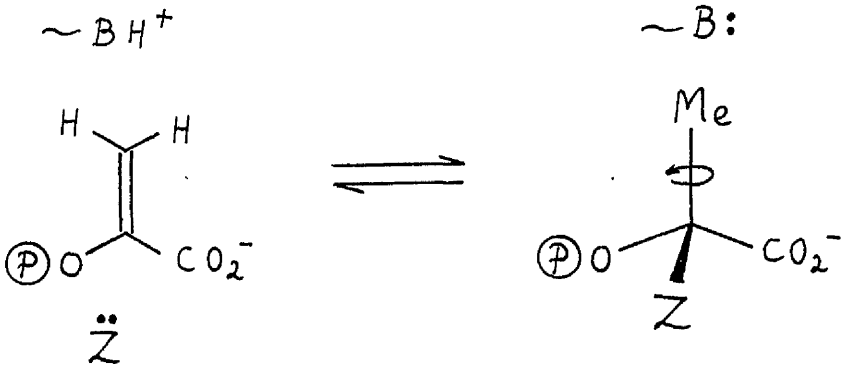


Figure 6.2 C-O bond cleavage and equal labelling of enolic hydrogens in the EPSP synthase reaction



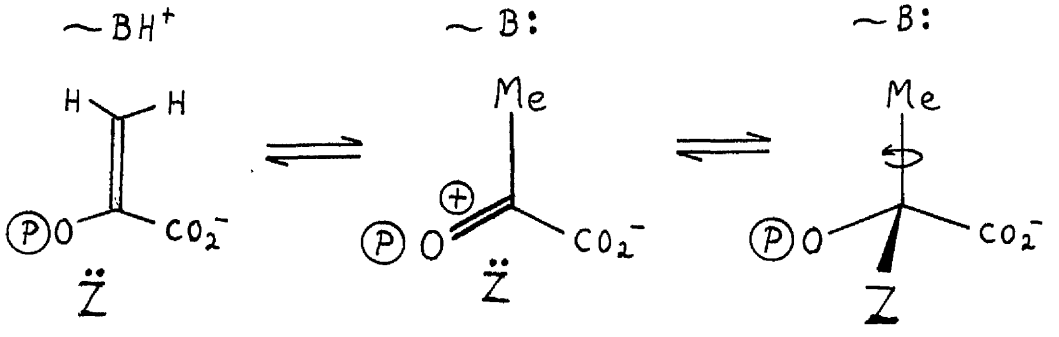


Figure 6.3 Two possible routes to an intermediate with a freely-rotating methyl group in the catalytic mechanism of EPSP synthase

good grounds for assuming that the PEP C2 position also adopts tetrahedral coordination during the reaction - it would otherwise be necessary to eliminate inorganic phosphate from a trigonal carbon centre.

Two contrasting chemical mechanisms have been proposed for the EPSP synthase reaction. Their common feature is the formation of an intermediate with a methyl group at the PEP C3 position and with tetrahedral geometry at the C2 position. Such an intermediate would be generated by protonation of the C3 position of PEP and addition of a nucleophile at the C2 position. (see Fig. 6.3a). The identity of the nucleophile has yet to be established. Two possible modes of addition across the PEP double bond can be distinguished - a concerted addition reaction (Fig. 6.3a), and a two-step reaction with a protonated intermediate (Fig. 6.3b).

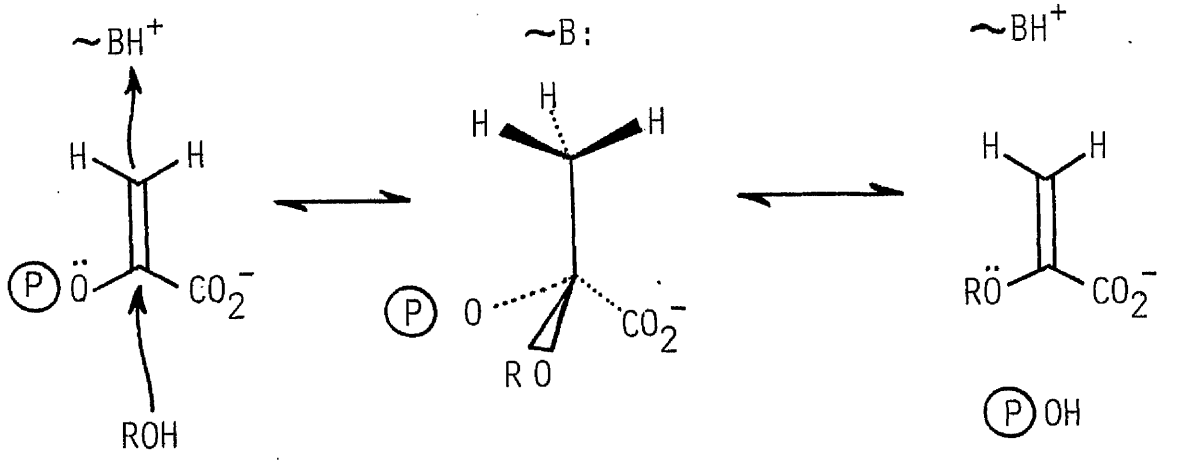
6.1.3.2 The tetrahedral intermediate hypothesis

Levin and Sprinson (1964) have suggested that protonation of C3 of PEP, and an attack on C2 of PEP by the 5-hydroxyl of shikimate 3-phosphate, create a "tetrahedral intermediate" that collapses in favour of products (see Fig. 6.4a). Most subsequent mechanistic studies on EPSP synthases have been interpreted in terms of this "tetrahedral intermediate" model. Levin and Sprinson (1964) initially favoured a protonated PEP species as a stepping stone to the central intermediate, but more recently a concerted reaction with a carbonium-like transition state has been preferred. (Grimshaw et al, 1982) (see Fig. 6.3). In either case electron donation from the ester oxygen could stabilise the cationic centre that is generated at the PEP C2 position.

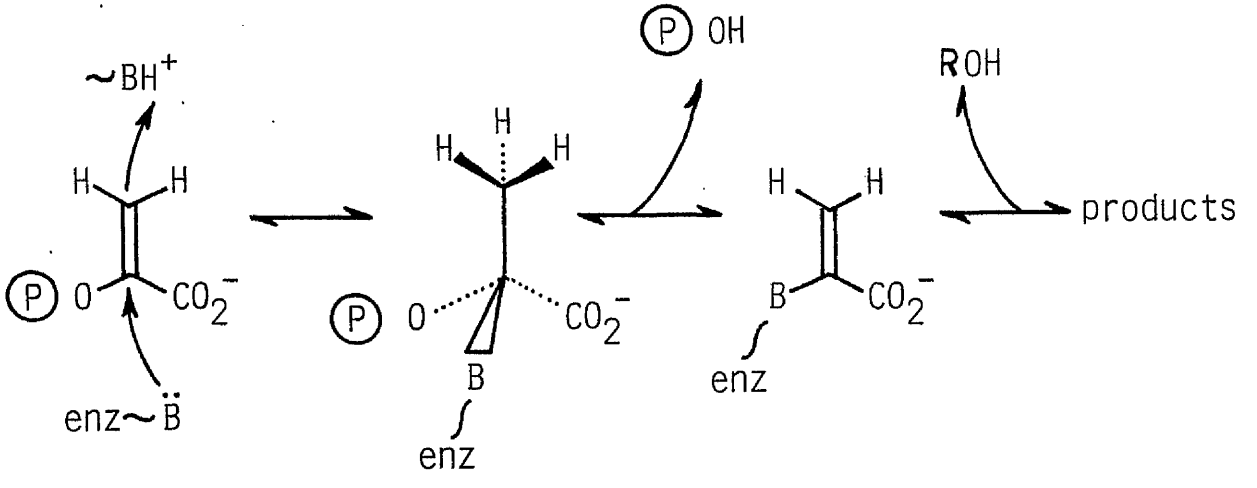
6.1.3.3 The covalent intermediate hypothesis

The chemical mechanism proposed for the UDPGlc-NAc pyruvyl transferase reaction is somewhat different; PEP is protonated at C3 and attacked by a cysteine thiol at C2, and a covalent enzyme-PEP adduct is

Figure 6.4 The "tetrahedral intermediate" and "covalent intermediate" models for EPSP synthase catalysis



Tetrahedral intermediate



Covalently-bound intermediates

formed. (see Fig. 6.4b). This species collapses by expelling phosphate to give an enzyme-pyruvate adduct; the UDPGlc-NAc skeleton is then added at C2 to give the covalently bound product. (Zemell & Anwar, 1974). Alternatively these two steps may be combined in a concerted substitution reaction at C2 that has the same overall effect. (Cassidy & Kahan, 1973). The two groups that have studied this enzyme have both presented evidence for a covalently bound PEP or pyruvate adduct, but they have worked with enzymes from different species of bacteria, and they disagree on some details of the mechanism. Both groups have suggested that their "covalent intermediate" mechanism may apply equally well to the EPSP synthase reaction. Others have argued that the "covalent intermediate" mechanism is intrinsically more plausible than the "tetrahedral intermediate" mechanism of Levin and Sprinson (1964), on the grounds that a thiol nucleophile would more readily initiate the attack on the PEP double-bond. (Ganem, 1978).

6.1.3.4 Isotope exchange reactions

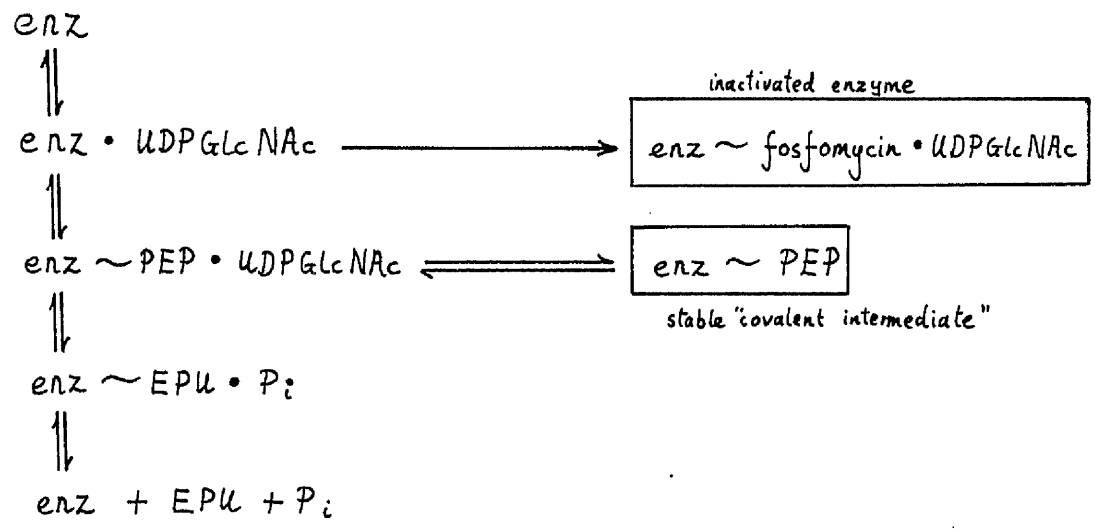
Since the overall EPSP synthase reaction is reversible the same must be true of its constituent elementary steps. A number of predictions can be made about isotope exchange reactions and kinetic isotope effects in the light of the proposed catalytic mechanisms.

At first sight a "covalent intermediate" mechanism would oblige the enzyme to catalyse the part reactions that are characteristic of a "ping-pong" scheme. (see Fig. 6.4b). EPSP synthase would therefore be expected to exchange ^{32}P label between inorganic phosphate and PEP and to incorporate ^{3}H label from water into the C3 hydrogens of PEP. No such exchange of label is observed when the bacterial EPSP synthase is incubated with PEP. (Bondinell et al, 1971). It is probably for this reason that the "covalent intermediate" idea was dismissed at an early stage as a candidate mechanism for EPSP synthase.

Table 6.1 Summary of mechanistic data for EPSP synthase and for UDPGlcNAc pyruvyl transferases

Catalytic activity	observed properties			predicted properties	
	EPSP synthase	pyruvyl transferases		tetrahedral intermediate mechanisms	covalent intermediate mechanisms
	E.coli	E.cloacae	M.leisodeikticus		
Exchange of isotopic label between:					
$^{32}P_i$, PEP		-		-	-
$^{3}H_2O$, PEP	-			+/-	-
*Shik3P, EPSP or *UDPGlcNAc, EPU		+		-	+/-
$^{3}H_2O$, EPSP or EPU	?-	+		+/-	+/-
Equal incorporation of $^{3}H_2O$ label into enolic hydrogens of product EPSP or EPU	+		+	+	+
Incorporation of isotopic label into "covalent intermediates":					
^{14}C of PEP		+	+	-	+
^{32}P of PEP		-	+	-	+/-

Sources : Zemell and Anwar (1974), Gunetileke and Anwar (1968), Cassidy and Kahan (1973), Bondinell et al (1971) Grimshaw et al (1982). The predictions listed for the "covalent intermediate" model are based on the catalytic mechanisms for UDPGlcNAc pyruvyl transferases proposed by Zemell and Anwar (1974) and Cassidy and Kahan (1973). Blank entries indicate that no data are available.



where • implies a Michaelis complex.
~ implies a covalent link to an enzyme sulphydryl group.

Figure 6.5 A catalytic mechanism proposed for UDPGlcNAc pyruvyl transferase (Cassidy & Kahan, 1973)

UDP Glc-NAc pyruvyl transferase also fails to catalyse any exchange reactions when it is incubated with PEP and phosphate in the absence of UDP Glc-NAc. (Gunetileke & Anwar, 1968). There is a trivial explanation for this result; the transferase is unable to interact with PEP unless UDP Glc-NAc is also present. (Cassidy & Kahan, 1973; fig 6.5). This example illustrates how easily misleading mechanistic conclusions are drawn when the order of substrate binding and activation is ignored.

Table 6.1 summarises the isotope exchange data that have been accumulated for the pyruvyl transferases and for EPSP synthase, and lists the results predicted by the two competing models for the chemical mechanism. The uncertainties in the predictions stem from uncertainties about the order of substrate binding and the concertedness of the protonation step in the "tetrahedral intermediate" mechanism of Levin and Sprinson (1964). Even a complete set of data for EPSP synthase would not allow one to discriminate unambiguously between the "tetrahedral intermediate" model and a "covalent intermediate" mechanism of the type proposed for the UDP Glc NAc pyruvyl transferases.

6.1.3.5 Kinetic isotope effects

Using tritium and deuterium-labelled PEP as substrates for the E.coli enzyme, Knowles and his coworkers have conducted an intensive study of kinetic isotope effects in the EPSP synthase reaction. (Grimshaw et al, 1982). These authors interpreted their results in terms of the tetrahedral intermediate mechanism of Levin and Sprinson (1964) (see Fig. 6.6); only some of their most important findings are discussed here.

It is clear that once a heavy hydrogen atom is incorporated into the methyl group that forms at the PEP C3 position during the course of the reaction there is a net discrimination against its removal in the steps leading to release of EPSP. The observed isotope effect (V_{H/V_D}= 3.0) is probably smaller than is expected for the intrinsic

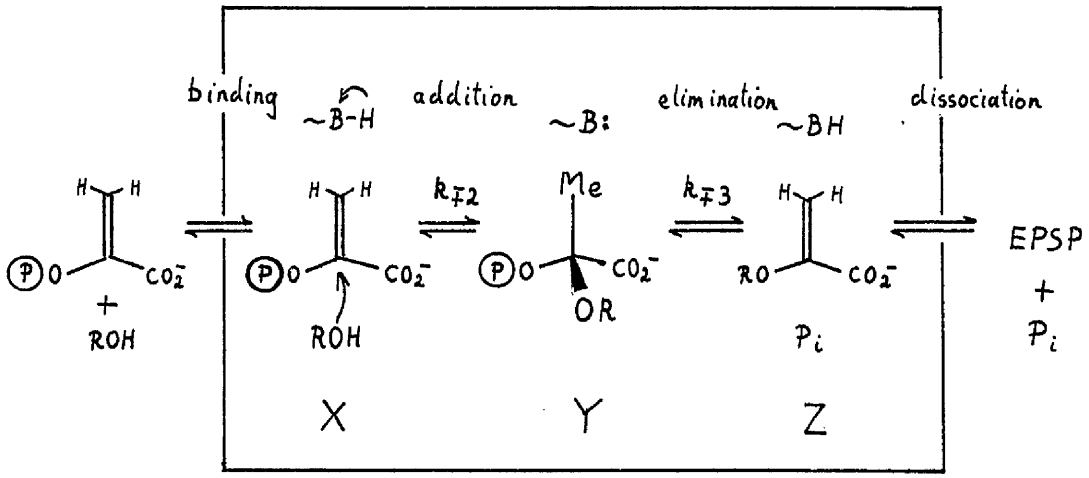


Figure 6.6 Elementary steps in the proposed catalytic mechanism of EPSP synthase discussed by Grimshaw et al (1982)

isotope effect on k_{+3} in the deprotonation step. (see Fig. 6.6). If the intermediates Y and Z interconvert faster than EPSP is released from the enzyme the observed isotope effect for the overall process Y ⟶ free EPSP can be smaller than the intrinsic kinetic isotope effect on k_{+3} for the individual step Y⟶ Z. It is therefore likely that there is significant equilibration of Y and Z if the mechanism in Figure 6.6 is correct.

Another way to explain the modest discrimination against the removal of the heavy isotopes would be to incorporate an additional protonation/deprotonation step into the mechanism. The data place no restriction on the number of protonation/deprotonation steps in the reaction sequence, and could certainly accommodate the requirement for four such steps in a "covalent intermediate" mechanism.

A second significant finding of Grimshaw et al is that only a small proportion of doubly-labelled PEP loses both deuterium atoms on conversion to EPSP. This result can be rationalised if the base that accepts the proton in step 3 is unable to exchange its proton with the solvent rapidly while the substrates remain bound to the enzyme. A labelled hydrogen atom removed in step +3 would then be returned to the substrate if this step were reversed. The mechanism must, however, allow for both labelled protons from PEP to be "washed out" of about 5% of the product, EPSP, before it is released from the enzyme. If the mechanism shown in Figure 6.6 is correct this means that the catalytic acid/base group in the enzyme must exchange protons with the solvent at a significant rate while the substrates are bound (in either X or Z). But it is also essential that there should be partial reversal of at least one of the protonation/deprotonation steps - and reversal of step 3 is only possible if the release of products is relatively slow. The second condition can be relaxed if EPSP synthase follows a "covalent

intermediate" mechanism, as there are then always two opportunities for the label to be removed during the course of the reaction.

It should be apparent that a knowledge of substrate binding and dissociation would greatly aid in the interpretation of the isotope results. The data presently available for the bacterial enzyme are fully consistent with both "covalent intermediate" and "tetrahedral intermediate" models for EPSP synthase catalysis.

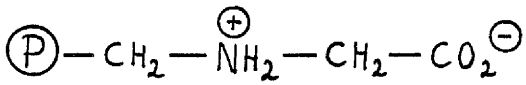
6.1.4 Steady state kinetic properties of EPSP synthases

The EPSP synthases of E.coli and N.crassa display saturation kinetics in the forward reaction, but no data relating to the order of substrate binding have been reported. (Levin & Sprinson, 1964; Welch & Gaertner, 1976).

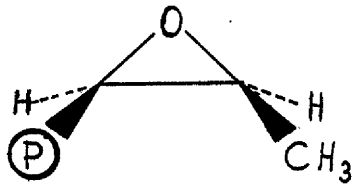
The rather unusual kinetic mechanism proposed for the bacterial UDP Glc-NAc pyruvyl transferases, (Fig. 6.5), is based not on steady-state kinetics, but on the results of isotope exchange and enzyme labelling experiments. (Zemell & Anwar, 1975; Cassidy & Kahan, 1973). The antibiotic fosfomycin inactivates the enzyme by covalent modification, and has been useful in the mechanistic studies. A striking feature of the proposed mechanism is that neither PEP nor fosfomycin is able to interact with the enzyme unless UDP Glc-NAc is also present. (see Fig. 6.5) (Kahan et al, 1974).

6.1.5 Glyphosate

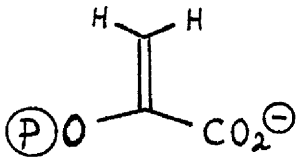
Glyphosate, N-phosphonomethyl glycine (Figure 6.7), was developed as a broadspectrum post-emergence herbicide by the Monsanto corporation. It has found worldwide applications in agriculture and horticulture. (Baird et al, 1971). Glyphosate is absorbed through leaf surfaces and appears to kill the plant by inducing chlorosis. A number of lines of evidence point to a primary site of action in the shikimate pathway. (Amrhein et al, 1980a). The EPSP synthase activity of crude



glyphosate



fosfomycin



phosphoenolpyruvate

Figure 6.7

extracts of Aerobacter aerogenes, and of mung beans, is highly sensitive to inhibition by glyphosate. (Amrhein et al, 1980b; Steinrucken & Amrhein, 1980). Two earlier steps in the shikimate pathway, the DHQ synthase and DAHP synthase reactions, have also been put forward as possible targets for glyphosate action. (Rubin et al, 1982; Roisch & Lingens, 1980). Much higher concentrations of glyphosate are required for significant inhibition of these activities. Many DAHP and DHQ synthases require transition metal cations for activity, and the inhibition may reflect non-specific metal-chelating properties of glyphosate. (Hasan & Nester, 1978; Nimmo & Coggins, 1981b; Knuuttila & Knuuttila, 1979).

So far as plants are concerned, it is generally agreed that all of the biochemical and physiological repercussions of glyphosate treatment can be attributed to a decline in EPSP synthase activity. (Cole, 1982). This view is not universally held, and it is possible that glyphosate has direct effects on porphyrin metabolism and auxin metabolism. (Kitchen et al, 1981; Lee, 1982).

A long-term objective of several groups working on the mechanism of glyphosate action is the production of glyphosate-resistant crop plants. This goal has been brought closer by the successful cloning of bacterial EPSP synthase genes (Duncan & Coggins, 1983; Rogers et al, 1983) and by preliminary indications that glyphosate-resistant mutant EPSP synthase gene products can be selected (Comai et al, 1983).

The interaction of glyphosate with EPSP synthase has not previously been investigated in detail. Glyphosate-like activity has not been reported for any related compound. It is therefore of interest to explain why this simple compound has such a specific and dramatic effect on EPSP synthase activity.

6.2 The EPSP synthase activity of the arom enzyme complex : summary and objectives

At the outset of the present study the arom enzyme complex was the only highly purified preparation of EPSP synthase available. It was therefore the most promising system for a preliminary study of the steady state kinetic properties of this enzyme. However, the EPSP synthase activity of the intact arom complex (Lumsden & Coggins, 1977) had not been characterised in any detail, and it was first necessary to demonstrate the copurification of this activity with the other enzymes of the complex (see Chapter 4)

The established methods for EPSP synthase assay are not generally convenient for routine use with crude cell extracts, and the large amounts of chorismate synthase and anthranilate synthase required for the sensitive coupled fluorimetric assay of Gaertner and De Moss (1970) are not readily available (J.R.Coggins and A.A.Coia, unpublished results). There was therefore a strong incentive to develop new assay systems and improve established methods, both for routine purification work, and for steady-state kinetics experiments.

The kinetic mechanism of the EPSP synthase of the arom complex was of interest for a number of reasons. Two main proposals for a sequence of catalytic intermediates must be considered, and only one of these, the "covalent intermediate" mechanism, may give rise to ping-pong kinetics. The order of substrate binding and release has important implications for the mechanistic interpretation of isotope tracer studies. Although the approach adopted by Sprinson and Knowles and their coworkers should reveal the stereochemical course of the synthase reaction, a detailed understanding of the association and dissociation of substrates from the enzyme is essential for a full analysis of the results of the kinetic isotope experiments. (Grimshaw et al, 1982).

Since the EPSP synthase reaction is reversible it can, in principle, be subjected to kinetic study in the forward and reverse directions. A combination of steady-state kinetic experiments, kinetic isotope studies and other approaches may eventually lead to a satisfactory description of EPSP synthase catalysis in terms of rate constants for the elementary steps in the reaction. A similar objective has already been achieved with the enzyme triose phosphate isomerase. (Knowles & Albery, 1977).

A further application for a detailed knowledge of the kinetic properties of the arom EPSP synthase arises when the kinetic properties of the combined shikimate kinase (E4) and EPSP synthase (E5) reactions are considered. Previous work on "substrate channeling" in the arom complex has been based on a somewhat cursory examination of the kinetic properties of the individual reactions. (Welch & Gaertner, 1976). It was therefore of some interest to look critically at the evidence for catalytic interactions between EPSP synthase and the other enzymes of the complex.

6.3 The development of new methods for the assay of EPSP synthase activity

The established method for the continuous assay of EPSP synthase activity is the coupled fluorimetric assay system of Gaertner and De Moss (1970) which uses chorismate synthase and anthranilate synthase to convert EPSP into the highly fluorescent compound anthranilic acid. This assay system was adapted for use in steady state kinetics experiments (see chapters 3 and 5) and proved invaluable. The spectrophotometric assay of Grimshaw et al (1982) is based on the small extinction changes at 230-240nm that accompany the EPSP synthase reaction, particularly at low pH. This assay system was used for certain product inhibition experiments, but poor linearity, low sensitivity and high background absorbances severely limit its value.

For most routine purposes and for certain steady state kinetics experiments the forward reaction was instead coupled to chorismate synthase in a very satisfactory continuous spectrophotometric assay that exploits the 275nm extinction maximum of chorismic acid (Gibson, 1970).

It was found that the most convenient and economical method for detecting EPSP activity is the new continuous coupled spectrophotometric assay of the reverse reaction, in which the release of PEP is coupled to the pyruvate kinase and lactate dehydrogenase reactions (see Chapter 3). This assay system has the advantage of being suitable for the quantitation of EPSP synthase activity in crude extracts of N.crassa, E.coli and pea seedlings. It has been used extensively throughout the present study and has recently been used to monitor the purification of the E.coli and pea seedling EPSP synthases and the overexpression of the cloned E.coli aro A gene (Lewendon & Coggins, 1983; Mousdale & Coggins, 1983; Duncan & Coggins, 1983). For certain steady state kinetics experiments a more sensitive continuous fluorimetric assay of the reverse reaction was obtained by coupling the release of PEP to the glucose 6-phosphate dehydrogenase reaction.

An assay buffer based on 50mM KCl, 50mM bis tris / HCl , 2.5mM $MgCl_2$, pH 7.00 (buffer A) was chosen for the steady state kinetics experiments because it was compatible with all of the spectrophotometric and fluorimetric assay systems listed above (except for the "direct" assay of Grimshaw et al, 1982). Full details of the enzyme assay methods are given in sections 3.5 and 3.6.

6.4 Determination of the equilibrium constant for the EPSP synthase reaction

It was considered desirable to estimate the equilibrium constant for the EPSP synthase reaction under the conditions employed in the steady state kinetics experiments. This was done by incubating the arom multifunctional enzyme with EPSP and P_i at a wide range of initial concentrations (details are given in section 3.11). The equilibrium concentrations of EPSP and PEP were determined by enzymatic conversion of these intermediates to pyruvate. The final concentration of shikimate 3-phosphate was taken to be equal to the final concentration of PEP, while the final concentration of P_i was calculated from the extent of conversion of EPSP to PEP. The estimates of substrate concentrations are thus based on the well-established extinction coefficient of NADH at 340nm, and on the formula weight of the anhydrous phosphate salt KH_2PO_4, and assume stoicheiometric conversion of EPSP and P_i to shikimate 3-phosphate and PEP (Levin & Sprinson, 1963).

The results shown in Table 6.2 indicate that for a wide range of initial substrate concentrations the observed equilibrium constant of the EPSP synthase reaction is essentially the same, as is expected in "ideal" dilute solutions (Atkins, 1978). The estimated equilibrium constant is:

$$K_{eq} = \frac{[EPSP].[P_i]}{[Shik3P].[PEP]} = 116$$

at 25°C in buffer A, pH 7.0. This estimate of the equilibrium constant differs substantially from the figure of 12-15 estimated at 37°C, pH 6.1 in 125mM tris/maleate, 25mMKF containing 0.5mg/ml partially purified A.aerogenes EPSP synthase (Levin & Sprinson, 1963). The differences in temperature, pH and ionisation state of the phosphate groups may well account for the discrepancy.

Table 6.2 Determination of K_{eq} for the EPSP synthase reaction

incubation	volume (μl)	initial [P_i] (mM)	final [PEP] (mM)	final [EPSP] (mM)	calculated K'_{eq}
1	240	8.33	.132	.248	117
			.130	.244	118
2	520	9.615	.0796	.0788	119
			.0796	.0780	117
3	1020	9.80	.0502	.0303	117
			.0498	.0303	119
4	260	15.39	.155	.177	112
			.161	.175	103
5	520	19.23	.0997	.0595	114
			.0981	.0595	118
6	1020	19.61	.0586	.0251	143
			.0611	.0217	114
			.0591	.0293	164

Incubation mixtures contained the stated initial concentrations of potassium phosphate, a range of initial concentrations of EPSP, and 2.5 mUnits of EPSP synthase activity (reverse reaction), all in buffer A, pH 7.00. After 15 hours incubation at 25°C samples were boiled for 2 minutes and aliquots were assayed for pyruvate, PEP and EPSP. The final concentration of Shik3P was taken to be identical to the PEP concentration.

$$K'_{eq} = \frac{[EPSP] \cdot [P_i]}{[Shik3P] \cdot [PEP]}$$

Following the example of Levin and Sprinson (1964) it is possible to calculate the free energy change for the synthase reaction in buffer A, and the free energy of EPSP hydrolysis, using the established value for the free energy of PEP hydrolysis quoted by Metzler (1977). Thus:

$$\text{Shik3P} + \text{PEP} \longrightarrow \text{EPSP} + P_i \qquad \Delta G' = -11.8\text{kJmol}^{-1}$$

$$\text{EPSP} + H_2O \longrightarrow \text{Shik3P} + \text{pyruvate} \qquad \Delta G' = -50.1\text{kJmol}^{-1}$$

$$\text{PEP} + H_2O \longrightarrow \text{Shik3P} + \text{pyruvate} \qquad \Delta G' = -61.9\text{kJmol}^{-1}$$

From a similar calculation Levin and Sprinson (1964) concluded that the enol ether linkage in EPSP is thermodynamically highly unstable to hydrolysis. The figures presented here indicate that at 25°C, pH 7.0 the free energy of EPSP hydrolysis is only some 12kJmol^{-1} less negative than the free energy of PEP hydrolysis. The enol ether linkage is nevertheless kinetically stable at pH 7.00; insignificant hydrolysis of EPSP occurred over 15 hours at 25°C in "blank" incubations (without enzyme) for the equilibrium constant experiment. It should also be noted that EPSP synthase does not catalyse the hydrolysis of either EPSP or PEP at a significant rate; it would otherwise be impossible to estimate an equilibrium constant from a set of results like those in Table 6.2.

6.5 Steady state kinetic properties of EPSP synthase

The discussion of the kinetic properties of EPSP synthase that follows is based on the general approach to steady state enzyme kinetics described by Cleland (1970) and Engel (1977. Much of the terminology used here also follows the conventions of Cleland (1970). Attention is drawn in particular to the use of the term "noncompetitive inhibitor" to cover the general case of an inhibitor that alters both the slope and intercept of a Lineweaver-Burke plot.

Two equivalent formulations of the general two-substrate linear hyperbolic rate equation are mentioned: the "K_M" form of Alberty (1953), and the "ϕ parameter" form of Dalziel (1957).

6.5.1 Initial velocities of the forward and reverse reactions

The initial velocity patterns for the forward reaction of arom EPSP synthase are shown in Figure 6.8. The primary and secondary plots are all linear within the chosen range of substrate concentrations, and the double reciprocal plots clearly show intersecting lines.

The determin ation of the initial velocity patterns for the reverse reaction presented greater technical difficulties; the results are shown in Figure 6.9. The linearity of the replots suggests that linear hyperbolic kinetics may be obeyed. An intersecting kinetic pattern for the reverse reaction was obtained in several independent experiments, but the K_M values for EPSP derived from the replot in Figure 6.8 can only be regarded as preliminary estimates. Technical difficulties arise because the limiting K_M's for EPSP are at the lower limit of the useful concentration range of the fluorimetric assay system. Figure 6.8 should, however, be compared with Figure 6.23, which displays the results of a related experiment where a greater range of EPSP concentrations is accessible.

Taken together these results constitute good preliminary evidence that linear hyperbolic kinetics are obeyed in both the forward and reverse reactions of EPSP synthase. The limiting kinetic parameters estimated for arom EPSP synthase are listed in Table 6.3. The values of the limiting K_M's for the forward reaction are very much lower than those reported for a partially purified arom preparation (Welch & Gaertner, 1976). The limiting K_M's of the homogeneous E.coli EPSP synthase are also much lower than those originally reported for

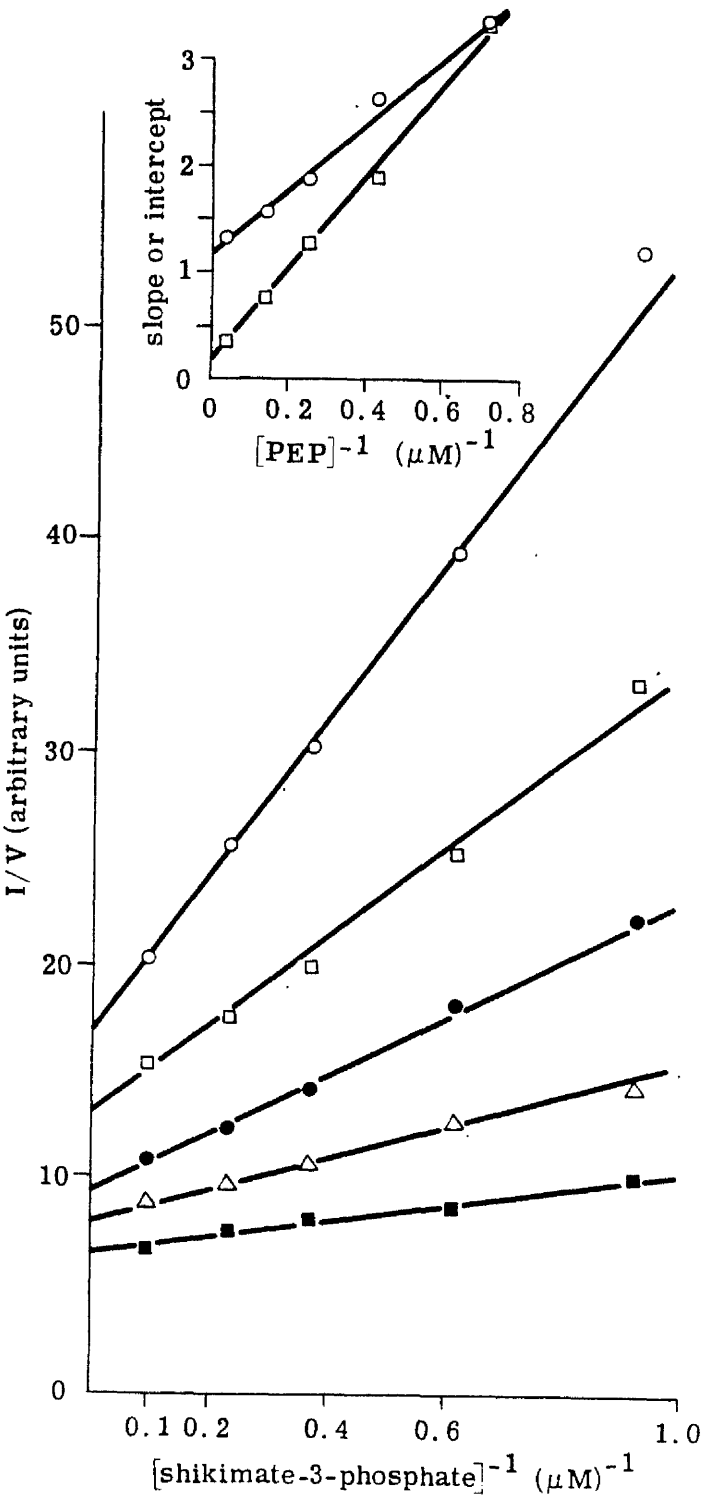


Figure 6.8 Initial velocities of the forward reaction of EPSP synthase

Double reciprocal plot of initial velocity versus [Shik3P] at a series of fixed PEP concentrations: ■ 28.7μM; △ 7.04μM; ● 4.03μM; □ 2.35μM; ○ 1.41μM. Inset: replot of slopes (□) and intercepts (○) as a function of [PEP]$^{-1}$. Fluorimetric assay system.

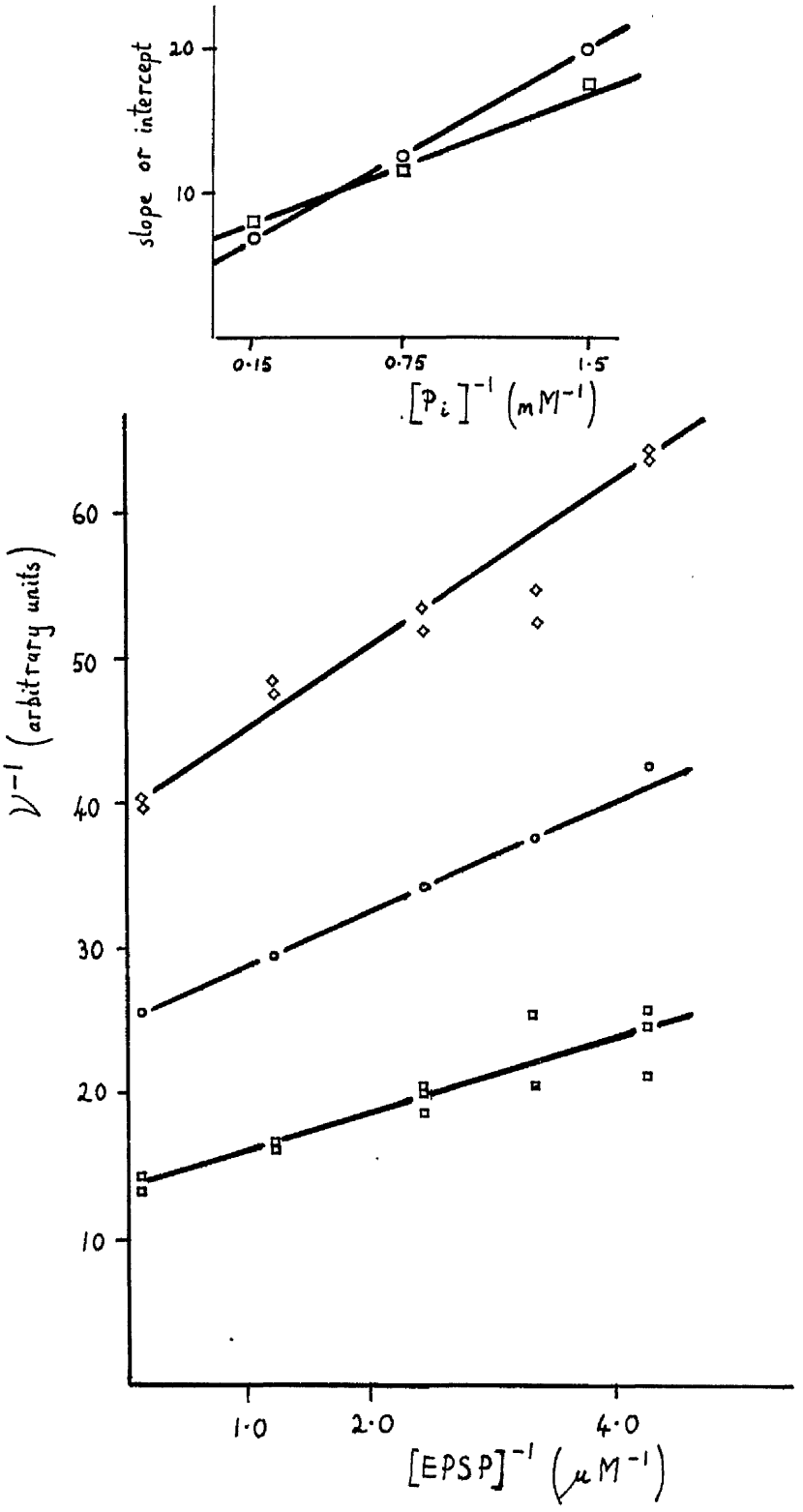


Figure 6.9 Initial velocities of the reverse reaction of EPSP synthase

Double reciprocal plot of initial velocity versus [EPSP] at a series of fixed concentrations of P_i: □ 6.7mM; ○ 1.3mM; ◇ 0.67mM. Inset: replot of slopes (□) and intercepts (○) as a function of $[P_i]^{-1}$. Fluorimetric assay system.

Table 6.3 Kinetic Constants for the EPSP Synthase Reactions

Reaction	Kinetic parameter	Dalziel notation	Estimated value		Data
Shik3P + PEP $\rightarrow$ EPSP + P_i	$\vec{V}_m$		7.66	u/mg	
	k_{cat}	ϕ_o^{-1}	21.1	s^{-1}	
	K_M(Shik3P)	ϕ_{Shik3P}/ϕ_o	0.362	μM	Fig. 6.8
	K_M(PEP)	ϕ_{PEP}/ϕ_o	2.6	μM	
	K_M^*(Shik3P)	$\phi_{Shik3P.PEP}/\phi_{PEP}$	2.88	μM	
	K_M^*(PEP)	$\phi_{Shik3P.PEP}/\phi_{Shik3P}$	21.1	μM	
EPSP + P_i $\rightarrow$ Shik3P + PEP	$\overleftarrow{V}_m$		4.24	u/mg	
	k_{cat}	ϕ_o^{-1}	11.6	s^{-1}	
	K_M(EPSP)	ϕ_{EPSP}/ϕ_o	0.219	μM	Fig. 6.9
	K_M(P_i)	ϕ_{P_i}/ϕ_o	1.8	mM	
	K_M^*(EPSP)	$\phi_{EPSP.P_i}/\phi_{P_i}$	0.114	μM	
	K_M^*(P_i)	$\phi_{EPSP.P_i}/\phi_{EPSP}$	0.94	mM	
PEP $\rightarrow$ pyruvate+P_i	V_m		6.79	u.mg	
at saturating	k_{cat}	ϕ_o^{-1}	18.7	s^{-1}	
concentrations	K_M(EPSP)	ϕ_{EPSP}/ϕ_o	0.175	μM	Fig. 6.23
of PEP	K_M(Asi)	ϕ_{Asi}/ϕ_o	3.14	mM	
	K_M^*(EPSP)	$\phi_{EPSP.Asi}/\phi_{Asi}$	0.079	μM	
	K_M^*(Asi)	$\phi_{EPSP.Asi}/\phi_{ESSP}$	1.42	mM	

The kinetic parameters are those estimated for a linear rate equation of the form

$$v^{-1} = \phi_o + \phi_A [A]^{-1} + \phi_B [B]^{-1} + \phi_{A.B} [A]^{-1}[B]^{-1}$$

The notation "K_M" and "K_M^*" is used as shorthand for the limiting K_M's at saturating and vanishing concentrations of the fixed substrate, respectively

Table 6.4a Haldane relation for EPSP synthase

The following Haldane relation is valid for any EPSP synthase that exhibits linear hyperbolic kinetics in the forward and reverse reactions:

$$\frac{K_M\ (EPSP)\ .\ K_M^*(P_i)}{K_M\ (Shik3P)\ .\ K_M^*\ (PEP)} \quad x \frac{\vec{V}_M}{\overleftarrow{V}_M} \quad = \quad K_{eq}'$$

Substituting the values of the kinetic parameters listed in Table 6.3:

$$\frac{0.219 \quad x \quad 0.94 \times 10^3}{0.362 \times 21.1} \quad x \quad \frac{7.66}{4.24} \quad = \quad 48.8$$

The value of K_{eq}' estimated by conventional means is 116 (see Table 6.2)

Table 6.4b Association and dissociation of Shik3P

If the proposed ordered mechanism is correct the following kinetic parameters may be calculated from the data of Table 6.3:

process			calculated value
$k_{on}(Shik3P)$	=	$k_{cat}/K_M(Shik3P)$	$5.8 \times 10^7\ M^{-1}s^{-1}$
$k_{off}(Shik3P)$	=	$k_{cat} \times K_M^*(Shik3P)/K_m(Shik3P)$	$168\ s^{-1}$
$K_D(Shik3P)$	=	$K_M^*(Shik3P)$	$2.88\ \mu M$

relatively crude material (A.Lewendon, unpublished, Levin & Sprinson, 1964). The discrepancies are probably due to the limitations of the assay systems used in the earlier studies.

6.5.2 Haldane relations

Since the kinetic constants for the forward and reverse reactions were all obtained under comparable conditions (in buffer A at pH 7.00, 25°C) they can be related to the equilibrium constant for the reaction by a Haldane relation. The apparent equilibrium constant calculated by substituting the kinetic constants into the Haldane relation is 49 (Table 6.4). The value of K_{eq}' estimated by conventional means is 116 (Table 6.2). There are several ways to explain the relatively small discrepancy between the two estimates of K_{eq}'. It is possible that the K_M's for the forward reaction have been underestimated, or that those for the reverse reaction have been overestimated. Alternatively it is possible that the kinetic constants are not completely independent of the cofactors included in the coupled assay systems. NADPH is needed for the forward assays, while $NADP^+$ and ADP are required for the reverse assays, and these charged species are likely to bind to the E3 and E4 sites of the arom enzyme complex.

The broad agreement between the two sides of the Haldane relation is taken as a weak validation of the internal consistency of the kinetic data, and of the assumption that the rate equations for the forward and reverse reactions are linear.

6.5.3 Sequential kinetic mechanisms for EPSP synthase

The intersecting kinetic patterns obtained for the forward and reverse reactions of arom EPSP synthase indicate that this enzyme has a sequential kinetic mechanism (see Figure 6.13). This does not necessarily rule out a catalytic mechanism with a "covalent intermediate"

similar to that proposed for UDPGlcNAc pyruvyl transferase; the specific catalytic mechanisms that have been put forward for UDPGlcNAc pyruvyl transferases all involve a ternary complex and would thus generate intersecting kinetics (Cassidy & Kahan, 1973; Zemell & Anwar, 1974). If EPSP synthase had a catalytic mechanism of the "covalent intermediate" type it would only display ping pong kinetics if P_i were released from the enzyme before shikimate 3-phosphate entered the kinetic sequence.

6.5.4 Inhibition of EPSP synthase by glyphosate

The herbicide glyphosate is a potent reversible inhibitor of arom EPSP synthase catalysis. The kinetic patterns of glyphosate inhibition are shown in Figures 6.10, 6.11 and 6.12 and summarised in Table 6.5.

Glyphosate is a linear competitive inhibitor with respect to PEP (Figure 6.10). A K_I for glyphosate of 1.1μM and an apparent K_M for PEP of 3.5μM were determined at saturation in shikimate 3-phosphate (177μM), giving a K_I/K_M ratio of 0.31. Glyphosate is a linear uncompetitive inhibitor with respect to shikimate 3-phosphate (Figure 6.11).

These inhibition patterns indicate that:

1. glyphosate inhibits the enzyme by binding to kinetic intermediates to which PEP can bind productively.
2. glyphosate binds only to kinetic intermediates downstream in the kinetic sequence from the point(s) where shikimate 3-phosphate binds to the enzyme.

Since all kinetic intermediates to which glyphosate binds in an inhibitory capacity must also bind PEP, it can be deduced that PEP and glyphosate compete for binding to an enzyme • shikimate 3-phosphate complex (see Figure 6.13).

Table 6.5 Glyphosate inhibition patterns

variable substrate	fixed substrate	inhibition pattern	figure
PEP	Shik3P(177μM)	linear competitive K_I = 1.1μM, K_M(PEP) = 3.5μM	6.10
Shik3P	PEP	linear uncompetitive	6.11
P_i	EPSP	complex competitive	6.12
EPSP	P_i	not competitive (?uncompetitive)	not shown (see 6.25)

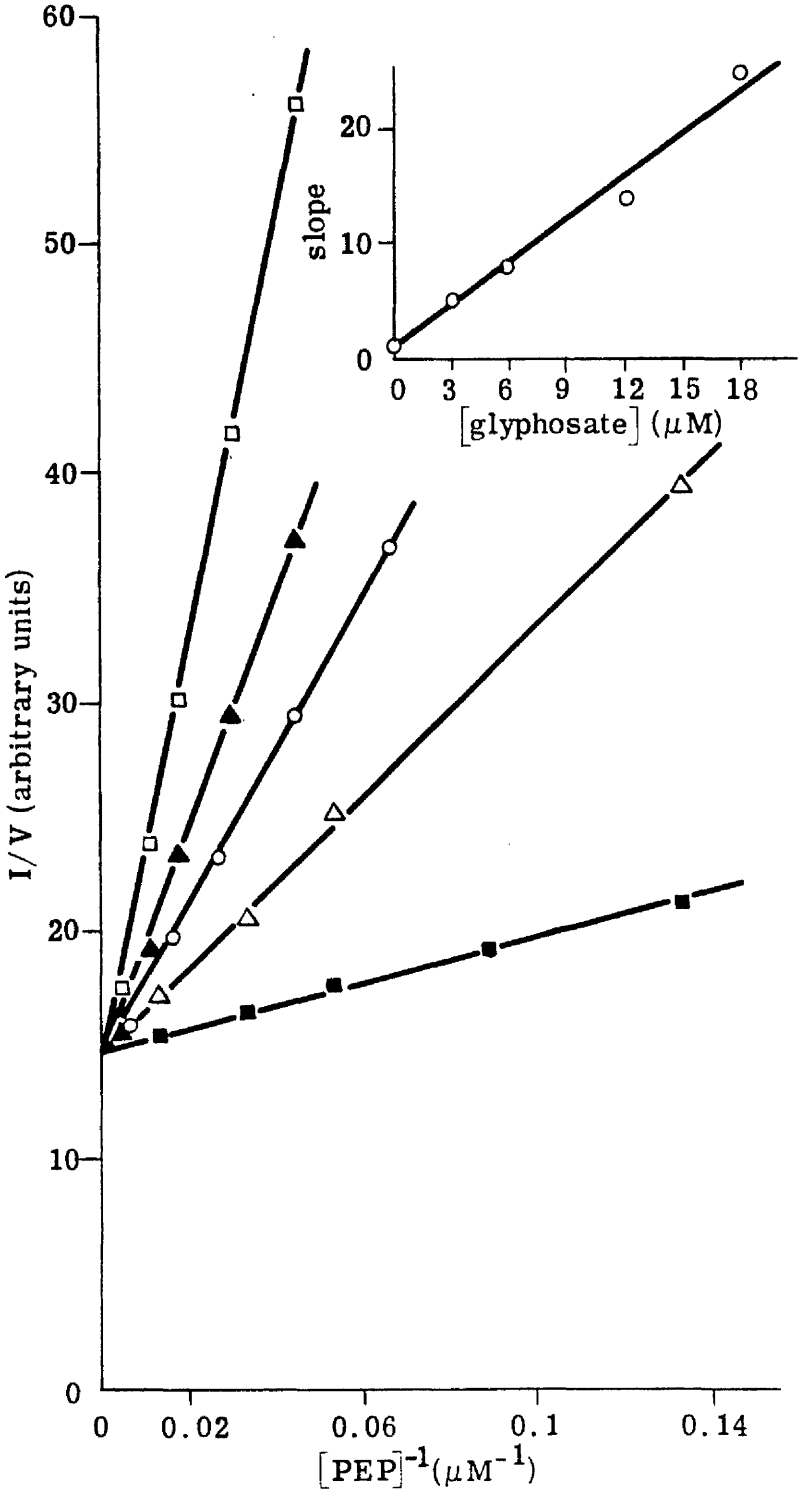


Figure 6.10 Inhibition of the forward reaction of EPSP synthase by glyphosate ([PEP] varied)

Double reciprocal plot of initial velocity versus [PEP] at fixed 177μM Shik3P, and glyphosate at: ■ 0; Δ 3μM; ○ 6μM; ▲ 12μM; □ 18μM. Inset: replot of slopes as a function of [glyphosate]. Fluorimetric assay system.

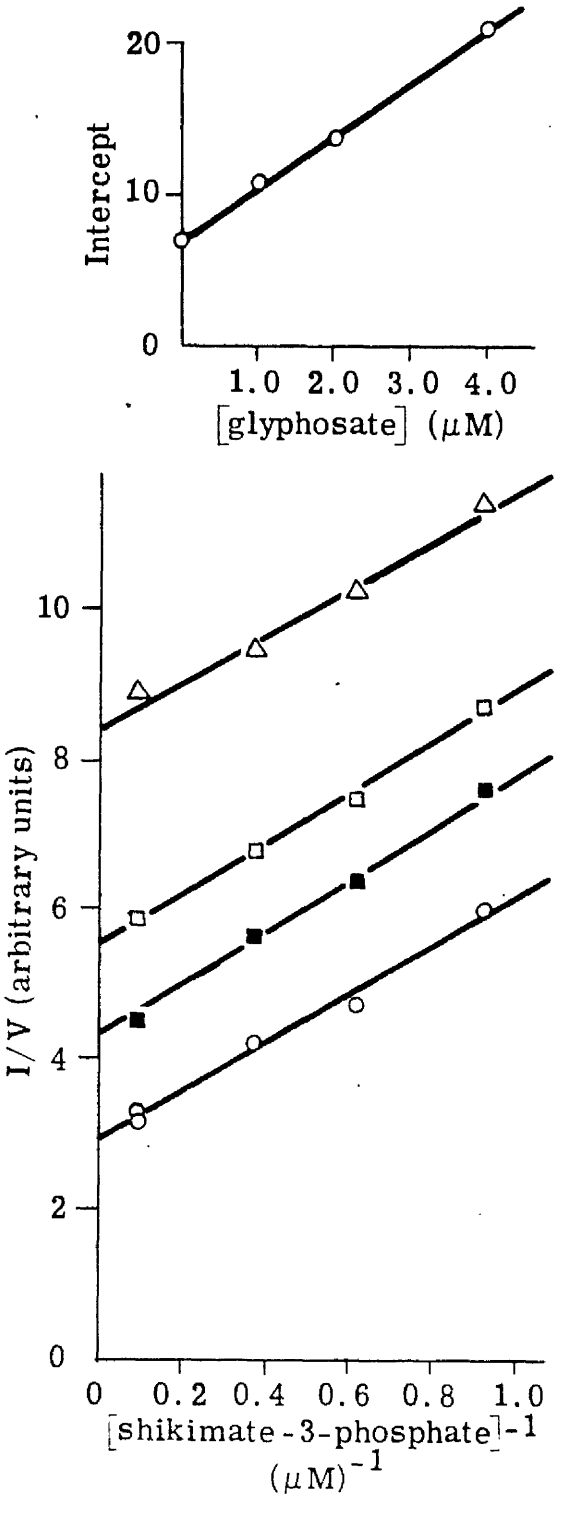


Figure 6.11 Inhibition of the forward reaction of EPSP synthase by glyphosate ([Shik3P] varied)

Double reciprocal plot of initial velocity versus [Shik3P] at fixed 5.63μM PEP and glyphosate at: o O; ■ 1μM; □ 2μM; Δ 4μM. Inset: replot of intercepts as a function of [glyphosate]. Fluorimetric assay system.

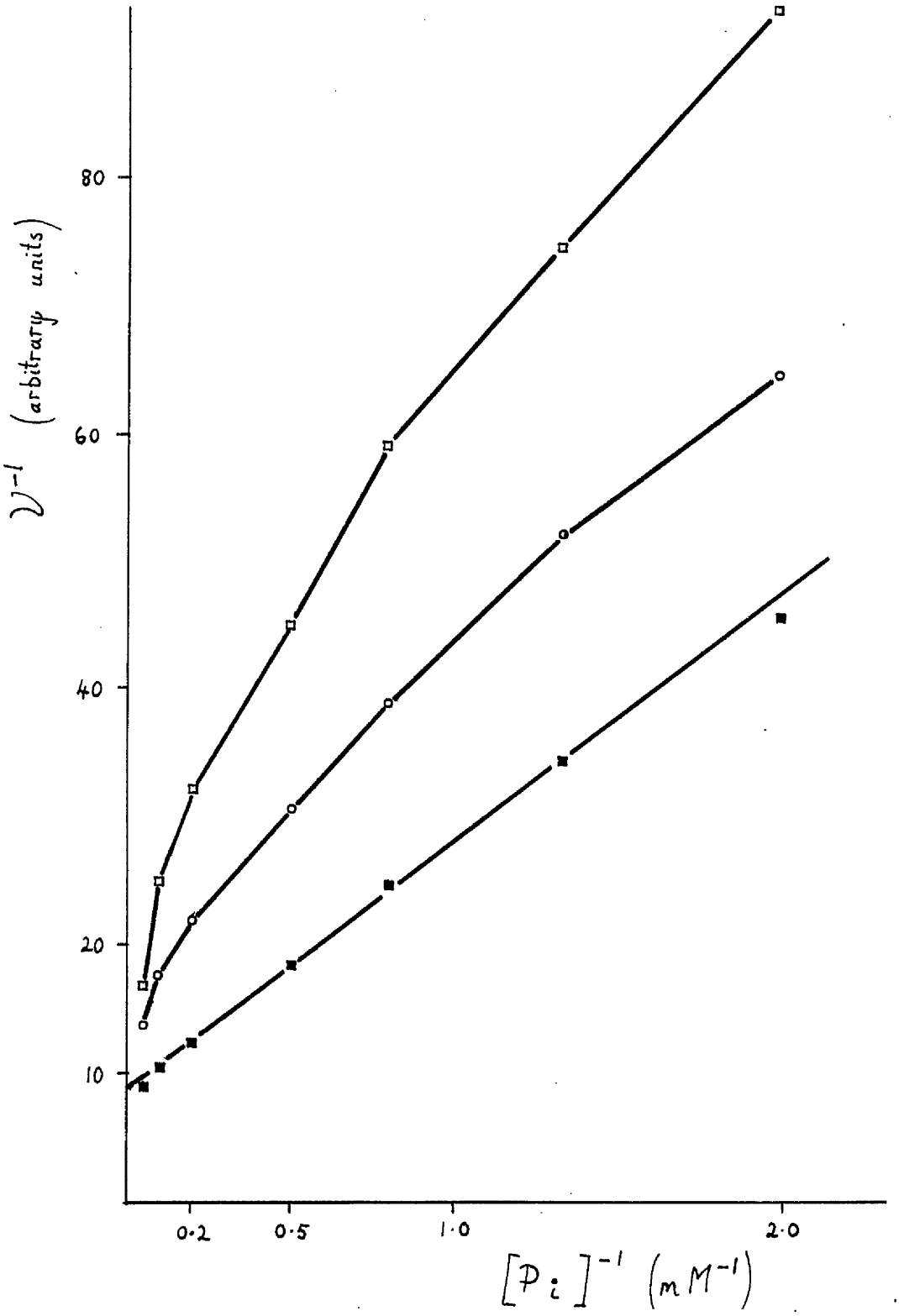


Figure 6.12 Inhibition of the reverse reaction of EPSP synthase by glyphosate ($[P_i]$ varied)

Double reciprocal plot of initial velocity versus $[P_i]$ at fixed 32.8μM EPSP and [glyphosate] at: ■ 0; o 48.2μM; □ 96.4μM. Spectrophotometric assay system.

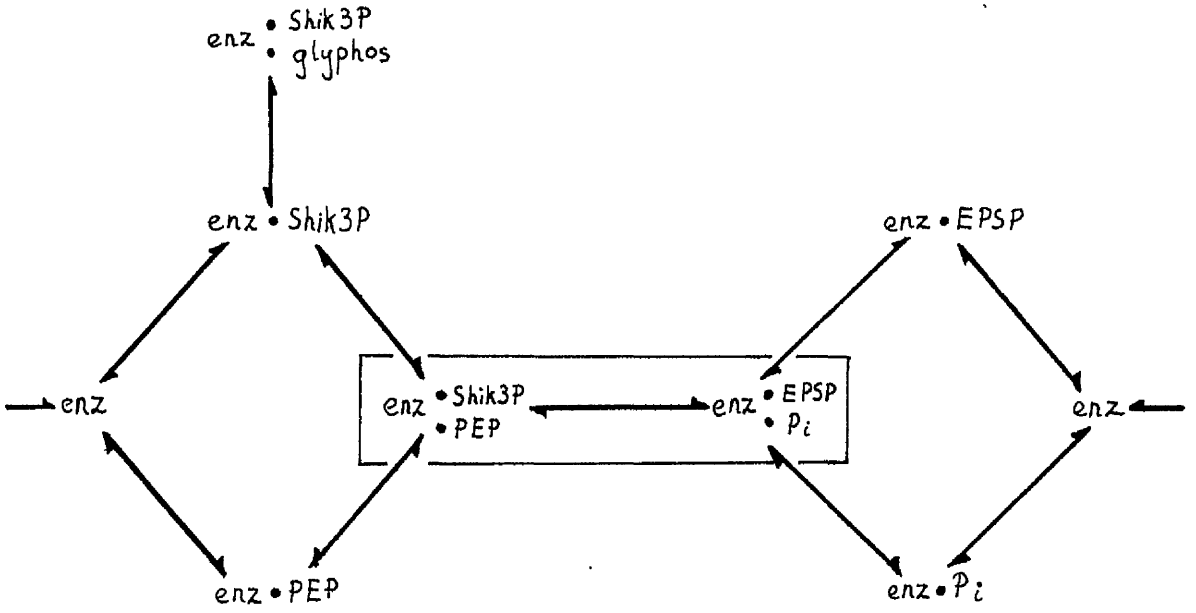


Figure 6.13 The general sequential mechanism for EPSP synthase

If glyphosate binds uniquely to the enzyme • shikimate 3-phosphate complex it may be predicted that the herbicide will act as an uncompetitive inhibitor with respect to both substrates in the reverse reaction of EPSP synthase. Glyphosate is not a competitive inhibitor with respect to EPSP in the reverse reaction; there is no significant relief of glyphosate inhibition at high saturating concentrations of EPSP ($\geq 20\mu M$). It is possible that glyphosate is indeed an uncompetitive inhibitor with respect to EPSP, but this pattern is difficult to establish because of the inconveniently low K_M's for EPSP. In a related experiment using arsenate in place of phosphate it is relatively easy to demonstrate that the apparent K_M for EPSP falls when the concentration of glyphosate is raised, and a very close approximation to an uncompetitive inhibition pattern is obtained (Figure 6.25).

The pattern of glyphosate inhibition with respect to P_i in the reverse reaction is somewhat complex (Figure 6.12). Three points should be noted:

1. significant inhibition is only observed at glyphosate concentrations much higher than required for inhibition of the forward reaction.
2. at low P_i concentrations a close approximation to an uncompetitive inhibition pattern is seen.
3. very high concentrations of P_i appear to relieve glyphosate inhibition of the reverse reaction.

The inhibition pattern can be rationalised if glyphosate binds uniquely to the enzyme • shikimate 3-phosphate complex; the pronounced competitive element of glyphosate inhibition at high P_i concentrations is explicable if P_i and glyphosate compete for binding to enzyme • shikimate 3-phosphate, and if shikimate 3-phosphate can be released from the dead-end complex enzyme • shikimate 3-phosphate • P_i.

Glyphosate is presumably a relatively weak inhibitor of the reverse reaction because the enzyme • shikimate 3-phosphate complex is only a minority species in the steady state.

The inhibition patterns discussed in this section strongly support the view that glyphosate interacts uniquely with an enzyme • shikimate 3-phosphate complex in such a way as to exclude productive binding of PEP; there is no evidence that glyphosate can bind to the free enzyme or to any of the other kinetic complexes. The binding of glyphosate to enzyme • shikimate 3-phosphate is fully reversible and the observed K_I of 1.1μM places an upper limit on the true dissociation constant for this process. Glyphosate thus displays the properties of a catalytically inert structural analogue of PEP.

6.5.5 Preliminary evidence for ordered binding of Shik3P and PEP

The evidence presented in the previous section indicates that glyphosate and PEP compete for binding to enzyme • Shik3P, and that glyphosate is unable to bind to the free enzyme. The question then arises of whether PEP is able to bind productively to the free enzyme.

The results considered above could be consistent with two types of kinetic mechanism for EPSP synthase:

1. a steady state ordered sequential mechanism in which Shik3P is the first substrate to bind to the enzyme.
2. a random-order sequential mechanism.

The intersecting initial velocity patterns of the forward and reverse reactions of EPSP synthase and the glyphosate inhibition patterns strongly suggest that Shik3P is the first substrate to bind to the enzyme in an ordered sequential mechanism. This type of mechanism provides the most satisfactory explanation of the striking uncompetitive pattern of glyphosate inhibition with respect to Shik3P. This uncompetitive

pattern can also be rationalised in terms of a random sequential mechanism, but it is then necessary to postulate that glyphosate interacts uniquely with just one of the two kinetic intermediates (free enzyme and enzyme • Shik3P) that bind PEP. (Figure 6.13). In view of the clear-cut competition between glyphosate and PEP for binding to enzyme • Shik3P a random mechanism may be considered unlikely.

6.5.6 Product inhibition patterns

Product inhibition studies were undertaken in an attempt to rule out random binding of PEP and Shik3P, and to define the order of release of products from the enzyme (Table 6.6).

The uncompetitive pattern of glyphosate inhibition with respect to Shik3P implies that Shik3P can bind to the free enzyme. EPSP is a competitive inhibitor with respect to Shik3P in the forward reaction, while Shik3P is a competitive inhibitor with respect to EPSP in the reverse reaction (Figures 6.14 & 6.15). These patterns indicate that Shik3P and EPSP are both able to bind to the free enzyme, and are mutually exclusive. Ordered sequential mechanisms in which Shik3P is the second substrate to bind to the enzyme, or EPSP is the first product to be released, are thus ruled out.

Three general types of sequential mechanism remain to be considered:

1. random-order mechanims.
2. ordered mechanimsm in which Shik3P is the first substrate to bind to the enzyme and P_i is the first product to be released.
3. ordered mechanisms in which Shik3P is bound before PEP, but EPSP and P_i are bound and released in a random order (and also the converse of this mechanism).

These three possibilities will be considered in turn.

Table 6.6 Product inhibition patterns

Reaction	Inhibitor	Variable substrate	Fixed substrate	Pattern	Assay buffer	Figure
→ E5	EPSP	Shik3P	PEP	Competitive	B	6.15
	EPSP	PEP	Shik3P	Noncompetitive	B	6.16
	P_i	Shik3P	PEP	Noncompetitive	A	not shown
	P_i	PEP	Shik3P	Noncompetitive	A	6.17
← E5	Shik3P	EPSP	P_i	Competitive	A	6.14
	Shik3P	P_i	EPSP	Noncompetitive	A	not shown

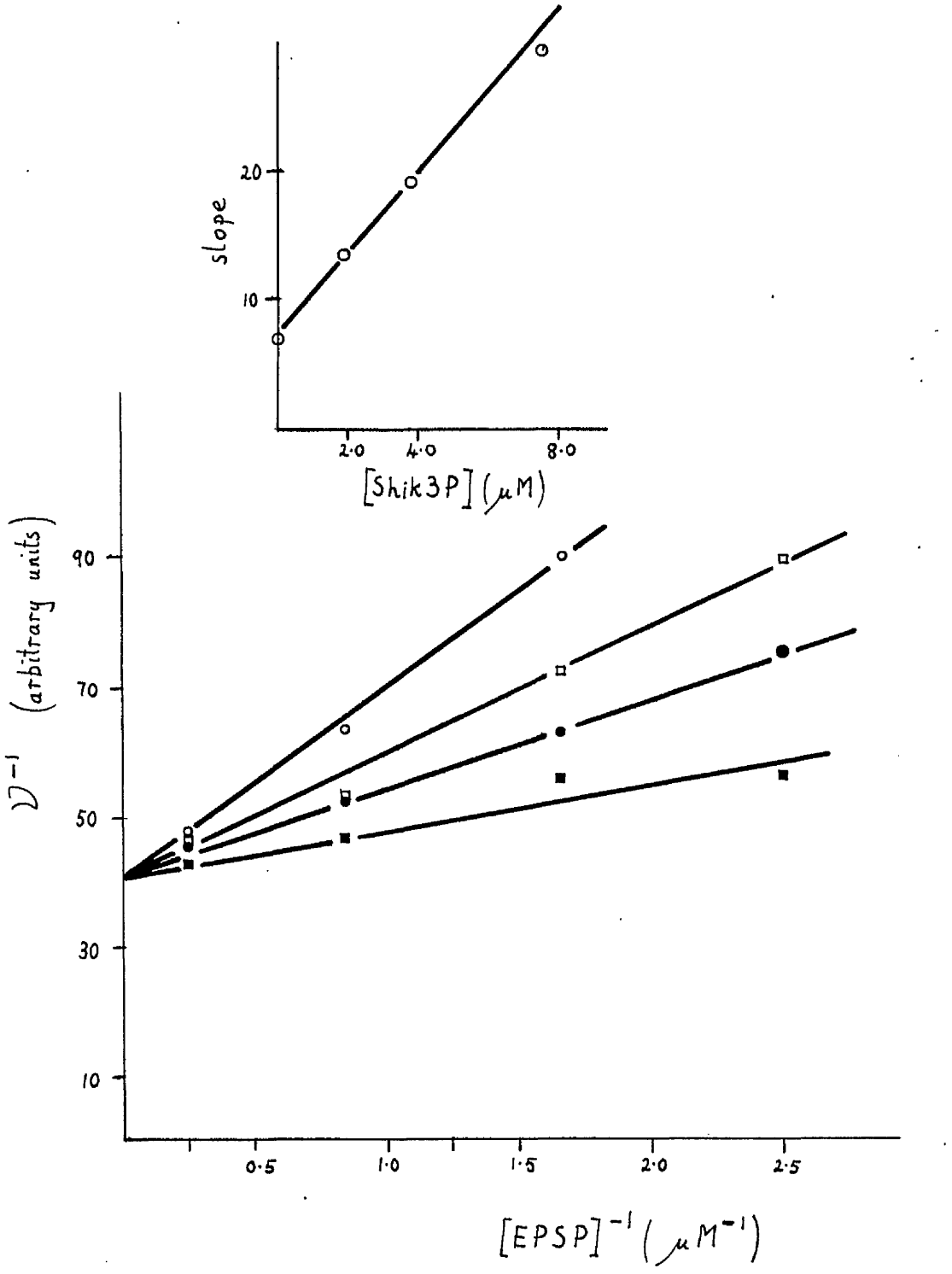


Figure 6.14 Inhibition of the reverse reaction of EPSP synthase by shikimate 3-phosphate ([EPSP] varied)

Double reciprocal plot of initial velocity versus [EPSP] at fixed 1mM P_i and [Shik3P] at: ■ 0; o 1.9μM; □ 3.8μM; o 7.6μM. Inset: replot of slopes as a function of [Shik3P]. Fluorimetric assay system.

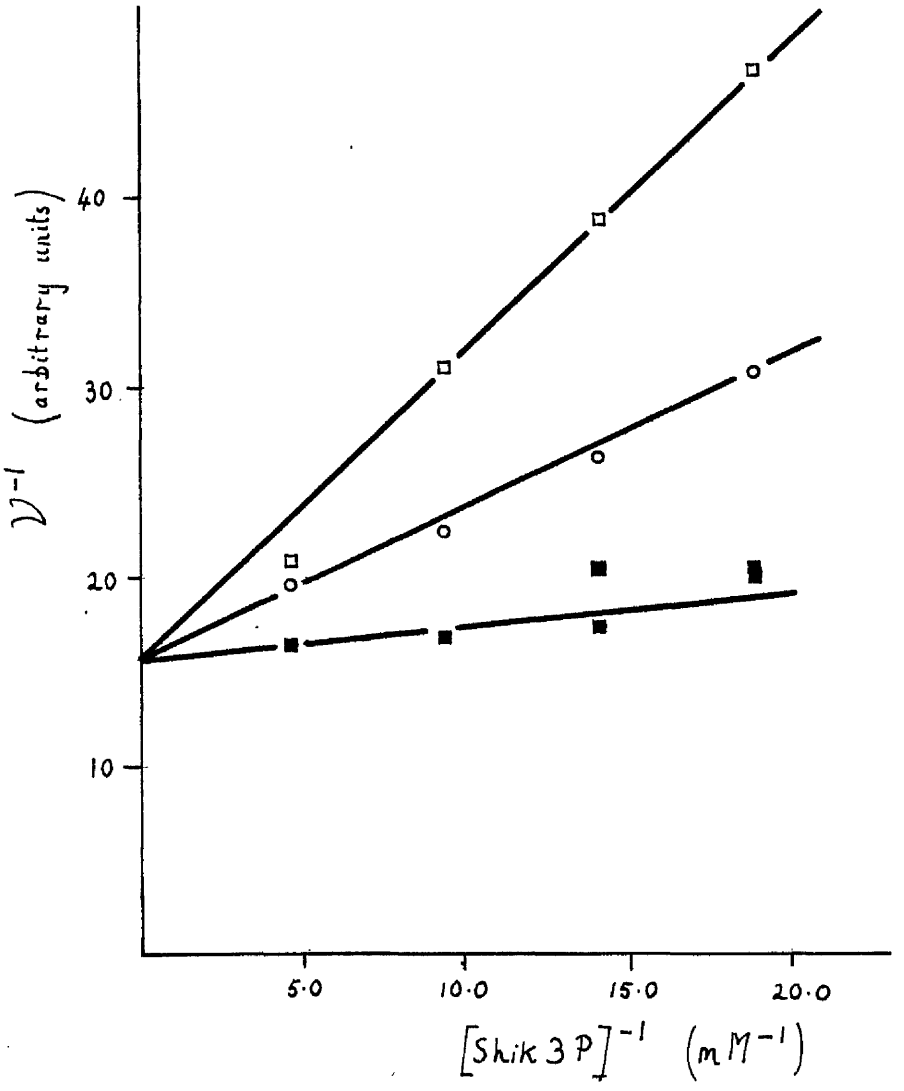


Figure 6.15 Inhibition of the forward reaction of EPSP synthase by EPSP [Shik3P] varied).

Double reciprocal plot of initial velocity versus [Shik3P] at fixed 203μM PEP and [EPSP] at: ■ 0; o 34.1μM; □ 68.2μM. Assays were performed in buffer C, pH 7.0, using the direct spectrophotometric assay.

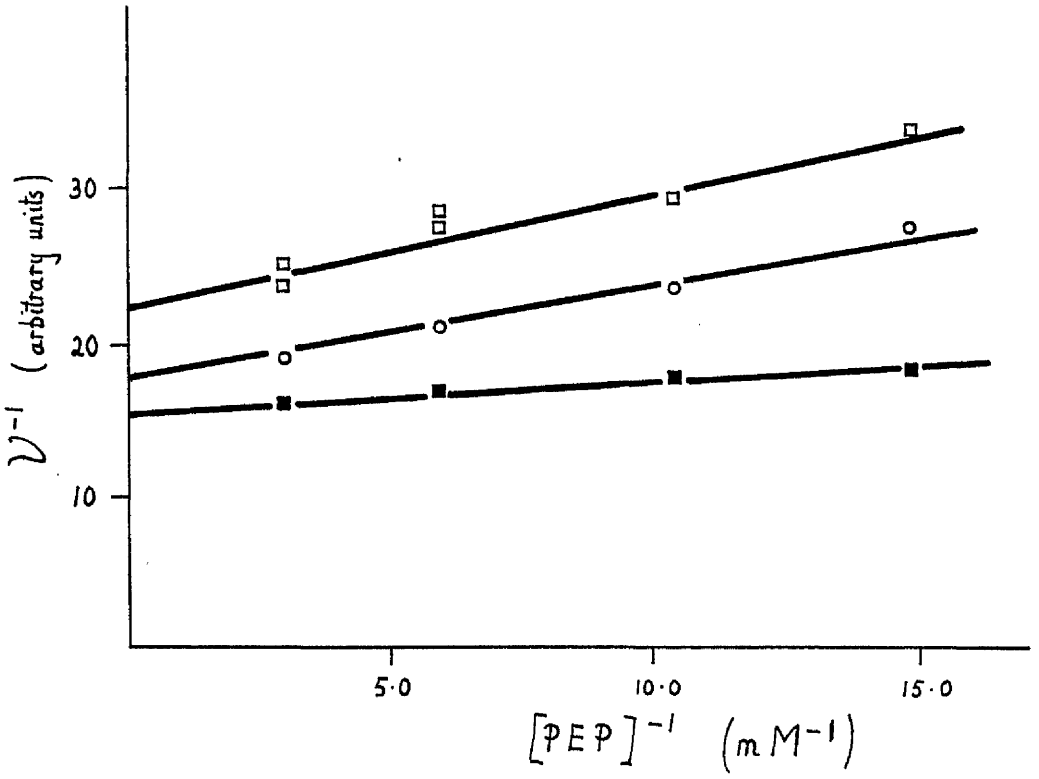


Figure 6.16 Inhibition of the forward reaction of EPSP synthase by EPSP ([PEP] varied)

Double reciprocal plot of initial velocity versus [PEP] at fixed 106μM Shik3P and [EPSP] at: ■ 0; O 34.1μM; □ 68.2μM. Assays were performed in buffer C, pH 7.0, using the direct spectrophotometric assay.

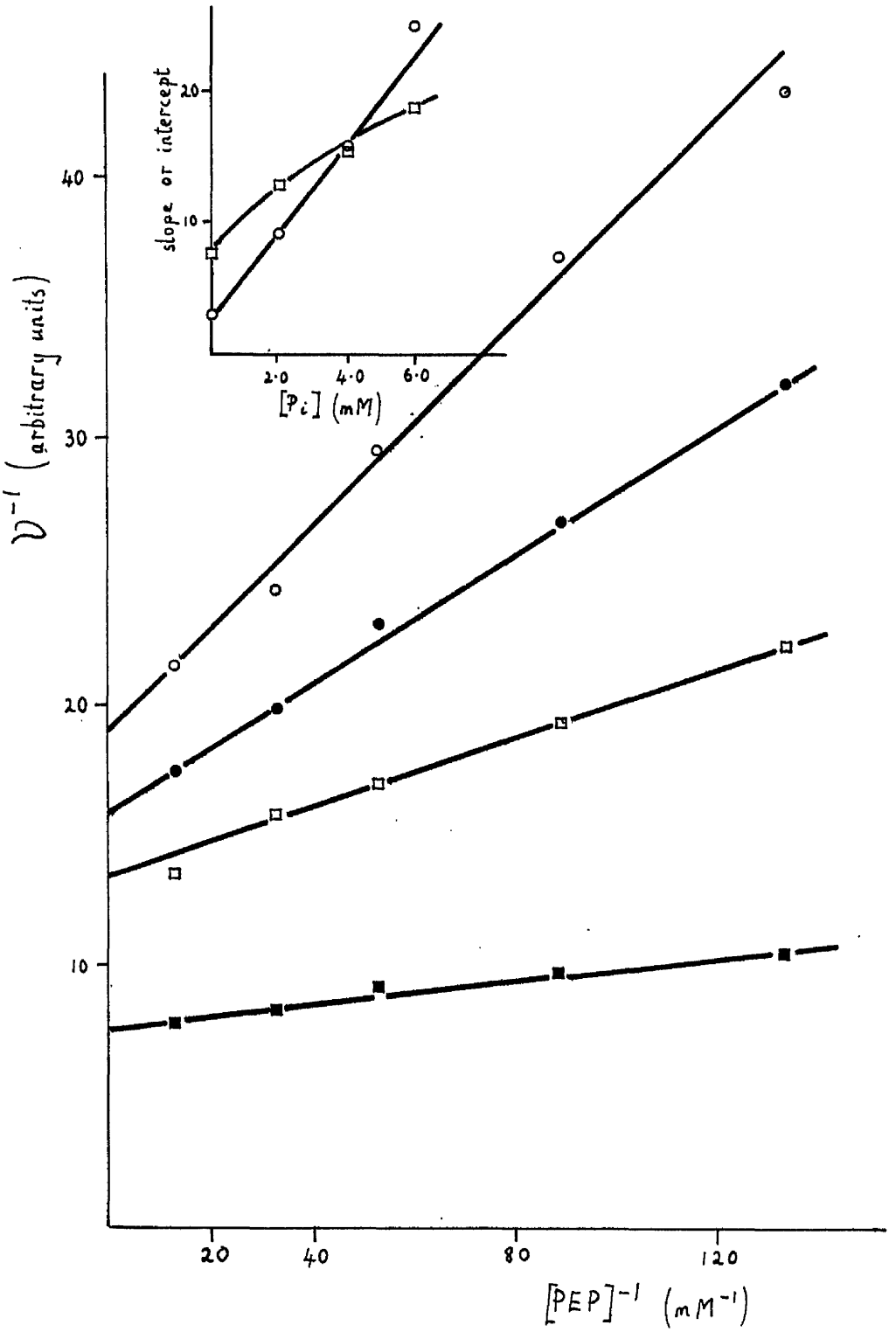


Figure 6.17a Inhibition of the forward reaction of EPSP synthase by P_i ([PEP] varied: low concentration range)

Double reciprocal plot of initial velocity versus [PEP] at fixed 177μM Shik3P and P_i at: ■ 0; □ 2mM; ● 4mM; ○ 6mM. Inset: replot of slopes (⊙) and intercepts (□) as a function of [P_i]. Fluorimetric assay system.

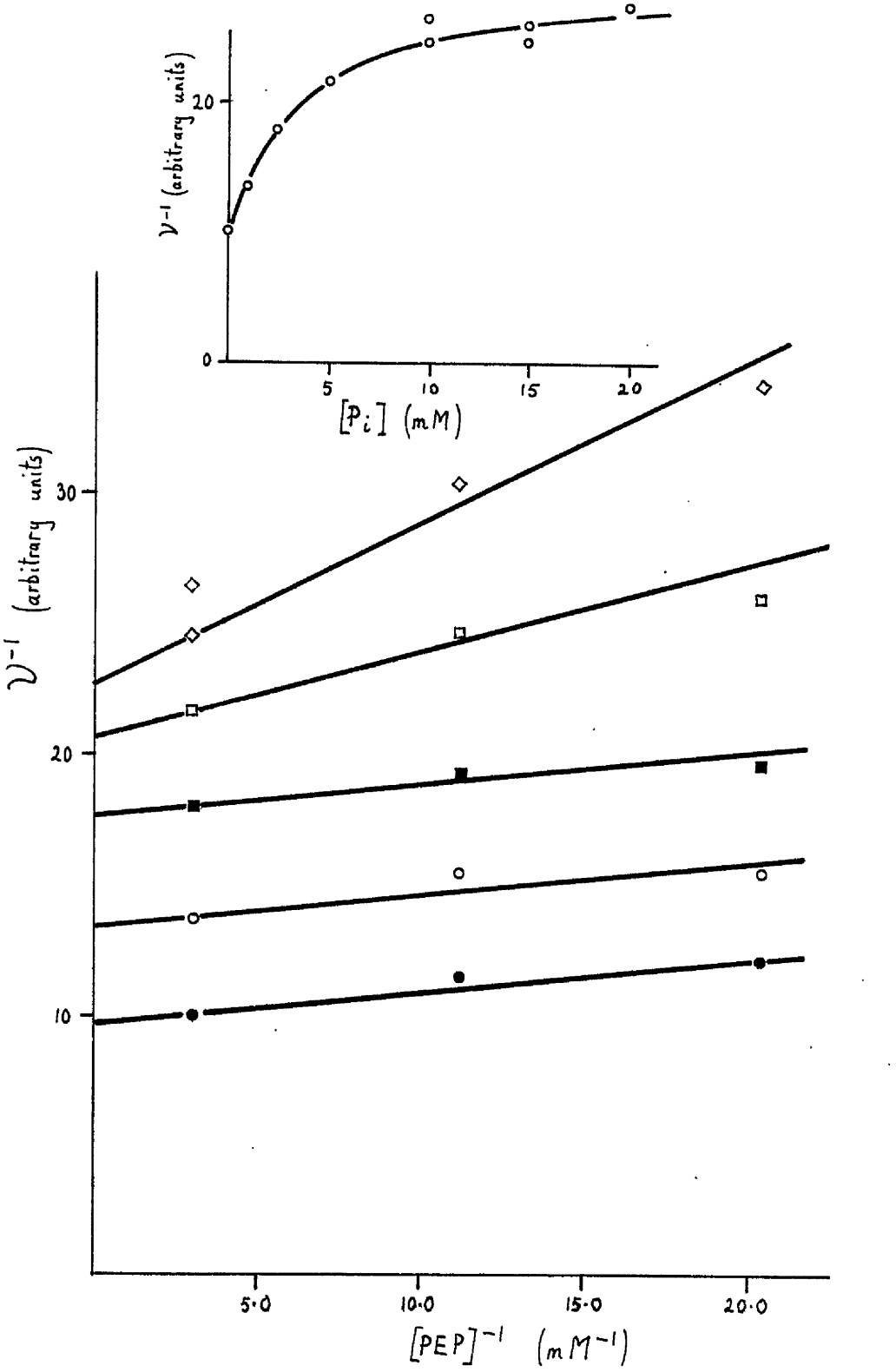


Figure 6.17b Inhibition of the forward reaction of EPSP synthase by P_i ([PEP] varied: high concentration range)

Double reciprocal plot of initial velocity versus [PEP] at fixed 255μM Shik3P and [P_i] at: ● 0; ○ 1mM; ■ 2.5mM; □ 5mM; ◇ 10mM. Inset: Dixon plot showing (initial velocity)$^{-1}$ as a function of [P_i] at fixed concentrations of 255μM Shik3P and 447μM PEP (the highest of the 3 concentrations used in this experiment). Spectrophotometric assay system.

6.5.7 Evidence against random binding of Shik3P and PEP

If EPSP synthase followed a rapid equilibrium ordered mechanism all product inhibition patterns would be competitive. On the strength of three clear-cut noncompetitive patterns a mechanism of this type can be ruled out with confidence (Table 6.6). The product inhibition patterns are accommodated only a little more easily within the framework of a steady state random mechanism. To explain the noncompetitive patterns of EPSP and P_i inhibition with respect to PEP it is necessary to postulate rate limiting dissociation of EPSP from enzyme • EPSP • P_i after reveral of the phosphate dissociation steps, and formation of an enzyme • EPSP • PEP dead-end complex. A steady state random mechanism always gives rise to nonlinear initial rate equations, but if the rate equations approach the rapid equilibrium limit the nonlinearity of the initial velocity patterns may not easily be detected; the product inhibition patterns should then closely resemble those predicted for the limiting case of a rapid equilibrium random mechanism (Cleland, 1970). A steady-state random mechanism for EPSP synthase is thus considered highly implausible for two reasons:

1. the formation of an enzyme • EPSP • PEP dead-end complex is likely to be stereochemically unreasonable
2. EPSP synthase displays neither the competitive product inhibition patterns characteristic of a mechanism close to the rapid equilibrium limit nor the nonlinear initial velocity patterns characteristic of other steady state mechanisms.

A number of lines of evidence strongly suggest that PEP cannot bind productively to any kinetic intermediate other than enzyme • ShiK3P:

1. Neither EPSP nor P_i acts as a competitive inhibitor with respect to PEP (evidence that both P_i and EPSP can bind to the free enzyme is presented in Section 6.5.9).

2. Glyphosate acts as a competitive inhibitor with respect to PEP but binds only to enzyme • Shik3P.

3. PEP cannot exclude productive binding of either EPSP or arsenate in the reverse reaction (see Section 6.6.3).

Kinetic mechanisms in which PEP is able to bind productively to the free enzyme will not be considered further.

6.5.8 Evidence for an ordered sequential mechanism

There are two special cases of the ordered sequential mechanism in which only Shik3P and EPSP bind to the free enzyme. These are the Theorell-Chance mechanism, which requires mutually competitive product inhibition by PEP and P_i, and the rapid equilibrium ordered mechanism, which requires that K_M/K_M^* for the first-bound substrate should approach zero. Both mechanisms can be ruled out on the basis of the initial velocity patterns and product inhibition data (Tables 6.3 & 6.6).

In the general case of a steady state ordered sequential mechanism for EPSP synthase in which Shik3P is bound first and P_i released first the following product inhibition patterns are predicted:

1. EPSP with respect to Shik3P : competitive
2. EPSP with respect to PEP : noncompetitive
3. P_i with respect to PEP : noncompetitive
4. P_i with respect to Shik3P : noncompetitive
   (but uncompetitive at high saturating concentrations of PEP)

All four patterns are seen in product inhibition experiments with arom EPSP synthase (Table 6.6 and Figures 6.15, 6.16 & 6.17). It is important to realise that these four patterns are not diagnostic for the fully ordered mechanism, but are also consistent with kinetic mechanisms in which Shik3P and PEP bind to the enzyme in the same compulsory order

and the products P_i and EPSP are released (and bound) in a random order. Although the kinetic properties of EPSP synthase discussed up to this point are consistent only with ordered sequential mechanisms in which Shik3P is the first substrate to bind to enzyme, further information is needed to define the order of product release.

6.5.9 Evidence that EPSP and P_i are bound in a random order

The major outstanding problem concerning the kinetic properties of EPSP synthase is to discriminate between steady state sequential mechanisms in which Shik3P is the first substrate to bind in the forward reaction and:

1. EPSP is the first substrate to bind in the reverse reaction.
2. EPSP and P_i are bound in a random order.

A diagnostic feature of the fully ordered mechanism (1) is the predicted conversion of the noncompetitive pattern of P_i inhibition with respect Shik3P into an uncompetitive pattern at highly saturating concentrations of PEP. This experiment has not been attempted with arom EPSP synthase because of the very low limiting K_M for Shik3P at saturation in PEP. A number of alternative approaches have been employed to distinguish between the two possible mechanisms listed above.

A steady state ordered sequential mechanism for EPSP synthase in which neither PEP nor P_i can bind productively to the free enzyme has a number of consequences:

1. the rate constant for dissociation of Shik3P from enzyme • Shik3P calculated from the kinetic parameters will exceed k_{cat} for the reverse reaction; the calculated rate constant for dissociation of EPSP will exceed k_{cat} for the forward reaction. This is the powerful maximum rate test for ordered mechanisms of Dalziel (1957).

2. P_i will act as a noncompetitive inhibitor with respect to PEP, and at high concentrations it will totally block release of EPSP from the enzyme. If the dead-end complex enzyme • Shik3P • P_i can form it is expected that the intercept and slope replots from the primary double reciprocal plot will be linear and parabolic, respectively.

3. glyphosate inhibition of the reverse reaction will not be relieved at high concentrations of P_i, since even if the dead-end complex enzyme • Shik3P • P_i is formed the release of Shik3P to yield a productive enzyme • P_i complex is forbidden.

Two further predictions can be made if the substitution of As_i for P_i does not affect the compulsory order of EPSP and P_i/As_i binding (Evidence that As_i can replace P_i without seriously perturbing the catalytic mechanism is presented in Section 6.6).

4. At saturation in Shik3P and PEP, high concentrations of As_i will totally block release of EPSP from the enzyme in the forward reaction.

5. In the "cyclic" arsenate- and EPSP-dependent PEP hydrolysis reaction catalysed by EPSP synthase the apparent K_M for EPSP will "vanish" at saturation in arsenate. This is because the association and dissociation of EPSP is not part of the catalytic cycle and enzyme • EPSP will be wholly converted into enzyme • EPSP • As_i (see Section 6.6.3).

Taking these predictions in turn:

1. EPSP synthase passes the Dalziel maximum rate test for the compulsory release of Shik3P as a second product in the reverse reaction; the calculated dissociation rate constant for Shik3P is 14-fold higher than k_{cat} for the reverse reaction. EPSP synthase fails the Dalziel maximum rate test for the release of EPSP as a second product in the forward reaction; the rate constant for dissociation of EPSP calculated on the assumption that the mechanism is fully ordered is only $6.1 S^{-1}$, while k_{cat} for the forward reaction is $21\ S^{-1}$ (see Table 6.7). These results indicate that EPSP and P_i cannot bind to the enzyme in a compulsory order. Because the reverse reaction is slower than the forward reaction, a compulsory order of substrate binding simply cannot account for the nearly parallel initial velocity patterns seen in the reverse reaction.

2. Phosphate is not a linear inhibitor of the forward reaction, and acts as a noncompetitive partial inhibitor with respect to PEP (Figure 6.17). This result cannot easily be rationalised unless EPSP is able to dissociate from enzyme • EPSP • P_i, and this would imply random binding of EPSP and P_i.

3. As discussion in Section 6.5.4, glyphosate inhibition of the reverse reaction appears to be relieved at high concentrations of P_i (Figure 6.12). This result is most easily rationalised if release of Shik3P from an enzyme • Shik3P • P_i dead-end complex yields a productive enzyme • P_i complex.

Table 6.7 Dalziel maximum rate tests for EPSP synthase

1. If Shikimate 3-phosphate is the second product to be released from the enzyme in the reverse reaction of EPSP synthase the following inequality should hold:

$$\overleftarrow{k_{cat}} \leqslant \overrightarrow{k_{cat}} \times K_M^*(\text{Shik3P})/K_M(\text{Shik3P})$$

Substituting the estimated values of kinetic parameters listed in Table 6.3:

$$11.6 \leqslant 21.1 \times (2.88/0.362)$$

The kinetic parameters are thus consistent with ordered binding of shikimate 3-phosphate as a first substrate in the forward reaction.

2. If EPSP is the second product to be released from the enzyme in the forward reaction of EPSP synthase the following inequality should hold:

$$\overrightarrow{k_{cat}} \leqslant \overleftarrow{k_{cat}} \times K_M^*(\text{EPSP})/K_M(\text{EPSP})$$

Substituting the estimated values of the kinetic parameters listed in Table 6.3:

$$21.1 \not\leqslant 11.6 \times (114/219)$$

The kinetic parameters are thus incompatible with ordered binding of EPSP as a first substrate in the reverse reaction.

4. At saturation in Shik3P and PEP, high concentrations of As_i do not totally block release of EPSP from the enzyme, although a productive ternary complex for the As_i-dependent reverse reaction forms under the same conditions (see Figure 6.22).

5. The apparent K_M for EPSP of the EPSP- and As_i-dependent PEP hydrolysis reaction does not vanish, and is in fact highest at saturation in As_i (see Figure 6.23).

These last two results clearly rule out a compulsory order for binding of EPSP and As_i, and indicate that As_i can bind productively to the free enzyme. Since P_i is a competitive inhibitor with respect to As_i in the As_i-dependent reverse reaction (Figure 6.20) this implies that P_i can also bind to the free enzyme.

In the light of these results it can be stated with confidence that the simplest type of ordered mechanism, in which only Shik3P and EPSP are able to bind to the free enzyme, is inconsistent with the observed kinetic properties of arom EPSP synthase. It is therefore proposed that Shik3P is the first substrate to bind in the forward reaction of EPSP synthase and that EPSP and P_i bind in a random order in the reverse reaction (Figure 6.18). This proposed kinetic mechanism appears to be unique in providing a straight forward explanation for all the catalytic properties of EPSP synthase discussed here.

6.5.10 The proposed kinetic mechanism of arom EPSP synthase

The proposed kinetic mechanism of arom EPSP synthase is shown in Figure 6.18. In addition to the productive kinetic complexes, two dead-end complexes, enzyme • Shik3P • glyphosate and enzyme • Shik3P • P_i, are required to explain the glyphosate and P_i inhibition patterns

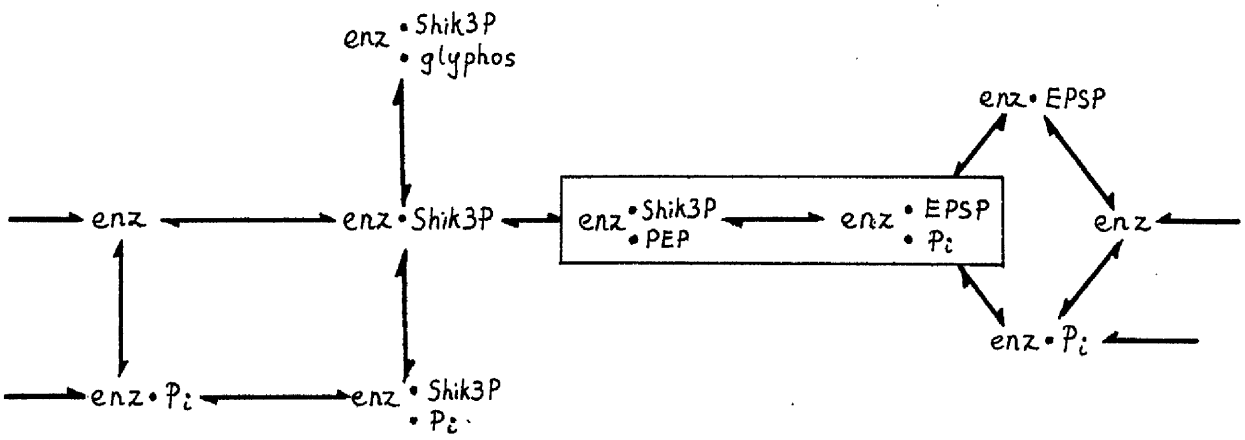


Figure 6.18 The proposed kinetic mechanism of arom EPSP synthase

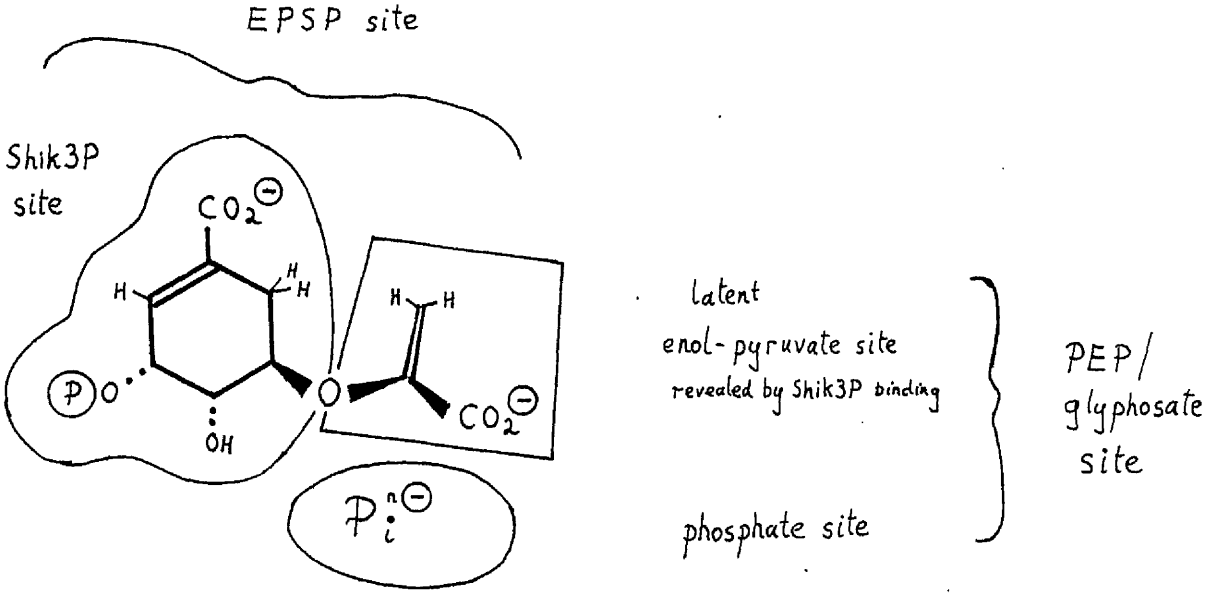


Figure 6.19 A pictorial view of the EPSP synthase catalytic site that is consistent with the observed kinetic properties of the arom enzyme complex

discussed in the previous sections. The active site of EPSP synthase may thus be imagined pictorially as shown in Figure 6.19. It is possible to speculate that the latent PEP/glyphosate binding site in the free enzyme is only unmasked by a conformational change that accompanies the binding of Shik3P to the free enzyme. If there is such a conformational change it must be extremely rapid, since the estimated bimolecular rate constant for formation of the productive enzyme • Shik3P complex is $5.8 \times 10^{7} M^{-1} S^{-1}$; this is very close to the diffusion controlled limit of $\approx 10^{9} M^{-1} S^{-1}$ (Eigen & Hammes, 1963; see Section 6.7.3 and table 6.4b)

6.6 Kinetic experiments using arsenate as a substrate for EPSP synthase

A major drawback of the pyruvate kinase-linked assay system for the authentic P_i-dependent reverse reaction of EPSP synthase is that PEP cannot be used as a product inhibitor. Kinetic experiments with arsenate as an alternative substrate were undertaken in the hope that this problem could be circumvented. It was also of some interest to see whether EPSP synthase would catalyse the "arsenolysis" of PEP.

6.6.1 The arsenate-dependent reverse reaction

Arsenate can replace phosphate as a substrate for the reverse reaction of EPSP synthase; the immediate products of the reaction are Shik3P and free pyruvate. Reaction traces are always linear and there is no evidence of a transient "lag" due to rate-limiting decomposition of arsenoenolpyruvate, the presumed product of the enzymatic reaction.

The arsenate-dependent reverse reaction exhibits linear saturation kinetics with respect to arsenate and has a limiting K_M for arsenate of 1.13mM and a calculated k_{cat} of $18.7 s^{-1}$. Like the

phosphate-dependent reverse reaction, the arsenate-dependent reverse reaction is saturated by inconveniently low concentrations of EPSP. Phosphate is a competitive inhibitor of the arsenate-dependent reaction, with a K_I of 1.6mM (Figure 6.20). Glyphosate also inhibits the reaction, in a manner highly reminiscent of glyphosate inhibition of the normal phosphate-dependent reaction (Figures 6.21 & 6.12).

Taken together these findings indicate that arsenate can substitute for phosphate without grossly altering the catalytic mechanism of EPSP synthase; arsenate is presumably incorporated into a productive ternary complex enzyme • EPSP • As_i analogous to that formed in the authentic reverse reaction.

A large number of enzymes are unable to discriminate between the chemically very similar arsenate and phosphate anions (Sutton, 1958). The arsenate-dependent reactions generally differ only in the kinetic instability to hydrolysis of the arsenate esters or mixed anhydrides that are formed. There is, however, clear evidence that the labile reaction products are frequently released from the enzyme surface before hydrolysis occurs. One of the best-characterised examples of an enzyme with a broad specificity for structural analogues of phosphate is glyceraldehyde 3-phosphate dehydrogenase, which can utilise arsenate, molybdate, tungstate and a variety of phosphonates, RPO_3^{2-} (Byers et al, 1979; Adams et al, 1983). EPSP synthase appears not to have as broad a substrate specificity as glyceraldehyde 3-phosphate dehydrogenase; catalytic activity was not evident when arom was incubated with 50μM EPSP and either 10mM molybdate, 10mM phosphite or 15mM phosphonoformate. Arsenate, however, is a good hyperbolic substrate for arom EPSP synthase, but is a rather poor substrate for glyceraldehyde 3-phosphate dehydrogenase (Byers et al, 1979).

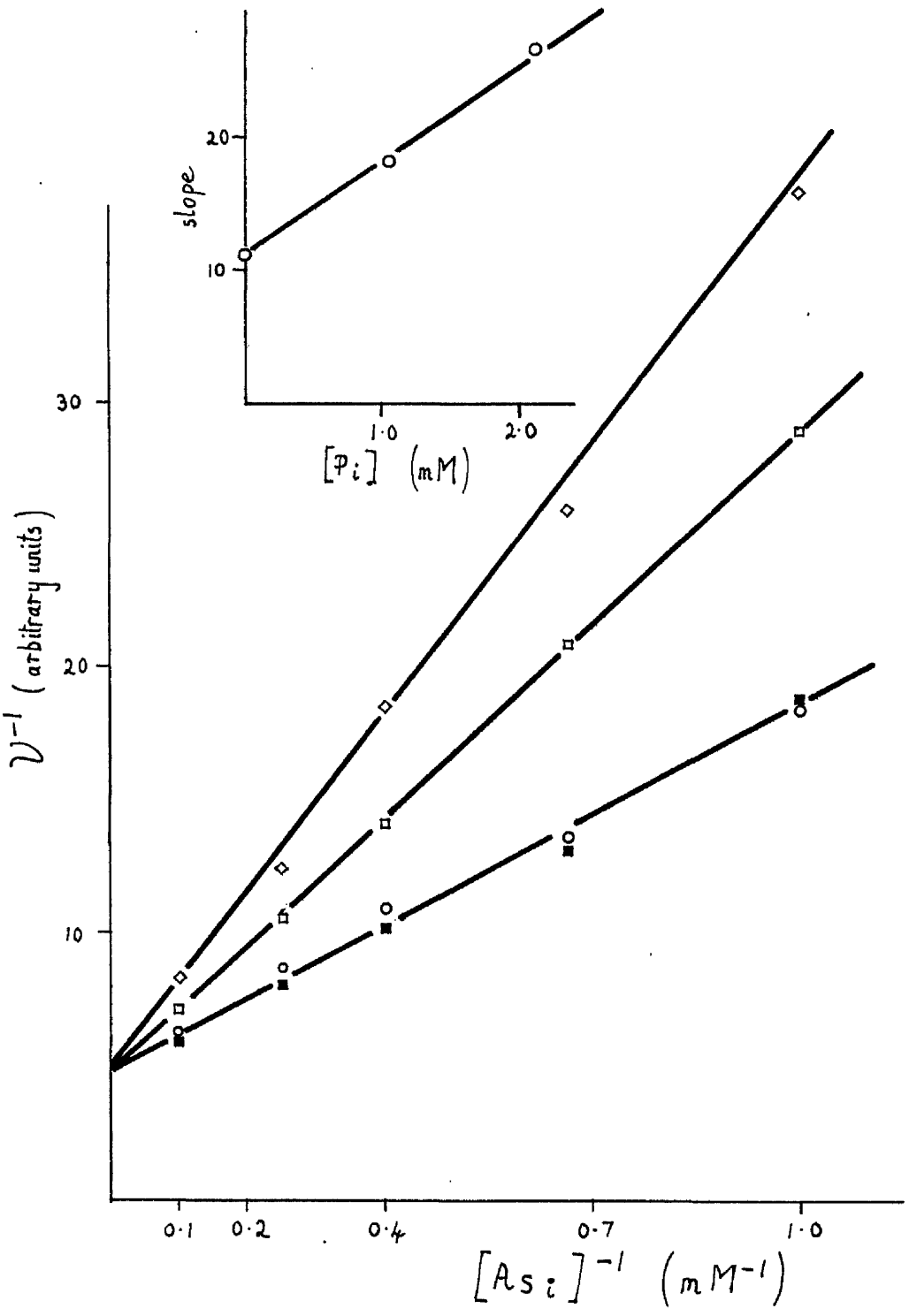


Figure 6.20 Inhibition of the As_i-dependent reverse reaction of EPSP synthase by P_i and by PEP

Double reciprocal plot of initial velocity of free pyruvate release versus $[As_i]$ at fixed 20μM EPSP and P_i at ■ O; □ 1.05mM; ◇ 2.1mM. The initial velocities under the same conditions at fixed 10μM EPSP, zero P_i, and 311μM PEP are also recorded (o). Inset: replot of slopes as a function of $[P_i]$.

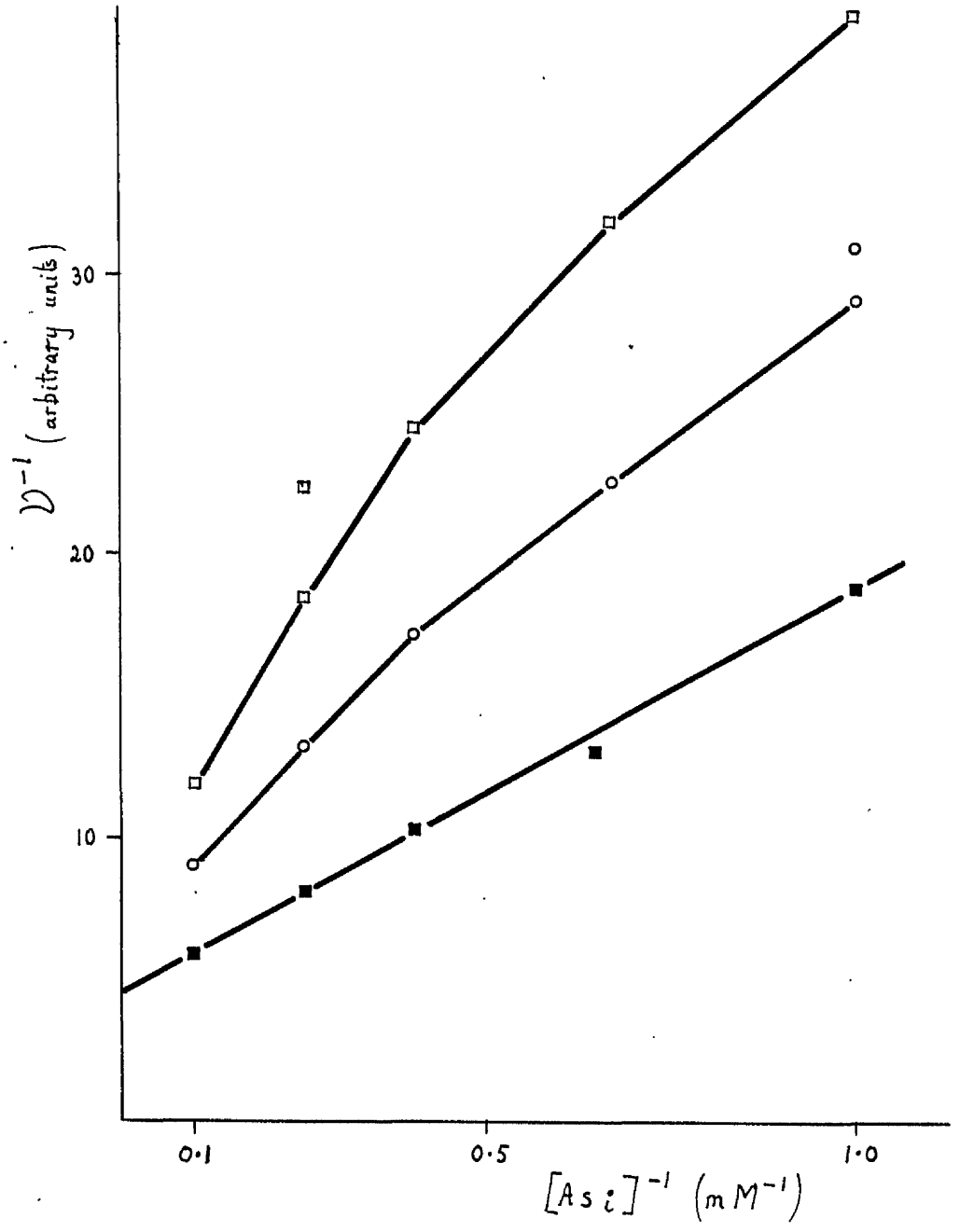


Figure 6.21 Inhibition of the As_i-dependent reverse reaction of EPSP synthase by glyphosate

Double reciprocal plot of initial velocity versus $[As_i]$ at fixed 10μM EPSP and glyphosate at ■ 0; o 96μM; □ 193μM.

The question of whether or not the arsenoylated product of the reverse reaction of EPSP synthase is hydrolysed before it leaves the enzyme is probably irrelevant to the discussion that follows. The hydrolysis of enzyme-bound arsenoenolpyruvate would yield arsenate and pyruvate; pyruvate does not displace PEP from its binding site (Table 6.9) and would presumably dissociate from the enzyme very rapidly. For the sake of clarity alone the dissociation of the elements of arsenoenolpyruvate will be referred to as "pyruvate release".

6.6.2 Arsenolysis of PEP

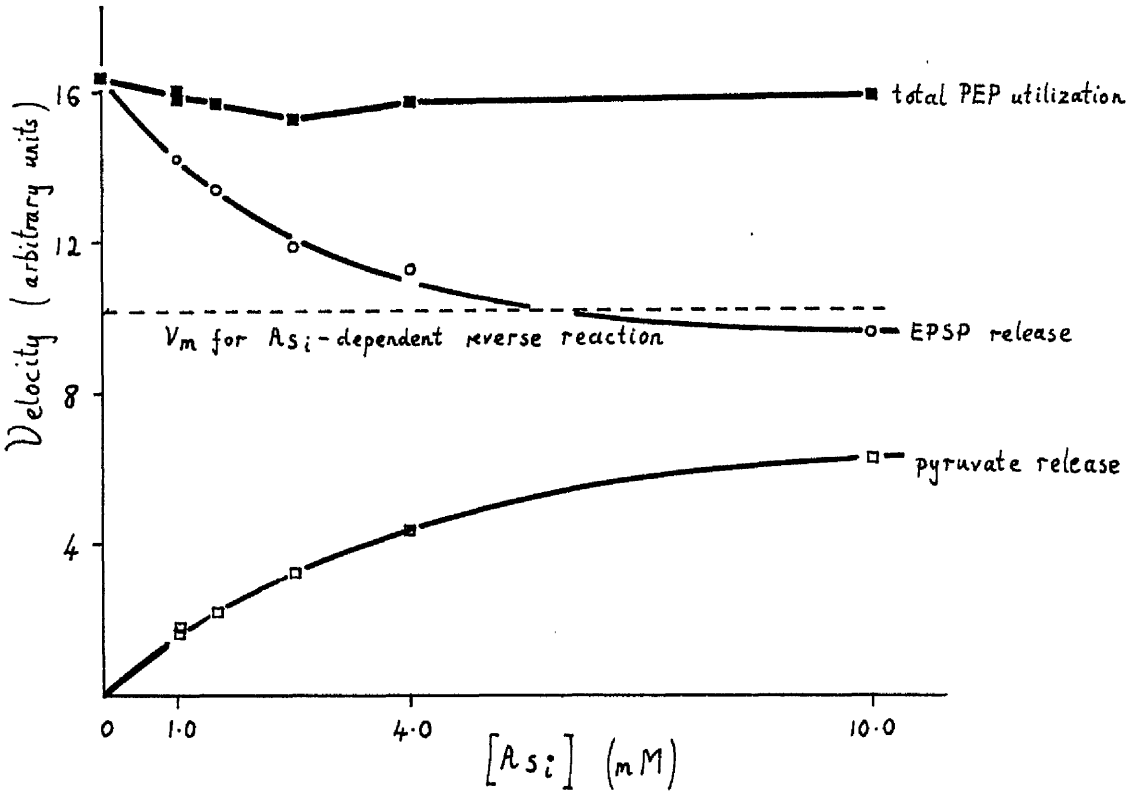
If the catalytic mechanism of arom EPSP synthase involved a covalently bound enzyme-pyruvate intermediate it might be expected that arom would catalyse the hydrolysis of PEP in the presence of As_i. No such hydrolysis of PEP is seen (e.g. Figure 6.23). Since As_i can substitute for P_i in the reverse reaction this experiment is equivalent to an attempt to exchange labelled phosphorus between PEP and P_i. The result confirms that arom EPSP synthase does not have a "covalent intermediate" mechanism of the ping-pong type. However, a "covalent intermediate" mechanism involving an enzyme • PEP • Shik3P ternary complex analogous to that proposed for UDPGlcNAc pyruvyl transferase cannot be ruled out on this evidence.

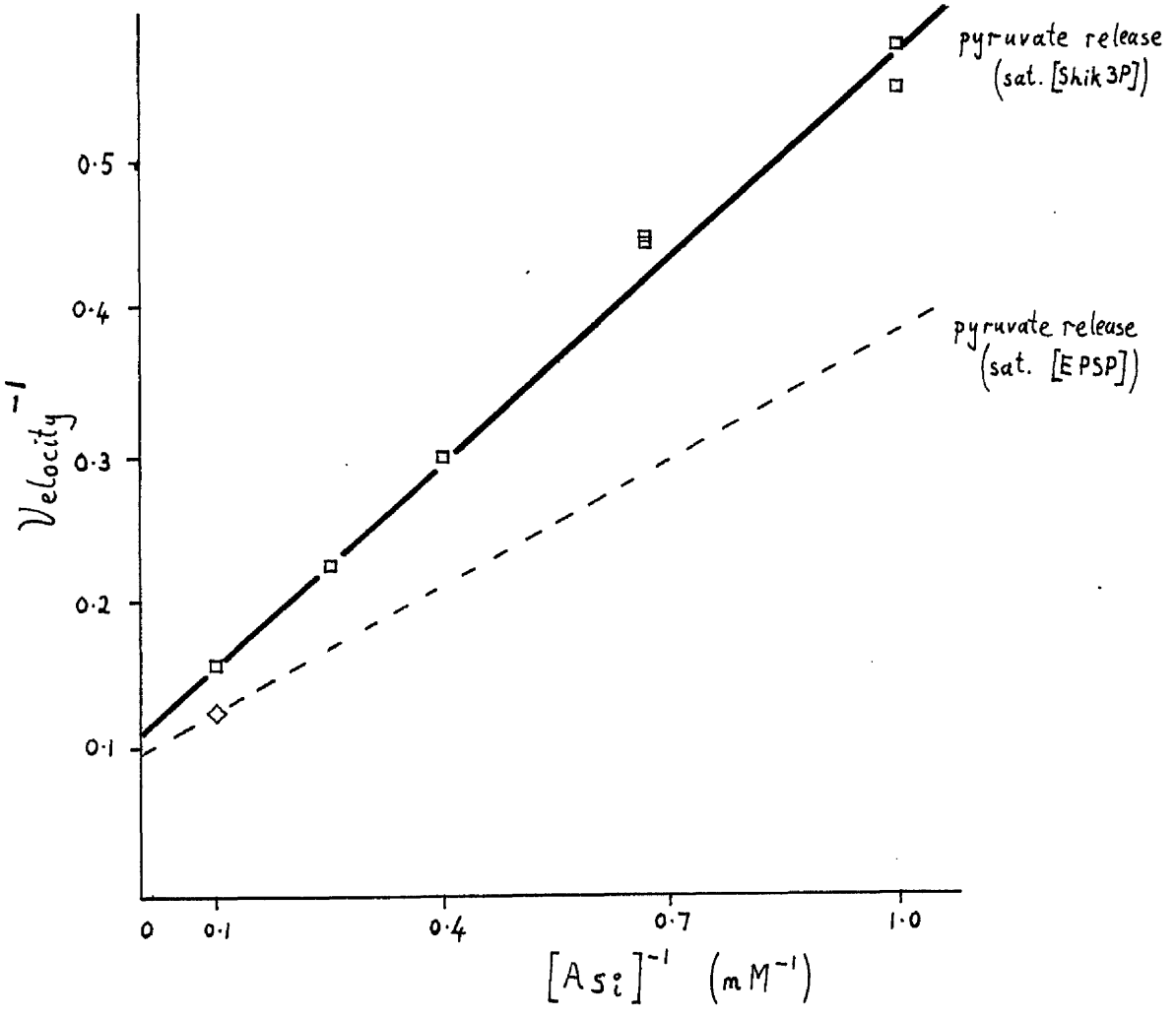
In the presence of arsenate and both substrates of the forward reaction of EPSP synthase arom does catalyse the hydrolysis of PEP. At high saturating concentrations of PEP and Shik3P, and in the presence of excess chorismate synthase activity, the initial rate of PEP hydrolysis is easily measured by coupling the release of pyruvate to the lactate dehydrogenase reaction. The rate of PEp hydrolysis saturates as the As_i concentration is raised (see Figure 6.22). At the same time arom continues to synthesise EPSP from PEP and Shik3P, although at a reduced

Figure 6.22 Utilisation of PEP by EPSP synthase in the presence of As_i and saturating concentrations of PEP and Shik3P

The upper plot shows the initial velocities of EPSP release from EPSP synthase as a function of [As_i] at fixed 311μM PEP and 255μM Shik3P, in the presence of 6mU/ml chorismate synthase, 10μM FMN and 20μM NADPH (○). The initial velocity of pyruvate release as a function of [As_i] was measured under identical conditions, except that the assays also contained 100μM NADH and 2.5U/ml lactate dehydrogenase (□). The total rate of PEP utilisation under these conditions, the sum of the rates of pyruvate and EPSP release, is plotted as a function of [As_i] (■), and the saturated velocity of the As_i-dependent reverse reaction, assayed under the same conditions, is also recorded.

The lower plot is a double reciprocal plot of the same data, showing the initial velocity of pyruvate release as a function of [As_i] (□). Also plotted on the same axes is the rate of pyruvate release observed under the same conditions at 311μM PEP, 10mM As_i and 10μM EPSP (◇). The line showing the rate of pyruvate release in the As_i-dependent reverse reaction as a function of [As_i] at saturation in [PEP] and [EPSP] is extrapolated from the data of figure 6.23.


Velocity (arbitrary units)
16
12
8
4
total PEP utilization
V_m for As_i-dependent reverse reaction
EPSP release
pyruvate release
0
1·0
4·0
10·0
$[As_i]$ (mM)


pyruvate release (sat. [Shik3P])
pyruvate release (sat. [EPSP])
$Velocity^{-1}$
0·5
0·4
0·3
0·2
0·1
0
0·1
0·4
0·7
1·0
$[As_i]^{-1}$ (mM^{-1})

rate. The total rate of PEP utilisation is hardly altered as the As_i concentration is raised (see Figure 6.22).

The results presented in section 6.6.1 strongly suggest that arsenate can take the place of phosphate at all points in the reverse reaction of EPSP synthase. It is thus logical to examine the possiblity that the inhibition of the forward reaction by arsenate at high saturating concentrations of PEP and Shik3P is analogous to the inhibition of the forward reaction by phosphate under similar conditions. The "intercept" component of P_i inhibition with respect to PEP (Figure 6.17) is ascribed to reversal of the step

$$\text{enzyme} \bullet \text{EPSP} \bullet P_i \longrightarrow \text{enzyme} \bullet \text{EPSP}$$

and diversion of the flow of intermediates into the alternative pathway for the release of products

$$\text{enzyme} \bullet \text{EPSP} \bullet P_i \longrightarrow \text{enzyme} \bullet P_i$$

"Reversal" of the P_i dissociation step by arsenate should lead to the ternary complex enzyme • EPSP • As_i, which is presumably an obligatory intermediate in the As_i-dependent reverse reaction. The release of pyruvate can then be seen as a natural consequence of the diversion of intermediates into the kinetic pathway of the As_i-dependent reverse reaction (see Figure 6.24*b*.).

At saturation in As_i, PEP and Shik3P the rate of pyruvate release reaches approximately 87% of the rate of the arsenate-dependent reverse reaction at saturation in As_i and EPSP (Figure 6.22). This result is consistent with the proposal that pyruvate is released from the enzyme only when intermediates are diverted into the kinetic pathway of the As_i-dependent reverse reaction. The Shik3P-dependent "arsenolysis" of PEP by EPSP synthase is thus easily explained within the framework of the proposed kinetic mechanism (Figures 6.24 & 6.18).

6.6.3 Hydrolysis of PEP in the presence of EPSP and As_i

A startling feature of the As_i-dependent reverse reaction is the apparent failure of PEP to act as an inhibitor (Figure 6.20). This is an illusion created by the assay system. The progress of the As_i-dependent reverse reaction is followed by coupling the release of pyruvate to the lactate dehydrogenase reaction. When one attempts to inhibit the reaction with PEP the rate of pyruvate release is indeed unchanged, but the source of the pyruvate is no longer EPSP, but PEP itself. This is most clearly evident when a very low concentration of EPSP (100nM) is used to initiate the reaction in the presence of arsenate and relatively high concentrations of PEP. Pyruvate release then begins immediately and continues at a constant rate while excess PEP remains available. The rate of PEP hydrolysis is sensitive only to the initial concentrations of EPSP and arsenate; the full kinetic pattern is illustrated in the double reciprocal plot, Figure 6.23.

The EPSP/As_i-dependent PEP hydrolysing activity of arom EPSP synthase has a number of very interesting properties. The double reciprocal plot, Figure 6.23, shows good linearity over a wide range of concentrations of EPSP and As_i, as do the replots of slopes and intercepts. This indicates that the reciprocal rate of the PEP-hydrolysing reaction is a linear function of the reciprocal concentrations of EPSP and As_i, despite the fact that neither EPSP nor As_i is consumed. The saturated rate of the PEP-hydrolysing reaction is essentially identical to that of the As_i-dependent reverse reaction; the limiting kinetic parameters are listed in Table 6.3.

There is a strong resemblance between the kinetic patterns of the authentic P_i-dependent reverse reaction and the EPSP/As_i-dependent PEP hydrolysis reaction (Figures 6.9 & 6.23). At saturation in EPSP the kinetic pattern of the PEP hydrolysis reaction is

identical to that of the As_i-dependent reverse reaction (Figure 6.20). The intersecting kinetic pattern of the PEP hydrolysis reaction indicates that a ternary complex enzyme • EPSP • As_i is an obligatory kinetic intermediate. It is natural to suppose that the fractional concentration of enzyme • EPSP • As_i determines the rate of the PEP hydrolysis reaction (and of the As_i-dependent reverse reaction) just as the fractional concentration of enzyme • EPSP • P_i determines the rate of the authentic P_i-dependent reverse reaction. At first sight this hypothesis fails to explain why EPSP is not consumed in the PEP hydrolysis reaction.

On the basis of the proposed kinetic mechanism of Figure 6.18 and the evidence that arsenate can substitute for phosphate it is in fact possible to predict that arom EPSP synthase will exhibit EPSP/As_i-dependent PEP-hydrolysing activity, as is illustrated in Figure 6.24a. When the ternary complex enzyme • EPSP • As_i is consumed in the As_i-dependent reverse reaction, arsenoenolpyruvate is released from the enzyme first, leaving enzyme • ShiK3P, an obligatory intermediate in the forward reaction sequence. Diversion of enzyme • Shik3P into enzyme • Shik3P • PEP will inhibit the As_i-dependent reverse reaction, but it need not substantially reduce the rate of pyruvate release if enzyme • Shik3P • PEP is rapidly converted into enzyme • EPSP via the catalytic steps of the forward reaction. A cyclic sequence is therefore possible, in which the overall reaction is simply the hydrolysis of PEP. In this sequence the elements of PEP are released in two stages, the carbon skeleton in the step leading to enzyme • Shik3P, and the phosphate group after the regeneration of EPSP (Figure 6.24). At saturation in PEP the cyclic reaction should dominate the catalytic proceedings and Shik3P will not be released from the enzyme at a significant rate.

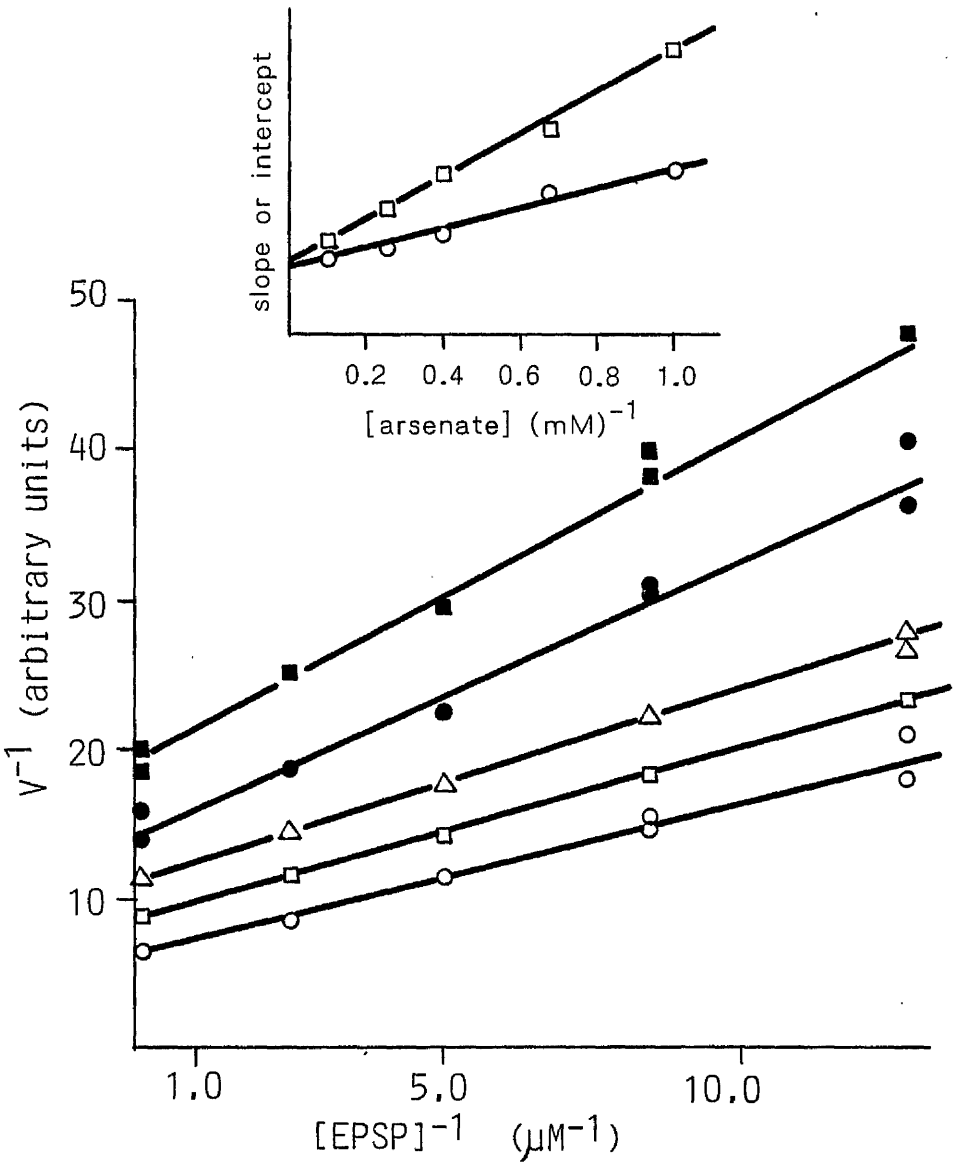
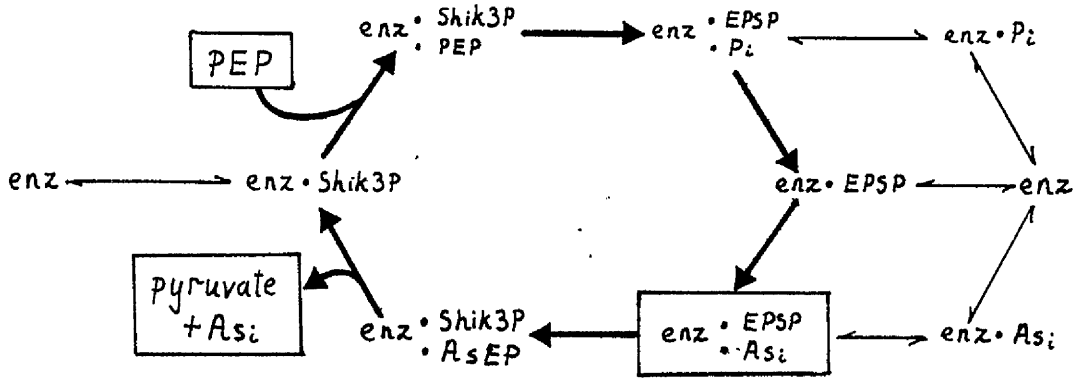


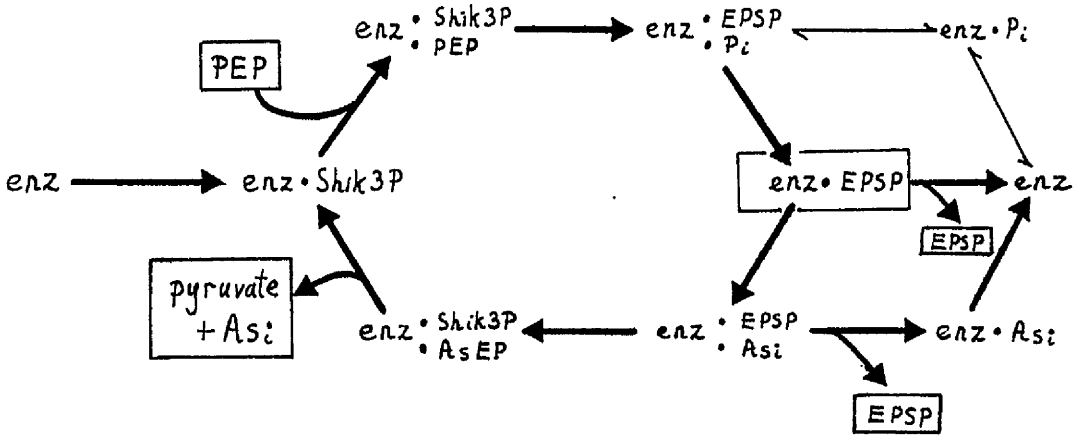
Figure 6.23 Initial velocities of the EPSP/As_i-dependent PEP-hydrolysing activity of EPSP synthase

Double reciprocal plot showing the initial reates of free pyruvate release as a function of [EPSP], at fixed 311μM PEP, at a series of fixed concentrations of As_i: O 10mM; □ 4mM; Δ 2.5mM; o 1.5mM; ■ 1mM. Inset: replot of slopes (O) and intercepts (□) as a function of [arsenate]$^{-1}$.

Figure 6.24 Models for the utilisation of PEP by EPSP synthase in the presence of As_i



a. Utilization of PEP at saturating [PEP] in the presence of EPSP and As_i : heavy arrows represent net flow of kinetic intermediates.



b. Utilization of PEP at saturating [Shik3P] and [PEP] in the presence of excess chorismate synthase ; heavy arrows indicate the net flow of kinetic intermediates.

The cyclic reaction scheme shown in Figure 6.24 successfully explains several key features of the kinetic properties of the EPSP/As_i-dependent PEP hydrolysing activity of EPSP synthase:

1. the dependence of the reaction rate on [As_i] and [EPSP]
2. the insensitivity of the reaction rate to [PEP]
3. the inhibition of the reaction by high concentrations of glyphosate, and the relief of inhibition at higher concentrations of PEP (not shown).
4. the close approximation to an uncompetitive pattern of glyphosate inhibition with respect to EPSP shown in figure 6.25.

The kinetic pattern of Figure 6.23 has already been cited as confirmatory evidence that PEP cannot bind to the free enzyme and that it cannot block productive binding of either EPSP or As_i, as is required in the proposed kinetic mechanism of arom EPSP synthase (Figure 6.18). The cyclic scheme in Figure 6.24 thus provides a fully satisfactory description of the EPSP/As_i-dependent PEP-hydrolysing activity of the enzyme.

6.6.4 As_i-dependent reactions catalysed by EPSP synthase

To summarise, it can be stated that the kinetic properties of the various As_i-dependent reactions catalysed by EPSP synthase are fully consistent with:

1. the proposed kinetic mechanism, Figure 6.18
2. the proposal that arsenate can replace phosphate in the kinetic steps of the reverse reaction

Further implications of the kinetic properties of the As_i-dependent activities of the enzyme will be explored in the next section.

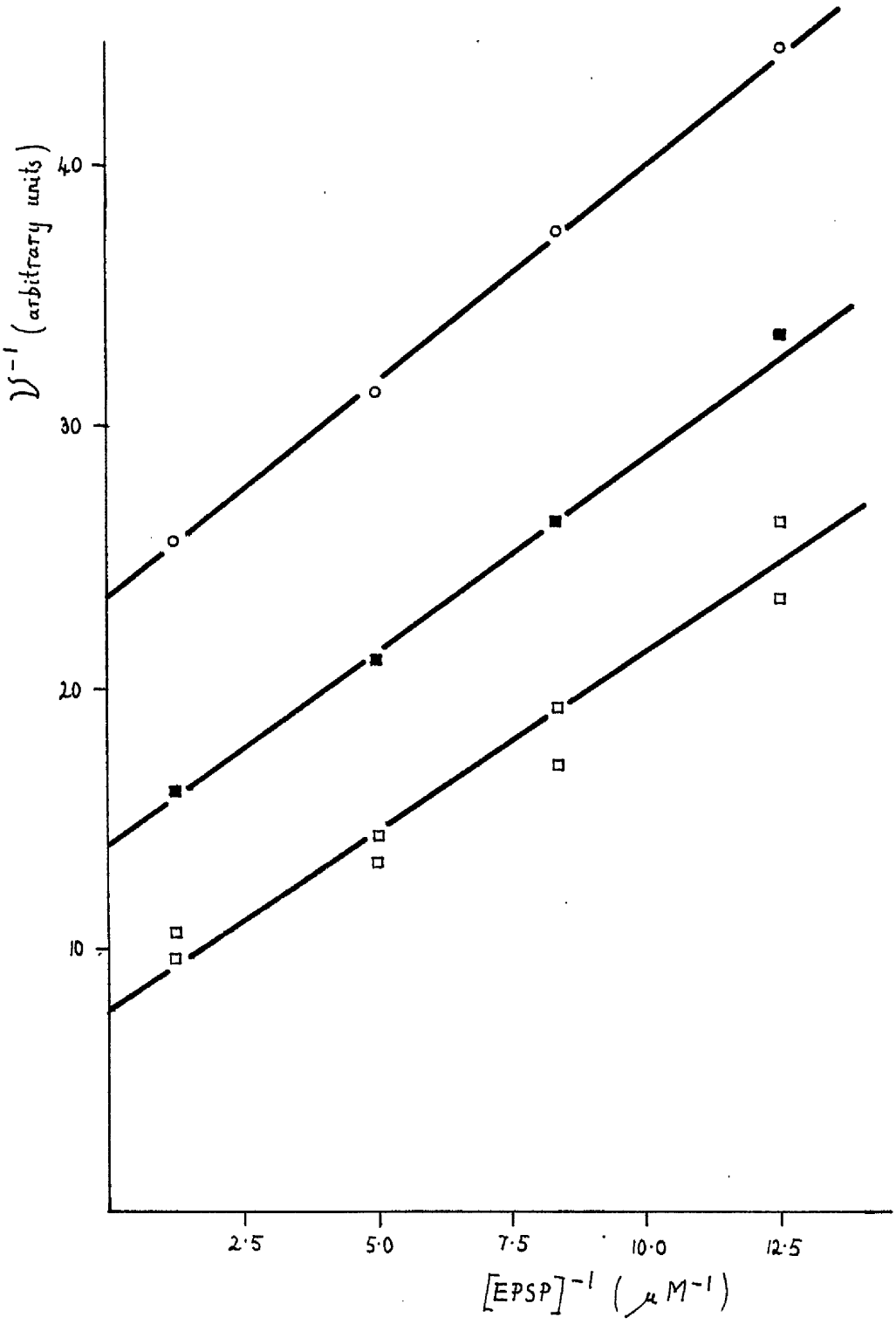


Figure 6.25 Inhibition of the EPSP/As_i-dependent PEP-hydrolysing activity of EPSP synthase by glyphosate ([EPSP] varied)

Double reciprocal plot showing the initial rates of free pyruvate release as a function of [EPSP], at fixed 203μM PEP and 5mM As_i, at a series of fixed concentrations of glyphosate: □ 0; ■ 200μM; o 400μM.

6.7 The relative rates of elementary steps in the EPSP synthase reaction

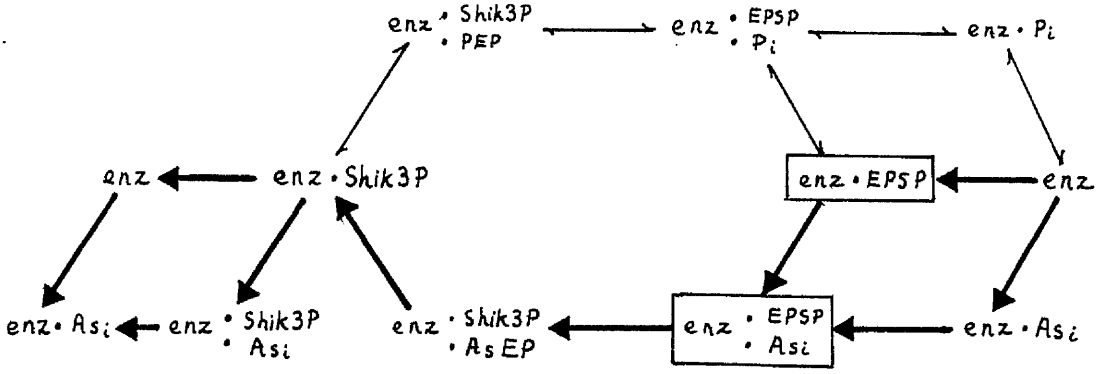
As was seen in the previous section, As_i can be used as a convenient "labelled" substitute for P_i in steady state kinetic experiments with EPSP synthase. A careful comparison of the kinetic experiments with arsenate summarised in Figures 6.20, 6.22 and 6.23 can therefore yield important information about the relative rates of different sections of the kinetic sequence.

6.7.1 Rapid synthesis of enzyme-bound EPSP

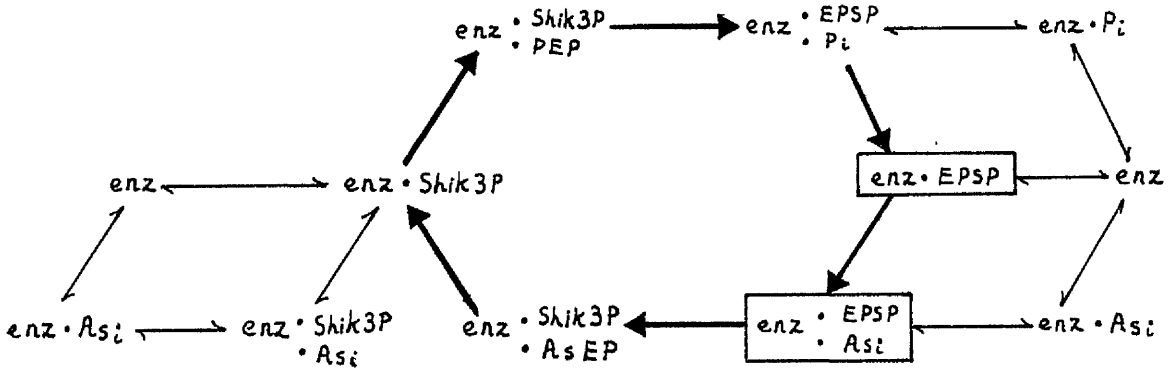
When the As_i-dependent reverse reaction (Figure 6.26a) is diverted into the EPSP/As_i-dependent PEP-hydrolysing cycle (Figure 6.26b) by saturating the enzyme with PEP, the rate of pyruvate release is not significantly altered over a wide range of As_i concentrations (see Figure 6.20). It follows that the fractional concentration of the ternary complex enzyme • EPSP • As_i also remains the same, since this determines the rate of "pyruvate" release (presumably as arsenoenolpyruvate) in an irreversible step. The diversion of the flow of kinetic intermediates therefore cannot necessitate a redistribution of a large proportion of the enzyme among new intermediates that are highly populated in the steady state.

The As_i-dependent reverse reaction and the cyclic PEP hydrolysis reaction differ only in the route taken in the regeneration of enzyme • EPSP from enzyme • Shik3P. The only first order step in the route taken in the As_i-dependent reverse reaction is the dissociation of Shik3P. It can be calculated from the kinetic parameters of the forward reaction that this process is approximately 14-fold faster than k_{cat} for the reverse reaction, and can therefore make no major demand on the distribution of kinetic intermediates in the reverse reaction.

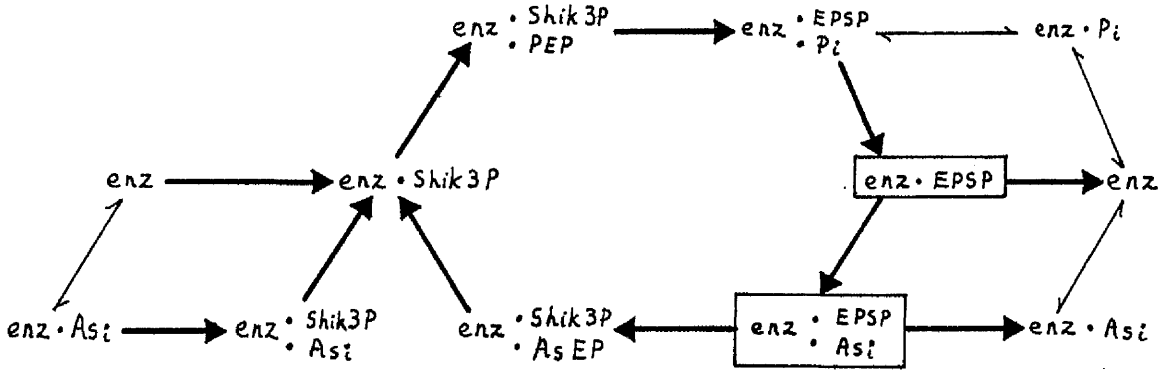
Figure 6.26 Models for the flow of catalytic intermediates in the As_i-dependent reactions of EPSP synthase



6.26 a As_i-dependent reverse reaction



6.26 b EPSP/As_i-dependent PEP hydrolysis reaction



6.26 c Forward reaction in the presence of As_i. Saturating [PEP], [Shik3P]; free EPSP removed with excess chorismate synthase.

Heavy arrows indicate the proposed net flow of kinetic intermediates.

Two conclusions follow from this analysis:

1. the alternative pathway for the regeneration of enzyme EPSP from enzyme • Shik3P, via the kinetic steps of the forward reaction, involves first order steps that are at least as fast as the dissociation of Shik3P from enzyme • Shik3P.
2. the saturated velocity of the As_i-dependent reverse reaction (and of the PEP hydrolysis reaction) is limited by the rate of conversion of enzyme • EPSP • As_i to enzyme • Shik3P.

The flow of intermediates in the cyclic EPSP/As_i-dependent PEP hydrolysis reaction (Figure 6.26b) can be perturbed by saturating the enzyme with Shik3P and PEP and removing free EPSP with chorismate synthase (Figure 6.26c). There is still no dramatic change in the rate of pyruvate release as a function of the As_i concentration (Figure 6.22). The total rate of PEP utilisation is now higher, as EPSP is also released from the enzyme. Since the rate of pyruvate release is determined only by the fractional concentration of enzyme • EPSP • As_i, these results imply that the fractional concentrations of the interconverting complexes enzyme • EPSP and enzyme • EPSP • As_i are little altered when the flow of intermediates is perturbed by saturating the forward reaction and removing EPSP. This is a quite unexpected outcome, because under the new conditions enzyme • EPSP and enzyme • EPSP • As_i are consumed in two competing processes:

1. dissociation of EPSP (now an "irreversible" step)
2. conversion to enzyme • Shik3P via the catalytic steps of the reverse reaction.

The results can only be explained if:

1. the synthesis of enzyme • EPSP at the full rate of the saturated forward reaction makes little demand on the distribution of kinetic intermediates.

2. the rate constants for the dissociation of EPSP from enzyme • EPSP and enzyme • EPSP • As_i are in the same range as the saturated velocity of the reverse reaction.

3. enzyme • EPSP • As_i partitions fairly equally between release of EPSP and conversion to enzyme • Shik3P.

Since the saturated velocity of the forward reaction is approximately twice that of the reverse reaction this implies that:

1. the slowest first order step in the forward reaction is the release of EPSP

2. the catalytic steps in the synthesis of enzyme-bound EPSP are relatively rapid.

6.7.2 The fate of enzyme • EPSP

It is of some interest to estimate the fractional concentration of the kinetic intermediate enzyme • EPSP that obtains in the steady state when the forward reaction is saturated with Shik3P and PEP. This can be done by comparing the relative rates of As_i-dependent pyruvate release seen:

1. at saturation in Shik3P and PEP (forward reaction)

2. at saturation in EPSP (As_i-dependent reverse reaction)

Inspection of the schemes in Figure 6.26 indicates that the rate of pyruvate release in the limiting case of vanishingly low As_i concentrations should be a reliable measure of the fractional concentration of the enzyme • EPSP complex. The correct kinetic parameter for making this comparison is

$$V_M \text{ } (As_i\text{-dependent pyruvate release})/K_M(As_i)$$

At saturation in Shik3P and PEP this parameter only falls to 66% of its value at saturation in EPSP (see Figure 6.22).

At saturation in EPSP the free enzyme is converted quantitatively into enzyme • EPSP. At saturation in Shik3P and PEP approximately two thirds of the enzyme is therefore present as enzyme • EPSP. This implies that only one third of the enzyme is sufficient to populate all the other catalytic intermediates at a level necessary to maintain the full saturated velocity of the forward reaction. A number of conclusions follow from this analysis:

1. The dissociation of EPSP from enzyme • EPSP is by far the slowest first order kinetic step in the forward reaction sequence, with a rate constant no greater than 150% of k_{cat} for the overall reactions, or about $30s^{-1}$.
2. The other first order steps in the forward reaction, the catalytic steps and the dissociation of P_i, are all somewhat faster, but make a significant demand on the distribution of kinetic intermediates.

6.7.3 Association and dissociation of EPSP and Shik3P

The limiting K_M for EPSP in the EPSP/As_i-dependent PEP hydrolysis reaction at vanishing $[As_i]$ has the very low value of 79×10^{-9}M. Inspection of the scheme in figure 6.26b suggests that this limiting K_M should be identical to the dissociation constant for binding of EPSP to the free enzyme. Since k_{ass}, the second order rate constant for the association of EPSP with free enzyme, cannot exceed the diffusion-controlled limit of approximately $10^9 M^{-1}s^{-1}$ a theoretical upper limit on k_{diss}, the first-order rate constant for the dissociation of EPSP from enzyme • EPSP, can be calculated:

$$k_{diss}/k_{ass} = K_D = 79 \times 10^{-9}M$$

$$k_{ass} \leqslant 10^9 M^{-1}s^{-1} \qquad \text{(Eigen \& Hammes, 1971)}$$

$$\Rightarrow k_{diss} \leqslant 79s^{-1}$$

The dissociation rate constant of $30s^{-1}$ deduced from the kinetic parameters of the enzyme is thus very close to the theoretical upper limit. This suggests that the association of EPSP with free enzyme is essentially a diffusion-controlled process. Accordingly the rate-limiting dissociation of EPSP in the forward reaction must be seen as an unavoidable consequence of the very tight binding of this intermediate. The partial patterns of inhibition of the forward reaction by high concentrations of As_i and P_i (Figures 6.17 & 6.22) strongly suggest that EPSP dissociates from enzyme • EPSP only slightly faster than it dissociates from enzyme • EPSP • As_i and enzyme • EPSP • P_i.

The rate constant for the association of Shik3P and the free enzyme, estimated from the kinetic parameters of the forward reaction, is $5.8 \times 10^7 M^{-1}S^{-1}$, which is again close to the diffusion controlled limit. Very fast association kinetics are not unusual for relatively tightly-bound substrates (Eigen & Hammes, 1963).

6.7.4 Rate limiting steps in the reactions of EPSP synthase

The results discussed in the previous sections clearly point to EPSP dissociation as a rate-limiting step in the forward reaction of EPSP synthase. For the reverse reaction the picture is less clear; the rate-limiting step(s) must reside within the kinetic sequence from enzyme • EPSP • P_i to enzyme • Shik3P. It is thus possible to speculate that the rate of the reverse reaction is limited by slow dissociation of PEP or its arsenate analogue. Alternatively the rate of the reverse reaction may be controlled by one of the central catalytic steps. These ideas are illustrated in Figure 6.27.

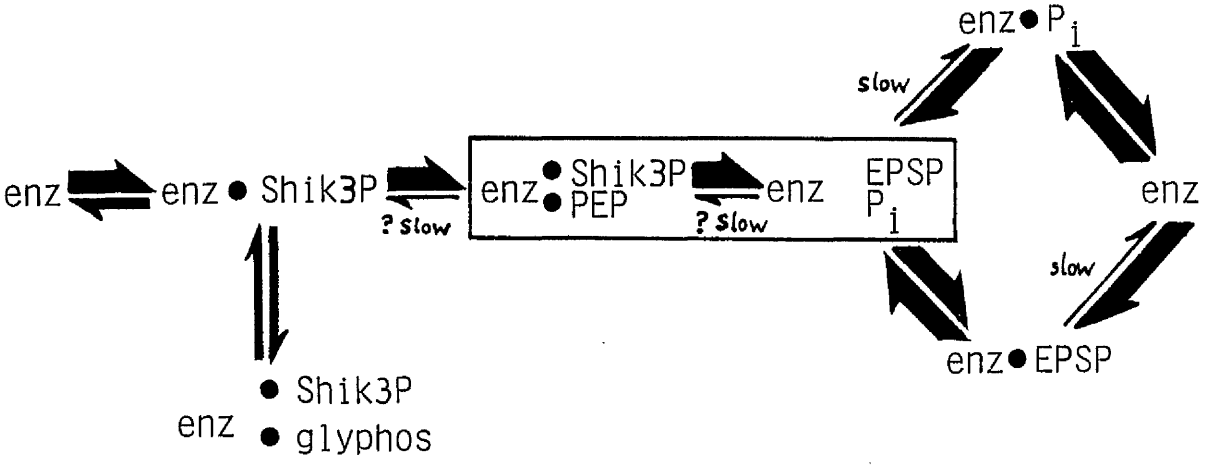


Figure 6.27 Possible rate-limiting steps in the catalytic mechanism of arom EPSP synthase

6.7.5 Kinetic isotope effects and the catalytic mechanism of EPSP synthase

During the course of the EPSP synthase reaction at least one catalytic intermediate with a freely-rotating methyl group is created by addition of a proton to the methylene carbon of PEP (Grimshaw et al, 1982; see Section 6.1.3.5). In the previous section it was pointed out that the rate of the forward and reverse reactions may be limited by slow dissociation of EPSP and PEP. A number of interesting predictions about hydrogen isotope effects on EPSP synthase catalysis can be made for the extreme case where the interconversion of the central catalytic complexes (e.g. X, Y, Z in Figure 6.6.) is very rapid relative to k_{cat}.

1. there will be no kinetic isotope effects on the overall reaction rate.
2. isotopic product ratios will not be determined by isotope effects on individual kinetic steps, but by isotope effects on kinetic equilibria.
3. conversion of PEP to EPSP may be accompanied by complete "washout" of heavy hydrogen isotopes from doubly labelled PEP methylene groups.

No kinetic isotope experiments have been conducted with arom EPSP synthase, but a very detailed study of the E.coli enzyme has been undertaken (Grimshaw et al, 1982). The results reported for the E.coli enzyme may be compared with the predictions listed above:

1. no kinetic isotope effects on the overall rate of the forward reaction have been documented
2. the observed product ratios of isotopically labelled EPSP strongly suggest that there may be significant equilibration of at least one protonation/deprotonation step in the mechanism

3. EPSP synthesis is accompanied only by partial "washout" of label from doubly-labelled PEP.

Preliminary steady state kinetics experiments on the E.coli EPSP synthase suggest that it may have a kinetic mechanism very similar to that of the arom EPSP synthase (A.Lewendon, unpublished work). It is thus possible to speculate that EPSP synthase catalysis follows a very similar course in the E.coli and N.crassa enzymes, and that in both cases the rapid conversion of enzyme • Shik3P • PEP into enzyme • EPSP is followed by relatively slow dissociation of EPSP.

A significant feature of the E.coli enzyme is that at saturation in Shik3P and PEP it does not catalyse rapid exchange of ^{3}H label from solvent water into "unreacted" PEP (Grimshaw et al, 1982). If the "tetrahedral intermediate" scheme of Grimshaw et al (Figure 6.6) is correct this implies that one of the following kinetic steps is slow relative to k_{+3}:

1. reversal of the first protonation step (k_{-2})
2. dissociation of PEP from enzyme • Shik3P • PEP, "X" (k_{-1})

The second alternative could be consistent with a model for EPSP synthase catalysis in which the interconversion of the central catalytic intermediates in very rapid and the dissociation of PEP is the rate limiting step in the reverse reaction. The observed partition of the protonated intermediates in favour of EPSP and P_i (Ife et al, 1976; Grimshaw et al, 1982) could then be explained in terms of rapid release of P_i from enzyme • EPSP • P_i, (Z), and slow dissociation of PEP from enzyme • Shik3P • PEP (X) (see Figures 6.6 & 6.27).

6.8 The chemical nature of the catalytic intermediates in the EPSP synthase reaction

Two general types of catalytic mechanism have been put forward for EPSP synthase (Levin & Sprinson, 1964; Cassidy & Kahan, 1973). In

the introduction to this chapter it was stressed that neither the "covalent intermediate" model nor the "tetrahedral intermediate" model can be ruled out on the basis of previously reported properties of any EPSP synthase. The results discussed in the previous sections clearly indicate that a "covalent intermediate" mechanism of the "ping-pong" type is inadmissible. Steady state kinetics cannot, however, provide any direct information about the chemical nature of the central ternary complexes in an ordered sequential mechanism of the type proposed for arom EPSP synthase. The steady state kinetic properties of this enzyme are thus equally compatible with:

1. an ordered, sequential mechanism involving an enzyme~PEP or enzyme~pyruvate "covalent intermediate" similar to that proposed for UDPGlcNAc pyruvyl transferase (Cassidy & Kahan, 1973).
2. a mechanism involving a central "tetrahedral intermediate" (Levin & Sprinson, 1964 ; fig. 6.4)

There are, however, a number of striking parallels between the properties of the arom EPSP synthase and the bacterial UDPGlcNAc pyruvyl transferases studied by Zemell and Anwar (1974) and Cassidy and Kahan (1973):

1. both enzymes have an ordered sequential mechanism in which PEP is the second substrate to bind to the enzyme.
2. in the presence of the first-bound substrate:
   - -EPSP synthase is reversibly inhibited by the presumed PEP analogue glyphosate. (fig 6.18)
   - -UDPGlcNAc pyruvyl transferase is covalently modified by the presumed PEP analogue fosfomycin. (fig 6.5)

3. both enzymes are highly sensitive to covalent modification by the thiol-directed reagent NEM. PEP protects UDPGlcNAc pyruvyl transferase from inactivation only in the presence of the first-bound substrate, (UDPGlcNAc), while glyphosate protects arom EPSP synthase from inactivation only in the presence of Shik3P (results not shown); similar results have been obtained with the E.coli EPSP synthase (A.Lewendon, unpublished work).

4. both enzymes are sensitive to oxidation and are stabilised by thiol reducing agents (see Chapter 4).

These properties suggest that EPSP synthase, like UDPGlcNAc pyruvyl transferase, may possess an active site thiol group that reacts with PEP to form a "covalent intermediate". Further work is needed to examine this possibility.

6.9 The interaction between glyphosate and EPSP synthase

Although glyphosate and PEP are both small acidic molecules containing a phosphoryl group and a carboxylate group, the structural resemblance between the two compounds is very limited. Glyphosate adopts an extended chain conformation in the crystalline phase (Knuuttila & Knuuttila, 1979). If the conformation of the herbicide in free solution is similar, the distance between the phosphoryl group and the carboxylate group will be very much greater than in PEP. At neutral pH glyphosate also carries an additional positive charge on the central amine nitrogen. Glyphosate has not been reported to interact with any enzyme other than EPSP synthase as a competitive inhibitor with respect to PEP. Rabbit muscle pyruvate kinase and the tryptophan-sensitive DAHP synthase of N.crassa, for example, are insensitive to inhibition by high concentrations of glyphosate (M.R.Boocock & A.A.Coia, unpublished

work). There is therefore no sound theoretical basis for the assumption that glyphosate necessarily acts as a structural analogue of PEP in the EPSP synthase reaction.

The kinetic properties of arom EPSP synthase discussed in Section 6.5 do, however, strongly suggest that the herbicide mimics PEP at least to the extent that it binds very tightly (K_I = 1.1μM) at the same site in the enzyme • Shik3P complex, and binds to no other kinetic intermediate. There is clear evidence that glyphosate, PEP and P_i all compete for binding to enzyme • Shik3P; of these three compounds only P_i can bind to the free enzyme (see Figure 6.18). It is thus reasonable to suppose that the phosphoryl groups of glyphosate, PEp and P_i share a common binding site, and that the carboxylate groups of glyphosate and PEP share a coomon binding site which only becomes available when Shik3P is bound to the enzyme.

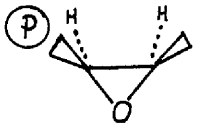
It has been shown in our laboratory that glyphosate also acts on the E.coli and pea seedling EPSP synthases as a competitive inhibitor with respect to PEP (A.Lewendon, unpublished work; Mousdale & Coggins, 1983). Amrhein et al (1982) have independently reported competitive inhibition of EPSP synthases from A.aerogenes and the plant Corydalis sempervirens, but have not specified how these results were obtained. The relative affinities of the enzymes for glyphosate and PEP can be compared using the parameter K_I/K_M. The K_I/K_M values estimated for five enzymes from highly diverse sources fall within a surprisingly narrow range, from 0.31 (N.crassa) to 0.01 (C.sempervirens) (see Table 6.8). By this criterion the two plant enzymes are the most sensitive to glyphosate. It is possible that competition between glyphosate and PEP for binding to an enzyme • Shik3P complex will prove to be a universal feature of EPSP synthases.

Table 6.8 Sensitivity of different EPSP synthases to glyphosate inhibition

Source of enzyme		Data	Buffer System	[Shik3P] (μM)	K_I'/K_M' (PEP)	K_I (glyphos.) (μM)	K_M (PEP) (μM)
Fungi	N.crassa	This study	buffer A	177	0.31	1.1	3.5
	N.crassa	This study	buffer B + 2.5mM P_i	200	0.32	4.8	15
Bacteria	E.coli	A.Lewendon	buffer A	200	0.08	1.0	12.5
	A.aerogenes	Amrhein et al (1982)	Not reported	5000	0.018	0.16	9.0
Planta	C.sempervirens	Amrhein et al (1982)	Not reported	5000	0.01	10.0	1000
	Pisum sativum	D.M.Mousdale	buffer B	100	0.015	0.08	5.2

The kinetic parameters of the A.aerogenes and C.sempervirens enzymes have been estimated from the data of Amrhein et al, 1982, assuming linear competitive inhibition with respect to PEP.

Table 6.9 Insensitivity of arom EPSP synthase to inhibition by structural analogues of glyphosate

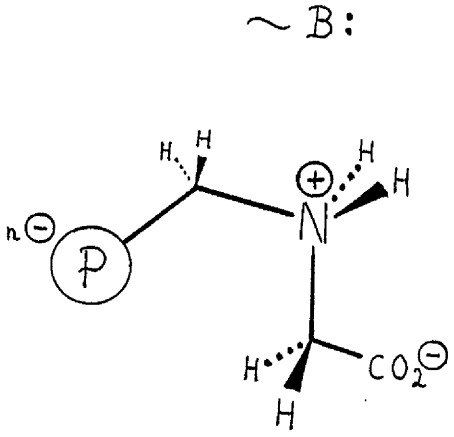
structure	trivial name	source	estimated $K_I'/K_M'(PEP)$
(P) $CH_2\ NH\ CH_2\ CO_2H$	glyphosate		0·32
(P) $CH_2\ NH\ CH_3$			>200
(P) $CH_2\ NH_2$		C	>30
$H_2N\ CH_2\ CO_2H$	glycine		>100
$Me\ CO\ CO_2H$	pyruvate	S	>100
$Me\ CH(OH)CO_2H$	L-lactate	B	>100
(P) $CH_2\ O\ CH_2\ CO_2H$			>60
(P) $CH_2\ S\ CH_2\ CO_2H$			>180
(P) $CH_2\ NH\ CO\ CCl_3$			>50
$Me\text{-}PO_2\text{-}CH_2\ NH\ CH_2\ CO_2H$			>50
(P) $CH_2\ N(Me)CH_2\ CO_2H$			>90
(P) $CH_2\ NH\ CH(Me)CO_2H$			>150
(P) $CH_2\ NH\ CH_2CH_2CO_2H$			>50
(P) $CH_2CH_2\ NH\ CH_2\ CO_2H$			>120
$(P)\ CH_2)_2\ NH\ CH_2\ CO_2H$	glyphosine	S	>4·0
(P) $CH_2\ CH(NH_2)\ CO_2H$		C	>200
(P) $O\ CH_2CH(NH_2)\ CO_2H$	phosphoserine		>20
(P) $CH_2CH_2\ CH(NH_2)\ CO_2H$			>50
(P) 	fosfomycin	S	>100

Assays were conducted in buffer B, pH 7·0 at saturating concentrations of Shik3P (200μM) with 10-50μM PEP and glyphosate analogue concentrations up to 1-5mM. The lower limits on $K_I'/K_M(PEP)$ assume linear competitive inhibition with respect to PEP. Sources of compounds: Calbiochem. (C), Sigma (S), BDH (B), Dr. S. Ridley of ICI (all others).

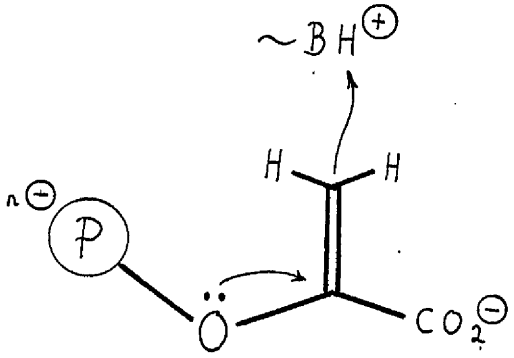
Very recently a number of groups have reported the isolation of glyphosate-tolerant plant cell lines and strains of bacteria (Amrhein et al, 1983; Rogers et al, 1983). In our own laboratory it has been shown that overexpression of the wild-type EPSP synthase confers glyphosate tolerance on E.coli (K.Duncan, unpublished work). No glyphosate-insensitive EPSP synthase has yet been fully characterised; it will be of great interest to see whether such an enzyme necessarily has a low affinity for PEP.

A large number of structural analogues of glyphosate and PEP have been tested as potential competitive inhibitors of arom EPSP synthase. The results listed in Table 6.9 indicate that none of the screened compounds interact strongly with the enzyme; many of the closest structural analogues of glyphosate bind to the enzyme over 500-fold less well than the parent compound. These results suggest that the geometry of the PEP/glyphosate binding site in the enzyme • Shik3P complex gives it a very high specificity for precise structural conformations of the substrate and inhibitor.

A speculative model for the interaction between glyphosate and EPSP synthase is shown in Figure 6.28. It is proposed that glyphosate binds to enzyme • Shik3P in a conformation that allows the phosphoryl and carboxylate groups to take the positions normally occupied by the corresponding groups of PEP; the positively charged secondary amino group could then interact with the catalytic base that transfers a proton to the methylene group of PEP. The distribution of electrostatic charges in the enzyme • Shik3P • glyphosate dead-end complex may thus resemble the charge distribution in catalytic intermediates downstream in the sequence from the enzyme • Shik3P • PEP ternary complex.



glyphosate



PEP.

Figure 6.28 Models for the interaction between EPSP synthase and PEP or glyphosate

CHAPTER 7 LIMITED PROTEOLYSIS AND THE STRUCTURE OF THE AROM MULTIFUNCTIONAL ENZYME

7.1 Introduction : The structure of the arom multifunctional enzyme

7.1.1 Subunit structure

The arom multifunctional enzyme consists of two identical pentafunctional 165kDa polypeptides (Lumsden & Coggins, 1977, 1978) and two atoms of tightly bound zinc that are essential for E1 activity (see Chapter 4; Lambert, Boocock & Coggins, in preparation). The ratio of the molecular weights of the native enzyme complex (270kDa) and of the component polypeptide subunits (165kDa), estimated from sedimentation velocity and SDS PAGE experiments, respectively, the results of protein cross-linking experiments (Lumsden & Coggins, 1977), and the occurrence of allelic complementation within the arom gene cluster (Case et al 1969a) are consistent with the proposed dimeric structure of the enzyme. The comigration of the component polypeptides in gel electrophoresis in the presence of either SDS or 8M urea (Lumsden & Coggins, 1977), the results of peptide mapping experiments (Lumsden & Coggins, 1978) and the genetics of the arom locus, particularly the occurrence of allelic complementation (Giles et al, 1967a) all point to the identity of the two subunits.

Detailed hydrodynamic measurements have been made on a preparation of the arom enzyme complex that was proteolytically nicked but appeared homogeneous by PAGE under native conditions (Gaertner, 1972). A molecular weight of 290kDa and a sedimentation coefficient of 10.6S were estimated from sedimentation equilibrium and sedimentation velocity results, respectively. From the same data Gaertner (1972) calculated a frictional coefficient f/fo of 1.5. The hydrodynamic properties of the intact arom complex are very similar to those of

proteolytically nicked material, with which it comigrates in native gel electrophoresis and sedimentation velocity experiments (Lumsden & Coggins, 1977; Smith, 1980). These results suggest that the native arom multifunctional enzyme is a compact globular protein.

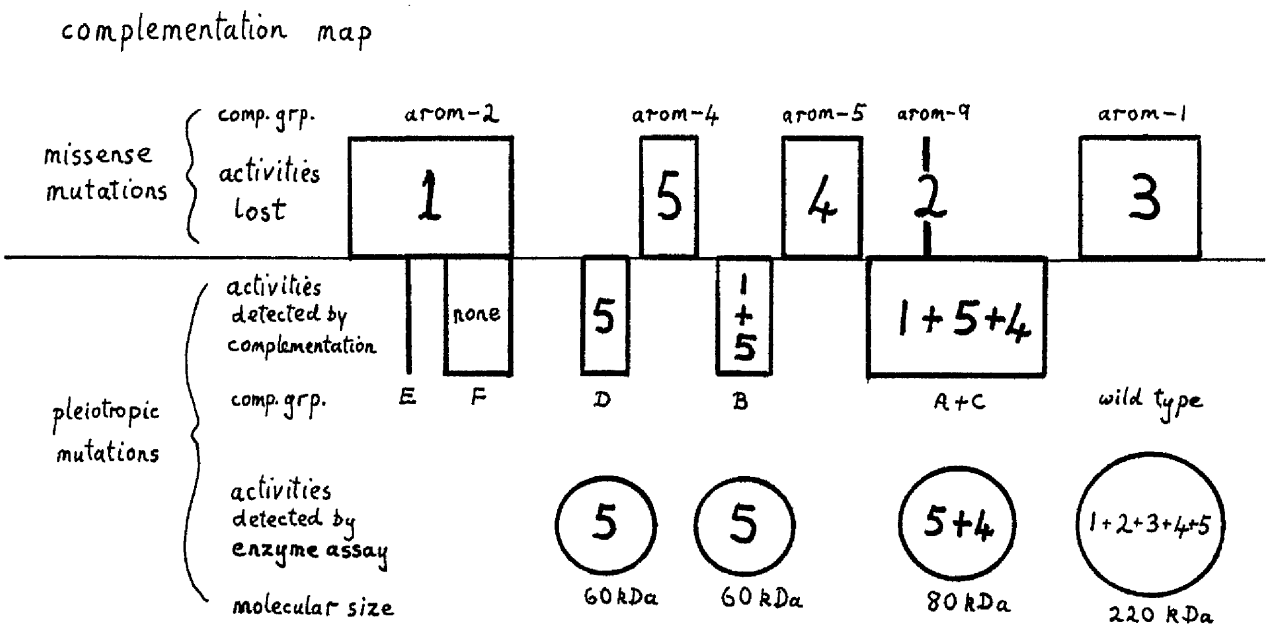
7.1.2 Genetics of the arom locus

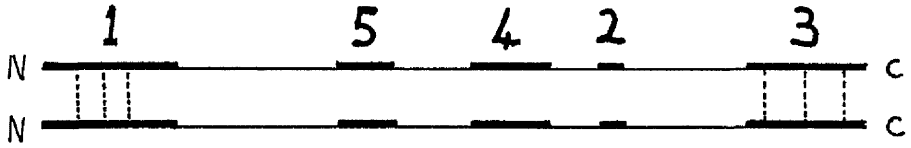
7.1.2.1 Arom Mutations

Auxotrophic strains of N.crassa that require the three aromatic amino acids phenylalanine, tyrosine and tryptophan as growth factors are known as arom mutants (Gross & Fein, 1960). These strains are deficient in one or more enzyme activities of the common aromatic pathway of chorismate biosynthesis. Arom mutations can be subdivided on the basis of genetic complementation analyses; arom mutations of different classes normally complement each other when they are present in a heterocaryon in the trans configuration, and the "hybrid" heterocaryon then recovers the ability to grow on unsupplemented minimal medium.

The clustering of "genes" associated with a number of distinct complementation groups of arom mutations was first recognised by Gross and Fein (1960). Subsequent fine structure genetic analysis by Giles et al (1967a) revealed five clear-cut complementation groups of arom mutations that are each associated with the loss of one of the five enzyme activities of the arom complex. These five classes of mutations map in five discrete non-overlapping sub-regions of the arom gene cluster on linkage group II (see Fig. 7.1). The arom-3, arom-6, arom-7 and arom-8 genes and the qa genes, which specifiy chorismate synthase (E6), three DAHP synthases (EO), and the catabolic quinate pathway enzymes, are not closely linked to the arom gene cluster (Rines et al, 1969; Doy, 1968). The remaining classes of arom mutations map within the arom gene cluster and are associated with the loss of two or more arom enzyme

Figure 7.1 Genetic map of the arom locus of N.crassa (Giles et al, 1967a; Rines et al, 1969; Case & Giles, 1971)





activities. The complementation map of these "pleiotropic" mutations exhibits a clear polarity, (see Fig. 7.1) which indicates that the arom gene cluster is transcribed as a single unit (Giles et al, 1967a).

7.1.2.2 Transcripts and translation products

The picture of the arom gene cluster that emerged from the early genetic and biochemical studies was of an operon-like polycistronic transcription unit. The five complementation regions in the fine structure map that are associated with defects in the five component activities of the arom enzyme complex were taken to be genes for five distinct polypeptide subunits (Rines et al, 1969). It is now clear that the arom region is the gene for a single pentafunctional polypeptide chain (Lumsden & Coggins, 1977). The fine structure mapping results can be reinterpreted in this light, and they yield a great deal of information about the structure of the arom enzyme complex.

7.1.2.3 Mis-sense mutations

About half of the arom mutations that map within the arom gene cluster fit into one of the five complementation classes that are associated with the loss of individual enzyme activities of the complex (Rines et al, 1969). These mutations are located in five discrete sub-regions of the linkage map that do not overlap (see Fig. 7.1). Mutant strains generally express the remaining four enzyme activities at wild-type levels in an aggregate that is indistinguishable from the normal arom enzyme complex in sedimentation velocity experiments (Giles et al, 1967a). It appears that the "single enzyme" mutations do not dramatically alter the quaternary structure of the arom enzyme complex and that in general only one of the five enzyme activities is severely affected.

The "single enzyme" mutations are most easily interpreted as mis-sense mutations that specify amino acid substitutions in the arom polypeptide (Strauss, 1979). The complementation patterns indicate that amino acid substitutions within a particular sub-region of the arom

polypeptide generally lead to the loss of one particular enzyme activity. The fine structure complementation map suggests that there are five discrete, non-overlapping sub-regions of the arom polypeptide that define the catalytic activities of the five component enzymes.

Many mis-sense mutations probably lead to locally incorrect folding of the polypeptide backbone of a protein (Crick & Orgel, 1964). There is little evidence that single mis-sense mutations in the arom gene can eliminate more than one enzyme activity. This suggests that the sub-regions of the arom polypeptide that are associated with particular enzyme activities are in general structurally autonomous. The complementation map of the arom locus is thus compatible with a "mosaic" model for the structure of the arom enzyme complex, in which five sub-regions of the polypeptide chain fold into five structural domains (or groups of domains) that carry the component enzyme activities. It should, however, be emphasised that a more complex tertiary structure cannot be ruled out on the strength of the genetic evidence presently available.

7.1.2.4 Subunit interactions and allelic complementation

While there is little genetic evidence of interactions between non-homologous sub-regions of the arom polypeptide there is good evidence that homologous sub-regions of the two polypeptide subunits of the native enzyme complex interact strongly. Allelic complementation within the classes of mutations associated with defects in E1 and E3 activities is very common; many pairs of arom-2 mutant alleles (and of arom-1 alleles) direct the synthesis of fully active arom enzyme complexes when they are present together in "hybrid" heterocaryons (Giles et al, 1967a). Complementation can also be seen in vitro; when partially purified arom enzyme complexes from two complementing arom-2 (E1 deficient) strains are incubated together under alkaline conditions substantial E1 activity is reconstituted (Case et al, 1969).

A minority of arom-1 (E3-deficient) strains express the remaining four enzyme activities in an arom complex of half the normal molecular weight. When the complementing alleles of this type are brought together in a heterocaryon, E3 activity is recovered, and is found in a full-size arom complex with all five enzyme activities (Case & Giles, 1971),

The only simple explanation of these results is that the sub-regions of the arom polypeptide associated with E1 and E3 activities interact with homologous regions of the other polypeptide sub-unit of the native arom multifunctional enzyme. It appears that the isolated subunits of the enzyme are able to express E1, E2, E4, E5 and perhaps E3 activities, and that interactions between the two homologous E3 sub-regions may be important in maintaining the quaternary structure of the enzyme complex.

7.1.2.5 Pleiotropic mutations and premature chain termination

The "pleiotropic" arom mutations which affect more than one catalytic activity of the enzyme complex can be sub-divided on the basis of complementation patterns (Fig.7.1). The complementation class E consists of arom mutations that eliminate all detectable arom activities and that fail to complement any other mutations in the arom gene cluster. All class E mutations are located within the E1 sub-region at one end of the linkage map of the arom cluster gene (Giles et al, 1967a). The phenotype of certain mutations of this class is suppressed in strains of N.crassa that are believed to carry a nonsense suppressor tRNA gene (Case & Giles, 1968). Taken together these results provide conclusive evidence that the arom region is transcribed as a single unit, and that the polarity of translation is from the E1 sub-region to the E3 sub-region; the loss of all five arom activities in class E mutants would thus be brought about by premature polypeptide chain termination at

a nonsense codon within the E1 sub-region of the arom message (Case & Giles, 1968; see Fig.7.1).

The other classes of pleiotropic arom mutations have not been characterised as nonsense mutations, but their phenotypes are probably best interpreted in terms of premature polypeptide chain termination (Case & Giles, 1971; see Fig. 7.1). Mutations in complementation class C lead to a total loss of E2 and E3 activities. E4 and E5 activities are present in cell extracts at much reduced levels, and co-sediment with an apparent molecular weight of 80kDa; E1 activity is only detectable by genetic complementation.

The properties of the pleiotropic arom mutations indicate that at least in the N-terminal region of the arom polypeptide the relationship between polypeptide function and gene structure may be rather complex. The schematic view of the structure of the arom multifunctional enzyme shown in Figure 7.1 should therefore be regarded as a simplified working model that is consistent with most of the genetic properties of the arom locus.

7.1.2.6 N-terminal fragments of the arom polypeptide

However one interprets the phenotypes of the pleiotropic arom mutations it is clear that certain small sub-fragments of the pentafunctional arom polypeptide can express relatively high E4 and E5 activities (Case & Giles, 1971). The genetic complementation results suggest that, in principle, it should be possible to isolate and characterise fragments of the arom complex that carry the enzyme activities associated with the N-terminal region of the polypeptide chain, E1, E5 and E4 (see Fig.1.1). Case and Giles (1971) were, however, unable to detect E1 activity in mycelial extracts of the pleiotropic mutants by biochemical assay.

7.1.3 Spatial independence of the five catalytic sites

Two independent lines of evidence indicate that the five reactions catalysed by the *arom* multifunctional enzyme take place at five discrete active sites. The genetic evidence that mis-sense mutations generally only inactivate a single enzyme activity has already been cited. Independent inactivation of each of the five enzyme activities can also be achieved by chemical or proteolytic modification.

Under defined conditions, the hydrolysis of probably a single peptide bond can specifically inactivate E4 or E5 without affecting the other four activities (Smith, 1980; this study). E5 is specifically inactivated by oxidation, and E1 by removal of the essential zinc atom (see Chapter 4). E2 is specifically inactivated by $NaBH_4$ in the presence of DHQ, while both E2 and E3 are inactivated by $NaBH_4$ in the presence of formaldehyde. Shikimate protects E3 from inactivation by $NaBH_4$/formaldehyde but does not protect E2 (Smith, 1980; Lumsden & Coggins, in preparation).

The results described above do not rule out a very close juxtaposition of the active sites in the native enzyme, but they strongly suggest that the substrate binding sites of the five enzyme activities are spatially distinct.

7.1.4 Limited proteolysis

The susceptibility of the *arom* multifunctional enzyme to limited proteolytic digestion by endogenous *N.crassa* proteases is one of the major obstacles encountered during the purification of the enzyme complex (Gaertner & Cole, 1976; Lumsden & Coggins, 1977). Under non-denaturing conditions endogenous proteases readily cut the 165kDa *arom* polypeptide into a number of large polypeptide fragments. These fragments generally remain tightly associated in an aggregate that can display all five enzyme activities and is indistinguishable from the intact *arom*

multifunctional enzyme in PAGE and sedimentation velocity experiments carried out under native conditions (Gaertner, 1972; Lumsden & Coggins, 1977). After very severe proteolytic degradation smaller active fragments are produced; these are usually associated with arom E3 activity (Jacobson et al, 1972; J.R.Coggins et al, unpublished work).

Limited proteolysis was the major technique employed in a detailed study of the structure of the arom enzyme complex undertaken by Smith (1980), who found that limited digestion of the native enzyme complex with trypsin generates a series of characteristic polypeptide fragments. The immediate products of an initial rapid cleavage of the intact 165kDa arom polypeptide are two fragments of apparent molecular weights 110kDa and 68kDa. E4 activity is destroyed in parallel with the disappearance of the 165kDa arom polypeptide, while E1, E2 and E3 activities are not affected by the initial proteolytic cleavage. E2 and E3 activities and the 68kDa polypeptide fragment are very resistant to further proteolytic degradation. The 110kDa fragment is somewhat less stable, and its breakdown appears to be accompanied by the destruction of E1 activity (Smith, 1980).

The major 68kDa and 110kDa polypeptide fragments generated by limited digestion of the arom complex by trypsin are not separated from each other by a number of chromatographic techniques, nor by gel electrophoresis under native conditions. They appear to be tightly associated in an aggregate that closely resembles the native arom enzyme complex in its physical properties (Smith, 1980). The two major polypeptide fragments can be resolved be gel electrophoresis under denaturing conditions. The 68kDa polypeptide fragment carries the active site lysine residue of arom E2 and after renaturation it can be stained for E3 activity. A similar 68kDa primary fragment generated by limited digestion of the arom multifunctional enzyme with subtilisin also carries the active-site lysine residue of arom E2 (Smith, 1980; Smith & Coggins, 1983).

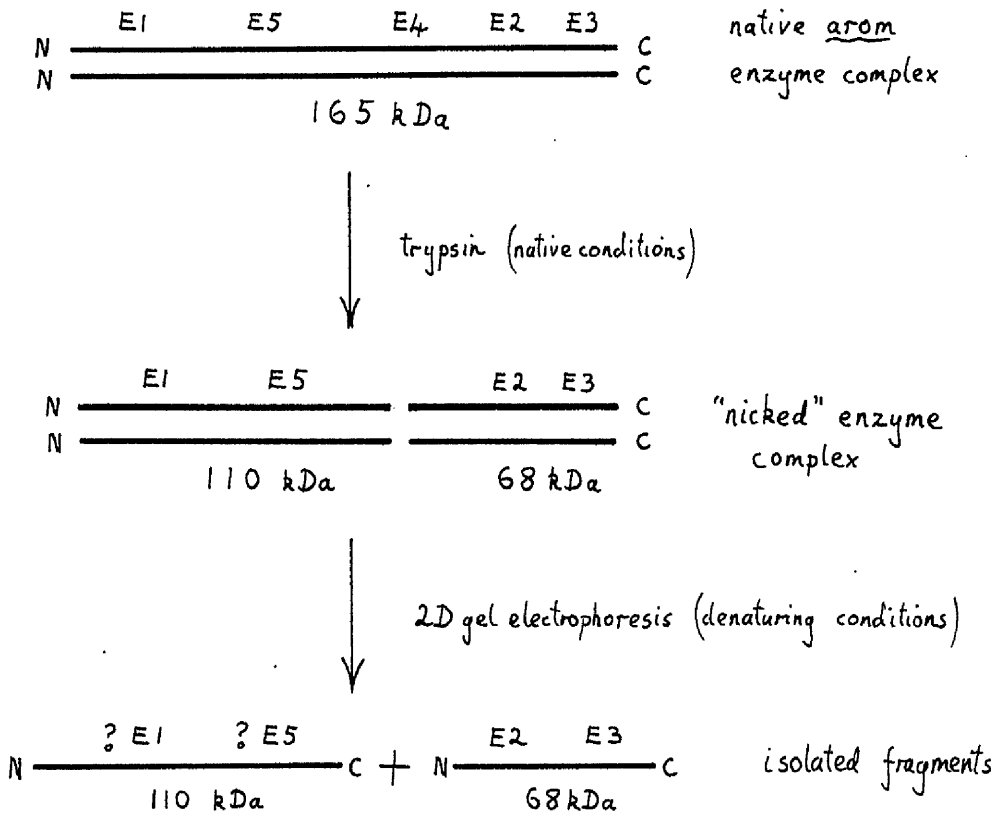


Figure 7.2 Isolation of a fragment of the arom polypeptide containing the E2 and E3 catalytic sites. (Smith, 1980; Smith & Coggins, 1983)

Smith (1980) concluded that there is an exposed loop of polypeptide in the native arom multifunctional enzyme that is highly sensitive to attack by a number of proteases, including trypsin, subtilisin, chymotrypsin and papain. Cleavage of the arom polypeptide in this region destroys E4 activity and yields two complementary polypeptide fragments of 110kDa and 68kDa, which remain tightly associated. Smith (1980) proposed that the 68kDa polypeptide fragment is a bifunctional domain from the C-terminal end of the arom polypeptide which carries the catalytic sites of E2 and E3. The assignment of polypeptide chain polarity was based on the polarity of the complementation map of the arom locus (Giles et al, 1967a). The arrangement of structural domains in the arom polypeptide envisaged by Smith (1980) is shown in Figure 7.2.

Vitto and Gaertner (1978) have reported that the substrate of arom E1, DAHP, "coordinately" protects the five enzyme activities of the arom multifunctional from proteolytic inactivation, but they did not characterise any of the polypeptide fragments.

7.2 Objectives of the present study

In Chapter 2 I listed a number of interesting hypotheses concerning the structure and function of the arom multifunctional enzyme that are amenable to experimental investigation. An amended list that takes into account the results discussed in the previous sections might be:

1. the arom multifunctional enzyme is made up of structural domains.
2. the five enzyme activities of the arom complex are associated with five structurally independent domains (or groups of domains).

3. the structural domains associated with the five enzyme activities are derived from five contiguous sub-regions of the arom polypeptide chain and correspond to the five major complementation regions of the arom gene cluster, arranged in the order and polarity shown in Figure 7.1.
4. The structural domains associated with the five enzyme activities attain their active folded conformations independently.
5. the quaternary structure of the enzyme complex is maintained by interactions between regions of the polypeptide chain that specify E3 activity.
6. The interactions between structural domains within the intact multifunctional enzyme complex are essential for certain of its catalytic properties.

The hypotheses numbered 1-4 are associated with a "mosaic" model for the structure of the arom gene, which requires that each one of five contiguous segments of the primary sequence encodes all the information needed to specify one of the five component activities of the enzyme complex. The genetic properties of the arom locus discussed in Section 7.1.2 are broadly consistent with hypotheses 1 to 5, but do not provide conclusive evidence on any of these points.

Limited proteolysis offers another powerful tool for dissecting the structure of the enzyme complex. The assignment of the E2 and E3 active sites to a 68kDa proteolytic fragment marked a significant advance (Smith, 1980; Smith & Coggins, 1983). The properties of the 68kDa fragment are essentially those predicted from hypotheses 1 to 4. Smith (1980) presented good evidence in support of the contention that the isolated 68kDa polypeptide carries all the information needed to specify the active conformation of the arom E3 catalytic site. The very reasonable supposition that E5 and E1 activities are specified by the

presumably complementary 110kDa polypeptide fragment (Smith, 1980) was based almost entirely on the evidence of the complementation map of the arom gene (Giles et al, 1967a).

In the light of these considerations it was important to define the regions of the arom polypeptide that are concerned with E1 and E5 activity. The isolation of polypeptide fragments of the arom enzyme complex under non-denaturing conditions was pursued in the hope that this approach would facilitate the assignment of enzyme activities and allow detailed comparisons between the isolated component enzymes and the native enzyme complex. Active fragments of the arom multifunctional enzyme that specify EPSP synthase (E5) activity were of particular interest because of the concurrent work on the mechanism of this enzyme.

7.3 Results and Discussion

7.3.1. Evidence that active fragments of the arom multifunctional enzyme can be isolated under nondenaturing conditions

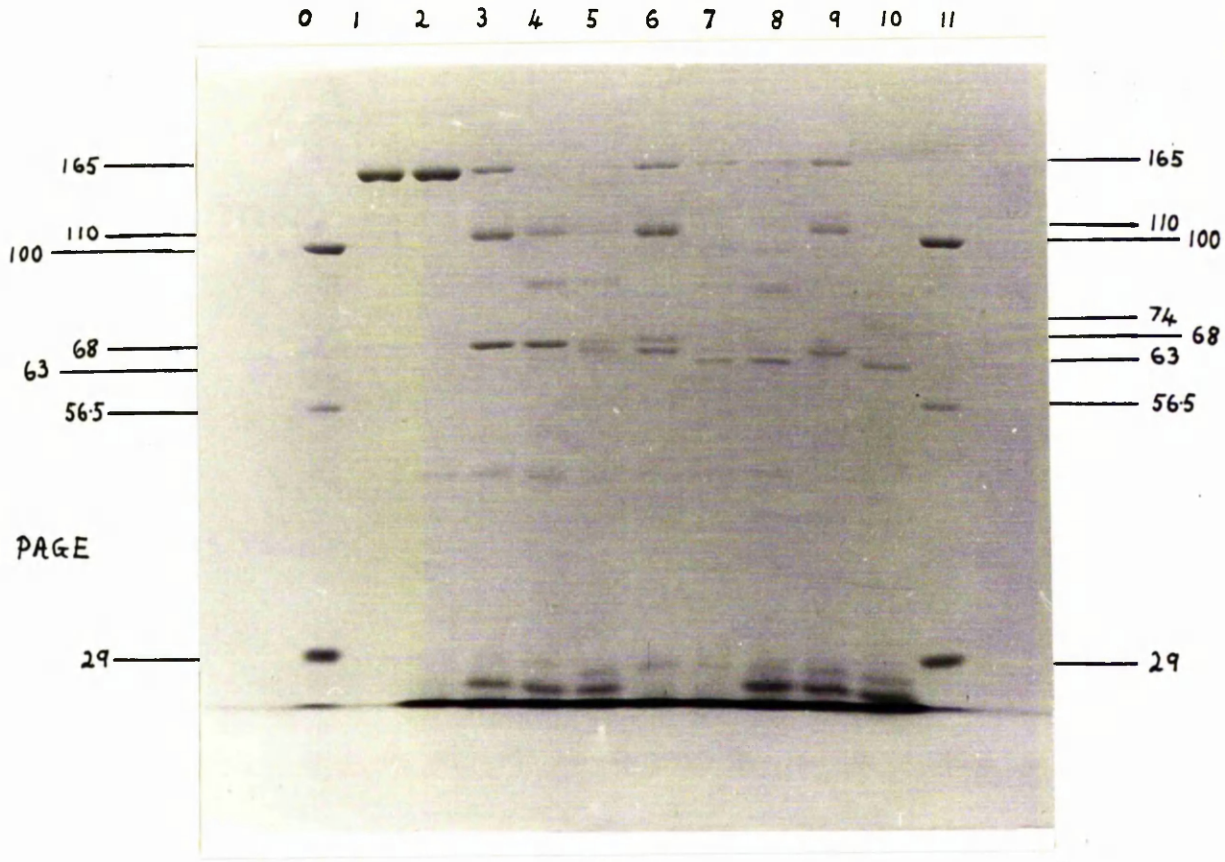
The homogeneous arom multifunctional enzyme isolated as described in Chapter 4 was subjected to limited proteolysis by trypsin under conditions very similar to those employed by Smith (1980) and Smith and Coggins (1983) (see Section 3.12). Brief treatment of the intact arom enzyme complex with trypsin yielded two large complementary polypeptide fragments (e.g. Fig. 7.3, lanes 4 and 5). This primary proteolytic cleavage was accompanied by the loss of E4 activity; the other four enzyme activities of the complex were retained quantitatively. These results are very similar to those obtained by Smith (1980), who used enzyme complex with only partial E1 activity purified by the "second generation" method discussed in Chapter 4. The molecular weights of the two primary polypeptide fragments estimated by calibrated SDS PAGE were 68kDa and 110kDa (Smith, 1980).

Figure 7.3 Resolution of active E3 species and polypeptide fragments after limited proteolysis of the arom enzyme complex by trypsin and chymotrypsin

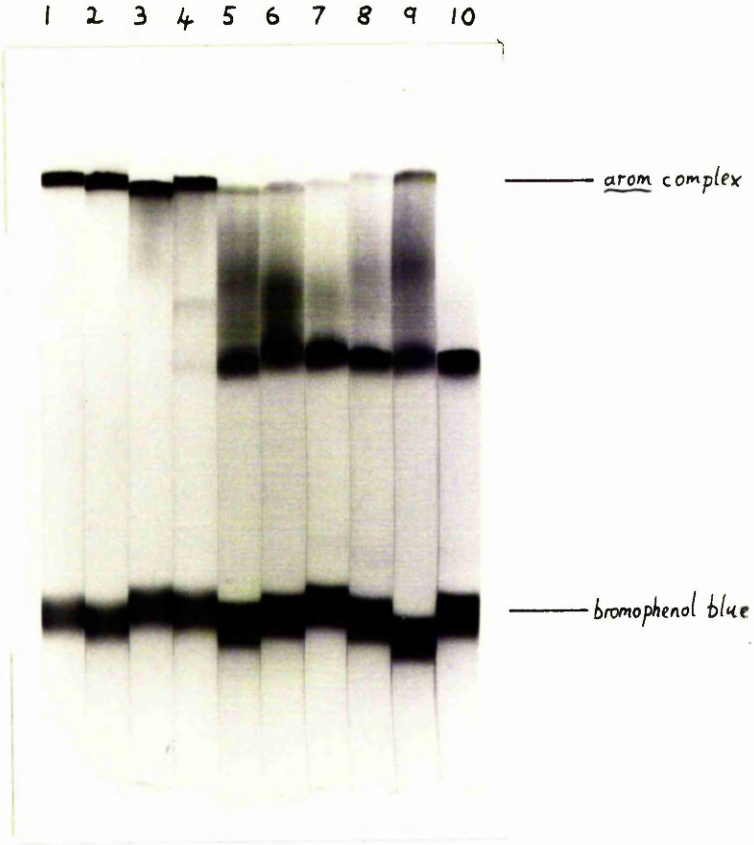
Arom enzyme complex was subjected to limited proteolysis under a variety of conditions: after inactivation of the proteases with lima bean trypsin inhibitor samples of the digestion products were subjected to SDS PAGE in a 10% Laemmli slab gel, which was stained for protein. Samples were also subjected to PAGE under nondenaturing conditions in 7% Davis gels, which were stained for E3 activity. The protease concentrations used were 100μg/ml trypsin (T) and 20μg/ml chymotrypsin(C). The samples were:

0, 11. E.coli pyruvate dehydrogenase, carbonic anhydrase
1. arom enzyme complex
2. arom + protease inhibitor
3-10. arom digested with:

3. T (2 min)
4. T (20 min)
5. T (20 min) then T + C (2 min)
6. C (2 min)
7. C (20 min)
8. C (20 min) then T + C (2 min)
9. T + C (2 min)
10. T + C (20 min)



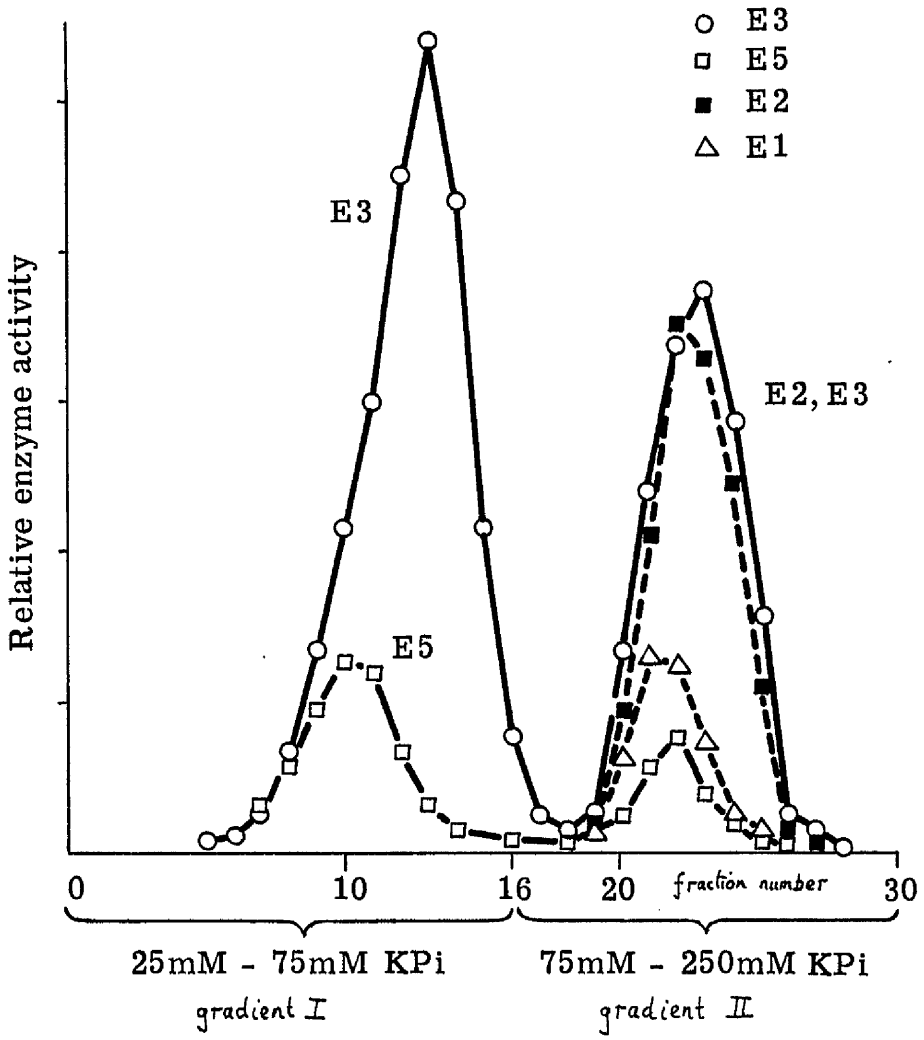
SDS PAGE



nondenaturing gels stained for arom E3 activity

Under nondenaturing conditions the 68kDa and 110kDa primary fragments were not separated from each other, nor from the intact arom multifunctional enzyme, by PAGE or by chromatography on DEAE Sephacel, phosphocellulose or Blue Dextran Sepharose. More extensive digestion of the enzyme complex by trypsin was accompanied by the destruction of a substantial proportion of the initial E1 activity and the degradation of the 110kDa primary fragment (e.g. Fig. 7.5, lanes 7 and 6). Three new high mobility species with E3 activity were seen in nondenaturing PAGE after prolonged digestion with trypsin (e.g. 2 hours at 100μg/ml trypsin). Material of this type was resolved into a number of species by chromatography on DEAE-Sephacel (Fig. 7.4). The major part of the residual E5 activity was not associated with any other arom activities, and coeluted from DEAE-Sephacel, phosphocellulose and Blue Dextran Sepharose with a major 74kDa polypeptide fragment which was derived from the 110kDa primary fragment. A substantial proportion of the E3 activity of the digested arom complex also appeared not to be associated with any other arom activities. The isolated E3 activity exhibited the highest mobility in nondenaturing PAGE, and preliminary evidence indicated that it was associated with a small ($\leqslant$30kDa) subfragment of the arom polypeptide. The remainder of the E3 activity eluted from DEAE-Sephacel, (and from other chromatographic media) in a peak of essentially constant E2:E3 ratio. This material consisted mainly of the 68kDa primary fragment and other large polypeptide fragments, and exhibited an intermediate mobility in nondenaturing PAGE. Residual material with essentially the same mobility in nondenaturing PAGE as the intact arom multifunctional enzyme eluted from the DEAE Sephacel matrix at the same ionic strength and was associated with E2, E3 and residual E1 and E5 activities, and with both the 68kDa primary fragment and the 110 kDa primary fragment and its proteolytic derivatives.

Figure 7.4 Elution of arom activities from DEAE Sephacel after prolonged treatment of the enzyme complex with trypsin (130 minutes, 100µg/ml trypsin)

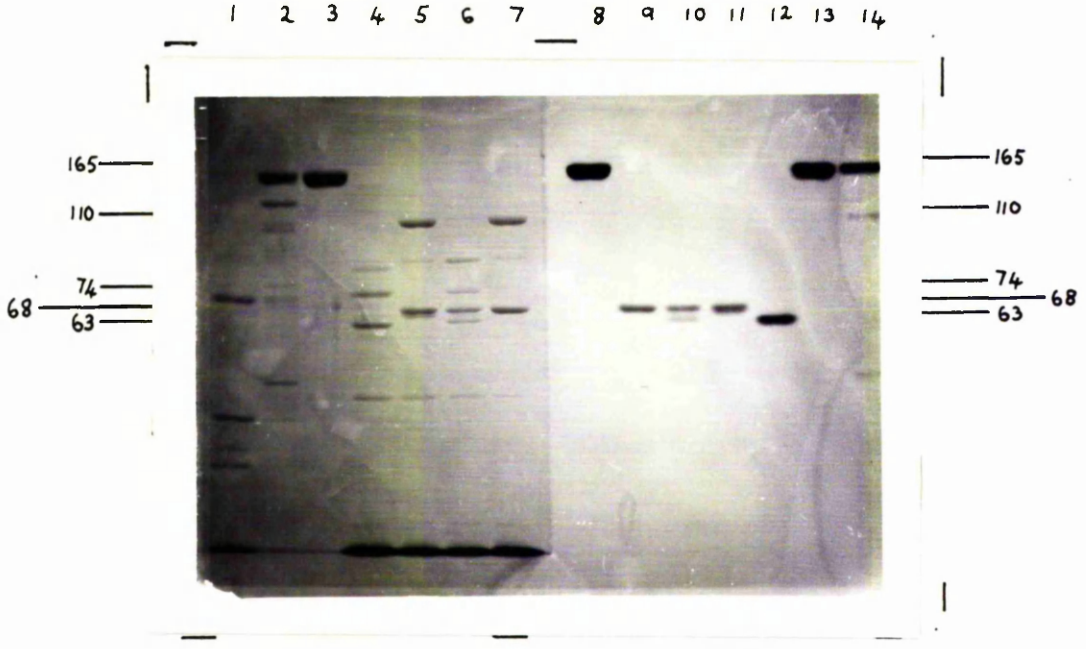


The preliminary results described above indicated that it should be possible to characterise active fragments of the arom complex that carry E5 alone, E3 alone and the combined E2/E3 activities. One problem with these preliminary experiments was that the active fragments of interest were difficult to separate from residual material that retained the arom quaternary structure. To identify appropriate conditions for the efficient degradation of all material with a quaternary structure similar to that of the intact arom complex, nondenaturing PAGE was used to resolve proteolytic digestion products with E3 activity. Digestion of the enzyme complex with 100μg/ml trypsin together with 10μg/ml chymotrypsin efficiently converted the native enzyme complex into a number of high mobility active species that were resolved in nondenaturing PAGE (see Fig.7,3, lanes 10 and 11). These proteolysis conditions were therefore investigated in more detail.

7.3.2. Limited Proteolysis by Trypsin and Chymotrypsin

Limited proteolysis of the arom enzyme complex by trypsin and chymotrypsin together yields products that closely resemble those generated by trypsin alone (e.g. Fig. 7.3 and Fig. 7.5). The results of a large-scale trypsin/chymotrypsin proteolysis and fractionation experiment are documented in Table 7.1 and Figures 7.6, 7.7 and 7.8. The initial products of limited trypsin/chymotrypsin proteolysis are two large polypeptide fragments which appear to be identical to the two primary fragments of 110kDa and 68kDa seen when trypsin alone is used. The 68kDa fragments and the intact 165kDa arom polypeptide can be stained for E3 activity after SDS PAGE (Fig. 7.5). The cleavage of the 165kDa arom polypeptide into two large primary fragments is accompanied by the loss of E4 activty; the other four enzyme activities are retained quantitatively (compare Figs. 7.6 and 7.7). There is no significant change in the mobility of the enzyme complex in nondenaturing PAGE nor in

Figure 7.5 E3 activity of fragments of the arom polypeptide isolated by SDS PAGE



SDS PAGE was through a 10% polyacrylamide gel.

Lanes 1-7 were stained for protein
Lanes 8-14 were stained for arom E3 activity

3, 8, 13 : arom enzyme complex
7, 9 : arom digested with 100μg/ml trypsin for 3 min.
6, 10 : arom digested with 100μg/ml trypsin for 40 min.
5, 11 : arom digested with 100μg/ml tryspin and 20μg/ml chymotrypsin for 3 min.
4, 12 : arom digested with 100μg/ml trypsin and 20μg/ml chymotrypsin for 40 min.

high performance gel filtration chromatgraphy (HPGFC) (Fig. 7.8). This last result indicates that the cleavage of the polypeptide chain into two large fragments has little effect on the quaternary structure of the enzyme complex.

After prolonged trypsin/chymotrypsin proteolysis all material that comigrates with the arom enzyme complex in nondenaturing PAGE and HPGFC is degraded. E1 activity is lost, but a large proportion of the initial E2, E3 and E5 activities remains. Two major high-molecular weight arom degradation products are seen in nondenaturing HPGFC (Fig. 7.8), with estimated sizes of 200kDa and 130kDa. Two major polypeptide species are seen in SDS PAGE, with apparent molecular weights of 74kDa and 63kDa (Fig. 7.6, lanes 5 & 6). The major 63kDa fragment is generated by proteolytic trimming of the 68kDa primary fragment (Fig. 7.6, lanes 3& 4; Fig. 7.3), and retains the capacity to recover E3 activity after SDS PAGE (Fig. 7.5, lanes 4 & 12). The major 74kDa fragment is derived from the 110kDa primary fragment; neither the 110kDa fragment nor the 74kDa sub-fragment exhibits E3 activity after SDS PAGE.

7.3.3 Fractionation of the proteolytic digestion products

In the experiment documented in Table 7.1 the arom digestion products were passed through a column of immobilised trypsin inhibitor, then treated with ethanolic PMSF (1.2mM final), diluted to a final concentration of 25mM KP_i pH 7.0, and applied to a column of DEAE Sephacel equilibrated with the same buffer. All arom enzyme activities were bound to the DEAE Sephacel column under these conditions; the proteolytic fragments were fractionated by elution with a series of steps of increasing concentrations of KP_i pH 7.0, 0.4mM DTT.

E5 activity and a small proportion of the E3 activity were eluted from the DEAE Sephacel column in 50mM KP_i. SDS PAGE of the eluted material showed that the predominant polypeptide species

comigrated with the major 74kDa component of the unfractionated digestion products (Fig. 7.6, lanes 6, 7, 8). The pooled fractions with E5 and E3 activity were applied to a column of phosphocellulose equilibrated with 50mM KP_i, pH 7.0, 0.4mM DTT. E5 activity bound to the column under these conditions, while E3 activity was not retarded. E5 activity was eluted from the phosphocellulose column with a step of 200mM KP_i, 0.4mM DTT.

E2 activity and the major part of the E3 activity were not eluted from the DEAE Sephacel column in 50mM KP_i or 75mM KP_i. These activities were recovered with an elution step of 200mM KP_i, pH 7.0.

7.3.4 Characterisation of an active E5 fragment of the arom complex

The material with E5 activity recovered after chromatography on DEAE Sephacel and phosphocellulose as described above was devoid of E2 and E3 activity. The EPSP synthase (E5) activity of the isolated fragment was almost indistinguishable from the EPSP synthase activity of the native arom complex by two criteria:

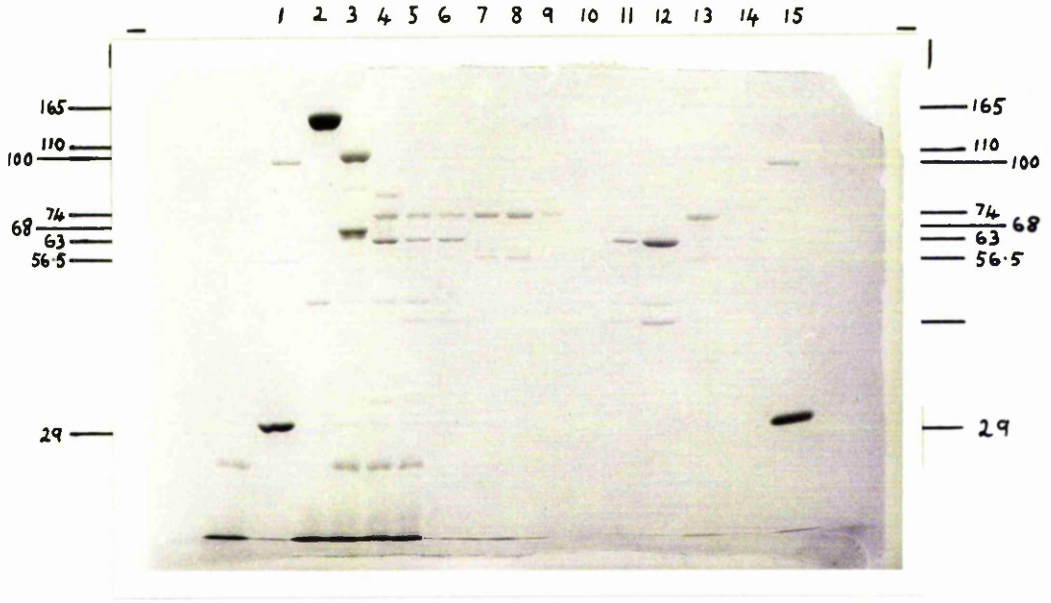
1. relative velocities of the forward and reverse reactions under standard saturated assay conditions.
2. sensitivity to glyphosate inhibition at a fixed concentration of PEP and shikimate 3-phosphate in buffer B.

Nondenaturing HPGFC of the purified material with E5 activity revealed a single high molecular weight species. At least 60% of the E5 activity applied to the column was recovered in a single peak that coincided with the peak of peptide material (Fig. 7.8). The mobility of the isolated E5 fragment was identical to that of the smaller of the two major high molecular weight species seen before fractionation of the digestion products; the molecular weight of the fragment under nondenaturing conditions estimated from the calibration plot of Figure 7.9 is 130kDa.

TABLE 7.1 Resolution of active fragments after limited proteolysis of the arom multifunctional enzyme by 100μg/ml trypsin and 20μg/ml chymotrypsin

Sample	V/ml	fig. 7.6 lane	units of enzyme activity E1	E2	E3	E4	E5	activity ratios E1	E2	E3	E4	E5
limited proteolysis												
0 mins	3.34	2	3.21	3.37	8.01	2.69	2.59	95.0	100	238	79.8	76.6
2.5 mins		3	2.48	3.32	6.26	0.03	2.78	74.5	100	188	0.7	83.3
35.0 mins		4	0.14	2.46	6.61	0.0	2.59	5.6	100	268	0.0	104
104.0 mins	3.74	5	0.0	1.65	4.63	0.0	2.22	0.0	100	279	0.0	134
trypsin-inhibitor-agarose												
	4.6	6	0.0	1.25	4.15	0.0	1.39	-	100	333	-	112
elution from DEAE-sephacel												
50mM KP_i	2.85	7,8,9	-	0.0	0.54	-	0.70	-	≤ 0.2	78	-	100
200mM KP_i (E2/E3 fragment)	2.30	12,13	-	0.99	2.24	-	0.0	-	100	239	-	≤ 0.02
elution from phosphocellulose												
200mM KP_i (E5 fragment)	1.95	14	-	0.0	0.0	-	0.38	-	< 0.05	≤ 0.2	-	100

Figure 7.6 Resolution of active fragments of the arom enzyme complex after limited proteolysis



Samples removed during the course of the limited proteolysis experiment documented in table 7.1 were subjected to SDS PAGE in a 10% Laemmli gel, and are numbered as specified in the table.

1, 15	E.coli pyruvate dehydrogenase, carbonic anhydrase
2.	arom enzyme complex + trypsin inhibitor
3, 4, 5	proteolysis timecourse (2.5, 35, 104 min)
6.	material recovered from trypsin inhibitor column
7, 8, 9	DEAE Sephacel 50mM KP_i elution
11, 12	DEAE Sephacel 200mM KP_i elution E2/E3 fragment
13, 14	phosphocellulose 200mM KP_i elution E5 fragment

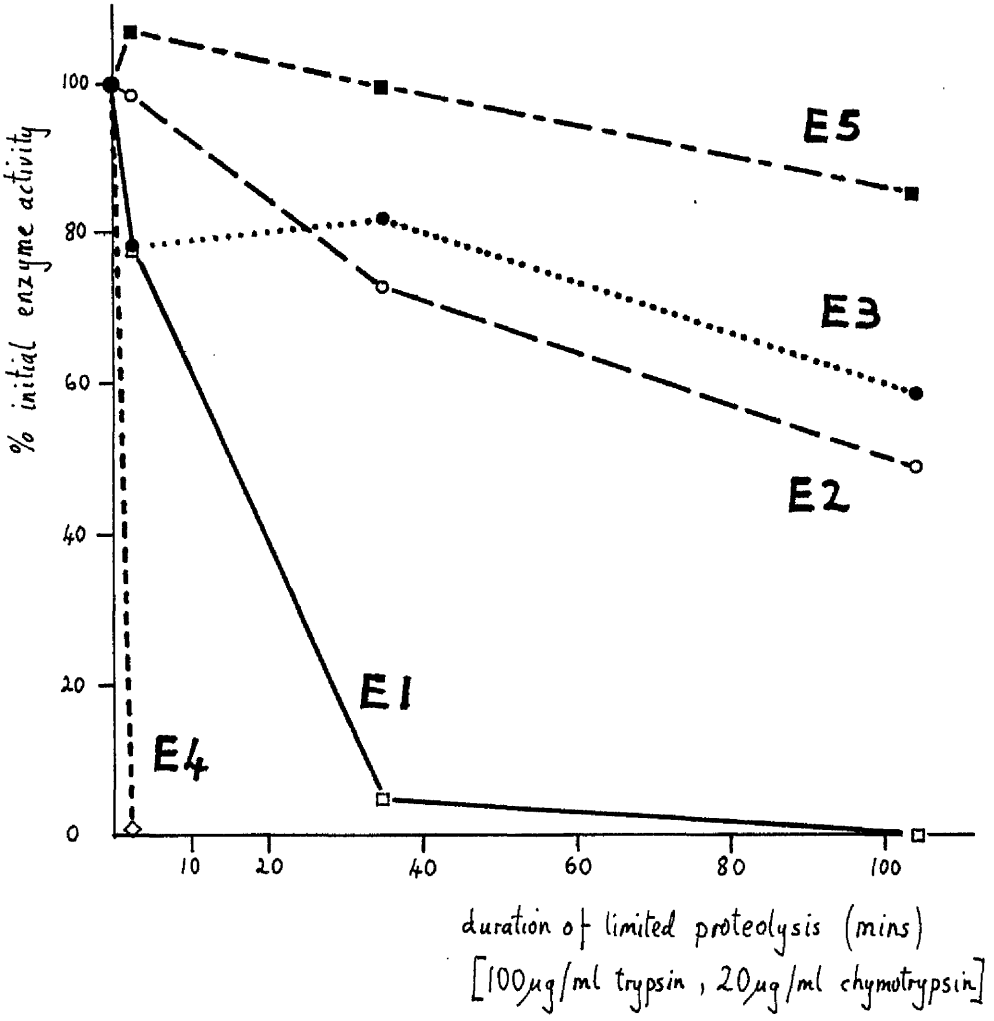


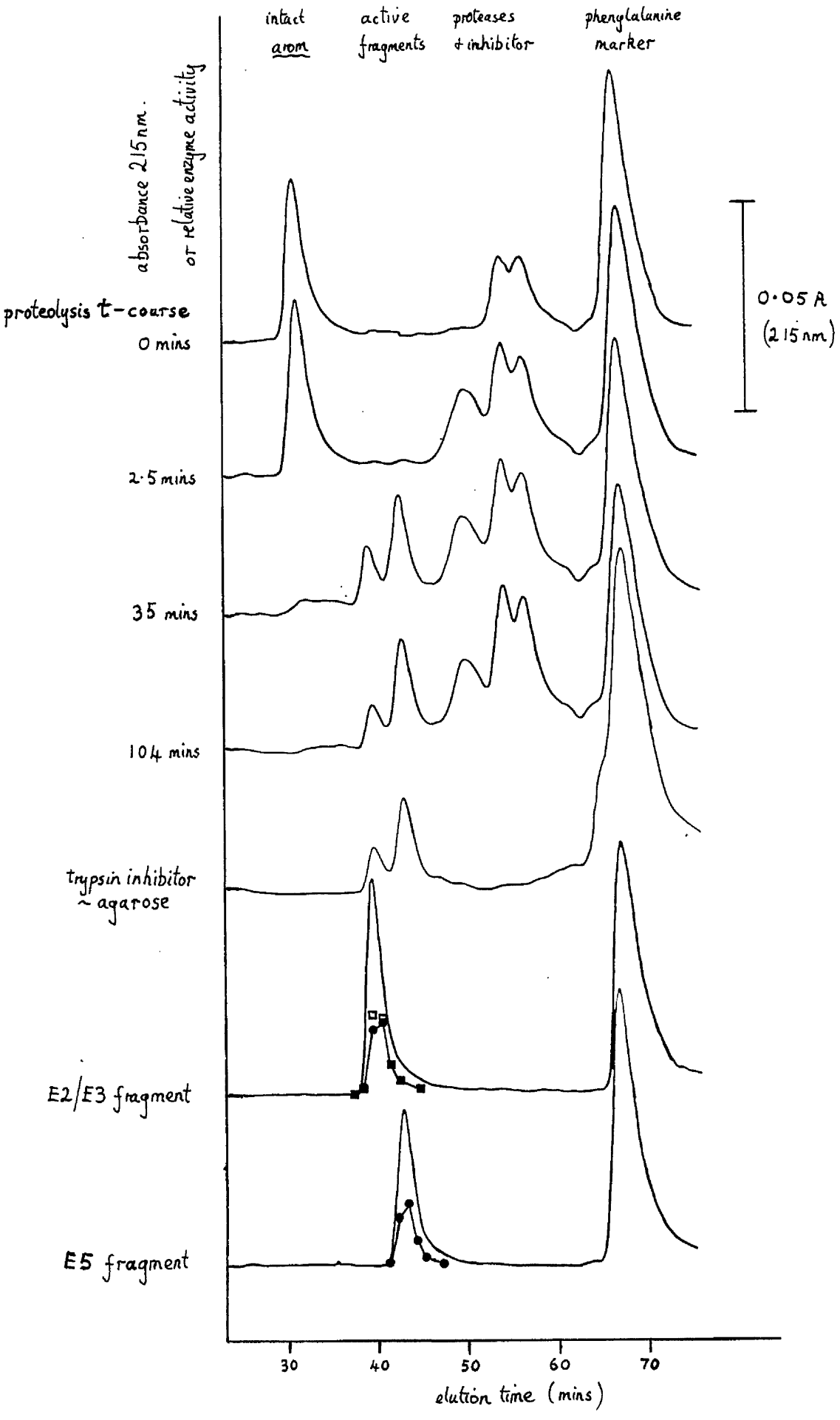
Figure 7.7 Resistance of arom activities to limited proteolysis (data of table 7.1)

Figure 7.8 Separation of active fragments of the arom enzyme complex by high performance GFC.

Samples removed during the course of the limited proteolysis experiment documented in table 7.1 were subjected to HPGFC as described in section 3.13, using phenylalanine as an internal marker. The absorbance of the column effluent was monitored continuously at 215nm; 0.4ml fractions were assayed for the appropriate enzyme activities.

● E5 activity or E2 activity (arbitrary units)
□ (E3 activity)/2 (arbitrary units)
■ E2 and (E3 activity)/2, data points superimposed

Identical samples were subjected to SDS PAGE (figure 7.6)


intact arom
active fragments
proteases + inhibitor
phenylalanine marker
absorbance 215 nm. or relative enzyme activity
proteolysis t-course
0 mins
2·5 mins
35 mins
104 mins
trypsin inhibitor ~ agarose
E2/E3 fragment
E5 fragment
0·05 A (215 nm)
30
40
50
60
70
elution time (mins)

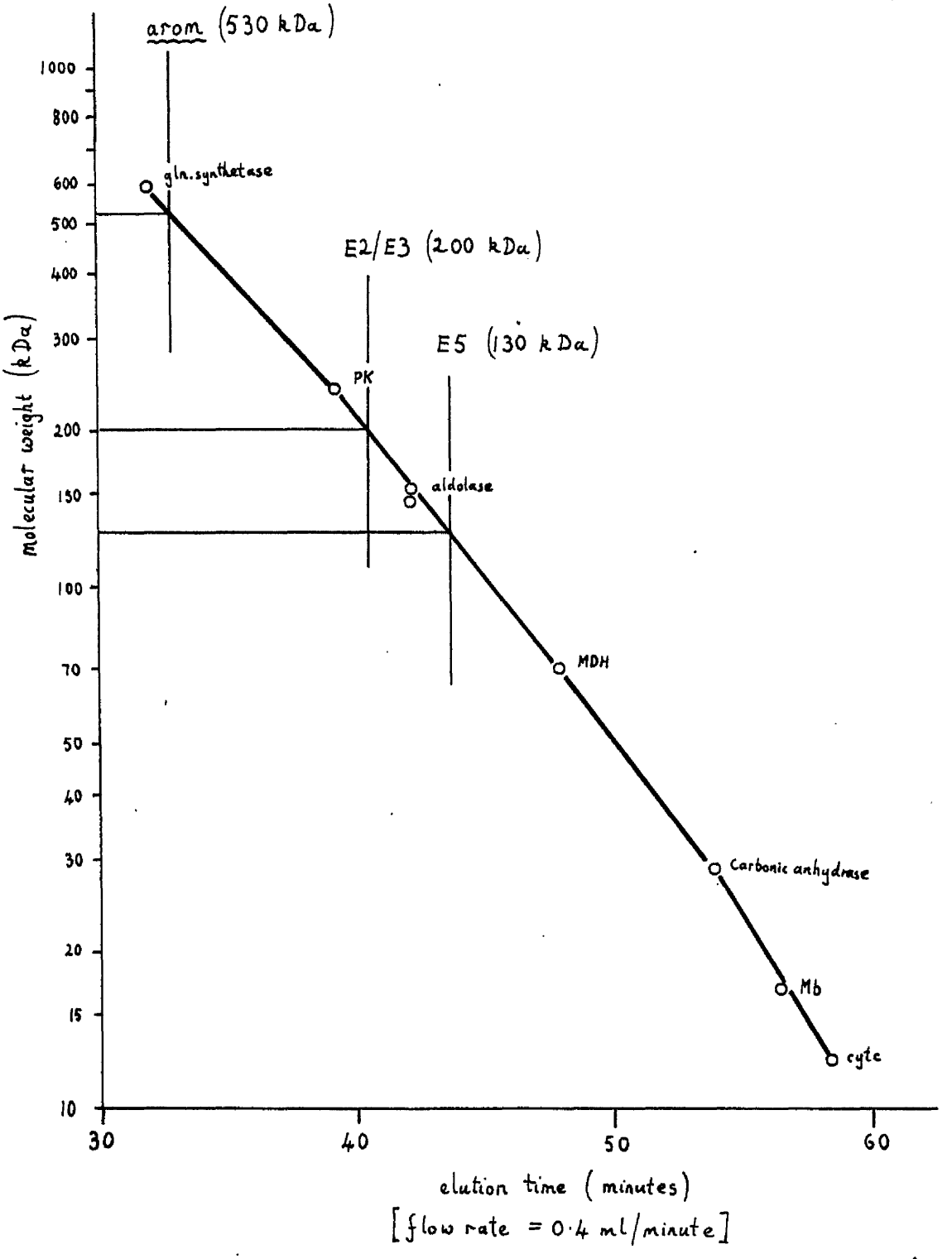


Figure 7.9 Molecular weight calibration plot for HPGFC experiments

SDS PAGE of the purified material with E5 activity showed that the predominant polypeptide species had an apparent molecular weight of 74kDa. A smaller amount of a satellite species of 57kDa was also present (Fig. 7.6, lane 13). The peak fractions of E5 activity collected after HPGFC of this material were subjected to SDS PAGE. The major 74kDa species and the 57kDa satellite species were present in essentially the same relative proportions. The 74kDa polypeptide fragment comigrates with, and is presumably identical to, the larger of the two major species seen in SDS PAGE of the unfractionated proteolytic digestion products. This species is derived from the 110kDa primary fragment in a complex sequence of proteolytic cleavage events that appear to bring about the disintegration of the enzyme complex into active fragments and the destruction of E1 activity. The remainder of the 110kDa primary fragment is digested into small peptides. The 57kDa satellite species is probably an internal subfragment of the major 74kDa fragment. This latter fragment is, however, comparatively stable in the presence of high concentrations of trypsin and chymotrypsin.

On the strength of the evidence outlined above it is concluded that the isolated E5 fragment carries an intact EPSP synthase catalytic site and consists of a 74kDa polypeptide subfragment of the 110kDa primary proteolytic fragment. The subunit structure of the active E5 fragment remains to be determined, but the molecular weight of 130kDa estimated by HPGFC under nondenaturing conditions strongly suggests that the active species is a dimer of 74kDa polypeptide chains.

7.3.5 Characterisation of an active E2/E3 fragment of the arom enzyme complex

The material with E2/E3 activity isolated by chromatography on DEAE Sephacel as described above was devoid of E5 activity. The E2:E3 activity ratio was very similar to that of the intact enzyme complex.

The specific E2 activity of the isolated E2/E3 fragment was greater than 10 units/E_{280} and was thus higher that that of the intact enzyme complex.

HPGFC of the isolated material with E2/E3 activity revealed a single high molecular weight species. At least 60% of the E2 and E3 activities applied to the column co-eluted in constant ratio in a single peak that coincided with the peak of polypeptide material. The mobility of the E2/E3 fragment was identical to that of the larger of the two major high molecular weight products of limited proteolysis; the apparent molecular weight of the active fragment estimated from the calibration plot of Figure 7.9 is 200kDa.

SDS PAGE of the material with E2/E3 activities recovered from the DEAE Sephacel column revealed that a 63kDa polypeptide was the major component. Two polypeptide species of 47kDa and 44kDa were also present in small amounts. The peak fractions of E2/E3 activity recovered after HPGFC of this material were subjected to SDS PAGE. The 63kDa fragment was again the predominant species; the two satellite bands were present in the same relative proportions as before.

The material with E2/E3 activity isolated after chromatography on DEAE Sephacel in a similar experiment was analysed by PAGE under native conditions; a single sharp band was seen when gels were stained for protein or for E3 activity. In this particular experiment SDS PAGE showed that the isolated E2/E3 fragment was essentially homogeneous with respect to the 63kDa polypeptide.

The smaller of the two major polypeptide products of extended trypsin/chymotrypsin digestion of the arom complex under native conditions is a 63kDa species which is derived from the 68kDa primary fragment characterised by Smith (1980). The evidence presented above indicates that a proteolytic fragment of the native arom enzyme complex

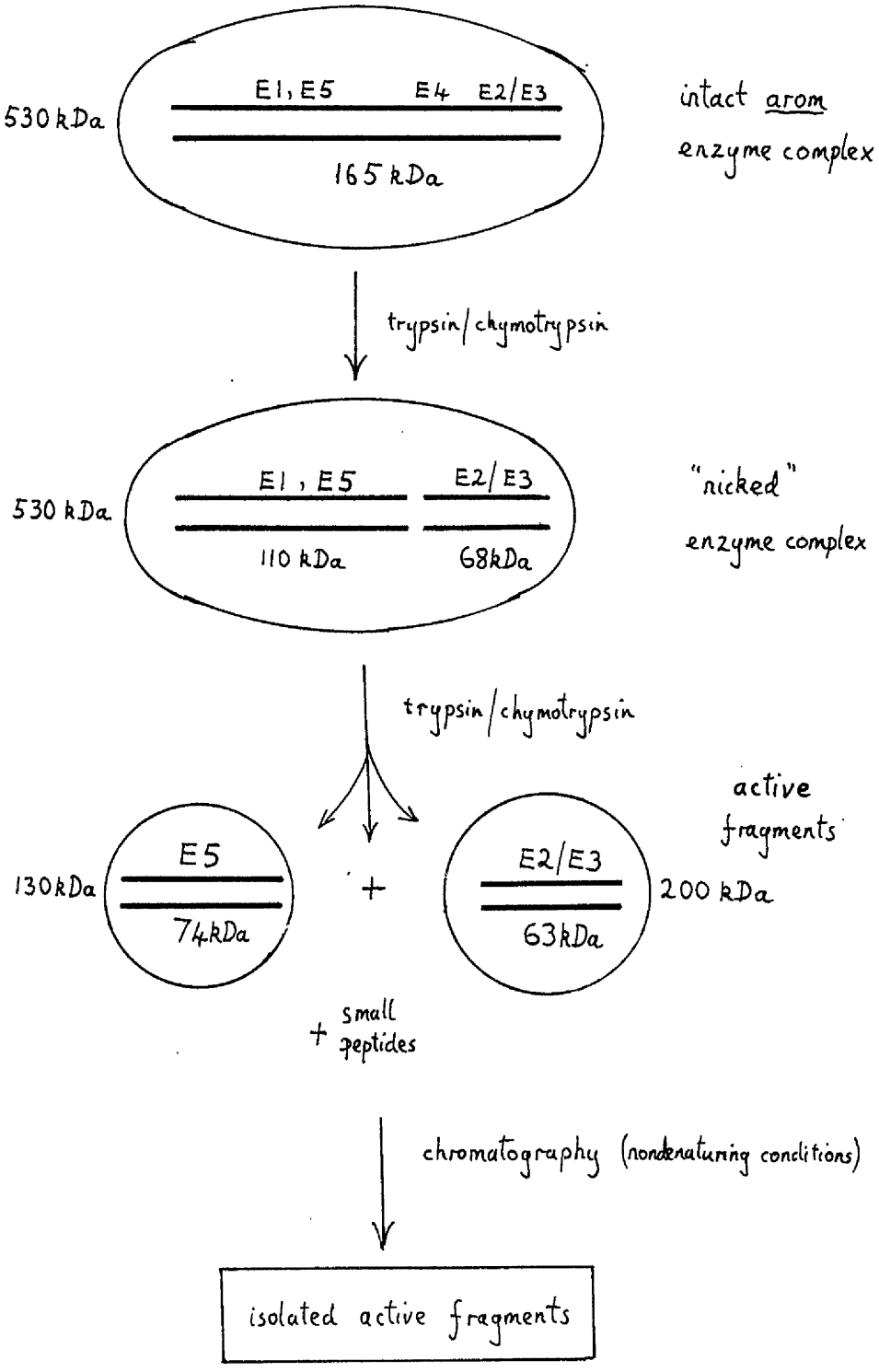
consisting of the same 63kDa polypeptide carries functional catalytic sites for the E2 and E3 reactions, can be isolated preparatively under nondenaturing conditions, and can recover E3 activity after SDS PAGE. These conclusions extend those of Smith (1980) who isolated the parent 68kDa fragment by gel electrophoresis in the presence of 8M urea, and showed that it could recover E3 activity, and that it contained the active-site lysine residue of E2. It is likely that the two satellite species associated with the 63kDa polypeptide fragment are the products of proteolytic "nicking" of the latter species, which is otherwise relatively stable in the presence of trypsin and chymotrypsin.

The subunit structure of the E2/E3 fragment has yet to be defined, but the molecular weight of 200kDa estimated under native conditions by HPGFC strongly suggests that it is a dimer of 63kDa polypeptides. A higher aggregation state is considered unlikely, since the native arom enzyme complex, which is a dimer of 165kDa polypeptide chains (Lumsden and Coggins, 1978), exhibits an anomalously high apparent molecular weight of 530kDa in HPGFC experiments.

7.3.6 Resolution of the arom multifunctional enzyme into active fragments carrying E5 activity and E2/E3 activities

Prolonged proteolytic digestion of the arom enzyme complex with trypsin and chymotrypsin under nondenaturing conditions yields two relatively stable high molecular weight fragments which carry a substantial proportion of the initial E2/E3 and E5 activities (Table 7.1 and Fig. 7.6). The experiments outlined above demonstrate that under nondenaturing conditions these two active fragments can be resolved by a variety of techniques, and that they correspond to major polypeptide species of 63kDa and 74kDa, respectively. The 63kDa and 74kDa polypeptides are derived from complementary primary fragments of the

Figure 7.10 Isolation of active fragments of the arom multifunctional enzyme



intact 165kDa *arom* polypeptide and thus comprise non-overlapping segments of the primary sequence of the polypeptide. It can therefore be concluded that the regions of the multifunctional enzyme essential for E5 catalysis, and the regions essential for E2/E3 catalysis, are structurally independent. It follows that the sub-regions of the *arom* polypeptide that are concerned with the expression of E5 activity and E2/E3 activities in the intact multifunctional enzyme are specified by two non-overlapping sub-regions of the *arom* gene.

The E2, E3, E4 and E5 activities of the native enzyme complex isolated by Burgoyne *et al* (1967) were partially reconstituted after vigorous treatment with denaturing agents such as guanidine • HCl, urea and SDS. Similar results have been obtained with the *arom* enzyme complex isolated in the present study. The primary structure of the 165kDa *arom* polypeptide thus presumably encodes all of the information necessary for the expression of at least four of the component enzyme activities.

The successful isolation of an active E5 species corresponding to a 74kDa sub-fragment of the 165kDa *arom* polypeptide does not necessarily indicate that the information needed to specify the correct tertiary structure of an E5 catalytic site is exclusively encoded within this sub-region of the primary sequence of the *arom* polypeptide. This is because the active fragment is isolated under nondenaturing conditions and may retain the folded conformation of the intact enzyme complex. However, the 63kDa polypeptide associated with the E2/E3 active fragment can recover E3 activity after it has been treated with 1% SDS and 1% βME at 100°C for 2 minutes prior to SDS PAGE (Fig. 7.5). This finding strongly suggests that the primary structure of the 63kDa sub-region encodes all of the information necessary for efficient refolding of the denatured polypeptide and expression of shikimate dehydrogenase (E3)

activity. It is not yet known whether the E2 and E5 activities of the isolated proteolytic fragments can be reconstituted after treatment with denaturing agents. It would therefore be premature to conclude that there are five non-overlapping sub-regions of the primary sequence that encode all of the information necessary for the expression of the corresponding enzyme activity, and that the arom gene is essentially a "mosaic" of monofunctional genes. The information presently available, namely the genetic data of Giles et al (see Section 7.1.2) and the limited proteolysis results of Smith (1980), and of this study, are fully consistent with the "mosaic" model but the picture is still very incomplete. Taking the E3 reconstitution results together with the results obtained with "pleiotropic" arom mutants (Case & Giles, 1971; see Section 7.1.2.5) it can be stated with some confidence that the information required to specify E5 activity is exclusively encoded within the primary sequence of the 110kDa N-terminal segment of the arom polypeptide, and that the information required to specify E3 activity is exclusively encoded within the complementary C-terminal 68kDa segment of the polypeptide.

7.3.7 Structural domains within the arom multifunctional enzyme

The relative stability of the E5 and E2/E3 fragments of the arom enzyme complex in the presence of high concentrations of trypsin and chymotrypsin suggests that they have a well-defined globular structure and contain few scissile peptide bonds that are readily accessible to the two proteases. The active fragments may therefore constitute structural "domains" (Rossmann & Argos, 1981) derived from non-overlapping regions of the intact enzyme complex. Since the isolated active fragments are likely to contain two identical polypeptide chains it is possible that they consist of pairs of identical and tightly associated structural domains. The occurrence of allelic complementation within the E3 region

of the arom gene (Case & Giles, 1971) suggests that interactions between the two polypeptide chains in the isolated E2/E3 fragment may be important for the expression of E3 activity.

It is of some interest to recall that E2 and E3 are the only two shikimate pathway activities that are tightly associated in plants, and that they are expressed as a bifunctional polypeptide of 48kDa in the moss Physcomitrella patens (Polley, 1978; see Fig. 1.4). The E2 and E3 activities of the E2/E3 fragment of the arom enzyme complex of N.crassa have not been resolved, and it is therefore impossible to decide whether the two component catalytic activities are associated with independent structural domains. The isolation of stable proteolytic fragments with E3 activity alone, probably associated with very small ($\leq$ 30kDa) polypeptide fragments (Section 7.3.1., Fig. 7.4), suggests that this could be the case, but no active E2 fragments of the complex devoid of E3 activity have yet been observed. The proposal that the E2 and E3 active sites are carried by a single bifunctional structural domain of the arom enzyme complex of N.crassa (Smith, 1980; Smith & Coggins, 1983) thus cannot lightly be dismissed. One may speculate that a homologous bifunctional structural domain catalyses the same two reactions in all plants and fungi.

7.3.8 Assignment of enzyme activities to sub-regions of the arom polypeptide

Under the nondenaturing conditions investigated in the present study it has not been possible to resolve active tryptic and chymotryptic fragments of the arom enzyme complex with E4 and E1 activities. E4 activity is destroyed in the primary proteolytic cleavage event; in the light of the genetic complementation map (Fig. 7.1) E4 functions can be tentatively assigned to the protease-hypersensitive region 110kDa from the N-terminus of 165kDa arom polypeptide (Smith, 1980). The complementation map would thus place E1 functions within the N-terminal

110kDa primary polypeptide fragment. This assignment is consistent with the observation that the degradation of the 110kDa polypeptide is accompanied by the loss of E1 activity and break-up of the enzyme complex into active fragments.

Cleavage of the arom enzyme complex by an endogenous N.crassa protease yields two large polypeptide fragments of 125kDa and 58kDa, and coincides with the destruction of E5 activity, but not of E1, E2, E3 or E4 activities (Section 4.3.7; Fig. 4.4). The large 125kDa fragment can be stained for E3 activity after SDS PAGE. These results, taken together with the complementation map constructed for the "pleiotropic" arom mutants (Fig. 7.1) strongly suggest that regions of the arom polypeptide within 58kDa of the N-terminus are associated with E5 activity. This deduction is difficult to rationalise in terms of the complementation map of the "single enzyme" mutants, which suggests that the N-terminal regions of the polypeptide are associated with E1 activity and not with E5 activity (see Fig. 7.1). The limited proteolysis results thus do not help to resolve the ambiguity in the ordering of E1 and E5 activities (see Fig. 1.1). It will clearly be of some interest to find out whether the 74kDa polypeptide associated with the isolated E5 active fragment is derived from the N-terminal region of the 110kDa primary fragment.

The most satisfactory model for the progress of limited trypsin/chymotrypsin digestion of the arom enzyme complex is shown in Figure 7.10.

7.3.9 The structure of the arom enzyme complex

The genetics of the arom locus, discussed in the introduction to this chapter, are consistent with a simple "beads-on-a-string" model for the tertiary and quaternary structure of the enzyme complex, in which five pairs of independently folding catalytic domains are held together

only by linking regions of the polypeptide backbone and by interactions between the two C-terminal (E3) regions and possibly the two N-terminal (E1) regions. The "beads-on-a-string" structure is one possible consequence of a "mosaic" model for the structure of the arom gene. The impressive resistance of the overall quaternary structure of the enzyme to proteolytic attack, the evidence that the isolated proteolytic fragments retain the dimeric structure of the native enzyme complex, and the hydrodynamic data for the highly purified enzyme complex (Gaertner et al, 1972), indicate that the "beads-on-a-string" model is probably an oversimplification. The enzymological evidence suggests that the arom enzyme complex is a highly condensed globular protein with extensive interchain and intrachain interactions and very few regions of poorly defined tertiary structure that are accessible to proteolytic attack. In other respects, however, the limited proteolysis results are broadly consistent with predictions about the structure of the enzyme complex made on the basis of the genetic properties of the arom locus. The results obtained at the polypeptide level thus complement the genetic data of Giles et al; viewed in isolation they constitute independent evidence that is consistent with a "mosaic" model for the structure of the arom gene.

CHAPTER 8 GENERAL DISCUSSION AND FUTURE PROSPECTS

8.1 Catalytic properties of the arom multifunctional enzyme

A comparison of the catalytic properties of the five component activities of the arom multifunctional enzyme reveals some interesting patterns. The saturated velocities of the five enzymes, assayed in the forward direction at neutral pH, fall within a very narrow range, corresponding to k_{cat} values of $15s^{-1}$ to $60s^{-1}$. In all cases the limiting K_M values are very low, particularly the limiting K_M values for the shikimate pathway intermediates (see Tables 8.1 amd 4.13).

The K_M values are, for instance, lower that those reported for the corresponding monofunctional E1, E2 and E5 activities of E.coli (J.M. Lambert & A. Lewendon, unpublished work) and are very much lower than those reported for the majority of mammalian glycolytic enzymes (e.g. Fersht, 1978). These patterns are at variance with theoretical predictions and experimental observations which suggest that the component enzymes of metabolic pathways normally operate below saturation with respect to the pathway intermediates, and that enzymes operate most efficiently if the K_M's are relatively high (e.g. Fersht, 1978; Page, 1977).

The evidence that has so far been presented in support of substrate "channelling" between component enzymes of the arom enzyme complex (Welch & Gaertner, 1977) has very serious shortcomings (see Section 2.4). Genetic evidence, particularly the complementation of mutant arom-9 (E2) alleles by qa-2 (catabolic dehydroquinase) alleles (Rines et al, 1969) suggests that catalytic interactions between the component enzymes are not essential for aromatic amino acid biosynthesis in vivo. However, the evidence that the shikimate pathway intermediates are relatively tightly bound to the arom complex and that the five enzyme activities have very similar saturated velocities may prompt a

TABLE 8.1 Comparison of the kinetic parameters of the five activities of the arom multifunctional enzyme

	$\xrightarrow{E1}$	$\xrightarrow{E2}$	$\xrightarrow{E3}$	$\xrightarrow{E4}$	$\xrightarrow{E5}$
relative V_m	98.0	100	263.9	94.4	173.5
specific activity (U/mg)	6.9	7.04	18.5	6.6	12.2
calculated k_{cat} (s^{-1})	19.0	19.4	51.2	18.3	33.6
K_M for pathway intermediate (μM) (at saturation in 2nd substrate)	1.4	5.0	18	(100)	0.36

The activity ratios are those estimated for a 4th generation preparation of arom (table 4.13). The specific activities and turnover numbers are calculated from the final E2 specific activity recorded in the 3rd generation experiment A7 (table 4.7). Kinetics constants were all estimated at pH 7.0 or pH 7.2, but in a variety of buffering systems. Unpublished kinetic data of J.M.Lambert (E1 & E2), S. Chaudhuri (E3) and G.A.Nimmo (E4).

re-examination of the integrated catalytic behaviour of the multifunctional enzyme. It should be noted that if the ordered sequential mechanism proposed for arom E5 is valid a very sensitive assay for the intramolecular transfer of shikimate 3-phosphate between the E4 and E5 catalytic sites is available, since at saturation in PEP the productive binding of shikimate 3-phosphate at the E5 active site is an "irreversible" step.

8.2 The structure of the arom multifunctional enzyme

Very recently Larimer et al (1983) have reported the cloning of the aro gene cluster of the yeast S.cerevisiae in a yeast/E.coli shuttle vector. Fine structure genetic mapping results suggest that this cluster gene is very similar to the arom cluster gene of N.crassa and the aro cluster of the fission yeast Schizosaccharomyces pombe (De Leeuw, 1967; Strauss, 1979; Giles et al, 1967a) and it probably encodes a pentafunctional 150kDa polypeptide which is functionally homologous to the arom polypeptide of N.crassa (Larimer et al, 1983). It is likely that the primary structure of either the yeast enzyme complex or the N.crassa arom polypeptide will soon be determined, and rapid progress may also be made in determining the tertiary structure by diffraction methods. It will also be possible to examine the properties of polypeptide fragments specified by defined regions of the arom gene, and this may eventually become the method of choice for resolving many of the issues addressed in the previous chapter.

When the primary structure of the arom polypeptide has been determined it should be possible to identify the regions that correspond to the two active fragments which have so far been characterised in detail, using modern small-scale polypeptide sequencing methods (e.g. Walker et al, 1982). The successful alignment of the genetic complementation maps, the primary sequence, and the proteolytic cleavage

map will be an important objective, and may be of particular interest if the gene structure is complex.

8.3 Evolution of the arom multifunctional enzyme

Three plausible models for the evolution of multifunctional enzymes, the "adaptation" model, the "gene fusion" model, and the "gene duplication" model, were discussed in Section 2.6. The resolution of the arom multifunctional enzyme into E5 and E2/E3 active fragments (Section 7.3.6) and the genetic evidence of the structural autonomy of the five catalytic sites (e.g. Giles et al, 1967a) strongly suggest that the enzyme has a modular structure, as is predicted by the "gene duplication" and "gene fusion" evolutionary models.

During the course of this study a number of interesting similarities between individual arom enzyme activities and the corresponding monofunctional enzymes of other organisms have come to light. Arom E1 is a metalloenzyme, and shares this feature, and several others, with the DHQ synthases of a variety of plants and bacteria (J.M. Lambert, M.R. Boocock and J.R.Coggins, in preparation; see Chapter 4). The herbicide glyphosate acts as a potent reversible inhibitor of arom E5 by binding to an enzyme • Shik3P intermediate in the kinetic sequence (Boocock & Coggins, 1983). An ordered sequential kinetic mechanism has been proposed for this enzyme (Section 6.5.10). There is good evidence that EPSP synthase is the principal target enzyme of the herbicide in a wide variety of plants and bacteria (e.g. Schulz & Amrhein, 1983). Preliminary evidence from studies on the monofunctional EPSP synthases of E.coli and pea seedlings indicates that they interact with glyphosate in a similar way to the N.crassa enzyme; several other catalytic properties of the pea and E.coli enzymes closely resemble those established for the N.crassa enzyme (Lewendon & Coggins, 1983; Mousdale & Coggins, 1983). Since the available evidence indicates that glyphosate does not act

indiscriminately as a conventional structural analogue of PEP (Section 6.9) the potent inhibition of enzymes from all sources may be taken as a strong indication that the catalytic sites of the monofunctional and multifunctional EPSP synthases are structurally and functionally homologous.

The isolated E5 and E2/E3 fragments of the arom complex are both somewhat larger than the corresponding monofunctional and bifunctioanl enzymes isolated from E.coli and peas, and from a moss, respectively, and probably differ in having a dimeric structure (Chapter 7; Lewendon & Coggins, 1983; Mousdale & Coggins, 1983; Polley, 1978). However the three E5 species share the unusual property of binding to both DEAE Sephacel and phosphocellulose at close to neutral pH, and the E2/E3 species both interact strongly with immobilised Cibacron Blue.

A number of other striking similarities between monofunctional and multifunctional shikimate pathway enzymes, particularly the dehydroquinases (E2), were discussed in Chapter 1. No functional or structural similarities between different component enzymes of the arom enzyme complex have been noted. The available evidence therefore favours a "gene fusion" origin for the arom multifunctional enzyme, and leads one to suppose that the component enzyme activities will prove to be structurally and functionally homologous to their monofunctional counterparts in other organisms. The adaptive significance of the multifunctional organisation of the arom enzyme complex in fungal aromatic amino acid biosynthesis remains to be explored.

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