PYRIDINE NUCLEOTIDE DEHYDROGENASES IN THE CYANOBACTERIUM *ANABAENA* PCC 7120 AND THE CHLOROPLASTS OF HIGHER PLANTS

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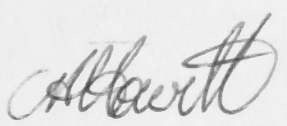
DECLARATION

The research presented in this thesis is my own work unless otherwise stated. This work was carried out in the Division of Biochemistry and Molecular Biology, School of Life Sciences, The Faculties, at The Australian National University. The material presented in this thesis has not been submitted for any other degree unless otherwise stated.



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ABBREVIATIONS

ABC	Adenosine triphosphate binding cassette
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate synthase
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toludene salt
bp	base pair
BSA	Bovine serum albumin
BTP	Bis-Tris propane
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
DBMIB	2,5-Dibromo-3-methyl-6-isopropylbenzoquinone
DCCD	Dicyclohexylcarbodiimide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
FAD	Flavine adenine dinucleotide
FCCP	Carbonyl cyanide p-trifluromethoxyphenylhydrazone
FeCN	Ferricyanide
FMN	Flavin mononucleotide
FNR	Ferredoxin $NADP^+$oxidoreductase
FP	Flavoprotein fraction
FQR	Ferredoxin plastoquinone reductase
GCG	Genetics Computer Group
GET	Glucose Ethylenediaminetetraacetic acid Tris
GST	Glutathione-S-transferase

HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IAC	Isoamyl alcohol chloroform
IP	Iron-sulfur protein fraction
IPTG	Isopropyl β-d-Thigalactopyranoside
kb	kilobase
KCN	Potassium cyanide
kDa	kilodaltons
LB	Luria broth
LMP	Low melting point
M.W.	Molecular weight
MES	2-[N-morpholino]ethanesulfonic acid
MOPS	3-[N-Morpholino]propanesulfonic acid
MTPBS	Mouse tonicity phosphate buffered saline
NADH	Nicotinamide adenine dinucleotide hydride
NADPH	Nicotinamide adenine dinucleotide phosphate hydride
NBT	pnitro blue tetrazolium chloride
NDH-1	Type 1 NADH dehydrogenase
NDH-2	Type 2 NADH dehydrogenase
n-PG	n-propyl gallate
ORF	Open reading frame
PCC	Paster Culture Collection
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl fluoride
PS I	Photosystem I
PS II	Photosystem II
Q1	Quinone 1
RNA	Ribonucleic acid

RNase	Ribonuclease
SHAM	Salicyl hydroxylic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STE	Sodium Tris EDTA
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline Tween-20
TCA	Tricarboxcylic acid
TE	Tris EDTA
TES	N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid
Tris	tris[hydroxymethyl]aminomethane
UV	ultraviolet light
UWGCG	University of Wisconsin genetics computer group
X-GAL	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

ABSTRACT

Pyridine nucleotide dehydrogenases have been implicated in the inorganic carbon concentrating mechanism cyanobacteria posses and they have also been implicated in cyclic electron transport around photosystem I. Recent studies on chloroplasts from higher plants, have shown that electrons can be donated to the intersystem chain from stromal components. A pyridine nucleotide dehydrogenase may be involved in this process. Until recently investigation of pyridine nucleotide dehydrogenases in cyanobacteria and chloroplasts has been neglected. The aim of this study was to investigate pyridine nucleotide dehydrogenases in soybean chloroplasts and the cyanobacterium *Anabaena* PCC 7120. The study would also aim to further investigate the apparent difference between the cellular location of type-1 pyridine dehydrogenases, and their functions in the cyanobacterium *Anabaena* PCC 7120 and *Synechocystis* PCC 6803. In *Anabaena* PCC 7120 the type-1 pyridine dehydrogenase is only present on the plasma membrane, while in *Synechocystis* PCC 6803 it is present on both the plasma and thylakoid membranes.

The gene encoding *ndhK* , which encodes a subunit of the type-1 NADH dehydrogenase, from *Synechocystis* PCC 6803 was amplified using PCR and cloned into the expression vector pGEX-2T. NdhK was overexpressed as a fusion protein with glutathione-S-transferase. The fusion protein was purified and antibodies were raised against it. Immunological analysis of membrane fractions from *Anabaena* PCC 7120, using the antibodies raised against the fusion protein, showed that a protein on the plasma membrane of *Anabaena* PCC 7120 cross-reacted specifically. No proteins on the thylakoid membrane cross-reacted specifically with the antibodies. These results confirm the plasma membrane specific location of the type-1 pyridine nucleotide dehydrogenase and are in agreement with inhibitor studies which showed that respiration on the plasma membrane was rotenone-sensitive, but that respiration on the thylakoid membrane was not.

Southern analysis of digested genomic DNA, using probes from *Synechocystis* PCC 6803, showed that the operon encoding *ndhC-K-J* was conserved in *Anabaena* PCC 7120. Using PCR, with degenerate primers designed against conserved regions of the amino acid sequence of NdhC and NdhJ from cyanobacteria and chloroplasts, part of the operon was cloned and sequenced. The proteins encoded by the genes in the operon had greater homology to the same proteins from *Synechocystis* PCC 6803, than to those from chloroplasts. However, the structure of the operon was more closely related to that from chloroplasts with the coding region for *ndhC* and *ndhK* overlapping, unlike

Synechocystis PCC 6803 where there is an intergenic spacer region between the two genes.

Southern analysis showed that *ndhK* was a single copy gene. A kanamycin resistance gene was inserted into *ndhK*, to inactivate it, and the construct was used to transform *Anabaena* PCC 7120. PCR revealed that twenty of the transformants obtained were unsegregated, containing both the wild type and the inactivated copy of *ndhK*. All of the unsegregated transformants died during attempts to segregate them. This indicates that *ndhK* is likely to be an essential gene in *Anabaena* PCC 7120 under the growth conditions used, as insertional inactivation resulted in an apparently lethal phenotype.

A very simple, rapid method was developed for screening of potential transformants of *Anabaena* PCC 7120 using PCR, with whole cells as the template. This greatly speeds up the screening process for transformants as it allows direct screening of single colonies from a plate. The method developed removes the need for growing cells in liquid culture and isolating genomic DNA which then can be used as a template for PCR or Southern analysis.

It was shown that soybean chloroplasts contain both NADH- and NADPH-quinone oxidoreductase activities. These activities were rotenone-sensitive, indicating that they might be catalysed by a type-1 pyridine nucleotide dehydrogenase. The activities were localised to the stroma lamellae.

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CHAPTER 1

INTRODUCTION

1.1 CYANOBACTERIA

Cyanobacteria (cyanophyte: formerly blue green algae) are gram negative photosynthetic bacteria (Binder 1982; Scherer *et al.* 1988a). As they are prokaryotic they do not contain chloroplasts or mitochondria and thus photosynthesis and respiration must occur in the same compartment. Much is known about the photosynthetic electron transport chain in cyanobacteria: it is located exclusively on the thylakoid membrane and is very similar to the photosynthetic electron transport chain found in the chloroplasts of photosynthetic eukaryotes (Haselkorn 1978; Scherer 1990). Comparatively little is known about the respiratory electron transport chain and there is still some doubt as to the components and their location in the cell, particularly pyridine nucleotide dehydrogenases (see Peschek 1987; Matthijs and Lubberding 1988; Scherer *et al.* 1988b; Scherer 1990 for reviews).This will be discussed more fully in section 1.3.

One problem in studying cyanobacteria is that they are a physiologically and structurally heterogeneous group which has been divided into five sections (Table 1.1) (Rippka *et al.* 1979). This diversity must be taken into account when comparing results from different genera and species.

Table 1.1: Classification of Cyanobacteria (after Rippka *et al.* 1979)

Section	Characteristics
I	Single celled divide by budding or binary fission
II	Single celled, divide by multiple fission
III	Filamentous, non-heterocystous. Divide by branching in one plane
IV	Filamentous, can form heterocysts Divide by branching in one plane
V	Filamentous, can form heterocysts. Divide by branching in more than one plane

Most of the work on respiration in cyanobacteria has been done on species from the genera *Synechocystis* and *Synechococcus* from section II and species from the genera *Anabaena* and *Nostoc* from section IV.

1.1.1 *Anabaena* PCC 7120

The organism used in this study was *Anabaena* PCC 7120 which is an obligate photoautotroph (Rippka *et al.* 1988), more properly assigned to the genus *Nostoc*

(Lachance 1981). It is filamentous and capable of forming heterocysts (Figure 1.1) and is classified into section IV. Heterocysts are specialised cells that form in the absence of combined nitrogen in the growth medium and can fix nitrogen (Wolk 1973; Wolk 1980). They are differentiated vegetative cells that have a multilayered envelope external to the cell wall, that retards diffusion of O_2 into the cell. Photosystem II (PS II) is lost during differentiation thus no O_2 is produced during photosynthesis, allowing the oxygen labile nitrogenase to function. There also seems to be one or more enzymatic scavenging systems to remove traces of O_2 (Haselkorn 1978).

The ultrastrusture of both vegetative cells and heterocysts of *Anabaena* PCC 7120 (the organism used in this study) are similar, and these have similar ultrastructures to vegetative cells from *Anabaena variabilis* M3, which is shown in Figure 1.2.

1.2 RESPIRATORY ELECTRON TRANSPORT IN CYANOBACTERIA

With the advent of methods to separate the plasma membrane from the thylakoid membrane our knowledge of respiration in cyanobacteria has advanced.

1.2.1 Components Of The Respiratory Chain In Cyanobacteria

The photosynthetic electron transport chain in cyanobacteria has been well studied, virtually all the components within the cell are known and have been located on the thylakoid membrane (Figure 1.3). In contrast, investigation of respiration in cyanobacteria is a neglected field and it is only in recent years that the components of the respiratory electron transport chain and their location within the cell have been resolved, and then only in part and only in some species.

Omata and Murata (1984) showed the presence of plastoquinone in both the plasma membrane and thylakoid membrane in *Anacystis nidulans* (now known as *Synechococcus* PCC 7942). Phylloquinone is also present, in smaller amounts, in the membrane fractions of cyanobacteria and can restore respiration in quinone depleted membranes (Peschek 1980a). Most workers favour plastoquinone as the respiratory quinone (Scherer 1990).

The cytochrome b_6/f complex in cyanobacteria is remarkably similar to the corresponding complex in plants and was shown to be present in the membrane fraction of *Anabaena*

variabilis (Krinner *et al.* 1982) and *Nostoc* sp. strain MAC (Peschek and Schmetterer 1982). Recent immunological studies on *A. nidulans*, *Synechocystis* PCC 6714, *A. variabilis* and *Nostoc* sp. strain MAC have shown the presence of the cytochrome b_6/f complex in both the thylakoid membrane and plasma membrane of all four species (Kraushaar *et al.* 1990).

Cytochrome c_{553} has been shown to be present in both the periplasmic space and the intra-thylakoidal lumen of *A. variabilis* (Serrano *et al.* 1990) and *Nostoc* sp. strain MAC (Obinger *et al.* 1990). Plastocyanin is also present in cyanobacteria, but cytochrome c_{553} is more common (Binder 1982). In *Nostoc* sp. cytochrome c_{553} has been shown to be the native electron donor to cytochrome oxidase (Alpes *et al.* 1984). While Sandmann (1986) showed that when copper was depleted cytochrome c_{553} was able to perform all the functions of plastocyanin in the 15 species studied. Recent studies on *Synechocystis* PCC 6803 suggest that there is a third, as yet unidentified, electron carrier that can function in the place of both cytochrome c_{553} and plastocyanin. In strains in which both carriers were missing photosynthetic and respiratory electron transport rates were comparable to those seen in wild type cells (Zhang *et al.* 1994).

Spectral studies and the effect of cyanide on the reaction catalysed by the terminal oxidase in nine different species first suggested that the terminal oxidase was of the cytochrome aa_3 type (Peschek 1981a; Peschek 1981b; Kienzl and Peschek 1982; Peschek *et al.* 1982; Peschek 1984), similar to that found in animals and plants. Fry and co-workers (1985) showed that the terminal oxidase of *Synechocystis* PCC 6311 contained copper that was firmly bound; again, this is similar to cytochrome aa_3 in animals and plants.

Molitor and co-workers (1987) showed, by immunological studies, that *A. nidulans* contained a cytochrome aa_3 in both plasma membranes and thylakoid membranes, that was homologous to the cytochrome aa_3 in *Paracoccus denitrificans*. This was also shown to be true of *Synechocystis* PCC 6714 and *A. variabilis* (Wastyn *et al.* 1987; Wastyn *et al.* 1988). The cytochrome oxidase from *A. variabilis* and *A. nidulans* has been isolated and characterised as a cytochrome aa_3 type oxidase (Häfele *et al.* 1988; Peschek *et al.* 1989).

1.2.2 Interaction Between Photosynthesis and Respiration in Cyanobacteria

As photosynthesis and respiration, which catalyse opposite reactions, both occur in the same uncompartmentalised cell, it is to be expected that there is some interaction between the two processes. This was first shown to be the case by Webster and Frenkel (1953) who showed that respiration in cyanobacteria is inhibited by light, suggesting a direct link between photosynthesis and respiration. Jones and Myers (1963) showed that this was

due to the competition of photosynthesis and respiration for electrons from a common pool.

The first component shown to be common to both the respiratory electron transport chain and the photosynthetic electron transport chain was plastoquinone (Hirano *et al.* 1980; Peschek 1980a; Aoki and Katoh 1982). Cytochrome c_{553} was also shown to be utilised for both respiration and photosynthesis as addition of cytochrome c_{553} (which is removed during the preparation of membranes) to membrane preparations reconstituted both photosynthetic and respiratory electron transport (Hirano *et al.* 1980; Lockau 1981; Peschek and Schmetterer 1982; Stürzl *et al.* 1982; Sandmann and Malkin 1984). Studies on a wide range of species have also shown cytochrome b_6/f to be common to photosynthesis and respiration (Hirano *et al.* 1980; Krinner *et al.* 1982; Peschek 1983; Matthijs *et al.* 1984a; Sandmann and Malkin 1984; Scherer *et al.* 1987). It has been suggested that ferredoxin-$NADP^+$ oxidoreductase (FNR), the last enzyme in the photosynthetic electron transport chain, may also function as an NADPH dehydrogenase in respiration (Houchins and Hind 1982; Alpes *et al.* 1985; Scherer *et al.* 1988c; Howitt *et al.* 1993). In *Synechocystis* PCC 6803, it has been shown that the NAD(P)H dehydrogenase on the thylakoid membrane is the main site of entry of electrons into the plastoquinone pool in the NAD(P)H mediated cyclic electron flow around PS-I (Mi *et al.* 1992a; Mi *et al.* 1994). This does not appear to be the case in *Anabaena* PCC 7120 (Mi *et al.* 1992b).

It is now accepted that all of the components of the respiratory electron transport chain on the thylakoid membrane, except for the terminal oxidase, are also involved in photosynthetic electron transport. However, recently it has been shown that in the absence of PS I electrons generated by PS II are utilised by a KCN-sensitive oxidase on the thylakoid membrane (Vermaas *et al.* 1994). This interaction between the two electron transport chains has been shown to be beneficial to the cyanobacterium, as *A. nidulans* has been shown to be more susceptible to photoinhibition when respiration is inhibited by sodium azide (Shyam *et al.* 1993) The components of the respiratory electron transport chain on the plasma membrane, on the other hand, do not interact with the photosynthetic electron transport chain. (Figure 1.4, for reviews see Peschek (1987), Matthijs (1988), Scherer (1988b) and Scherer (1990)).

1.3 PYRIDINE NUCLEOTIDE DEHYDROGENASES

Pyridine nucleotide dehydrogenases catalyse the first step in the respiratory electron transport chain. Catalysing the oxidation of NAD(P)H and the reduction of a species of

quinone. In cyanobacteria, as in the respiratory chains of most organisms, the pyridine nucleotide dehydrogenases are the components about which least is known. This is because there are often multiple forms present in the respiratory chain and one class of these, if present, is the most complex enzyme in the respiratory electron transport chain.

1.3.1 Types of Pyridine Nucleotide Dehydrogenases

Bacterial pyridine nucleotide dehydrogenases can be divided into two classes, named type-1 (NDH-1) and type-2 (NDH-2) (Anraku 1988; Yagi 1991; Yagi 1993), which have a number of distinguishing features.

a) Type-1 Pyridine Nucleotide Dehydrogenases

NDH-1 shows homology to complex I found in the respiratory chains of animals and plants (see Weiss *et al.* 1991; Weiss and Friedrich 1991; Walker 1992; Walker *et al.* 1992 for reviews of complex I) and has a number of distinctive features. In particular, it is sensitive to low concentrations of the poison rotenone and it translocates protons across the membrane. It is a multisubunit complex that has a minimal form of 14 subunits (Walker 1992; Weidner *et al.* 1993). In the mitochondria of eukaryotes, NDH-1 (complex I) is much larger and consist of about 35 subunits in *Neurospora crassa,* while NDH-1 in bovine heart mitochondria consists of 42 subunits, all of which have been cloned and sequenced (Chomyn *et al.* 1985; Chomyn *et al.* 1986; Fearnley *et al.* 1989; Pilkington and Walker 1989; Runswick *et al.* 1989; Dupuis *et al.* 1991a; Dupuis *et al.* 1991b; Pilkington *et al.* 1991a; Pilkington *et al.* 1991b; Skehel *et al.* 1991; Arizmendi *et al.* 1992a; Arizmendi *et al.* 1992b; Walker *et al.* 1992). NDH-1 contains 4-6 iron sulfur clusters and has a flavin mononucleotide (FMN) as the prosthetic group. NADPH is unable to be used as a substrate by NDH-1; deamino NADH can be used as a substrate but the sensitivity to rotenone may be lost (Yagi 1991). Recently an operon encoding 14 subunits of NDH-1 and 6 unidentified reading frames has been cloned and sequenced from *P. denitrificans* (Xu *et al.* 1991a; Xu *et al.* 1991b; Xu *et al.* 1992a; Xu *et al.* 1992b; Xu *et al.* 1993). An operon encoding the same 14 subunits in the same order has been cloned and sequenced from *Esherischia coli* (Weidner *et al.* 1993), but it does not contain any unidentified open reading frames.

Weiss *et al.* (1991) have proposed that NDH-1 arose as a result of modular evolution, from an *en bloc* association of pre-existing smaller complexes that had different functions. According to this theory, complete sections of the electron pathway and the mechanisms of proton translocation would have evolved independently as separate structural modules

and then come together to form complex I (Weiss *et al.* 1991; Walker 1992). This theory is supported by the findings that complex I, at least in *N. crassa*, assembles as two independent sub-complexes that aggregate to form complex I (Tuschen *et al.* 1990; Nehls *et al.* 1992). Subunits within each of these two sub-complexes have been shown to have significant homology with other simpler bacterial enzymes, further strengthening the modular theory of evolution for complex I. The module made up of the three subunits 24(FP), 51(FP) and 75(IP) in bovine complex I (Walker 1992) and NUO5, NUO6 and NUO7 in *E. coli* (Weidner *et al.* 1993), contains the binding sites for NADH, FMN and up to five FeS clusters. These three subunits show significant homology to the diaphorase part of the NAD^+-reducing hydrogenase of *Alcaligenes eutrophus,* which contains covalently bound FMN and an NADH oxidoreductase (Pilkington and Walker 1989; Runswick *et al.* 1989; Pilkington *et al.* 1991a; Pilkington *et al.* 1991b). Similarly, subunits of the second sub-complex show significant homology to a different bacterial enzyme. The 49(IP), PSST, TYKY, ND1 and ND5 subunits of bovine complex I and the NUO4, NUO2, NOU9, NUO8 and NUO12 subunits of *E. coli* complex I are related to the proteins Hyc E,G,F,D and C, respectively, of the hydrogenase 3 part of the *E. coli* formate hydrogenlyase (Weiss *et al.* 1991; Fearnley, 1992).

b) Type-2 Pyridine Nucleotide Dehydrogenases

The second class of NADH dehydrogenases have been named NDH-2. They are insensitive to rotenone and do not contain an energy coupling site (i.e. they do not translocate protons) (Anraku 1988; Yagi 1991). They do not contain iron sulfur clusters and flavin adenine dinucleotide (FAD) is the prosthetic group. They consists of a single subunit that cannot use NADPH as a substrate, but may be able to utilise deamino NADH and are inhibited by flavone (Yagi 1991).

1.3.2 Pyridine Nucleotide Dehydrogenases In Cyanobacteria

Little is known about the pyridine nucleotide dehydrogenases in cyanobacteria and there are some conflicting reports from studies on the same species as well as on different species. The evidence for pyridine nucleotide dehydrogenases in cyanobacteria has developed along two lines, biochemical studies and the cloning of genes coding for homologues of subunits of complex I.

a) Biochemical Evidence

Activity staining for NAD(P)H dehydrogenase activity has indicated that the majority of the activity is found on the thylakoid membrane but there is also some activity on the plasma membrane (Peschek *et al.* 1984; Selyakha and Gusev 1990). Initial reports indicated that there were at least four types of pyridine nucleotide dehydrogenases present in cyanobacteria, but they are not all present in one species. A number of groups provided evidence for the existence of at least two different membrane bound pyridine nucleotide dehydrogenases: one for NADH and another for NADPH (Peschek 1980b; Binder *et al.* 1984; Matthijs *et al.* 1984b; Stürzl *et al.* 1984; Molitor and Peschek 1986) and two different types of soluble pyridine nucleotide dehydrogenases, discussed below.

In *Aphanocapsa* Sandmann and Malkin (1983) identified two different pyridine nucleotide dehydrogenases, both acting as quinone oxidases. One was located in the soluble fraction of the cell free extract and was specific for NADH. It was also sensitive to rotenone, an inhibitor of NDH-1. The second was membrane bound and could utilise both NADH and NADPH as substrates, and had a lower sensitivity to rotenone than the enzyme in the soluble fraction. The membrane bound enzyme was partly purified and EPR signals were indicative of it containing an iron-sulfur centre. The partly purified enzyme had a greater affinity for NADH than NADPH, but the opposite was true in the unpurified fraction. This could indicate that there are two enzymes and the one oxidising NADH is purified to a greater degree or that there is a single enzyme that is undergoing some sort of modification during purification.

In *Microcystis aeruginosa* a pyridine nucleotide dehydrogenase was isolated from the soluble fraction after high speed centrifugation of cell extracts (Viljoen *et al.* 1985). It consisted of a non-covalently bound aggregate of 8 identical subunits. The octomer had a native molecular weight of 41 kDa and could transfer electrons from NADH and NADPH to membrane particles, with NADPH being the superior electron donor. Fluorescence spectra indicated the presence of a flavinoid, possibly FAD (Viljoen *et al.* 1985). It is possible that this enzyme has a peripheral location on the membrane and is removed during preparation of cell free extracts.

A number of researchers have suggested that FNR is the NADPH dehydrogenase on the thylakoid membrane. Houchins and Hind (1982), working on heterocysts of *Anabaena* PCC 7120, proposed that NADPH dehydrogenation was mediated by FNR directly reducing oxygen. Alpes *et al.* (1985), working on *A. variabilis*, partly purified an NADPH dehydrogenase, tentatively identified it as FNR and proposed that it fed electrons into the respiratory chain.

Scherer *et al.* (1988c) confirmed this identification after purifying thylakoid membranes from *A. variabilis*, by showing that the active fraction after purification had a K_m for NADPH of 5 μM, identical to that of purified FNR. The solubulised NADPH dehydrogenase was completely inhibited by an antibody raised against purified FNR, and $NADP^+$ affected the NADPH supported rate. The activity staining pattern of extensively washed and solubulised membranes on a native gel for NADPH was identical to that seen for FNR, and the purified enzyme was a single poylpeptide with an apparent molecular weight of 34 kDa, similar to that of purified FNR. The kinetics of NADPH oxidation by purified thylakoid membranes indicated that this was the only NADPH dehydrogenase present, as a straight Lineweaver Burk plot was obtained from kinetic studies. Work on *Anabaena* PCC 7120 indicated that FNR may be the NADPH dehydrogenase on the thylakoid membrane (Howitt *et al.* 1993) because in native gels an identical banding pattern was seen for the NADPH oxidising activity as was seen by Scherer *et al.* (1988c).

There is some controversy over this identification, as a different study showed that there was an NADPH dehydrogenase in *Anabaena variabilis*, but it was located on the plasma but not thylakoid membrane (Wastyn *et al.* 1988). This difference has not been explained but the culture conditions of Scherer *et al.* (1988c) were very different from those of Wastyn *et al.* (1988). This may have had some bearing on the result, as it has been shown for other types of bacteria that culture conditions can have a dramatic effect on the redox protein composition (Meyer and Cusanovich 1989). The membranes prepared by Wastyn *et al.* (1988) were extensively washed and this may have removed FNR from the thylakoid membrane, as Serrano *et al.* (1986) have shown that FNR can easily be removed from the thylakoid membrane of *Anabaena* PCC 7119 during preparation. Alternatively, the extensive washing may have damaged the NAD(P)H dehydrogenases, as Stürzl *et al.* (1984) have shown that this can occur in *A. variabilis*.

Work has also progressed on the identification of NADH dehydrogenase present in cyanobacteria. Alpes *et al.* (1989) purified and characterised an NADH dehydrogenase that was tightly bound to the thylakoid membrane of *A. variabilis* and used quinones as electron acceptors. The enzyme had a K_m for NADH of 22 μM and a V_{max} of 17.4 μmol min^{-1} mg^{-1}protein. The purified enzyme contained FAD covalently bound, was poorly inhibited by rotenone and consisted of one major subunit of 17 kDa and a minor subunit of 52 kDa. These are all characteristics of NDH-2. The kinetics of NADH oxidation by the thylakoid membrane indicated that this was the only NADH dehydrogenase present. Work on *Anabaena* PCC 7120 (Howitt *et al.* 1993) also indicated that the thylakoid membrane contained an NDH-2, which was poorly inhibited by rotenone and partially inhibited by flavone, while a native gel indicated there was probably only one NADH dehydrogenase on the thylakoid membrane of this species.

Berger *et al.* (1991) showed that both purified plasma and thylakoid membranes from *Synechocystis* PCC 6803 catalysed rotenone-sensitive NADH oxidation, suggesting the presence of a type-1 NADH dehydrogenase on both membranes. Further to this they showed that antibodies raised against fusion proteins containing the products encoded by *ndhK* and *ndhJ* (genes encoding homologues of subunits from complex I, the first enzyme in the respiratory chain of animal mitochondria), cross reacted with proteins found on both the plasma and thylakoid membrane, providing evidence for a multisubunit NADH dehydrogenase on both the plasma and thylakoid membranes of *Synechocystis* PCC 6803. Dzelzkalns *et al.* (1994) have also shown the presence of a polypeptide homologous to NdhK on the plasma and thylakoid membrane of *Synechocystis* PCC 6803. These results conflict with results from Ogawa (1992a), that show that the product of the *ndhI* gene is only found on the thylakoid membrane of *Synechocystis* PCC 6803.

The results indicating *Synechocystis* PCC 6803 contains an NDH-1 on either the plasma and thylakoid membrane or only the thylakoid membrane, are in contrast to the results of Howitt *et al.* (1993), showing that *Anabaena* PCC 7120 contained an NDH-1 on the plasma membrane but not on the thylakoid membrane. This identification was made using antibodies raised against isolated complex I from the red beet mitochondria and on the sensitivity of NADH oxidation by the plasma membrane to rotenone. Native gels indicated that this was the only NADH dehydrogenase on the plasma membrane and that it was not the same as the NADH dehydrogenase on the thylakoid membrane (Howitt *et al.* 1993). One interesting feature was that this NADH dehydrogenase may be a novel type, as NADPH oxidation on the plasma membrane was sensitive to rotenone and the native gel indicated that the same dehydrogenase on the plasma membrane may be responsible for NADH and NADPH oxidation. Oxidation of NADPH by bacterial type-1 NADH dehydrogenases has not been reported before, although complex I in plants can oxidise NADPH at about 15% of the NADH rate (Wiskich and Menz 1993).

Recently, part of a multisubunit enzyme (7 subunits) has been purified from *Synechocystis* PCC 6803. It was shown to contain the products encoded for by the *ndhK*, *ndhJ* and *ndhH* genes, providing further evidence for the presence of NDH-1 in cyanobacteria (Berger *et al.* 1993a). On the basis of this work Berger *et al* (1993a) proposed that NdhK may be part of a central subcomplex of the NDH-1 like complex in cyanobacteria, that is involved in holding the whole complex together. A similar model was also proposed by Weidner *et al.* (1993) as a way of explaining why NdhK is the most highly conserved of all the subunits of complex I from different species.

b) Genetic Evidence

Twelve *ndh* genes have been cloned and sequenced in cyanobacteria. Eleven of these are homologues of genes encoding subunits of complex I (Table 1.2). Most of these have been identified in *Synechocystis* PCC 6803. Steinmüller *et al.* (1989) cloned and sequenced a gene cluster encoding *ndhC-psbG-ORF157*. *psbG* has now been renamed *ndhK* (Berger *et al.* 1991; Arizmendi *et al.* 1992a; Whelan *et al.* 1992) and *ORF157* has been renamed *ndhJ* (Dupuis *et al.* 1991a). Mayes et al. (1990) identified a second copy of *psbG* from *Synechocystis* PCC 6803, but this has been shown to be inactive in wild type cells and is only activated when the first copy has been inactivated (Steinmüller *et al.* 1991). While Anderson and McIntosh (1991) identified an operon encoding *ndhE-psaC-ndhD*, in *Synechocystis* PCC 6803, probing experiments indicated that the 3' end of *ndhD* was absent from both *Synechocystis* PCC 6803 and *Anabaena* PCC 7120, leaving the question as to what its function is in cyanobacteria. Steinmüllers group have also cloned and studied the transcription of *ndhH* (Steinmüller 1992) and an operon encoding *ndh(A-I-G-E)* and *ndhD* (Ellersiek and Steinmüller 1992). The operon encoding *ndh*(*A-I-G-E*) has also been cloned and sequenced from *Plectonema boryanum* and all the genes in the operon have been shown to be single copy genes that are cotranscribed (Takahashi *et al.* 1991). The gene encoding *ndhF* has been cloned and sequenced from *Synechococcus* PCC 7002 (Schluchter *et al.* 1993). Recently a second copy of *ndhD*, named *ndhD-2*, has been cloned from *Synechocystis* PCC 6803 (Dzelzkalns *et al.* 1994). *ndhD-2* is only 54% identical to *ndhD-1* at the amino acid level.

As yet, no homologues to the three subunits thought to be involved in NADH and FMN binding (24, 51 and 75 kDa subunits of bovine complex I) have been found in cyanobacteria. An extensive study in *Synechocystis* PCC 6803, using southern blotting and PCR with degenerate primers, has failed to show the presence of homologues of the 51 and 75 kDa proteins (Funk *et al.* 1994). This has led these workers to suggest that the part of the enzyme responsible for oxidising NAD(P)H in the cyanobacterial form of NDH-1 has a different structure from that of complex I. NDH-1 in cyanobacteria must have some mechanism of NAD(P)H oxidation though, as Mi *et al* (1992a) have shown that in cells of *Synechocystis* PCC 6803 in which *ndhB* has been inactivated, NAD(P)H-mediated cyclic electron transport is lost. Suggesting that the *ndh* genes play a role in NAD(P)H oxidation in cyanobacteria. This different mechanism of NAD(P)H oxidation by cyanobacterial NDH-1 is further supported by studies on *Anabaena* PCC 7120 in which it was shown that the plasma membrane NDH-1 apparently could oxidise both NADH and NADPH, with the NADPH rate being about 20% of the NADH rate (Howitt *et al.* 1993). Bacterial and mammalian NDH-1 can only oxidise NADPH at about 2% of the NADH rate (Yagi 1991).

The *ndh* genes have also been implicated in the inorganic carbon concentrating mechanism in the cyanobacterium *Synechocystis* PCC 6803, which will be discussed in more detail in section 1.3.3. It has been shown that mutants with severe defects in this mechanism contained mutations in the *ndhB* and *ictA* genes (Ogawa 1991b; Ogawa 1991c). *ictA* has now been identified as an *ndh* gene and renamed *ndhL* (Ogawa 1992a).

1.3.3 The Role Of Pyridine Nucleotide Dehydrogenases In Cyanobacteria

The most obvious role of the pyridine nucleotide dehydrogenases in cyanobacteria is their role in respiration, as the entry point for electrons into the electron transport chain. Respiration in cyanobacteria is thought to have a number of roles. Firstly it maintains the cell's energy balance during periods of darkness and allows vital cellular processes to continue. Respiration on the plasma membrane is also thought to be involved in maintenance of the cell's osmotic balance. The proton gradient generated across the membrane would be used to actively extrude cations (eg. Na^+) from the cell. This is supported by the findings that respiration rates in whole cells and on the plasma membrane of cyanobacteria can increase up to ten fold when the cells are subjected to salt stress (Nitschmann and Peschek 1982; Fry *et al.* 1986; Jeanjean *et al.* 1993). In filamentous species of cyanobacteria that fix nitrogen, heterocysts must rely on pyridine nucleotide dehydrogenases on the thylakoid membrane to provide electrons to PS I to drive cyclic electron transport and the generation of ATP needed by nitrogenase. Respiration in heterocysts may also play a role in oxygen scavenging to ensure that nitrogenase is not inhibited by oxygen.

The NDH-1 like enzyme in cyanobacteria appears to have a number of functions besides its role in respiration, as it has been shown that the products encoded by the genes *ndhB*, *ndhC*, *ndhK* and *ndhL* are involved in the inorganic carbon concentration mechanism in *Synechocystis* PCC 6803 (Ogawa 1990; Ogawa 1991a; Ogawa 1991b; Ogawa 1991c;Ogawa 1992a). Recently the *ndhB* gene product has been shown to be involved in the inorganic carbon concentrating mechanism in *Synechococcus* PCC 7942 (Marco *et al.* 1993), but in *Synechococcus* PCC 7002 insertional inactivation of *ndhF* had no effect on the CO_2 concentrating mechanism (Schluchter *et al.* 1993). For reviews of the inorganic carbon concentrating mechanism in cyanobacteria see Badger (1987), Coleman (1991), Kaplan, (1991) and Badger and Price (1992). Ogawa (1991c) has shown that respiration is repressed in cells of *Synechocystis* PCC 6803 in which *ndhB* has been inactivated. This is also the case in *Synechococcus* PCC 7942 in which *ndhB* has been inactivated (Marco *et al.* 1993) and in *Synechococcus* PCC 7002 in which *ndhF* has been inactivated (Schluchter *et al.* 1993), indicating that the gene products are involved in respiration. Since they show homology to genes encoding subunits of complex I, they are probably

part of an NAD(P)H dehydrogenase. Not all *ndh* mutants repress the rate of respiration though. In *Synechocystis* PCC 6803, a second copy of *ndhD* has been cloned and inactivated (Dzelzkalns *et al.* 1994), and this resulted in cells in which respiration rates on the plasma membrane increased five fold. These cells contained normal amounts of NdhK on the plasma membrane, but a reduced sensitivity to rotenone. These results suggest that *ndhD-2* plays a role in regulation of the NAD(P)H dehydrogenase on the plasma membrane, but is not essential for assembly or activity of the complex.

The NDH-1 appears to play a role in cyclic electron transport around PS I in some species of cyanobacteria. Mi *et al* (1992a) have shown that a mutant in *Synechocystis* PCC 6803 in which *ndhB* has been inactivated did not show any NAD(P)H mediated cyclic electron transport, but it was present in wild type cells. Based on studies, on dark starved spheroplasts of wild type *Synechocystis* PCC 6803 and an NDH-1 defective mutant, in which the effect of addition of NADH and NADPH on chlorophyll fluorescence and the redox change of P700 was monitored Mi and co-workers (1994) have proposed that the NDH-1 mediated cyclic electron transport is an NADPH specific reaction. Similar studies on isolated thylakoid membranes, from wild type cells and an NDH-1 defective mutant, have confirmed that in *Synechocystis* PCC 6803 the NDH-1 present mediates cyclic electron transport in an NADPH specific manner (Mi *et al.* 1995 498). Mi *et al.* (1992b) have also shown that in *Synechococcus* sp. strain PCC 7002 electrons from cytosolic components were donated to the intersystem chain in an $HgCl_2$ sensitive manner, suggesting the involvement of an NDH-1. Yu *et al.* (1993) studying an *ndhF*$^-$ strain of *Synechococcus* sp. strain PCC 7002 have shown that electrons from the NADH dehydrogenase are able to reduce P700. Wild type *Anabaena* PCC 7120 did not show any NAD(P)H mediated cyclic electron transport (Mi *et al.* 1992b) and thus it would appear that the NDH-1 like enzyme in *Anabaena* PCC 7120 has a different function from that in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7002. This difference is consistent with the different location of the NAD(P)H dehydrogenase in *Anabaena* PCC 7120 and *Synechocystis* PCC 6803. NDH-1 is on the plasma membrane in *Anabaena* PCC 7120 (Howitt *et al.* 1993) and on the thylakoid membrane in *Synechocystis* PCC 6803 (Berger *et al.* 1991; Ogawa 1992a; Dzelzkalns *et al.* 1994) as well as on the plasma membrane.

1.4 EVOLUTION OF CHLOROPLASTS

The idea that chloroplasts evolved from an endosymbiotic cyanobacteria was first proposed in 1883 by Schimper (cited in Whatley (1993)). This was based on the

observations that chloroplasts and cyanobacteria had essentially similar structure and function, and that chloroplasts never arose *de novo* but, like cyanobacteria, reproduced by fission. The hypothesis was extended to include mitochondria evolving from an endosymbiotic bacteria (Mereschowsky 1905, 1920) (cited in Whatley (1993)). With the discovery of DNA in mitochondria and chloroplasts (Ris and Plaut 1962) the theory was revived. This renewed interest culminated in the publication of "The Origin of the Eukaryotic Cell" (Margulis 1970) in which the endosymbiotic theory of evolution of chloroplasts and mitochondria was restated. It is now believed that cyanobacteria were the ancestors of chloroplasts, for reviews of the literature see Gray and Doolittle (1982), Gray (1988), Gray (1989) and Whatley (1993). The question now is whether chloroplasts had a monophyletic or polyphyletic origin.

As chloroplasts evolved from an ancestral cyanobacterium it would be expected that chloroplasts would show a number of features similar to those of cyanobacteria, one of these is that chloroplasts also contain genes that encode for proteins homologous to subunits of complex I and may contain a type-1 NADH dehydrogenase (Meng *et al.* 1986; Ohyama *et al.* 1986; Shinozaki *et al.* 1986; Fearnley *et al.* 1989; Dupuis *et al.* 1991a; Pilkington *et al.* 1991b; Arizmendi *et al.* 1992a).

1.4.1 Evidence for a Chlororespiratory NAD(P)H Dehydrogenase in Chloroplasts

The presence of a respiratory chain (oxidative electron transport in the thylakoids) in the chloroplast was first suggested by Goedheer (1963). Since 1980 there has been a growing body of biochemical evidence to support this claim, that complements the molecular evidence discussed below. First it was shown that NADH could act as an electron donor to the photosynthetic membrane of *Chlamydomonas reinhardtii* in a rotenone-sensitive manner, but relatively high concentrations of rotenone were required to achieve inhibition (Godde and Trebst 1980). Godde (1982) partly purified an NADH plastoquinone oxidoreductase from the thylakoid membrane of *C. reinhardtii* and the spectral properties indicated it contained a flavoprotein and probably contained iron-sulfur clusters. Bennoun (1982) demonstrated the generation of an electrochemical gradient across the thylakoid membrane in the dark; by using a mutant deficient in PS I it was shown that this electrochemical gradient was not due to the action of the Mehler reactions or photorespiration. He called this process chlororespiration. In *C. reinhardtii* the dark oxidation of the plastoquinone pool was inhibited by high concentrations of cyanide and azide, but not by inhibitors of the alternative oxidase (i.e. SHAM and n-PG) of plant mitochondria, while in *Chlorella pyrenoidosa* the dark oxidation of the plastoquinone pool was inhibited by SHAM, but not by cyanide, indicating the terminal oxidase for

chlororespiration varies from one organism to another (Bennoun 1982). It has been shown that plastoquinone is the only common link between photosynthesis and chlororespiration in *C. reinhardtii* and that there is probably a coupling site between NADH/NADPH and plastoquinone (Bennoun 1983). Chlororespiration in *C. reinhardtii* is thought to be a plastoquinone-mediated pathway that is involved in the degradation of starch (Gfeller and Gibbs 1985). Peltier *et al.* (1987) demonstrated that there was a light dependent inhibition of chlororespiration in *C. reinhardtii*. Willeford *et al.* (1989) showed that isolated chloroplasts of *C. reinhardtii* did not exhibit cytochrome oxidase activity and succinate could also act as a donor to chlororespiration.

It has been shown that in nitrogen-limited cells the level of NADH plastoquinone oxidoreductase activity in chloroplasts increases (Peltier and Schmidt 1991). The level of two novel cytochromes, H_1 and H_2, were also shown to increase at the same time as the NADH plastoquinone oxidoreductase activity. Ravenal and Peltier (1991) showed that antimycin A and myxothiazol inhibited chlororespiration. The last two results have been brought into question recently as H_1 and H_2 have been shown to be due to mitochondrial contamination (Atteia *et al.* 1992). Bennoun (1994) has shown that the effects of myxothiazol were due to inhibition of mitochondrial respiration as mutants whose mitochondria are resistant to myxothiazol no longer show the effect Ravenal and Peltier (1991) saw. He postulated that the effect seen on addition of myxothiazol was due to a specific interaction between the chloroplasts and mitochondria.

Most of the evidence for chlororespiration has come from the eukaryotic algae *C. reinhardtii*, but recently evidence for chlororespiration in plant chloroplasts has emerged. Oxidation of the plastoquinone pool in the dark in tobacco chloroplasts has been demonstrated (Garab *et al.* 1989) and recently chlororespiration has been implicated in the aerobic degradation of carbohydrates in intact chloroplasts of spinach and *C. reinhardtii* (Singh *et al.* 1992). Donation of electrons to the intersystem chain from stromal components has been demonstrated in both spinach and tobacco (Asada *et al.* 1992; Asada *et al.* 1993). Using oxygen stable isotope discrimination Gruszecki *et al.* (1994) have demonstrated the presence of light dependant oxygen uptake in the chloroplasts of tobacco, which they attributed to chlororespiration. This is the first direct evidence for an oxidase in the chloroplasts of higher plants or eukaryotic algae. They also concluded that the plastoquinone pool was the component shared between the photosynthetic and chlororespiratory electron transport chains.

Higher plant chloroplasts are now known to contain 11 genes in their genome that encode for proteins homologous to subunits of complex I. Seven of these are homologous to the seven subunits of complex I encoded for by the mitochondrial genome, they are *ndhA, ndhB, ndhC, ndhD, ndhE, ndhF and ndhG*, they have been found in liverwort (Ohyama *et al.* 1986), tobacco (Shinozaki *et al.* 1986), rice (Hiratsuka *et al.* 1989) and a number of

other species (Meng *et al.* 1986). Matsubayashi (1987) has shown that these genes are not pseudo-genes as they are transcribed in tobacco. *ndhC-K-J* have also been shown to be transcribed in maize (Schantz and Bogorad. 1988; Steinmüller *et al.* 1989). Vera *et al.* (1990) have shown that the *ndh* genes are expressed during senescence in barley leaves, but poorly expressed during chloroplast biogenesis. Four genes encoding subunits homologous to nuclear encoded subunits of mitochondrial complex I have also been identified in chloroplast genomes. They are homologues to the 49 kDa (IP) subunit named *ndhH* (Fearnley *et al.* 1989), the 30 kDa (IP) subunit named *ndhI* (Pilkington *et al.* 1991b), TYKY a 23 kDa subunit named *ndhJ* (Dupuis *et al.* 1991a) and PSST a 20 kDa subunit named *ndhK* formerly *psbG* (Arizmendi *et al.* 1992a). TYKY contains two iron-sulfur binding motifs (Dupuis *et al.* 1991a) and PSST contains three conserved cysteine residues which may be involved in binding an iron-sulfur cluster (Arizmendi *et al.* 1992a). These genes are arranged in two operons which encode *ndh H-A-I-G-E* and *D* and *ndh C-K-J*, with the other two genes *ndh*B and *ndhF* being encoded elsewhere in the genome. This organisation of the *ndh* genes is identical to that seen in cyanobacteria. It is unknown if these genes encode a functional NAD(P)H dehydrogenase as no genes encoding homologues of the subunits thought to be involved in the binding of NADH and FMN (75 kDa, 51 kDa and 24 kDa) have been identified in chloroplast genomes (Walker 1992). These are the same three subunits that have not been found in cyanobacteria and it may be that the chloroplast enzyme is similar to the cyanobacterial enzyme and has a different mechanism for NAD(P)H oxidation than complex I. Alternatively these three genes may be nuclear encoded and their products imported into the plastid.

In the parasitic flowering plant *Epifugus virginiana* which has completely lost the ability to perform photosynthesis, all the *ndh* genes have been lost from the chloroplast genome along with those genes encoding for proteins involved in photosynthesis (dePamphilis and Palmer 1990). This suggests that the role of the *ndh* genes is in some way linked to photosynthesis, and it may be via chlororespiration. Recently it has been shown that the *ndh* genes have also been lost from the chloroplast genome of another parasitic plant, *Cuscuta reflexa* (Haberhausen and Zetsche 1994). However the photosynthetic genes studied are apparently present in a functional form in *C. reflexa* (Haberhausen *et al.* 1992). This difference between *E. virginiana* and *C. reflexa* may be due to their different mode of parasitism. *E. virginiana* is a root parasite and is not reliant on photosynthesis for survival while *C. reflexa* is a stem parasite and may require photosynthesis during the seedling stage. Alternatively *E. virginiana* may represent a more advanced evolutionary stage of parasite than *C. reflexa* and the *ndh* genes are lost first before the photosynthetic genes. The actual role, if any, of the *ndh* genes in chloroplasts is further confused by the recent finding that the chloroplast genome of *Pinus thunbergii* has also lost the *ndh* genes. Four of the genes have been lost while the other seven are obvious pseudo genes, with many in-frame stop codons (Wakasugi *et al. 1994*). This opens the question as to

whether the *ndh* genes are needed, for a functional chloroplast, or is it only in *P thunbergii* that they are not essential. Alternatively the *ndh* genes may have been transferred to the nucleus in *P thunbergii* and their products imported into the plastid. Recently it has been shown that the *ndh* genes are also absent from the chloroplast genomes of the red algae *Porphrya purpurea* (Reith and Munholland 1994) and from the cyanelles of *Cyanophora paradoxa* (Stirewalt *et al.* 1995).

1.4.2 Function of NADH Dehydrogenases in Chloroplasts.

Two functions have been proposed for the putative chloroplast NAD(P)H dehydrogenase. Firstly it has been proposed that the NAD(P)H dehydrogenase may coordinate the initiation of DNA replication with available reducing power *in situ*, as NdhI has been shown to bind to the replicative origin of chloroplast DNA (Wu *et al.* 1989). Secondly it may function in chlororespiration. The function of chlororespiration itself is unknown, but a number of theories have been put forward:

a) Chlororespiration uses reducing equivalents produced by starch degradation to regenerate electron acceptors for triose phosphate formation (Bennoun 1982).

b) Chlororespiration allows more efficient carbon dioxide fixation on re-illumination by poising the reduction level of the photosynthetic apparatus under aerobic or microaerobic conditions, resulting in improved growth and survival under extreme conditions (Maione and Gibbs 1986).

c) Chlororespiration keeps the ATPases in functional form by maintaining the proton gradient across the thylakoid membrane thus allowing more efficient ATP production on reillumination (Peltier *et al.* 1987).

d) Chlororespiration contributes to acetate metabolism in the dark (Peltier *et al.* 1987).

e) Chlororespiration is a way of dissipating energy when reducing power is high (Ravenal and Peltier 1992).

f) Chlororespiration through the activity of the NAD(P)H dehydrogenase may act as a regulators for the coordination between photosynthetic electron flow and ATP and NADPH production (Berger *et al.* 1993b).

g) If chlororespiration generates NADP it would aid in the protection against photoinhibiton by serving as a sink for photosynthetically-generated reducing equivalents (Fork and Herbert 1993).

h) Chlororespiration may play a role in controlling state transitions (Bulte *et al.* 1990).

As yet the presence of a complex I like enzyme in the thylakoid membrane has not been demonstrated. The genomes of chloroplasts contain eleven *ndh* genes, of which the proteins encoded by two, *ndhK* and *ndhH*, have been shown to occur on the stroma lamella through immunological studies (Nixon *et al.* 1989; Berger *et al.* 1993b). An NADH-plastoquinone oxidoreductase from *C. reinhardtii* chloroplasts has been partially purified, it was partially sensitive to rotenone and had spectral properties indicating it contained a flavoprotein and probably iron-sulfur clusters (Godde 1982). Taken together these findings suggest that there is a complex I like enzyme in the thylakoid membrane of chloroplasts, but it has not been isolated.

1.5 AIMS AND RATIONALE OF THE PROJECT

The aim of this project was to investigate NAD(P)H dehydrogenases in cyanobacteria and the chloroplasts of higher plants (soybean). In cyanobacteria the aim was to further characterise the apparent difference in the cellular location of NDH-1 in *Anabaena* PCC 7120 and *Synechocystis* PCC 6803. This difference in the cellular location of NDH-1 was confirmed and studies were carried out to try and characterise the function of NDH-1 in *Anabaena* PCC 7120 in order to compare its function in with that of NDH-1 in *Synechocystis* PCC 6803. In order to do this *ndhK* from *Anabaena* PCC 7120 was cloned and sequenced. Constructs to inactivate *ndhK* were made and *Anabaena* PCC 7120 was transformed with them. As *ndhK* is thought to encode a central subunit of complex I involved in holding the complex together, inactivation of *ndhK* should inactivate the whole complex and thus impair any function of NDH-1. Therefore by characterising any transformants produced and comparing them with wild type the function of NDH-1, in *Anabaena* PCC 7120, can be elucidated.

In chloroplasts the aim was to demonstrate the presence of a rotenone-sensitive NAD(P)H dehydrogenase, as one has been shown to be present in the chloroplasts of the eukaryotic algae *C. reinhardtti*, but not in the chloroplasts of higher plants. If such an enzyme was found different chloroplast fractions would be isolated and assayed in order to determine the location of the enzyme within the chloroplast.

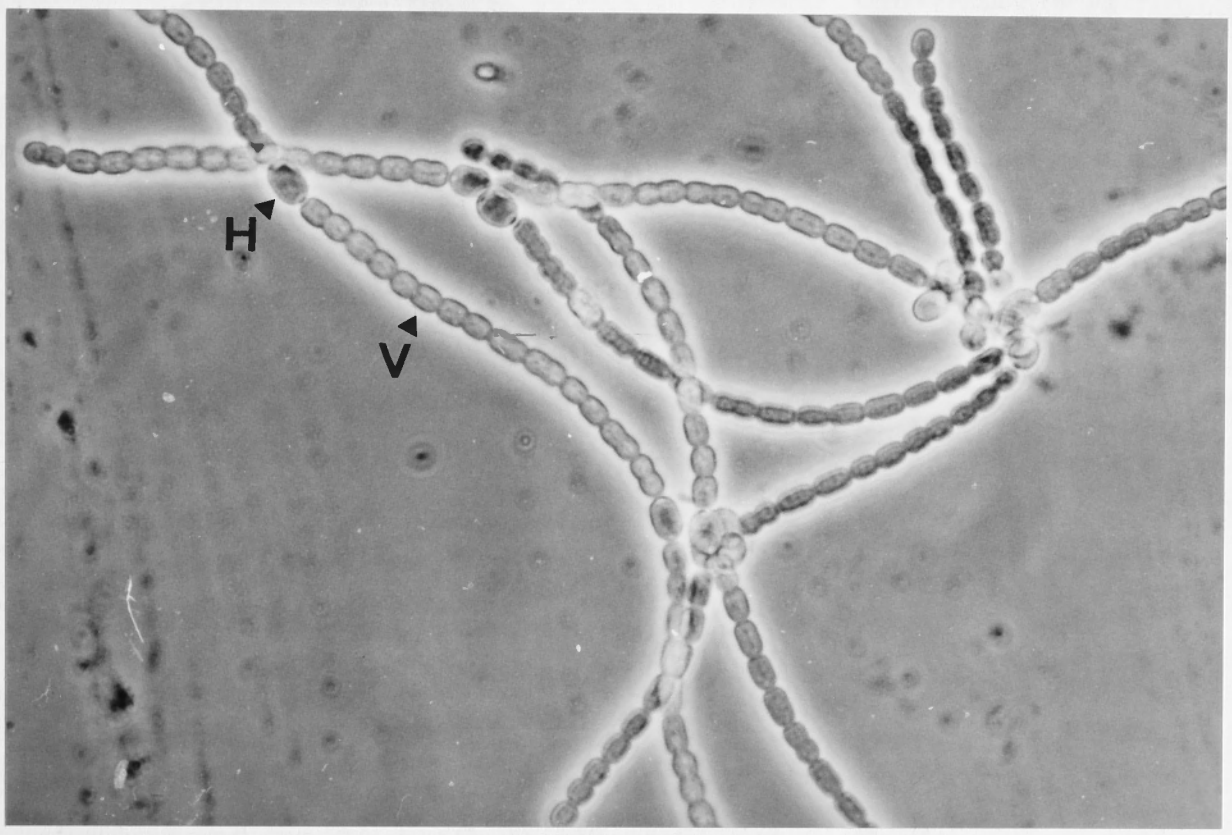


Figure 1.1 : Whole Filaments of *Anabaena* PCC 7120.

V = Vegetative cell

H = Heterocyst

Anabaena variabilis M3

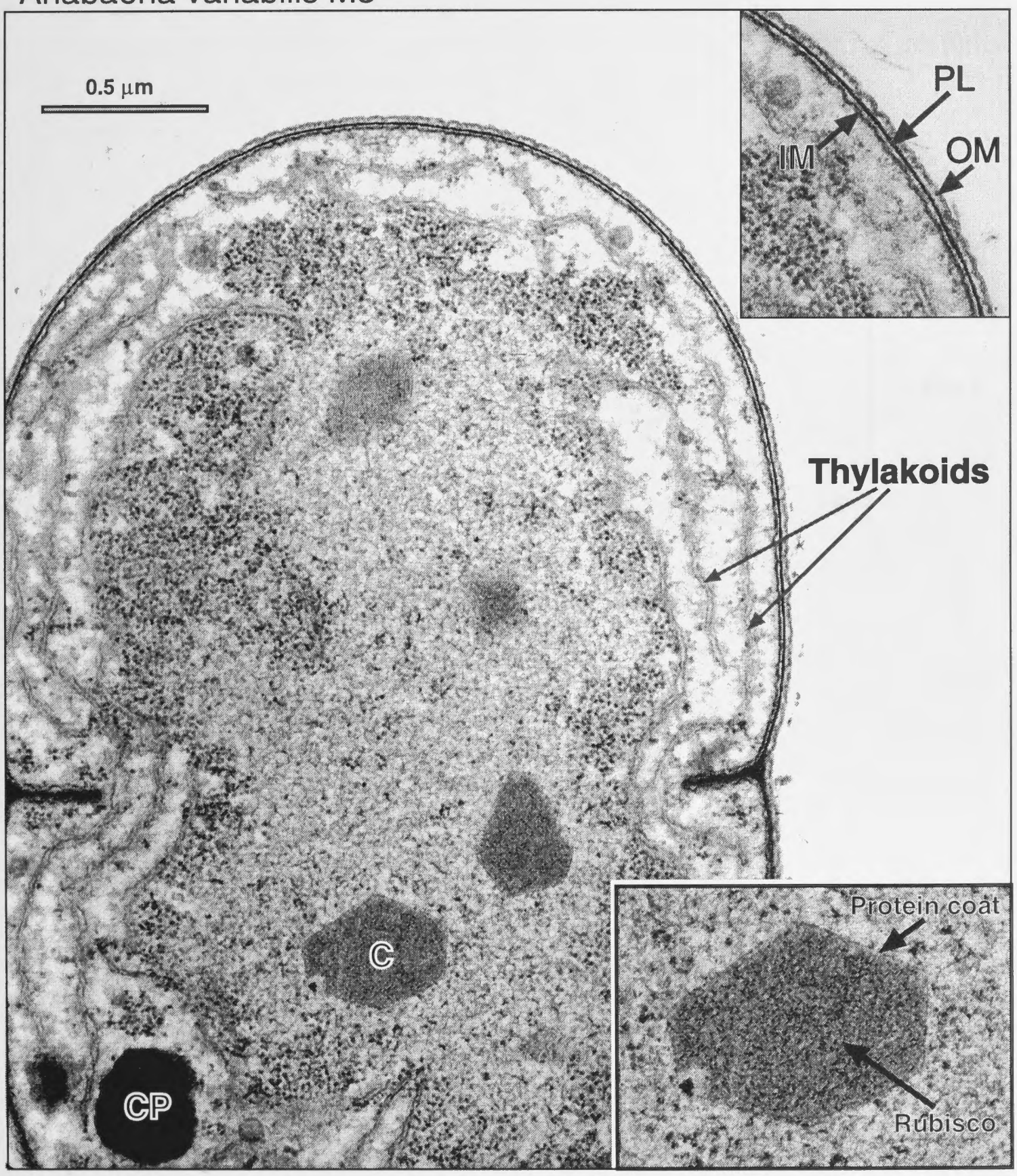


Figure 1.2: Ultrastructure of a vegetative cell of *Anabaena variabilis* M3

Abbreviations: OM = outer membrane, PL = plasma lemma, IM = inner membrane, C = carboxysome, CP = cyanophycin.

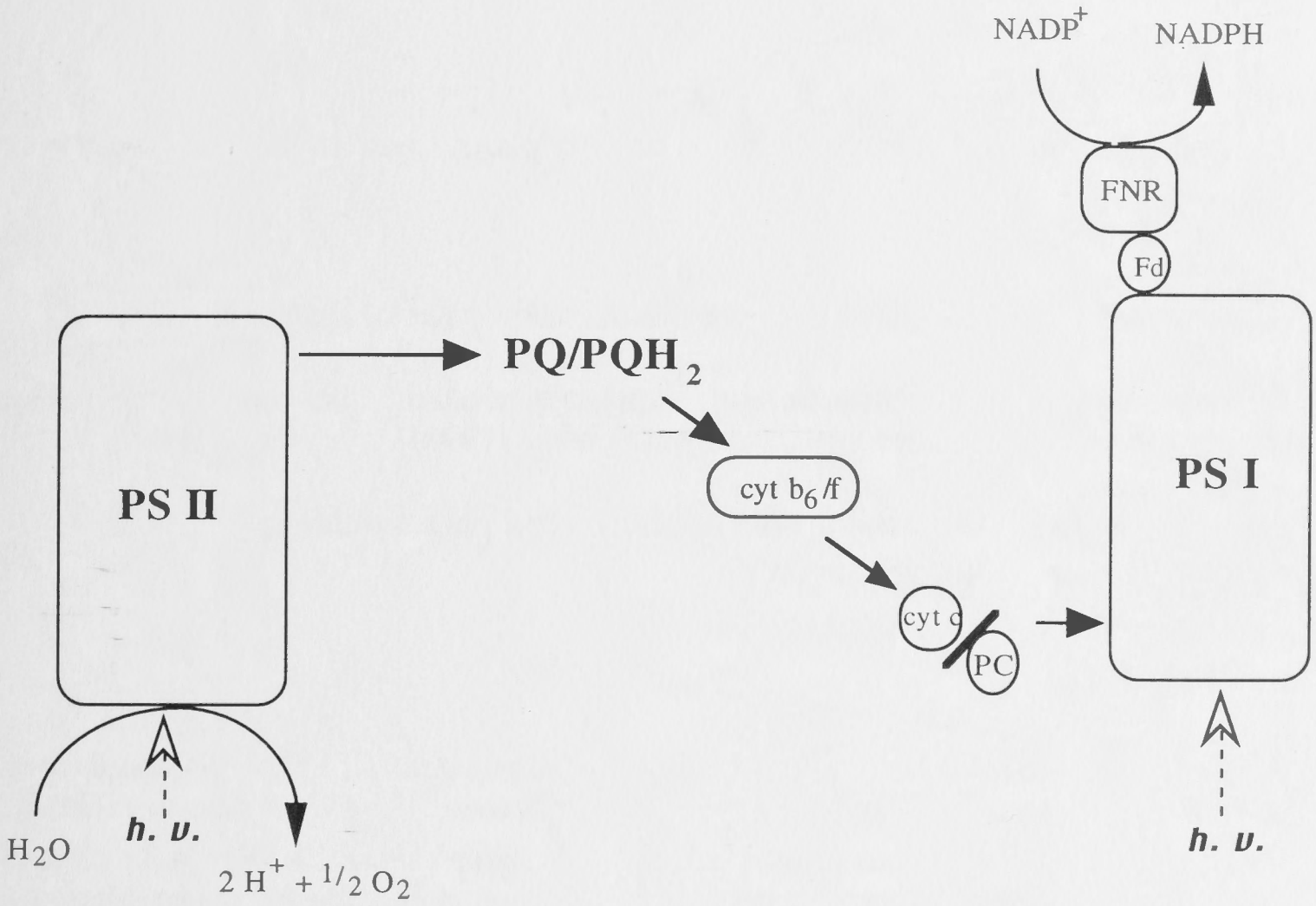


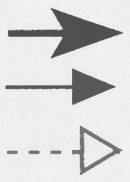
Figure 1.3: Photosynthetic Electron Transport Chain in Cyanobacteria.
(Scherer, 1990)

Abbreviations: PSI = Photosystem I, PS II= Photosystem II, PQ = plastoquinone, cyt b_6/f, = cytochrome b_6/f complex, cyt c = cytochrome c, PC = plastocyanin, Fd = ferredoxin, FNR = Ferredoxin $NADP^+$ oxidoreductase.

Figure 1.4: Electron transport chains on the plasma and thylakoid membranes of cyanobacteria

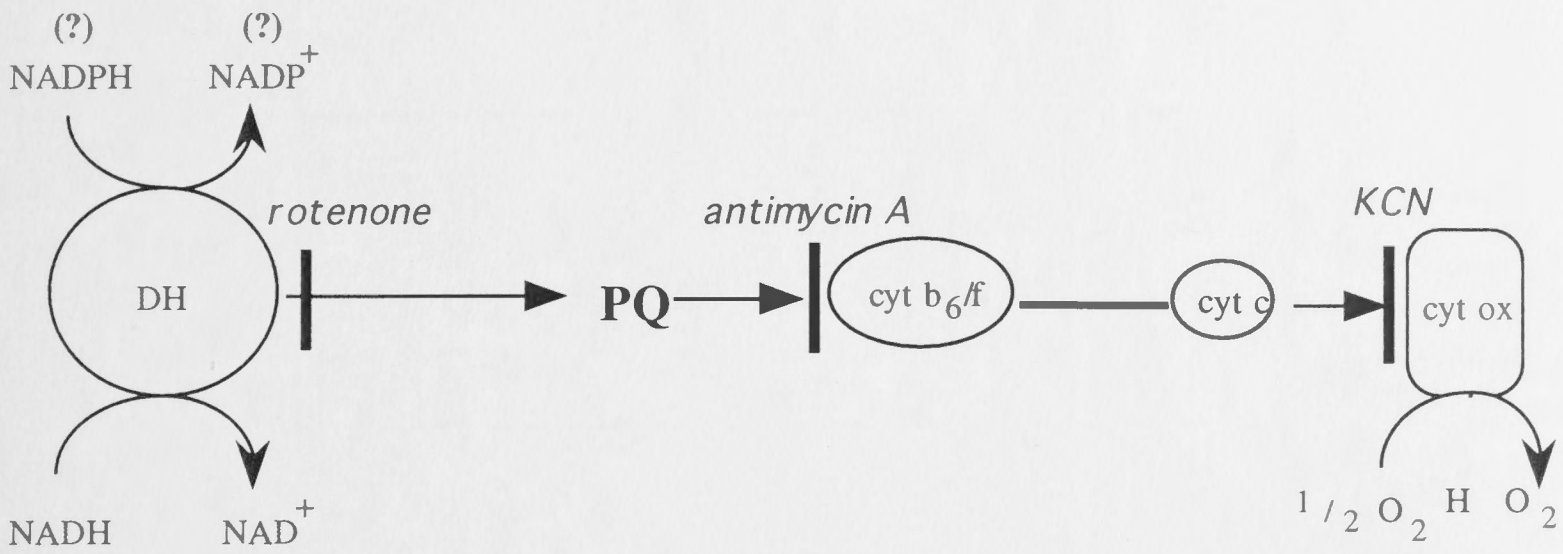
A. Respiratory electron transport on the plasma membranes of cyanobacteria

B. Interaction of photosynthetic and respiratory electron transport on the thylakoid membrane of cyanobacteria (Adapted from Scherer (1990))

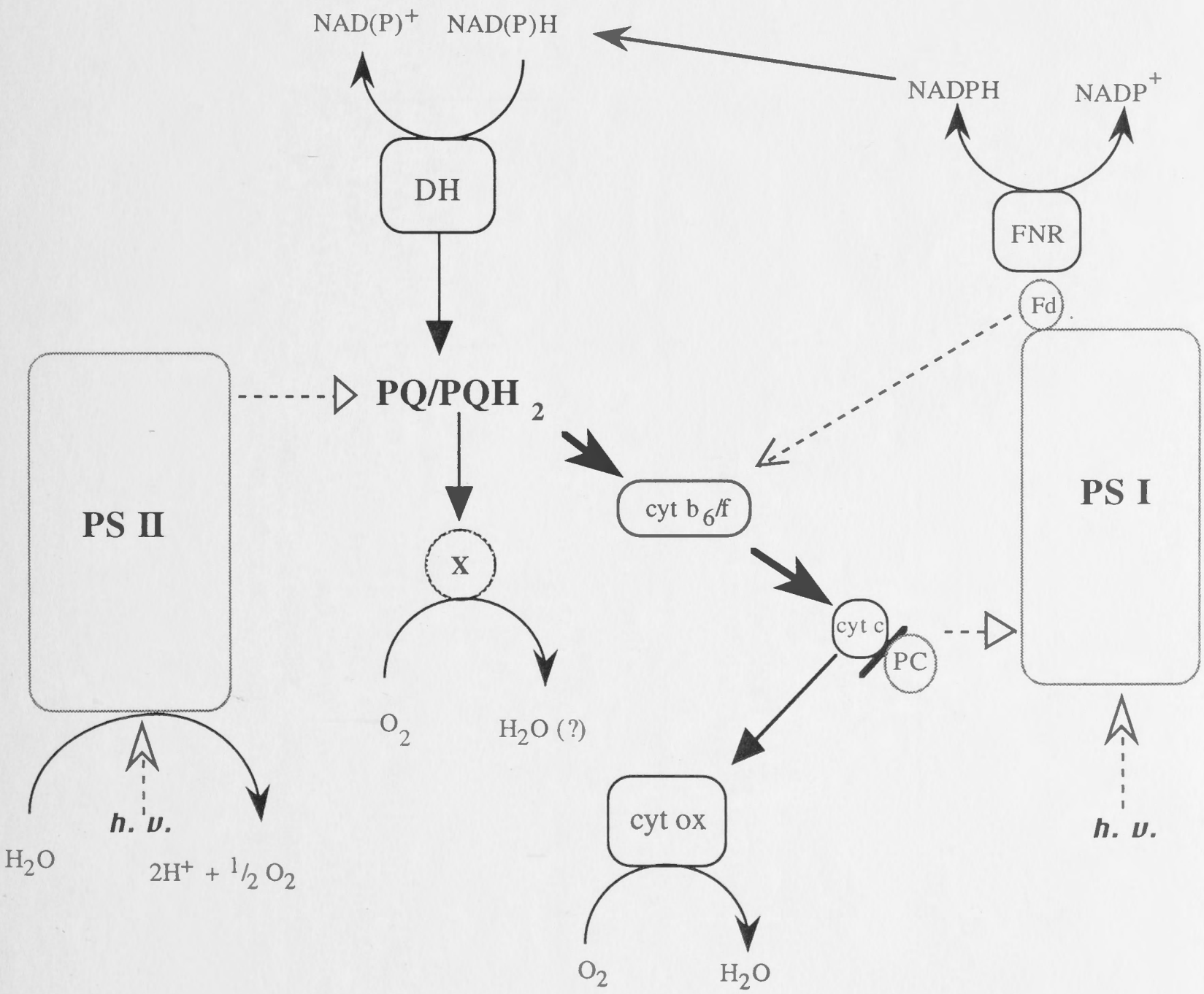


Abbreviations: PSI = Photosystem I, PS II= Photosystem II, PQ = plastoquinone, cyt b_6/f, = cytochrome b_6/f complex, cyt c = cytochrome c, PC = plastocyanin, Fd = ferredoxin, FNR = Ferredoxin $NADP^+$ oxidoreductase, DH = NAD(P)H dehydrogenase, cyt ox = cytochrome oxidase, X cause of cyanide insensitive respiration.

A.


(?)
NADPH
(?)
NADP+
rotenone
antimycin A
KCN
DH
PQ
cyt b6/f
cyt c
cyt ox
NADH
NAD+
1/2 O2 H O2

B.


NAD(P)+
NAD(P)H
NADPH
NADP+
DH
FNR
Fd
PQ/PQH 2
PS II
PS I
X
cyt b6/f
cyt c
PC
O2
H2O (?)
h. υ.
H2O
2H+ + 1/2 O2
cyt ox
O2
H2O
h. υ.

References [1] (Weidner *et al.* 1993), [2](Anderson *et al.* 1982), [3](Chomyn *et al.* 1985), [4](Arizmendi *et al.* 1992a), [5](Pilkington *et al.* 1991b), [6](Fearnley *et al.* 1989), [7](Pilkington and Walker 1989), [8](Pilkington *et al.* 1991a), [9](Runswick *et al.* 1989),[10](Dupuis *et al.* 1991a), [11](Xu *et al.* 1992), [12](Xu *et al.* 1991a), [13](Xu *et al.* 1991b), [14](Xu *et al.* 1992), [15](Xu *et al.* 1993), [16](Steinmüller *et al.* 1989), [17](Steinmüller 1992), [18](Ellersiek and Steinmüller 1992), [19](Dzelzkalns *et al.* 1994), [20](Ogawa 1991c), [21](Ogawa 1990), [22](Ogawa 1992a), [23](Schluchter *et al.* 1993), [24](Marco *et al.* 1993), [25](Takahashi *et al.* 1991), [26](Shinozaki *et al.* 1986), [27](Ohyama *et al.* 1986), [28](Hiratsuka *et al.* 1989), [29](Videira *et al.* 1990), [30](Wu *et al.* 1989)

(IP) iron-sulphur protein fraction.
(FP) flavoprotein fraction.

[a] *Synechocystis* PCC 6803 contains 2 copies of *ndhK* (Mayes *et al.* 1990; Steinmüller and Bogorad 1990) the second gene is a cryptic gene located on a plasmid.

[b] originally called *psbG* and has been renamed *ndhK* (Berger *et al.* 1991; Arizmendi *et al.* 1992a; Whelan *et al.* 1992).

[c] a region containing*ndhE* and a truncated version of *ndhD* has also been sequenced from *Synechocystis* PCC 6803 (Anderson and McIntosh 1991).

[d] *ndhL* from *Synechocystis* PCC 6803 contains no significant homology to any sequence in the database at either the DNA or protein level.

[e] *ndh(H-I-J-K)* in chloroplasts were identified by their homology to sequences encoding subunits of Bovine complex I.

[f] Sequence from tobacco chloroplast genome.

[g] Sequence from Marchantia chloroplast genome.

[h] Sequence from rice chloroplast genome.

Table 1.2 Names for genes encoding the same subunits of complex I from different organisms, using the genes for the minimal form of the complex from *E.coli* as the reference genes.

Escherichia coli	*Bos taurus* mitochondria	*Paracoccus denitrificans*	Cyanobacteria				Chloroplasts [e]
			Synechocystis PCC 6803	*Synechococcus* PCC 7002	*Synechococcus* PCC 7942	*Plectonema boryanum*	
nuo1[1]	ND3[2,3]	*nqo7*[11]	*ndhC*[16]				*ndhC*[26f,27g,28h]
nuo2[1]	PSST[4]	*nqo6*[11]	*ndhK*[16 a b]				*ndhK*[4]
nuo3[1]	30(IP)[5]	*nqo5*[11]	*ndhJ*[16]				*ndhJ*[5,29]
nuo4[1]	49(IP)[6]	*nqo4*[11]	*ndhH*[17]				*ndhH*[6]
nuo5[1]	24(FP)[7]	*nqo2*[12]					
nou6[1]	51(FP)[8]	*nqo1*[13]					
nuo7[1]	75(IP)[9]	*nqo3*[14]					
nuo8[1]	ND1[2,3]	*nqo8*[15]	*ndhA*[18]			*ndhA*[25]	*ndhA*[26,27,28]
nuo9[1]	TYKY[10]	*nqo9*[15]	*ndhI*[18]			*ndhI*[25]	*ndhI*[10,30]
nuo10[1]	ND6[2,3]	*nqo10*[15]	*ndhG*[18]			*ndhG*[25]	*ndhG*[26,27,28]
nuo11[1]	ND4L[2,3]	*nqo11*[15]	*ndhE*[18 c]			*ndhE*[25]	*ndhE*[26,27,28]
nuo12[1]	ND5[2,3]	*nqo12*[15]		*ndhF*[23]			*ndhF*[26,27,28]
nuo13[1]	ND4[2,3]	*nqo13*[15]	*ndhD-1*[18 c]				*ndhD*[26,27,28]
			ndhD-2[19]				
nuo14[1]	ND2[2,3]	*nqo14*[15]	*ndhB*[20]		*ndhB*[24]		*ndhB*[26,27,28]
			ndhL[21,22 d]				

CHAPTER 2
MATERIALS AND METHODS

2.1 REAGENTS AND MATERIALS PROVIDED

2.1.1 Reagents

Reagents used for the biochemical work were from Sigma Australia and reagents for the molecular work were from Sigma Australia or Boehringer Mannheim Australia unless otherwise stated. Enzymes used in the molecular studies were from Boehringer Mannheim unless otherwise stated.

2.1.2 Materials Provided

Soybean mitochondria were kindly provided by A. Harvey Millar (Division of Biochemistry and Molecular Biology, School of Life Sciences, Australian National University, Canberra, Australia). Polyclonal antibodies against the F_1 portion of the F_1F_0 ATPase from spinach thylakoid were raised in rabbits and kindly provided by Dr Paul Whitfield (Division of Plant Industry, CSIRO, Canberra, Australia). Polyclonal antibodies raised against Glutathione-S-Transferase (GST) in rabbits, were kindly provided by Dr G. Dean Price (Research School of Biological Sciences, Australian National University, Canberra, Australia). Ubiquinone-1 was a gift from Dr Joseph. T. Wiskich (Department of Botany, University of Adelaide, Adelaide, Australia). Purified thylakoid and envelope membranes from spinach chloroplasts were generous gift from Dr Jacques Joyard (Laboratoire de Physiologie Cellulaire Végétale, Department de Biologie Moléculaire et Structurale, Centre d'etudts Nucléaires de Grenoble and Université Joseph Fourier, France). The operon encoding *ndhC-K-J* from *Synechocystis* PCC 6803 was kindly provided by Dr Klaus Steinmüller (Institut für Entwicklungs und Molekularbiologie der Pflazen, Heinrich-Heine-Universität Universitätsstraße 1, Düsseldorf, Deutschland). The plasmids for transformation of *Anabaena* PCC 7120, pRL25, pRL271, pRL443, and pRL528, were kindly provided by Dr Geoff D. Smith (Division of Biochemistry and Molecular Biology, School of Life Sciences, Australian National University, Canberra, Australia)

2.2 GROWTH OF CYANOBACTERIA AND PLANTS

2.2.1 Cyanobacteria

Anabaena PCC 7120 was grown in one-eighth strength (Allen and Arnon 1955) medium, containing full strength phosphate and four times the specified amount of nickel (see Appendix A for final concentrations used). Liquid cultures were sparged continuously

with air or 5% CO_2 (v/v) in air that was passed through an 0.22 µm filter before entering the culture. Plates (1% agar) were grown in air, or in sealed clear topped boxes through which 5% CO_2 (v/v) in air was passed. Liquid cultures were grown in bottles (60 ml, 2 l and 10 l), continuously stirred at 25°C and subjected to 24 h light or a 12 h light/dark cycle. The light intensity at the surface of the culture bottle was 150 microeinsteins m^{-2} s^{-1}. Cells were harvested during mid logarithmic growth phase (see Appendix B for growth curve). The concentration of the cells was measured using a Klett-Summerson colourimeter (Malette 1969). Cells grown on the light/dark cycle were always harvested at the end of the 12 h light cycle. The purity of the cultures was checked using phase contrast microscopy, and only cultures of the highest purity were used.

Synechocystis PCC 6803 was grown under identical conditions to *Anabaena* PCC 7120, except that BG-11 (Allen 1968) was the growth media used

2.2.2. Soybeans

Soybean (*Glycine max* {L.} cv. Stevens) plants were propagated in trays of vermiculite in a growth cabinet (16 h light period, 28°C). Leaves were harvested after 12-14 days of growth and used for preparation of thylakoids and subthylakoidal particles. Plants used for envelope preparation were grown in naturally illuminated glasshouses in pots (30 cm) of sand. Plants were watered, on alternate days, with either water or a 1 in 4 dilution of Herridge solution (Herridge 1977; Delves *et al.* 1986). Leaves used for preparations of envelope membranes were harvested 5-6 weeks after planting.

2.3 PREPARATION OF CELLULAR FRACTIONS

2.3.1 Total Membranes from Cyanobacteria

These were prepared by a modified method of Houchins and Hind (1982). Filaments were harvested (10 l) by rapid self-sedimentation (allowed to settle for 1 h). The excess medium was siphoned off and the cells centrifuged (4,000 g, 10 min, 4°C). The pellet was washed in 300 ml of 40 mM HEPES-NaOH (pH 7.5), 10 mM Na_2EDTA buffer and centrifuged as above. The pellet was resuspended in 40 ml of 40 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$, 300 mM mannitol, and 2 mg ml^{-1} lysozyme, and incubated at 30°C for 30 min (to disrupt the vegetative cells while leaving the heterocysts intact). After the incubation the solution was passed through a chilled French pressure cell at 40 MPa

and centrifuged (2,000 g, 5 min, 4°C) to remove the heterocysts. The supernatant was centrifuged (300,000 g, 1 h, 4°C) and the pellet was resuspended in a minimum volume of 40 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$, 300 mM mannitol and assayed as total membranes of vegetative cells.

2.3.2 Isolation of Plasma Membranes and Thylakoid Membranes from Cyanobacteria

Plasma membranes and thylakoid membranes were isolated by the method of Murata and Omata (1988). Cultures (10 l) were harvested as above and the cells weighed. Each 3 g of cells (wet weight) were washed with 5 mM TES-NaOH (60 ml, pH 7.0), and resuspended in 10 mM TES-NaOH (30 ml, pH 7.0) containing 600 mM sucrose, 2 mM Na_2 EDTA, and 0.03 % lysozyme. The cell suspension was incubated for 2 h at 30°C under room light. The lysozyme treated cells were collected by centrifugation (5,000 g, 10 min) and washed twice with 20 mM TES-NaOH (pH 7.0), containing 600 mM sucrose, by resuspension and centrifugation. All of the above steps were carried out at room temperature, and subsequent steps were carried out on ice.

The pelleted cells were suspended in 20 mM TES-NaOH (30 ml, pH 7.0), containing 600 mM sucrose and passed through a chilled French pressure cell at 40 MPa. 10 mM sodium acetate (0.05 ml, pH 5.6) containing 1 mM $MgCl_2$, and 0.1 ml of 3% phenylmethylsulfonyl fluoride (PMSF) in methanol was added to the homogenate. This was incubated for 15 min and then centrifuged (5,000 g, 10 min) to remove unbroken cells.

The supernatant was made up to a concentration of 50% sucrose (w/v) by adding 0.74 volumes of 90% (w/v) sucrose solution containing 10 mM TES-NaOH (pH 7.0), 5 mM Na_2 EDTA, 10 mM NaCl and 1 mM PMSF. A 17 ml aliquot was placed in the bottom of a 35 ml centrifuge tube. This was overlaid with 8 ml of 39%, 3 ml 30%, and 7 ml 10% sucrose solutions (w/v), each containing 10 mM TES-NaOH (pH 7.0), 5 mM Na_2 EDTA, 10 mM NaCl and 1 mM PMSF. The gradients were centrifuged (130,000 g, 16 h, 4°C) in a swinging bucket rotor, to separate the membrane fractions. The plasma membranes (yellow) formed a band in the 30% sucrose layer and the thylakoid membranes (green) formed a band at the interface of the 39% and 50% sucrose layers (see Appendix C for a schematic representation).

The plasma membranes and thylakoid membranes were withdrawn from the gradient with a Pasteur pipette and diluted 3 fold with 10 mM TES-NaOH (pH 7.0), 10 mM NaCl, and collected by centrifugation (300,000 g, 1 h, 4°C). The pellets were resuspended in a

minimum volume of 10 mM TES-NaOH (pH 7.0). The purity of the plasma membranes was determined by the level of detectable chlorophyll.

2.3.3 Isolation of Thylakoid Membranes from Soybean

Thylakoid membranes from soybeans were isolated by the method of Yu and Woo (1988). All steps were carried out at 4°C using chilled solutions and apparatus. Approximately 30 g of leaves from 12-15 day old soybean seedlings were chilled and homogenised, 3 times for 1 second at power setting 6, in 250 ml of chloroplast isolation medium (0.33 M sorbitol, 10 mM NaCl, 2 mM Na_2EDTA, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM MES, 0.1% (w/v) BSA, just prior to use ascorbate was added to 10 mM and the pH adjusted to 6.1 using KOH) using a Polytron blender (Kinematica GmbH Switzerland) fitted with a PG35/2M aggregate. The homogenate was filtered through 4 layers of Miracloth and 4 layers of cheesecloth pre-wet with isolation medium.

The filtrate was centrifuged at 4,000 g for 30 s in a swing out rotor. The resultant crude chloroplast pellets were resuspended in 6 ml of wash medium (0.33 M sorbitol, 10 mM NaCl, 2 mM Na_2EDTA, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 40 mM HEPES (pH 7.5)) and layered over four 15 ml corex tubes containing 3.5 ml 25% Percoll (Pharmacia) in wash medium. The gradients were centrifuged at 1,000 g for 90 s in a swing out rotor (brake off). The pellets were resuspended in 6 ml of wash medium and layered onto gradients and centrifuged as described above. The pellets containing intact chloroplasts were washed with 10 ml of wash medium and centrifuged at 6000 g for 30 s in a swing out rotor.

The outer chloroplast envelope was osmotically ruptured (as determined by phase contrast microscopy). The chloroplast pellets were resuspended in 15 ml of dH_2O vortexed briefly and incubated for 5 min. An equal volume of 2X wash buffer was added and the thylakoids pelleted, (8,000 g, 2 min) in a swing out rotor. The pellet was resuspended in 10 ml of wash medium and centrifuged (8,000 g, 2 min) in a swing out rotor. The pellet was resuspended in 0.5 ml of resuspension medium (0.33 M sorbitol, 10 mM NaCl, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM Tricine (pH 7.8)).

Sub-thylakoid particles were prepared by diluting the thylakoid suspension to 2.5 ml with resuspension medium and sonicating the thylakoids 5 times at maximum power for 10 s with 15 s intervals between sonications, using an MSE 100 watt ultrasonic disintegrator. The larger particles were pelleted in a microfuge 10 min 12,000 g and the supernatant used for assays.

2.3.4 Fractionation of Soybean Thylakoids

Preparations enriched in PS I and PS II were prepared by a modified method of Berthold *et al.* (1981). Thylakoids were prepared as described in Section 2.3.3. The chlorophyll concentration was adjusted to 2 mg ml^{-1} and Triton X-100 was added to a final concentration of 25 mg ml^{-1}. The sample was incubated on ice for 30 min and centrifuged (33,000 g, 30 min, 4°C). The supernatant was saved and the pellet was resuspended in resuspension media (2.3.3) to a chlorophyll concentration of 2 mg ml^{-1}. Triton X-100 was added to a final concentration of 5 mg ml^{-1} and the sample centrifuged as above. The supernatant was saved and the pellet was resuspended in resuspension media (Section 2.3.3). All three fractions collected were assayed.

2.3.5 Isolation of Chloroplast Envelope Membranes

A modified method of Douce and Joyard (1982) was used in attempts to isolate envelope membranes from soybean chloroplasts. The wash medium and the thylakoid resuspension medium were identical to the solutions used for the isolation of thylakoid membranes from soybean (Section 2.3.3) except that the wash medium was at pH 7.8. The chloroplast medium used was the same as that described in Section 2.3.3 except that 50 mM Tricine was used instead of 50 mM MES and the solution was at pH 7.8. All steps were carried out at 4°C using prechilled solutions and apparatus.

a) Chloroplast Isolation

Seven hundred grams of leaves were homogenised, in 2 l of isolation medium, for 3 s in a Waring Blender at low speed. The homogenate was filtered through 6 layers of muslin and 1 layer of Miracloth, and the filtrate centrifuged (10 min, 4°C, 1,500 g in a Sorvall GS-3 rotor). Each pellet was resuspended in 4 ml of wash medium and filtered through Miracloth. The Miracloth was washed with 10 ml of wash medium. The resulting solution contains a crude chloroplast suspension.

b) Purification of Intact Chloroplasts

Method 1; more than 50 mg chlorophyll in the crude chloroplast suspension.

For each 8-9 ml of crude chloroplast suspension one discontinuous Percoll gradient was used. Each gradient consisted of 7 ml of 80% Percoll solution (80% (v/v) Percoll, 300

mM Mannitol, 10 mM MOPS (pH 7.8)) which had been overlayed with 16 ml of 40% Percoll solution (40% (v/v) Percoll, 300 mM Mannitol, 10 mM MOPS (pH 7.8)). The crude chloroplast suspension was loaded onto the top of the gradients and the gradients were centrifuged (15 min, 4°C, 6,000g in a swing out rotor). The stripped chloroplasts were removed by aspiration from the 0/40% interface, while intact chloroplasts were recovered from the 40/80% interface. The intact chloroplasts were diluted with 10 volumes of wash medium and pelleted by centrifugation (90 s, 4°C, 3,500 g). The wash was repeated and the intact chloroplasts pelleted as above.

Method 2; less than 50 mg chlorophyll in the crude chloroplast suspension.

Thirty four ml of a 50% Percoll solution (50% (v/v) Percoll, 300 mM Mannitol, 10 mM MOPS (pH 7.8)) was loaded into a centrifuge tube and the gradient was centrifuged (100 min, 4°C, 10,000 g). This produced a continuous percoll gradient. Two ml (4-6 mg chlorophyll) of the crude chloroplast suspension was then loaded onto each gradient and the gradients centrifuged (10 min, 4°C, 5,000g). The stripped chloroplasts were removed from the sample/gradient interface by aspiration, and the intact chloroplasts were recovered from near the bottom of the gradient. The intact chloroplasts were then treated in the same way as the intact chloroplasts isolated by method 1.

c) Isolation of Envelope Membranes

The intact chloroplasts were resuspended in 90 ml of swelling medium (10 mM Tricine (pH 7.8), 4 mM $MgCl_2$) and the chloroplasts were allowed to swell for a few seconds, and then layered onto discontinuous sucrose gradients. Each gradient consists of 12 ml of 31.8% sucrose solution (31.8% (w/v) sucrose, 10 mM Tricine (pH 7.8), 4 mM $MgCl_2$) onto which 12 ml of 20.5% sucrose solution had been layered (20.5% (w/v) sucrose, 10 mM Tricine (pH 7.8), 4 mM $MgCl_2$). The gradients were then centrifuged (1 h, 4°C, 72,000g in a swing out rotor). The top fraction which contains the stroma was collected and stored, the envelope membranes were removed from the interface of the sucrose layers and the thylakoids which pellet at the bottom of the tube were resuspended in resuspension media. The envelope membranes were diluted to 0.2-0.3 M sucrose and the solution centrifuged (40 min, 4°C, 113,000 g in a swing out rotor). The supernatant was discarded and the pellet resuspended in resuspension medium using a Potter-Elvehjem homogeniser with a loose fitting pestle.

2.4 ASSAYS

2.4.1 Total Chlorophyll

Chlorophyll was determined by the method of Arnon (1949). Samples were diluted in 80% (v/v) acetone and centrifuged briefly. The absorbance of the samples was measured at 710 nm, 663 nm and 645 nm against a blank of 80% acetone. The A_{710} reading was subtracted from the A_{663} and A_{645} readings and the chlorophyll content determined using the following formula

$$\text{mg Chl ml}^{-1} = \{(8.02 * A_{663}) + (20.2 * A_{645})\} * \text{dilution factor.}$$

2.4.2 Total Protein

The protein concentration of samples was determined by the method of Lowry *et al.* (1951). Stock solutions (a) 2% (w/v) Na_2CO_3 in 0.1 M NaOH, (b) 1% (w/v) Na tartrate and (c) 0.5% (w/v) $CuSO_4$ were mixed in the ratio a:b:c = 50:1:1. 5-10 µl of the samples to be assayed were made up to 400 µl with distilled H_2O. A standard curve was prepared using 0-100 µg of BSA which were also made up to 400 µl with distilled H_2O. All samples and the standard curve were prepared in duplicate. To all tubes, 2 ml of the solution containing a, b and c, and SDS to a final concentration of 0.25% (w/v) were added. The tubes were mixed and allowed to stand for 10 min at room temperature. To each tube 100 µl of Folin's reagent was added, the tube was mixed immediately and left to stand at room temperature for 30 min. The absorbance of the solution was read at 750 nm against a blank consisting of an equal volume of the medium that the samples were resuspended in and prepared in the same way as were the samples.

2.4.3 Oxygen Consumption

Oxygen consumption was measured in a Rank Bros. (Cambridge, U.K) electrode at 25°C in 2 ml of reaction medium (0.3 M mannitol, 10 mM TES-NaOH (pH 7.0), 10 mM $MgCl_2$, 6 mM Na_2HPO_4, and 0.1% (w/v) fatty acid free BSA). Data shown in subsequent chapters are means from a number (n) of different preparations. All assays were carried out in the dark so that photosynthetic oxygen production would not interfere with the respiratory measurements. Cytochrome c_{553} (oxidised) was added to all assays which used NAD(P)H, deamino NADH or duroquinol as substrates in order to reconstitute the electron transport chain, as it has been shown that cytochrome c_{553} is removed during the preparation of membranes. For most species of cyanobacteria

studied, horse heart ferrocytochrome c is an adequate substitute (Krinner *et al.* 1982; Lockau and Pfeffer 1982; Stürzl *et al.* 1982).

2.4.4 Oxidation of NADH

NADH oxidation was followed spectrophotmetrically at 340 nm using an absorbance coefficient of 6.22 mM cm^{-1}. Cuvettes (1 ml) were used and the reaction media was identical to the medium used for oxygen consumption assays (section 2.4.3). Substrates, acceptors and inhibitors were added to the concentrations indicated in the results sections.

2.4.5 Succinate Dehydrogenase Assay

Succinate dehydrogenase was followed by monitoring oxygen consumption as in 2.4.3. Membranes were added to the reaction media, followed by succinate (10 mM final concentration) and then ATP (1 mM final concentration) to ensure any succinate dehydrogenase present was activated (Dr D.A. Day Pers. Comm.).

2.4.6 Cytochrome c Oxidase Assay

Oxidation of reduced horse heart ferrocytochrome c was followed by dual wavelength spectrophotometry at 25°C using an absorption coefficient of 19.5 per mM per cm at 550 nm (Wastyn *et al.* 1988). Cuvettes (1 ml) were used and the reaction medium was identical to the medium used for oxygen consumption assays (section 2.4.3).

2.5 GEL ELECTROPHORESIS

2.5.1 Sample Preparation and Electrophoresis

Membrane proteins were solubulized in up to 60 µl of sample buffer (2% (w/v) SDS, 62.5 mM Tris (pH 6.8), 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 50 mM dithiothreitol (DTT) or 10 % β-Mercaptoethanol) and boiled for 3-5 min. The samples were centrifuged (12,000 g, 10 min, room temperature) to remove aggregates. Electrophoresis was carried out in a manner similar to that of Laemmli (1970) using a 7% polyacrylamide stacking gel and a 12% resolving gel (see Appendix D for compositions).

Electrophoresis was performed in 1X running buffer (25 mM Tris-HCl (pH 8.3), 0.2 M glycine, 0.05% (w/v) SDS) using a BioRad Protean II slab gel apparatus. Gels were run at 12 mA until the dye front was about 1 cm from the bottom of the gel. Promega mid-range molecular weight marker proteins, or Novex Mark 12 wide range molecular weight markers were used.

2.5.2 Staining

After removing the gel from the running apparatus and cutting off the stacking gel the resolving gel was stained

a) Coomassie Brilliant Blue

The resolving gel was incubated in Coomassie Blue staining solution (0.2% (w/v) Coomassie Blue, 45% (v/v) methanol, 10% (v/v) glacial acetic acid, 42% (v/v) distilled H_2O and 3% (v/v) glycerol) for 2 h at room temperature. The gel was destained (in 25% (v/v) ethanol, 10% (v/v) glacial acetic acid and 3% (v/v) glycerol) until blue bands were visible on an otherwise clear gel.

b) Copper Staining

Copper staining was performed using the method of Lee *et al.* (1987). The resolving gel was washed for 30 s in distilled water, with several changes of water during this period. The gel was incubated, with agitation, in at least five volumes of 0.3 M $CuCl_2$ for 5 minutes and then washed for several minutes in distilled water. Regions of the gel that do not contain protein become opaque, while proteins remain clear.

2.5.3 Native Gels

a) Sample Preparation

A modified version of Kuonen *et al.* (1986) was used. Samples were solubilized at a concentration of 10 mg ml^{-1} by addition of equal volume of 20% (w/v) Triton X-100 solution, incubated on ice for 20 min and then centrifuged (100,000 g, 4^0 C, 1 h), to remove any insoluble material. Samples were stored at -70^0 C until use.

b) Gel Preparation and Running

3-22% (w/v) polyacrylamide gels were prepared by placing 17.5 g of heavy and light solutions (see Appendix E for compositions) into the chambers of a standard two chamber gradient mixer. The heavy solution was placed in the chamber from which the mixture was withdrawn. The gel was poured at a flow rate of 3 ml min^{-1}. Immediately after this, a stacking gel of light solution (6 ml) was poured on top at a rate of 2 ml min^{-1}. This was left to polymerise for at least 3 h.

Before loading, samples were diluted two fold with sample buffer (50 mM Tris-glycine buffer pH 9.0, 0.4 % (w/v) Triton X-100 and 0.002% (w/v) bromophenol blue).

Electrophoresis was done in a BioRad Protean II slab gel apparatus containing 100 mM Tris-glycine buffer (pH 9.0) and 0.1% (w/v) Triton X-100 at 15 mA (10 mA mm^{-1} thickness of gel) at 4^0 C for 3 h.

c) Staining

After electrophoresis the gel was incubated in 100 mM MOPS-NaOH (pH 7.0) for 30 min. The gel was then stained overnight in 100 mM MOPS-NaOH (pH 7.0) containing 1 mM NAD(P)H or 1 mM deamino NADH and 500 µg ml^{-1} NBT. After staining the gel was returned to the incubation buffer.

2.6 WESTERN BLOTTING

2.6.1 Antibodies

Polyclonal antibodies against NdhK from *Synechocystis* PCC 6803 were raised in rabbits according to the method described in Section 2.7. Polyclonal antibodies against Glutathione-S-Transferase (GST) from the over-expressed protein in pGEX2-T, were raised in rabbits and kindly supplied by Dr. G.D. Price (Research School of Biological Sciences, Australian National University, Canberra, Australia).

2.6.2 Method

a) Chemiluminescence

A modified version of the method of (Towbin *et al.* 1979) was employed. Proteins were transferred from polyacrylamide gels to Amersham Hybond-C extra filters in a Milliblot

dry blot system. The gel was soaked in transfer buffer (40 mM glycine, 50 mM Tris-HCl (pH 8.9-9.0), 0.375% (w/v) SDS and 20% (v/v) methanol) for 1 h before transfer. A sheet of Hybond-C extra filter was cut to exactly the same size as the gel and soaked in transfer buffer for 10 minutes. Ten sheets of Whatman 3-M filter paper, cut to exactly the same size as the membrane, were soaked in transfer buffer for 5 minutes before transfer. Five sheets of the pre-wetted Whatman 3-M filter paper were placed on the anode and the sheet of Hybond-C extra on top of them. The gel was placed on the membrane and the remaining 5 sheets of pre-wetted Whatman 3-M filter paper were placed on top of the gel. Air bubbles were removed by rolling a 10 ml test-tube over the surface of the stack. The cathode was placed over the stack and proteins were transferred from the gel to the membrane at 0.8mA cm^{-2} for 90 min.

All of the following steps were carried out with gentle agitation. The membrane was stained in Ponceau Red (0.2% (w/v) Ponceau red in 3% (w/v) trichloroacetic acid) for 2 min and destained in distilled water until bands appeared. The destained membrane was blocked in Tris-buffered saline-Tween (TBS-T, 0.9% (w/v) NaCl, 0.1% (w/v) Tween-20 and 10 mM Tris-NaOH (pH 7.4)), containing 3% (w/v) BSA, for 1 h at room temperature or overnight at 4°C . The blocked membrane was rinsed once in TBS-T and washed twice for 5 min in TBS-T. The washed membrane was incubated with primary antibodies (diluted as indicated on each blot in TBS-T) for 1 h at 25°C, rinsed twice and washed once for 15 min and twice for 5 min in TBS-T. After the washes the membrane was incubated for 1 h at 25°C with Anti-Rabbit Ig, horseradish peroxidase linked whole antibody (from donkey, Amersham) diluted as indicated on each blot in TBS-T. The membrane was rinsed three times and washed once for 15 min and twice for 5 min in TBS-T. The washed membrane was incubated for 1 min with chemiluminescence western blotting reagents (Boehringer Mannheim) and proteins that cross-reacted with the antibodies were detected by autoradiography. Forty five min before use aliquots of the chemiluminescence western blotting reagents, Solution A and Solution B, were taken out of the fridge and incubated at room temperature for 15 min. The solutions were mixed in a ratio of 100:1 (A:B) and incubated for 30 min at room temperature before use. One hundred and twenty five µl of the mixture (A:B) was used per square centimetre of membrane. If the membrane was to be reprobed, it was stripped by incubating it in 0.8% (v/v) β-mercaptoethanol, 2%(w/v) SDS, 0.1% (v/v) Tween-20, 112.5 mM Tris-HCl (pH8.9-9.0) and 0.9% (w/v) NaCl at 50°C for 30 minutes with occasional shaking, rinsed twice for 10 minutes in TBS-T, reexposed to check the stripping had been successful and then blocked overnight at 4°C in 3% (w/v) BSA in TBS-T. Immunodetection was carried out as described above.

b) Colour Development

A modified version of the method of Towbin *et al.* (1979) was employed. Proteins were transferred from polyacrylamide gels to Millipore Immobilon filters in a Milliblot dry blot system at 25 mA cm^{-2} for 35 min. The filter was then stained in Ponceau Red (0.2% (w/v) Ponceau red in 3% (w/v) Trichloroacetic acid) for 2 min and destained in distilled water until bands appeared. The filter was then soaked (in 3% (w/v) BSA, 0.9% (w/v) NaCl and 10 mM Tris-NaOH (pH 7.4)) for at least 2 h and then washed (in 0.1% (v/v) Tween-20, 0.9% (w/v) NaCl and 10 mM Tris-NaOH (pH 7.4)) five times for 5 min each. The filter was then incubated with primary antibodies (diluted as indicated on each blot in 3% (w/v) BSA, 0.9% (w/v) NaCl and 10 mM Tris-NaOH (pH 7.4)) for 3 hours at 25^0 C. The 0.1% (v/v) Tween-20, 0.9% (w/v) NaCl and 10 mM Tris-NaOH (pH 7.4) washes were repeated and the filter incubated for 1 h at 25^0 C in a 1:1,000 dilution of goat anti-rabbit alkaline phosphate conjugate or a 1:3,000 dilution of goat anti-mouse alkaline phosphate conjugate diluted in 1% (w/v) BSA, 0.9% (w/v) NaCl and 10 mM Tris-NaOH (pH 7.4). The 0.1% (v/v) Tween-20, 0.9% (w/v) NaCl and 10 mM Tris-NaOH (pH 7.4) washes were repeated and the filter incubated with the p-nitro blue tetrazolium chloride (NBT)/ 5-bromo 4-chloro 3-indolyl phosphate p-toluidine salt (BCIP) colour development system until bands appeared.

2.7 ANTIBODY PRODUCTION

2.7.1 Induction of fusion protein expression and breaking of *E. coli*

E. coli containing the expression vector was grown overnight at 37°C with shaking in LB (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl, pH 7.0 with NaOH) to which the appropriate antibiotic had been added. The culture was added to 9 volumes of LB containing the appropriate antibiotic and grown at 37°C with shaking for 1 h. Isopropylthio-β-galactosidase (IPTG) was added to a final concentration of 2 mM and the cells grown for 3 h with shaking at 37°C. The cells were harvested by centrifugation (10,000 g, 5 min, 4°C) and the pellet resuspended in a minimum volume of lysis buffer (20mM TES-NaOH (pH 7.0), 5 mM EDTA, 50 µl 1% (w/v) PMSF in methanol). The cells were sonicated 4 times for 20 s, with 20 s between sonications, using a Sonifier Cell Disruptor B-30 on setting 3. The disrupted cells were pelleted as above, the supernatant saved and the pellet resuspended in a minimum volume of *E. coli*

busting buffer. Samples of both fractions were run on polyacrylamide gels to determine which fraction the fusion protein was in.

2.7.2 Purification of GST-fusion proteins using affinity chromatography

a) Solubilisation of the fusion protein

As the fusion protein was in the insoluble cell fraction the method of Fish and Hoare (1988) was used to solubulise it before affinity chromatography. The insoluble cellular fraction was prepared as described in Section 2.7.1, but the final pellet was resuspended in 7 volumes of 8 M urea instead of lysis buffer. The sample was incubated at room temperature for 15-30 min and then the urea concentration was diluted to 2 M with MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) over the course of 2 h with continuous mixing. The sample was centrifuge (39,000 g, 45 min, 20°C) and the supernatant collected.

b) Affinity Chromatography

Affinity chromatography was performed using a 2 ml Glutathione Sepharose® 4B column (Pharmacia). The column was washed with 10-20 ml of MTPBS and the gel bed equilibrated by passing 6 ml of MTPBS containing 1% (w/v) Triton X-100 through the column. The sample, prepared as described in section 2.7.2.a, was applied to the column and the eluent discarded. The column was washed twice with 10 ml of 2 M urea in MTPBS. 2 ml of MTPBS containing 10 u ml^{-1} thrombin was applied to the column and the column incubated for 6 h at 4°C with shaking. The sample was eluted and collected. The bound glutathione was eluted by the addition of 10 ml of elution buffer (5 mM Glutathione, 50 mM Tris-HCl (pH 8.0))

c) Regeneration and storage of column

The column was regenerated by washing once with MTPBS containing 3 M NaCl, twice with 5 bed volumes of MTPBS and twice with 5 bed volumes of 20% ethanol. Columns were stored at 4°C in 20% ethanol.

2.7.3 Purification of inclusion bodies from *E. coli*

a) Method 1 (Harlow and Lane 1988)

E. coli was grown as described in Section 2.7.1 and the bacterial cells were pelleted by centrifugation (7,000 g, 5 min, 4°C). The pellet was resuspended in 9 volumes of lysis buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 8.0)) and lysozyme added to 1 mg ml^{-1}. The solution was incubated at room temperature for 20 min and the lysed cells pelleted (5,000g, 10 min, 4°C). The supernatant was removed and discarded and the pellet was transferred to ice and the spheroplasts resuspended in ice-cold lysis buffer containing 0.1% sodium deoxycholate. This was incubated on ice with occasional mixing for 10 minutes and $MgCl_2$ was added to a final concentration of 8 mM and DNase I to 10 µg ml^{-1}. This was incubated at 4°C with occasional mixing until the viscosity disappeared. Inclusion bodies were removed from the suspension by centrifugation (10,000 g, 10 min, 4°C). The pellet was washed once in lysis buffer containing 1% NP-40, once in lysis buffer and resuspended in a minimum volume of lysis buffer. Samples were subjected to polyacrylamide gel electrophoresis (Section 2.5) to determine purity.

b) Method 2 (Sambrook *et al.* 1989)

E. coli was grown as described in Section 2.7.1, all subsequent steps, unless otherwise stated, were carried out in a cold room using pre-chilled apparatus and solutions. The bacterial cells were pelleted by centrifugation (500 g, 15 min, 4°C). The supernatant was discarded and the *E. coli* pellet weighed. For each gram of *E. coli* (wet weight) 3 ml of lysis buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 8.0)) was added. To this 8 µl of 50 mM PMSF and 80 µl of lysosyme (10 mg ml^{-1}) was added for each gram of *E. coli*. The solution was incubated for 20 min with occasional stirring. Four mg of deoxycholic acid was added per gram of *E. coli* while stirring continuously. The solution was placed at 37°C and stirred with a glass rod. Once the lysate had become viscous 20 µl of DNase I (1 mg ml^{-1}) was added per gram of *E. coli*. The lysate was incubated at room temperature until it was no longer viscous (about 30 min). The lysate was centrifuged (12,000 g, 15 min, 4°C) in a microfuge, the supernatant discarded and the pellet resuspended in 9 volumes of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA (pH 8.0). This was incubated at room temperature for 5 min and then centrifuged (12,000 g, 15 min, 4°C) in a microfuge. The supernatant was decanted and saved and the pellet resuspended in 100 µl of H_2O. Samples were subjected to polyacrylamide gel electrophoresis (Section 2.5) to determine purity.

2.7.4 Preparation of Antigen for Injection

The purified fusion protein was separated from any contaminating proteins on a 12% polyacrylamide gel (Section 2.5). The gel was stained with copper chloride (Section 2.5.2.b), the fusion protein excised, using a scalpel, and rinsed in distilled water with continuous gentle agitation for 30 min. The water was removed and the sample divided into 3 (approximately 500 µg each). Two samples were stored a 4°C to be used later for primary and secondary boosts. The third sample was ground in a mortar and pestle with PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, (pH 7.3)) to a paste and the paste was transferred to an Eppendorf tube and PBS added to 500 µl. This was sucked up into a 2 ml glass syringe and 500 µl of Freunds Complete Adjuvunct (Gibco BRL) was added. The syringe was connected to a second syringe through a luer lock fitting taking care to ensure no air bubbles were present in the solution. The solution was pumped back and forth between the two syringes until a thick emulsion had formed. The emulsion was injected into a rabbit. Samples used for the primary and secondary boosts were prepared as above but Freunds Incomplete Adjuvunct (Gibco, BRL) was used instead of Freunds Complete Adjuvunct (Gibco, BRL).

2.7.5 Injection of Antigen

The antigen prepared as described in Section 2.7.4, was injected subcutaneously into rabbits. The primary injection and both the primary and secondary boosts consisted of two 500 µl injections to the back of the neck. The primary boost was given one month after the primary injection and the secondary injection was one month after that. Test bleeds were performed 14 days after the primary and secondary boosts and the final bleed was 16 days after the secondary boost.

2.7.6 Bleeding of Rabbits and Preparation of Serum

All bleeds were performed on the marginal ear vein. The rabbit was wrapped in a hessian sack and the ear placed under a heat lamp for 2 minutes. The marginal ear vein was cut with a sterile scalpel blade and the blood allowed to drip into a sterile 50 ml Falcon tube. The blood was left to clot for 30-60 min at 37°C. The clot was separated from the sides of the tube using a wooden rod and incubated a 4°C overnight to allow the clot to contract. The serum was decanted from the clot and any remaining insoluble material removed by centrifugation (10,000 g, 10 min, 4°C). The serum was used for western analysis as described in Section 2.6

2.8 PHENOL/CHLOROFORM EXTRACTION OF DNA

Phenol (ICN ultrapure) was water saturated by mixing with deionised water (at least 12.4 ml per 100 g phenol) until a fine emulsion had been formed. The emulsion was incubated at 4°C until the phases had separated. The aqueous phase was decanted and an equal volume 0.5 M Tris-HCl (pH 8.0) was added to the water saturated phenol. The phases were mixed and allowed to separate at room temperature. The aqueous phase was removed and an equal volume of 0.1 M Tris-HCl (pH 8.0) was added to the phenol, mixed and allowed to separate. The pH of the aqueous phase was checked using pH paper and the above step repeated until the pH of the aqueous phase was between 7.8 and 8.0.

For phenol/chloroform extractions 0.5 volumes of Phenol and 0.5 volumes of chloroform isoamyl alcohol (IAC, chloroform and isoamyl alcohol mixed in the ratio of 24:1) was added to the solution containing the DNA and mixed. The two phases were separated by centrifugation (12,000 g, 1 min in a microfuge) and the aqueous phase removed and subjected to a chloroform extraction to remove traces of phenol. An equal volume of IAC was added to the aqueous phase and the solution mixed, the phases separated by centrifugation as above and the aqueous phase retained.

2.9 ISOLATION OF DNA

2.9.1 Isolation of Genomic DNA from *Anabaena* PCC 7120

The method used was a modified version of Mazur *et al.* (1980). All steps were carried out using wide-bore pipettes. Cells in the mid to late-logarithmic growth phases were harvested by centrifugation (4,000 g, 10 min, 4°C) and washed twice in water. The pellet was weighed and the cells were resuspended in 1.6 ml of STE1 (50 mM Tris (pH 8.5), 50 mM NaCl, 50 mM EDTA) per gram of cells (wet weight), and incubated for 1 h at 37°C in the presence of lysozyme (10 mg ml^{-1}). The cells were chilled on ice and the solution made up to 67.5 mM EDTA and 2% (w/v) SDS and incubated at 65°C for 15 min. The lysed cells were subjected to two phenol/chloroform extractions and one chloroform extraction (in each of the extractions the two phases were mixed gently for 15 min, by placing the sealed tube on a wheel rotating at 20 rpm, before separating the

phases by centrifugation). Two volumes of chilled ethanol (-20°C) was added and the solution mixed gently, and the DNA collected by centrifugation. The pellet was rinsed in 70% (v/v) ethanol and resuspended in STE2 (100 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA). RNase A was added to a final concentration of 400 μg ml^{-1} and the solution incubated for 30 min at 37°C. Proteinase K was added to a final concentration of 100 μg ml^{-1} and the solution incubated for 2 h at 37°C. The solution was extracted twice with phenol/chloroform and once with chloroform. The DNA was collected by ethanol precipitation (0.1 volume of 3 M ammonium acetate (pH 5.2) and 2 volumes of chilled ethanol were added and the DNA collected by centrifugation (12,000 g, 15 min, 4°C)), washed in 70% ethanol and resuspended in sterile deionised water.

The DNA that was used to for the Southern analysis to determine if the *ndhC-K-J* operon was conserved in *Anabaena* PCC 7120 was further purified using caesium chloride gradients (Ausubel *et al.* 1994). After the 70% ethanol wash the DNA was resuspended in 9 ml of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and 9.7 g of CsCl was added and mixed gently until it dissolved. The solution was incubated on ice for 30 min and centrifuged (7,500 g, 10 min, 4°C), the supernatant was saved and 0.5 ml of 10 mg ml^{-1} ethidium bromide was added. This was incubated on ice for 30 min and then centrifuged (7,500 g, 10 min, 4°C). The supernatant was transferred to quick seal ultracentrifuge tubes and centrifuged (300,000 g, overnight, 20°C). The band of DNA was collected using a large-bore needle and syringe. The ethidium bromide was removed by repeatedly extracting the DNA with isopropanol that had been equilibrated over a CsCl-saturated aqueous phase. Two volumes of water and 6 volumes of ethanol were added to the DNA solution and mixed. This was incubated for 1 h at -20°C and the DNA collected by centrifugation (7,500 g, 10 min, 4°C). The DNA was resuspended in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) ethanol precipitated and resuspended in TE.

2.9.2 Isolation of Genomic DNA from *Synechocystis* PCC 6803

The method used was a modified version of Dzelzkalns and Bogorad (1986). Eighty ml of cells were harvested by centrifugation (4,000 g, 10 min, 4°C), and the pellet washed once with growth medium and centrifuged as above. The pellet was snap frozen in liquid nitrogen and thawed on ice and resuspended in 3 ml of 50 mM Tris-HCl (pH 7.8), 40 mM Na_2EDTA and 100 μl of 20% (w/v) SDS and 50 μl of 3 M sodium acetate were added. The solution was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was thawed at 37°C and phenol/chloroform extracted 3 times, with the two phases being mixed by slow agitation for 15 min in each extraction. The DNA was ethanol precipitated and resuspended in TE. The resuspended DNA was passed through a Sephadex G-50 column and the eluent, which contains the DNA, was saved.

2.9.3 Isolation of Plasmid DNA from *E. coli*

a) Alkaline lysis mini-prep

A modified version of Birboim and Doly (1979) was used. 1 ml of culture was centrifuged (12,000 g, 10 s, room temperature) and washed once with GET (50 mM Glucose, 10 mM Na_2EDTA, 25 mM Tris-HCl (pH 8.0)) and resuspended in 150 µl of GET. Five µl RNase A (10 mg ml^{-1}) was added and the solution was vortexed. Cells were lysed by the addition of 350 µl of 0.2 M NaOH, 1% (w/v) SDS and incubated on ice for 5 min, followed by the addition of 250 µl of 3 M sodium acetate (pH 5.0) and incubation on ice for a further 20 min. Cell debris were removed by centrifugation (12,000 g, 15 min, 4°C). The supernatant was transferred to a new Eppendorf tube, snap frozen in liquid nitrogen, thawed and centrifuged as above. The supernatant was transferred to a fresh tube and the DNA precipitated by the addition of 750 µl of isopropanol and collected by centrifugation (12,000 g, 5 min, 4°C). The DNA was washed once in 70% (v/v) ethanol and resuspended in 20 µl of water.

b) Large Scale Preparation

The method used is based on the method of Birboim and Doly (1979). Transformed cells from a 100 ml culture were pelleted by centrifugation (5,000 g 5 min 4°C) and the pellet resuspended in 4.8 ml of GET. The cells were lysed by the addition of 200 µl of 25 mg ml^{-1} lysosyme and incubating at room temperature for 10 min. Genomic DNA and RNA were denatured by the addition of 10 ml of 0.2 M NaOH, 1% (w/v) SDS; the solution vortexed and incubated on ice for 10 min. Denatured nucleic acids and cell debris were removed by the addition of 7.5 ml of 3 M sodium acetate (pH 5.2), the solution was incubated on ice for 30 min with occasional mixing. The precipitate was removed by centrifugation (20,000 g, 15 min 4°C, brake off). The supernatant was decanted into a fresh tube and the plasmid DNA precipitated by addition of 0.8 volume of isopropanol and incubation at room temperature for 10-30 min. The DNA was recovered by centrifugation (12,000 g, 15 min, 4°C). The DNA was resuspended in 5 ml of 2 M ammonium acetate and then centrifuged (17,000 g, 15 min, 4°C). The supernatant was decanted to a fresh tube and the DNA precipitated by the addition of 1 volume of isopropanol. The DNA was collected by centrifugation (8,000 g, 15 min, 4°C) and

washed with 70% ethanol and resuspended in 500 µl of water. The resuspended DNA was treated sequentially with RNase A (final concentration 25 µg ml^{-1}) for 10 min at 37°C and Proteinase K (final concentration 20 µg ml^{-1}) for 30 min at 37°C, phenol/chloroform extracted once and chloroform extracted once. The DNA was ethanol precipitated, washed with 70 % ethanol and resuspended in 100-500 µl of water. DNA from this preparation was sufficiently pure for sequencing.

2.10 ENZYMATIC REACTIONS

2.10.1 Digestion of DNA with Restriction Endonucleases

DNA was digested in buffers provided by the manufacturers (Boehringer Mannheim, Promega) in which the enzyme used had maximal activity at the concentration recommended. All digests were carried out at the optimal temperature for the enzyme used. In double digestions where the enzymes used operated maximally in different buffers the DNA was first digested with one enzyme and the reaction stopped by heating the reaction to 65°C or addition of EDTA to a final concentration of 12.5 mM. The DNA was extracted once with phenol /chloroform and once with chloroform and the DNA recovered by ethanol precipitation. The DNA was resuspended in 1X concentration of the appropriate buffer and digestion with the second enzyme carried out. Digestion was monitored by agarose gel electrophoresis (Section 2.13).

2.10.2 Ligation of DNA Molecules

Ligation of DNA was carried out with T4 DNA ligase which catalyses the formation of a phosphodiester bond between a 5' phosphate and a 3' hydroxyl of DNA with the concomitant hydrolysis of ATP. The reaction mixture contained 1 unit of ligase for cohesive ends, 3 units for blunt ends, and 1X T4 DNA ligase buffer (66 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM ATP (Boehringer Mannheim)). The reaction was incubated 3-20 h at 15°C for cohesive ends and 1-5 h at room temperature for blunt ends. In order to maximise ligation of the desired inserts to plasmid DNA over plasmid-plasmid ligations insert and vector DNA were added to the reaction at a molar ratio of 3:1. The T4 DNA ligase was denatured by heating to 65°C for 5-10 min prior to transformation.

2.10.3 Repair of DNA with 5'-overhanging Termini

In order to ligate DNA fragments with non-complimentary termini it is necessary to repair or "fill-in" these termini to produce blunt ends. This was done by using the 5'-3' polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. DNA (up to 1 μg) was incubated at 22°C for 20-30 minutes in a reaction mixture containing 2-4 units of Klenow, 0.1 volume of 10X nick translation buffer (0.5 M Tris-HCl (pH 7.5), 0.1 M $MgSO_4$, 0.5 mg ml^{-1} BSA, 2 mM DTT) and 0.04 volume of 2 mM solution of all four dNTP's. The reaction was terminated by heating to 70°C for 5 min.

2.10.4 Treatment of Digested DNA with Calf Alkaline Phosphatase

DNA to be treated with calf alkaline phosphatase was digested with the appropriate restriction enzyme (2.10.1), phenol chloroform extracted once and ethanol precipitated. The precipitated DNA was resuspended in Milli Q water and 10X alkaline phosphatase buffer (0.5 M Tris-HCl (pH 8.0), 1 mM EDTA) was added to give a final concentration of 1X. Calf alkaline phosphatase was then added (1 u 100 $pmol^{-1}$ ends of DNA) and the mixture incubated at 37°C for 5 min. EDTA (pH 8.0) was then added to a final concentration of 5 mM and the mixture incubated at 75°C for 10 min. The DNA was then phenol chloroform extracted once, ethanol precipitated and resuspended. An aliquot of the resuspended DNA was then used for ligation reactions (2.10.2)

2.11 AMPLIFICATION OF SPECIFIC REGIONS OF DNA WITH THE POLYMERASE CHAIN REACTION (PCR)

2.11.1 PCR

The thermal stability of a number of DNA polymerases isolated from thermophilic bacteria enables specific regions of DNA to be amplified using an automated thermal cycler. The DNA polymerase from *Termus aquaticus, Taq* polymerase was used in this study. Specific primers homologous to known sequences which bind to the template DNA provide the starting point for an extension. The reaction sequence involves (i) denaturation of the target DNA to single strands by heating to 95°C, (ii) cooling to an annealing temperature at which the primers will bind, and (iii) heating to 72°C for the extension reaction. This sequence is repeated for an appropriate number of cycles to achieve amplification of the target DNA molecule.

PCR was carried out using a Corbitt Research FTS-1 capillary thermocycler. Reactions were done in sealed capillary tubes containing 10 µl reaction volumes. A typical reaction contained 1 µl BSA (1 mg ml^{-1}), 1 µl 10X Amplification buffer containing Mg^{2+} (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl (Boehringer Mannheim)), 0.8 µl 2.5 mM dNTP's, 20 pmol of each primer, 100 ng of template DNA and 0.5 u *Taq* polymerase. The following program was used for amplification of genomic DNA from cyanobacteria, 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 80°C for 15 s, 65°C for 15 s, 55°C for 1.5 min, 65°C for 15 sec, 72°C for 2.5 min and 85°C 15 s. This was followed by one cycle of 94°C for 1 min, 72°C for 5 min and 25°C for 1 min. PCR products were visualised by ethidium bromide staining after agarose gel electrophoresis.

2.11.2 Purification of PCR Products

PCR products were either purified by agarose gel electrophoresis (Section 2.13), or by direct purification using the Wizard PCR Purification System (Promega). One hundred µl of direct purification buffer was added to the PCR reaction and the mixture vortexed. To this mixture1 ml of purification resin was added and the solution vortexed briefly 3 times over 1 min and then pipetted into a 2 ml syringe to which a mini-column had been attached. The solution was passed through the column and the eluent discarded. The column was washed with 2 ml of 80% (v/v) isopropanol and then centrifuged (12,000 g, 20 s, room temperature) to dry the column and the eluent discarded. The DNA was eluted from the column by addition of 50 µl of water, incubation for 1 min at room temperature and then the column was centrifuged as above. The eluent from the column contained the DNA, if the fragment of DNA to be extracted from the agarose was larger than 3 kb the water was heated to 65°C before being added to the column.

2.11.3 Cloning of Purified PCR Products

The T-Vector System (Promega) was used to clone PCR products. pGEM-T is pGEM5Zf(+) which has been cut at the *Eco RV* site and a single 3' terminal thymidine base is added to both ends. These single 3'-T overhangs take advantage of the non-template dependant addition of a single deoxyadenosine to the 3'-end of PCR products by certain thermostable polymerases. In a 0.5 ml tube the PCR product was mixed with 50 ng of pGEM-T vector to a molar ratio of 1:1 (insert:vector), to this 1 µl of T4 DNA Ligase 10X buffer (300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100mM DTT, 5 mM ATP) and 3 u of T4 Ligase were added. Water was added to give a final volume of 10 µl. The mixture was incubated overnight at 15°C, heated to 70°C for 10 minutes and

allowed to cool to room temperature. An aliquot of the ligation mixture was transformed into competent *E. coli* (Section 2.12)

2.12 INTRODUCTION OF RECOMBINANT DNA MOLECULES INTO *E. coli*

2.12.1 Preparation of Competent *E. coli* Cells by $CaCl_2$ Treatment

Treatment of *E. coli* cells with $CaCl_2$ renders them "competent" to take up exogenous DNA. Competent cells were prepared by the method of Morrison (1979). A single colony from an M9 or LB plate incubated overnight at 37°C was picked and used as the inoculum for a 50 ml LB culture. Cells were grown to an absorbance (650 nm) of 0.6-0.8, chilled in an ice water bath and harvested by centrifugation (4,400g, 5 min, 4°C). The pellet was resuspended in 0.25 volume of ice-cold 0.1 M $MgCl_2$ and the cells pelleted by centrifugation as above. The supernatant was decanted and the pellet resuspended in 0.05 volume of ice-cold 0.1 M $CaCl_2$, incubated on ice for 30 min and the cells pelleted as above. The pellet was resuspended in 0.25 volume of ice-cold 0.1 M $CaCl_2$, 15 % (v/v) glycerol and dispensed into 200 µl aliquots to pre-chilled Eppendorf tubes which were snap frozen in liquid nitrogen and stored at -70°C until required.

2.12.2 Transformation of $CaCl_2$ Treated *E. coli*

An appropriate dilution (1-10 µl) of a ligation reaction or an isolated plasmid was added to a 200 µl aliquot of competent *E. coli* cells of the desired strain (pre-thawed on ice) and incubated on ice for 30 min. The mixture was heat-shocked at 42°C for 90 s and incubated on ice for a further 2 min. One ml of room temperature LB was added and the cells incubated at 37°C for 1 h with vigorous shaking. Aliquots of this mixture were spread onto LB plates supplemented with the appropriate antibiotic(s) to select for the presence of the plasmid(s).

2.12.3 Screening for Recombinant Plasmids

Screening for recombinant plasmids, i.e. plasmids which contained inserts, was performed by α-complementation. The pUC and pGEM vectors used in this study carry the *lacZ* gene encoding the N-terminal portion of β-galactosidase which can complement *E. coli* hosts deficient in β-galactosidase production. The multiple cloning site of these plasmids, into which insert DNA is ligated, lies within the *lacZ* coding region. Introduction of insert DNA into this region abolishes expression of *lacZ*. Plasmid

encoded *lacZ* can be induced by IPTG and β-galactosidase can act upon the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to produce a blue product. Thus α-complementation of *E. coli* in the presence of IPTG (150 μg ml^{-1}) and X-gal (40 μg ml^{-1}) results in blue colonies (see Sambrook *et al.* (1989) for details). Colonies containing plasmids with inserts, i.e. in which *lacZ* is disrupted, are white and thus white colonies are diagnostic for the presence of an insert.

The vectors used for transformation of *Anabaena* PCC 7120 do not contain *lacZ* and thus screening for recombinant plasmids was done purely by selection on appropriate antibiotics.

2.13 AGARAOSE GEL ELECTROPHORESIS

2.13.1 Separation of DNA Fragments by Agarose Gel Electrophoresis

DNA fragments were separated on the basis of size by electrophoresis in agarose gels (concentrations varied from 0.7-2.0% (w/v) agarose). For routine work high melting point agarose (Progen) was used, but when DNA fragments were to be isolated from the gel high purity, low melting point agarose (LMP, Progen) was used. Gels were of the horizontal slab type and electrophoresis was performed in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.0)) or 1X TBE (90 mM Tris-borate, 1 mM EDTA (pH 8.0)) at 20-100 V for 1-18 h. Prior to loading the gel the DNA was mixed with 0.25-1 volume of loading dye (50 mM Tris-HCl (pH 7.8), 25% (v/v) glycerol, 1% (w/v) SDS, 5 mM EDTA, 0.2% (w/v) bromophenol blue). The gels contained ethidium bromide (0.01 mg ml^{-1}) and the DNA was visualised and photographed on a UV light box (302 nm). Bacteriophage λ DNA digested with *Hind III* or bacteriophage SPP1 digested with *Eco RI* were used as molecular weight markers on agarose gels.

2.13.2 Extraction of DNA from Agarose Slices

If fragments of DNA were to be excised from the gel, LMP gels were run using sterile TAE. Gels were placed on a perspex sheet to reduce the intensity of the UV light during visualisation, and the fragment(s) of interest excised with a sterile scalpel blade. DNA was extracted from the agarose using the Wizard PCR Purification System (Promega). The gel slice (approximately 300 μl) was incubated at 65°C until it had melted and 1 ml of purification resin was added. The solution was shaken for 20 seconds and then pipetted

into a 2 ml syringe to which a mini-column had been attached. The solution was passed through the column and the eluent discarded. The column was then washed with 2 ml of 80% (v/v) isopropanol and centrifuged (12,000g, 20 s, room temperature) to dry the column, and the eluent discarded. The DNA was eluted from the column by addition of 50 µl of water, incubation for 1 min at room temperature and then the column was centrifuged as above. The eluent from the column contained the DNA, if the fragment of DNA to be extracted from the agarose was larger than 3 kb the water was heated to 65°C before being added to the column.

2.14 DNA HYBRIDISATION PROCEDURES

2.14.1 Southern Blotting of DNA from Agarose Gels

DNA fragments separated by agarose gel electrophoresis (Section 2.13) were transferred to Hybond-N+ (Amersham) using methods based on that of Southern (1975).

Transfer of the DNA to Hybond-N+ (Amersham) was performed using a Vacugene apparatus (Pharmacia). A piece of Hybond-N+ was cut so that it was 1 cm larger than the gel on all sides and wet with distilled water. The wetted membrane was placed on the surface of the blotter and the plastic mask placed over the blotter, with the hole in the mask over the membrane. The gel was placed over the membrane so that it also overlapped the mask and the frame was clipped onto the blotter. The gel was covered with denaturing solution (1.5 M NaCl, 0.5 M NaOH), the blotter turned on and the pump adjusted to give a suction pressure of 50 mBar. After 15 min the denaturing solution was removed and replaced with neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA) and incubated for a further 15 minutes. The neutralising solution was replaced with 20X SSC (3 M NaCl, 0.3 M sodium citrate) and incubated for 45 min. The vacuum was turned off the membrane removed from the apparatus and fixed by incubating it on 2 sheets of Whatman 3-M filter paper soaked in 0.4 M NaOH for 5 min. The membrane was neutralised in 5X SSC for 10 seconds and then placed between 2 sheets of Whatman 3-M filter, wrapped in saran wrap and stored a 4°C until used for Southern analysis.

2.14.2 Transfer of DNA from Bacteriophage Plaques or Bacterial Colonies to Hybond-N$^+$

Discs and plates were asymetrically marked to allow alignment, identification and retrieval of potential positive clones. Filters were placed on the plates and transfer allowed to proceed for 1 min. The filter was then placed colony side up on Whatman 3-M filter paper soaked in denaturing solution and incubated for 7 min. The filter was then placed colony side up on Whatman 3-M filter paper soaked in neutralising solution for 3 min. The neutralising step was repeated and then the filters were washed in 2X SSC, and placed colony side up on Whatman 3-M filter paper to dry. DNA was fixed to the membrane as described above (Section 2.14.1).

2.14.3 Radioactive Labelling of DNA Probes

Probe template was labelled using the Megaprime labelling kit (Amersham). Probe DNA was denatured by boiling for 4 min, 25 µl of probe (~400 ng) was added to 5 µl of primer solution (random hexanucleotides) and boiled for a further 4 min. The following reagents were added: 4 µl of each of unlabelled dATP, dGTP, dTTP, 5 µl of Reaction buffer and 2 µl of Klenow DNA polymerase. The solution was spun down and 4 µl of [α-^{32}P] dCTP (40 µCi) added. The labelling reaction was incubated at 37°C for 1 h. Unincorporated nucleotides were removed by passage through a Sephadex G-50 spun column.

2.14.4 Hybridisation of Radioactive Probes to DNA

Hybond-N$^+$ filters were prehybridised and hybridised in 6X SSC, 5% (w/v) dextran sulphate, 0.2% (w/v) SDS, 0.05% (w/v) Blotto (Boehringer Mannheim), 10 mM EDTA, 0.02 mg ml^{-1} salmon sperm DNA (boiled for 5 min before addition). Filters were pre-hybridised for 6-18 h at the appropriate temperature, with gentle shaking. The probe DNA was boiled for 4 min immediately prior to addition to the hybridisation mixture. Hybridisation was allowed to proceed for 16-20 h, with gentle shaking. After hybridisation the filter was rinsed once with an appropriate concentration of SSC, 0.1% (w/v) SDS at room temperature and then twice for 10 min with the same solution at room temperature. The membrane was then washed twice for 15 min with wash solution that had been pre-heated to the hybridisation temperature. Filters were dried briefly wrapped in saran wrap and exposed to X-ray film at -70°C for 30 min to 2 weeks.

Probe DNA could be removed from Hybond-N^+ by adding boiling 0.1% (w/v) SDS to the membrane and allowing the solution to cool to room temperature while agitating gently, thus enabling the blot to be reprobed with different probes.

2.15 DNA SEQUENCING AND ANALYSIS

2.15.1 DNA Sequencing with *Taq* DNA Polymerase and Dye Labelled Primers

The dideoxy chain termination method (after Sanger *et al.* (1977)) was used in this study, using double-stranded plasmid DNA as a template. Dideoxy sequencing relies on the ability of DNA polymerase to incorporate dideoxy-nucleotides into growing DNA sequences, and once incorporated no further extension of that strand can occur. Thus a population of DNA fragments varying by one nucleotide in length are generated, and separation of these fragments allows the sequence to be read.

Sequencing was carried out using dye labelled primers and conducting incorporation reactions in a thermal cycler (Corbitt Research) with *Taq* polymerase. For each of the termination reactions the primer is labelled with a different dye. The primers used were SP6 and T7 (Applied Biosystems).

Sequencing was carried out using a *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystems). The kit uses the analogue of dGTP c^7dGTP as this reduces the likelihood of GC compressions due to secondary structure which render sequence information difficult to interpret. One µg of template DNA in 6 µl was used for each sequence determination and reactions were set up under low light in 0.5 ml tubes and then transferred to the capillary thermocycler tubes and heat sealed. For ddATP and ddCTP the reactions contained 1 µl of DNA template, 1 µl of d/ddNTP mix, 1 µl (0.4 pmol) of the appropriately labelled dye primer, 1 µl of 5X cycle sequencing buffer (0.4 M Tris-HCl (pH 8.9), 0.1 M $(NH_4)_2$ SO_4, 25 mM $MgCl_2$), 1 µl of diluted AmpliTaq *Taq* polymerase (0.6 u). ddGTP and ddTTP reactions contained double the amount of every component.

Thermal cycling was carried out in a Corbitt Research FTS-1 Capillary Thermocycler with the ramp rate set on 3 using the following parameters: 95°C for 1 min, 53°C for 15 s, 72°C for 1 min (one cycle), followed by 30 cycles of 94°C for 15 s, 53°C for 15 s, 72°C for 1 min, and one cycle of 72°C for 3 min, 25°C for 1 min. After the reactions were completed the four reactions were pooled and precipitated by the addition of 80 µl

of 95% (v/v) ethanol and 1.5 µl of sodium acetate and placed on wet ice for 15-30 min. The DNA was pelleted by centrifugation (12,000 g, 15 min, 4°C) and washed once with 250 µl of 70% (v/v) ethanol. The pellet was dried in a vacuum centrifuge (Savant Speed Vac, SC100) for 2 min. Precipitated samples were processed by the Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, using an applied Biosystems automated DNA sequencer (Model 370A).

2.15.2 Sequence Analysis

DNA sequences were analysed using programs of the DNA analysis package produced by the Genetics Computer Group (GCG) of the University of Wisconsin (Devereux *et al.* 1984) using a VAX computer at the Research School of Biological Sciences, Australian National University.

2.16 LIBRARY CONSTRUCTION

2.16.1 Preparation of DNA

Genomic DNA (250-500 µg) was partially digested with *Sau 3A* so that the majority of fragments were between 15 and 23 kb, as determined by agarose gel electrophoresis. The digested DNA was phenol/chloroform extracted twice and chloroform extracted once, ethanol precipitated, washed in 70% (v/v) ethanol and resuspended in 500 µl TE and stored at 4°C.

2.16.2 Ligation and Packaging of DNA

An overnight culture of *E. coli* strain LE392 was grown in 50 ml of LB supplemented with 0.5 ml of 20% (w/v) maltose and 0.5 ml of 1 M $MgSO_4$, at 30°C with shaking. One ml of this was used to inoculate 50 ml of supplemented LB media and the cells grown as above until the $O.D._{600}$ reached 0.6. The cells were stored at 4°C until required.

The digested DNA was ligated into EMBL3 *Bam HI* arms (Promega). Each ligation reaction contained 2 µg of EMBL3 *Bam HI* arms, 1 µl 10X ligase buffer (400 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 500 µg ml^{-1} BSA), 1 u T4 DNA ligase, insert DNA and water to 10 µl. The insert DNA was added to the ligation in quantitites that gave molar insert:arms ratios of 1:1 or 2:1. To test the background due to

the vector arms alone the above reaction was set up but the insert was omitted. The ligation mixtures were incubated for 3 h at room temperature.

The ligated DNA was packaged using the Packagene packaging *in vitro* system (Promega). The Packagene extract was thawed on ice and the entire ligation reaction was added to the extract and the tube mixed by gently tapping the bottom several times. The mixture was incubated at 22°C for 2 h, 445 µl of phage buffer (200 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgSO_4$) and 25 µl of chloroform were added to the mixture. The tube was mixed gently by inversion and the chloroform allowed to settle to the bottom. The packaging extract was serially diluted in phage buffer to 1 in 100,000 and 100 µl of the diluted phage (from each dilution) was added to 100 µl of LE392 and the phage allowed to adsorb for 30 min at 37°C. 3 ml of molten (45°C) top agar (1% (w/v) Bacto-Tryptone, 0.5% (w/v) NaCl, 0.8% (w/v)Bacto-Agar) was added to the adsorbed phage and immediately poured onto LB plates. The top agar was allowed to set and the plates were incubated inverted overnight at 37°C. The number of plaques were counted the next morning and the titre of the phage calculated using the formula:

plaque forming units (pfu) = (number of plaques on plate) X dilution factor X 10

The last factor converts the 100 µl of packaging extract plated to a per ml basis

The remaining packaging extract was plated out as above at an appropriate dilution and the library screened as described in section 2.14.

2.17 TRANSFORMATION OF *Anabaena* PCC 7120

2.17.1 Transformation By Conjugal Transfer

Conjugal transfer was performed using the method of Elhai and Wolk (1988), with the adaption of Cai and Wolk (1990) used to screen for double recombinants.

a) Preparation of *E. coli*

For each transformation plate 10 ml of cells containing the conjugal plasmid (conjugal strain) and 10 ml of cells containing the cargo and helper plasmids (cargo strain) were grown in LB containing the appropriate antibiotics. The cells were harvested in late exponential phase by centrifugation (5,000 g, 5 min, 20°C) and the cells resuspended in

10-25 ml of LB without antibiotics, the conjugal strain must not be vortexed. Equal volumes of the conjugal and cargo strains were mixed, harvested by centrifugation and resuspended, without vortexing, in LB without antibiotics (200 µl per plate). The mixture was allowed to stand at room temperature while the cyanobacteria were prepared.

b) Preparation of Cyanobacteria

The growth phase of the cyanobacteria does not appear to matter, so cultures were harvested in the mid-logarithmic growth phase. Forty ml of culture is sufficient for several plate matings. Cultures were harvested by centrifugation (4,000g, 10 min, 20°C) and resuspended in a minimal volume of growth medium that was easily pipettable. If the filaments were to be fragmented to one or two cells in length, prior to transformation, the resuspended filaments were sonicated for 8 seconds on setting 3 with 50% duty power using a Sonifier Cell Disruptor B-30. The cells were pelleted by centrifugation (12,000 g, 1 min, 20°C) in a microfuge and resuspended in the same volume of growth media. Disruption of the filaments was checked using phase contrast microscopy. The cells were serially diluted 1:10 up to 1:100,000 in order to obtain single colonies, with the volume of the dilution being 100 µl or less.

c) Conjugation

A sterile filter (Nucleopore REC-85) was placed on the mating plate containing cyanobacterial growth medium without antibiotic. For each plate, 200 µl of the *E. coli* suspension (conjugal strain plus cargo strain) was mixed with 10 µl of cyanobacteria, there was one plate for each dilution of cyanobacteria. The mixture was spread onto the filter and the filter incubated for 24 h under optimal growth conditions to allow expression of the antibiotic resistance gene. The filter was then transferred to a plate containing cyanobacterial growth medium and the appropriate antibiotic at the pre-determine minimal inhibitory concentration required to kill untransformed cyanobacteria. The plate was incubated under optimal growth conditions. When using a cargo plasmid that contains *sacB* (eg pRL271) colonies that were present after 3 weeks of growth were streaked onto plates containing cyanobacterial growth medium supplemented with 5% (w/v) sucrose and the appropriate antibiotic and incubated for a further 3 weeks under optimal growth conditions. Colonies that survive sucrose selection should be double recombinants. Sucrose tolerant colonies were either transferred to liquid culture to make

them axenic and genomic DNA was isolated for analysis, or used as the template for PCR reactions (as discussed in Chapter 6)

2.17.2 Transformation by Electroporation

Attempts were also made to transform *Anabaena* PCC 7120 using electroporation (Thiel and Poo 1989). *Anabaena* PCC 7120 was harvested during mid-logarithmic growth phase by centrifugation (4,000g, 10 min 20°C), washed twice in 1 mM HEPES (pH 7.2) and resuspended in the above buffer to a cell density of approximately 10^9 cells ml^{-1} and chilled on ice. Plasmid DNA was isolated from the cargo strain used in the conjugal transfer experiments (Section 6.3.3) using the method described in Section 2.9.3.b. The plasmid DNA was resuspended in 1 mM HEPES (pH 7.2). Plasmid DNA was added to 40 µl of cyanobacterial cells to give final concentrations of between 1 and 10 µl ml^{-1}. The mixture was added to an electroporation cuvette with a 2 mm gap between the electrodes and the sample electroporated. A 25 µF capacitor was used and the resistor was changed to give different time constants (100 Ω resistor for a time constant of 2.5 ms and a 200 Ω resistor for a time constant of 5 ms). Cells were given a single pulse and immediately diluted and rinsed out of the cuvette with 2 ml of growth medium. The cells were harvested by centrifugation (4,000 g, 10 min 20°C) washed once with 5 ml of growth media and resupended in 10 ml of growth media. Cells were grown in liquid culture without antibiotics for 24 h and then harvested by centrifugation (4,000 g, 10 min 20°C) and resuspended in a minimal volume of growth media. The cells were serially diluted 1:10 to 1:100,000 and plated out onto growth media, containing the appropriate antibiotic. After 3 weeks colonies that appeared were transferred to plates containing growth media, 5% (w/v) sucrose and the appropriate antibiotic. Sucrose tolerant colonies were treated in the same way as the sucrose tolerant colonies that arose from conjugal transfer (Section 2.17.1.c).

CHAPTER 3

STUDIES ON PYRIDINE NUCLEOTIDE DEHYDROGENASES FROM SOYBEAN CHLOROPLASTS AND *ANABAENA* PCC 7120

3.1 INTRODUCTION

A large body of evidence has emerged for the existence of a respiratory pathway (chlororespiration) in chloroplasts (Section 1.4), but, there is no direct evidence for the presence of a dehydrogenase, to drive chlororespiration, in the chloroplasts of higher plants. Eleven *ndh* genes are encoded within the chloroplast genome and a number of these have been shown to be expressed (Matsubayashi *et al.* 1987; Schantz and Bogorad 1988; Steinmüller *et al.* 1989; Vera *et al.* 1990). The products of two NdhK and NdhH have been shown to be targeted to the stroma lamellae (Nixon *et al.* 1989; Berger *et al.* 1993b). Taken together these findings suggest that there may be an enzyme homologous to mitochondrial complex I in the stroma lamellae of higher plant chloroplasts. This is supported by the finding that there is a rotenone-sensitive NAD(P)H quinol oxidase present in the chloroplasts of *C. reinhardtii* (Godde and Trebst 1980; Godde 1982).

While this study was in progress other workers, working on spinach, demonstrated the presence of a rotenone-sensitive NADH quinol oxidoreductase on both the thylakoid and envelope membranes (D. Day, P. Vottero, A.-J. Dorne, M. Block and J. Joyard, unpublished results). These workers have also shown that the stromal fraction from spinach chloroplasts contains a rotenone-sensitive NADH quinol oxidoreductase. This finding confuses the results somewhat, as it is unknown if the activities seen in the thylakoids, envelope membranes and stromal fraction are catalysed by different enzymes or if one or more of the activities can be attributed to contamination from one of the other fractions. It was hoped that the studies on soybean, presented here, which were being conducted at the same time as the studies discussed above, would help to clarify the issue. Soybean was chosen for two reasons: firstly, a project characterising the mitochondrial pyridine nucleotide dehydrogenases was being carried out in the laboratory and secondly, other workers in the laboratory had cloned and sequenced *ndhK* from soybean chloroplasts (Whelan *et al.* 1992). If soybean chloroplasts proved suitable for studying pyridine nucleotide dehydrogenases, antibodies could then be raised against NdhK in order to determine if it was present in the same part of the chloroplast that any pyridine nucleotide dehydrogenase activity was found.

Evidence for the existence of complex-I like enzymes in cyanobacteria is very strong (Section 1.3.2). During the course of my honours year, I presented evidence that this enzyme is confined to the plasma membrane of *Anabaena* PCC 7120: during the initial phase of my Ph.D I confirmed and extended these results which were subsequently published (Howitt *et al.* 1993). A summary of the main results are included in this chapter.

3.2 RESULTS

3.2.1 Analysis of NAD(P)H Dehydrogenase Activity in Soybean Thylakoids

Thylakoid membranes from soybeans were assayed for NAD(P)H→Q_1 activity (Table 3.1). Both activities were present in the membrane, although the level of activity was extremely low (3-7 nmol NAD(P)H min^{-1} mg^{-1} protein), and both were partially sensitive to rotenone. The sensitivity to rotenone was a specific effect of rotenone and not the solvent ethanol, as in controls in which ethanol alone was added no inhibition of the NAD(P)H→Q_1 activity was seen (data not shown). The membrane fractions that showed activity were assayed for cytochrome c oxidase activity (2.4.6) and succinate dehydrogenase activity (2.4.5) to determine if the activity seen was due to mitochondrial contamination in the thylakoid preparation. No cytochrome oxidase activity or succinate dehydrogenase activity was detected in any of the preparations, indicating that the preparations were not contaminated with mitochondria.

The above results suggest that soybean chloroplasts may contain an NDH-1. To try and confirm this, immunoblotting was carried out with antibodies raised against complex I isolated from red beet mitochondria. Figure 3.1 shows that the antibodies cross reacted strongly with the positive control, soybean cotyledon mitochondria (lane a). Nine proteins, with apparent molecular weights of 61, 55, 54, 52, 43, 41, 28, 27 and 19 kDa (minor bands are indicated with arrows), from soybean thylakoids cross-reacted with the antibodies raised against complex I (lane b), providing further evidence for an enzyme homologous to complex I in the soybean thylakoids.

The F_1 portion of the F_1F_0 ATPase is a common contaminant of complex I preparations (Ragan 1985), and it is possible that some of the bands seen in Figure 3.1 were due to cross-reaction with the CF_1 in soybean chloroplasts. Therefore a second western blot was carried out with antibodies raised against the F_1 portion of spinach thylakoid ATPase. Five proteins from spinach thylakoids were immunoreactive with the antibodies (Figure 3.1, lane c). These had apparent molecular weights of 60, 56, 44, 42 and 32 kDa(minor bands are indicated with arrows). Four of these have almost identical molecular weights to proteins that cross reacted with the antibodies raised against complex I and thus they can not be identified. Nonetheless, 5 other cross-reacting proteins, with apparent molecular weights of 54, 52, 28, 27 and 19 kDa, were seen in the complex I blot suggesting that there are proteins homologous to subunits of mitochondrial complex I in soybean chloroplasts. This is in agreement with the inhibitor studies (Table 3.1)

A native gel (non-denaturing)(2.5.3), which allows the separation of membrane proteins in their functional form, was run to try and determine if a single dehydrogenase catalysed both NADH and NADPH oxidation, or if there were separate dehydrogenases for both substrates. Lane a (Figure 3.2) contains soybean thylakoid membranes that were stained for NADH dehydrogenase activity, while lane b contains thylakoid membranes that were stained for NADPH dehydrogenase reductase activity. By comparing the two it can be seen that the banding pattern is different for both substrates, indicating that the two activities are probably catalysed by different enzymes.

3.2.2 Localisation of the NAD(P)H Dehydrogenase Activity within Soybean Chloroplasts

The products of two of the *ndh* genes have been shown to be located on the stroma lamella (Nixon *et al.* 1989; Berger *et al.* 1993b), it was decided to fractionate soybean thylakoids and assay all of the fractions for the rotenone-sensitive NAD(P)H$\rightarrow Q_1$ activity. The method of Berthold *et al,* (1981) (2.3.4, Figure 3.3) was used to fractionate the thylakoid membranes. This method is based on the fact that the grana stacks are less susceptible to detergent solubilisation than the unstacked stroma lamellae. Therefore, if the concentration of detergent added is correct, the stroma lamellae will be solubilised but the grana stacks will remain intact and can be removed from the solubilised stroma lamellae by centrifugation. No activity was found in either the pellet, which is predominantly grana, or the supernatant from the first detergent solubilisation step. The supernatant from the second detergent solubilisation step, which is predominantly stroma lamellae, showed both NADH and NADPH$\rightarrow Q_1$ (Table 3.2). These activities had higher rates than those seen in thylakoid membranes (20-50 nmol NAD(P)H min^{-1} mg^{-1} protein) and both were partially sensitive to rotenone (Table 3.2). This activity was not inhibited when ethanol was added to the same concentration, indicating the inhibition seen by rotenone was not an ethanol effect. No succinate dehydrogenase or cytochrome oxidase activity could be detected in the fraction that showed the activity, indicating no mitochondrial contamination was present in the sample.

Attempts were also made to isolate envelope membranes from soybean chloroplasts using the method of Douce and Joyard (1982) (2.3.5). Small amounts of purified envelope membrane were isolated, however the quantities were too small to be useful for further study. This area was not pursued any further.

3.2.3 Pyridine Nucleotide Dehydrogenases in the Cyanobacterium *Anabaena* PCC 7120

The results presented in this section are confirmation and extensions of the work I did in my honours year and have been published (Howitt *et al.* 1993). Plasma and thylakoid membranes from *Anabaena* PCC 7120 were isolated using the method of Murata and Omata (1988) (2.3.2). Chlorophyll could not be detected in the plasma membrane samples used for analysis. The ability of both membranes to oxidise NADH, NADPH and deamino NADH was tested; the sensitivity of these activities to rotenone was also tested.

Both NADH and deamino NADH were readily oxidised by isolated plasma membrane (728 and 806 nmol O_2 h^{-1} mg^{-1} protein respectively) the oxidation of NADH was almost completely sensitive to the respiratory poison rotenone, while deamino NADH oxidation was only partially sensitive to rotenone (Table 3.3). Both of these features are characteristics of a type-1 NADH dehydrogenase (Yagi 1991). The ability of the plasma membrane to oxidise NADH in a rotenone-sensitive manner is in agreement with studies on *Synechocystis* PCC 6803 (Berger *et al.* 1991; Dzelzkalns *et al.* 1994). NADPH was also oxidised in a rotenone-sensitive manner by the plasma membrane, but the rate was about one fifth of the rate of NADH oxidation (Table 3.3). In contrast to this, oxygen uptake by the thylakoid membrane with NADH, NADPH and deamino NADH as substrates was not inhibited by rotenone (Table 3.3) and occurred at much lower rates (14, 12 and 6 nmol O_2 h^{-1} mg^{-1} protein respectively), indicating that the pyridine nucleotide dehydrogenases on the thylakoid membrane are different from those on the plasma membrane.

The ability of the plasma membrane of *Anabaena* PCC 7120 to oxidise NADPH in a rotenone-sensitive manner indicated it may contain a novel type of NDH-1, as bacterial type-1 NADH dehydrogenases are unable to oxidise NADPH (Yagi 1991). Therefore native gels were run to determine if the same dehydrogenase that oxidised NADH and deamino NADH could also oxidise NADPH. It was also hoped that this would give some indication as to whether the plasma and thylakoid membranes used different dehydrogenases as the inhibitor studies indicated (Table 3.3).

Figure 3.4 indicates that the plasma and thylakoid membranes were pure. There were no similarities between the banding patterns for the thylakoid and plasma membranes (lanes a and b on each gel) for any of the activity stains, indicating there was no cross contamination between the membrane fractions.

The top band in the thylakoid membranes for each gel (lane a) is the chlorophyll front and this masks a slight band of activity with both deamino NADH and NADH (lanes 1a and

2a). This banding pattern is quite different from that seen when NADPH was the substrate (lane 3a), indicating that the thylakoid membrane contains a separate dehydrogenase for NADH and NADPH. The multiple bands with NADPH have been observed in *A. variabilis* (Scherer *et al.* 1988c) and identified as ferredoxin $NADP^+$-oxidoreductase. The plasma membrane of *Anabaena* PCC 7120 showed a single band of activity with deamino NADH, NADH and NADPH as the substrates, indicating that only one dehydrogenase, which can oxidise all three substrates, was present.

Table 3.4 shows that respiration on the thylakoid membrane with NADH as the substrate was inhibited by flavone. This, combined with the fact that rotenone did not inhibit, suggests that the NADH dehydrogenase on the thylakoid membrane is a type-2 NADH dehydrogenase (Yagi 1991).

The results of the inhibitor studies indicated that the plasma membrane of *Anabaena* PCC 7120 contained an NDH-1. To try and confirm this immunoblotting was carried out with antibodies raised against complex I isolated from red beet mitochondria. Figure 3.5 shows that the antibodies cross react strongly with proteins from soybean cotyledon mitochondria (positive control (lane 2a). There was some cross-reactivity with the proteins from *Anabaena* PCC 7120 thylakoid membranes (lane 2b; 2 major bands with apparent molecular weights of 51 and 56 kDa and 5 minor bands (indicated with arrows) with apparent molecular weights of 61, 40, 38, 35 and 34 kDa). The plasma membrane from *Anabaena* PCC 7120 (lane 2c) showed a higher degree of cross-reactivity with the antibodies, 11 bands being visible with apparent molecular weights of 62, 59, 56, 54, 52, 48, 41, 36, 34 and 31 kDa.

The F_1 portion of spinach thylakoid F_1F_0 ATPase is a common contaminant of the complex I preparations from mitochondria (Ragan 1985), and it is possible that some bands seen in Figure 3.5 were due to cross reactivity with the CF_1 of *Anabaena* PCC 7120. Therefore a second western blot was carried out with an antibody raised against the CF_1 portion of spinach thylakoid ATPase (kindly provided by Dr P. Whitfield). The result is shown in Figure 3.6, as can be seen with the plasma membrane fraction, two major bands were immunoreactive with the ATPase antibodies (lane a). These bands had apparent molecular weights of 58 and 55 kDa, and therefore confuse the identity of two of the bands seen in the blot with complex I antibodies (Figure 3.5, lane 2c). Nonetheless, several other bands were seen in the complex I blot and it is apparent the plasma membrane of *Anabaena* PCC 7120 contains an enzyme homologous to complex I from plants. This is in agreement with the inhibitor studies (Table 3.3).

The thylakoid membrane from *Anabaena* PCC 7120 also showed cross-reactivity with the antibodies raised against spinach ATPase (Figure 3.6, lane b); five bands were seen with apparent molecular weights of 60, 55, 40, 38 and 36 kDa. These sizes are very close to

5 of the bands seen in the complex I blot (Figure 3.5, lane b). Thus it is difficult to assign identities to these bands and no conclusion can be drawn as to the identity of the NADH dehydrogenase on the thylakoid membrane.

3.3 DISCUSSION

3.3.1 Analysis of NAD(P)H Dehydrogenase Activity in Soybean Thylakoids

The rotenone-sensitive NAD(P)H-quinone oxidoreductase activity reported here, along with similar findings in spinach (D. Day, P. Vottero, A.-J. Dorne, M. Block and J. Joyard, unpublished results), are the first reports of such activity in the chloroplasts of higher plants. The level of activity detected was extremely low (Table 3.1). When different thylakoid fractions were assayed for this activity it was found that the activity was only present in a stroma lamellae enriched fraction (Table 3.2). The activity in this fraction was higher than that seen in thylakoid membranes. This increase probably represents a partial purification of the enzyme particularly as the level of rotenone-sensitivity seen was similar. There was no cytochrome oxidase or succinate dehydrogenase activity in any of the preparations that showed this activity, therefore the possibility that the activity was due to mitochondrial contamination can be ruled out.

Unfortunately attempts to isolate envelope membranes were unsuccessful and it could not be determined if there was an NAD(P)H quinone oxidase-reductase present on these membranes, as has been seen in spinach (D. Day,P. Vottero, A.-J. Dorne, M. Block and J. Joyard, unpublished results). Small quantities of envelope membrane were isolated from soybean, but the quantities isolated were insufficient for either activity studies or western analysis. There were a number of reasons behind the failure to isolate envelope membranes from soybean. Firstly, due to space limitations insufficient amounts of starting material were used, 25 pots of plants (250 plants) only yielded 700 g of leaves. Secondly, compared to spinach leaves soybean leaves are relatively dry thus larger volumes of isolation medium need to be used. Thirdly, intact chloroplasts are extremely difficult to isolate from soybean. Less than 50% of the chloroplasts were isolated intact. Attempts to increase the yield of intact chloroplasts by grinding for shorter times at lower speeds were unsuccessful. Intact chloroplasts are required for successful isolation of envelope membranes (Douce and Joyard 1982) and the low yield of intact chloroplasts significantly reduced the potential yield of envelope membranes.

The products of two of the *ndh* genes encoded in chloroplasts, NdhK and NdhH, have been localised to the stroma lamellae (Nixon *et al.* 1989; Berger *et al.* 1993b), raising the possibility that the rotenone-sensitive NAD(P)H-quinone oxidoreductase activity reported here is due to the activity of a chloroplast encoded NDH-1. The results of the western blot with antibodies raised against complex I (Figure 3.1) support this as they indicate that soybean thylakoids contain proteins that are homologous to subunits of mitochondrial complex I. This NAD(P)H-quinone oxidoreductase may be the site of entry of electrons for the chlororespiratory pathway postulated to be present in the chloroplasts of higher plants. This will be discussed in more detail in Chapter 7.

While this is the first report of rotenone-sensitive NAD(P)H-quinone oxidoreductase activity in the chloroplasts of higher plants, an NADH-plastoquinone oxidoreductase activity has been shown to occur in the thylakoids of *C. reinhardtii* chloroplasts (Godde and Trebst 1980). This enzyme was partially purified, shown to contain flavins and could utilise both NADH and NADPH (Godde 1982). The sensitivity of NADPH oxidation to rotenone was not tested. Further studies on the enzyme present in soybean chloroplasts will be needed to determine if it is similar to the enzyme in *C. reinhardtii.*

An NAD(P)H-ferricyanide (FeCN) oxidoreductase activity has been shown to be present in barley thylakoids (Vera *et al.* 1990; Cuello *et al.* 1995), and this activity has been shown to be sensitive to rotenone, but very high concentrations of rotenone were required to see inhibition (Cuello *et al.* 1995). Studies in which both NADH and NADPH were added together indicated that the rates were additive suggesting that the activities were catalysed by 2 different enzymes, one for each substrate (Cuello *et al.* 1995). The activity staining for NADH and NADPH dehydrogenase activity in soybean thylakoids (Figure 3.2) suggest that these two activities, present in the membrane, are catalysed by different enzymes as the banding pattern is different for both substrates. It is unknown if the enzymes catalysing the NADH and NADPH dehydrogenase activity in soybean thylakoids are also catalysing the rotenone-sensitive NAD(P)H-quinone oxidoreductase activity. Nor is it known if the enzyme(s) catalysing the rotenone-sensitive NAD(P)H-quinone oxidoreductase activity in soybeans are homologous to the enzyme(s) in barley thylakoids catalysing the rotenone-sensitive NAD(P)H-ferricyanide (FeCN) oxidoreductase activity. If it is, it would suggest that either the enzymes are not homologous to mitochondrial complex I or the mechanism of NAD(P)H oxidation is different, as has been suggested to be the case in cyanobacteria (Funk *et al.* 1994). In mitochondrial complex I (NDH-1) the NADH-ferricyanide reductase activity is a partial activity of the peripheral flavoprotein fragment (Hatefi 1985) and is insensitive to rotenone (Menz *et al.* 1992; Friedrich *et al.* 1994a; Friedrich *et al.* 1994b).

Soybean chloroplasts had proven to be a an extremely difficult system to work with, with respect to characterisation of NDH-1, and attempts to isolate pure envelope and thylakoid

membranes were unsuccessful, therefore it was decided not to pursue this area any further. Instead it was decided to pursue characterisation of NDH-1 in photosynthetic organisms in the cyanobacterium *Anabaena* PCC 7120. This was chosen as it is relatively easy to purify plasma and thylakoid membranes from cells of *Anabaena* PCC 7120, both membranes have been characterised with respect to the pyridine nucleotide dehydrogenases (Howitt *et al.* 1993) and reliable transformation systems have been developed (Elhai and Wolk 1988; Thiel and Poo 1989; Cai and Wolk 1990). This would enable the NDH-1 to be manipulated at a molecular level and its functions within the cell to be determined.

3.4 SUMMARY

The results presented in this chapter showed that a rotenone-sensitive NAD(P)H-quinone oxidoreductase activity was present on the stroma lamellae of soybean thylakoids. This work and the unpublished results of D. Day, P. Vottero, A.-J. Dorne, M. Block and J. Joyard, on spinach, are the first demonstrations of such an activity in the chloroplasts of higher plants.

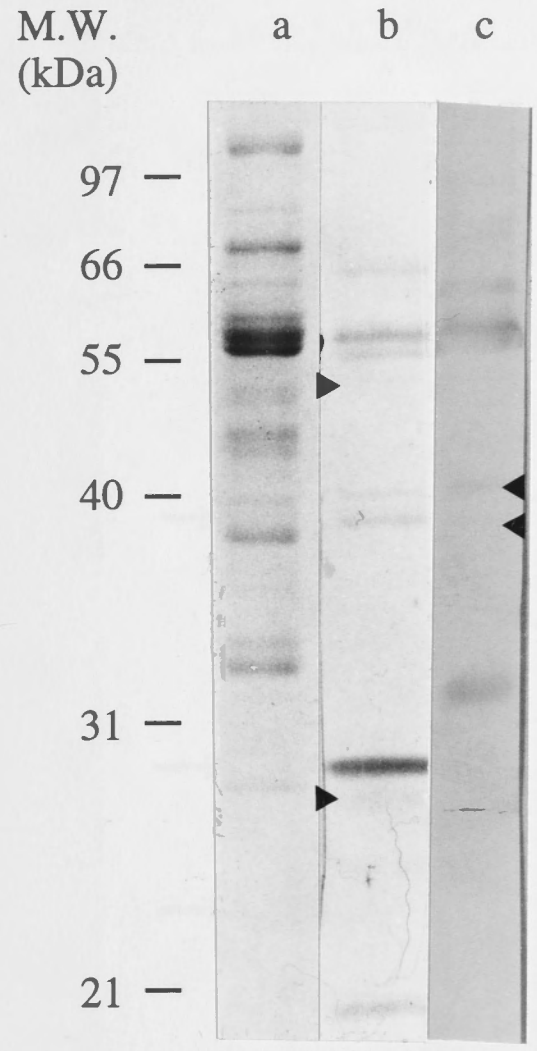


Figure 3.1: Immunological analysis of thylakoid membrane proteins from soybean, using antibodies raised against purified complex I from red beet and antibodies raised against the F_1 portion of the F_1F_0 ATPase from spinach thylakoids.

Proteins were separated by SDS-PAGE (Section 2.5), transferred to Hybond-C^+ and incubated with the antibodies (Section 2.6). The filters shown in lanes a and b were incubated with the antibodies raised against purified complex I from red beet mitochondria using 1 in 2,500 and 1 in 1,000 dilutions respectively. The filter shown in lane c was incubated with the antibodies raised against the F_1 portion of the F_1F_0 ATPase from spinach thylakoids diluted 1 in 50.

Lane a, 20 μg soybean cotyledon mitochondria proteins.
Lane b, 30 μg soybean thylakoid proteins.
Lane c, 30 μg soybean thylakoid proteins.

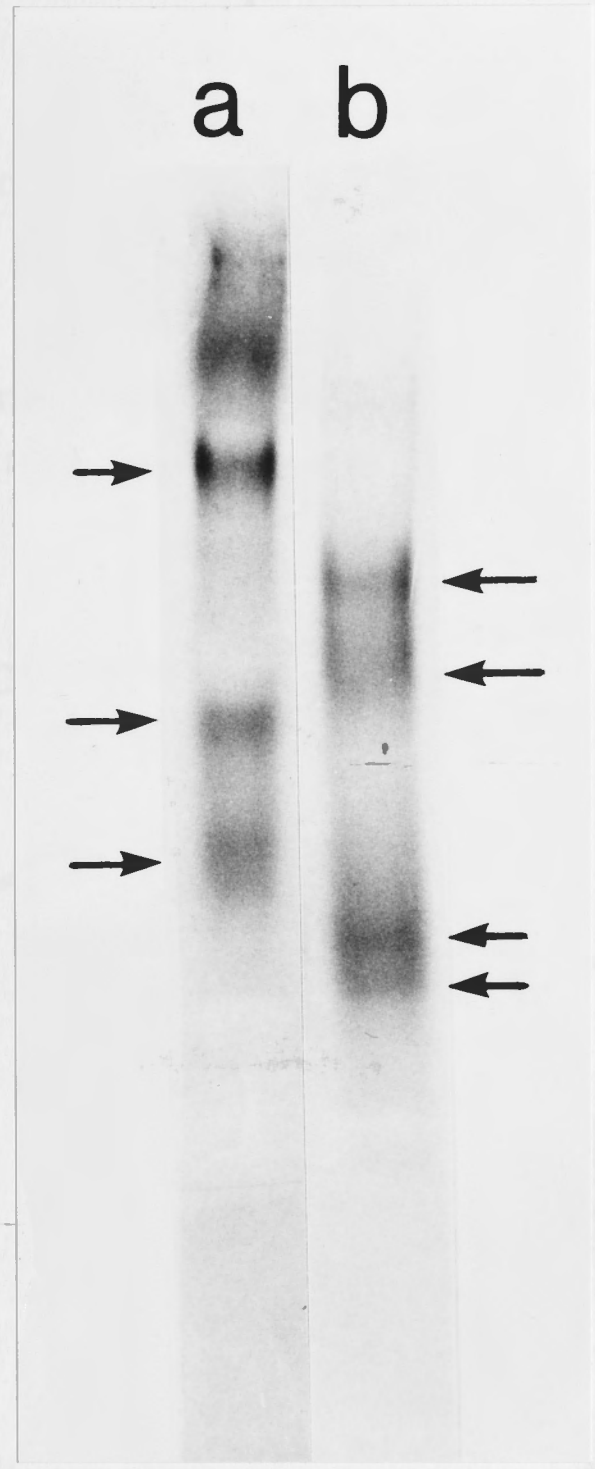


Figure 3.2: Native gel of soybean thylakoid proteins solubilised in Triton X-100 and stained for NADH- (**a**) and NADPH- (**b**) dehydrogenase activity. The arrows indicate the bands of activity. Each lane contains 10 μg of protein.

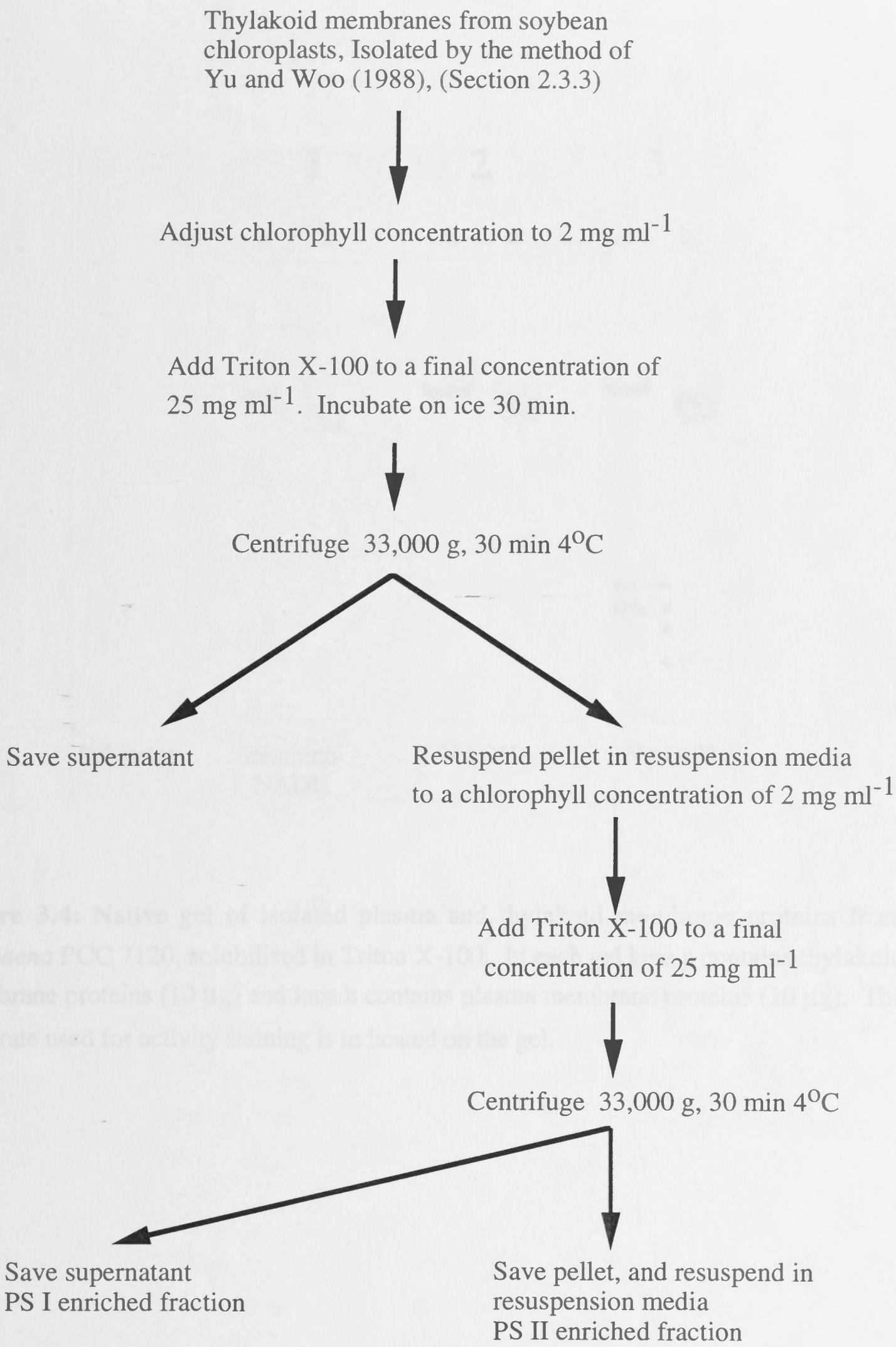


Figure 3.3: Fractionation of thylakoids into PS I and PS II enriched fractions by the method of Berthold *et al.* (1981)

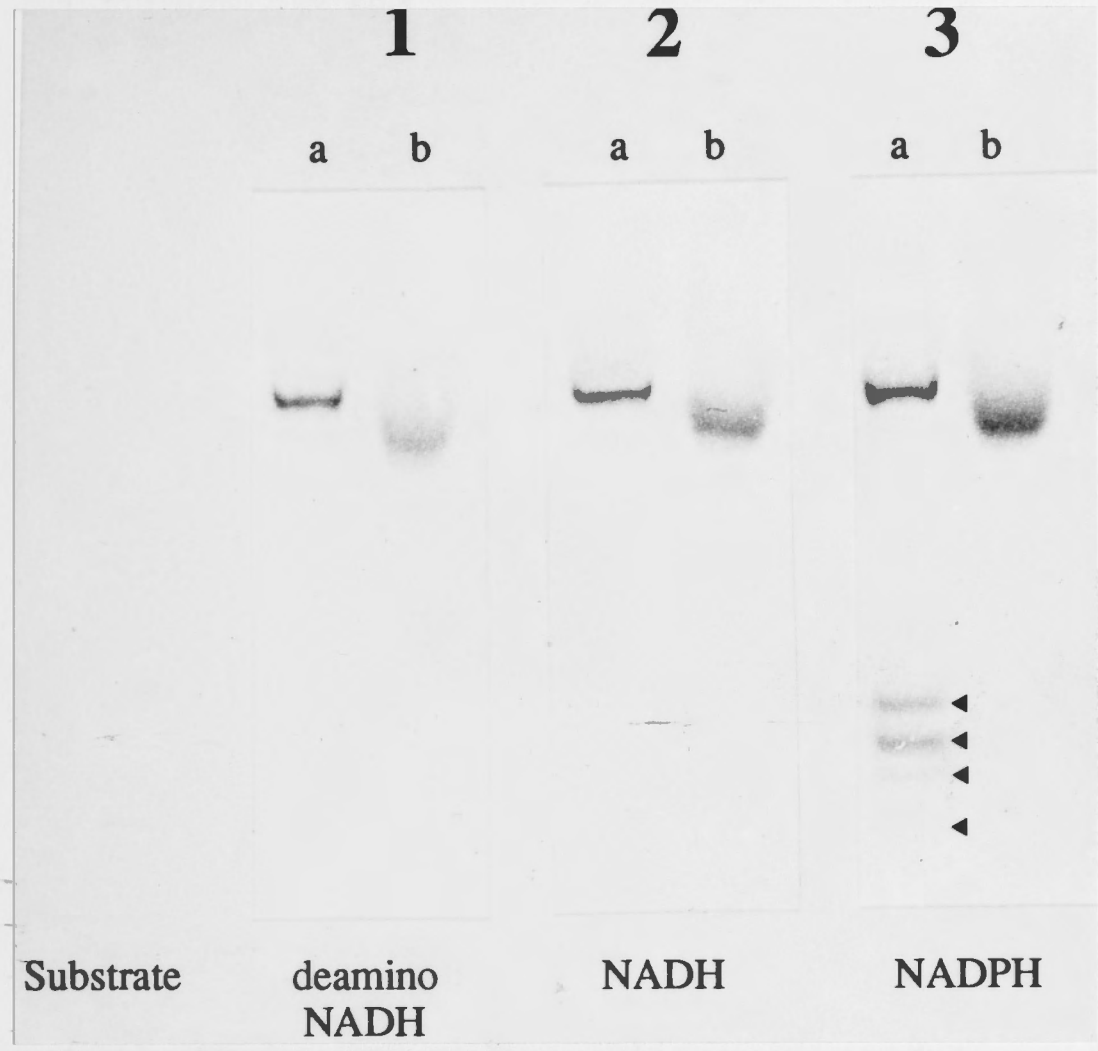


Figure 3.4: Native gel of isolated plasma and thylakoid membrane proteins from *Anabaena* PCC 7120, solubilised in Triton X-100. In each gel lane a contains thylakoid membrane proteins (10 μg) and lane b contains plasma membrane proteins (10 μg). The substrate used for activity staining is indicated on the gel.

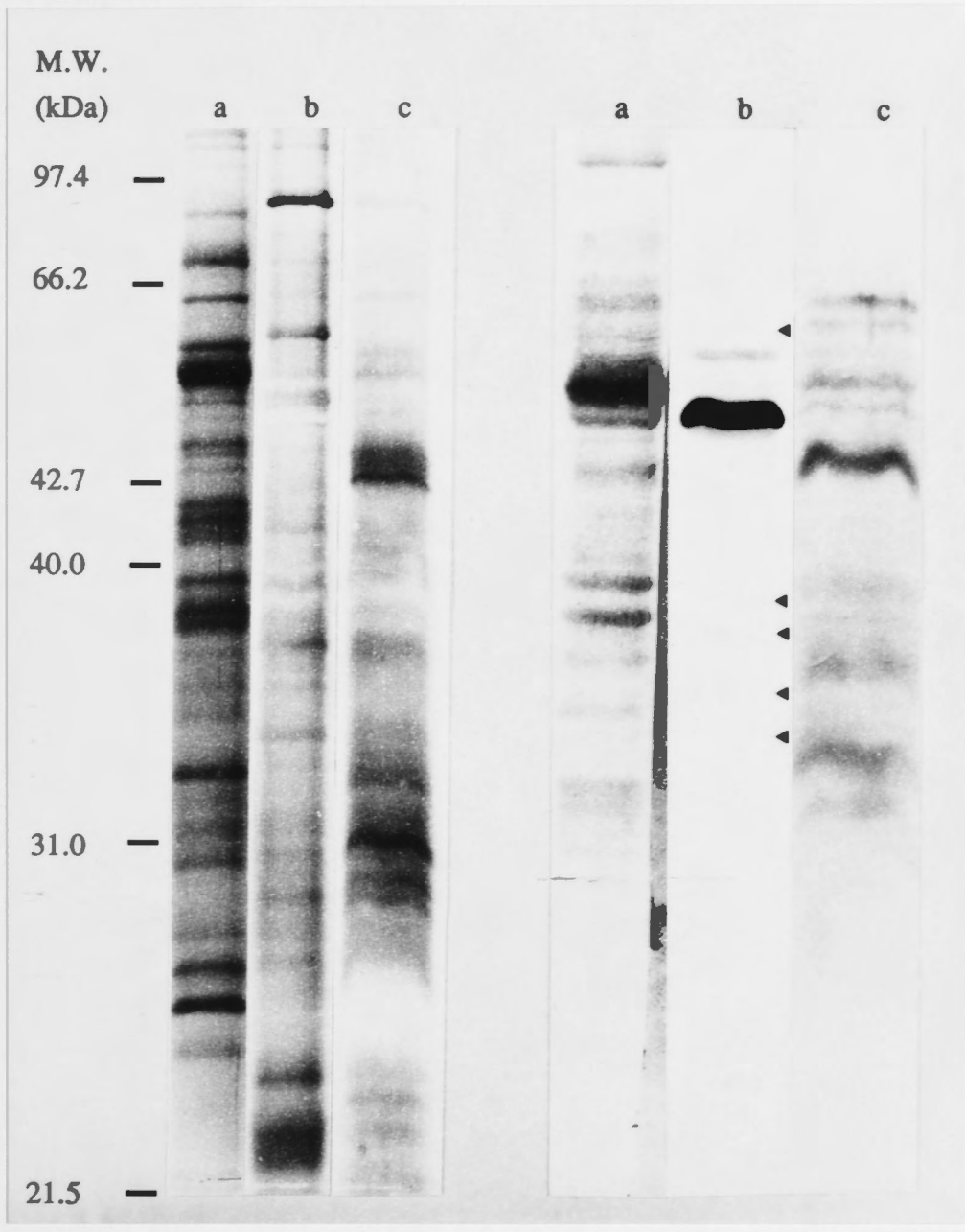


Figure 3.5: Western blot of plasma and thylakoid membrane proteins from *Anabaena* PCC 7120 using antibodies raised against purified complex I from red beet.

Plasma and thylakoid membranes were separated by SDS-PAGE (Section 2.6) and then transferred to Hybond-C$^+$ (Amersham). The filters were incubated with antibodies raised against complex I from red beet mitochondria. Antibodies were diluted 1 in 2,500 for soybean cotyledon mitochondria and 1 in 1,000 for all other lanes.

Panel 1: Coomassie blue stained SDS-PAGE, lane a 20 µg soybean cotyledon mitochondria proteins: lane b, 29 µg thylakoid membrane proteins from *Anabaena* PCC 7120: lane c, 22 µg plasma membrane proteins from *Anabaena* PCC 7120.

Panel 2: Western blot, protein concentrations were as in panel 1.

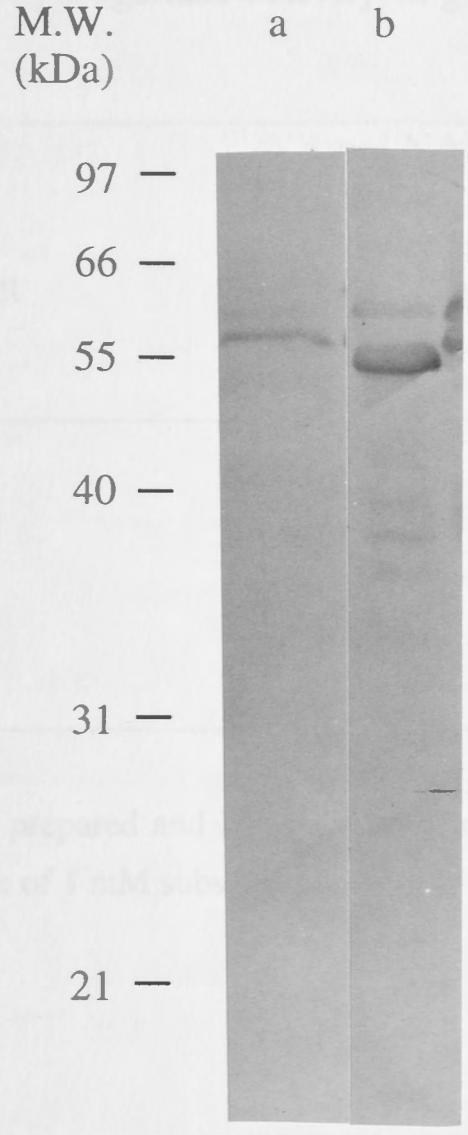


Figure 3.6: Immunological analysis of plasma and thylakoid membrane proteins from *Anabaena* PCC 7120, using antibodies raised against the F_1 portion of the F_1F_0 ATPase from spinach thylakoids.

Proteins were separated by SDS-PAGE (Section 2.5), transferred to Hybond-C^+ and incubated with the antibodies (Section 2.6). The filter shown was incubated with the antibodies raised against the F_1 portion of the F_1F_0 ATPase from spinach thylakoids diluted 1 in 50.

Lane a, 22 µg plasma membrane proteins from *Anabaena* PCC 7120.
Lane b, 29 µg thylakoid membrane proteins from *Anabaena* PCC 7120.

Table 3.1 NAD(P)H dehydrogenase activity in grana-enriched particles from soybean chloroplasts.

Substrate	Experiment Number	nmol NAD(P)H min^{-1} mg^{-1} protein	
		Control	+ rotenone
NADH	1	4	2
	2	7	4
	3	3	2
NADPH	1	7	5
	2	7	4

Thylakoid membranes were prepared and NADH oxidation measured as described in Chapter 2. The control rate is in the presence of 1 mM substrate and 80 μM Q_1, while "+ rotenone" is in the presence of 25 μM rotenone.

Table 3.2 NAD(P)H dehydrogenase activity in the stroma lamellae from soybean chloroplasts.

Substrate	Experiment Number	nmol NAD(P)H min^{-1} mg^{-1} protein	
		Control	+ rotenone
NADH	1	50	37
	2	37	25
NADPH	1	28	20
	2	20	16

Thylakoid membranes were prepared and NADH oxidation measured as described in Chapter 2. The control rate is in the presence of 1 mM substrate and 80 μM Q_1, while "+ rotenone" is in the presence of 25 μM rotenone.

Table 3.3 Effect of rotenone on the respiration of plasma and thylakoid membranes of Anabaena PCC 7120. (Howitt *et al.*. 1993)

	Oxygen Uptake			
	Plasma membrane		Thylakoid membrane	
Substrate	control	+ rotenone	control	+ rotenone
	(nmol O_2 h^{-1} mg^{-1}protein)			
NADH	728 ± 134	78 ± 13	14 ± 4	14 ± 3
Deamino-NADH	806 ± 214	564 ± 25	6 ± 3	6 ± 1
NADPH	149 ± 4	13 ± 1	12 ± 3	11 ± 1

Oxygen consumption was measured as described in Chapter 2. All substrates were added to give a final concentration of 1 mM, and 30 μM cyt c was present in all assays. Rotenone was added at a final concentration of 20 μM. Values shown are means ± S.E. of separate experiments (n = 3-4).

Table 3.4 Effect of Flavone on NADH oxidation by isolated thylakoid membranes from Anabaena PCC 7120. (Howitt *et al.*. 1993)

Sequential additions to reaction vessel	Oxygen uptake (nmol O_2 h^{-1} mg^{-1} protein)
Thylakoid membrane plus NADH (1 mM)	11.6 ± 1.6
Cyt c (30 mM)	17.2 ± 2.1
Flavone (1 mM)	11.2 ± 2.5

Oxygen consumtion was measured as described in Chapter 2. Values shown are means ± S.E. (n = 4) of separate experiments.

CHAPTER 4

PRODUCTION AND USE OF ANTIBODIES RAISED AGAINST NdhK FROM *SYNECHOCYSTIS* PCC 6803

4.1 INTRODUCTION

As discussed earlier (Section 1.3.2 and Section 3.2.3), inhibitor studies and western blots with antibodies raised against purified complex I from red beet mitochondria indicated that NDH-1 was only present on the plasma membrane in *Anabaena* PCC 7120 (Howitt *et al.* 1993). This is apparently not the case in *Synechocystis* PCC 6803 where NDH-1 is found on both the plasma membrane and the thylakoid membrane (Berger *et al.* 1991; Dzelzkalns *et al.* 1994). These analyses were performed using inhibitors and antibodies raised against a subunit of NDH-1. To further investigate this difference, and confirm that NDH-1 is only on the plasma membrane in *Anabaena* PCC 7120 it was decided to raise antibodies against a subunit of NDH-1 from *Synechocystis* PCC 6803. Recently a subcomplex of NDH-1 from *Synechocystis* PCC 6803 has been purified and the product of *ndhK* was shown to be present (Berger *et al.* 1993a). Therefore it was decided to raise antibodies against NdhK from *Synechocystis* PCC 6803. These antibodies would also be useful in future studies in which attempts would be made to insertionally inactivate *ndhK* in *Anabaena* PCC 7120.

The first step in making antibodies against a protein when the gene which encodes it is available, is to clone it into an expression vector, overexpress it and purify the protein. The expression vector chosen for this study was pGEX-2T (Pharmacia) (Smith and Johnson 1988). When the gene of interest is cloned into pGEX-2T and overexpressed the desired protein will be expressed as a fusion protein with glutathione-S-transferase (GST). Often the fusion protein will display domain behaviour: ie each of the two proteins that make up the fusion protein will retain their structure and properties (Gray *et al.* 1982; Slilaty *et al.* 1990). This allows the properties of GST to be utilised to rapidly purify the fusion protein. GST binds reduced glutathione, therefore the fusion protein can be rapidly purified, provided it is soluble, by using affinity chromatography. The soluble cellular extract is passed through a column which contains agarose beads that have reduced glutathione bound to them. The fusion protein should bind to the reduced glutathione allowing the rest of the cellular proteins to be eluted from the column. Once the contaminating proteins have been eluted from the column, reduced glutathione can be added to elute the fusion protein. Alternatively thrombin can be added and the column incubated to allow cleavage of the fusion protein, as a thrombin recognition site is present at the carboxy terminus of GST. Once cleavage is complete the purified protein of interest can be eluted from the column.

4.1.1 Aims

The aim of this part of the project was to clone *ndhK* from *Synechocystis* PCC 6803 using PCR. This would then be cloned into an expression vector, overexpressed and purified. Antibodies would then be raised using standard techniques and these would be used to study the distribution of NdhK in *Anabaena* PCC 7120.

4.2 RESULTS

4.2.1 Production of Antibodies

a) Cloning of *ndhK* from *Synechocystis* PCC 6803

In order to clone *ndhK* from *Synechocystis* PCC 6803, primers were designed to the published sequence (Steinmüller *et al.* 1989). As shown in Figure 4.1 the 5' primer (SNDHK5') was designed with an in frame *Bam HI* site in the middle of the primer. This was to allow the PCR product to be cloned into the expression vector pGEX-2T (Pharmacia) in frame. The 3' primer (SNDHK3') contained an *Eco RI* site down stream of the stop codon of *ndhK,* also to allow cloning of the PCR product into the expression vector. SNDHK5' was designed to a region 12 amino acids downstream of the start of NdhK, as primers designed to incorporate the start codon had the potential to bind to SNDHK3'. *Synechocystis* PCC 6803 was grown and genomic DNA isolated as described in Sections 2.2.1 and 2.9.2 The genomic DNA was then used as template for amplification using PCR. A sample of the reaction run on an agarose gel showed a single product of approximately 720 bp, as expected (Figure 4.2. lane 1a). To confirm the correct region of DNA had been amplified the PCR product was partially restriction mapped (*ndhK* from *Synechocystis* PCC 6803 contains an *Ava I* site 100 bp from the 3' end). The digested PCR product was subjected to Southern analysis using a partial clone of *ndhK* from the chloroplasts of soybeans (Whelan *et al.* 1992) as a probe. Figure 4.2 shows the partial restriction map of the PCR product: lane 1a contained the uncut PCR product giving a 720 bp fragment as expected; lane 1b contained the PCR product digested with *Ava I* and the fragment produced was approximately 100 bp shorter than the fragment in lane 1a, indicating that *ndhK* from *Synechocystis* PCC 6803 had been amplified. The results of the Southern analysis confirmed this as both the uncut PCR product (lane 2a) and the *Ava I* digested PCR product (lane 2b) hybridised with the probe.

To confirm the PCR product was from *Synechocystis* PCC 6803 and not an amplification of a contaminant in either the genomic DNA preparation or the PCR reaction, it was purified using a Wizard DNA purification kit (Promega) (Section 2.11.3). The purified DNA was then digested sequentially with *Eco RI* and *Bam HI* and cloned into pGEM3Zf(+) (Promega) cut with *Eco RI* and *Bam HI*, giving plasmid pSNDHK. pSNDHK was sequenced from one end. Figure 4.3 shows an alignment of the sequence from the cloned PCR product with the published sequence of *ndhK* from *Synechocystis* PCC 6803 (Steinmüller *et al.* 1989). This confirmed that *ndhK* from *Synechocystis* PCC 6803 had been cloned.

b) Over expression and purification of NdhK from *Synechocystis* PCC 6803

The *Bam HI, Eco RI* fragment was cloned into pGEX-2T (Pharmacia), giving pGEX-SNDHK. Competent *E. coli* strain JM109 was transformed with pGEX-SNDHK, the transformed cells were grown, and expression of the fusion protein was induced, as described in section 2.7.1 Cells were disrupted, the soluble and insoluble cellular fractions prepared as described in section 2.7.1, and the proteins separated by polyacrylamide gel electrophoresis (2.13). Figure 4.4 shows a comparison of proteins from cells in which expression of the fusion protein was induced and those in which expression was not induced. The insoluble cellular fraction of cells in which expression of the fusion protein had been induced contained a protein with an apparent molecular weight of 44 kDa, that was present in quantities in excess of any other protein (lane c). This protein was absent from the insoluble cellular fraction of cells in which expression of the fusion protein had not been induced (lane d). This protein was the correct size to be the NdhK-GST fusion protein. The gel also showed that the fusion protein was insoluble.

Purification of GST fusion proteins requires the protein to be in a soluble form so that the properties of GST can be used to purify the protein of interest (Section 4.1.1). A modified version of the method of Fish and Hoare (1988) was used to solubilise the fusion protein (Section 2.7.2). The insoluble cellular proteins were solubilised using 8 M urea and then the urea concentration was slowly lowered by dilution, in an attempt to get the fusion protein to refold correctly. The solubilised fraction was then subjected to affinity chromatography using a Glutathione Sepharose 4B column (Pharmacia) as described in section 2.7.2 As can be seen in Figure 4.5 the technique successfully solubilised the fusion protein (lane a), but it can also be seen that the fusion protein did not bind to the column and washed through with the other solubilised cellular debris (lane b). No proteins of a similar molecular weight to NdhK were eluted from the column after cleavage with thrombin (lane d). Further modification of the method in which the urea

concentration was diluted to 1 M and/or Triton X-100 added to the sample to a final concentration of 1% (w/v), also proved unsuccessful. Solubilisation of the fusion protein using Triton X-100 also proved unsuccessful. As can be seen in Figure 4.6 the majority of the proteins from the insoluble cellular fraction, including the fusion protein, remained insoluble even after 2 hours incubation with 2% (w/v) Triton X-100 (lane b) and there was no fusion protein in the soluble fraction after Triton X-100 solubilisation (lane a).

Purification of the fusion protein using affinity chromatography had proven unsuccessful, therefore it was decided to first purify inclusion bodies from *E. coli* and then make an antibody against the complete fusion protein rather than just NdhK. Initially the method of Harlow and Lane (1988) was used (2.7.3.a). The majority of the insoluble cellular fraction co-purified with the fusion body (Figure 4.7, lane a). Therefore the method of Sambrook *et al.* (1989) was tried (2.7.3.b). As can be seen in Figure 4.7 (lane b), the inclusion body was not completely purified, but the purification was better than that seen using the method of Harlow and Lane (1988), with fewer proteins of lower molecular weight being present. Therefore it was decided to modify the method further to ascertain if the purification could be improved.

A number of modifications to this method were tried. The method finally used involved the addition of two steps to the method of Sambrook *et al*, (1989) described in section 2.7.3.b. After the incubation with 0.5% Triton X-100 on ice, the sample was centrifuged for 30 s at 250 g at 4°C. The supernatant was saved and the pellet resuspended in a minimum volume of lysis buffer. Both the supernatant and the resuspended pellet were passed through a 1.2 μm filter (Millipore). The filtrate from both samples was then taken, separately through the remaining steps in the purification procedure (Section 2.7.3.b). In this case a large proportion of the fusion protein remained unpurified in the pellet from the 250 g spin (Figure 4.7 lane d), but a proportion was present in an almost pure form in the fraction that was pelleted from the supernatant from the 250 g spin (Figure 4.7 lane f). There were two contaminating proteins of lower molecular weight present in the purified sample. As the yield using this procedure was relatively low the preparation was scaled up in order to allow sufficient fusion protein to be purified to raise antibodies against. Approximately 1.5 mg of fusion protein was prepared using this method from a 1 l culture of *E. coli* in which the fusion protein was overexpressed. The partially purified inclusion bodies were run on a 12% polyacrylamide gel and the fusion protein excised as a final purification step in order to prevent antibodies being raised against the two contaminating proteins. Polyclonal antibodies were raised in rabbits according to the method outlined in Section 2.7.

4.2.2 Immunological Investigations Using Antibodies Raised Against NdhK from *Synechocystis* PCC 6803

a) Investigation of membrane fractions from *Anabaena* PCC 7120

The antibodies raised against NdhK from *Synechocystis* PCC 6803 were used to probe preparations of total membranes from *Anabaena* PCC 7120 to determine if growth conditions had any effect on the level of expression of NdhK. A protein with an apparent molecular weight of 35 kDa cross-reacted with the antibodies in all 10 growth conditions tested (Figure 4.8 Panel 1), and it is of approximately equal intensity in all lanes suggesting that growth conditions did not affect expression of the cross-reacting protein. As the antibodies were raised against a fusion protein of NdhK with GST, the membrane was stripped and reprobed with polyclonal antibodies raised in rabbits against GST alone. As can be seen a protein with an apparent molecular weight 35 kDa also cross-reacted with the antibodies raised against GST in all conditions tested (Figure 4.8 Panel 2). It is, therefore, unlikely that the protein cross-reacting with the antibodies was homologous to NdhK.

The plasma membrane only represents a very small proportion of the total membranes present in a cell preparation. Therefore, plasma and thylakoid membranes were purified and these were subjected to western analysis with antibodies raised against the fusion protein and GST alone. Cells grown under four different growth regimes were used for these studies. The antibodies against the fusion protein cross-reacted with a protein with an apparent molecular weight of 35 kDa in the thylakoid membrane fraction from cells grown under three of the growth regimes tested (Figure 4.9 lanes 1d, 1f, and 1h). A protein with an apparent molecular weight of 28 kDa in the plasma membrane fraction from the same three growth conditions also cross-reacted with the antibodies against NdhK (Figure 4.9 lanes 1c, 1e, 1g). The cross-reacting band was of approximately equal intensity in all three conditions. This is the correct size to be the protein encoded by *ndhK* from *Anabaena* PCC 7120 (as discussed in Chapter 5), and was the same size as a the only protein from a total membrane preparation from *Synechocystis* PCC 6803 that cross-reacted with the antibodies (data not shown). The blot was stripped and reprobed with the antibodies against GST. A protein with an apparent molecular weight of 35 kDa in the thylakoid membrane fractions from the three growth conditions in which a protein of the same size cross-reacted with the antibodies against the fusion protein also cross-reacted with the antibodies raised against GST (Figure 4.9, lanes 2d, 2f and 2h). No other proteins cross-reacted with the antibodies raised against GST. These results confirm the plasma membrane specific location of NDH-1 in *Anabaena* PCC 7120. As no proteins in the purified plasma or thyalkoid membranes from the fourth growth condition tested cross-reacted with either antibody it suggests that the samples had degraded. This is supported by the finding that proteins from total membrane

preparations from the same growth condition cross-reacted with both antibodies (Figure 4.8 lane 1c and 2c).

b) Investigation of membrane fractions from the chloroplasts of higher plants

The presence of a partially rotenone-sensitive NAD(P)H→Q_1 activity on the stroma lamellae (3.2.2) suggests that there is an NDH-1 in this region of the thylakoid membrane from higher plant chloroplasts. To try and confirm this, immunoblotting was carried out on the three different thylakoid fractions, isolated using the method of Berthold *et al.* (1981), that had been assayed for the NAD(P)H→Q_1 activity, with the antibodies raised against the NdhK-GST fusion protein. Figure 4.10 shows that the antibodies cross-reacted with proteins from all three samples (lanes 1a: 2 bands with apparent molecular weights of 53 and 42 kDa; 1b: 1 band with an apparent molecular weight of 53 kDa; 1c: 1 band with an apparent molecular weight of 29 kDa). One of the proteins, from the PS II enriched fraction (Figure 4.10, lane 1c), that cross-reacted with the antibody was in the correct size range to be NdhK (~ 27 kDa, by analogy to NdhK from other plant chloroplasts). Spinach thylakoid and envelope membranes (kindly provided by Dr J. Joyard) were also run on the gel. A number of proteins in both samples cross-reacted with the antibodies against the NdhK-GST fusion protein (thylakoids lane 1d: 1 band with an apparent molecular weight of 29 kDa; envelope membranes lane 1e 2 bands with apparent molecular weights of 67 and 56 kDa). One of the proteins from the spinach thylakoids that cross reacted with the antibodies (Figure 4.10, lane d) was in the correct size range to be NdhK (~27 kDa).

The blot was stripped and reprobed with antibodies raised against GST. As can be seen by comparing Panel 1 and Panel 2 (Figure 4.10), for every protein from thylakoid membranes of both soybean and spinach that cross reacted with the NdhK-GST fusion protein antibodies, a protein with a similar apparent molecular weight also cross-reacted with the antibodies raised against GST. It is difficult to positively assign identities to these proteins, but it is more likely that they are cross-reacting with antibodies raised against the GST part of the fusion protein rather than NdhK. One protein with an apparent molecular weight of 67 kDa, from spinach envelope membranes cross-reacted with the antibodies raised against GST (Figure 4.10 lane 2e). This had a similar apparent molecular weight to one of the two proteins from spinach envelope membranes that cross-reacted with the antibodies raised against the NdhK-GST fusion protein. Therefore it is difficult to assign an identity to this protein. A protein from the envelope membrane of spinach with an apparent molecular weight of 56 kDa only cross-reacted with the NdhK-GST fusion protein antibodies; therefore it is likely that it was cross-reacting with antibodies raised against the NdhK part of the fusion protein. No identity can be assigned to this protein though, as it is twice the predicted size of NdhK from chloroplasts of higher plants (~27 kDa).

4.3 DISCUSSION

4.3.1 Purification of NdhK

NdhK from *Synechocystis* PCC 6803 was expressed as a fusion protein with GST. Often fusion proteins with GST display domain behaviour (Gray *et al.* 1982; Slilaty *et al.* 1990), thereby allowing the properties of GST to be used to purify the fusion protein. As can be seen from the results presented in Section 4.2.1 this was not the case. The properties of GST could not be used to purify the fusion protein even after it had been solubilised and the urea concentration dropped to a level that should allow refolding. This suggests that the fusion protein was not displaying domain behaviour. In hindsight this is not surprising as NdhK is thought to be a central subunit of an enzyme that is embedded in the membrane (Berger *et al.* 1993a; Weidner *et al.* 1993). Thus it would appear that the folding of NdhK interfered with folding of GST, a soluble protein, such that the fusion protein did not display the properties of GST. No attempts were made to grow induced cultures at lower temperatures to see if this improved solubility of the fusion protein.

It is unknown why the methods used to purify inclusion bodies from *E. coli* did not work. As NdhK is associated with the membrane this may have resulted in the fusion protein being associated or partially embedded in the membrane thus interfering with the purification. A number of modifications were made to the method used in order to get a nearly pure fusion protein, but the yield was low. Low yield of purified protein can be overcome, when using an overexpression system, simply by increasing the amount of *E. coli* overexpressing the protein used.

4.3.2 Immunological Studies of Membranes from *Anabaena* PCC 7120

The immunological analysis of total membranes from *Anabaena* PCC 7120 showed that only a single protein with an apparent molecular weight of 35 kDa cross-reacted with the antibodies raised against the NdhK-GST fusion protein (Figure 4.8, Panel 1). However a protein of identical apparent molecular weight also cross-reacted with antibodies raised against GST alone (Figure 4.8, Panel 2). It is likely that this protein is cross-reacting with epitopes raised against the GST part of the fusion protein rather than the NdhK part. Also this protein was the wrong size to be the protein encoded by *ndhK* from *Anabaena* PCC 7120 (see Chapter 5).

The 35 kDa protein was shown to be a thylakoid membrane specific protein (Figure 4.9). On the other hand, the plasma membrane contained a protein (28 kDa) that only cross-reacted with the antibodies raised against the NdhK-GST fusion protein and not those raised against GST. This protein is almost certainly NdhK. This identification is strengthened as the protein that cross-reacted with the antibodies had an apparent molecular weight that was the correct size to be the protein encoded by *ndhK* from *Anabaena* PCC 7120 (27.5 kDa, Chapter 5). The plasma membrane specific location of NdhK confirms the results of previous studies, using inhibitors to NDH-1 (Table 3.3) and antibodies raised against purified complex I from red beet mitochondria (Figure 3.5), which showed that NDH-1 in *Anabaena* PCC 7120 was located on the plasma membrane and not the thylakoid membrane (Chapter 3; Howitt *et al.* 1993). The presence of a protein of the correct size to be NdhK (~28 kDa) on the plasma membrane, that cross-reacts specifically with the antibodies raised against the fusion protein and not GST alone, is verification that the gene for *ndhK* from *Anabaena* PCC 7120 cloned (see Chapter 5) codes for a protein and is not a pseudogene. The intensity of the cross-reacting protein does not differ between samples from cells grown under different growth regimes (Figure 4.9) indicating that the growth conditions tested do not appear to effect the level of expression of *ndhK* in *Anabaena* PCC 7120.

The western blots of total membranes from *Anabaena* PCC 7120 (Figure 4.9) did not show the presence of the 28 kDa plasma membrane specific protein. This may be due to the fact that the majority of the membranes present in a total membrane preparation are thylakoid membranes; there is approximately 50 times more thylakoid than plasma membrane per cell (Wastyn *et al.* 1987; Murata and Omata 1988; Jeanjean *et al.* 1990).

The absence of the 28 kDa protein in the blots of total membrane fractions is further evidence for the plasma membrane specific location of NdhK and thus NDH-1. If NdhK was present on the thylakoid membrane, a protein that only cross-reacted with the NdhK-GST fusion protein antibodies, would have been expected in the total membrane preparations as they are predominantly thylakoid membranes.

4.3.3 Immunological Studies of Membranes from Higher Plant Chloroplasts

Figure 4.10 shows that there were no proteins in any of the fractions isolated from soybean thylakoids that cross-reacted specifically with the antibodies raised against the NdhK-GST fusion protein. All of the proteins that cross-reacted also cross-reacted with the antibodies raised against GST. One of the proteins, from the PS II enriched fraction (Figure 4.10 lane c), that cross-reacted was the correct size to be NdhK (approximately

27 kDa by analogy to NdhK from other plants). However, a protein with the same apparent molecular weight also cross-reacted with the antibodies raised against GST so these proteins can not be positively identified. These results differ from those of other workers Nixon *et al* (1989), using antibodies raised against NdhK from wheat chloroplasts, who showed that NdhK was located on the stroma lamellae of pea chloroplasts. Antibodies raised against the carboxy-terminal end of NdhH from rice chloroplasts have been shown to cross-react with a protein located on the stroma lamellae of spinach, beans, maize and sorghum (Berger *et al.* 1993b). Both of these studies used antibodies raised against chloroplast encoded copies of the Ndh proteins, while this study used antibodies raised against a cyanobacterial encoded Ndh protein. The reason why no proteins of the correct size for NdhK and specific for the NdhK-GST fusion protein antibody were seen might be that there is insufficient homology between NdhK from *Synechocystis* PCC 6803 and NdhK from soybean chloroplasts for the antibodies to recognise NdhK in soybean chloroplasts. At the amino acid level, NdhK from *Synechocystis* PCC 6803 (Steinmüller *et al.* 1989) has 60% identity to the partial sequence available for the chloroplast encoded NdhK from soybean (Whelan *et al.* 1992). The region of sequence missing from the chloroplast encoded copy of NdhK from soybean is the region that shows the lowest degree of homology between NdhK from *Synechocystis* PCC 6803 and NdhK from the chloroplasts of other higher plants (see Figure 5.13). Therefore the two proteins probably show less than 60% identity over their entire length. The proteins encoded by the *ndh* genes in chloroplasts of higher plants are very highly conserved (see Tables 5.2 and 5.3 for conservation of NdhK and NdhJ). The higher degree of conservation of the proteins among chloroplasts than between chloroplasts and cyanobacteria may explain why other workers have seen cross-species hybridisation when using antibodies raised against chloroplasts encoded proteins, while this study did not when using antibodies raised against a cyanobacterial protein.

No proteins from spinach thylakoids cross-reacted specifically with the antibodies raised against the NdhK-GST fusion protein and not to antibodies against GST (Figure 4.10). As was the case for the soybean thylakoids a protein of the correct size to be NdhK (~27 kDa) cross-reacted with both antibodies. Thus no identity can be assigned to this protein.

Interestingly a protein of approximately 56 kDa from the envelope membranes of spinach chloroplasts cross-reacted specifically with the antibodies raised against NdhK from *Synechocystis* PCC 6803 (Figure 4.10). This protein is approximately twice the predicted size of NdhK from the chloroplasts of plants in which *ndhK* has been cloned and sequenced. Berger *et al,* (1993b) showed that there were no proteins in the envelope of spinach thylakoids that cross-reacted with antibodies raised against the carboxy-terminal end of NdhH from rice. It is most likely, therefore, that the 56 kDa protein seen here contains an epitope similar to a sequence in NdhK.

4.4 SUMMARY

Antibodies were raised against NdhK from *Synechocystis* PCC 6803 and these were used to show that NdhK in *Anabaena* PCC 7120 is a plasma membrane specific protein, confirming the results of previous studies, which had shown that NDH-1 in *Anabaena* PCC 7120 was located only on the plasma membrane. This also confirms that *ndhK*, cloned from *Anabaena* PCC 7120 (Chapter 5), codes for a protein of the expected size and is not a pseudo gene. No proteins in the thylakoid membranes of soybean or spinach, of the correct size for NdhK, were seen that cross-reacted specifically with the antibodies raised against NdhK. A protein that was twice the predicted size of NdhK was present on the envelope membranes of spinach chloroplasts but no identity could be assigned to this protein.

SNDHK5'

ndhK S 6803 5' T GCT ACC CCC ACT GAC GTC GAA CGG G

! ! ! ! ! ! !

SNDHK5' 5' T GCT ACC CCC GGA TCC *ATG* GAA CGG G

Bam HI

SNDHK5' 5' T GCT ACC CCC GGA TCC ATG GAA CGG G

SNDHK3'

ndhK S 6803 5' GGC *TGA* GGA AGT GAA CTC CCC CAA

! !

5' GGC *TGA* GGA ATT CAA CTC CCC CAA

3' CCG ACT CCT TAA GTT GAG GGG GTT

Eco RI

SNDHK3' 5' TTG GGG GAG TTG AAT TCC TCA GCC

Figure 4.1: Primer design for amplification of *ndhK* from *Synechocystis* PCC 6803.

ndhK S 6803 = published sequence from *ndhK* from *Synechocystis* PCC 6803 (Steinmüller *et al.* 1989), triplets shown are in frame. SNDHK5' and SNDHK3' = sequence of primers used to amplify *ndhK* from *Synechocystis* PCC 6803. ! = nucleotides changed to introduce restriction sites and an in frame start codon into the primers; *ATG*, *TGA* = start and stop codons; XXX XXX = restriction sites designed within primers to facilitate cloning of *ndhK* into the expression vector in frame.

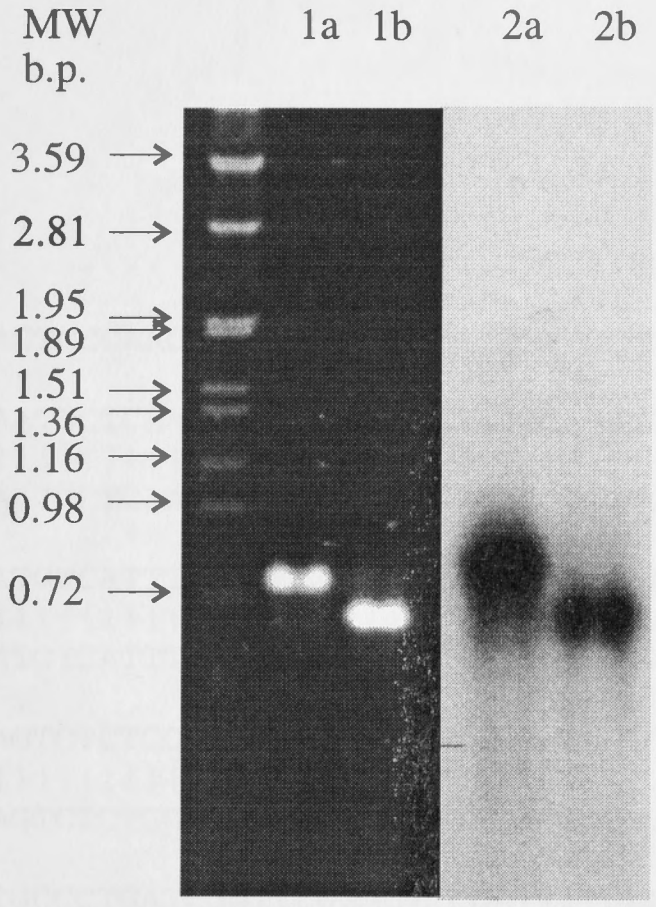


Figure 4.2: Southern analysis of genomic DNA from *Synechocystis* PCC 6803 amplified using PCR.

Genomic DNA (100 ng) was amplified using the primers SNDHK5' and SNDHK3'. The PCR product(s) were separated by gel electrophoresis (2.13), transferred to Hybond-N^+ (Amersham) (2.13) and subjected to Southern analysis using *ndhK* from soybean chloroplasts as the probe (2.14). The blot was washed at 50^0C using 0.5X SSC.

Panel 1: Agarose gel electrophoresis, lane a PCR product, lane b PCR product digested with *Ava* I.

Panel 2: Southern blot, loadings are as for panel 1.

```
                .         .         .         .         .
pSNDHK        1 ..........................CCATGGAACGGGTCGCCACAGC 22
                                           |  ||||||||||||||||||
S6803       521 ATGAGTCCCAACCCTGCTAACCCCACTGACCTGGAACGGGTCGCCACAGC 570
                .         .         .         .         .
pSNDHK       23 CAAAATTCTCAACCCCGCCAGCCGTAGCCAGGTCACCNAAGACCTTTCGG 72
                ||||||||||||||||||||||||||||||||||||||:|||||||||||
S6803       571 CAAAATTCTCAACCCCGCCAGCCGTAGCCAGGTCACCCAAGACCTTTCGG 620
                .         .         .         .         .
pSNDHK       73 AAAATGTCATTTTAACCACGGTGGATGACCTCTACAATTGGGCCAAACTT 122
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
S6803       621 AAAATGTCATTTTAACCACGGTGGATGACCTCTACAATTGGGCCAAACTT 670
                .         .         .         .         .
pSNDHK      123 TCTAGTCTCTGGCCGTTGTTGTATGGCACTGGTTGTTGCTTCATCGAATT 172
                |||||||||||||||||||||||||| ||||||||||||||||||||
S6803       671 TCTAGTCTCTGGCCGTTGTTGTATGGCACTGCTTGTTGCTTCATCGAATT 720
                .         .         .         .         .
pSNDHK      173 TGCCGCCCTGATCGGTTCGCGCTTCGATTTTGACCGATTTGGTTTGGTGC 222
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
S6803       721 TGCCGCCCTGATCGGTTCGCGCTTCGATTTTGACCGATTTGGTTTGGTGC 770
                .         .         .         .         .
pSNDHK      223 CCCGTTCTAGCCCCCGGCAAGCGGATTTAATTATAACC..........  260
                ||||  |||||||||||||||||||| |||||  |||
S6803       771 CCCGCTCTAGCCCCCGGCAAGCGGATTTGATTATCACCGCCGGAACCATC 820
```

Figure 4.3: Alignment of the partial sequence of pSNDHK with *ndhK* from *Synechocystis* PCC 6803.

The two sequences show 96% identity to each other.

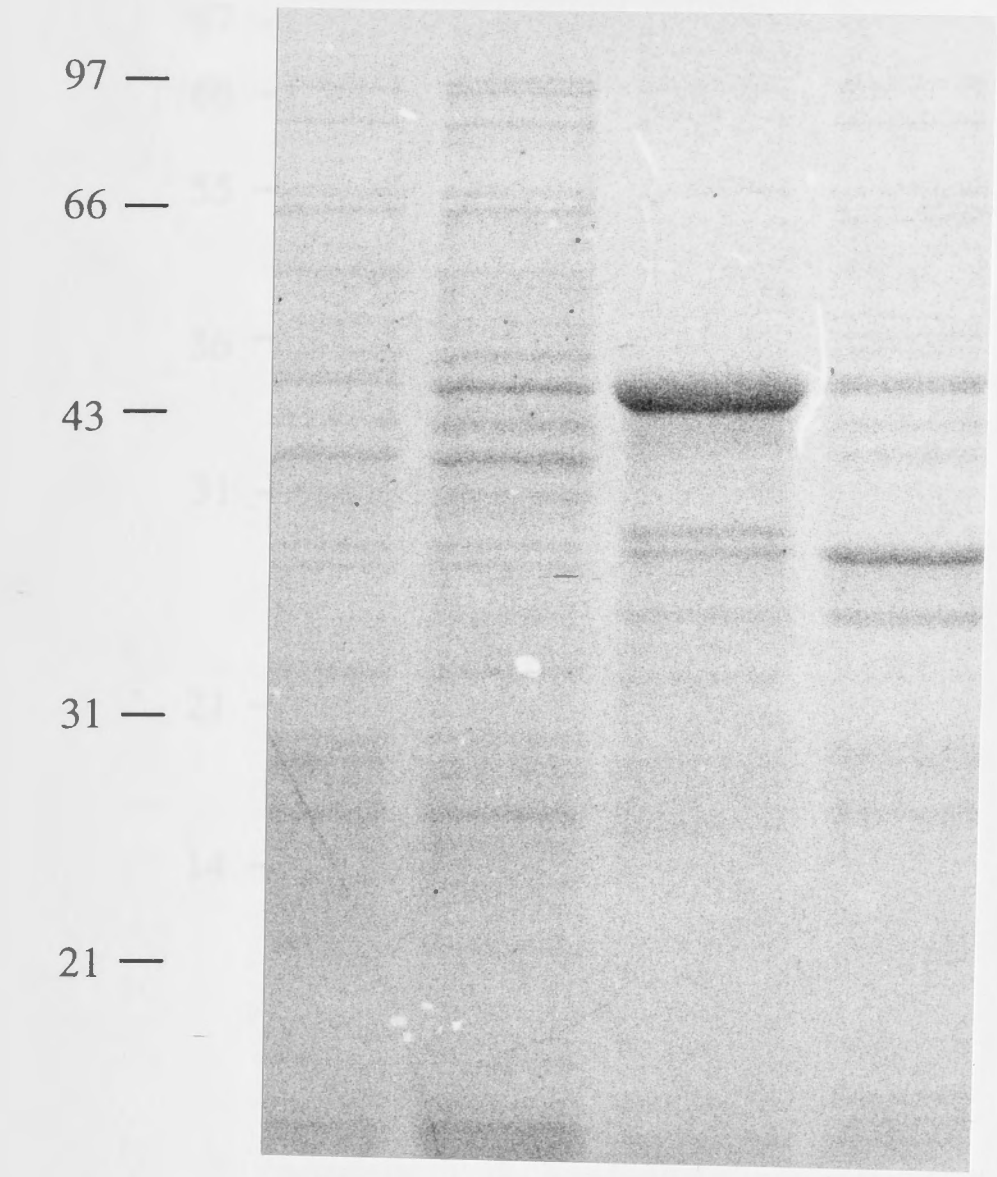


Figure 4.4: Comparison of protein content from the soluble and insoluble fractions from cells in which expression of pGEX-SNDHK had been induced and cells in which expression was not induced.

Expression of the fusion protein was induced and insoluble and soluble cellular fractions were prepared as described in section 2.7.1. Proteins were separated by SDS-PAGE and stained with coomassie blue (2.13). Each lane contains 10 µl of sample.

Lane a, soluble fraction from cells in which expression of the fusion protein had been induced.
Lane b, soluble fraction from cells in which expression of the fusion protein had not been induced.
Lane c, insoluble fraction from cells in which expression of the fusion protein had been induced.
Lane d, soluble fraction from cells in which expression of the fusion protein had not been induced.

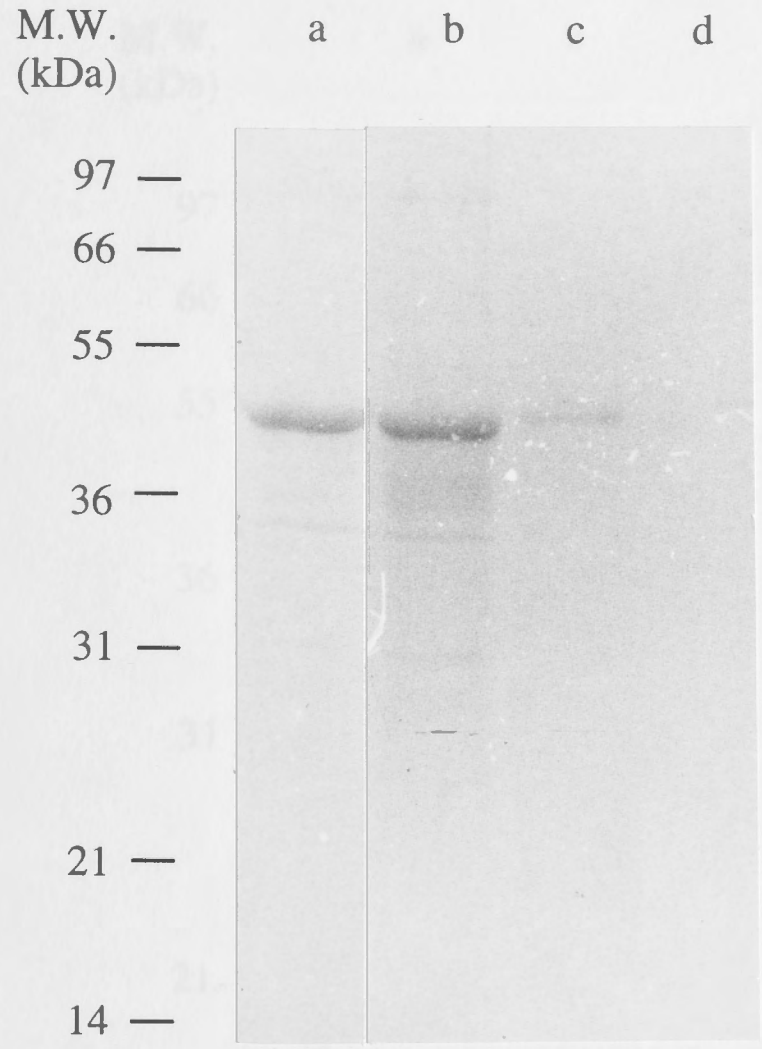


Figure 4.5: Urea soubilisation of the fusion protein and eluents from the glutathione speharose 4B column.

The insoluble fraction containing the fusion protein was soubilised in 8 M urea, and then the urea concentration was lowered to 2 M (2.7.2). The solubilised fraction was then subjected to affintiy chromatography using a glutathione sepharose 4B column (2.7.2), and the eluents from the column separated by SDS-PAGE and the proteins stained with coomassie blue (2.13). Each lane contains 10 µl of sample.

Lane a, Urea solubilised proteins from the insoluble cellular fraction containing the fusion protein.
Lane b, Proteins that did not bind to the glutathione sepharose 4B column.
Lane c, Proteins eluted from the column by the first wash.
Lane d, proteins eluted from the column after clevage with thrombin.

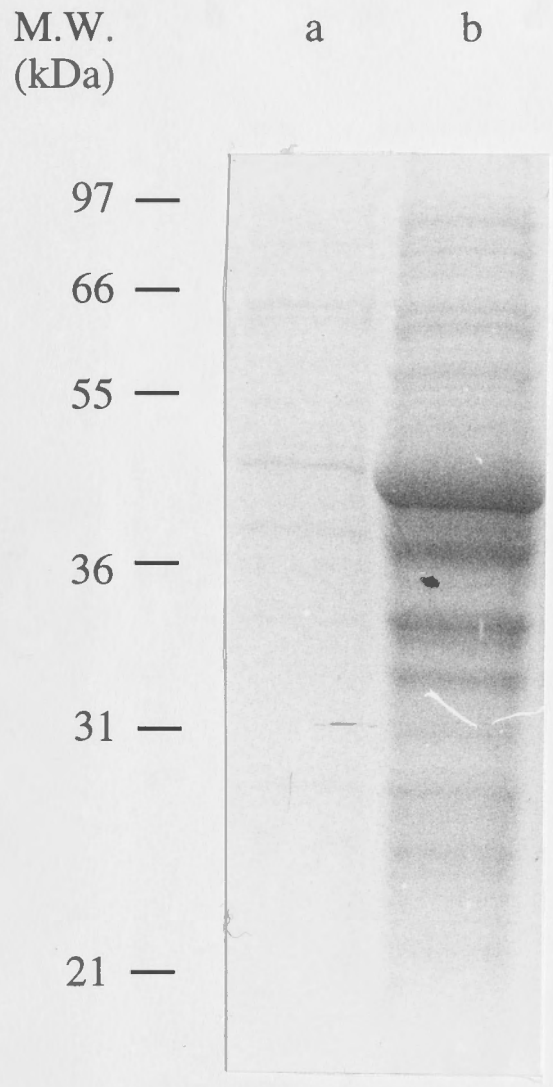


Figure 4.6: Triton X-100 soubilisation of the overexpressed fusion protein.

The insoluble fraction containing the fusion protein was incubated with 1% (v/v) Triton X-100 for 2 h, the soluble and insoluble fractions separated by centrifugation. The proteins in each fraction were separated by SDS-PAGE and the proteins stained with coomassie blue (2.13). Each lane contains 10 µl of sample.

Lane a, Proteins in the soluble fraction after Triton X-100 solubilisation.
Lane b, Proteins that remained insoluble after Triton X-100 solubilisation.

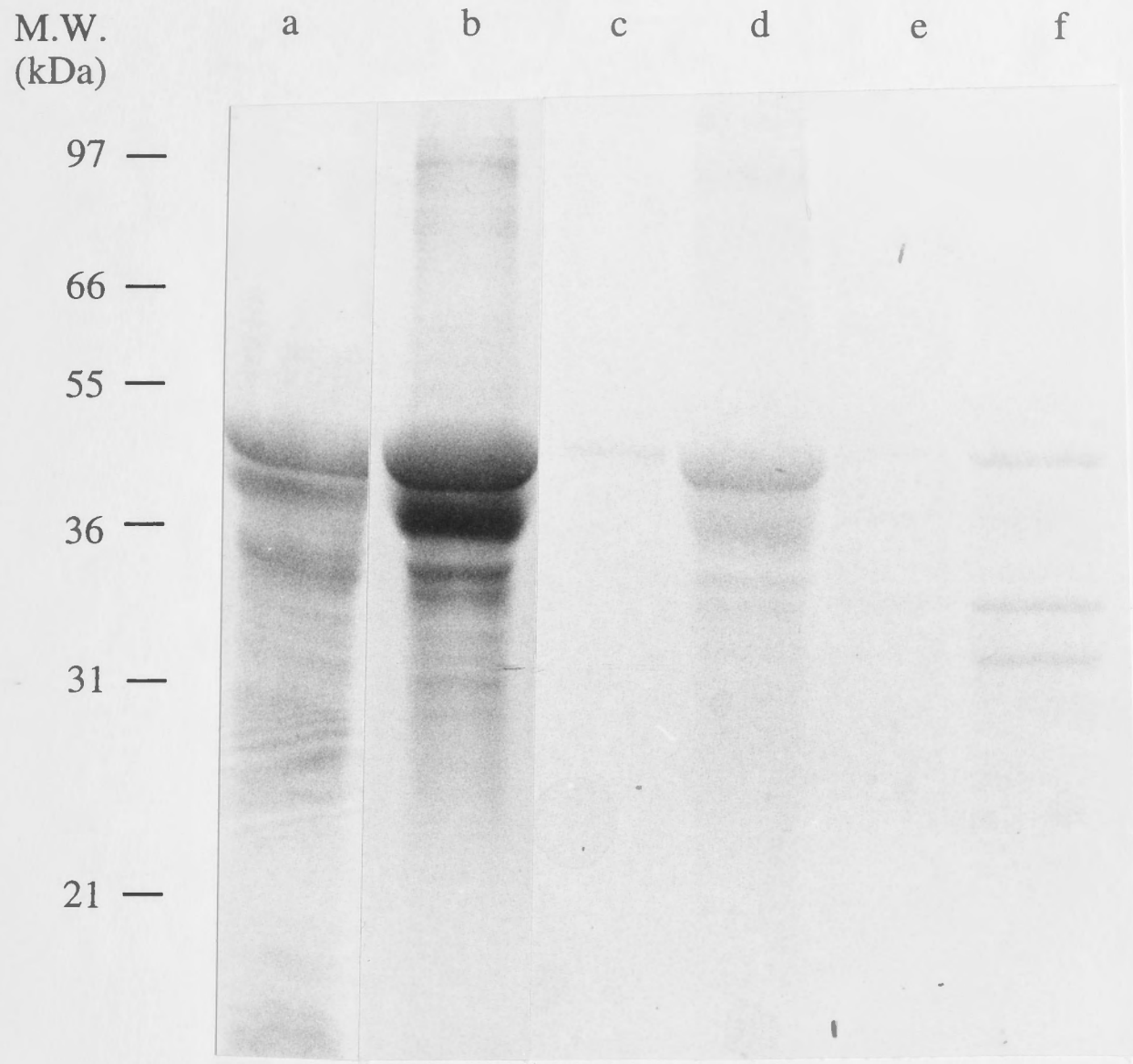


Figure 4.7: Purification of inclusion bodies from cells in which the fusion protein had been overexpressed.

Inclusion bodies were purified as described in sections 2.7.3.a and 2.7.3.b. Modifications to the second method used are as described in section 4.2.1.b. Proteins from the different purification steps were separated by SDS-PAGE and the proteins stained with coomassie blue (2.13). Each lane contains 10 µl of sample.

Lane a, Inclusion body purification using the method of Harlow and Lane (1988).
Lane b, Inclusion body purification using the method of Sambrook *et al.* (1989).
Lane c, Soluble proteins from the pellet from the 250 g spin.
Lane d, Insoluble proteins from the pellet from the 250 g spin.
Lane e, Soluble proteins from the supernatant from the 250 g spin.
Lane f, Insoluble proteins from the supernatant from the 250 g spin.

Figure 4.8: Immunological analysis of total membrane preparations from *Anabaena* PCC 7120, to determine the effect of growth conditions on the level of NdhK.

Total membrane proteins were separated by SDS-PAGE (2.5) and then transferred to Hybond-C$^+$ (Amersham) and subjected to western analysis (2.6)

Panel 1: Western blot using antibodies raised against the NdhK-GST fusion protein. The primary antibody was used at a 1 in 1,000 dilution and the secondary antibody was used at a 1 in 5,000 dilution. Each lane contained 55 µg of protein.
Lane a, continuous illumination, + air, without combined nitrogen in the growth medium.
Lane b, continuous illumination, + air, with combined nitrogen in the growth medium.
Lane c, continuous illumination, + 5% (v/v) CO_2, without combined nitrogen in the growth medium.
Lane d, continuous illumination, + 5% (v/v) CO_2, with combined nitrogen in the growth medium.
Lane e, 12 h light/dark cycle, + air, without combined nitrogen in the growth medium.
Lane f, 12 h light/dark cycle, + air, with combined nitrogen in the growth medium.
Lane g, 12 h light/dark cycle, + 5% (v/v) CO_2, without combined nitrogen in the growth medium.
Lane h, 12 h light/dark cycle, + 5% (v/v) CO_2, with combined nitrogen in the growth medium.
Lane c, continuous illumination, + 5% (v/v) CO_2, without combined nitrogen in the growth medium, + 50 mM NaCl.
Lane d, continuous illumination, + 5% (v/v) CO_2, with combined nitrogen in the growth medium, + 100 mM NaCl.

Panel 2: The membrane used for the blot shown in Panel 1 was stripped (2.6) and reprobed with antibodies raised against GST. The primary antibody was used at a 1 in 1,000 dilution and the secondary antibody was used at a 1 in 5,000 dilution.
The loadings in each lane were as in panel 1.

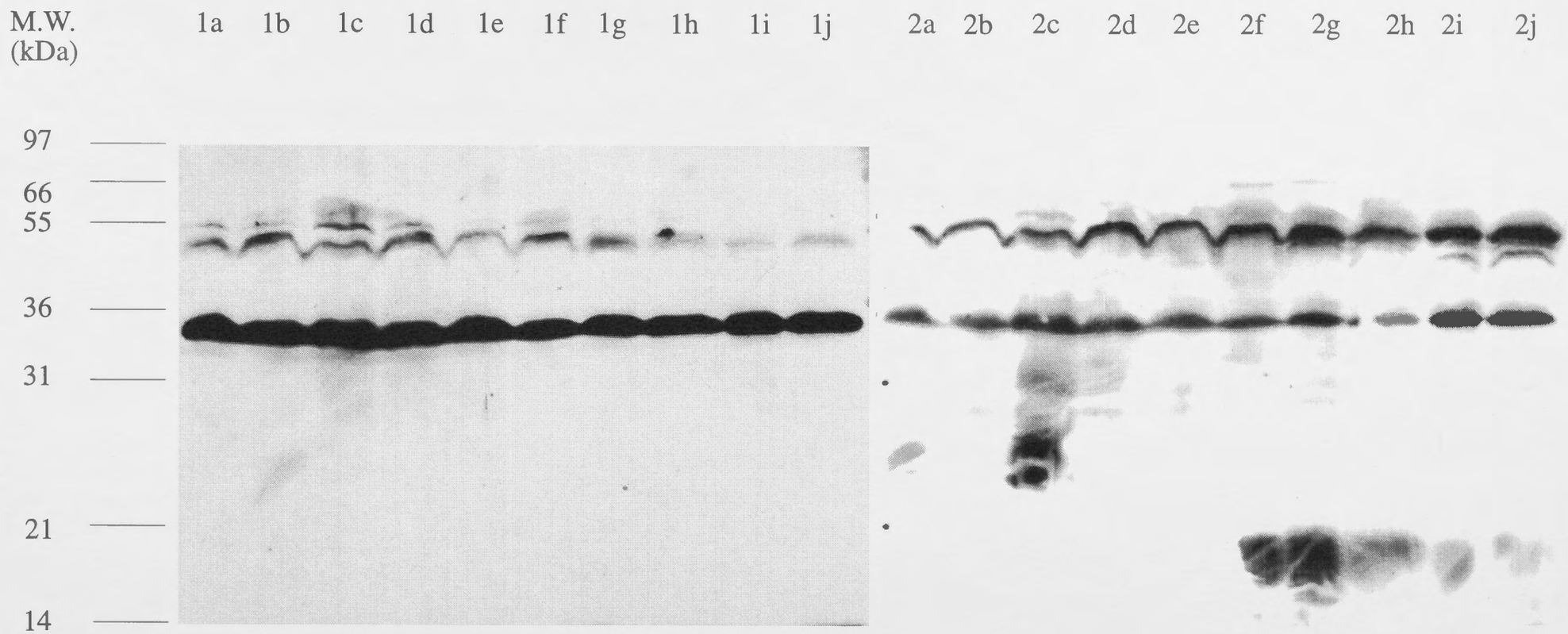

M.W.
(kDa)
1a
1b
1c
1d
1e
1f
1g
1h
1i
1j
2a
2b
2c
2d
2e
2f
2g
2h
2i
2j
97
66
55
36
31
21
14

Figure 4.9: Immunological analysis of isolated plasma and thylakoid membrane preparations from *Anabaena* PCC 7120, to determine the effect of growth conditions on the level of NdhK.

Plasma and thylakoid membrane proteins were separated by SDS-PAGE (2.5) and then transferred to Hybond-C+ (Amersham) and subjected to western analysis (2.6)

Panel 1: Western blot using antibodies raised against the NdhK-GST fusion protein. The primary antibody was used at a 1 in 1,000 dilution and the secondary antibody was used at a 1 in 5,000 dilution.
Lane a, 25 µg of plasma membrane protein from cells grown under continuous illumination, + 5% (v/v) CO_2, without combined nitrogen in the growth medium.
Lane b, 55 µg of thylakoid membrane protein from cells grown under continuous illumination, + 5% (v/v) CO_2, without combined nitrogen in the growth medium.
Lane c, 25 µg of plasma membrane protein from cells grown under continuous illumination, + air, without combined nitrogen in the growth medium.
Lane d, 55 µg of thylakoid membrane protein from cells grown under continuous illumination, + air, without combined nitrogen in the growth medium.
Lane e, 25 µg of plasma membrane protein from cells grown under light/dark cycle, + 5% (v/v) CO_2, without combined nitrogen in the growth medium.
Lane f, 55 µg of thylakoid membrane protein from cells grown light/dark cycle, + 5% (v/v) CO_2, without combined nitrogen in the growth medium.
Lane g, 25 µg of plasma membrane protein from cells grown light/dark cycle illumination, + air, without combined nitrogen in the growth medium.
Lane h, 55 µg of thylakoid membrane protein from cells grown light/dark cycle , + air, without combined nitrogen in the growth medium.

Panel 2: Western blot using antibodies raised against the GST fusion protein. The primary antibody was used at a 1 in 1,000 dilution and the secondary antibody was used at a 1 in 5,000 dilution.
The loadings in each lane were as in Panel 1

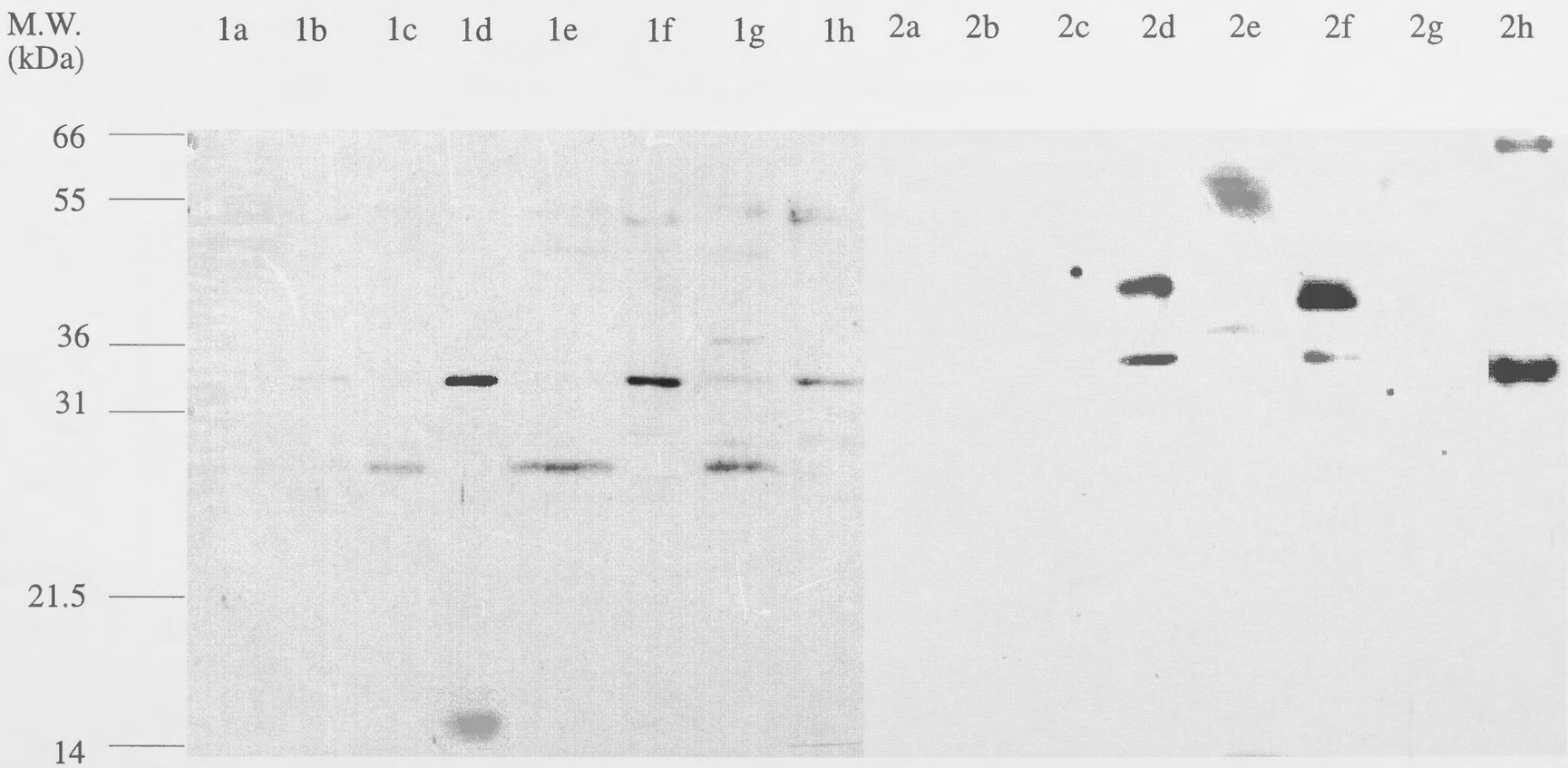
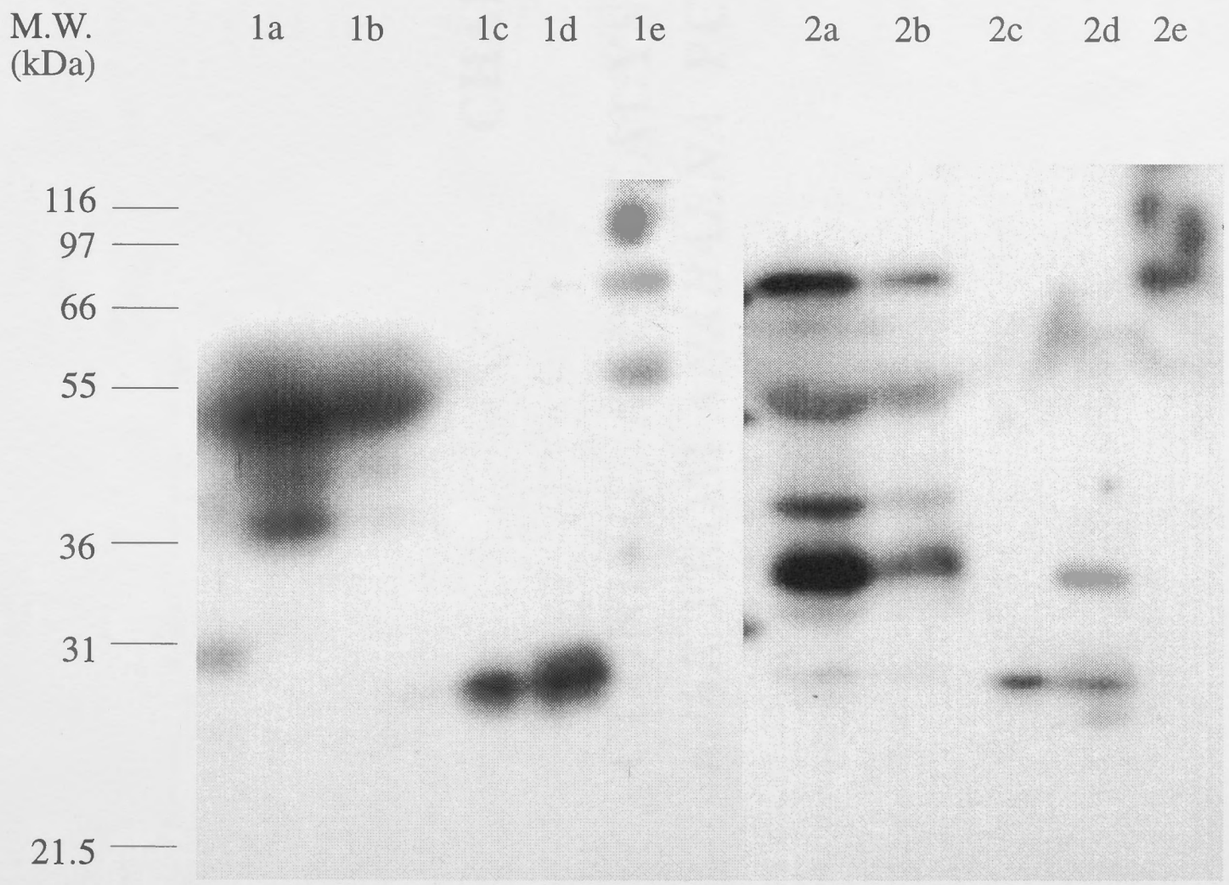

M.W.
(kDa)
1a
1b
1c
1d
1e
1f
1g
1h
2a
2b
2c
2d
2e
2f
2g
2h
66
55
36
31
21.5
14

Figure 4.10: Immunological analysis of different membrane fractions from soybean and spinach chloroplasts.

Membrane fractions were separated by SDS-PAGE (2.5) and then transferred to Hybond-C^+ (Amersham) and subjected to western analysis (2.6)

Panel 1: Western blot using antibodies raised against the NdhK-GST fusion protein. The primary antibody was used at a 1 in 1,000 dilution and the secondary antibody was used at a 1 in 5,000 dilution.
Lane a, 50 mg of protein from soybean thylakoid membranes isolated from the supernatant of the first Triton X-100 solubilisation step, using the method of Berthold *et al.* (1981).
Lane b, 50 mg of protein from soybean thylakoid membranes isolated from the supernatant of the second Triton X-100 solubilisation step, using the method of Berthold *et al.* (1981).
Lane c, 50 mg of protein from soybean thylakoid membranes that remained insoluble after both Triton X-100 solubilisation steps, using the method of Berthold *et al.* (1981).
Lane d, 50 mg of protein from purified spinach thylakoid membranes, kindly provided by Dr J. Joyard.
Lane e, 50 mg of protein from purified spinach chloroplast envelope membranes, kindly provided by Dr J. Joyard

Panel 2: Western blot using antibodies raised against the GST protein. The primary antibody was used at a 1 in 1,000 dilution and the secondary antibody was used at a 1 in 5,000 dilution.
The loadings in each lane were as in Panel 1


M.W.
(kDa)
1a
1b
1c
1d
1e
2a
2b
2c
2d
2e
116
97
66
55
36
31
21.5

CHAPTER 5

CLONING AND ANALYSIS OF *ndhK* FROM *ANABAENA* PCC 7120

5.1 INTRODUCTION

Anabaena PCC 7120 has an NDH-1 enzyme in the plasma membrane but not the thylakoid membrane (Chapter 4, Howitt *et al.* 1993). This is different from *Synechocystis* PCC 6803 which has an NDH-1 on the thylakoid membrane (Berger *et al.* 1991; Ogawa 1992a; Dzelzkalns *et al.* 1994) and another on the plasma membrane (Berger *et al.* 1991; Dzelzkalns *et al.* 1994). The difference in location of NDH-1 in *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 suggests that the NDH-1 has different functions in these cyanobacteria. This is strengthened by the findings that wild type *Synechocystis* PCC 6803 has cyclic electron transport around PS I, but this is absent in a strain in which *ndhB* has been inactivated (Mi *et al.* 1992a). Wild type *Anabaena* PCC 7120 do not show any cyclic electron transport around PS I (Mi *et al.* 1992b).

In order to further characterise this difference, it was decided to clone and sequence an *ndh* gene from *Anabaena* PCC 7120, determine the gene copy number, and use this for insertional inactivation studies if it was a single copy gene. The *ndhK* gene was chosen as the target gene for two reasons: firstly, it is the most highly conserved of the *ndh* genes cloned to date (Weidner *et al.* 1993); secondly, *ndhK* is the central gene in an operon encoding three *ndh* genes (*ndhC-K-J*). This operon has only been cloned and sequenced from one other cyanobacterium, *Synechocystis* PCC 6803 (Steinmüller *et al.* 1989), but has been identified in the chloroplasts of higher plants. The complete operon has been cloned and sequenced from the chloroplasts of liverwort (Ohyama *et al.* 1986), tobacco (Shinozaki *et al.* 1986), rice (Hiratsuka *et al.* 1989) and maize (Steinmüller *et al.* 1989). Partial sequence of the operon has also been obtained from wheat (Nixon *et al.* 1989) and soybean (Whelan *et al.* 1992). The order of the genes in the *ndh* operon is also conserved in organisms in which the operon encodes all of the subunits of NDH-1, such as *P. denitrificans* (Xu *et al.* 1993) and *E. coli* (Weidner *et al.* 1993)

Synechocystis PCC 6803 contains a second copy of *ndhK* in its genome (Mayes *et al.* 1990; Steinmüller and Bogorad 1990). The second copy is located on a plasmid and has been shown to be a cryptic gene that is not activated unless the first copy is inactivated (Steinmüller *et al.* 1991).

5.1.1 Aims

The aim of this part of the project was to determine if the operon encoding *ndh C-K-J* is conserved in *Anabaena* PCC 7120. If this was the case, *ndhK* would be cloned and

sequenced and then used to reprobe genomic DNA to determine the copy number in *Anabaena* PCC 7120.

5.2 RESULTS

5.2.1 Is the *ndhC-K-J* operon conserved in *Anabaena* PCC 7120?

In order to determine if the operon encoding *ndhC-K-J* is conserved in *Anabaena* PCC 7120, Southern analysis was performed. Genomic DNA was digested with *Eco RI* and *Hind III*, separately and together, and separated by agarose gel electrophoresis (2.13.1). DNA was transferred to Hybond-N^+ (Amersham) (2.14.1). The blot was then probed with *ndhK*, *ndhC*, and *ndhJ* from *Synechocystis* PCC 6803 (2.14.4), and washed at low stringency (50°C, 0.5X SSC). The probes for *ndhC* and *ndhJ* were a 400 bp *Ava II* fragment and a 360 bp *Hinc II* fragment, respectively, from the operon encoding *ndhC-K-J* from *Synechocystis* PCC 6803 (kindly provided by Dr. K Steinmüller). The probe for *ndhK* was the PCR product described in Chapter 4.

Figure 5.1 shows that there are homologues of all three genes in the genome of *Anabaena* PCC 7120. *ndhK* hybridised to a 2.6 kb *Eco RI*, a 3.0 kb Hind III, and a 1.6 kb *Eco RI-Hind III* fragment (lanes 2a, 2b, 2c). Fragments of the same size in each of the three digests also hybridised with *ndhJ* (lanes 3a, 3b, 3c). *ndhC* also hybridised to a 3 kb *Hind III* fragment (lane 4b), but also to a 2.8 kb *Eco RI* fragment (lane 4a) and a 1.4 kb *Eco RI-Hind III* fragment (lane 4c). As all three probes hybridised to a 3 kb *Hind III* fragment from *Anabaena* PCC 7120 suggesting that an operon encoding *ndhC-K-J* exists in *Anabaena* PCC 7120. A map of the operon encoding *ndhC-K-J* in *Anabaena* PCC 7120, based on the Southern analysis, is shown in Figure 5.2.

5.2.2 Construction of a genomic library from *Anabaena* PCC 7120

In order to clone *ndhK* from *Anabaena* PCC 7120, attempts were made to construct a genomic library. Genomic DNA was partially digested with *Sau 3A* so that a large proportion of the DNA was digested into fragments of between 15 and 23 kb.(Figure 5.3, lane b). The DNA was phenol extracted, ethanol precipitated, resuspended to a concentration of 0.5 $\mu g\ ul^{-1}$, and ligated into *Bam HI* cut EMBL3 Arms (Promega), treated with calf alkaline phosphatase to prevent religation of the vector to itself, with an insert : Arms ratios of 1:1 and 2:1 (2.16.2). The ligated DNA was then packaged into *E. coli* strain NM539 using a packaging kit from Promega according to the manufacturer's instructions (2.16.2). The 1:1 ligation produced no plaques, while the 2:1 ligation

produced a library of approximately 2,500 plaques. The probability of isolating an *ndhK*-homologous recombinant from this library was calculated using the following equation (Sambrook *et al.* 1989):

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

where N is the number of clones, P is the probability of isolating a unique clone and f is the proportion of the genome represented in each clone. *Anabaena* PCC 7120 has a genome size of 6.4 megabases (Herdman *et al.* 1979; Bancroft *et al.* 1988). Assuming an insert size of 15 kb, screening 2500 plaques represents a 99% probability of isolating the desired clone.

The library was screened as described in section 2.14 at the same stringency as the genomic Southerns, using *ndhK* from *Synechocystis* PCC 6803 as a probe. No positively hybridising plaques were found. Further attempts at library construction and screening proved unsuccessful.

5.2.3 Construction of subgenomic libraries from *Anabaena* PCC 7120

As construction and screening of genomic libraries proved unsuccessful, it was decided to try and clone *ndhK* from *Anabaena* PCC 7120 by constructing and screening subgenomic libraries. Two different strategies were pursued, one involved cloning the 3 kb *Hind III* fragment that contained *ndhC*, *ndhK* and *ndhJ* as determined by Southern analysis (Section 5.2.1), while the other involved cloning the 1.6 kb *Eco RI - Hind III* fragment that contained *ndhK* and *ndhJ* (Section 5.2.1).

a) Construction of a *Hind III* subgenomic library from *Anabaena* PCC 7120

Genomic DNA from *Anabaena* PCC 7120 was digested to completion with *Hind III* and separated by electrophoresis. Fragments between 2.5 kb and 3.5 kb were excised from the gel and purified using the Wizard purification system (Promega) (Section 2.13.2). The purified DNA was ligated into pGEM 3Zf(+) (Promega) cut with *Hind III*, that had been treated with calf alkaline phosphatase (Section 2.10.4). Competent *E. coli* strain DH5α MCR (Gibco BRL) was transformed with the ligation mixture (Section 2.10.2). DH5α MCR was chosen as the host as it is an *mcrB*$^-$ strain and will not restrict DNA carrying 5-methylcytosine, which is important as genomic DNA from Anabaena PCC 7120 is methylated at *Ava I* and *Ava II* sites (Elhai and Wolk 1988). The transformed cells were subjected to blue-white screening (Sambrook *et al.* 1989) to determine the

proportion carrying plasmids with inserts. Cells were plated out and transferred to Hybond-N^+ discs (Amersham) and probed with *ndhK* from *Synechocystis* PCC 6803, using the same conditions as for the genomic Southerns. A number of potential positive clones were found. These clones were amplified and plasmid DNA isolated (Section 2.9.3.a). The plasmid DNA was cut with *Hind III* and separated by electrophoresis, transferred to Hybond-N^+ (Amersham) and subjected to Southern analysis, with the same probe used for the initial screen. All clones tested were found to be false positives (data not shown).

Approximately 7,000 white colonies (colonies containing plasmids with inserts) were screened. Assuming that the whole genome of *Anabaena* PCC 7120, when digested to completion with *Hind III,* restricts into fragments of 2.5 kb to 3.5 kb in size, screening 7,000 colonies represents a 97% chance of the correct insert being found. In reality only a small proportion of the genome falls into the size range selected, upon complete digestion with *Hind III* as can be seen from Figure 5.1 (lane 1b). Effectively this means that a much smaller genome is being screened, and the probability of the correct fragment being present should have approached 100%.

b) Construction of a *Eco RI - Hind III* subgenomic library from *Anabaena* PCC 7120

The strategy used for constructing the *Eco RI - Hind III* subgenomic library was the same as that described above for the *Hind III* subgenomic library, except that genomic DNA was digested to completion with *Eco RI* and *Hind III* and the fragments selected for subcloning were between 1.3 and 1.9 kb. These fragments were subcloned into pGEM 3Zf(+) cut with *Eco RI* and *Hind III*. Approximately 2,000 white colonies were screened and no positives were found. 2,000 colonies represents a 99% chance of the desired insert being present, assuming one tenth of the genome falls into the size range 1.3 to 1.9 kb after digestion to completion with *Eco RI* and *Hind III*. The assumption that only one tenth of the genome falls within the desired size range is based on the roughly even spread of fragments between 1 kb and 8 kb when *Anabaena* PCC 7120 genomic DNA is digested to completion with *Eco RI* and *Hind III* (Figure 5.1 lane 1c).

5.2.4 Cloning of *ndhK* from *Anabaena* PCC 7120 using PCR

As construction and screening of genomic and subgenomic libraries had proven unsuccessful, it was decided to try and clone *ndhK* from *Anabaena* PCC 7120 using PCR. As the operon encoding *ndhC-K-J* was shown to be conserved in *Anabaena* PCC 7120 (Section 5.2.1) it was, therefore, decided to design primers to highly conserved

regions in *ndhC* and *ndhJ*. As *ndhC* and *ndhJ* flank *ndhK,* the ensuing PCR product should contain part of the coding regions for *ndhC* and *ndhJ* and the complete coding region of *ndhK*.

a) Primer Design

Some organisms display a marked bias in the use of some codons (Aota *et al.* 1988) and this phenomenon may be used to reduce degeneracy within a primer by avoiding infrequently used codons (Compton 1990). In order to optimise primer design a codon preference table was constructed for *Anabaena* PCC 7120 (Table 5.1) using the UWGCG program "Codonfrequency" (version 7.1) (Devereux *et al.* 1984). All the sequences of genes from *Anabaena* PCC 7120 available in the genebank were used in constructing the codon preference table (60 at the time of construction of the table).

The amino acid sequences of NdhC and NdhJ available from cyanobacteria and chloroplasts were aligned using the "Pileup" program from the UWGCG sequence analysis package (Devereux *et al.* 1984). As can be seen in Figures 5.4 and 5.5, both NdhC and NdhJ are highly conserved among cyanobacteria and chloroplasts. Figure 5.4 shows that there are three large blocks of conserved amino acids within NdhC, against which primers could be designed. Figure 5.5 shows that there is only one region of conserved amino acids long enough to design a primer within NdhJ. Each of these regions was back translated using the codon preference table constructed for *Anabaena* PCC 7120 (Table 5.1). The absolutely conserved block of amino acids, shown in green in Figure 5.4 gave the least degenerate primer amongst the three possible regions from NdhC. The complete region, when back translated, gave a 36 mer. This was "trimmed" to give a 29 mer that had G's and C's at the 3' end where binding is critical for subsequent extension. The G:C pairing results in tighter binding at the priming site (Compton 1990; Bej *et al.* 1991). This primer was called 5'ANDHK. The conserved block of amino acids from NdhJ (shown in green in Figure 5. 5) was back translated to give a 27 mer which contained two G's at the 3' end; this was "trimmed" to a 26 mer as the T at the 5' end was not conserved. This primer was called 3'ANDHK. The sequences of the two primers used are shown below;

5' ANDHK 5' GTA GAA ACC GTA TTT TTA TAC CCT TGG GC

3' ANDHK 5' ATG TAA TCT TTA CGT AAA GGC CAA CC

Both primers were made at The Biomolecular Resources Unit at the Australian National University.

b) PCR amplification of *ndhK* using genomic DNA from *Anabaena* PCC 7120

The above primers were used to amplify genomic DNA from *Anabaena* PCC 7120, as described in Section 2.11.1 with an annealing temperature of 50°C. The result is shown in Figure 5.6. Genomic DNA from *Synechocystis* PCC 6803 amplified with the primers SNDHK5' and SNDHK3' (Section 4.2.1.a) was run as a positive control (lane 1a). A single band of about 750 bp was seen, as expected. Lane 1b contained genomic DNA from *Synechocystis* PCC 6803 amplified with 5'ANDHK and 3'ANDHK, resulting in multiple PCR products. Lane 1c contained genomic DNA from *Anabaena* PCC 7120 amplified with 5'ANDHK and 3'ANDHK, resulting in multiple PCR products. To determine if any of these products were *ndhK* the DNA was transferred to Hybond-N^+ (Amersham) (2.14.1) and subjected to Southern blot analysis using *ndhK* from *Synechocystis* PCC 6803 as the probe. Figure 5.6 shows that, as expected, the probe hybridised to itself (lane 2a); interestingly none of the bands in lane 2b, which contained genomic DNA from *Synechocystis* PCC 6803, hybridised with the probe indicating that, under the conditions used, 5'ANDHK and 3'ANDHK can not be used to amplify *ndhK* from *Synechocystis* PCC 6803. This suggests that *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 have different frequencies of codon usage. A fragment of about 1.4 kb from *Anabaena* PCC 7120 hybridised with the probe (lanes 2c), suggesting it contained *ndhK* from *Anabaena* PCC 7120. This fragment was gel purified as described in Section 2.13.2 and used as a template for a second round of PCR using the same primers. The second round PCR gave two products, one of approximately 1.4 kb and one of approximately 500 bp (Figure 5.7). As the template for the second round PCR was a gel purified band, the smaller fragment may have resulted from an internal priming site within the larger fragment but outside the region the probe is homologous to. Therefore it was decided to clone and sequence both fragments.

c) Cloning and Sequencing of *ndhK* from *Anabaena* PCC 7120

Both fragments from the second round PCR were cloned into pGEM-T (Promega) (Section 2.11.3) giving clones AKL (*Anabaena ndhK* long) and AKS (*Anabaena ndhK* short). Two clones of each, from separate primary and secondary PCR reactions, were sequenced completely in both directions by subcloning fragments, using restriction sites identified by restriction mapping or earlier sequencing runs, into pGEM 3Zf(+) or pGEM 5Zf(+) (Promega). The sequencing strategy used for both clones is shown in Figure 5.8. In regions where the sequences differed between clones, a third clone was sequenced, in at least one direction, to verify the sequence.

The sequence of AKS is shown in figure 5.9, with a putative translation also shown. It was compared to the sequences in GenBank using the UWGCG program "Fasta" (Devereux *et al.* 1984). At the nucleic acid level it showed no homology to any other sequence. The sequence was translated in all six frames and they were used to search the protein databases. One frame contains an open reading frame that runs through the entire length of the clone (Figure 5.9). This putative coding region has 67% similarity and 45% identity to a region towards the carboxy terminus of the protein encoded by *rbsA* from *E. coli* (Figure 5.10). *rbsA* is part of an operon that encodes the proteins of the high affinity ribose transport system in *E. coli* (Bell *et al.* 1986). The protein encoded by *rbsA* is a member of the ATP-binding transport protein family (ABC transporters) and is homologous to the proteins encoded by *hisP*, *malK*, and *pstB* components of the histidine, maltose and phosphate high affinity transport systems. The translation shown in Figure 5.9 contains two motifs, known as Walker motifs A and B (WA and WB) that are characteristic of many nucleotide binding proteins (Walker *et al.* 1982). The spacing between these two motifs is correct for the translation shown to be part of a functional ABC transporter (Higgins 1992). These proteins are thought to bind the ATP necessary for active transport of the substrate (Buckel *et al.* 1986; Higgins 1992; Dogie and Ames 1993; Fath and Kolter 1993; Tam and Saier 1993). Analysis of this clone was not taken any further.

The sequence of AKL is shown in Figure 5.11: the translations of the three open reading frames encoding *ndhC*, *ndhK* and *ndhJ* are shown. The sequence encodes the 37 amino acids at the carboxy terminus of *ndhC*, the complete sequence of *ndhK,* and all but the last 20 amino acids of *ndhJ,* as determined by homology to NdhJ from chloroplasts and cyanobacteria which all terminate at exactly the same point (see Figure 5.14). Potential ribosomal binding sites for 16 S ribosomal RNA (Shine and Dalgarno 1974) are shown for *ndhK* and *ndhJ*. At the nucleic acid level, the partial sequence of *ndhC* has 72% similarity to *ndhC* from *Synechocystis* PCC 6803. *ndhK* has 69% similarity to *ndhK* from *Synechocystis* PCC 6803, while the partial sequence of *ndhJ* has 69% similarity to *ndhJ* from *Synechocystis* PCC 6803. At the amino acid level, the partial sequence of NdhC has 95% identity and 97% similarity to NdhC from *Synechocystis* PCC 6803. NdhK has 77% identity and 86% similarity to NdhK from *Synechocystis* PCC 6803, while the partial sequence of NdhJ has 74% identity and 85% similarity to NdhJ from *Synechocystis* PCC 6803. This confirms that the operon encoding *ndhC-K-J* is conserved in *Anabaena* PCC 7120.

5.2.5 Determination of copy number of *ndhK* in *Anabaena* PCC 7120

To determine the copy number of *ndhK* in *Anabaena* PCC 7120, genomic DNA was digested to completion with *Eco RI* and *Hind III*, separately and together. The DNA was separated by electrophoresis and transferred to Hybond-N^+ membrane (Amersham) (Section 2.14.1). The membrane was then probed with a 440 bp *Eco RI, Acc I* fragment of *ndhK* from *Anabaena* PCC 7120. The blot was washed at low stringency (42°C and 0.5X SSC). As can be seen (Figure 5.12) the pattern of bands hybridising to the probe is identical to that seen in Figure 5.1, lanes 2a, 2b and 2c, when *ndhK* from *Synechocystis* PCC 6803 was used as the probe. This suggests that, unlike *Synechocystis* PCC 6803 which contains a second copy of *ndhK* (Mayes *et al.* 1990; Steinmüller and Bogorad 1990), *Anabaena* PCC 7120 contains only one copy.

5.3 DISCUSSION

5.3.1 Failure of genomic and subgenomic libraries to yield a positive clone

It is not known why a positive clone was not obtained from the screening of the genomic and subgenomic libraries constructed. However, one interesting fact noted from cloning *ndhK* by PCR may in part explain this. Full length clones of the PCR product were always in the antisense orientation with respect to *lacZ*, suggesting that the sense orientation is lethal to the host cell. This is further supported by the fact that undirectional subclones of this region were also found only in the antisense orientation, while some fragments could not be cloned in the sense orientation in directed subcloning. If clones of this region in the sense orientation with respect to *lacZ* are lethal in *E. coli,* this could partially explain why the clone was not found in the *Hind III* subgenomic library constructed. The 3 kb *Hind III* fragment may not have been found simply due to insufficient colonies being screened; if half of the desired clones are lethal, then a larger number colonies would need to be screened to have a 99% chance of finding a positive clone. This does not explain why the 1.6 kb *Eco RI - Hind III* in pGEM 3Zf(+) was not found as it would be in the antisense direction with respect to *lacZ*. Nor does it explain why construction and screening of the genomic library proved unsuccessful.

5.3.2 Analysis of *ndhK* from *Anabaena* PCC 7120

ndhK from *Anabaena* PCC 7120 encodes a protein of 245 amino acids with a predicted size of 27.5 kDa. This is 5 amino acids smaller than the predicted protein from *Synechocystis* PCC 6803 (Steinmüller *et al.* 1989). An alignment of the putative protein encoded by *ndhK* in *Anabaena* PCC 7120, with both copies of NdhK from *Synechocystis* PCC 6803 and five chloroplast sequences, is shown in Figure 5.13. As can be seen, the central region of the proteins are highly conserved. Ninety two amino acids are conserved across all the sequences aligned (shown in red). A further 20 amino acids are conserved except in the second copy of NdhK from *Synechocystis* PCC 6803 (shown in green). The second copy of NdhK has been shown to be encoded by a cryptic gene that is not expressed in wild type cells (Steinmüller *et al.* 1991), thus changes would be expected in this sequence as there is no selective pressure to maintain the absolutely conserved amino acids. The cryptic copy can be activated when the copy expressed in the wild type is inactivated (Steinmüller *et al.* 1991). Excluding the second copy of NdhK from *Synechocystis* PCC 6803 from the alignment, 122 amino acids are conserved, and 119 of these are conserved in the partial sequence of NdhK available from soybean (Whelan *et al.* 1992). Fifty of these 122 amino acids are conserved in the sequences of the homologues of NdhK from *E. coli*, *P. denitrificans* and bovine heart mitochondria (indicated by the stars under the amino acids in the alignment). Of these 50, 3 are cysteine residues (positions 103, 167 and 198 in the alignment, Figure 5.13) and it has been proposed that they may be involved in binding an Fe-S cluster (Arizmendi *et al.* 1992a; Albracht 1993). However, as yet there is no evidence to corroborate this. These three cysteine residues are also conserved in HycG from the *E. coli* formate hydrogen lyase (Weiss *et al.* 1991; Fearnley and Walker 1992) and in the subunit of the NAD^+-reducing hydrogenase from *A. eutrophus* (Albracht 1993). Both of these enzymes show significant homology to subcomplexes of complex I (as discussed in Section 1.3.1)

Three amino acids that are conserved in the chloroplast and *Synechocystis* PCC 6803 sequences are changed in *Anabaena* PCC 7120 (shown in blue in Figure 5.13). All three changes are conservative: at position 148 leucine is replaced by methionine, both are neutral non-polar amino acids; at position 177 aspartic acid is changed to glutamic acid, while at position 219 glutamic acid is changed to aspartic acid; both aspartic acid and glutamic acid are acidic amino acids.

Table 5.2 shows the sequence identity of the proteins used in the alignment (Figure 5.13). As can be seen from the table, NdhK from *Anabaena* PCC 7120 has greatest identity to NdhK from *Synechocystis* PCC 6803. It has approximately 60% identity with NdhK from all the chloroplast sequences and 42%, 43% and 42% identity to the sequences of the homologues from *E. coli, P. denitrificans* and bovine heart

mitochondria, respectively. It can also be seen that NdhK is extremely highly conserved among the monocots, with the sequences from *O. sativa* (rice), *Z. mays* (maize) and *T. aestivum* (wheat) having at least 95% identity. A high degree of amino acid sequence identity is shown across all of the sequences in the table with the lowest degree of identity being shown between the proteins from *E. coli* and *M. polymorpha* (37%) and *E. coli* and *N. tobacum* (37%). This high degree of conservation can not be explained by the possibility that NdhK is an iron-sulphur protein (as discussed above), and has led other workers to suggest that NdhK may be a central subunit in NDH-1 that closely interacts with other subunits (Weidner *et al.* 1993). Recently, a subcomplex of the NAD(P)H-plastoquinone oxidoreductase from *Synechocystis* PCC 6803 has been purified and shown to contain NdhK (Berger *et al.* 1993a). These workers proposed that the subcomplex was a connecting part holding the two arms of the enzyme together. Thus two independent lines of evidence have developed to suggest NdhK is a vital subunit of NDH-1

5.3.3 Analysis of *ndhJ* from *Anabaena* PCC 7120

As in *Synechocystis* PCC 6803 (Steinmüller *et al.* 1989), *ndhJ* in *Anabaena* PCC 7120 is judged to have a GTG start codon. It is located 6 bp downstream from a ribosomal binding site (Shine and Dalgarno 1974) (Figure 5.11). In *E. coli* ATG, GTG and TTG are used as initiation codons in the frequency 91:8:1 (Gold and Stormo 1987) and all are translated or modified to methionine. A GTG start codon has been found for the chloroplast encoded ribosomal protein RPS19 in maize (Schwartz *et al.* 1981; McLaughlin and Larrina 1987) and a number of other species (Zurawski and Clegg 1987).

The partial sequence of *ndhJ* from *Anabaena* PCC 7120 encodes 139 amino acids of a predicted 159 by homology to other NdhJ sequences, as they all stop in exactly the same place relative to the last absolutely conserved amino acid (Figure 5.14). As can be seen from the alignment (Figure 5.14) NdhJ, like NdhK, is highly conserved among cyanobacteria and chloroplasts, with 70 conserved amino acids (shown in red) in the sequences aligned. Unlike NdhK this high degree of conservation does not extend through to the homologues in *E. coli*, *P. denitrificans* and bovine heart mitochondria. Only 16 (23%) of the 70 conserved amino acids are conserved in the homologues from *E. coli*, *P. denitrificans* and bovine heart mitochondria (indicated by stars below the amino acids), while 40% of the conserved amino acids from cyanobacterial and chloroplastic NdhK are also conserved in the homologues from the three species listed above (Figure 5.13). This lower degree of conservation of NdhJ is bourne out in Table 5.3, where it can be seen that cyanobacterial and chloroplastic NdhJ have 25-34%

identity with the sequences from *E. coli*, *P. denitrificans* and bovine heart mitochondria, whereas Table 5.2 shows that the equivalent comparisons for NdhK have 37-43% identity. It can also be seen that NdhJ from *Anabaena* PCC 7120 is more closely related to its homologue in *Synechocystis* PCC 6803 than its chloroplast homologues. Again the species with the highest identity between their sequences are the monocots *O. sativa* (rice) and *Z. mays* (maize).

Three amino acids that are conserved in NdhJ from the cyanobacterium *Synechocystis* PCC 6803 and the chloroplasts of rice, maize, tobacco and liverwort are changed in *Anabaena* PCC 7120 (Figure 5.14, shown in blue). Glutamine is changed to lysine (position 41), an unconservative change as glutamine is a neutral non-polar amino acid while lysine is basic. Alanine to glycine (position 67) is a conservative change as both are neutral non-polar amino acids, while tyrosine to isoleucine (position 68) represents the replacement of a polar amino acid with a non-polar amino acid.

5.3.4 Features of the *ndhC-K-J* Operon from *Anabaena* PCC 7120

The sequence presented here contains no transcription initiation or termination sequences. This is not unexpected as it has been shown in *Synechocystis* PCC 6803, maize (Steinmüller *et al.* 1989) and tobacco (Matsubayashi *et al.* 1987) that the whole operon is co-transcribed. If the operon is co-transcribed in *Anabaena* PCC 7120 the sequences upstream of *ndhC* and downstream of *ndhJ* would be needed for the transcription initiation and termination sequences to be identified.

The coding regions for *ndhC* and *ndhK* overlap by 7 bp in *Anabaena* PCC 7120 (Figure 5.11 and 5.15). This overlap is identical to that seen in the chloroplasts of liverwort (Ohyama *et al.* 1986), maize (Steinmüller *et al.* 1989) and rice (Hiratsuka *et al.* 1989), while in wheat the overlap is 28 bp (Nixon *et al.* 1989) and in tobacco it is 118 bp (Shinozaki *et al.* 1986). This is markedly different from the case in *Synechocystis* PCC 6803 which has a 71 bp non-coding intergenic spacer region between *ndhC* and *ndhK* (Figure 5.15). This suggests that, in terms of operon structure, *Anabaena* PCC 7120 is more closely related, in evolutionarily terms, to chloroplast genomes than is *Synechocystis* PCC 6803. However, *Synechocystis* PCC 6803 contains a cryptic copy of *ndhK* in its genome (Mayes *et al.* 1990; Steinmüller and Bogorad 1990), while the chloroplast genomes, sequenced to date, and *Anabaena* PCC 7120 only have a single copy (Section 5.2.5). Thus the non-coding intergenic spacer region seen in *Synechocystis* PCC 6803 may be a result of duplication of *ndhK* and the subsequent movement of one copy elsewhere in the genome after the divergence of the lineages that gave rise to *Anabaena* PCC 7120, *Synechocystis* PCC 6803 and chloroplasts. The

proteins encoded within the operon are more closely related to the equivalent proteins from *Synechocystis* PCC 6803 than chloroplasts.

5.4 SUMMARY

The results presented in this chapter show that the operon encoding *ndhC-K-J*, found in chloroplast genomes and the cyanobacterium *Synechocystis* PCC 6803, is also conserved in *Anabaena* PCC 7120. It was also shown that there is only a single copy of *ndhK* in the genome of *Anabaena* PCC 7120. PCR amplification, using primers designed to absolutely conserved regions from the amino acid sequences of NdhC and NdhJ of *Synechocystis* PCC 6803 and chloroplasts, was used to amplify and clone part of the operon encoding *ndhC-K-J* from *Anabaena* PCC 7120. This operon is structurally more closely related to the equivalent operon in chloroplasts than in *Synechocystis* PCC 6803. However, the proteins encoded within the operon have greater identity with the equivalent proteins from *Synechocystis* PCC 6803 than chloroplasts.

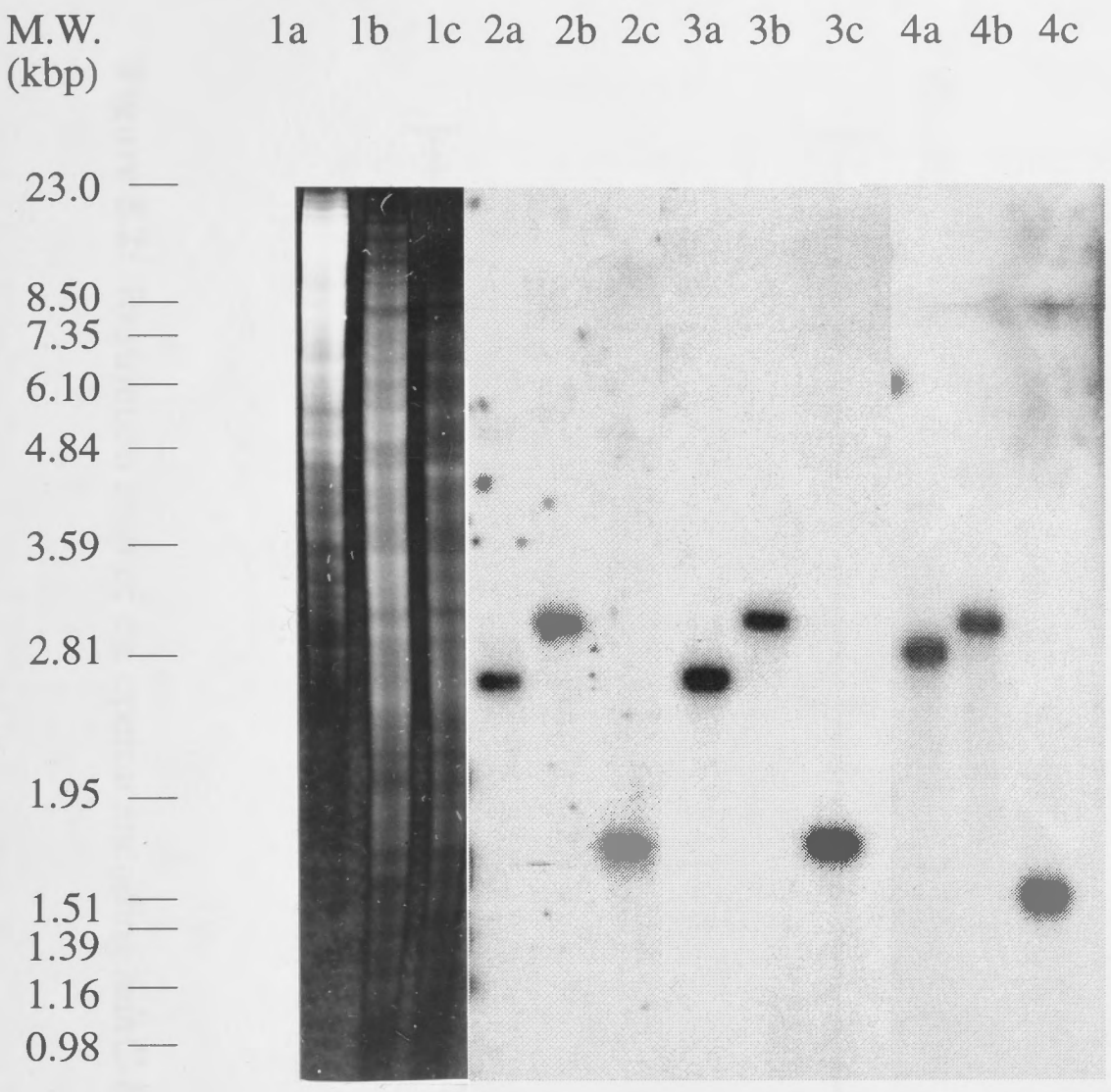


Figure 5.1: Southern analysis of genomic DNA from *Anabaena* PCC 7120 using fragments of *ndhC, ndhK* and *ndhJ* from *Synechocystis* PCC 6803 as the probes.

Genomic DNA was digested, separated by agarose gel electrophoresis (2.13.1), transferred to Hybond-N^+ (Amersham) and probed (2.14) with fragments of DNA from the operon encoding *ndhC-K-J* in *Synechocystis* PCC 6803.

Panel 1: Digest of genomic DNA using different restriction enzymes. Each lane contains 5 μg of genomic DNA. Lane a, Genomic DNA digested with *Eco RI*. Lane b, genomic DNA digested with *Hind III*. Lane c, genomic DNA digested with *Eco RI* and *Hind III*.

Panel 2: Digested genomic DNA probed with *ndhK* from *Synechocystis* PCC 6803. Loadings are as in panel 1.

Panel 3: Digested genomic DNA probed with *ndhJ* from *Synechocystis* PCC 6803. Loadings are as in panel 1.

Panel 4: Digested genomic DNA probed with *ndhC* from *Synechocystis* PCC 6803. Loadings are as in panel 1.

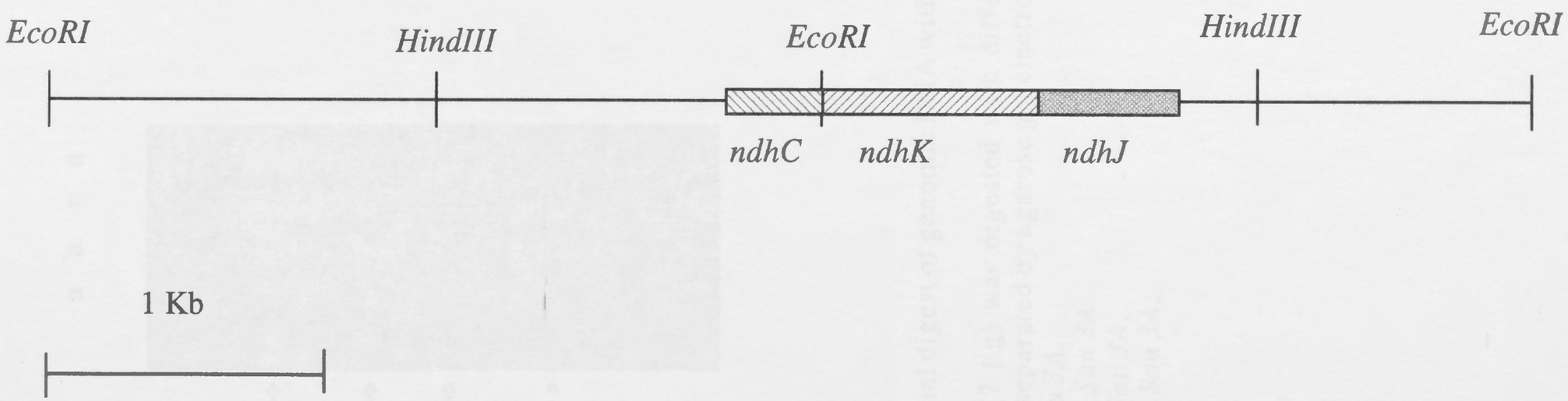


Figure 5.2: Restriction map of the operon encoding *ndhC-K-J* in *Anabaena* PCC 7120, based on the results of the Southern analysis.

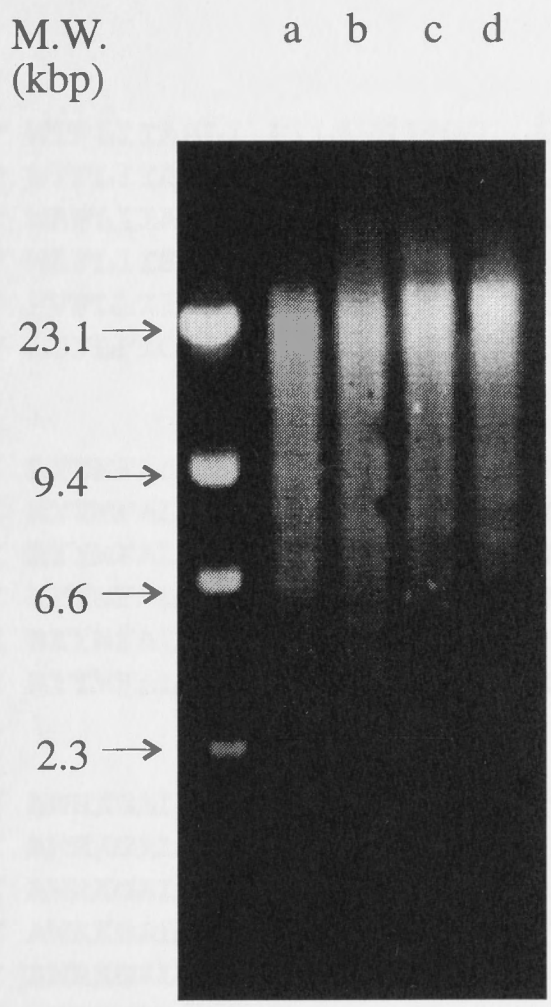


Figure 5.3: Partial digest of genomic DNA with *Sau 3A*.

Genomic DNA (2 μg) was digested with different amounts of *Sau 3A* and then separated by agarose gel electrophoresis (2.13).
Lane a, 1 unit *Sau 3A*.
Lane b, 0.75 unit *Sau 3A*.
Lane c, 0.5 unit *Sau 3A*.
Lane d, 0.25 unit *Sau 3A*.

```
                1                                                  50
    Wheat       MFLLHEYDIF WTFLIIASLI PILAFSISGL LAPVSEGPEK LSSYESGIEP
    Maize       MFLLHEYDIF WTFLIIASLI PILVFWISGL LAPVSEGPEK LSSYESGIEP
     Rice       MFLLHEYDIF WAFLIIASLI PILAFWISAL LAPVREGPEK LSSYESGIEP
  Tobacco       MFLLYEYDFF WAFLIISILV PILAFLISGV LAPISKGPEK LSTYESGIEP
Liverwort       MFLLQKYDYF FVFLLIISFF SILIFSLSKW IAPINKGPEK FTSYESGIEP
    S6803       MFVLTGYEYF LGFLFICSLV PVLALTASKL LRPRDGGPER QTTYESGMEP

                51                                                 100
    Wheat       MGGAWVQFRI RYYMFALVFV VFDVETVFLY PWAMSFDVLG VSVFIEALIF
    Maize       MGGAWLQFRI RYYMFALVFV VFDVETVFLY PWAMSFDVLG VSVFIEAFIF
     Rice       MGGAWLQFRI RYYMFALVFV VFDVETVFLY PWAMSFDVLG ISVFIEAFIF
  Tobacco       MGDAWLQFRI RYYMFALVFV VFDVETVFLY PWAMSFDVLG VSVFIEAFIF
Liverwort       MGEACIQFQI RYYMFALVFV IFDVETVFLY PWAMSFYNFG ISSFIEALIF
    S6803       IGGAWIQFNI RYYMFALVFV VFDVETVFLY PWAVAFNQLG LLAFVEALIF

                101             120
    Wheat       VLILVVGLVY AWRKGALEWS
    Maize       VLILVVGLVY AWRKGALEWS
     Rice       VLILVVGLVY AWRKGALEWS
  Tobacco       VLILIIGLVY AWRKGALEWS
Liverwort       ILILIIGLVY AWRKGALEWS
    S6803       IAILVVALVY AWRKGALEWS
```

Figure 5.4: Alignment of NdhC from chloroplasts and cyanobacteria.

Sequences were aligned using the "Pileup" program in the UWGCG sequence analysis package (Devereux *et al.* 1984). Conserved amino acids are shown in red and green. The sequence shown in green is the sequence used to design 5'ANDHK.

*S*6803 = *Synechocystis* PCC 6803

References for the sequences are: wheat Nixon *et al.* (1989); maize and *Synechocystis* PCC 6803 Steinmüller *et al.* (1989); rice Hiratsuka *et al.* (1989); tobacco Shinozaki, *et al.* (1986); liverwort Ohyama *et al.* (1986).

```
            1                                                  50
    S6803   .......... ..VGPVSTWL TTNGFEHQSL TADHLGVEMV QVEADLLLPL
     Rice   .......... MQQGWLSNWL VKHEVVHRSL GFDHRGIETL QIKAEDWDSI
    Maize   .......... MQQGWLSNWL VKHDVVHRSL GFDHRGVETL QIKAGDWDSI
  Tobacco   .......... .MQGRLSAWL VKHGLIHRSL GFDYQGIETL QIKPEDWHSI
Liverwort   MLNILKNNNN KIQGRLSIWL IKHNLKHRPL GFDYQGIETL QIRSEDWPSL

            51                                                 100
    S6803   CTALYAYGFN YLQCQGAYDE GPGKSLVSFY HLVKLTEDTR NPEEVRLKVF
     Rice   AVILYVYGYN YLRSQCAYDV APGGSLASVY HLTRIQYGID NPEEVCIKVF
    Maize   AVILYVYGYN YLRSQCAYDV APGGSLASVY HLTRIQYGID NPEEVCIKVF
  Tobacco   AVIFYVYGYN YLRSQCAYDV APGGLLASVY HLTRIEDGVD QPEEVCIKVF
Liverwort   AVALYVYGFN YLRSQCAYDV EPGGLLASVY HFTKITDNAD QPEEICIKIF

            101                                                150
    S6803   LPRENPVVPS VYWIWKAADW QERECYDMFG IVYEGHPNLK RILMPEDWVG
     Rice   AQKDNPRIPS VFWIWRSSDF QERESFDMVG ISYDNHPRLK RILMPESWIG
    Maize   AQKDNPRIPS VFWVWRSADF QERESYDMVG ISYDNHPRLK RILMPESWIG
  Tobacco   ASRRNPRIPS VFWVWKSVDF QERESYDMLG ISYDNHPRLK RILMPESWIG
Liverwort   ILRKNPKIPS IFWVWKSADF QERESYDMFG IFYENHPCLK RILMPDSWLG

            151              170
    S6803   WPLRKDYISP DFYELQDAY*
     Rice   WPLRKDYITP NFYEIQDAH*
    Maize   WPLRKDYITP NFYEIQDAH*
  Tobacco   WPLRKDYIAP NFYEIQDAH*
Liverwort   WPLRKDYIVP NFYELQDAY*
```

Figure 5.5: Alignment of NdhJ from chloroplasts and cyanobacteria.

Sequences were aligned using the "Pileup" program in the UWGCG sequence analysis package (Devereux *et al.* 1984). Conserved amino acids are shown in red and green. The sequence shown in green is the sequence used to design 3'ANDHK.

*S*6803 = *Synechocystis* PCC 6803

References for the sequences are: maize and *Synechocystis* PCC 6803 Steinmüller *et al.* (1989); rice Hiratsuka *et al.* (1989); tobacco Shinozaki, *et al.* (1986); liverwort Ohyama *et al.* (1986).

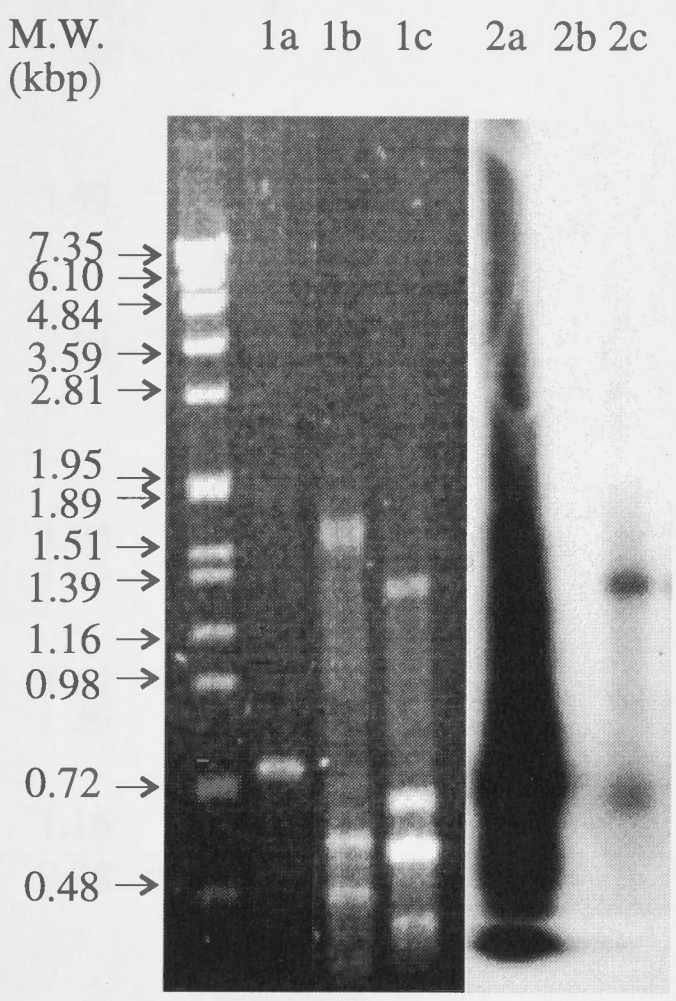


Figure 5.6: Amplification of part of the operon encoding *ndhC-K-J* from *Anabaena* PCC 7120 using PCR.

Panel 1: Agarose gel electrophoresis of PCR products.

Lane a, positive control genomic DNA (100 ng) from *Synechocystis* PCC 6803 using the primers SNDHK5' and SNDHK3' (4.2.1.a).

Lane b, amplification of genomic DNA (100 ng) from *Synechocystis* PCC 6803 using the primers 5'ANDHK and 3'ANDHK (5.2.4.a).

Lane c, amplification of genomic DNA (100 ng) from *Anabaena* PCC 7120 using the primers 5'ANDHK and 3'ANDHK (5.2.4.a).

Panel 2: Southern analysis of PCR products. The PCR products were transferred to Hybond-N^+ (Amersham) and probed with *ndhK* from *Synechocystis* PCC 6803. Loadings are as for panel 1.

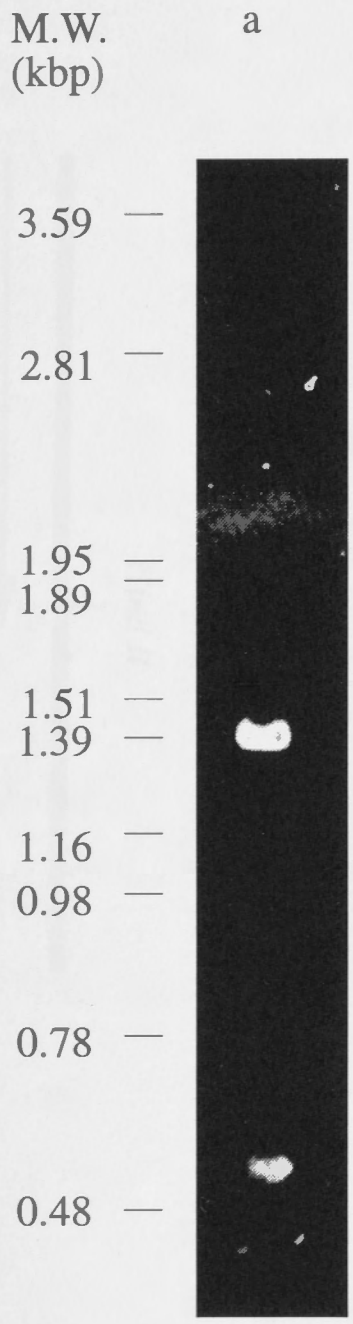


Figure 5.7: Second round amplification of *ndhK* from *Anabaena* PCC 7120, using a gel purified sample of the fragment that hybridised with *ndhK* from *Synechocystis* PCC 6803, in the first round, as the template for PCR.

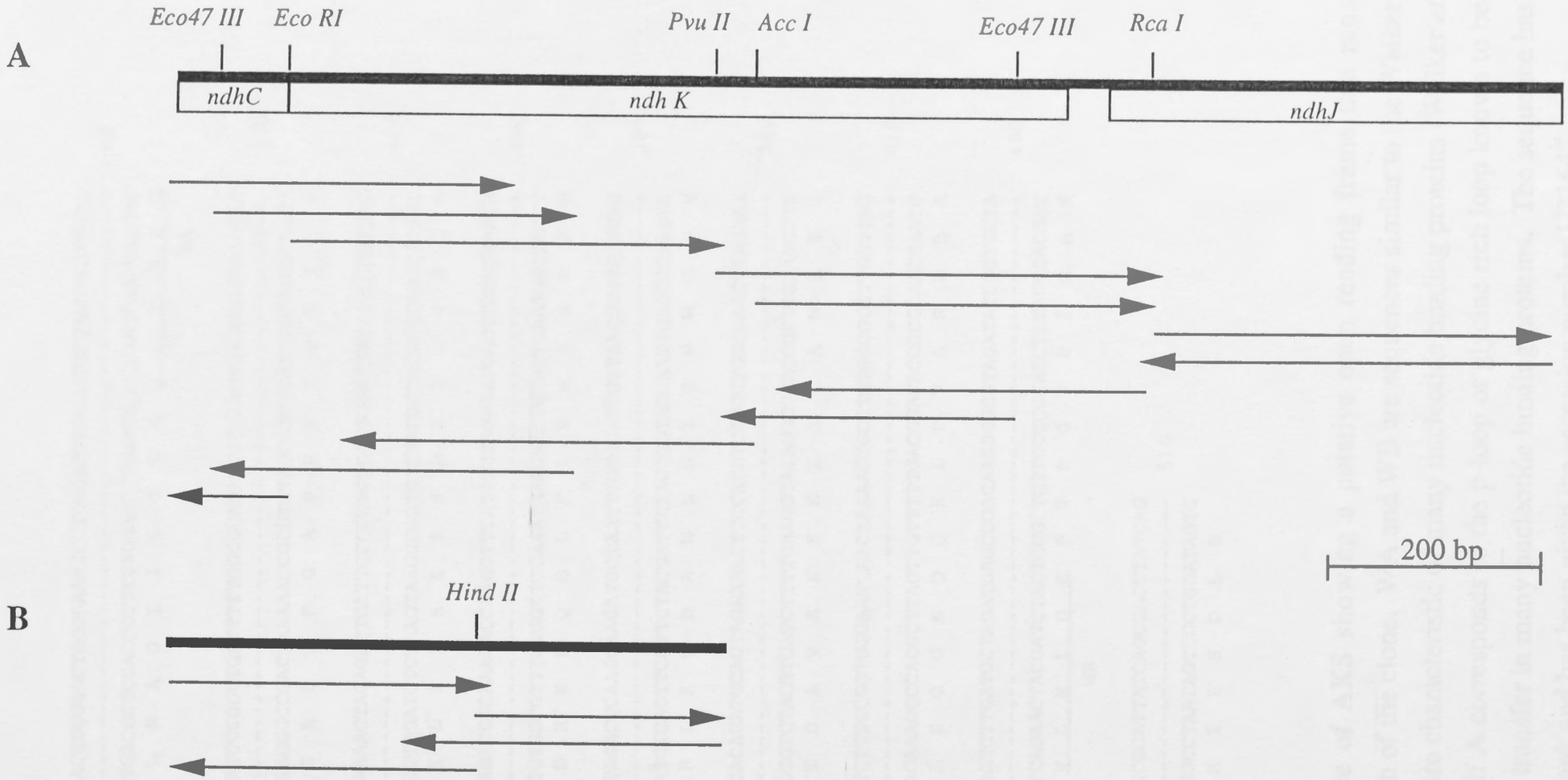


Figure 5.8 Physical map and schemes used in sequencing AKL and AKS. Boxes shown below the maps show open reading frames. Restriction enzyme sites used to generate fragments for sequencing are shown above the line. The direction and extent of individual sequence analyses are indicated by arrows below the map. The scale is the same for both A and B

A) Physical map of AKL.
B) Physical map of AKS.

```
    CAGCTAGTTTCAAACTGCGGGCTGGGGAAATCCTCGGACTAGCGGGGTTAGTTGGTGCAG
1   ---------+---------+---------+---------+---------+---------+   60
    GTCGATCAAAGTTTGACGCCCGACCCCTTTAGGAGCCTGATCGCCCCAATCAACCACGTC
      A  S  F  K  L  R  A  G  E  I  L  G  L  A  G  L  V  G  A  G
                                                   WA

    GACGCACAGAGGTATCCCGGCTGATTTTTGGCGCAGATCGCAAAGTAAGCGGTGAAGTAT
61  ---------+---------+---------+---------+---------+---------+  120
    CTGCGTGTCTCCATAGGGCCGACTAAAAACCGCGTCTAGCGTTTCATTCGCCACTTCATA
      R  T  E  V  S  R  L  I  F  G  A  D  R  K  V  S  G  E  V  F

    TTTTAAATGGCAAAAAACTAGAGATTCATTCCCCCAGTGATGCGATCGCCGTCGGTATTG
121 ---------+---------+---------+---------+---------+---------+  180
    AAAATTTACCGTTTTTTGATCTCTAAGTAAGGGGGTCACTACGCTAGCGGCAGCCATAAC
      L  N  G  K  K  L  E  I  H  S  P  S  D  A  I  A  V  G  I  G

    GCTATGTCCCAGAAGACCGCAAAGACCAAGGTTTATTTCTGGAGATGAGTTCCCGTAAGA
181 ---------+---------+---------+---------+---------+---------+  240
    CGATACAGGGTCTTCTGGCGTTTCTGGTTCCAAATAAAGACCTCTACTCAAGGGCATTCT
      Y  V  P  E  D  R  K  D  Q  G  L  F  L  E  M  S  S  R  K  N

    ACATTGGACTCAATAGACTCAAGCAAGATGCTAATTTGGGCATCGTTAACTGGGGTTCAG
241 ---------+---------+---------+---------+---------+---------+  300
    TGTAACCTGAGTTATCTGAGTTCGTTCTACGATTAAACCCGTAGCAATTGACCCCAAGTC
      I  G  L  N  R  L  K  Q  D  A  N  L  G  I  V  N  W  G  S  V

    TGAATAAGGTTGCCACAGATGCAGTAGAAAACTTCCATATCCGCCTGGCGAACTTAGAAA
301 ---------+---------+---------+---------+---------+---------+  360
    ACTTATTCCAACGGTGTCTACGTCATCTTTTGAAGGTATAGGCGGACCGCTTGAATCTTT
      N  K  V  A  T  D  A  V  E  N  F  H  I  R  L  A  N  L  E  I

    TTAGAGCCGTGGATCTTTCCGGTGGGAATCAACAAAAACTGCCTGTGGCGCGTTGGTTAG
361 ---------+---------+---------+---------+---------+---------+  420
    AATCTCGGCACCTAGAAAGGCCACCCTTAGTTGTTTTTGACGGACACCGCGCAACCAATC
      R  A  V  D  L  S  G  G  N  Q  Q  K  L  P  V  A  R  W  L  A

    CCATTAACCCCAGAGTCTTGATGCTAGATGAGCCGACAAGGGGCGTAGATATCGGTGCTA
421 ---------+---------+---------+---------+---------+---------+  480
    GGTAATTGGGGTCTCAGAACTACGATCTACTCGGCTGTTCCCCGCATCTATAGCCACGAT
      I  N  P  R  V  L  M  L  D  E  P  T  R  G  V  D  I  G  A  K
                        WB

    AAAGCGAAATTTACCGGATTATCAGCGACCTATCAG
481 ---------+---------+---------+------  516
    TTTCGCTTTAAATGGCCTAATAGTCGCTGGATAGTC
      S  E  I  Y  R  I  I  S  D  L  S
```

Figure 5.9: Sequence of AKS showing a putative open reading frame that runs through the entire length of the clone. WA and WB are sequences similar to the Walker motifs A and B which are characteristic of many nucleotide binding proteins (Walker *et al.* 1982). Walker motif A corresponds to the p-loop or glycine rich loop known to be involved in phosphoryl transfer in many nucleotide binding proteins. The sequence has been submitted to Genbank and has been given the accession number U31647.

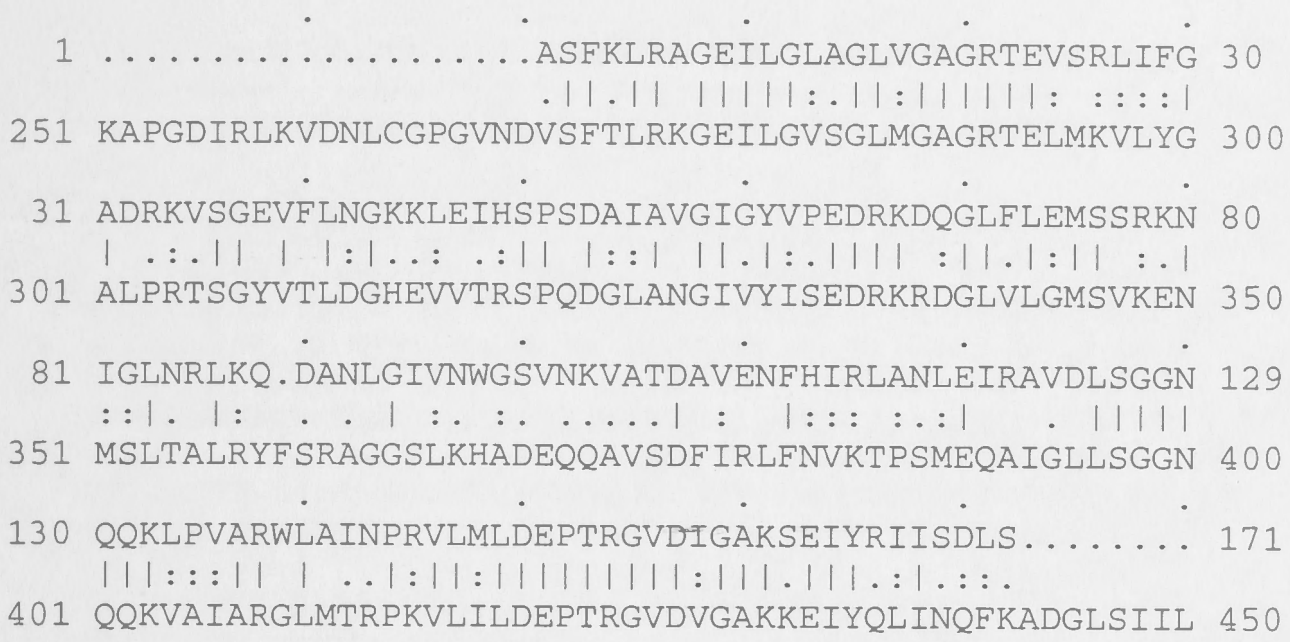


Figure 5.10: Alignment of the amino acid sequence of the putative open reading frame from AKS with the ribose transport protein (RbsA) from *Escherichia coli* (Bell *et al.* 1986). The sequences have 67% similarity and 45% identity.

```
    AGTTGCTTTTAACCGTCTGGGGCTATTGGCATTCATTGAAGCGCTGATTTTTATTGCAAT
1   ---------+---------+---------+---------+---------+---------+   60
    TCAACGAAAATTGGCAGACCCCGATAACCGTAAGTAACTTCGCGACTAAAAATAACGTTA
     V  A  F  N  R  L  G  L  L  A  F  I  E  A  L  I  F  I  A  I

                                                S.D.
    TCTTGTAGTCGCCCTAGTTTACGCATGGCGTAAAGGAGCATTGGAATGGTCTTGAATTCT
61  ---------+---------+---------+---------+---------+---------+  120
    AGAACATCAGCGGGATCAAATGCGTACCGCATTTCCTCGTAACCTTACCAGAACTTAAGA
     L  V  V  A  L  V  Y  A  W  R  K  G  A  L  E  W  S  *
                                                   M  V  L  N  S

    GATTTAACTACTCAGGACATAGAGCGCATCATCAACCCCATTGAACGTCCTACAGTCACT
121 ---------+---------+---------+---------+---------+---------+  180
    CTAAATTGATGAGTCCTGTATCTCGCGTAGTAGTTGGGGTAACTTGCAGGATGTCAGTGA
     D  L  T  T  Q  D  I  E  R  I  I  N  P  I  E  R  P  T  V  T

    CAAGACCTTTCGGAAAACGTTATTTTAACCACGGTTGATGACCTCTACAACTGGGCTAGG
181 ---------+---------+---------+---------+---------+---------+  240
    GTTCTGGAAAGCCTTTTGCAATAAAATTGGTGCCAACTACTGGAGATGTTGACCCGATCC
     Q  D  L  S  E  N  V  I  L  T  T  V  D  D  L  Y  N  W  A  R

    CTTTCGAGTCTGTGGCCGTTGTTATTTGGTACTGCTTGCTGCTTTATTGAGTTTGCAGCC
241 ---------+---------+---------+---------+---------+---------+  300
    GAAAGCTCAGACACCGGCAACAATAAACCATGACGAACGACGAAATAACTCAAACGTCGG
     L  S  S  L  W  P  L  L  F  G  T  A  C  C  F  I  E  F  A  A

    CTAATTGGTTCCCGTTTTGACTTTGACCGCTTCGGGTTAATTCCCCGTTCCAGTCCCCGT
301 ---------+---------+---------+---------+---------+---------+  360
    GATTAACCAAGGGCAAAACTGAAACTGGCGAAGCCCAATTAAGGGGCAAGGTCAGGGGCA
     L  I  G  S  R  F  D  F  D  R  F  G  L  I  P  R  S  S  P  R

    CAAGCCGATTTGATTATTACAGCTGGAACAATCACCATGAAGATGGCTCCTCAAATGGTG
361 ---------+---------+---------+---------+---------+---------+  420
    GTTCGGCTAAACTAATAATGTCGACCTTGTTAGTGGTACTTCTACCGAGGAGTTTACCAC
     Q  A  D  L  I  I  T  A  G  T  I  T  M  K  M  A  P  Q  M  V

    CGTCTTTATGAAAAAATGCCCGAACCTAAGTATGTAATTGCGATGGGCGCTTGTACAATT
421 ---------+---------+---------+---------+---------+---------+  480
    GCAGAAATACTTTTTTACGGGCTTGGATTCATACATTAACGCTACCCGCGAACATGTTAA
     R  L  Y  E  K  M  P  E  P  K  Y  V  I  A  M  G  A  C  T  I

    ACGGGCGGGATGTTCAGTGTCGAATCACCTACAGCTGTGCGTGGAGTTGACAAGTTAATT
481 ---------+---------+---------+---------+---------+---------+  540
    TGCCCGCCCTACAAGTCACAGCTTAGTGGATGTCGACACGCACCTCAACTGTTCAATTAA
     T  G  G  M  F  S  V  E  S  P  T  A  V  R  G  V  D  K  L  I

    CCCGTGGATGTCTACCTACCTGGTTGTCCTCCCCGGCCTGAAGCAATTATCGACGCAATG
541 ---------+---------+---------+---------+---------+---------+  600
    GGGCACCTACAGATGGATGGACCAACAGGAGGGGCCGGACTTCGTTAATAGCTGCGTTAC
     P  V  D  V  Y  L  P  G  C  P  P  R  P  E  A  I  I  D  A  M

    ATTAAGCTGCGGAAGAAGATTGCTAACGATTCCATGCAGGAACGGAGTCTGATTCGCCAA
601 ---------+---------+---------+---------+---------+---------+  660
    TAATTCGACGCCTTCTTCTAACGATTGCTAAGGTACGTCCTTGCCTCAGACTAAGCGGTT
     I  K  L  R  K  K  I  A  N  D  S  M  Q  E  R  S  L  I  R  Q

    ACCCACCGTTTCTACAGCACGACTCATAACTTGAAGCCAGTGGCAGAAATCTTAACTGGT
661 ---------+---------+---------+---------+---------+---------+  720
    TGGGTGGCAAAGATGTCGTGCTGAGTATTGAACTTCGGTCACCGTCTTTAGAATTGACCA
     T  H  R  F  Y  S  T  T  H  N  L  K  P  V  A  E  I  L  T  G
```

```
      AAGTATATGCAGTCTGAGACTCGCTTCAACCCACCAAAAGAATTGACAGAAGCGATCGGT
721,  ---------+---------+---------+---------+---------+---------+   780
      TTCATATACGTCAGACTCTGAGCGAAGTTGGGTGGTTTTCTTAACTGTCTTCGCTAGCCA
      K  Y  M  Q  S  E  T  R  F  N  P  P  K  E  L  T  E  A  I  G

      CTTCCTGTTCCCCCAGCGCTGCTGACATCACAAACACAGAAGGAGGAACAAAAACGTGGC
781   ---------+---------+---------+---------+---------+---------+   840
      GAAGGACAAGGGGGTCGCGACGACTGTAGTGTTTGTGTCTTCCTCCTTGTTTTTGCACCG
      L  P  V  P  P  A  L  L  T  S  Q  T  Q  K  E  E  Q  K  R  G

                                                S.D.
      TGATGAAGAATTAAAACCAGTACCCGCAGCAGCAGAGGCTATAGTGCCATCTGGGCCGAC
841   ---------+---------+---------+---------+---------+---------+   900
      ACTACTTCTTAATTTTGGTCATGGGCGTCGTCGTCTCCGATATCAAGGTAGACCCGGCTG
      *                                          M  P  S  G  P  T

      TTCTCAGTGGCTAACGGAAAATGGCTTTGCTCATGAATCCTTGGCGGCTGACAAAAATGG
901   ---------+---------+---------+---------+---------+---------+   960
      AAGAGTCACCGATTGCCTTTTACCGAAACGAGTACTTAGGAACCGCCGACTGTTTTTACC
       S  Q  W  L  T  E  N  G  F  A  H  E  S  L  A  A  D  K  N  G

      TGTAGAGATAATTAAAGTGGAACCAGATTTTATGCTTCCCATCGCTACAGCCCTGTATGC
961   ---------+---------+---------+---------+---------+---------+  1020
      ACATCTCTATTAATTTCACCTTGGTCTAAAATACGAAGGGTAGCGATGTCGGGACATACG
       V  E  I  I  K  V  E  P  D  F  M  L  P  I  A  T  A  L  Y  A

      TTACGGGTTTAATTATCTTCAGTTTCAAGGCGGTATTGATCTCGGCCCAGGACAGGATTT
1021  ---------+---------+---------+---------+---------+---------+  1080
      AATGCCCAAATTAATAGAAGTCAAAGTTCCGCCATAACTAGAGCCGGGTCCTGTCCTAAA
       Y  G  F  N  Y  L  Q  F  Q  G  G  I  D  L  G  P  G  Q  D  L

      AGTCAGTGTGTATCACTTGGTGAAAGTGAGTGACAACGCTGATAAGCCTGAAGAAGTCAG
1081  ---------+---------+---------+---------+---------+---------+  1140
      TCAGTCACACATAGTGAACCACTTTCACTCACTGTTGCGACTATTCGGACTTCTTCAGTC
       V  S  V  Y  H  L  V  K  V  S  D  N  A  D  K  P  E  E  V  R

      AGTGAAGGTGTTTTTACCACGGGAAAATCCTGTAGTACCTTCGGTTTACTGGATTTGGAA
1141  ---------+---------+---------+---------+---------+---------+  1200
      TCACTTCCACAAAAATGGTGCCCTTTTAGGACATCATGGAAGCCAAATGACCTAAACCTT
       V  K  V  F  L  P  R  E  N  P  V  V  P  S  V  Y  W  I  W  K

      AACCGCAGACTGGCAAGAACGCGAATCTTACGATATGTTCGGTATTATCTACGAAGGACA
1201  ---------+---------+---------+---------+---------+---------+  1260
      TTGGCGTCTGACCGTTCTTGCGCTTAGAATGCTATACAAGCCATAATAGATGCTTCCTGT
       T  A  D  W  Q  E  R  E  S  Y  D  M  F  G  I  I  Y  E  G  H

      CCCCAATTTGAAACGCATTTTGATGCCAGGAGATTGGGTA
1261  ---------+---------+---------+---------+  1300
      GGGGTTAAACTTTGCGTAAAACTACGGTCCTCTAACCCAT
       P  N  L  K  R  I  L  M  P  G  D  W  V
```

Figure 5.11: Nucleic acid sequence of AKL showing the open reading frames for *ndhC, ndhK* and *ndhJ*. S.D. indicates the position of the ribosomal binding sites (Shine-Dalgarno sequence) for *ndhK* and *ndhJ*. The order of the genes within the operon is *ndhC, ndhK, ndhJ*. The sequence has been submitted to Genbank and been given the accession number U31208.

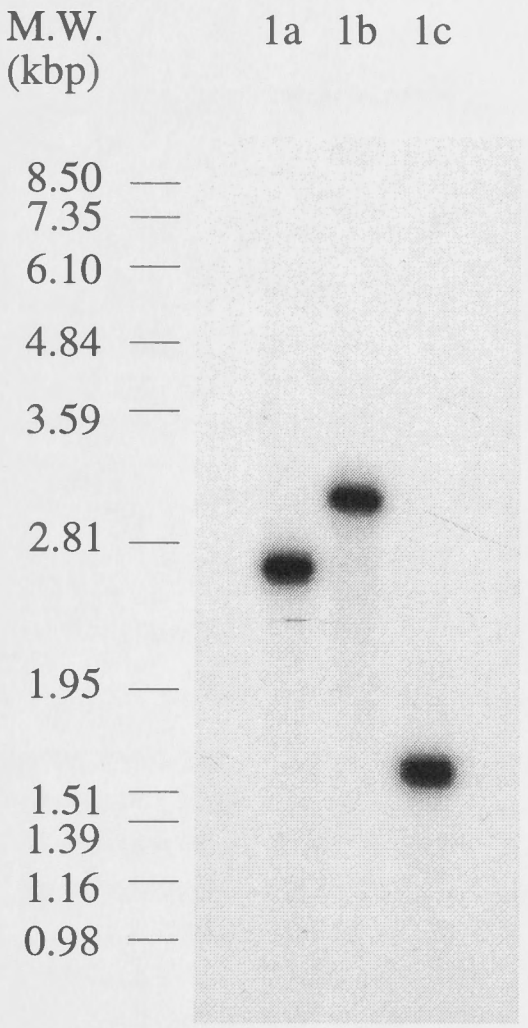


Figure 5.12: Southern analysis of genomic DNA from *Anabaena* PCC 7120 to determine the copy number of *ndhK*.

Genomic DNA was digested, separated by agarose gel electrophoresis (2.13.1), transferred to Hybond-N^+ (Amersham), probed (2.14) with a 440 bp *Eco RI, Acc I* fragment of *ndhK* from *Anabaena* PCC 7120, and washed at low stringency (42°C, 0.5X SSC).

Panel 1: Southern analysis of genomic DNA digested with different restriction enzymes. Each lane contains 5 µg of genomic DNA. Lane a, Genomic DNA digested with *Eco RI*. Lane b, genomic DNA digested with *Hind III*. Lane c, genomic DNA digested with *Eco RI* and *Hind III*.

```
                1                                                   50
      A7120                                                      MVLNSD
      S6803                                                   MSPNPANPTD
    S6803-2                                                         M
       Rice                                                 MVL T-EYSDKKKK
      Maize                                                 MVL T-EYSEKKKK
      Wheat                                          MAKRSLGMVL T-EYLD-KKK
    Tobacco  MGNEFRRIGC ICIYRSFHFR AYLNYWFSLC MAKGGIGMVL APEYSDNKKK
Liverwort                                               MVL NFKFFTCENS

               51                                                  100
      A7120  LTTQDIERII NPIERPTVTQ DLSENVILTT VDDLYNWARL SSLWPLLFGT
      S6803  LERVATAKIL NPASRSQVTQ DLSENVILTT VDDLYNWAKL SSLWPLLYGT
    S6803-2  STSTHALTLQ NPIQAPQVTK ELSENVILTC LDDIYNWARL STLYPMMFGT
       Rice  EGKDSIKTVM SLIEFPLLDQ RSSNSVISTT LKDLSNWSRL SSLWPLLYGT
      Maize  EGKDSIETIM SLIEFPLLDQ TSSNSVISTT PNDLSNWSRL SSLWPLLYGT
      Wheat  EGKDSIETVM NLIEFPLLDQ TSSNSVISTT PNDLSNWSRL SSLWPLLYGT
    Tobacco  NGKNKIETVM NSIQFPLLDR TTQNSVISTT LNDLSNWSRL SSLWPLLYGT
Liverwort  LEDNSTTMLK NSIESSFINK TLTNSIILTT FNDFSNWARL SSLWPLLYGT
                                                  *  **     *      *

              101                                                  150
      A7120  ACCFIEFAAL IGSRFDFDRF GLIPRSSPRQ ADLIITAGTI TMKMAPQMVR
      S6803  ACCFIEFAAL IGSRFDFDRF GLVPRSSPRQ ADLIITAGTI TMKMAPALVR
    S6803-2  ACCFMEFMAA FGPRFDLERF GSIPRATPRQ ADLMITAGTI TMKYAPALVQ
       Rice  SCCFIEFASL IGSRFDFDRY GLVPRSSPRQ ADLILTAGTV TMKMAPSLVR
      Maize  SCCFIEFASL IGSRFDFDRY GLVPRSSPRQ ADLILTAGTV TMKMAPSLVR
      Wheat  SCCFIEFASL IGSRFDFDRY GLVPRSSPRQ ADLILTAGTV TMKMAPSLVR
    Tobacco  SCCFIEFASL IGSRFDFDRY GLVPRSSPRQ ADLILTAGTV TMKMAPSLVR
Liverwort  SCCFIEFASL IGSRFDFDRY GLVPRSSPRQ ADLIITAGTV TMKMAPSLVR
             **  *          *  *  *   * **** *    ***    ****

              151                                                  200
      A7120  LYEKMPEPKY VIAMGACTIT GGMFSVESPT AVRGVDKLIP VDVYLPGCPP
      S6803  LYEEMPEPKY VIAMGACTIT GGMFSSDSTT AVRGVDKLIP VDVYIPGCPP
    S6803-2  LYEQIPEPKY VIAMGACTIT AGMFSADSPT AVRGVDKLIP VDVYIPGCPP
       Rice  LYEQMPEPKY VIAMGACTIT GGMFSTDSYS TVRGVDKLIP VDVYLPGCPP
      Maize  LYEQMPEPKY VIAMGACTIT GGMFSTDSYS TVRGVDKLIP VDVYLPGCPP
      Wheat  LYEQMPEPKY VIAMGACTIT GGMFSTDSYS TVRGVDKLIP VDVYLPGCPP
    Tobacco  LYEQMPEPKY VIAMGACTIT GGMFSTDSYS TVRGVDKLIP VDVYLPGCPP
Liverwort  LYEQMPEPKY VIAMGACTIT GGMFSTDSYT TVRGVDKLIP VDIYLPGCPP
             *  * **   ** ** *    **          * * *   * ** * *****

              201                                                  250
      A7120  RPEAIIDAMI KLRKKIANDS MQERSL--IR QTHRFYSTTH NLKPVAEILT
      S6803  RPEAIFDAII KLRKKVANES IQERAI--TQ QTHRYYSTSH QMKVVAPILD
    S6803-2  RPEAVIDGII KLRKKVAGES RQDYTE--DL QTHRFHAVRH RMKPVSPILT
       Rice  KPEAVIDALT KLRKKISREI VEDRTL--SQ KKNRCFTTSH KLYVRRSTNT
      Maize  KPEAVIDALT KLRKKIAREI IEDRTLCQSQ KKNRSFTTRH KLYVRRSTHT
      Wheat  KPEAVIDALT KLRKKISREI VEDRTL--SQ NKNRCFTTSH KLYVRRSTHT
    Tobacco  KPEAVIDAIT KLRKKISREL YEDRI--RSQ RANRCFTTNH KFHVQHSIHT
Liverwort  KPEAIIDAII KLRKKIAQEI YEEKKIL--K KGTRFFTLNH QFNFFSNLDN
             **         *
```

```
            251                                                  292
A7120       GKYMQSETRF NPPKELTEAI GLPVPPALLT SQTQKEEQKRG*
S6803       GKYLQQGTRS APPRELQEAM GMPVPPALTT SQ-QKEQLNRG*
S6803-2     GQYLRHHEDL TPHHD----- -----PLLIK *
Rice        GTY.EQELLY QSPSTLDISS ETFFKSKSPV SSYKLVN*
Maize       GTY.EQELLY QSPSTLDISS ETFFKSKSSV SSYKLVN*
Wheat       GTY.EQELLY QSPSTLDISS ETFFKSKSSV PSYKLVN*
Tobacco     GNY.DQRVLY QPPSTSEIPT EIFFKYKNSV SSPELVN*
Liverwort   PKLTSSNQFF QSKKTSKVLL ETSLTFKEKE NL*
```

Figure 5.13: Alignment of NdhK from *Anabaena* PCC 7120 with NdhK from chloroplasts and cyanobacteria.

Sequences were aligned using the "Pileup" program in the UWGCG sequence analysis package (Devereux *et al.* 1984). Conserved amino acids are shown in red; amino acids that are conserved in all sequences except the second copy of NdhK from *Synechocystis* PCC 6803 are shown in green. Amino acid which are conserved in all sequences in the alignment except *Anabaena* PCC 7120 are shown in blue. * under conserved amino acids indicates that the amino acid is also conserved in the homologues of NdhK in complex I from *E. coli*, *P. denitrificans* and bovine heart mitochondria.

*A*7120 = *Anabaena* PCC 7120; *S*6803 = *Synechocystis* PCC 6803; *S*6803-2 = second copy of NdhK from *Synechocystis* PCC 6803, which is cryptic and located on a plasmid.

References for the sequences are: *Anabaena* PCC 7120 this study; maize and *Synechocystis* PCC 6803 Steinmüller *et al.* (1989); *S*6803-2 Mayes *et al.* (1990; Steinmüller and Bogorad (1990); rice (Hiratsuka *et al.* (1989); tobacco Shinozaki, *et al.* (1986); liverwort Ohyama *et al.* (1986).

```
            1                                                   50
A7120       .......... VPSGPTSQWL TENGFAHESL AADKNGVEII KVEPDFMLPI
S6803       .......... ..VGPVSTWL TTNGFEHQSL TADHLGVEMV QVEADLLLPL
Rice        .......... MQQGWLSNWL VKHEVVHRSL GFDHRGIETL QIKAEDWDSI
Maize       .......... MQQGWLSNWL VKHDVVHRSL GFDHRGVETL QIKAGDWDSI
Tobacco     .......... .MQGRLSAWL VKHGLIHRSL GFDYQGIETL QIKPEDWHSI
Liverwort   MLNILKNNNN KIQGRLSIWL IKHNLKHRPL GFDYQGIETL QIRSEDWPSL

            51                                                  100
A7120       ATALYAYGFN YLQFQGGIDL GPGQDLVSVY HLVKVSDNAD KPEEVRVKVF
S6803       CTALYAYGFN YLQCQGAYDE GPGKSLVSFY HLVKLTEDTR NPEEVRLKVF
Rice        AVILYVYGYN YLRSQCAYDV APGGSLASVY HLTRIQYGID NPEEVCIKVF
Maize       AVILYVYGYN YLRSQCAYDV APGGSLASVY HLTRIQYGID NPEEVCIKVF
Tobacco     AVIFYVYGYN YLRSQCAYDV APGGLLASVY HLTRIEDGVD QPEEVCIKVF
Liverwort   AVALYVYGFN YLRSQCAYDV EPGGLLASVY HFTKITDNAD QPEEICIKIF
                               *           *                   *

            101                                                 150
A7120       LPRENPVVPS VYWIWKTADW QERESYDMFG IIYEGHPNLK RILMPGDWV.
S6803       LPRENPVVPS VYWIWKAADW QERECYDMFG IVYEGHPNLK RILMPEDWVG
Rice        AQKDNPRIPS VFWIWRSSDF QERESFDMVG ISYDNHPRLK RILMPESWIG
Maize       AQKDNPRIPS VFWVWRSADF QERESYDMVG ISYDNHPRLK RILMPESWIG
Tobacco     ASRRNPRIPS VFWVWKSVDF QERESYDMLG ISYDNHPRLK RILMPESWIG
Liverwort   ILRKNPKIPS IFWVWKSADF QERESYDMFG IFYENHPCLK RILMPDSWLG
                                  ***  *  *       *  *  **

            151               170
A7120       .......... ..........
S6803       WPLRKDYISP DFYELQDAY*
Rice        WPLRKDYITP NFYEIQDAH*
Maize       WPLRKDYITP NFYEIQDAH*
Tobacco     WPLRKDYIAP NFYEIQDAH*
Liverwort   WPLRKDYIVP NFYELQDAY*
             * ***
```

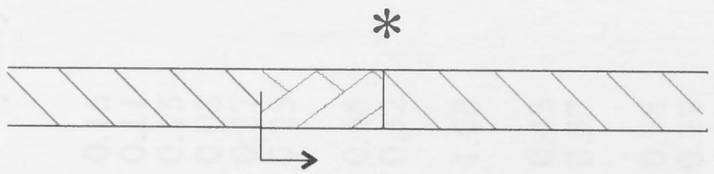
Figure 5.14: Alignment of NdhJ from *Anabaena* PCC 7120 with NdhJ from chloroplasts and cyanobacteria.

Sequences were aligned using the "Pileup" program in the UWGCG sequence analysis package (Devereux *et al.* 1984). Conserved amino acids are shown in red. Amino acid which are conserved in all sequences in the alignment except *Anabaena* PCC 7120 are shown in blue. * under conserved amino acids indicates that the amino acid is also conserved in the homologues of NdhJ in complex I from *E. coli*, *P. denitrificans* and bovine heart mitochondria.

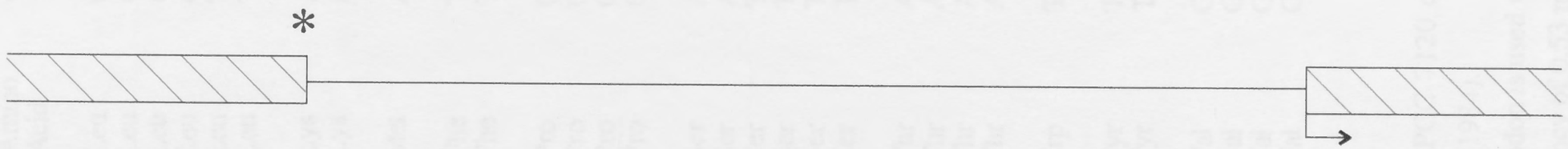
*A*7120 = *Anabaena* PCC 7120; *S*6803 = *Synechocystis* PCC 6803;

References for the sequences are: *Anabaena* PCC 7120 this study; maize and *Synechocystis* PCC 6803 Steinmüller *et al.* (1989); rice Hiratsuka *et al.* (1989); tobacco Shinozaki, *et al.* (1986); liverwort Ohyama *et al.* (1986).

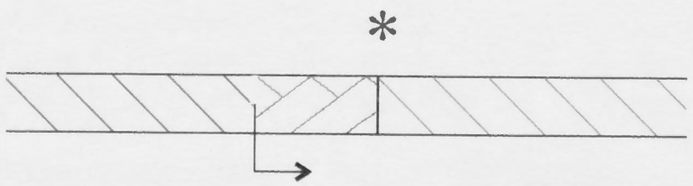
Anabaena PCC 7120



Synechocystis PCC 6803



Liverwort, Rice, Maize, Wheat (28), Tobacco (118)



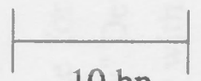


Figure 5.15: Map comparing the structure of the operon encoding *ndh(C-K-J)* from cyanobacteria and chloroplasts at the boundary between *ndhC* and *ndhK*.
The numbers in brackets after wheat and tobacco represent the size of the overlap in these species.

Amino Acid	Codon	Frequency
Ala	GCA	0.26
Ala	GCC	0.21
Ala	GCG	0.14
Ala	GCT	0.39
Arg	AGA	0.14
Arg	AGG	0.04
Arg	CGA	0.11
Arg	CGC	0.28
Arg	CGG	0.13
Arg	CGT	0.30
Asn	AAC	0.52
Asn	AAT	0.48
Asp	GAC	0.39
Asp	GAT	0.61
Cys	TGC	0.45
Cys	TGT	0.55
End	TAA	0.43
End	TAG	0.31
End	TGA	0.26
Gln	CAA	0.70
Gln	CAG	0.30
Glu	GAA	0.78
Glu	GAG	0.22
Gly	GGA	0.17
Gly	GGC	0.23
Gly	GGG	0.13
Gly	GGT	0.47
His	CAC	0.55
His	CAT	0.45
Ile	ATA	0.12
Ile	ATC	0.36
Ile	ATT	0.53
Leu	CTA	0.15
Leu	CTC	0.11
Leu	CTG	0.15
Leu	CTT	0.08
Leu	TTA	0.27
Leu	TTG	0.25
Lys	AAA	0.68
Lys	AAG	0.32
Met	ATG	1.00
Phe	TTC	0.43
Phe	TTT	0.57
Pro	CCA	0.28
Pro	CCC	0.29
Pro	CCG	0.08
Pro	CCT	0.35
Ser	AGC	0.17
Ser	AGT	0.18
Ser	TCA	0.15
Ser	TCC	0.16
Ser	TCG	0.08
Ser	TCT	0.26
Thr	ACA	0.31
Thr	ACC	0.32
Thr	ACG	0.08
Thr	ACT	0.29
Trp	TGG	1.00
Tyr	TAC	0.54
Tyr	TAT	0.46
Val	GTA	0.32
Val	GTC	0.17
Val	GTG	0.21
Val	GTT	0.30

Table 5.1: Codon frequency table for *Anabaena* PCC 7120 constructed using the UWGCG program "codonfrequency" Devereux *et al.*(1984).
Frequency represents the frequency with which the codon is used within its synonymous family, expressed as a fraction of 1: ie a codon frequency of 0.53 means that the codon is used in 53% of the cases in which the amino acid it encodes is used.

	A 7120	*S* 6803	S 6803-2	*T. a*	*Z. m*	*N. t*	*M. p*	*O. s*	*E. c*	*P. d*	*B. t*
B. t	42	41	45	41	41	38	41	43	68	71	100
P. d	43	43	45	41	41	42	43	41	46	100	
E. c	42	40	39	40	41	37	37	41	100		
O. s	59	54	52	96	95	83	63	100			
M. p	61	60	51	63	65	64	100				
N. t	58	55	52	84	83	100					
Z. m	60	55	53	96	100						
T. a	60	55	52	100							
S 6803-2	64	65	100								
S 6803	77	100									
A 7120	100										

Table 5.2 Percentage identity of NdhK from various organisms at the amino acid level.

Abbreviations: *B. t = Bos taurus, P. d = Paracoccus denitrificans, E. c = Escherichia coli, O. s = Oryza sativa, M. p = Marchantia polymorpha, N. t = Nicotiana tobacum, Z. m = Zea mays, T. a = Triticum aestivum,* *S* 6803 = *Synechocystis* PCC 6803, *A* 7120 = *Anabaena* PCC 7120

References: *B.t.* Arizmendi *et al.* (1992a); *P.d.* Xu *et al.* (1992b); *E.c.* Weidner *et al* (1993); *O.s., M.p.* and *N.t.* a chloroplast encoded ORF was identified as NdhK by Arizmendi *et al.* (1992a); *Z.m.* and *S*-6803 Steinmüller *et al.* (1989); *T.a.* Nixon et al. (1989); *S*-6803-2 Mayes *et al.* (1990) and Steinmüller and Bogorad (1990).

	A 7120	*S* 6803	*Z. m*	*N. t*	*M. p*	*O. s*	*E. c*	*P. d*	*B. t*
B. t	34	29	29	29	29	27	30	48	100
P. d	33	29	27	29	28	26	40	100	
E. c	29	32	26	26	28	25	100		
O. s	48	54	96	84	70	100			
M. p	51	55	71	77	100				
N. t	51	53	84	100					
Z. m	49	55	100						
S 6803	74	100							
A 7120	100								

Table 5.3: Percentage identity of NdhJ from various organisms at the amino acid level.

Abbreviations: *B. t = Bos taurus, P. d = Paracoccus denitrificans, E. c = Escherichia coli, O. s = Oryza sativa, M. p = Marchantia polymorpha, N. t = Nicotiana tobacum, Z. m = Zea mays, S* 6803 = *Synechocystis* PCC 6803 *A* 7120 = *Anabaena* PCC 7120

References: *B.t.* Pilkington *et al.* (1991b); *P.d.* Xu *et al.* (1992b); *E.c.* Weidner *et al* (1993); *O.s., M.p.* and *N.t.* a chloroplast encoded ORF was identified as NdhJ by Pilkington *et al.* (1991b); *Z.m.* and *S*-6803 Steinmüller *et al.* (1989).

CHAPTER 6

INSERTIONAL INACTIVATION OF *ndhK* FROM *ANABAENA* PCC 7120

6.1 INTRODUCTION

Chapter 5 described the cloning of part of the operon encoding *ndhC-K-J* from *Anabaena* PCC 7120. As discussed, *ndhK* and its homologues are highly conserved across a broad range of species. This suggests that it should have a similar function within NDH-1 in all of these species. However, the different cellular location of NDH-1 in *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 (see Chapter 4) suggests that the enzyme has a different function in these two species. Besides its role in respiration NDH-1 has been shown to play a role in the CO_2 concentrating mechanism of *Synechocystis* PCC 6803 (Ogawa 1990; Ogawa 1991a; Ogawa 1991b; Ogawa 1991c; Ogawa 1992a; Ogawa 1992b) as well as in cyclic electron transport around PS I (Mi *et al.* 1992a; Mi *et al.* 1994; Mi *et al.* 1995). It is unknown if the NDH-1 of *Anabaena* PCC 7120 has any other functions besides its role in respiration on the plasma membrane. Therefore experiments were carried out to insertionally inactivate *ndhK* to try and elucidate the function of NDH-1 in *Anabaena* PCC 7120.

Two methods have been developed by which DNA can be introduced into the cyanobacterium *Anabaena* PCC 7120. Conjugation, a process whereby DNA is transfered from one cell to another by a mechanism requiring cell to cell contact (Willetts and Skurray 1980), has been used and appears to be a general means to introduce DNA from *E. coli* into cyanobacteria (Wolk *et al.* 1984). More recently electroporation has been also been used (Thiel and Poo 1989). Electroporation is the reversible permeabilization of a cells membranes by a high voltage potential across the membrane (Knight 1981; Knight and Scrutton 1986). Conjugation requires the presence of a conjugal plasmid which encodes all of the genes necessary for the conjugal apparatus (Willetts and Skurray 1980; Thomas and Smith 1987). Conjugal plasmids are quite large (pRL443 is approximately 57 kb) and some smaller plasmids have evolved to take advantage of the presence of a conjugal plasmid. Two features are necessary for the smaller plasmid to be mobilised by the conjugal plasmid (Willetts and Wilkins 1984). Firstly, the plasmid must contain a segment of DNA called the *bom* site (basis of mobility) and, secondly, the plasmid must encode or be provided with a DNA-nicking protein, encoded by the *mob* gene, that specifically recognises the *bom* site (Finnegan and Sherratt 1982; Willetts and Wilkins 1984). The *mob* gene does not have to be present on the plasmid to be transferred (cargo plasmid), and can be provided *in trans* by a helper plasmid (Finnegan and Sherratt 1982).

One problem with transfer of DNA by conjugation is that it favours meridiploid formation (single recombination) over gene replacement (double recombination) (Van Haute *et al.* 1983). Therefore a positive selection mechanism for double recombinants is required. An elegant system has been developed by Cai and Wolk (1990), in which a conditionally

lethal gene is included on a suicide plasmid (a cargo plasmid that is unable to replicate in the host to which it is transferred) that also contains the DNA with which the cyanobacterium is to be transformed. The conditionally lethal gene used is *sacB*, from *Bacillus subtilis*, which encodes for levansucrase, a 50 kDa secretory protein. Transcription of *sacB* is induced by sucrose. Cai and Wolk (1990) have shown that the conditional lethality of *sacB* in *Anabaena* sp. enables direct selection for double recombinants on sucrose-containing medium. Cells in which gene replacement has occurred will lose *sacB* as it is on a suicide plasmid that can not replicate in the host and therefore growth in the presence of sucrose will be not be lethal. Cells in which a single recombination event has occurred will still contain *sacB* as it has been partially integrated into the genome, and growth in the presence of sucrose will be lethal. The lethality may be due to transfructosylation from sucrose to various metabolically important acceptors or to accumulation of unsecreted protein in the cell membranes because of inadequate cleavage and export (Gay *et al.* 1983).

The cells that survive sucrose selection will be unsegregated. Each colony will contain a population of filaments that contain transformed and untransformed cells. As *Anabaena* PCC 7120 contains multiple genome equivalents, as calculated from their genetic complexity (Herdman *et al.* 1979; Bancroft *et al.* 1988), the transformed cells will probably contain both mutant and wild type copies of the gene within their genome. Thus segregation of the transformants requires segregation of the genome of the cells containing both the mutant and wild type copies of the gene, and physical disjunction of adjacent transformed and untransformed cells within the filament. Generally this can be achieved by succesive streakings of the colonies onto plates containing the appropriate antibiotic(s) (Elhai and Wolk 1988; Cai and Wolk 1990).

6.1.1 Aims

The aim of this part of the project was firstly, to determine the best growth conditions for selection of an NDH-1 mutant and secondly, to insertionally inactivate *ndhK* in *Anabaena* PCC 7120. Biochemical analysis would then be performed on resultant mutants to try and determine the effect of inactivating *ndhK* and thus determining the functions of NDH-1 in *Anabaena* PCC 7120.

6.2 TRANSFORMATION PLASMIDS USED

pRL443 — Conjugal plasmid that contains ampicillin and tetracycline resistance genes, approximately 57 kb (Elhai and Wolk 1988).

pRL528 — Helper plasmid that contains a chloramphenicol resistance gene. Carries *mob* and genes for *Ava I* and *Eco47 II* methylases, which methylate *Ava I* and *Ava II* sites respectively, thereby preventing restriction by *Ava I* and *Ava II,* which are found in a wide variety of cyanobacteria (Tandeau de Marsac and Houmard 1987) Approximately 16.8 kb (Figure 6.1) (Elhai and Wolk 1988).

pRL271 — Suicide vector useful for generating gene replacements. Contains chloramphenicol and erythromycin resistance genes and *sacB*. Has an extensive polylinker (Figure 6.2), 6.4 kb. Related to the plasmids described in (Cai and Wolk 1990).

pRL25 — Cargo vector that can replicate in *Anabaena* PCC 7120 containing a kanamycin/neomycin resistance gene 10.14 kb (Figure 6.3) (Wolk *et al.* 1988). It is based on pDU1 a plasmid isolated from *Nostoc* PCC 7125 and contains the 2.3 kb *Not I - Sca I* fragment necessary for replication in *Anabaena* PCC 7120 (Wolk *et al.* 1984; Wolk *et al.* 1985; Schmetterer and Wolk 1988).

6.3 RESULTS

6.3.1 Analysis of Respiration in Total Membrane Preparations of *Anabaena* PCC 7120

Before attempts to insertionally inactivate *ndhK* in *Anabaena* PCC 7120 could be made it needed to be determined if growth conditions affected the level of NDH-1 catalysed respiration. Growth conditions have been shown to affect the composition of redox proteins within the cell in other bacteria (Meyer and Cusanovich 1989). In *Anabaena* PCC 7120 NDH-1 is only present on the plasma membrane and it is the only pyridine nucleotide dehydrogenase on the membrane (Howitt *et al.* 1993; Chapter 3). Respiration on the plasma membrane is almost completely rotenone-sensitive but respiration on the thylakoid membrane is rotenone-insensitive (Howitt *et al.* 1993; Chapter 3). The amount of rotenone sensitivity of respiration in total membrane preparations is, therefore a good

indicator of respiration catalysed by the NDH-1 on the plasma membrane. If the level of rotenone-sensitive respiration differed among the growth conditions tested, the one with the lowest level of rotenone-sensitive respiration is likely to be the best condition to select for mutants in which an *ndh* gene has been inactivated. The effects of the different growth conditions on the levels of Antimycin A and cyanide-insensitive respiration would also be studied.

Four different growth conditions were studied, they were: growth under continuous illumination sparged with air or 5% (v/v) CO_2 in air and growth under 12 hours of illumination followed by 12 hours of darkness (light/dark cycle) sparged with air or 5% (v/v) CO_2 in air. These conditions were tested for two reasons; firstly, continuous illumination and the light/dark cycle were chosen as when the cells are illuminated it is thought that the majority of the cells energy requirements will be met by photophosphorylation rather than oxidative phosphorylation (Scherer 1990), therefore under conditions of continuous illumination respiratory rates and hence the level of NDH-1 catalysed respiration may be lower. Secondly, the different CO_2 concentrations were chosen as the products of the *ndh* genes have been implicated in the CO_2 concentrating mechanism in *Synechocystis* PCC 6803 (Ogawa 1990; Ogawa 1991a; Ogawa 1991b; Ogawa 1991c; Ogawa 1992a; Ogawa 1992b), therefore different CO_2 concentrations may effect the level of NDH-1 catalysed respiration. In the assays horse heart ferrocytochrome c was added to reconstitute the electron transport chain, as cytochrome c_{553} is removed during membrane preparation. For most species of cyanobacteria horse heart ferrocytochrome c is an adequate substitute (Lockau 1981; Krinner *et al.* 1982; Stürzl *et al.* 1982). The difference between the level of respiration prior to the addition of cytochrome c, in the assays to determine the effect of growth conditions on respiration (Figures 6.4-6.9), probably just reflects different degrees of removal of cytochrome c_{553} during membrane preparation.

The effect of rotenone on the rate of NADH catalysed respiration by total membranes is shown in Figure 6.4. The data have been normalised to allow for the variations seen in the respiratory rates of different preparations. Approximately 60% of the NADH catalysed respiratory rate was insensitive to rotenone when the cells were grown on the light/dark cycle sparged with 5% CO_2 in air. In the other three conditions approximately 80% of the respiratory rate was insensitive to rotenone. Using a students T test with a confidence level of 95%, it was shown that level of rotenone-sensitive respiration was significantly lower in the cells grown on the light/dark cycle sparged with 5% CO_2 in air when compared to cells grown on the light dark cycle sparged with air ($p=0.03$) and continuous illumination sparged with air ($p=0.04$). The was no significant difference in the level of rotenone-sensitive NADH catalysed respiration between the cells grown on the light/dark cycle sparged with 5% CO_2 in air, and those grown on continuous

illumination sparged with 5% CO_2 in air. None of the other growth conditions showed any significant difference between the level of rotenone-sensitive NADH catalysed respiration.

The effect of rotenone on NADPH catalysed respiration in total membrane preparations was also tested (Figure 6.5). Approximately 70-90% of the respiratory rate was insensitive to rotenone under the four conditions tested. However, the degree of rotenone-sensitivity when the cells were grown under continuous illumination and sparged with air was significantly lower than when the cells were grown under continuous illumination and sparged with 5% CO_2 in air ($p=0.01$). There was no significant difference between the level of rotenone-sensitivity for any of the other growth conditions tested.

The sensitivity of both NADH and NADPH catalysed respiration to Antimycin A was also tested (Figures 6.6 and 6.7). As can be seen approximately 75% of both the NADH (Figure 6.6) and NADPH (Figure 6.7) catalysed respiratory rates were insensitive to Antimycin A. A students T-test, using a confidence level of 95%, showed that there was no significant difference between the level of Antimycin A sensitivity for either NADH or NADPH catalysed respiration under any of the culture conditions tested.

Figures 6.8 and 6.9 show the effect of cyanide on NADH and NADPH catalysed respiration respectively. As can be seen there was a large difference between the effect of cyanide on NADH and NADPH catalysed respiration. NADH catalysed respiration was much more sensitive to cyanide, with only 20-40% of the respiratory rate being insensitive to cyanide across the four conditions tested, while NADPH catalysed respiration was between 70 and 80% insensitive to cyanide. There was no significant difference between the level of cyanide-insensitive respiration in any of the four growth conditions tested for either NADH or NADPH catalysed respiration.

On the whole these studies have shown that growth conditions appear to have very little effect on respiration in *Anabaena* PCC 7120. No significant difference was seen between the effect of Antimycin A or cyanide on either NADH or NADPH mediated respiration in any of the growth conditions tested.

6.3.2 Rationale behind choice of growth conditions for selection of mutants

From the results presented above it can be seen that in total membrane preparations oxidation of NADH was more sensitive to cyanide than was NADPH oxidation (Figures 6.8 and 6.9). This is in agreement with studies on membrane preparations from

Plectonema boryanum (Matthijs *et al.* 1984a; Matthijs *et al.* 1984b). It has been shown that respiration on the plasma membrane, but not the thylakoid membrane, of *Anabaena* PCC 7120 is completely inhibited by cyanide (Howitt *et al.* 1993), therefore it would appear that the plasma membrane oxidises NADH preferentially to NADPH, hence the greater sensitivity to cyanide when NADH is the substrate. The converse is true of the thylakoid membrane. Thus when assessing the growth conditions to ascertain which one is the best for selection of *ndh* mutants more emphasis should be placed on the effect of rotenone on NADH mediated respiration, as NDH-1 in *Anabaena* PCC 7120 has been shown to be located only on the plasma membrane (Chapter 4; Howitt *et al.* 1993).

Of the four growth conditions tested only membranes from cells grown on the light/dark cycle and sparged with 5% CO_2 (v/v) in air had a significantly higher level of rotenone-sensitive NADH mediated respiration than any of the other three (Figure 6.4). There was no significant difference between the degree of rotenone-sensitivity of any of the other three conditions tested. Therefore, any of these could be used to select for *ndh* mutants.

It was decided to select for potential transformants under continuous illumination with nitrate in the growth medium and in an atmosphere enriched in CO_2 (5%). This condition was chosen as the *ndh* genes have been implicated in the CO_2 concentrating mechanism of some cyanobacteria, as discussed in section 1.3.3. Pyridine nucleotide dehydrogenases are the entry point for electrons into the respiratory chain and also provide electrons for cyclic electron transport around PS I used to generate ATP for nitrogenase in heterocysts of *Anabaena* PCC 7120. By growing the potential transformants under the above conditions it was hoped that they would compensate for any loss of respiration, nitrogen fixation and a functional CO_2 concentrating mechanism to ensure survival of the potential transformants.

6.3.2 Engineering Constructs for Transformation of *Anabaena* PCC 7120

The first step in building a construct to insertionally inactivate a gene is to insert an antibiotic resistance gene into the gene of interest. *ndhK* from *Anabaena* PCC 7120 contains an *Hinc II* restriction site near the centre of the gene and this site was used to insert the antibiotic resistance gene. A chloramphenicol resistance gene under the control of the *psbA* promoter (kindly provided by Dr G. D. Price, Research School of Biological Sciences, Australian National University) was used in initial studies (Figure 6.10). This was chosen because *psbA* has been shown to be a strong promoter in *Anabaena* PCC 7120 (Elhai 1993). A *Sma I* fragment containing the chloramphenicol resistance gene was cloned into the *Hinc II* site of *ndhK* in both the sense and antisense orientation with respect to *ndhK* (Figure 6.11), giving plasmids pAKL1C(+) and pAKL1C(-). Both

plasmids were digested with *Sph I* and *Pst I* and the fragments containing the disrupted *ndhK* gene were cloned into the *Sph I* and *Pst I* sites of pRL271, giving plasmids pRL271C(+) and pRL271C(-) (Figure 6.6).

6.3.4 Transformation of *Anabaena* PCC 7120

The critical concentration of chloramphenicol for *Anabaena* PCC 7120 was found to be 15 μg ml^{-1} for solid media and 5 μg ml^{-1} for liquid media. Initial selection was carried out on plates containing 15 μg ml^{-1} chloramphenicol, but this was raised to 25 μg ml^{-1} for the sucrose selection step and all subsequent platings, to ensure colonies seen were not due to background levels of resistance. Liquid cultures were grown in 7 μg ml^{-1} chloramphenicol.

a) Conjugal Transfer

Both pRL271C(+) and pRL271C(-) were transformed into *E. coli* strain DH5α MCR that had already been transformed with the helper plasmid pRL528 giving rise to the cargo strains. Triparental matings were carried out as described in Section 2.17.1 with both cargo strains using both sonicated and unsonicated *Anabaena* PCC 7120. No chloramphenicol resistant colonies arose from the transformation using sonicated *Anabaena* PCC 7120, but controls in which the sonicated cells were plated out onto growth media without chloramphenicol showed that the cells were still viable.

Forty eight colonies arose from the transformations of unsonicated cells and twenty of these survived selection on sucrose. These colonies were plated out through successive generations until they were free of *E. coli*. All 20 were transferred to liquid culture and genomic DNA isolated (Section 2.9.1) from the 17 that grew. To determine if the strains were fully segregated, PCR was carried out using the primers shown below.

ANDHK5'2 ACC GTC TGG GGC TAT TGG CA

ANDHK3'2 GCA TCA AAA TGC GTT TCA AA

These primers are 100% homologous to regions of *ndhC* and *ndhJ* respectively and produce a fragment of approximately 1.3 kb when used in a PCR reaction with genomic DNA from wild type *Anabaena* PCC 7120.

A typical result from reactions using these primers is shown in Figure 6.13. Lane 1a is the positive control using genomic DNA from wild type and a band of 1.3 kb can be seen

as expected. Surprisingly the 1.3 kb wild type fragment was also seen in lanes 1b to 1g and there is no band at 2.5 kb as would be expected if the strains contained the inactivated copy of *ndhK*. This banding pattern was exactly the same as that seen for the other 11 chloramphenicol resistant strains (data not shown). The DNA was transferred to Hybond-N$^+$ (Amersham) (2.14.1), subjected to Southern analysis and the blot washed at high stringency (55°C, 0.1X SSC). Figure 6.13 lanes 2a-2g shows that the 1.3 kb fragment in all lanes was the only fragment that hybridised when a 430 bp *Eco RI - Acc I* fragment of *ndhK* from *Anabaena* PCC 7120 was the probe, indicating that all of the chloramphenicol resistant strains were wild type for *ndhK*. No positively hybridising bands were seen when the blot was probed with a *Bam HI* fragment of the chloramphenicol resistance gene (Figure 6.13 lanes 3a-3g).

To determine if the chloramphenicol resistance gene had integrated elsewhere in the genome, genomic DNA from the six samples as shown in Figure 6.13 was digested separately with *Eco RI* and *Hind III*, separated by electrophoresis, transferred to Hybond-N$^+$ (Amersham) and probed with the *Bam HI* fragment of the chloramphenicol resistance gene. No positively hybridising bands were seen, suggesting that the resistance to chloramphenicol was not due to the presence of the chloramphenicol resistance gene and may have been the result of a spontaneous mutation giving rise to a chloramphenicol resistant phenotype. No attempts were made to determine the basis of the resistance seen.

b) Electroporation

Electroporation was carried out as described in section 2.17.2 using plasmid DNA isolated from the cargo strain used for the conjugal transfer experiments. Only one chloramphenicol resistant colony was found and this died during selection for double recombinants on sucrose.

6.3.5 Redesigning Constructs for Transforming *Anabaena* PCC 7120

As *Anabaena* PCC 7120 is apparently able to develop natural resistance to chloramphenicol, it was decided to redesign the constructs used for transformation. This time a kanamycin resistance gene was used in place of the chloramphenicol resistance gene. Two kanamycin resistance genes were available in the laboratory, one a derivative of Tn5 and the other of Tn903 (pUC-4K, Pharmacia). Nothing is known about the expression of the Tn903 derived kanamycin resistance gene but it is known that the Tn5 derived kanamycin resistance gene is poorly expressed in *Anabaena* PCC 7120 (Elhai

1993); therefore, it was decided to use the Tn903 derivative, which works well in other cyanobacteria.

The plasmid pUC-4K was digested with *Hinc II* and the 1.3 kb fragment containing the kanamycin resistance gene was cloned into the *Hinc II* site of *ndhK* in both the sense and antisense orientation with respect to *ndhK* (Figure 6.14), giving plasmids pAKL1K(+) and pAKL1K(-). Both plasmids were digested with *Sph I* and *Spe I* and the fragments containing the disrupted *ndhK* gene were cloned into the *Sph I* and *Spe I* sites of pRL271, giving plasmids pRL271K(+) and pRL271K(-) (Figure 6.15).

6.3.6 Transformation of *Anabaena* PCC 7120

The critical concentration of kanamycin for *Anabaena* PCC 7120 was found to be 6 μg ml^{-1} for solid media and 1 μg ml^{-1} for liquid media. Initial selection was carried out on plates containing 8 μg ml^{-1} kanamycin, but this was raised to 12 μg ml^{-1} for the sucrose selection step and all subsequent platings. Liquid cultures were grown in 2 μg ml^{-1} kanamycin.

a) Conjugal Transfer

Both pRL271K(+) and pRL271K(-) were transformed in to *E. coli* strain DH5α MCR that had already been transformed with the helper plasmid pRL528 giving rise to the cargo strains. Triparental matings were carried out as described in Section 2.17.1 with both cargo strains using both sonicated and unsonicated *Anabaena* PCC 7120. No kanamycin resistant colonies arose from the transformation using sonicated *Anabaena* PCC 7120, but controls in which the sonicated cells were plated onto growth media without kanamycin showed that the cells were still viable.

Transformation using unsonicated cells produced a large number of potential transformants. Fourteen colonies were present on the 1 in 10,000 dilution plate of the transformation with pRL271K(+) and 12 on the equivalent plate for the transformation with pRL271K(-). Approximately 10 times as many colonies were seen on the 1 in 1,000 dilution plates for both constructs. In controls, on the same preparation of cyanobacteria, in which the conjugal plasmid had been omitted from the mating, no colonies were seen on the 1 in 10,000 dilution and only 2 colonies were present in the 1 in 1,000 dilution for the pRL271K(+) control and 3 for the pRL271K(-) control. These results indicate that the majority of kanamycin resistant colonies seen were due to transfer of the suicide plasmid to *Anabaena* PCC 7120 from the cargo strain of *E. coli*. Fifty nine

colonies were seen on the 1 in 10,000 dilution plate in a positive control in which *Anabaena* PCC 7120 was transformed with pRL25, a plasmid capable of replicating in *Anabaena* PCC 7120 (Section 6.2) The same preparation of cyanobacteria and conjugal plasmid was used for this control as for the transformations with pRL271K(+) and pRL271K(-). Some of these colonies were transferred to liquid culture and were able to grow. This indicates that the colonies seen were real transformants and that the growth was not a background level provided by pRL25 being present in the cargo strain and inactivating the kanamycin in the plates, thereby allowing growth of *Anabaena* PCC 7120 (Elhai and Wolk 1988). Genomic DNA was isolated from one of the kanamycin tolerant strains, from the positive control, and digested with *Hind III*. The DNA was then subjected to Southern analysis, using the kanamycin resistance gene from pRL25 as the probe. The blot was washed at high stringency (55°C, 0.1X SSC). The probe did not hybridise to any of the fragments in the wild type control (Figure 6.16 lane a). A fragment of 3.0 kb from the kanamycin tolerant strain hybridised to the probe (Figure 6.16 lane b). This fragment was exactly the same size as the fragment from the positive control (pRL25 digested with *Hind III*) that hybridised with the probe (Figure 6.16 lane c). This provides conclusive proof that the transformation system was working.

Forty eight colonies from each transformation were streaked onto plates containing 5% (w/v) sucrose and 12 μg ml^{-1} kanamycin. Forty four of the colonies from the transformation using pRL271K(+) and 43 from the transformation using pRL271K(-) grew on media containing sucrose. These colonies should have been free of *E. coli* as expression of *sacB* is lethal to *E. coli* when grown on 5% (w/v) sucrose (Gay *et al.* 1983) and the conjugal and helper plasmids do not contain a kanamycin resistance gene. Therefore, growth on kanamycin and 5% (w/v) sucrose should kill both the conjugal and cargo strains. This was confirmed by plating randomly selected samples on to LB without selection and incubating at 37°C overnight: no bacterial colonies were seen.

With such a large number of potential transformants to be screened a faster screening method had to be developed than growing the cultures in liquid and isolating genomic DNA and using that for PCR. It was decided to try and use whole cells as a template for PCR.

A colony of wild type *Anabaena* PCC 7120 was resuspended in 100 μl of sterile Milli Q water and this was used as a template for PCR using ANDHK5'2 and ANDHK3'2 as the primers. PCR was carried out as described in Section 2.11.1. The results are shown in Figure 6.17. Wild type genomic DNA that had been passed through caesium chloride gradients was the template in lane a and as expected a single product of approximately 1.3 kb, which has previously been identified as *ndhK* (Section 6.3.3), was seen. Lanes b, c and d show the results when 1, 3 and 5 μl of the cell suspension was used as the template:

2 major products were seen a fragment of approximately 1.3 kb and one of approximately 500 bp. Some minor products of lower molecular weight were also seen in lanes b, c and d that were not present in lane a. This indicates that there was some non-specific priming when whole cells were used as a template. The presence of the 1.3 kb fragment, which is seen when genomic DNA is used as the template in the whole cell reactions, indicates that this technique can be used to rapidly screen large numbers of potential transformants. Interestingly, it was found that the yield of the PCR reaction decreased with increasing time after resuspension of the cells in water. The experiment described above was repeated 24 h after resuspension of the cells and the major product could only just be visualised after staining with ethidium bromide. However the product was clearly visible in the control lane with genomic DNA as the template (data not shown). This method has potential and as far as I am aware is the first report of whole cyanobacterial cells being used as a template for amplification of DNA by PCR in cyanobacteria.

PCR as described above was carried out on all 87 potential transformants that had survived selection on sucrose, using 3 µl of cells as the template. The 1.3 kb product representing the wild type copy of *ndhC-K-J* was seen in all samples in which the PCR worked. The non-specific 500 bp product was also seen in all reactions, including reactions in which wild type cells were the template. Only one potential transformant (M76) also contained a PCR product of approximately 2.6 kb, the size expected for the inactivated copy of *ndhK*. A typical result is shown in Figure 6.18; lane 1a is the control using wild type cells as the template and lanes 1b to 1r are potential transformants. M76 is in lane 1q. The PCR products from all 87 potential transformants were transferred to Hybond-N$^+$ (Amersham) and subjected to Southern analysis using a *Pst I* fragment of the kanamycin resistance gene as the probe. The washes were carried out at high stringency (65°C using 0.1X SSC). Twenty of the 87 potential transformants had positively hybridising bands at approximately 2.6 kb, suggesting that the PCR had amplified the insertionally inactivated copy of *ndhK*, but the product was present in insufficient amounts to be visualised by ethidium bromide staining. No positively hybridising bands were seen in any of the wild type controls, which were present on all gels. The result of the Southern blot of the gel shown in Figure 6.18 is also shown (Panel 2). As can be seen there were positively hybridising bands in lanes 2d, 2i, 2m, but no corresponding band in the equivalent lane in the ethidium bromide stained lane (Panel 1). There was a very strongly hybridising band in lane 2q that corresponds to the 2.6 kb band seen in lane 1q. This suggests that M76 is an unsegregated mutant and that the other potential transformants in which a positively hybridising band of approximately 2.6 kb was seen when probed with the kanamycin resistance gene were also unsegregated mutants.

As a control, PCR amplification was also performed on 19 samples which had not been passed through sucrose selection, but had been freed of *E. coli*. As can be seen in Figure

6.19 (Panel 1) the 1.3 kb product is present in all but four lanes of ethidium bromide stained gels, but none of the samples contained the 2.6 kb inactivated copy. Southern analysis (Figure 6.19 Panel 2) shows that all the samples, which contained the 1.3 kb product, except the wild type control (lane 2a) contained the 2.6 kb product that hybridised with the kanamycin resistance gene, but it was present in insufficient quantities to visualise with ethidium bromide staining.

All of the unsegregated mutants died during subsequent platings, suggesting that the product of *ndhK* is essential for the survival of *Anabaena* PCC 7120, at least under the selection conditions used.

6.4 DISCUSSION

6.4.1 Resistance of *Anabaena* PCC 7120 to chloramphenicol

The apparent ability of some colonies of *Anabaena* PCC 7120 to survive chloramphenicol containing medium in the absence of a chloramphenicol resistance gene, as determined by Southern analysis (Section 6.3.3), suggests that spontaneous mutants that are resistant to chloramphenicol were selected. Chloramphenicol acts by binding to the 50S ribosomal subunit and blocking translation (Sambrook *et al.* 1989). There are two types of mutations which could prevent inhibition of protein synthesis by chloramphenicol :-

a) a mutation which prevents uptake of chloramphenicol by the cell

b) a ribosomal mutation which prevents binding of chloramphenicol to 50S ribosomal subunit.

The basis of the chloramphenicol resistance is unknown, but it was noted that when the cells grown in liquid culture were pelleted by centrifugation, the pellets held together better than pellets from wild type cells, and there was a mucus associated with the pellets not seen with pellets of wild type cells. This may be an indication that the resistance was based on the inability of chloramphenicol to enter the cell, but no studies were carried out to determine if this was the case.

6.4.2 Rapid screening for potential mutants using PCR

A very simple, rapid method was developed for screening of potential transformants of *Anabaena* PCC 7120 using PCR, with whole cells as the template. This method removes the need for growing cells in liquid culture and isolating genomic DNA which then can be used as a template for PCR or Southern analysis, to test if the desired gene has been inactivated and the cells are fully segregated. This greatly speeds up the screening process. Also, it is the first successful amplification of genomic DNA from whole cyanobacterial cells using PCR that I am aware of. If this method can be applied to other cyanobacteria it may speed up mutagenic studies and also cloning of genes through the use of homologous primers.

6.4.3 *ndhK* is an essential gene in *Anabaena* PCC 7120

The results presented in this chapter indicate that inactivation of *ndhK* in *Anabaena* PCC 7120 is apparently lethal. Firstly, the control in which *Anabaena* PCC 7120 was transformed with pRL25, produced colonies that were able to grow in liquid media in the presence kanamycin. This indicates that these are real transformants and not due to background kanamycin resistance from *E. coli* (Elhai and Wolk 1988), and shows that the transformation system was working properly. The result of the Southern analysis (Figure 6.16) confirms this as it shows the kanamycin resistance gene was present in genomic DNA preparations from the kanamycin tolerant strains generated by the positive control transformations. As this control was done at the same time and using the same preparation of cyanobacteria and conjugal plasmid as the transformations with pRL271K(+) and pRL271K(-), it can be assumed that these two plasmids were transferred into *Anabaena* PCC 7120.

This is supported by the results of the negative controls in which the conjugal plasmid was left out of the triparental matings. Approximately 50 fold fewer colonies were seen in the negative controls when compared to the transformations in which the conjugal plasmid was included. This means that some of the kanamycin resistant colonies seen would not be transformants, but the majority were due to the transfer of the shuttle vector into *Anabaena* PCC 7120. A large proportion of the colonies survived sucrose selection, indicating that they were double recombinants. Their survival on sucrose is further evidence for the colonies not being the result of background levels of resistance provided by *E. coli* present on the plate. Expression of *sacB* in *E. coli* can be induced by 5% (w/v) sucrose in the growth medium and is lethal (Gay *et al.* 1983). Therefore, the cargo strain of *E. coli* should die during sucrose selection. As the helper plasmid does not contain a resistance gene for kanamycin the cargo strain of *E. coli* can not survive if the

suicide plasmid to be transferred is lost. The conjugal plasmid does not contain a kanamycin resistance gene either, therefore the conjugal strain should also die on kanamycin. This was verified, as no bacterial colonies grew when samples of the transformed cyanobacteria were plated onto LB and incubated overnight at 37°C.

PCR using primers 100% homologous to regions in genes either side of *ndhK* revealed that all of the sucrose resistant, kanamycin resistant colonies contained a wild type copy of *ndhK*. One of these (M76) also produced a PCR product that was the correct size to be the inactivated copy. Southern analysis with the kanamycin resistance gene revealed that the product of approximately 2.6 kb contained the kanamycin resistance gene. As this product was the correct size to be the inactivated copy of *ndhK*, positively hybridised with the kanamycin resistance gene and was amplified with primers 100% homologous to regions of genes either side of *ndhK*, its identity as the inactivated gene was confirmed. The 2.6 kb fragment that hybridised with the kanamycin resistance gene was not seen in any of the wild type controls, further supporting the identification of the 2.6 kb fragment as the inactivated gene that had been introduced using conjugal transfer. The presence of both the 1.3 kb wild type copy and the 2.6 kb inactivated copy of *ndhK* in M76 indicates that this strain was unsegregated. The Southern analysis also revealed that 19 other sucrose tolerant colonies were unsegregated. As the cells did not grow in liquid culture genomic DNA could not be isolated and Southerns analysis of the genomic DNA could not be performed. Therefore, the possibility that the inactivated gene had been randomly integrated into the genome can not be ruled out.

The unsegregated transformants died during successive streakings to segregate them. This indicates that the protein encoded by *ndhK* is probably essential for survival of *Anabaena* PCC 7120, under the growth conditions used. As cells of *Anabaena* PCC 7120 contain multiple genome equivalents, as calculated from their genetic complexity (Herdman *et al.* 1979; Bancroft *et al.* 1988) it is likely that the cells contained both the wild type copy and the inactivated copy within their genome. Also as *Anabaena* PCC 7120 is filamentous, it is likely that there are cells in the filament that only contain wild type copies of *ndhK* within their genome. The unsegregated population would survive as the wild type copy of *ndhK* would allow a functional NAD(P)H dehydrogenase to form and the expression of the kanamycin resistance gene would give resistance to kanamycin. The kanamycin resistance gene encodes an aminoglycoside phosphotransferase which phosphorylates kanamycin (Sambrook *et al.* 1989). Phosphorylation is thought to interfere with the transport of kanamycin into the cell. Therefore a localised area of phosphorylated kanamycin could be generated on the plate allowing the survival of the wild type cells in the filament. Successive streakings would see segregation towards all wild type or all inactivated copies of *ndhK* in the genome of cells within the filament. The cells containing the inactivated copies of *ndhK* would be unable to grow without a

functional NAD(P)H dehydrogenase on the plasma membrane. As these cells could not grow, no localised kanamycin free areas could be generated on the plates allowing growth of the wild type cells.

The above scenario is supported by the finding that all cells tested before selection on sucrose contained both a wild type and an inactivated copy of *ndhK* (Figure 6.18). The fact that no transformants were found when the filaments were disrupted, by sonication, to 1 or 2 cells in length before transformation, even though the cells were still viable as they survived on plates of growth media that did not contain kanamycin, lends weight to the theory that the transformants contained a mixed population of untransformed and transformed cells with unsegregated genomes within the filaments.

The inability to completely segregate mutants of essential genes in *Anabaena* PCC 7120 has been seen before. Muro-Pastor and Florencio (1994) cloned and inactivated the gene for $NADP^+$-Isocitrate dehydrogenase (*idc*), but could not get complete segregation even when grown in media supplemented with substrates that should have made the presence of a functional $NADP^+$-Isocitrate dehydrogenase unnecessary.

6.5 SUMMARY

The results presented in this chapter showed that spontaneous mutants of *Anabaena* PCC 7120 can apparently provide resistance to chloramphenicol. This means that resistance to chloramphenicol can not be used as a reliable marker for transformation of *Anabaena* PCC 7120. Secondly, a new method for rapidly screening large numbers of potential transformants, using PCR was developed. This greatly speeds up the screening process and allows selection of transformants for further study at an earlier stage. Thirdly, it is likely that *ndhK* is an essential gene in *Anabaena* PCC 7120 under the growth conditions used, as insertional inactivation resulted in an apparently lethal phenotype. This conclusion must stand until it can be verified. Unfortunately it is very difficult to verify if a gene is essential or not. The possible basis for the lethal phenotype will be discussed in Chapter 7.

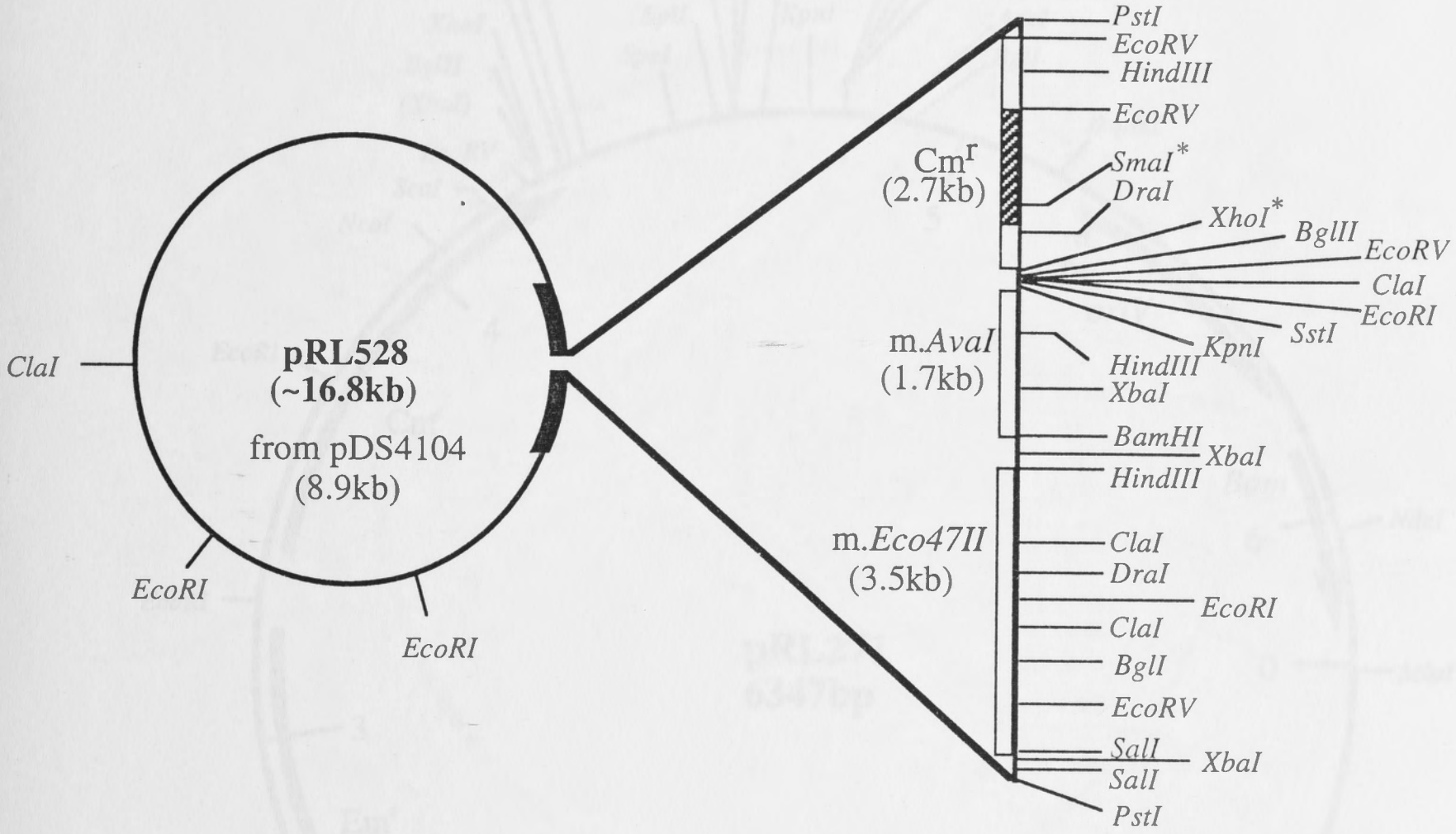


Figure 6.1: Helper plasmid pRL528 (Elhai and Wolk 1988)

▬▬ remnant of Tn1

pDS4104 is also known as ColK::Tn1

*SmaI** and *XhoI** are methylated in pRL528 and can not be cut

mob$^+$ from ColK

Cm^r = chloramphenicol resistance gene

m.*AvaI* methylates against *AvaI* (& *SmaI* & *XhoI*)

m.*Eco47II* methylates against *AvaII* (& *Sau96I*)

Will only grow in *mcrB*$^-$ hosts

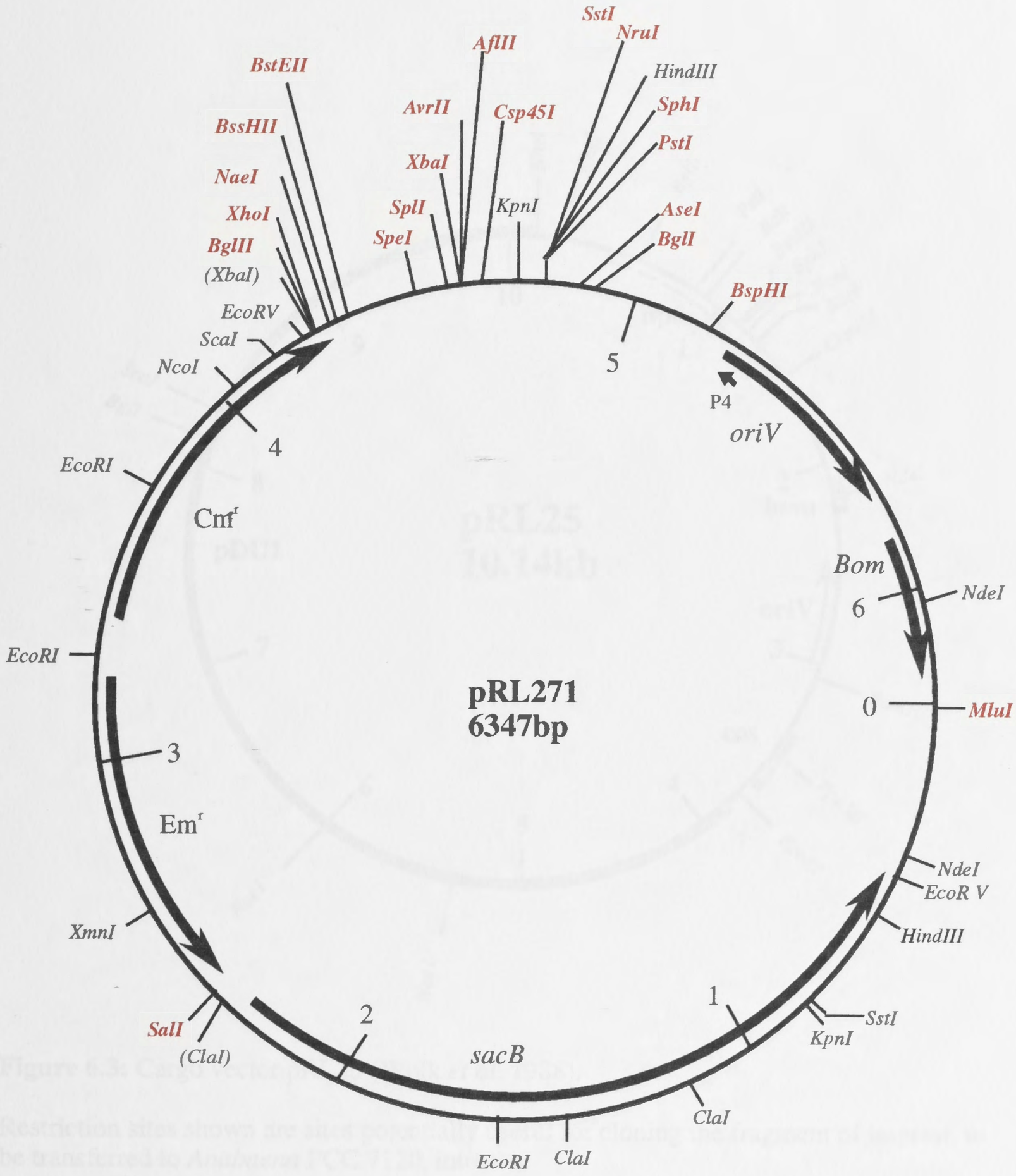


Figure 6.2: Map of suicide vector pRL271 (Cai and Wolk, 1990)
Em^r = Erythromycin resistance gene, Cm^r = chloramphenicol resistance gene, *bom* = basis of mobility, *sacB* = encodes levansucrase from *Bacillus subtilis*, P4 = strong promoter from pBR322, *(Xxx X)* = site of dam methylation, *oriV* = origin of replication, *Xxx X* = restriction site suitable for cloning fragment of DNA to be transferred to *Anabaena* PCC 7120 into.

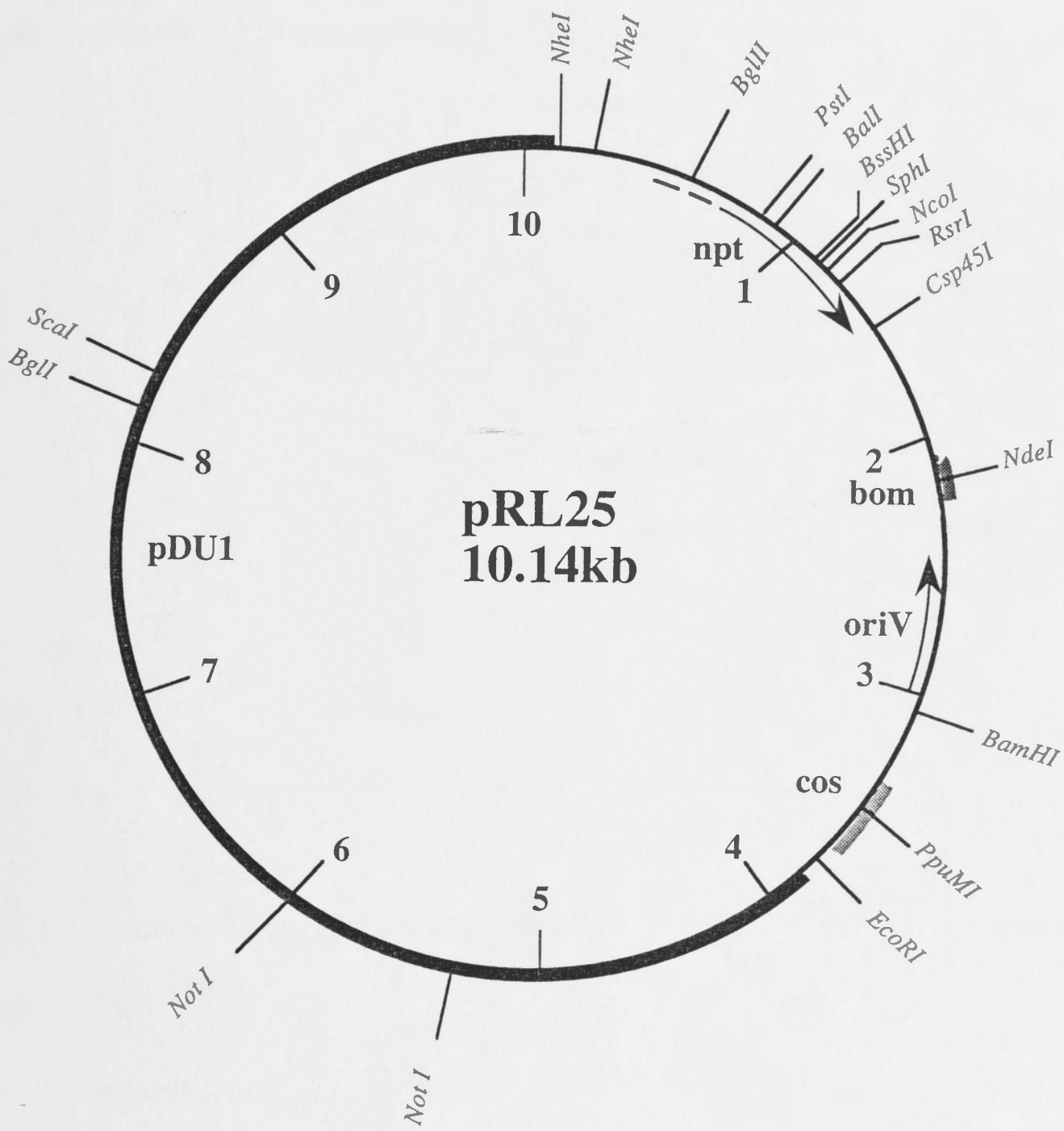


Figure 6.3: Cargo vector pRL25 (Wolk *et al.* 1988).

Restriction sites shown are sites potentially useful for cloning the fragment of interest, to be transferred to *Anabaena* PCC 7120, into.

bom = basis of mobility, npt = neomycin/kanamycin resistance gene, *oriV* = Origin of replication, *cos* = region of DNA required for packaging into bacteriophage l, pDU1 = plasmid isolated from *Nostoc* PCC 7125 that is able to replicate in *Anabaena* PCC 7120

Figure 6.4 Effect of rotenone on NADH catalysed respiration in total membrane preparations from *Anabaena* PCC 7120

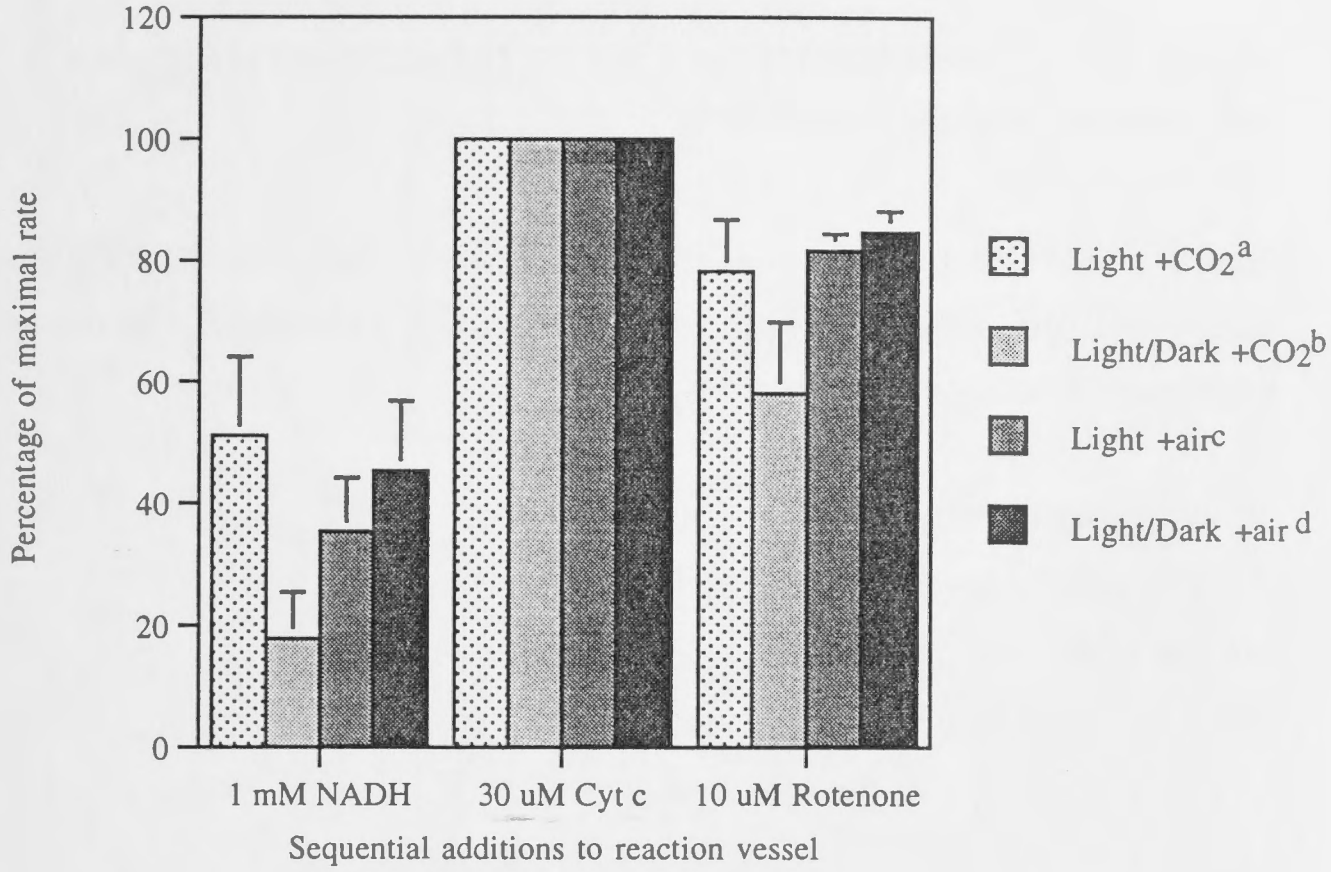
Membranes were prepared and oxygen consumtion was measured as described in chapter 2.
Data shown is the mean value ± standard error, expressed as a percentage of the rate after addition of horse heart ferrocytochrome c.

a) n = 5, 100% = 166 ± 21 nmols O_2 h^{-1} mg^{-1} protein
b) n = 5, 100% = 104 ± 27 nmols O_2 h^{-1} mg^{-1} protein
c) n = 5, 100% = 141 ± 46 nmols O_2 h^{-1} mg^{-1} protein
d) n = 5, 100% = 147 ± 42 nmols O_2 h^{-1} mg^{-1} protein

Figure 6.5 Effect of rotenone on NADPH catalysed respiration in total membrane preparations from *Anabaena* PCC 7120

Membranes were prepared and oxygen consumtion was measured as described in chapter 2.
Data shown is the mean value ± standard error, expressed as a percentage of the rate after addition of horse heart ferrocytochrome c.

a) n = 4, 100% = 86 ± 20 nmols O_2 h^{-1} mg^{-1} protein
b) n = 3, 100% = 69 ± 33 nmols O_2 h^{-1} mg^{-1} protein
c) n = 5, 100% = 104 ± 13 nmols O_2 h^{-1} mg^{-1} protein
d) n = 5, 100% = 74 ± 17 nmols O_2 h^{-1} mg^{-1} protein


120
100
80
60
40
20
0
Percentage of maximal rate
1 mM NADH
30 uM Cyt c
10 uM Rotenone
Sequential additions to reaction vessel
Light +CO2 a
Light/Dark +CO2 b
Light +air c
Light/Dark +air d

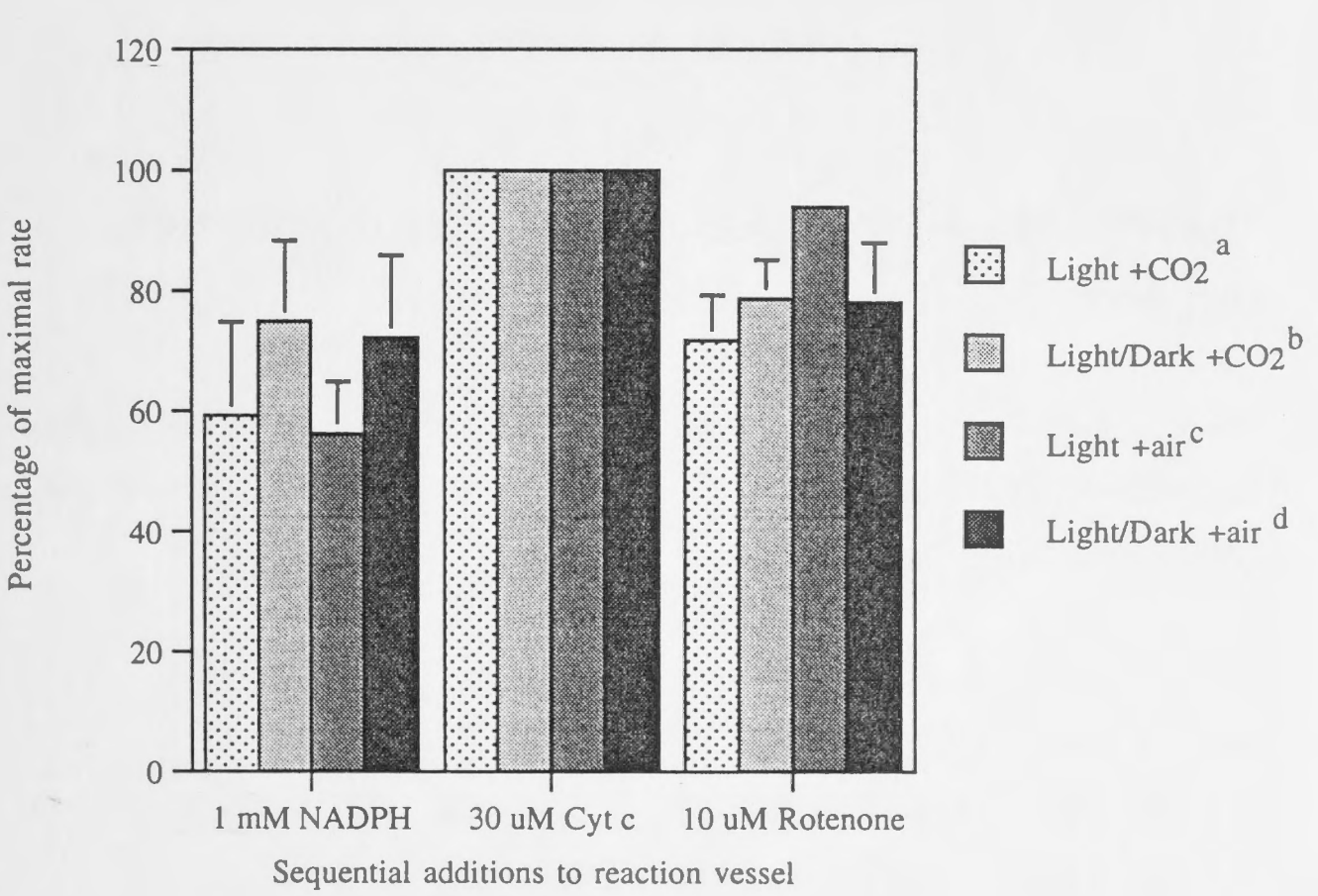

120
100
80
60
40
20
0
Percentage of maximal rate
1 mM NADPH
30 uM Cyt c
10 uM Rotenone
Sequential additions to reaction vessel
Light +CO2 a
Light/Dark +CO2 b
Light +air c
Light/Dark +air d

Figure 6.6 Effect of Antimycin A on NADH catalysed respiration in total membrane preparations from *Anabaena* PCC 7120

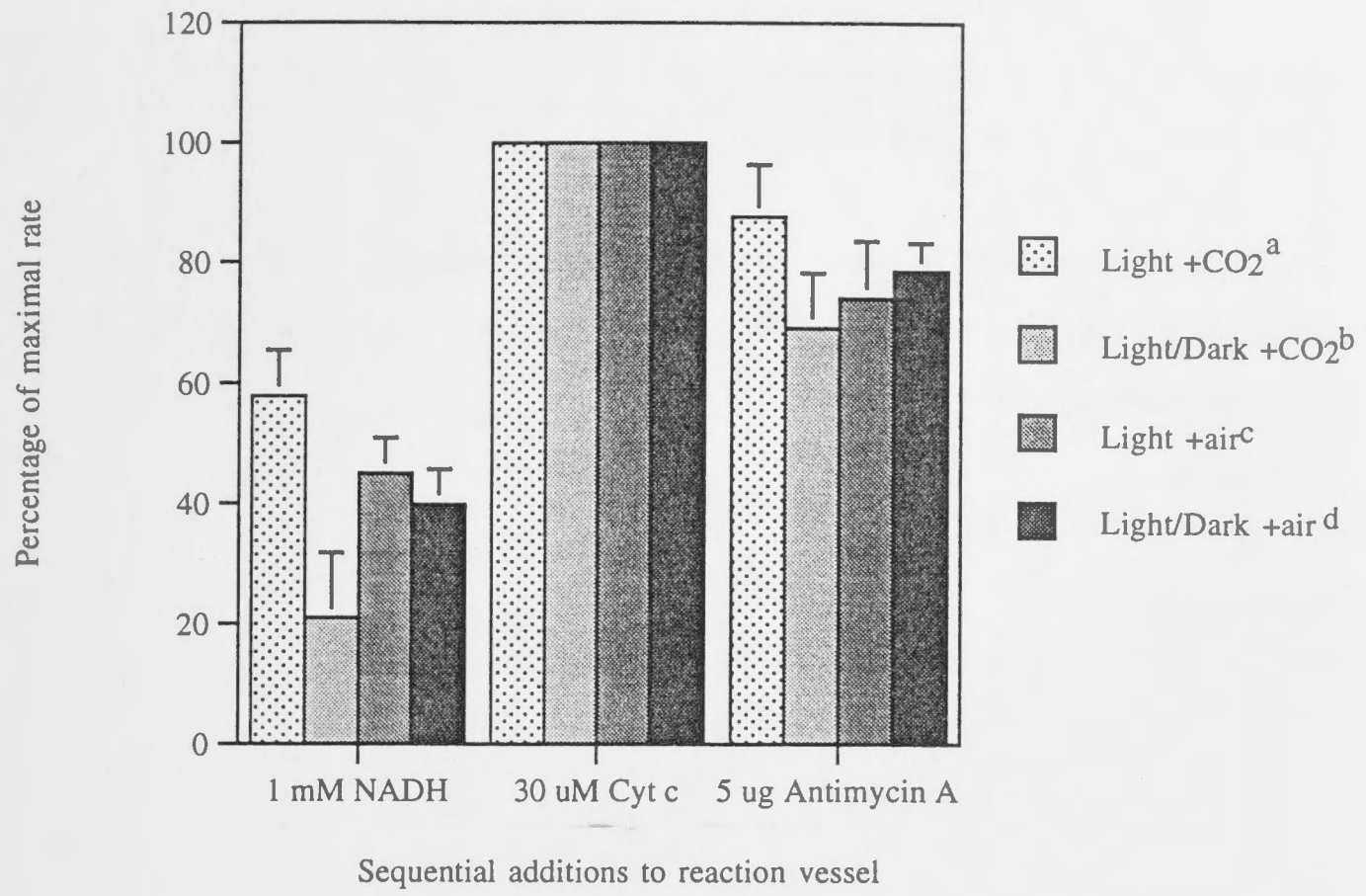
Membranes were prepared and oxygen consumtion was measured as described in chapter 2.
Data shown is the mean value ± standard error, expressed as a percentage of the rate after addition of horse heart ferrocytochrome c.

a) n = 3, 100% = 187 ± 24 nmols O_2 h^{-1} mg^{-1} protein
b) n = 5, 100% = 81 ± 15 nmols O_2 h^{-1} mg^{-1} protein
c) n = 6, 100% = 153 ± 39 nmols O_2 h^{-1} mg^{-1} protein
d) n = 4, 100% = 142 ± 17 nmols O_2 h^{-1} mg^{-1} protein

Figure 6.7 Effect of Antimycin A on NADPH catalysed respiration in total membrane preparations from *Anabaena* PCC 7120

Membranes were prepared and oxygen consumtion was measured as described in chapter 2.
Data shown is the mean value ± standard error, expressed as a percentage of the rate after addition of horse heart ferrocytochrome c.

a) n = 3, 100% = 92 ± 10 nmols O_2 h^{-1} mg^{-1} protein
b) n = 3, 100% = 107 ± 47 nmols O_2 h^{-1} mg^{-1} protein
c) n = 5, 100% = 104 ± 17 nmols O_2 h^{-1} mg^{-1} protein
d) n = 5, 100% = 93 ± 33 nmols O_2 h^{-1} mg^{-1} protein


120
100
80
60
40
20
0
Percentage of maximal rate
1 mM NADH
30 uM Cyt c
5 ug Antimycin A
Sequential additions to reaction vessel
Light +CO2 a
Light/Dark +CO2 b
Light +air c
Light/Dark +air d

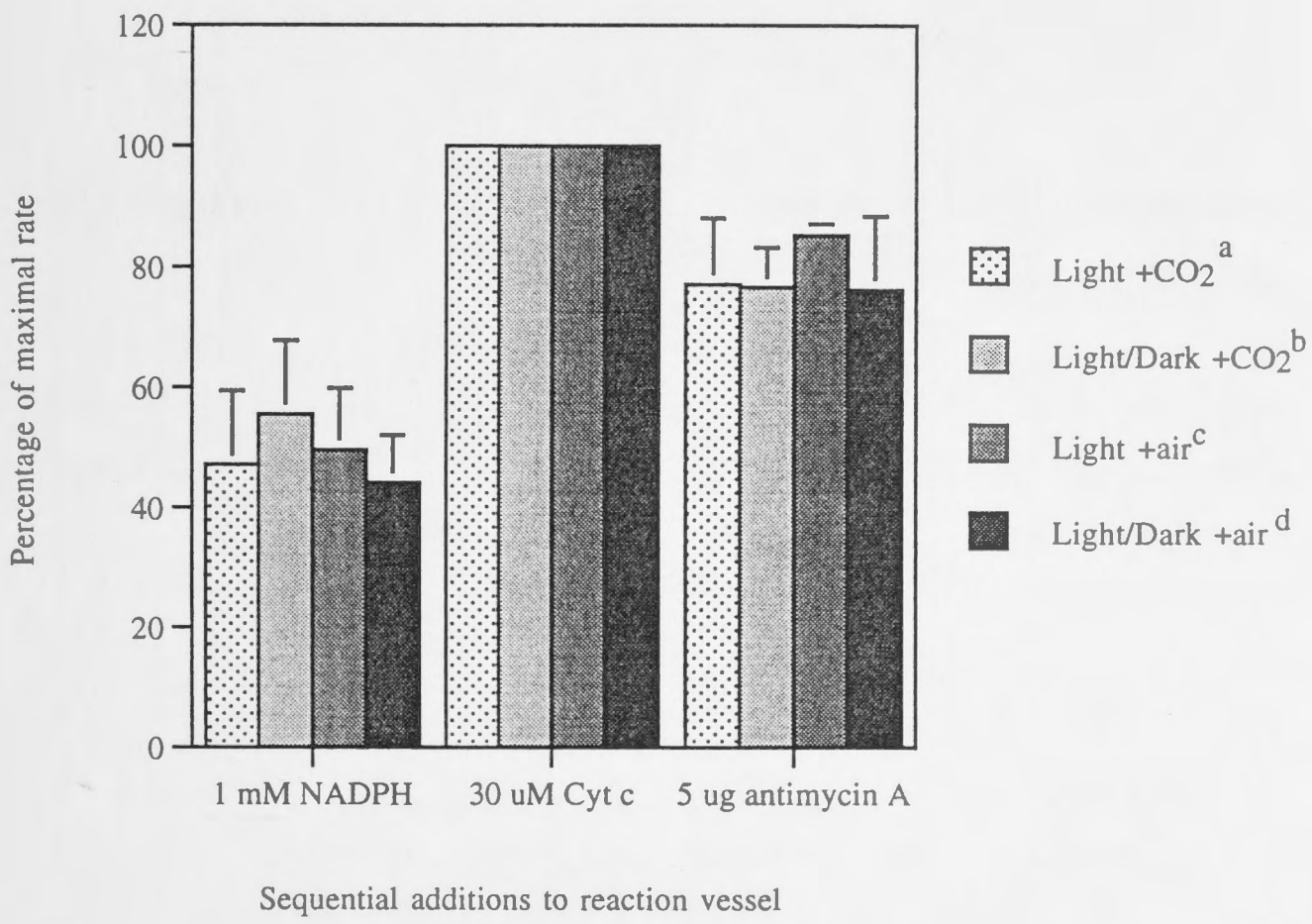

120
100
80
60
40
20
0
Percentage of maximal rate
1 mM NADPH
30 uM Cyt c
5 ug antimycin A
Sequential additions to reaction vessel
Light +CO2 a
Light/Dark +CO2 b
Light +air c
Light/Dark +air d

Figure 6.8 Effect of cyanide on NADH catalysed respiration in total membrane preparations from *Anabaena* PCC 7120

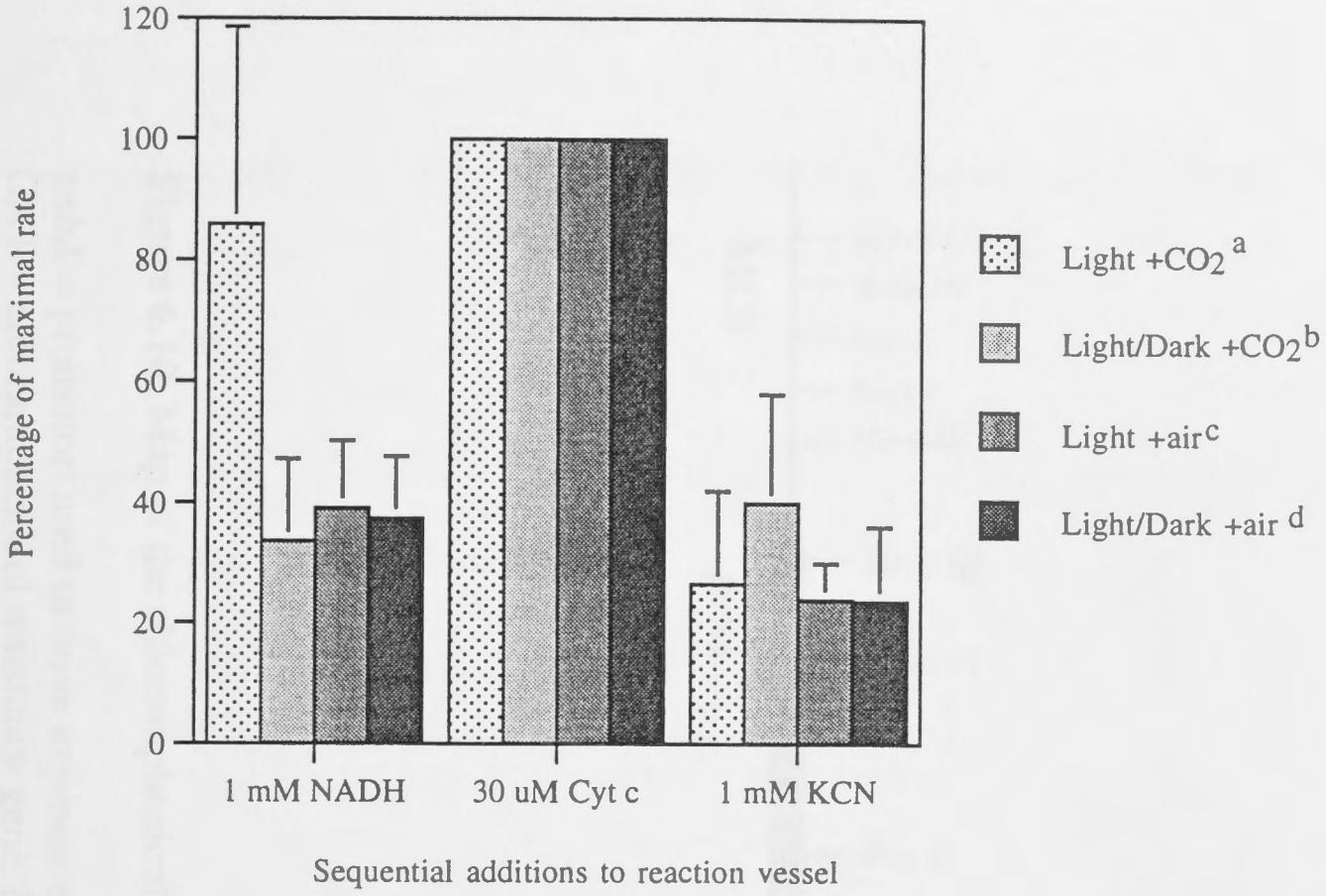
Membranes were prepared and oxygen consumtion was measured as described in chapter 2.
Data shown is the mean value ± standard error, expressed as a percentage of the rate after addition of horse heart ferrocytochrome c.

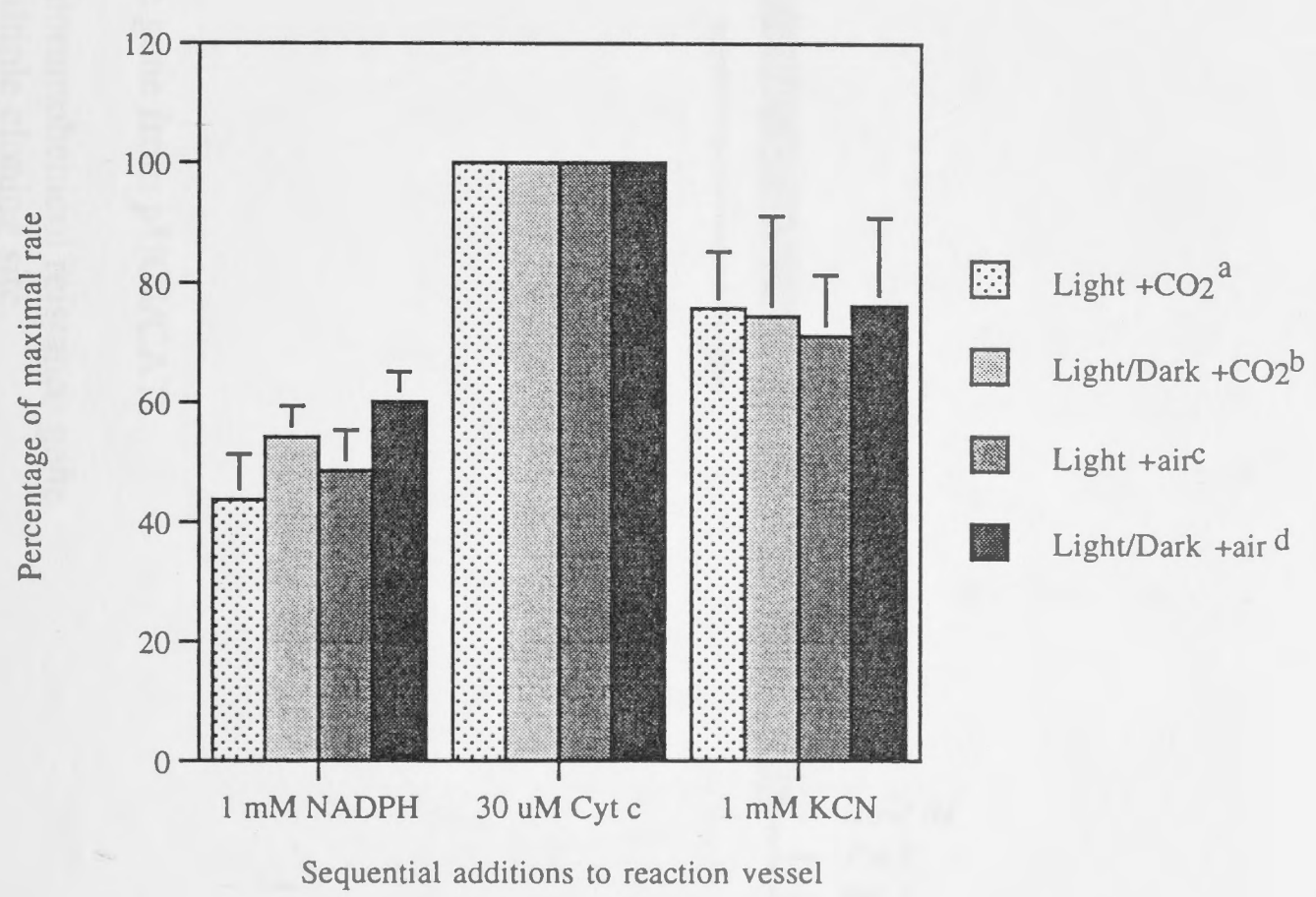
a) n = 4, 100% = 121 ± 46 nmols O_2 h^{-1} mg^{-1} protein
b) n = 5, 100% = 73 ± 16 nmols O_2 h^{-1} mg^{-1} protein
c) n = 5, 100% = 190 ± 54 nmols O_2 h^{-1} mg^{-1} protein
d) n = 5, 100% = 124 ± 33 nmols O_2 h^{-1} mg^{-1} protein

Figure 6.9 Effect of cyanide on NADPH catalysed respiration in total membrane preparations from *Anabaena* PCC 7120

Membranes were prepared and oxygen consumtion was measured as described in chapter 2.
Data shown is the mean value ± standard error, expressed as a percentage of the rate after addition of horse heart ferrocytochrome c.

a) n = 3, 100% = 93 ± 8 nmols O_2 h^{-1} mg^{-1} protein
b) n = 3, 100% = 99 ± 22 nmols O_2 h^{-1} mg^{-1} protein
c) n = 6, 100% = 118 ± 17 nmols O_2 h^{-1} mg^{-1} protein
d) n = 5, 100% = 116 ± 34 nmols O_2 h^{-1} mg^{-1} protein


120
100
80
60
40
20
0
Percentage of maximal rate
1 mM NADH
30 uM Cyt c
1 mM KCN
Sequential additions to reaction vessel
Light +CO2 a
Light/Dark +CO2 b
Light +air c
Light/Dark +air d


120
100
80
60
40
20
0
Percentage of maximal rate
1 mM NADPH
30 uM Cyt c
1 mM KCN
Sequential additions to reaction vessel
Light +CO2 a
Light/Dark +CO2 b
Light +air c
Light/Dark +air d

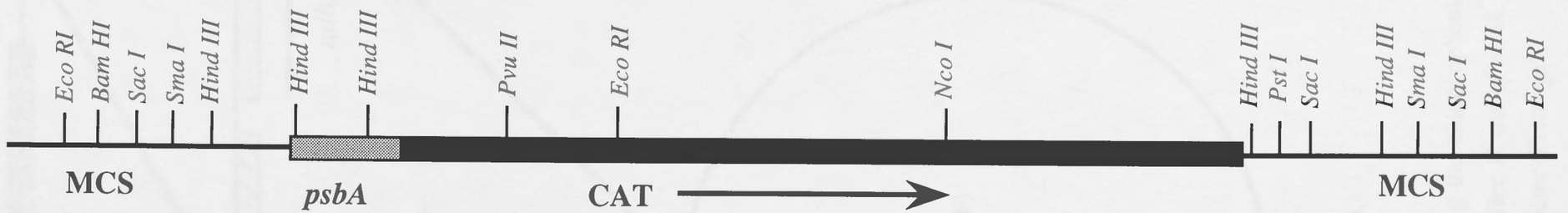


Figure 6.10: Map of the chloramphenicol resistance gene from pUC4/CAT.

psbA = promotor used to drive expression of the chloramphenicol reistance gene,
CAT = chloramphenicol resistance gene, MCS = multiple cloning site.
NB multiple cloning site is not drawn to scale.

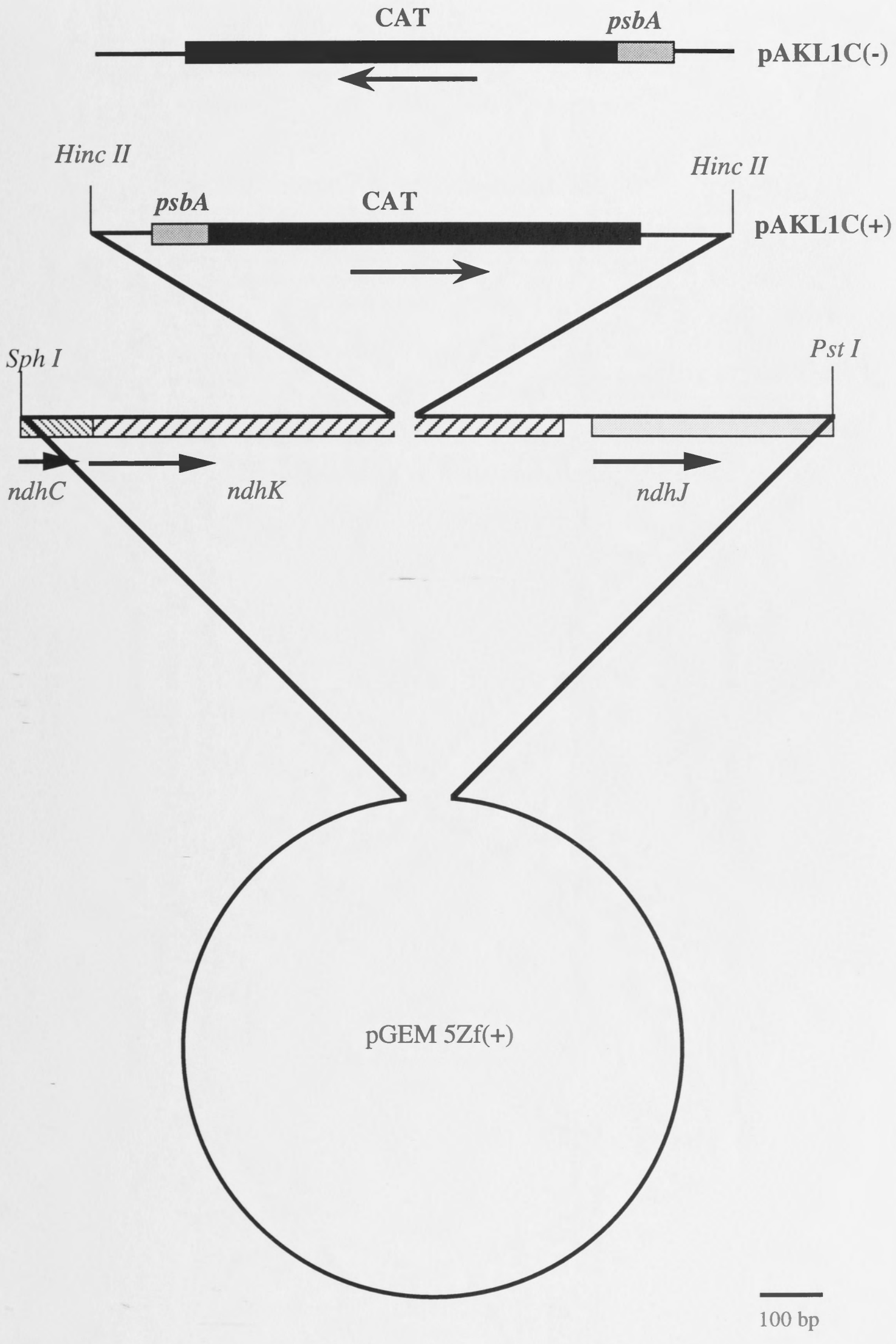


Figure 6.11 Map of pAKL1C(+) and pAKL1C(-).

pAKL1C(+) and pAKL1C(-) were constructed by cloning the chloramphenicol resistance gene from pUC4/CAT (Figure 6.10), cut with *SmaI* into the *Hinc II* site of *ndhK*. The PCR product containing *ndhC-K-J* had previously been cloned into T-Vector (Promega).

CAT = chloramphenicol resistance gene from pUC4/CAT

psbA = promotor from *psbA*

NB pGEM5Zf(+) is not drawn to scale.

Figure 6.12 Map of pRL271C(+) and pPL271C(-).

pRL271C(+) and pRL2711C(-) were constructed by cloning the *Sph I, Pst I* fragment from pAKL1C(+) or pAKL1C(-) (Figure 6.11),into the *Sph I* and *Pst I* sites of pRL271 (Figure 6.2).

CAT = chloramphenicol resistance gene from pUC4/CAT

psbA = promotor from *psbA*

NB pRL271 is not drawn to scale.

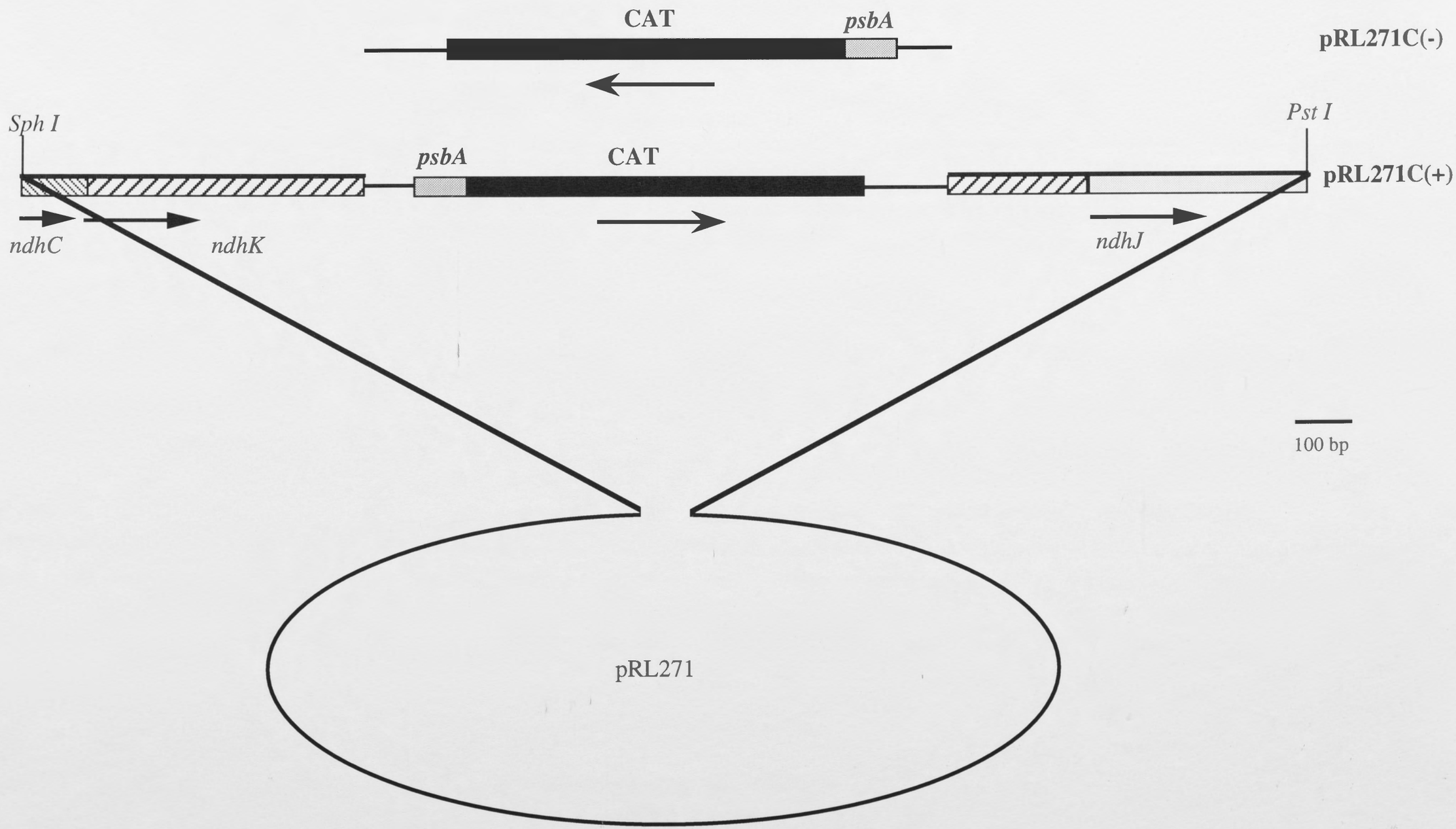

CAT
psbA
pRL271C(-)
Sph I
Pst I
psbA
CAT
pRL271C(+)
ndhC
ndhK
ndhJ
100 bp
pRL271

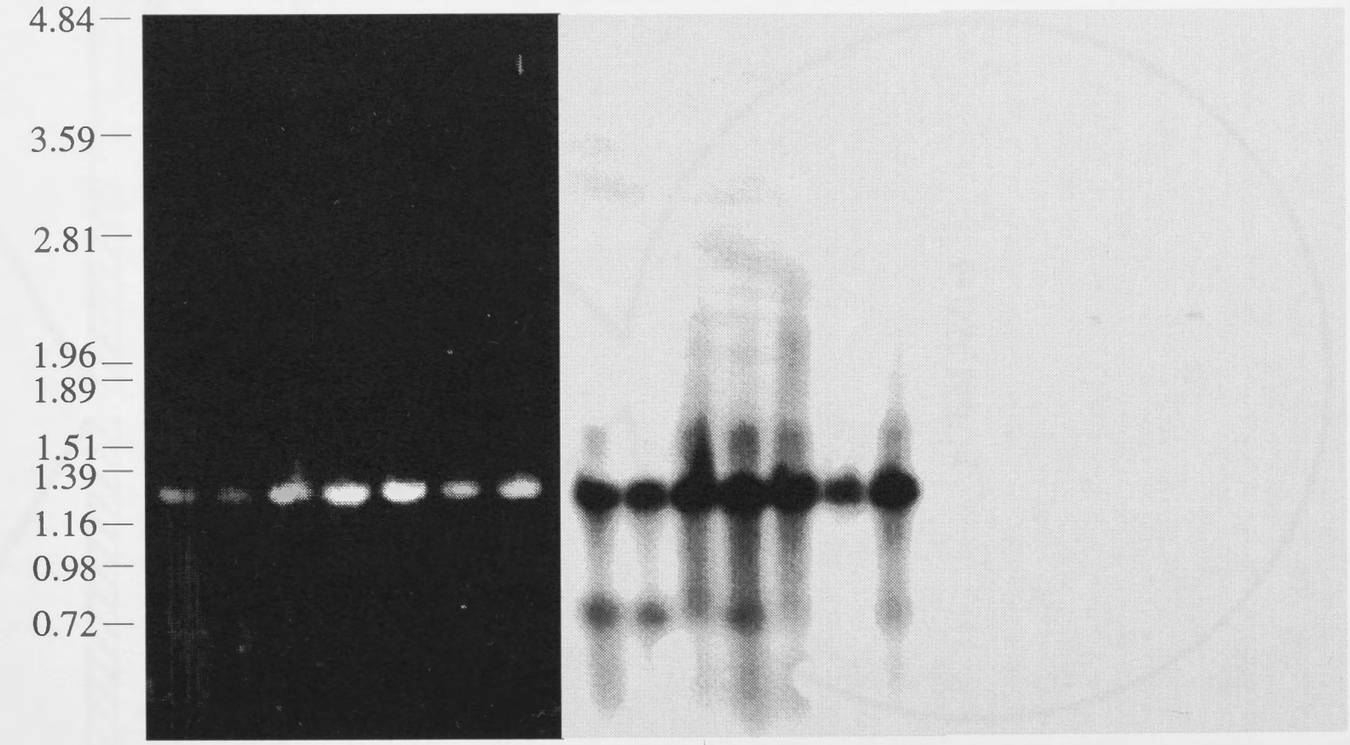
Figure 6.13: Amplification and Southern analysis of *ndhK* from chloramphenicol resistant strains of *Anabaena* PCC 7120 using PCR.

Genomic DNA was isolated (2.9.1) from the chloramphenicol resistant strains and used as the template for PCR (2.11). The PCR products were separated by agarose gel electrophoresis (2.13) transferred to Hybond-N^+ (Amersham) and probed with a 430 bp *Eco RI, Acc I* fragment of *ndhK* from *Anabaena* PCC 7120 (2.14). The blot was then stripped and reprobed with a *Bam HI* fragment of the chloramphenicol resistance gene.

Panel 1: Ethidium bromide stained gel showing PCR products. Each reaction contained 100 ng genomic DNA as the template. Lane a, wild type control. Lanes b-g, PCR using genomic DNA from chloramphenicol resistant strains as the template.

Panel 2: Southern analysis of the gel shown in panel 1, using a 430 bp *Eco RI, Acc I* fragment of *ndhK* from *Anabaena* PCC 7120 as the probe.

Panel 3: Southern analysis of the gel shown in panel 1, using a *Bam HI* fragment of the chloramphenicol resistance gene as the probe.


M.W.
(kbp)
1a 1b 1c 1d 1e 1f 1g 2a 2b 2c 2d 2e 2f 2g 3a 3b 3c 3d 3e 3f 3g
4.84
3.59
2.81
1.96
1.89
1.51
1.39
1.16
0.98
0.72

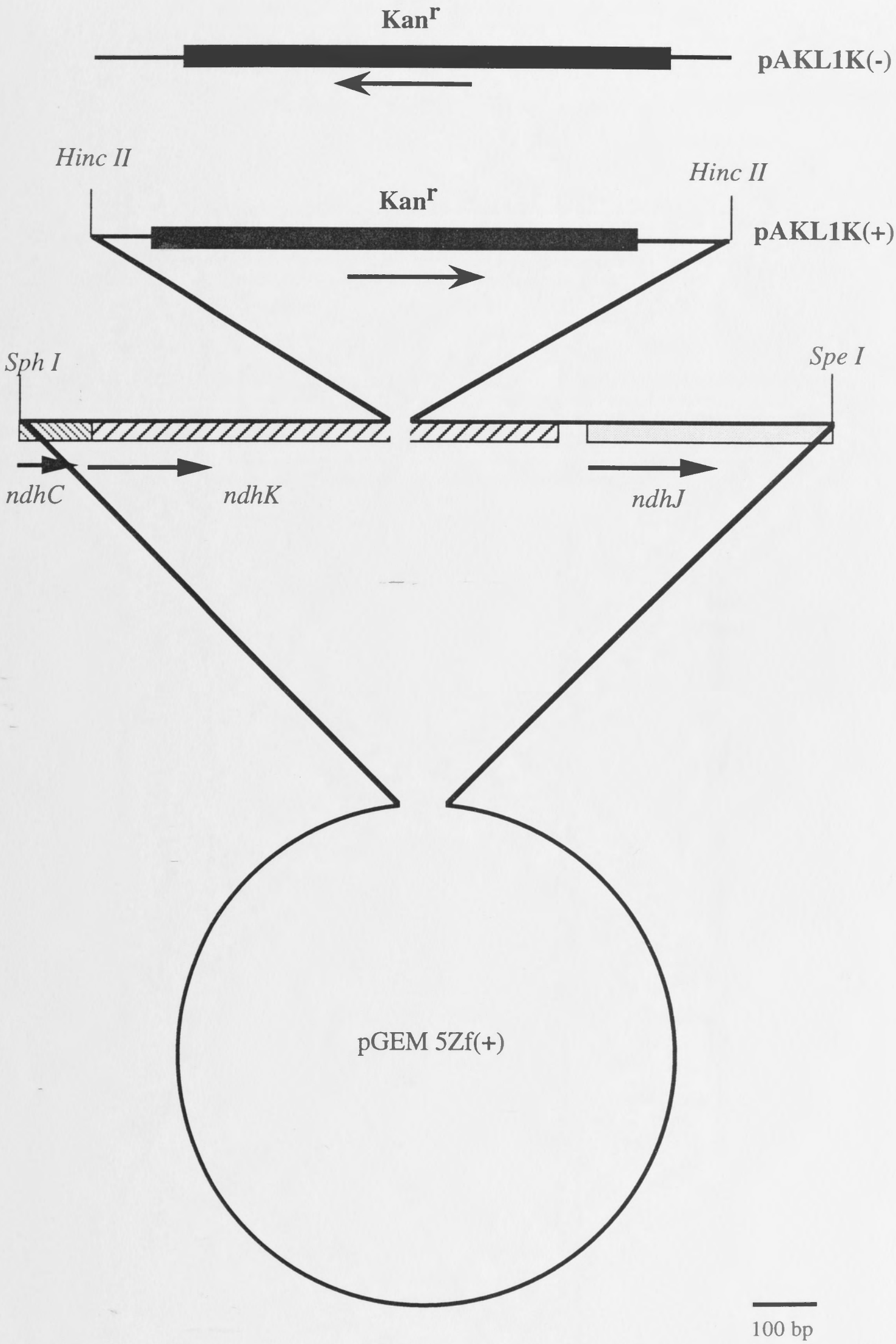


Figure 6.14 Map of pAKL1K(+) and pAKL1K(-).

pAKL1K(+) and pAKL1K(-) were constructed by cloning the Kanamycin resistance gene from pUC4K (Pharmacia), cut with *Hinc II* into the *Hinc II* site of *ndhK*. The PCR product containing *ndhC-K-J* had previously been cloned into T-Vector (Promega).

Kan^r = kanamycin resistance gene derived from Tn903

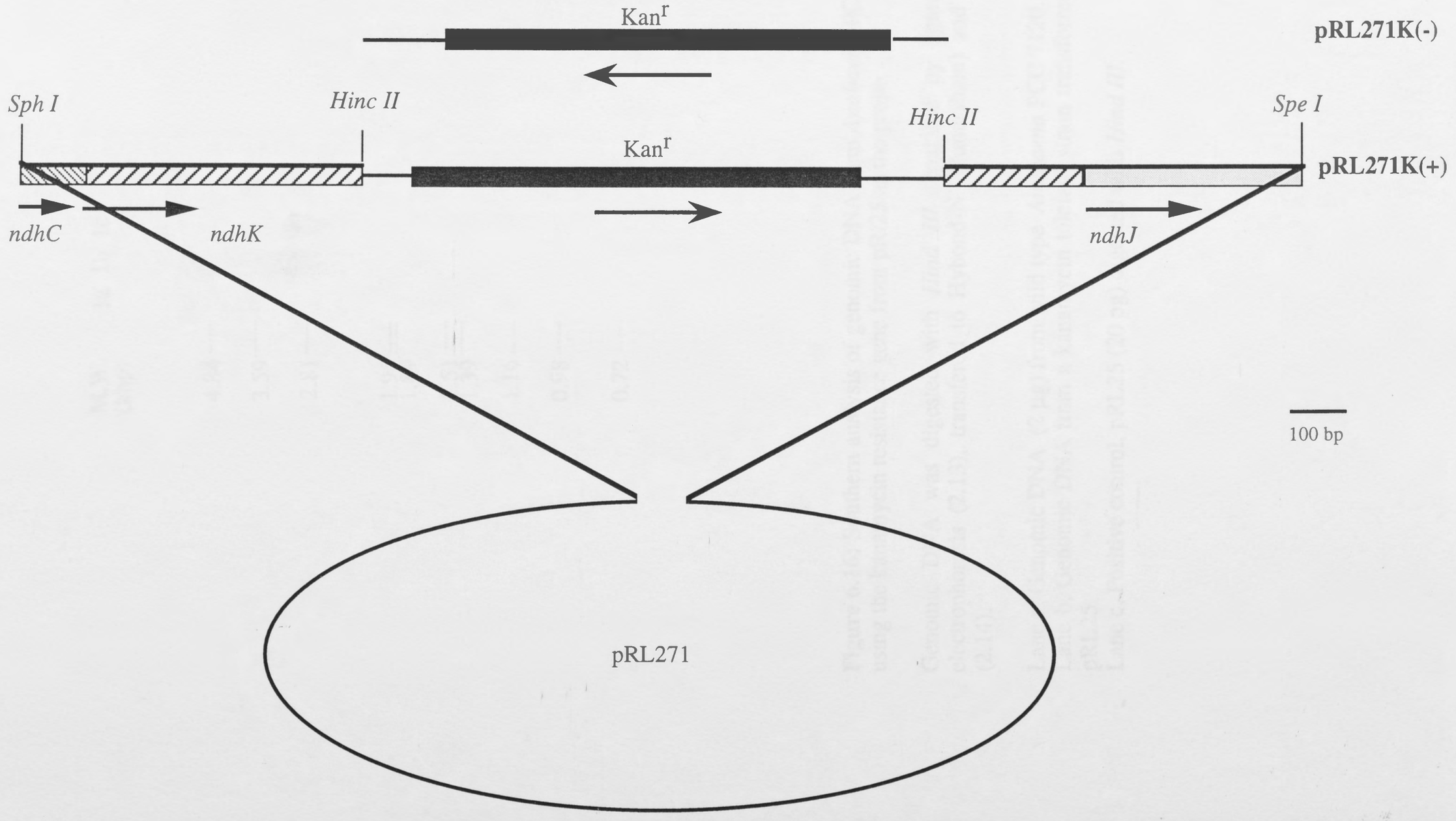
NB pGEM5Zf(+) is not drawn to scale.

Figure 6.15 pRL271K(+) and pPL271K(-).

pRL271K(+) and pRL2711K(-) were constructed by cloning the *Sph I, SpeI* fragment from pAKL1K(+) or pAKL1K(-) (Figure 6.13), into the *Sph I* and *Spe I* sites of pRL271 (Figure 6.2).

Kan^r = kanamycin resistance gene derived from Tn903

NB pRL271 is not drawn to scale.


Kan^r
pRL271K(-)
Sph I
Hinc II
Hinc II
Spe I
Kan^r
pRL271K(+)
ndhC
ndhK
ndhJ
100 bp
pRL271

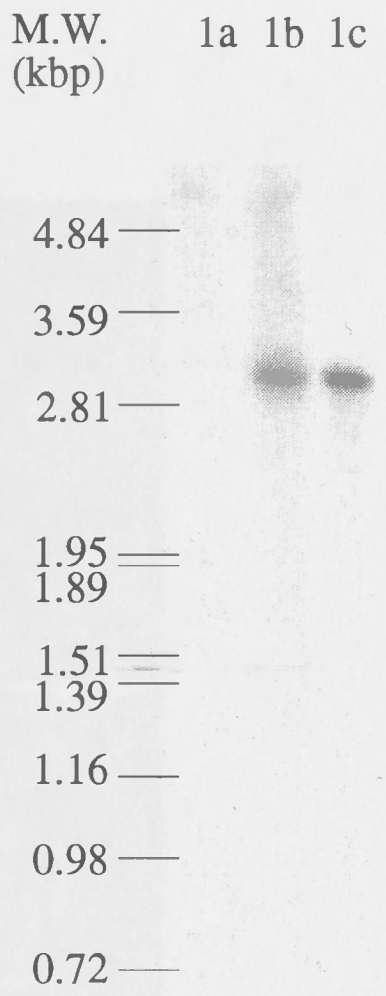


Figure 6.16: Southern analysis of genomic DNA from *Anabaena* PCC 7120 using the kanamycin resistance gene from pRl25 as the probe.

Genomic DNA was digested with *Hind III* separated by agarose gel electrophoresis (2.13), transfered to Hybond-N^+ (Amersham) and probed (2.14).

Lane a, Genomic DNA (2 μg) from wild type *Anabaena* PCC 7120.
Lane b, Genomic DNA from a kanamycin tolerant strain transformed with pRL25.
Lane c, Positive control, pRL25 (20 pg) digested with *Hind III.*

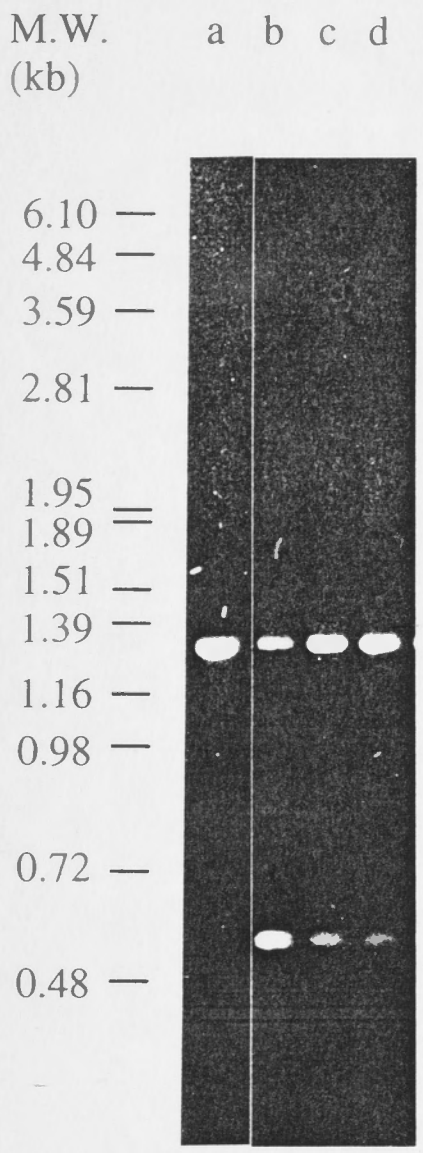


Figure 6.17: Amplification of *ndhK* from *Anabaena* PCC 7120 by PCR, using whole cells as the template.

A single colony of *Anabaena* PCC 7120 from a plate was resuspended in 100 µl of Milli Q water and aliquots of the cell suspension were used as the template for PCR (Section 2.11.1).

Lane a, 100 ng of cesium chloride purified genomic DNA from *Anabaena* PCC 7120 was used as the template.
Lane b, 1 µl of the cell suspension was used as the template.
Lane c, 3 µl of the cell suspension was used as the template.
Lane d, 5 µl of the cell suspension was used as the template.

Figure 6.18: Amplification and Southern analysis of *ndhK* from kanamycin resistant strains of *Anabaena* PCC 7120 using PCR.

Whole cells from the kanamycin resistant strains were used as the template for PCR (2.11). The PCR products were separated by agarose gel electrophoresis (2.13) transferred to Hybond-N^+ (Amersham) and probed with a *Pst I* fragment from the kanamycin resistance gene.

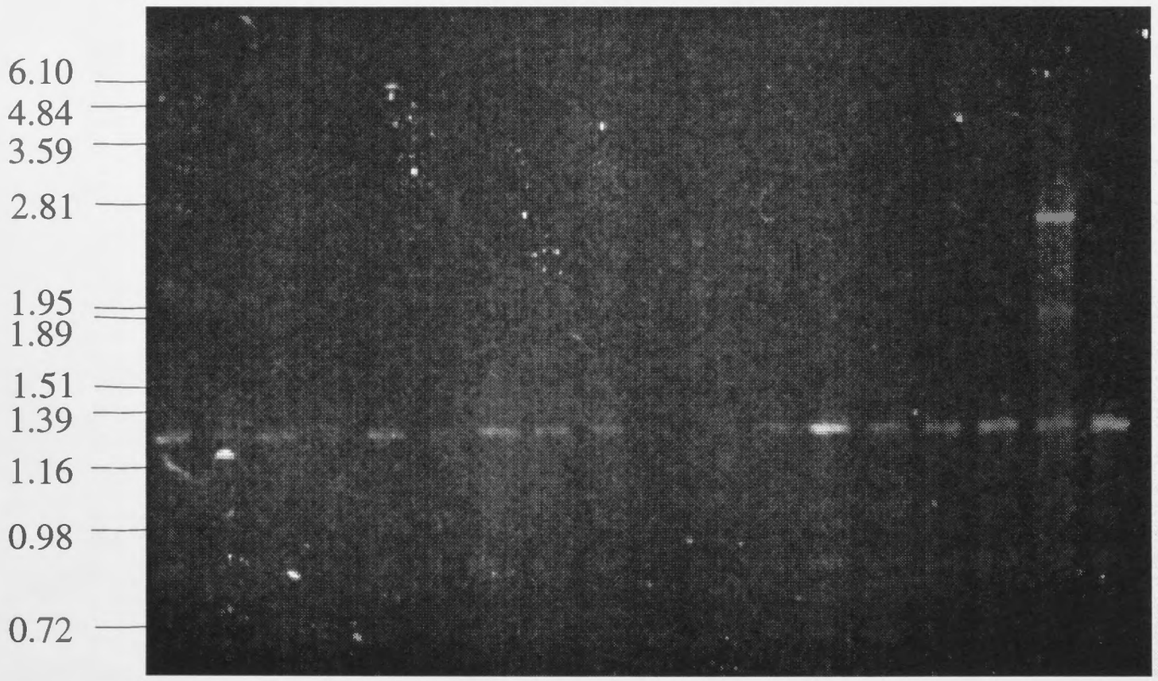
Panel 1: Ethidium bromide stained gel showing PCR products. Each reaction contained 100 ng genomic DNA as the template. Lane a, wild type control. Lanes b-r, PCR using whole cells from kanamycin resistant strains as the template.

Panel 2: Southern analysis of the gel shown in panel 1, using a *Pst I* fragment from the kanamycin resistance gene as the probe.

M.W. (kbp)

1a 1b 1c 1d 1e 1f 1g 1h 1i 1j 1k 1l 1m 1n 1o 1p 1q 1r

2a 2b 2c 2d 2e 2f 2g 2h 2i 2j 2k 2l 2m 2n 2o 2p 2q 2r



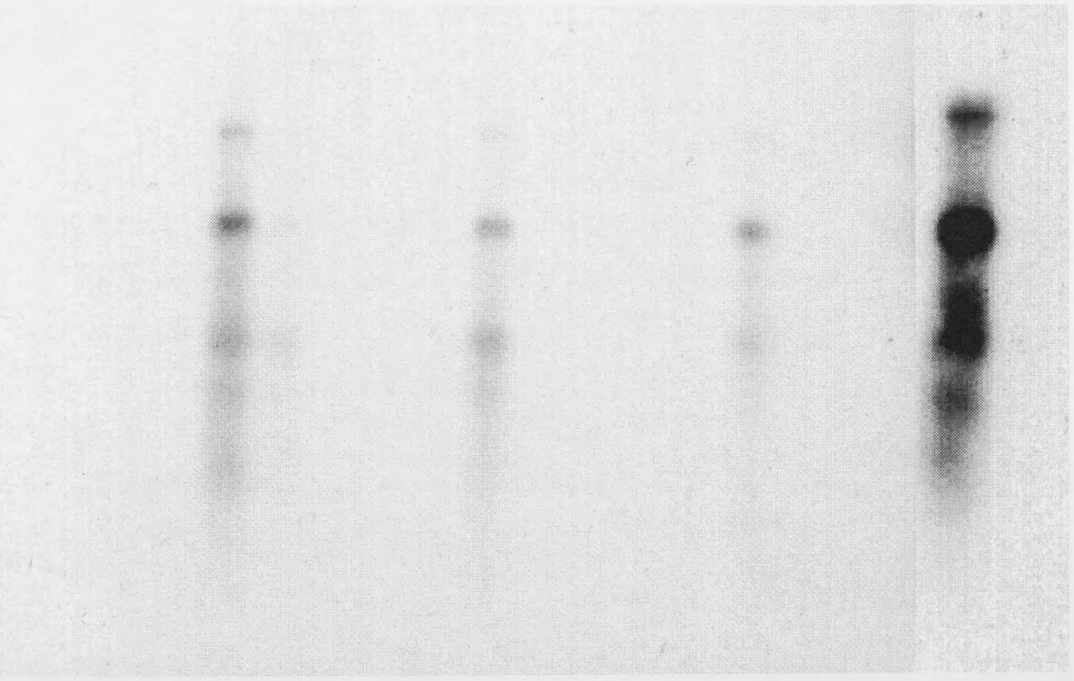


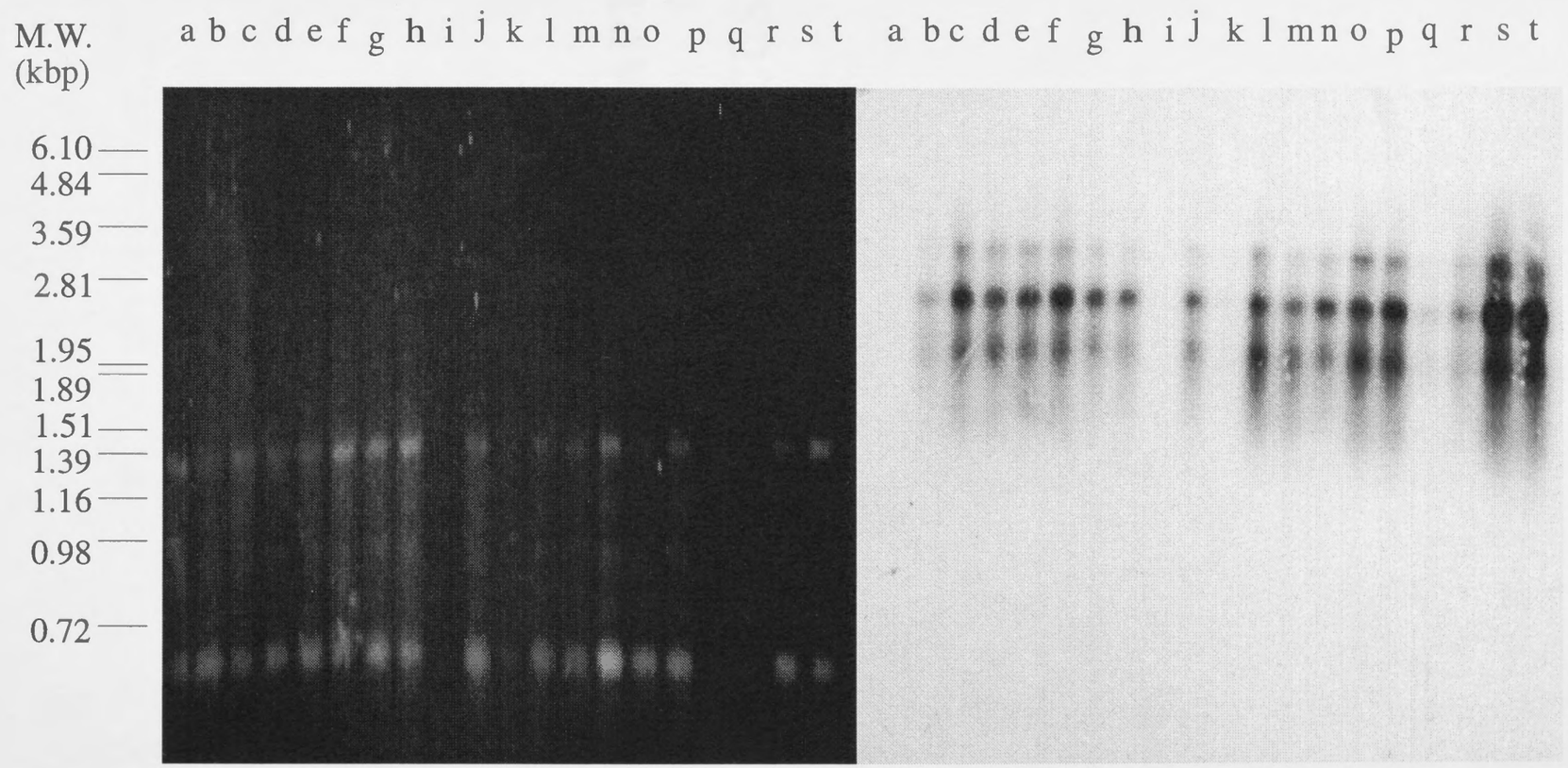
Figure 6.19: Amplification and Southern analysis of *ndhK* from kanamycin resistant strains of *Anabaena* PCC 7120, that had not been subjected to selection on sucrose, using PCR.

Whole cells from the kanamycin resistant strains were used as the template for PCR (2.11). The PCR products were separated by agarose gel electrophoresis (2.13) transferred to Hybond-N^+ (Amersham) and probed with a *Pst I* fragment from the kanamycin resistance gene.

Panel 1: Ethidium bromide stained gel showing PCR products. Each reaction contained 100 ng genomic DNA as the template. Lane a, wild type control. Lanes b-s, PCR using whole cells from kanamycin resistant strains, that had not been subjected to selection on sucrose, as the template.

Panel 2: Southern analysis of the gel shown in panel 1, using a *Pst I* fragment from the kanamycin resistance gene as the probe.

PANEL 1 **PANEL 2**



CHAPTER 7

GENERAL DISCUSSION

7.1 CLONING AND ANALYSIS OF *ndhK* FROM *ANABAENA* PCC 7120

The cloning of part of the operon encoding *ndhC-K-J* reported here is only the second report of cloning and sequencing of *ndh* genes from filamentous cyanobacteria (Sections III, IV and V, see Table 1.1). An operon encoding *ndhA-I-G-E* has been cloned and sequenced from the filamentous cyanobacterium *Plectonema boryanum* (Takahashi *et al.* 1991). The operon encoding *ndhC-K-J* has only been cloned from one other cyanobacterium, *Synechocystis* PCC 6803, (Steinmüller *et al.* 1989). Interestingly the structure of the operon encoding *ndhC-K-J* from *Anabaena* PCC 7120 shows greater similarity to the structure of the same operon from higher plant chloroplasts than to that in *Synechocystis* PCC 6803. In *Anabaena* PCC 7120 the coding regions for *ndhC* and *ndhK* overlap by 7 bp (Figures 5.11 and 5.15). This overlap is identical to that seen in the chloroplasts of liverwort (Ohyama *et al.* 1986), maize (Steinmüller *et al.* 1989) and rice (Hiratsuka *et al.* 1989), while in wheat the overlap is 28 bp (Nixon *et al.* 1989) and in tobacco, 118 bp (Shinozaki *et al.* 1986). This is markedly different from the case in *Synechocystis* PCC 6803 which has a 71 bp non-coding intergenic spacer region between *ndhC* and *ndhK* (Figure 5.15). This suggests that, with respect to operon structure, *Anabaena* PCC 7120 is more closely related, in evolutionary terms, to chloroplast genomes than is *Synechocystis* PCC 6803. However, the proteins encoded within the operon are more closely related to the equivalent proteins from *Synechocystis* PCC 6803 than chloroplasts. In recent years an increasing body of evidence has been emerging for a monophyletic origin of chloroplasts from cyanobacteria (Cavalier-Smith 1992; Morden *et al.* 1992; Palmer 1993; Wolfe *et al.* 1994 and references therein). The conserved structural features of the operon encoding *ndhC-K-J* in *Anabaena* PCC 7120 and the chloroplasts of higher plants, but not *Synechocystis* PCC 6803, suggest that the ancestral cyanobacterium from which *Anabaena* PCC 7120 arose was more closely related to the ancestral cyanobacterium from which chloroplasts arose, than was the ancestral cyanobacteria from which *Synechocystis* PCC 6803 arose.

7.2 THE PROBABLE ROLE OF *ndhK* IN *ANABAENA* PCC 7120

The results presented in Chapter 6 indicate that, under the selection conditions used, inactivation of *ndhK*, and hence the NDH-1 on the plasma membrane is apparently lethal

to *Anabaena* PCC 7120. This also is consistent with the results of previous studies (Howitt *et al.* 1993) which indicated that the NDH-1 on the plasma membrane was the only NDH on the membrane. Further, it provides evidence for the NDH-1 being the only electron donor to the respiratory electron transport chain on the plasma membrane. As selection for transformants was carried out on media containing nitrate, there was no requirement for the cells to fix nitrogen; therefore, the apparently lethal phenotype can not be attributed to impairment of nitrogen fixation by inactivation of *ndhK*.

Selection was also carried out at an elevated CO_2 concentration (5% (v/v) in air). This concentration is sufficiently high to alleviate the block in inorganic carbon uptake caused by inactivating *ndh* genes in *Synechocystis* PCC 6803 (Ogawa 1990; Ogawa 1991a; Ogawa 1991b; Ogawa 1991c; Ogawa 1992a; Ogawa 1992b) and *Synechococcus* PCC 7942 (Marco *et al.* 1993). Therefore it is unlikely that the apparent lethality of inactivating *ndhK* in *Anabaena* PCC 7120 was due to an effect on the CO_2 concentrating mechanism, unless *Anabaena* PCC 7120 has a barrier that prevents diffusion of CO_2 into the cell that is not present in *Synechocystis* PCC 6803 or *Synechococcus* PCC 7942. In studies on the inactivation of *ndhK* from *Synechocystis* PCC 6803, it has been shown that selection at elevated CO_2 concentrations is essential if a strain with a high CO_2 requiring phenotype is to be produced. In attempts to inactivate *psbG*, renamed *ndhK* (Berger *et al.* 1991; Arizmendi *et al.* 1992a; Whelan *et al.* 1992), Steinmüller *et al.* (1991) selected for transformants at air levels of CO_2 and produced a mutant in which a cryptic copy of *ndhK*, located on a plasmid, had been activated. On the other hand, Ogawa (1992b) inactivated *ndhK* and selected for transformants at elevated CO_2 concentrations, producing a strain in which the cryptic copy had not been activated and the cells were clearly unable to grow at air levels of CO_2.

In this study the cells were kept under continuous illumination during selection for the transformants. This should have allowed most of the cell's energy requirements to be met by photosynthetic ATP production, thereby making respiratory ATP production unnecessary.

7.2.1 Possible causes for the apparent lethality of inactivating *ndhK*

As the apparent lethality of inactivating *ndhK*, and thus NDH-1 on the plasma membrane, in *Anabaena* PCC 7120 can not be attributed to an effect on nitrogen fixation, impairment of the CO_2 concentrating mechanism, or lack of ATP, it must then be due to some other function that the NDH-1 is involved in. One possibility is that respiration on the plasma membrane is involved in energisation of that membrane which is essential for the maintenance of the cell's ionic/osmotic balance. Inactivation of NDH-1, the only

NAD(P)H dehydrogenase present on the membrane (Howitt *et al.* 1993), by insertional mutagenisis of *ndhK*, would inactivate respiration on the plasma membrane, and thus prevent the generation of a proton gradient across the membrane. Possible roles of the NDH-1 are discussed below.

a) Sodium cycle

The proton motive force generated across the plasma membrane may be used to maintain the concentration of Na^+ in the cell at low levels. All living cells maintain a Na^+ cycle across the cytoplasmic membrane and this cycle is driven by Na^+ extruding systems that excrete the ion and maintain a concentration gradient directed inwards (see Krulwich 1986; Padan and Schuldiner 1993; Schuldiner and Padan 1993; Krulwich *et al.* 1994; Padan and Schuldiner 1994a; Padan and Schuldiner 1994b for reviews). In many plants, algae, fungi and bacteria, the cycle is maintained by primary proton pumps which pump protons out. The proton gradient across the membrane is then utilised to drive Na^+ extrusion via an Na^+/H^+ antiporter (see Krulwich 1986; Padan and Schuldiner 1993; Schuldiner and Padan 1993; Krulwich *et al.* 1994; Padan and Schuldiner 1994a; Padan and Schuldiner 1994b for reviews). In the experiments reported here *Anabaena* PCC 7120 was grown in media containing 40 mM NaCl. In the cyanobacterium *A. nidulans* (*Synechococcus* PCC 7942), the internal Na^+ has been shown to be 5 mM (Dewar and Barber 1973). As in other bacteria this is maintained through the extrusion of Na^+ via an Na^+/H^+ antiporter (Paschinger 1977; Nitschmann and Peschek 1982; Blumwald *et al.* 1984; Erber *et al.* 1986), as discussed below. Figure 7.1 (adapted from (Peschek 1987)) shows a model for this system in cyanobacteria.

Under conditions of salt stress (NaCl) the level of respiratory activity in cyanobacteria increases significantly, under both light and dark conditions (Fry *et al.* 1986; Wastyn *et al.* 1987; Jeanjean *et al.* 1990). In particular the level of activity of cytochrome oxidase has been shown to increase to a greater extent than the activity of the other respiratory complexes (Fry *et al.* 1986; Molitor *et al.* 1986; Wastyn *et al.* 1987; Jeanjean *et al.* 1990; Molitor *et al.* 1990; Jeanjean *et al.* 1993). In studies on *Synechococcus* PCC 6311 the sodium-driven proton (antiporter) gradients, in salt stressed cells under both light and dark conditions, were sensitive to cyanide but not dicyclohexylcarbodiimide (DCCD) indicating a direct role of respiratory electron transport in maintaining low intracellular sodium levels (Fry *et al.* 1986). In *A. nidulans* measurement of cytochrome oxidase activity in purified plasma and thylakoid membranes showed that the increase in the level of cytochrome oxidase activity, under conditions of salt stress, was plasma membrane specific. At the same time the rate of whole cell respiration (measured polographically)

and the capacity for Na^+/H^+ exchange (measured by following the fluorescence quenching of acridine orange), were shown to increase 3-7 and 5 fold respectively (Molitor *et al.* 1986; Molitor *et al.* 1990). In *Synechocystis* strains PCC 6803 and PCC 6714, salt stress induces increases in the respiratory activities of both plasma and thylakoid membranes (Wastyn *et al.* 1987; Jeanjean *et al.* 1990).

It is thought that the proton gradient across the plasma membrane (acidic outside) that is utilised by the sodium-proton antiporter can be generated in one of two ways, or possibly a combination. Firstly, the proton gradient can be generated directly by the coupling sites present in the respiratory chain on the plasma membrane (Nitschmann and Peschek 1982; Erber *et al.* 1986; Molitor *et al.* 1986); secondly, it may be generated by an H^+-translocating ATPase that is powered by ATP generated elsewhere in the cell (Nitschmann and Peschek 1982; Wastyn *et al.* 1987). It has been proposed that the ATP necessary to drive the second process can come from either of three processes, linear or cyclic photophosphorylation and oxidative phosphorylation (Wastyn *et al.* 1987; Jeanjean *et al.* 1990).

In *Synechocystis* PCC 6803 it appears that both, mechanisms described above, operate to generate a proton gradient across the plasma membrane during adaptation to salt stress. Using chlorophyll a fluorescence emission profiles, it has been shown that the PS I:PS II ratio increases under conditions of salt stress (Schubert and Hagemann 1990). This has been shown to be due to synthesis of PS I reaction centres and a concomitant increase in the level of cyclic electron transport through PS I (Jeanjean *et al.* 1993), giving the cell greater capacity for ATP production. No change in the amount of PS II present in the cell was observed, during adaptation to salt stress (Jeanjean *et al.* 1993). At the same time the amount of cytochrome oxidase in the cell increases and so does the level of whole cell respiration (Jeanjean *et al.* 1993).

The cyclic electron transport around PS I, and donation of electrons to the intersystem chain from cytosolic components, have been shown to be mediated by an NADPH specific NDH-1, in *Synechocystis* PCC 6803 (Mi *et al.* 1992a; Mi *et al.* 1994; Mi *et al.* 1995) (as discussed in Section 1.3.3). By monitoring changes in the redox state of $P700^+$ during illumination with far-red and saturating multiple-turnover light, Mi *et al.* (1992b) showed that there was no significant donation of electrons to $P700^+$ from the cytosolic components or cyclic electron transport around PS I via the intersystem chain in *Anabaena* PCC 7120. However, cyclic electron transport has been demonstrated around PS I in isolated heterocysts from *Nostoc muscorum* (*Anabaena* PCC 7119) (Almon and Böhme 1982). Mi *et al.* (1992b) grew *Anabaena* PCC 7120 with nitrate in the growth media and thus cyclic electron transport in *Anabaena* sp. may only occur under nitrogen fixing conditions or perhaps in heterocysts. As there is apparently no cyclic electron

transport around PS I in *Anabaena* PCC 7120, no ATP can be generated by this cycle. In the absence of ATP production via cyclic electron transport around PS I, the respiratory chain on the plasma membrane probably plays a significant role in the generation and maintenance of the proton gradient across the membrane, as is the case in *A. nidulans* (Peschek *et al.* 1985; Erber *et al.* 1986; Molitor *et al.* 1986). In *Anabaena* PCC 7120 an ATP-hydrolase may also play a role in the maintenance of the proton gradient across the membrane. In *A. variabilis* it has been shown that on the addition of vanadate, to cells in the dark, oxidative phosphorylation is not inhibited but there is a 40% decrease in the acidification of the external medium (Scherer *et al.* 1984; Nitschmann and Peschek 1985). However, Scherer *et al.* (1984) also showed that KCN inhibited both oxidative phosphorylation and acidification of the external medium indicating that respiratory electron transport is essential for the maintenance of a proton gradient across the plasma membrane. Inactivation of *ndhK* and thus the NDH-1 on the plasma membrane, the only NDH present on that membrane (Chapter 3; Howitt *et al.* 1993), would inactivate respiration on the plasma membrane and reduce the potential for generation of a proton gradient across the membrane. With a lowered proton gradient across the membrane the cells would be unable to maintain their ionic/osmotic balance. This may be the basis of the apparent lethality of inactivating *ndhK* in *Anabaena* PCC 7120, under the selection conditions used.

b) pH homeostasis

The proton gradient generated across the plasma membrane, by respiratory electron transport, may also be involved in maintenance of the pH within the cell. Inactivation of this activity on the plasma membrane, by inactivating *ndhK*, may impair the cell's ability to maintain the pH within the cell at physiological levels. This effect may be the result of an inability to maintain a Na^+ gradient across the plasma membrane as Na^+/H^+ antiporters have been implicated in the maintenance of pH homeostasis in other cells (see Padan and Schuldiner 1993; Schuldiner and Padan 1993; Krulwich *et al.* 1994; Padan and Schuldiner 1994b for reviews). A change in the pH within the cell may impair or inhibit metabolic reactions essential for the cell's survival.

c) Transport of compounds into and out of the cell

Alternatively the proton gradient generated across the plasma membrane, or the ATP produced by oxidative phosphorylation, may be essential to drive the import of substrates

into the cell and/or export of potentially harmful waste products. As discussed in Chapter 5, part of a gene encoding an ABC transporter was cloned and sequenced. By analogy with similar proteins found in other bacteria (see Higgins 1992; Dogie and Ames 1993; Fath and Kolter 1993; Tam and Saier 1993 for reviews), the product of this gene is probably located on the plasma membrane as part of a complex used to actively transport substrates into the cell or export products from the cell. The active transport requires the hydrolysis of ATP (Higgins 1992; Dogie and Ames 1993; Fath and Kolter 1993; Tam and Saier 1993). Inactivation of the respiratory chain on the plasma membrane may upset the cell's energy balance sufficiently to impair the activity of the ABC transporter, and the apparent lethality may be due to lack of an essential substrate or build up of a waste product, within the cell.

d) Photoinhibition

Upsetting the cells energy balance by inactivation of respiration on the plasma membrane may also lead to increased levels of photoinhibition. In *A. nidulans*, inhibition of respiration by addition of sodium azide leads to increased levels of photoinhibition (Shyam *et al.* 1993). The basis of this is unknown, but, Shyam *et al.* (1993) proposed that respiratory electron transport may play an essential role in ATP production to provide energy necessary for rapid synthesis of the D1 protein, as addition of FCCP or CCCP also led to increased levels of photoinhibition.

The apparent lethality of inactivating *ndhK* may be due to one of the reasons discussed above or as a result of a combination of these. Alternatively the effect may be due to the inhibition of an, as yet, unknown function of the NDH-1 in *Anabaena* PCC 7120.

7.2.2 Inactivation of NDH-1 in other bacteria

E. coli contains both NDH-1 and NDH-2 (Matsushita *et al.* 1987). Strains in which both NDH-1 and NDH-2 have been inactivated are unable to oxidise NADH, produce smaller colonies and have longer doubling times when grown on "rich media" and can not grow on minimal media containing mannitol as the sole carbon source (Young and Wallace 1976; Calhoun and Gennis 1993). In contrast, strains containing mutations in only one of the NADH dehydrogenases were indistinguishable from wild type with respect to both phenotypes (Calhoun and Gennis 1993). In more extensive studies on strains defective in NDH-1 it has been shown that as the cells began the transition to stationary phase their growth rates slowed abruptly, unlike wild type cells (Zambrano and Kolter 1993; Prüß *et*

al. 1994). Using HPLC it was shown that wild type cells consumed L-glutamine, glycine, L-threonine and L-alanine during the transition to stationary phase, but mutant strains utilised these amino acids poorly (Prüß *et al.* 1994). The mutant strains were also shown to grow poorly when acetate is their sole carbon source, and when grown in tryptone broth they produced more acetate than wild type cells (Prüß *et al.* 1994). Mutants of *Salmonella typhimurium* deficient in NDH-1 also grow poorly on media with acetate as the sole carbon source (Archer *et al.* 1993). Prüß *et al.* (1994) have proposed that all of the effects seen in NDH-1 deficient mutants can be explained by increased $NADH/NAD^+$ ratios within the cell. High $NADH/NAD^+$ ratios inhibit allosterically both citrate synthase and malate dehydrogenase, and therefore mutant cells have difficulty utilising amino acids which require a functional TCA cycle for degredation e.g. L-glutamate. Large $NADH/NAD^+$ ratios also affect the reaction equilibria of threonine dehydrogenase and alanine dehydrogenase: thus the mutant cells should also experience difficulty utilising L-threonine and L-alanine. The apparent lethality of inactivation of NDH-1 in *Anabaena* PCC 7120 may, therefore, be the result of the cell's inability to degrade certain amino acids. However, it is unlikely that the effect is due to inhibition of the TCA cycle as studies on *A. variabilis* have shown that it does not have a complete TCA cycle (Pearce *et al.* 1969).

7.3 NDH-1 IN *ANABAENA* PCC 7120 AND *SYNECHOCYSTIS* PCC 6803

At the outset of this study one of the aims was to further characterise the NDH-1 from *Anabaena* PCC 7120 in order to compare its function with that of the NDH-1 present in *Synechocystis* PCC 6803, since the NDH-1 in *Synechocystis* PCC 6803 had been implicated in the CO_2 concentrating mechanism (Ogawa 1990; Ogawa 1991a; Ogawa 1991b; Ogawa 1991c; Ogawa 1992a; Ogawa 1992b). During the course of this study other workers have shown that the NDH-1 is involved in NADPH mediated cyclic electron transport around PS I in *Synechocystis* PCC 6803 but not in *Anabaena* PCC 7120 (Mi *et al.* 1992a; Mi *et al.* 1992b; Mi *et al.* 1994; Mi *et al.* 1995). The results of this study have confirmed that the NDH-1 is only present on the plasma membrane of *Anabaena* PCC 7120 and is probably essential for the cell's survival because inactivation of *ndhK* is apparently lethal, at least under the selection conditions used. Mutants of *Synechocystis* PCC 6803 in which *ndhK* has been inactivated are able to survive under the conditions used for selection of transformants in this study (Ogawa 1992b), suggesting the NDH-1 in *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 have

different functions presumably reflecting their different localisations in the cell. It is unknown if the NDH-1 in *Anabaena* PCC 7120 has any other functions besides its role in respiration.

The fact that inactivation of *ndhK* in *Synechocystis* PCC 6803 is not lethal suggests that either the NDH-1 on the plasma membrane is not inactivated, or it contains other enzymes that can provide the function in the place of the NDH-1. Unfortunately, as yet, no studies have been carried out on the *ndh* mutant strains, constructed by Ogawa, to see whether respiration on the plasma membrane is also affected. In a separate study a second gene for *ndhD* from *Synechocystis* PCC 6803 has been cloned and inactivated (Dzelzkalns *et al.* 1994). Inactivation of this gene only affected respiration on the plasma membrane. This result suggests that the NDH-1 on the plasma membrane may be encoded for by an entirely separate set of genes from the NDH-1 on the thylakoid membrane. Further studies will be needed to determine if this is the case, as *ndhD* is the only *ndh* gene in *Synechocystis* PCC 6803 for which two functional copies have been found.

7.4 FUTURE EXPERIMENTS

In experiments designed to elucidate the function of NDH-1 in *Anabaena* PCC 7120 an antisense construct to *ndhK* was designed and constructed (Figure 7.2). Unfortunately time constraints did not permit this construct to be used. A 440 bp *Eco RI, Acc I* fragment of *ndhK* from *Anabaena* PCC 7120 was cloned into the *Eco RI, Acc I* sites of pUC19, such that the fragment was in the antisense orientation with respect to the lac promoter (P_{lac}). Expression of this construct from P_{lac} will produce an antisense fragment of *ndhK*. The construct was digested with *Ssp I*, to linearise it, and cloned into the *Sca I* site of pRL25 (Figure 7.2). If time had permitted, this construct would have been used to transform *Anabaena* PCC 7120. Expression of the antisense fragment, in strains transformed with this construct, could then be induced by addition of IPTG to the growth medium. The phenotype of cells that had reduced levels of NDH-1 could then have been studied in order to determine the function(s) of NDH-1 in *Anabaena* PCC 7120. The plasmid pUC19 was chosen to drive expression of the antisense construct as it contains a complete *lacI* region, a *lac* represser, which represses expression from P_{lac}. Addition, of the inducer, IPTG causes the repressor to drop off, allowing the polymerase to bind and resulting in transcription from the promoter under the control of *lacI* (Sambrook *et al.* 1989). The repression of expression from the promoter under the

control of *lacI* has been shown to function in *Anabaena* PCC 7120 (Elhai 1993) and *Synechococcus* PCC 7942 (Price and Badger 1989; Geerts *et al.* 1995). On addition of IPTG, expression from the promoter under the control of *lacI* has been shown to increase 5 fold in *Anabaena* PCC 7120 (Elhai 1993), while in *Synechococcus* PCC 7942, one study, using *lacIQ* to repress expression, has shown a 12-33 fold increase (Price and Badger 1989) and another, using *lacI* to repress expression, has shown a 36 fold increase (Geerts *et al.* 1995). IPTG concentrations of up to 4 mM apparently have no effect on the growth of *Synechococcus* PCC 7942 (Geerts *et al.* 1995) and 0.5-1 mM IPTG has been shown to be sufficient to induce high levels of expression in *Synechococcus* PCC 7942 (Price and Badger 1989; Geerts *et al.* 1995). The repression by *lacI* is not complete though and there will be some expression from the promoter under its control, even in the absence of IPTG (Price and Badger 1989). These findings suggest that the strategy outlined above should produce strains of *Anabaena* PCC 7120 in which the level of NDH-1 can be reduced, through expression of the antisense construct. These strains should be useful in elucidating the function of NDH-1 in *Anabaena* PCC 7120, providing homologous crossover does not selectively remove the antisense fragment from the vector.

Another line along which future research should be directed is the cloning, sequencing and inactivation of other *ndh* genes from *Anabaena* PCC 7120. In *Synechocystis* PCC 6803 inactivation of *ndhC* did not inhibit respiration to the same extent that inactivation of *ndhK* did, and did not effect the CO_2 concentrating mechanism to the same extent (Ogawa 1992b). Therefore *ndhC* may prove to be an ideal target to inactivate in future studies to determine the function of the NDH-1 on the plasma membrane of *Anabaena* PCC 7120.

As yet, no homolouges for the genes encoding the three subunits thought to be involved in binding NADH and FMN (*nuo5-6-7* from *E. coli*) in type-1 NADH dehydrogenases have been found in cyanobacteria (nor in chloroplasts). An extensive study, in *Synechocystis* PCC 6803, using Southern analysis and PCR with degenerate primers, has failed to show the presence of homolouges of *nuo6* and *nuo7* (Funk *et al.* 1994). This led these workers to suggest that the part of the enzyme responsible for oxidising NAD(P)H in the cyanobacterial form of NDH-1 had a different structure from that of complex I. An alternative explanation is that the homolouges of these genes in cyanobacteria have insufficient homology with the probes to by detected by the methods used. One way to determine if these genes are present in cyanobacteria is to try and clone them by complementation. As the gene encoding NDH-2 and all the genes encoding subunits of NDH-1 have been cloned and sequenced from *E. coli* (Young and Wallace 1976; Weidner *et al.* 1993), mutants can be constructed which lack NDH-2 and a specific subunit of NDH-1. Mutants of *E. coli* that lack both NDH-1 and NDH-2 are unable to

oxidise NADH, produce smaller colonies and have longer doubling times when grown on "rich media" and can not grow on minimal media containing mannitol as the sole carbon source (Young and Wallace 1976; Calhoun and Gennis 1993). Each strain constructed would have to be tested to determine if it had this phenotype. Complementation tests with a genomic library from the cyanobacterial strain of interest could then be performed on the mutant strains of *E. coli*. Clones which rescue these strains and allow them to grow on minimal media containing mannitol as the sole carbon source could then be characterised. This method of cloning *ndh* genes has a number of advantages: firstly it allows genes encoding specific subunits of NDH-1 to be cloned; secondly some of the clones that complement the strain may contain the gene for NDH-2, a subject about which very little is known in cyanobacteria; thirdly once constructed the strains could be used to clone *ndh* genes from any organism. If a gene for an NDH-2 was cloned and sequenced strains could then be constructed in which it has been inactivated in order to determine its function.

7.5 NAD(P)H DEHYDROGENASES IN THE CHLOROPLASTS OF HIGHER PLANTS

The rotenone-sensitive NAD(P)H→Q_1 activity in the stroma lamella of soybean thylakoids reported here (Tables 3.1 and 3.2), along with the finding of a similar activity in spinach thylakoids (D.A. Day, P. Vottero, A.-J. Dorne, M. Block and J. Joyard, unpublished results), are the first reports of such an activity in the chloroplasts of higher plants. The rotenone-sensitivity of the activity suggests that it may be catalysed by a type-1 NAD(P)H dehydrogenase. The products of two of the *ndh* genes, NdhK and NdhH, have been shown to be present in the stroma lamella of thylakoids (Nixon *et al.* 1989; Berger *et al.* 1993). This raises the possibility that the enzyme may be encoded for by the *ndh* genes present in chloroplast genomes. Chloroplast *ndh* genes encode proteins homologous to subunits of complex I from mitochondria, an NDH-1 (as discussed in Section 1.4.1).

While this is the first report of a rotenone-sensitive NAD(P)H‡Q_1 activity in higher plant chloroplasts it is not the first report of rotenone-sensitive NADH and NADPH oxidising activities in these organelles. Rotenone-sensitive NADH and NADPH→ferricyanide activities have been shown to occur in barley thylakoids (Cuello *et al.* 1995). Comparison of the reaction rates with the two electron donors, alone and together, indicated that the rates were additive, suggesting that the activities were catalysed by two

different enzymes (Cuello *et al.* 1995). As discussed in Chapter 3, very high concentrations of rotenone (50-100 μM giving 10-50% inhibition respectively) had to be used to see inhibition, and no inhibition of either activity was seen with 25 μM rotenone, the concentration used in this study. The effect of ethanol alone on both activities was not determined, and it is unknown if the inhibition seen was an artifact of the high concentrations of ethanol in the assays (1-2% (v/v)). If the inhibition was not an artifact it indicates that the activities are not catalysed by an enzyme homologous to mitochondrial complex I, or the mechanism of NADH oxidation is different. The NADH→ferricyanide activity of mitochondrial complex I is not inhibited by rotenone (Menz *et al.* 1992; Friedrich *et al.* 1994a; Friedrich *et al.* 1994b). Further studies will need to be carried out to determine whether the rotenone-sensitive NAD(P)H→Q_1 activity reported here is catalysed by one or two enzymes and if it is catalysed by an enzyme(s) similar to those reported to be present in barley thylakoids. However, the results of the native gels in which the banding patterns were different when NADH and NADPH dehydrogenase activities were assayed for (Figure 3.2) suggest that the activities are catalysed by different enzymes in soybean chloroplasts. If the same enzyme catalysed both reactions the banding pattern on the native gels would have been expected to be identical.

The rotenone-sensitive NAD(P)H→Q_1 activity reported here may function as the dehydrogenase in the chlororespiratory pathway that has been postulated to be present in the chloroplasts of algae and higher plants (Goedheer 1963; Bennoun 1982). Bennoun (1983), using mutant strains of *C. reinhardtii* that lacked plastocyanin or cytochrome f in the chloroplasts, demonstrated that the only interaction between the chlororespiratory and the photosynthetic electron transport chains was at the level of plastoquinone. He also showed that addition of the ionophore dicyclohexyl-18-crown-6 (a synthetic ionophore with the specificity $K^+>Rb^+>Cs^+>Na^+>Li^+$ (Izatt *et al.* 1971)) induced reduction of the plastoquinone pool in the dark. This was interpreted as indicating the existence of a coupling site in the chlororespiratory pathway between NAD(P)H and plastoquinone. An NDH-1, such as that postulated to be responsible for the rotenone-sensitive NAD(P)H→Q_1 activity reported here, would provide a coupling site between NAD(P)H and plastoquinone.

Using oxygen stable isotope discrimination in experiments in which the partial pressure of $^{18}O_2$ and $^{16}O_2$ was measured, a light-dependent $^{18}O_2$ oxygen uptake process was shown to occur in tobacco chloroplasts (Gruszecki *et al.* 1994). The assay contained water in the $H_2{}^{16}O$ form only, and $^{18}O_2$ was added exogenously in the molecular form and brought to equilibrium between the water and gas phases. Therefore upon illumination the photosynthetic water splitting reaction results in the production of $^{16}O_2$ and any oxygen uptake process can be measured by a decrease in the partial pressure of $^{18}O_2$. The light dependent $^{18}O_2$ uptake process observed was the first direct evidence for

an oxidase in the chloroplasts of higher plants or eukaryotic algae. This process was attributed to chlororespiration. The light dependent $^{18}O_2$ uptake was completely inhibited by the addition of SiMo, which accepts electrons from the primary quinone acceptor in PS II (Q_A), and by DBMIB an inhibitor of photosynthetic reoxidation of the plastoquinone pool by the cytochrome b_6f complex (Gruszecki *et al.* 1994). These results suggest that a respiratory process in which plastoquinone is involved as an electron carrier might be responsible for the $^{18}O_2$ uptake. The inhibition of the $^{18}O_2$ uptake by SiMo or DBMIB rules out the possibility that it was due to photorespiration, as photorespiration would not be inhibited by the addition of either of these compounds. The demonstration of an NAD(P)H-quinone oxidoreductase in this study means that all the necessary components for a functional chlororespiratory pathway have been shown to be present in the chloroplasts of higher plants. Further study will be required to determine if both NADH and NADPH are able to donate electrons to the chlororespiratory pathway or if one substrate is favoured over the other. In the only study, to date, on electron donation to the chlororespiratory pathway it has been shown that in the green alga, *Dunaliella salina,* that NADPH is oxidised more efficiently than NADH (Harrison and Allen 1993).

Studies on a wide range of species, using chlorophyll a fluorescence, have demonstrated a non-photochemical reduction of the plastoquinone pool in the chloroplast following light to dark transitions (Groom *et al.* 1993). The underlying mechanism of the dark reduction of the plastoquinone pool is unknown, but it may involve chlororespiration through the action of an NAD(P)H-plastoquinone oxidoreductase. The NAD(P)H-quinone oxidoreductase demonstrated in this study may play a role in this. However, the non-photochemical reduction of the plastoquinone pool on light to dark transitions is not seen in soybean (Groom *et al.* 1993). This leaves open the question as to what the function of the NAD(P)H-quinone oxidoreductase in soybean is, because if it acts in chlororespiration then non-photochemical reduction of the plastoquinone pool would be expected. One possibility is that in soybean the chlororespiratory process is light dependent as has been shown to be the case for the oxygen uptake process in tobacco chloroplasts (Gruszecki *et al.* 1994) (discussed above), hence non-photochemical reduction of plastoquinone would not be seen on light to dark transitions. Alternatively the process may only occur in young leaves in soybean. Leaves from 12-14 day old plants were used for this study, while Groom *et al.* (1993) used mature plants in their study. Further studies need to be carried out to determine if the age (stage of development) of the leaves has an effect on the two activities discussed above.

The presence of an NAD(P)H-quinone oxidoreductase in a fraction enriched in PS I means that it may play a role in an NADPH mediated cyclic electron transport pathway around PS I. As discussed in section 1.3.3, an NADPH mediated cyclic electron

transport pathway around PS I has been shown to occur in the cyanobacterium *Synechocystis* PCC 6803 (Mi *et al.* 1992a; Mi *et al.* 1994; Mi *et al.* 1995) and there is evidence for a similar cycle in *Synechococcus* PCC 7002 (Mi *et al.* 1992b). The presence of a similar cycle in the chloroplasts of higher plants could explain the observations that electrons can be donated to the intersystem chain from stromal components (Asada *et al.* 1992; Asada *et al.* 1993), with the NAD(P)H-quinone oxidoreductase donating electrons to plastoquinone (Figure 7.3). In maize the donation of stromal components to the intersystem chain was shown to be NADPH mediated (Asada *et al.* 1993), as is the case in *Synechocystis* PCC 6803 (Mi *et al.* 1995). This theory should be treated with caution, however, as other workers have suggested the donation of electrons to the intersystem chain can be adequately explained by the reversal of the ferredoxin-$NADP^+$ reductase (FNR) reaction followed by reduction of the plastoquinone pool through ferredoxin-plastoquinone reductase (FQR) (Bendall and Manasse 1995). Further confusing the issue is the finding that donation of electrons from stromal components to the intersystem chain is not seen in soybeans (Asada *et al.* 1993).

The function of the NAD(P)H-quinone oxidoreductase activity in the stroma lamellae of soybean thylakoids remains unknown. If it donates electrons to the plastoquinone pool the studies of Groom *et al.* (1993) and Asada *et al.* (1993) should have been able to detect some effect on the redox state of the plastoquinone pool, unless it is a light dependent process that operates at levels so low that the effect can not be distinguished from the PS II mediated reduction of plastoquinone.

As discussed in Section 1.4.1 the actual role, if any, of the products of the chloroplast encoded *ndh* genes is confused by the findings that the chloroplast genomes of *Pinus thunbergii* (Wakasugi *et al.* 1994), the red algae *Porphrya purpurea* (Reith and Munholland 1994) and the cyanelles of *Cyanophora paradoxa* (Stirewalt *et al.* 1995), do not contain *ndh* genes. A report from the V^{th} Plant Molecular Biology Meeting (Amsterdam, 1994) showed that the *ndh* genes are also absent from the chloroplast genome of *C. reinhardtii* (Dr M. Badger Pers. Comm.). These findings open the question as to whether the *ndh* genes are needed, for a functional chloroplast, or is it only in these organisms that they are not essential as the have an alternative way of providing the function that the products of the *ndh* genes are involved in. Alternatively the *ndh* genes may have been transferred to the nucleus and their products imported into the plastid, in these organisms. This seems unlikely though, as algae, cyanophora and gymnosperms are generally considered to be less advanced, in evolutionary terms, than angiosperms. To clarify this, studies need to be carried out in these species to determine if proteins that are Ndh homologues are present in the chloroplasts. Another possibility is that the *ndh* genes play a role in leaf senescence. A region of the barley chloroplast

genome that contains *ndh* genes has been shown to be transcribed during senescence of barley leaves (Vera *et al.* 1990). Therefore the *ndh* genes may not be essential in the chloroplasts of organisms which do not undergo senescence. In order to determine if this is a possibility, studies need to be carried out on more evergreen higher plants to determine if their chloroplasts contain *ndh* genes or not.

The inner envelope membrane of spinach chloroplasts has been shown to contain a rotenone-sensitive NAD(P)H→Q_1 activity (D.A. Day, P. Vottero, A.-J. Dorne, M. Block and J. Joyard, unpublished results). It is unknown if this is catalysed by an NDH-1. However, immunological studies, with antibodies raised against NdhK from *Synechocystis* PCC 6803 (Chapter 4) and the carboxy terminal end of NdhH from rice (Berger *et al.* 1993), did not show any cross-reactivity with proteins of the correct size to be the proteins encoded by *ndhK* or *ndhH*. Further studies will be needed to determine the nature of the enzyme that catalyses this activity and what its function is. One possibility is that the enzyme provides the reducing power necessary to drive the desaturation of fatty acids that occurs in the inner envelope membrane (see Jaworski 1987; Joyard and Douce 1987 for reviews). A possible scheme for this is shown in Figure 7.4.

Future studies to determine if there is an NDH-1 in the chloroplasts of higher plants that is encoded by the *ndh* genes should be carried out in tobacco. Tobacco is an ideal plant to use for two reasons: firstly, a light dependent oxygen uptake process, that has been attributed to chlororespiration, has been demonstrated (Gruszecki *et al.* 1994); and secondly, a reliable transformation system for tobacco chloroplasts has been developed (Svab and Maliga 1993). This would allow experiments to be performed in which a chloroplast encoded *ndh* gene was inactivated. Any transformants could then be studied to determine the effect of inactivating an *ndh* gene on chloroplast metabolism, the redox state of the plastoquinone pool and on light dependant oxygen uptake. These studies would determine the function of the chloroplast *ndh* genes and whether their products participated in chlororespiration and/or donation of stromal components to the intersystem chain, thus elucidating the function of the *ndh* genes in higher plant chloroplasts.

7.6 CONCLUSIONS

This study has shown that there is a rotenone-sensitive NAD(P)H-quinone oxidoreductase activity present in the stroma lamellae of soybean thylakoids. The products of two *ndh* genes, NdhK and NdhH, have been shown to be present in the

stroma lamellae of higher plant chloroplasts (Nixon *et al.* 1989; Berger *et al.* 1993). This raises the possibility that the activity described in this study is catalysed by an enzyme, homologous to type-1 NADH dehydrogenases, that is encoded by the *ndh* genes present in chloroplast genomes. This is the first report of such activity in the chloroplasts of higher plants. It is unknown what the function of this activity is or whether it is the entry point for electrons into the chlororespiratory pathway.

Using antibodies raised against NdhK from *Synechocystis* PCC 6803, the plasma membrane specific location of the NDH-1 in *Anabaena* PCC 7120 was confirmed. The operon encoding *ndhC-K-J* was shown to be conserved in *Anabaena* PCC 7120 and part of this operon was cloned and sequenced using PCR. The structure of this operon was more closely related to the structure of the same operon in chloroplasts of higher plants than the cyanobacterium *Synechocystis* PCC 6803, but the proteins encoded by the genes were more closely related to the proteins encoded by the same genes in *Synechocystis* PCC 6803 than in chloroplasts. The protein on the plasma membrane that cross-reacted specifically with the antibodies raised against NdhK from *Synechocystis* PCC 6803 had an apparent molecular weight of 28 kDa. This is the correct size to be the protein encoded by *ndhK*, thus verifying that *ndhK* is a functional gene and not a pseudogene.

Studies in which *ndhK* and thus the NDH-1 in *Anabaena* PCC 7120 was inactivated indicated that the NDH-1 may be essential for survival of the cell, as the mutant had an apparently lethal phenotype. This conclusion must stand until it can be verified. Unfortunately it is extremely difficult to verify if a gene is essential for the cell's survival. The apparent lethality of inactivating *ndhK* in *Anabaena* PCC 7120, under the selection conditions used, provides support to the theory that the NDH-1 in *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 have different functions. Inactivation of *ndhK* in *Synechocystis* PCC 6803 is not lethal when the cells are grown under the same growth conditions used in this study. The nature of this apparent difference in function still remains to be elucidated.

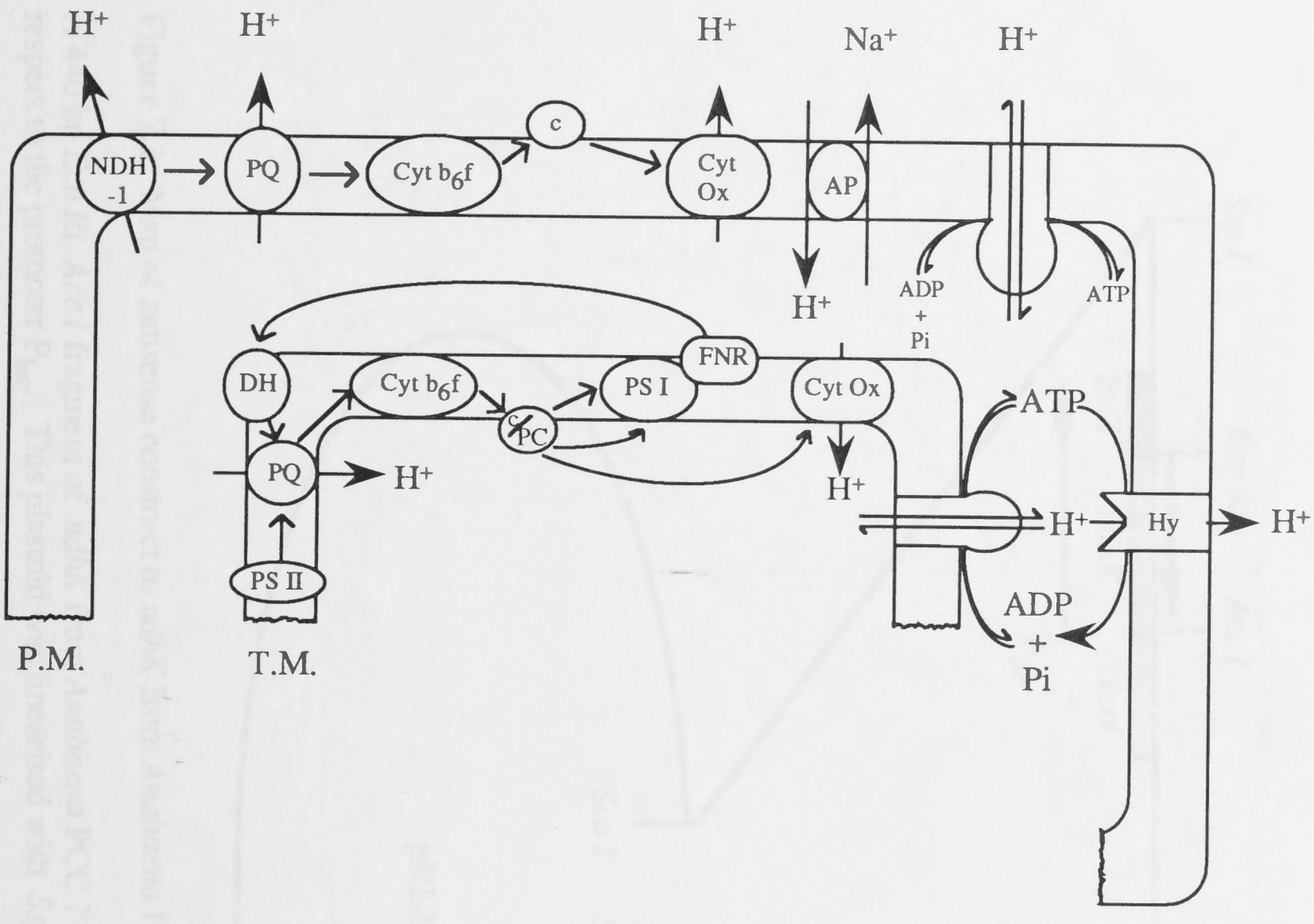


Figure 7.1: Polarity and sidedness of proton translocation, electron transport, and ATP synthesis/hydrolysis in the energy transducing membranes of cyanobacteria (Adapted from Peschek 1987).

Abbreviations: P.M. = plasma membrane; T.M. = Thylakoid membrane; NDH-1 = type 1 NAD(P)H dehydrogenase, DH = NAD(P)H dehydrogenase, PS I and PS II = photosystems I and II; PQ = plastoquinone; Cyt b_6f = cytochrome b_6f; Cyt Ox = cytochrome oxidase (aa_3); PC = plastocyanin; c = soluble c-type cytochrome; FNR = ferredoxin $NADP^+$-oxidoreductase; Hy = unidirectional ATP hydrolase; AP = antiporter.

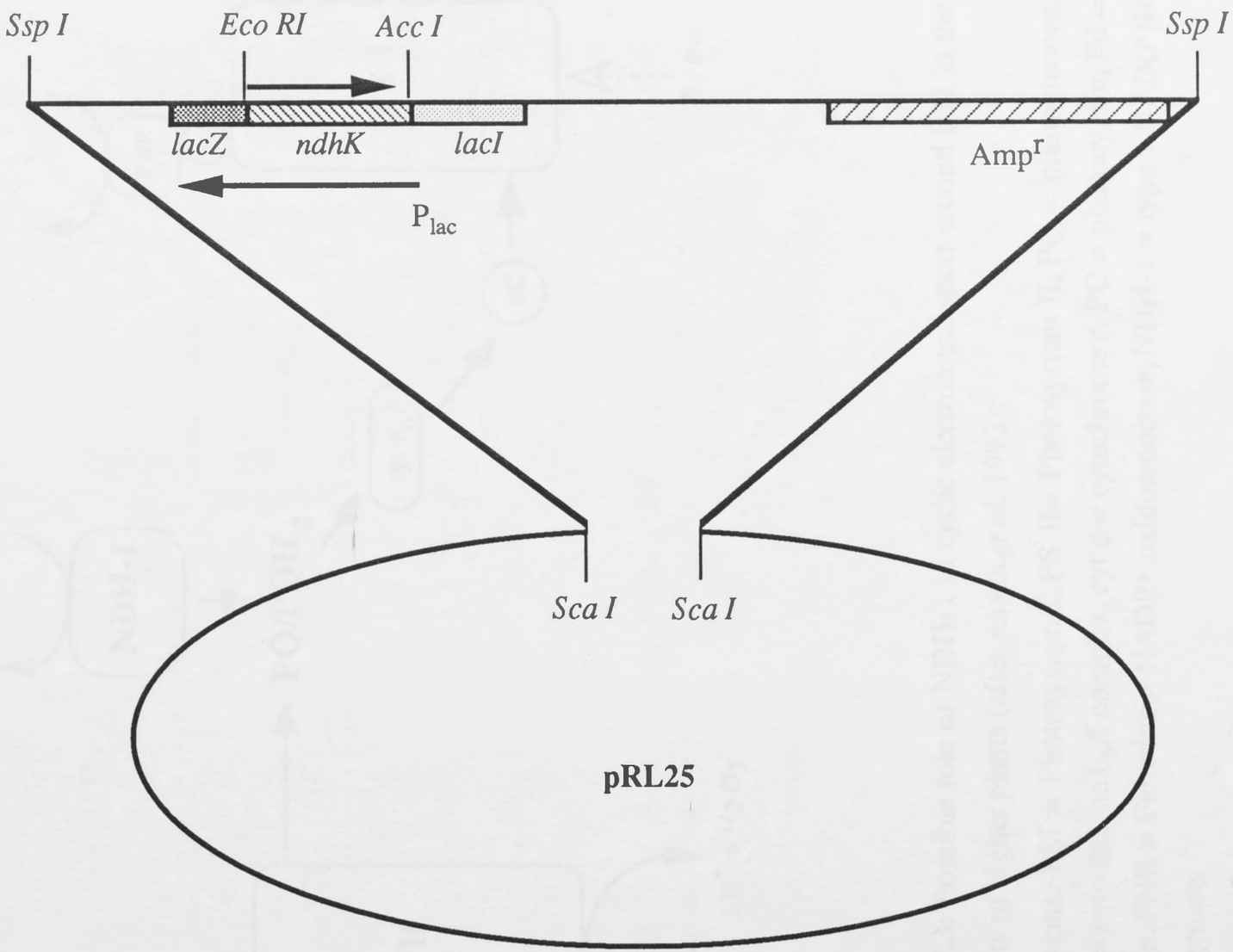


Figure 7.2: Map of antisense construct to *ndhK* from *Anabaena* PCC 7120.

A 440 bp *Eco RI, Acc I* fragment of *ndhK* from *Anabaena* PCC 7120 was cloned into pUC19 in the antisense orientation with respect to the promoter P_{lac}. This plasmid was linearised with *Ssp I* and cloned into the *Sca I* site of pRL25 (Figure 6.3).

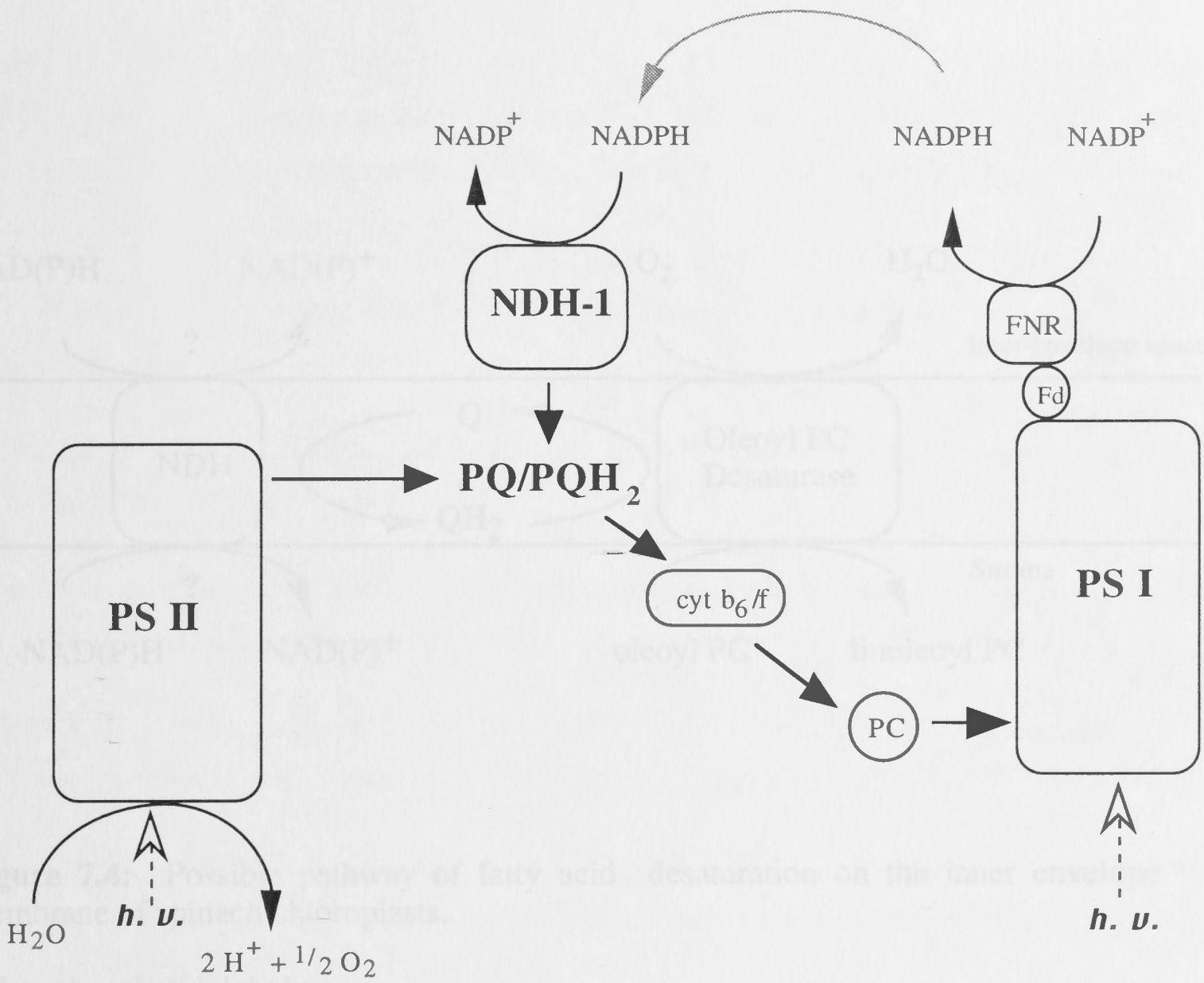


Figure 7.3: Possible role of NDH-1 in cyclic electron transport around PSI in the chlorolasts of higher plants (after Asada *et al.* 1993).

Abbreviations: PSI = Photosystem I, PS II= Photosystem II, PQ = plastoquinone, cyt b_6/f, = cytochrome b_6/f complex, cyt c = cytochrome c, PC = plastocyanin, Fd = ferredoxin, FNR = Ferredoxin $NADP^+$ oxidoreductase, NDH-1 = type 1 NAD(P)H dehydrogenase.

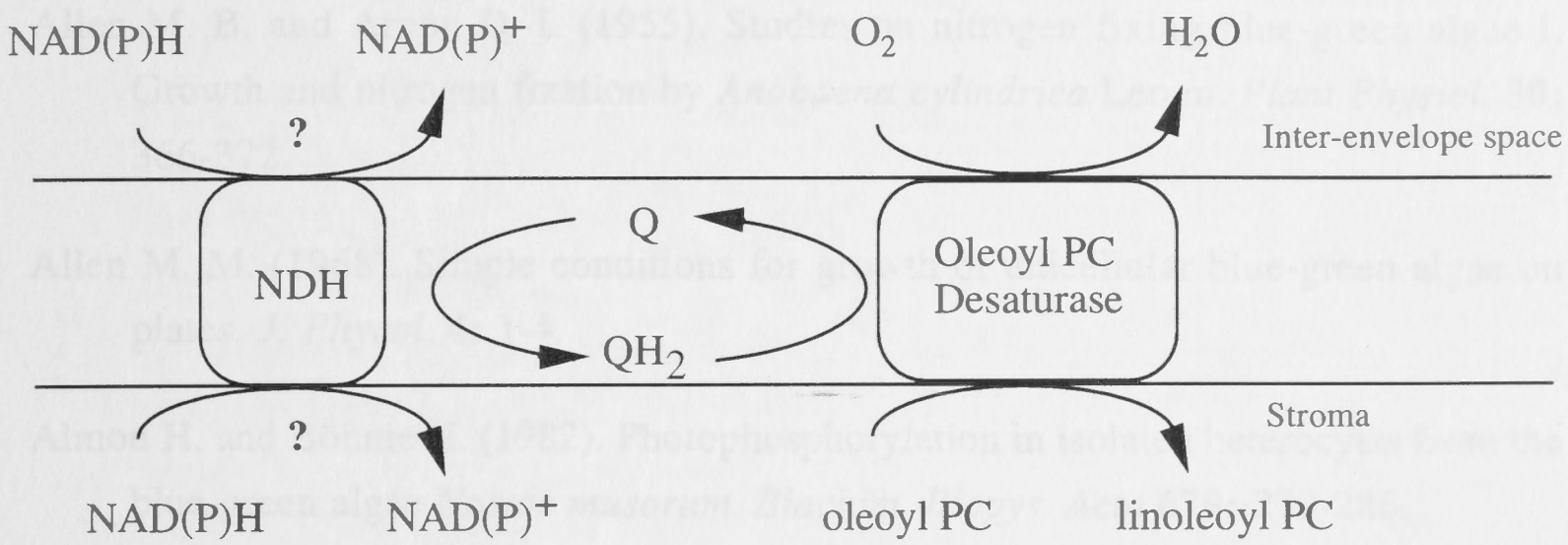


Figure 7.4: Possible pathway of fatty acid desaturation on the inner envelope membrane of spinach chloroplasts.

PC = phosphatidylcholine
NDH = NADH dehydrogenase

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APPENDIX A

Composition of 1/8 Allen and Arnon (1955) media containing full Pi and four times Ni.

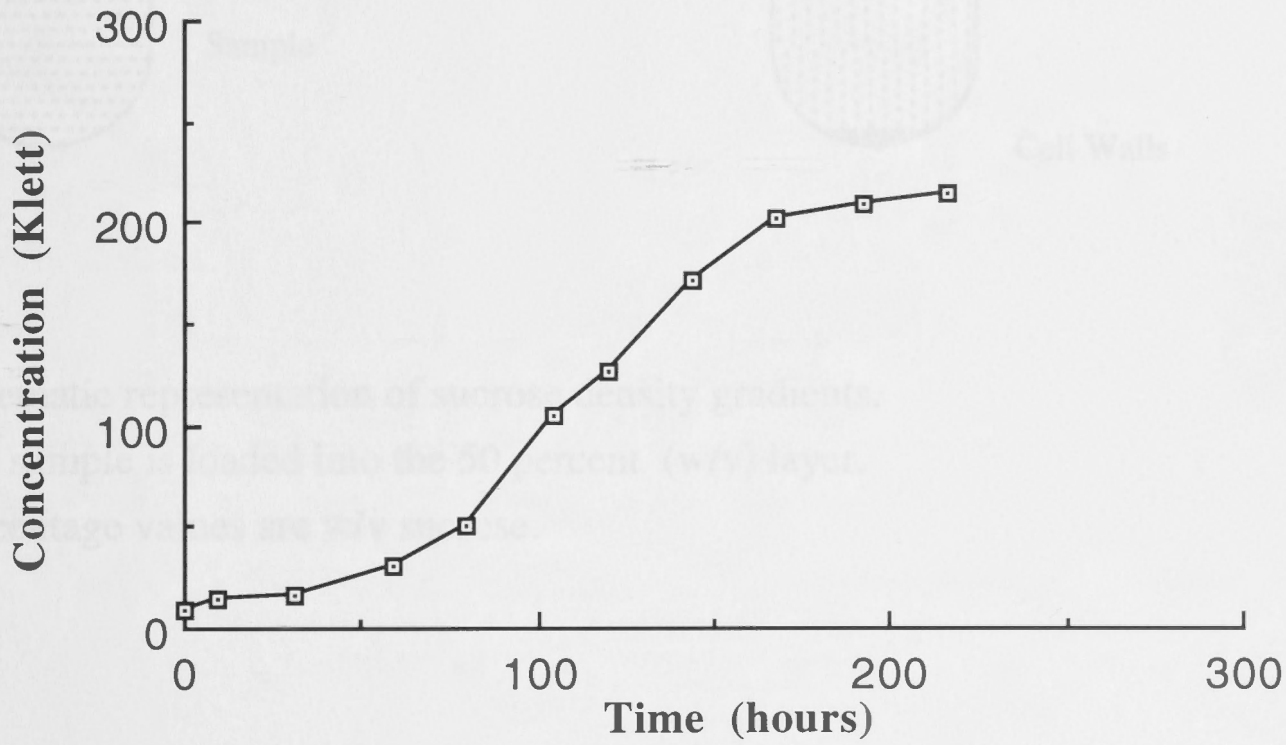
Element	Present as	Final Concentration
Macronutrients		
Mg	$MgSO_4$	125.0 µM
Ca	$CaCl_2$	62.5 µM
Na	NaCl	40.0 mM
P	K_2HPO_4	250.0 µM
Micronutrients		(ppb)
Fe	$(NH_4)_2SO_4FeSO_4.6H_2O$	500.0
Mn	$MnSO_4.4H_2O$	60.0
Mo	MoO_3	12.5
Zn	$ZnSO_4.4H_2O$	6.25
Cu	$CuSO_4.5H_2O$	2.5
B	H_3BO_3	62.5
V	NH_4VO_3	1.25
Co	$Co(NO_3)_2.6H_2O$	1.25
Ni	$NiSO_4.6H_2O$	10.0
Cr	$Cr_2(SO_4)_3K_2SO_4.24H_2O$	1.25
W	$Na_2WO_4.2H_2O$	1.25
Ti	$(NH_4)_2TiO(C_2O_4).H_2O$	1.25

KNO_3 was added to a final concenration of 4 mM when cells were grown in the presence of nitrate

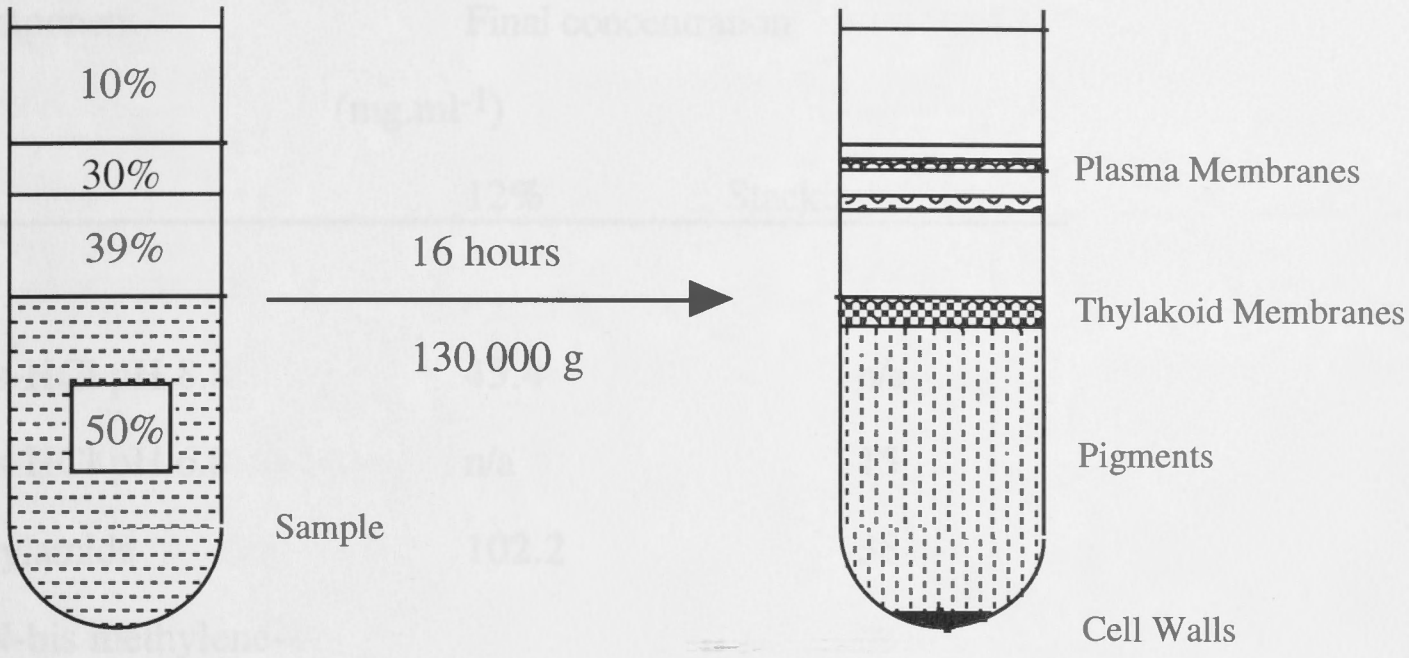
Plates contained 10 mM TES (pH 8.0) and liquid media contained 10 mM BTP (pH 8.0)

APPENDIX B

Growth curve for *Anabaena* PCC 7120



APPENDIX C



Schematic representation of sucrose density gradients.
The sample is loaded into the 50 percent (w/v) layer.
Percentage values are w/v sucrose.

APPENDIX D

Composition of SDS-PAGE gels

Component	Final concentration ($mg.ml^{-1}$)	
	12%	Stacking
Tris-HCl pH 8.8	45.4	n/a
Tris-HCl pH 6.8	n/a	15
Acrylamide	102.2	38
N,N-bis methylene-acrylamide	3.2	1
SDS	0.875	1
AMPS	0.5	0.5

APPENDIX E

Solutions for the preparation of gradient polyacrylamide gels

	percentage of gel solution (by volume)	
Solution	Light	Heavy[a]
40% (w/v) Acrylamide and 1% (w/v) bisacrylamide	8.0	62.5
200 mM Tris-glycine buffer pH 9.0,containing 0.4% (w/v) Triton X-100	25.0	25.0
10% (w/v) Ammonium persulfate	1.0	1.0
N,N,N',N'-Tetramethyl-p-persulfate	0.016	0.01
Water	66.0	11.7

[a] 15 % (w/v) sucrose was also added to the heavy solution