Characterisation of the sodium-dependent NADH-ubiquinone oxidoreductase from *Vibrio harveyi*.

Andrew Stevenson

Thesis presented for the degree of Master of Philosophy,
University of Edinburgh,
November, 1994.

Declaration.

I declare that this thesis was composed by myself and the research presented is my own except where otherwise stated.

Andrew Stevenson,

November, 1994.

Acknowledgements.

I would like to thank Bruce Ward and David Leach for their help and discussion during my project and the preparation of this thesis. I would also like to thank all members of ICMB both past and present who have helped me, in particular Margaret Daniel, John Connolly and Dave Dryden for their help during protein purification and for lending me columns to try. I would also like to thank Pam Beattie for her help especially during large scale growth of bacterial cells. Finally I would like to thank my family and friends for their support and Karen for her constant love and sense of humour!

Contents

Figures and Tables

Page No.

Chapter 1

Page No.

Page No.

Chapter 3

Chapter 4

Chapter 5

Page No.

Abbreviations

A	absorbance
ADP	adenosine diphosphate
APS	ammonium persulphate
ATP	adenosine triosphosphate
°C	degrees Celsius
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CHAPS	3-[(3cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	centimetre
DCCD	dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
DES	diethylstilbestrol
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
g	grams or standard acceleration of gravity
h	hours
HCl	hydrochloric acid
HQNO	2-heptyl-4-hydroxyquinoline-N-oxide
KCN	potassium cyanide

l	litres
kb	kilobases
kDa	kilodaltons
kg	kilogram
M	molar
mA	milliamps
Mega 8	octanoyl-N-methylglucamide
Mega 9	nonanoyl-N-methylglucamide
min	minutes
mg	milligrams
ml	millilitres
µl	microlitres
mM	millimolar
µM	micromolar
mm	millimetres
NADH	nicotinamide adenine dinucleotide
dNADH	deamino nicotinamide adenine dinucleotide
NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
NEM	N-ethylmaleimide
NQNO	2-nonyl-4-hydroxyquinoline-N-oxide
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonylfluoride
RNAase	ribonuclease
Rif^r	rifampicin resistance
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N',-tetramethylethylenediamine

Tris	tris (hydroxymethyl)-aminomethane
UV	ultraviolet
V	volts
V/cm	volts per cm
v/v	volume per volume
w/v	weight per volume

Abstract.

A sodium-dependent NADH-ubiquinone oxidoreductase has been discovered in the marine bacteria *Vibrio harveyi*. This enzyme acts as a primary sodium pump and can utilise the substrates NADH and dNADH and its activity is stimulated by sodium ions. The enzyme activity is inhibited reversibly by Zn^{2+} and Pb^{2+} and is irreversibly inhibited by Ag^{+}, Cu^{2+} and Cd^{2+}. The sodium-dependent NADH-ubiquinone oxidoreductase has an approximate molecular weight of 254 kDa and is extremely thermolabile and sensitive to acidic pH.

Transposon mutagenesis was used to isolate sodium-pump defective mutants as strains unable to grow in the presence of the proton gradient uncoupler CCCP, at pH 8.5. Only bacteria with a functional sodium-dependent NADH-ubiquinone oxidoreductase should be able to survive by utilising a sodium gradient during aerobic growth. A total of 16 CCCP sensitive transposon mutant strains were isolated. However all of these had normal sodium-dependent NADH-ubiquinone oxidoreductase activity. The NADH-ubiquinone oxidoreductase gene (*ndh*) from *E. coli* was used as a probe to determine whether a similar enzyme could be identified in *Vibrio* species. No homologues of *ndh* were identified in either *V. harveyi* or *V. alginolyticus* as determined by Southern blotting of chromosomal DNA and hybridisation using *ndh* as a probe.

The sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* was purified successively on columns of Sephacryl 300HR, DEAE Sepharose fast flow, hydroxylapatite and Mono Q. A 75 fold increase in specific activity and 40% recovery of total activity was achieved. Small amounts of relatively pure sodium-dependent NADH-ubiquinone oxidoreductase were obtained using preparative electrophoresis. Although these samples were not purified to homogeneity a number of polypeptides were identified which appear to be part of the sodium-dependent

NADH-ubiquinone oxidoreductase complex. These included a 66 kDa (ghost band), 62 kDa, 52 kDa (doublet), 36 kDa and 34 kDa polypeptide bands which are all in the correct size range.

Chapter 1

Introduction.

1.1. Aerobic respiratory chains.

The main function of aerobic respiratory chains is the the translocation of protons across bacterial or mitochondrial membranes at the expense of chemical energy. The coupling of proton translocation and electron transport was first proposed by Mitchell (1966) in his chemiosmotic theory. Respiratory enzymes convert the free energy from the oxidation of hydrogen containing substrates into a transmembrane proton electrochemical gradient ($\Delta\mu_H$). This gradient which is referred to as a proton motive force, consists of two components; a proton concentration gradient (ΔpH) and a transmembrane electrical potential ($\Delta\psi$). The proton motive force is used to drive ATP synthesis, secondary active solute transport, and cell motility.

In mitochondria and respiring bacteria, ubiquinone and its analogues are a confluence point for the collection of reducing equivalents from multiple dehydrogenases, linking metabolism to cellular energetics. Quinones are mobile electron carriers in the mitochondrial or bacterial plasma membranes, and are oxidised by membrane bound cytochrome complexes which in turn transfer electrons to the terminal electron acceptor, molecular oxygen. Two types of cytochrome complex participate in respiration, linking electron transfer to proton translocation. They are the cytochrome bc_1 complex and the terminal oxidases. The bc_1 complex does not transfer electrons to oxygen, while the terminal oxidases do.

In the mitochondria of most species the oxidation of ubiquinone by molecular oxygen is catalysed by the sequential action of two enzymes, the cytochrome bc_1 complex, which oxidises quinol and reduces cytochrome c, and the cytochrome c oxidase complex, which oxidises cytochrome c and reduces oxygen to water (Figure

1.1. and 1.2.a.). There are exceptions to this linear arrangement in mitochondria. *Saccharomyces cerevisiae* contain a lactate-cytochrome b_2 oxidoreductase which transfers electrons directly from L-lactate to cytochrome c (De Vries and Marres, 1987) and protozoae possess a quinol oxidase which transfers electrons from ubiquinol directly to oxygen (Clarkson *et al.*, 1989).

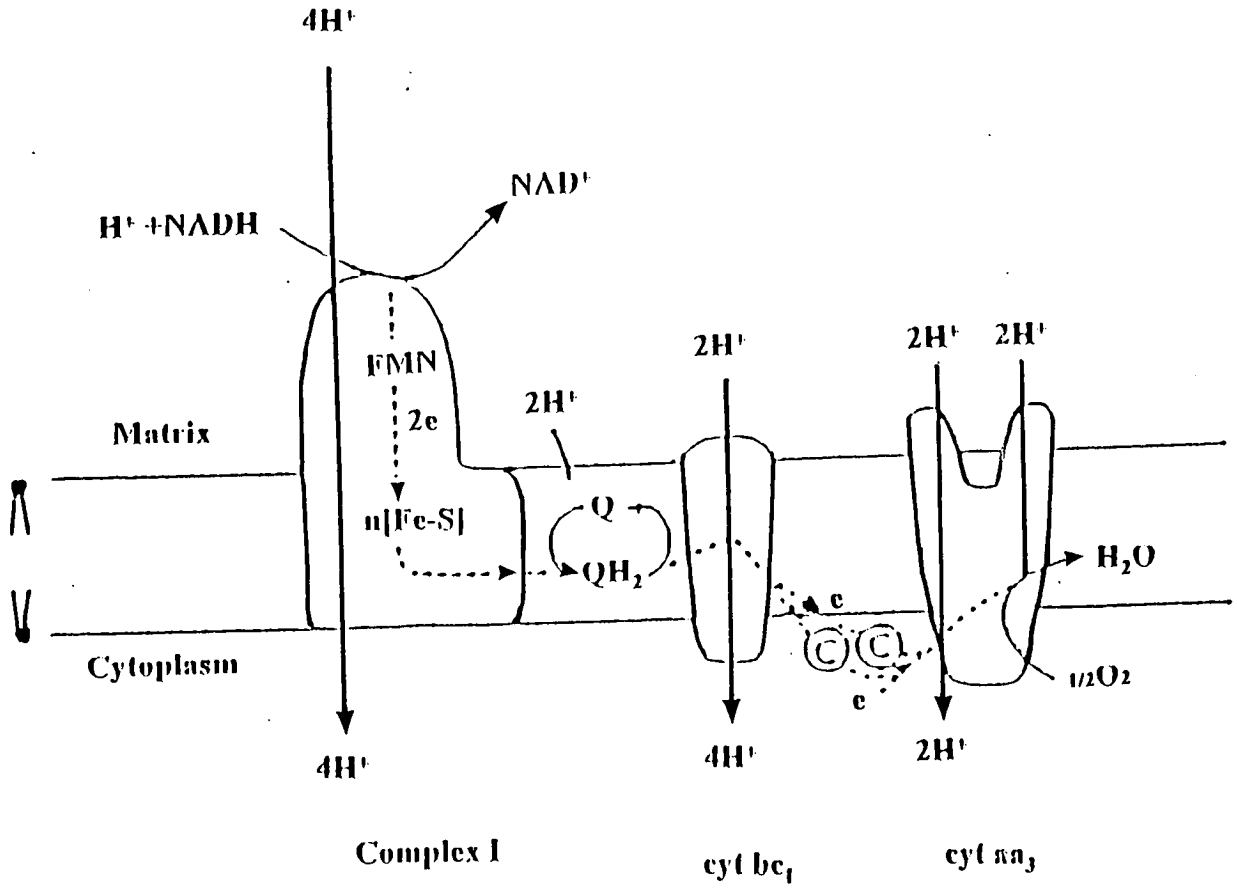
The simplest arrangement of respiratory cytochrome complexes in bacteria is found in *Escherichia coli*, which lacks a bc_1 complex and oxidises quinol directly with molecular oxygen by a quinol oxidase as illustrated in Figure 1.2.b.

The simplest means of forming a proton gradient is that used by the cytochrome bd ubiquinol oxidase from *E. coli* (Figure 1.3.a.). This enzyme has two partially separated active sites. The ubiquinol oxidation site is located near the outer surface of the membrane, while the oxygen reducing site appears to be on the inner surface of the membrane. The two electron oxidation of ubiquinol releases two substrate derived protons to the periplasm per ubiquinol, and electrons are passed to the second active centre, where oxygen is reduced using protons from the cytoplasm. There is no proton channel associated with this protein and it is not considered to be a proton pump (Trumpower and Gennis, 1994).

The cytochrome bo ubiquinone oxidase from *E. coli* generates a proton electrochemical gradient across the membrane as shown in Figure 1.3.b. Cytochrome bo is a member of the heme-copper oxidase superfamily, and is related to the mitochondrial cytochrome c oxidase. Cytochrome bo deposits one proton per electron into the periplasm from oxidation of quinol, and consumes protons from the cytoplasm in forming water just like cytochrome bd. In cytochrome bo one proton is translocated via a proton conducting channel for every electron transfered through the enzyme (Trumpower and Gennis, 1994).

Figure 1.1. Electron transport coupled to proton translocation in the mitochondrial electron transfer chain.

The redox energy difference (1.2 V at neutral pH) is converted into an electrochemical proton potential gradient $\Delta\mu_{H^+}$ by translocation of protons across the membrane which is harnessed by ATPase synthase, another multisubunit enzyme in the membrane, to drive the synthesis of ATP from ADP and inorganic phosphate. n [Fe-S] denotes at least five iron sulphur clusters (From Walker, 1992).


4H+
NAD+
H+ +NADH
FMN
2e
2H+
2H+
2H+
2H+
Matrix
n[Fe-S]
Q
QH2
H2O
e
e
1/2O2
Cytoplasm
4H+
4H+
2H+
Complex I
cyt bc1
cyt aa3

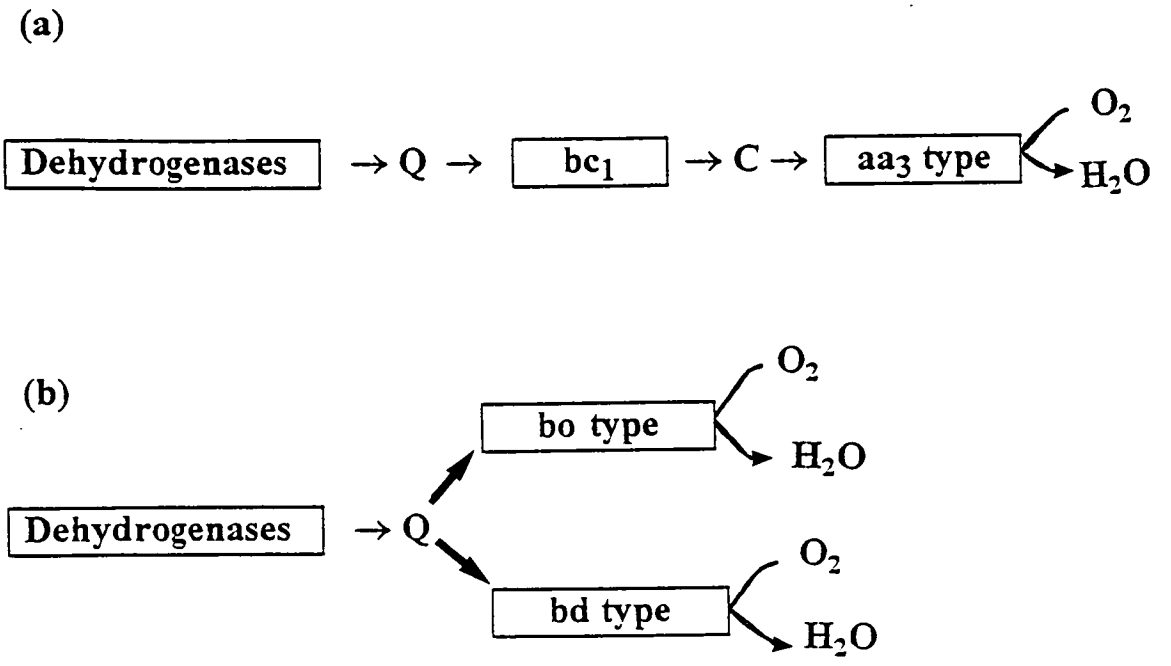
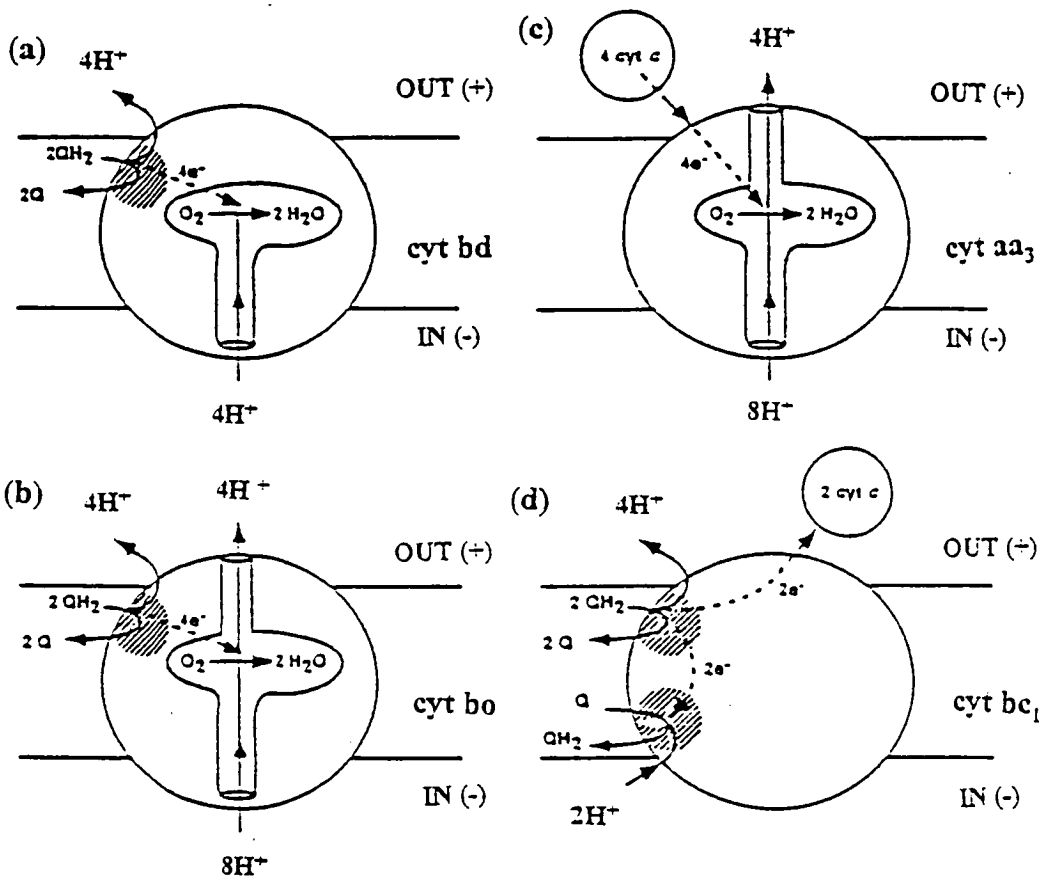


Figure 1.2. Energy-transducing cytochrome complexes in mitochondrial and bacterial respiration.

The diagram summarises the relationships between the cytochrome complexes, dehydrogenases, quinone (**Q**) and cytochrome c (**C**) involved in respiration in (a) mitochondria and (b) *E. coli*.

Figure 1.3. Schemes by which respiratory cytochrome complexes generate a transmembrane proton electrochemical gradient.

In each case the enzyme is shown within a membrane. The electrochemically positive, outer side corresponds to the mitochondrial intermembrane space or the bacterial periplasm. Protons are taken up from the either the mitochondrial matrix or the bacterial cytoplasm. (a) Cytochrome bd ubiquinol oxidase from *E. coli*. Protons that appear on the outside are derived from the substrate. (b) Cytochrome bo ubiquinol from *E. coli*. Half of the protons that appear on the outside are transported through a proton-conducting channel through the enzyme. (c) Cytochrome c oxidases from prokaryotes and mitochondria. The protons that appear on the outside are actively transported through a transmembrane channel. (d) Cytochrome bc_1 complex from prokaryotes and mitochondria (Modified fromTrumpower and Gennis, 1994)..


(a)
4H+
OUT (+)
2QH2
2Q
4e-
O2
2 H2O
cyt bd
IN (-)
4H+
(c)
4 cyt c
4H+
OUT (+)
4e-
O2
2 H2O
cyt aa3
IN (-)
8H+
(b)
4H+
4H +
OUT (+)
2 QH2
2 Q
4e-
O2
2 H2O
cyt bo
IN (-)
8H+
(d)
4H+
2 cyt c
OUT (+)
2 QH2
2e-
2 Q
2e-
Q
QH2
cyt bc1
2H+
IN (-)

The eukaryotic cytochrome c oxidases and several prokaryotic homologues pump one proton per electron. There are no substrate protons released at the outer surface, but protons from the inside are required for translocation via the pump and for the formation of water (Figure 1.3.c.) (Trumpower and Gennis, 1994).

The cytochrome bc_1 complex oxidises quinol by a mechanism that deposits two protons outside of the membrane for each electron transferred to cytochrome c, without a transmembrane proton channel. This accomplished by recycling electrons through separate quinol oxidation and quinone reduction sites within the the same enzyme, but on opposite sides of the membrane (Figure 1.3.d.) (Trumpower and Gennis, 1994).

1.2. NADH-ubiquinone oxidoreductases.

NADH-ubiquinone oxidoreductase is the first enzyme in aerobic respiratory electron transfer chains from NADH to oxygen. This enzyme complex is found in the membranes of mitochondria and bacteria, and transfers electrons from NADH to the mobile electron carrier ubiquinone. In bacteria and yeast there are at least two different types of NADH-ubiquinone oxidoreductase, known as NDH-1 and NDH-2. In mitochondrial membranes (excluding yeast) only one type of NADH-ubiquinone oxidoreductase is present, and it is known as complex I (Walker, 1992).

For both complex I and NDH-1 electron transfer is coupled to the transport of ions across the membrane. Generally protons are the coupling ion used but more recently sodium-translocating NADH-ubiquinone oxidoreductases have been discovered in a number of marine bacteria (Tokuda and Unemoto, 1981, 1983; Tsuchiya and Shinoda, 1985; Ken-Dror *et al.*, 1986). No ion transport is associated with the transfer of electrons through the NDH-2 type enzymes. (Yagi, 1991).

One of the most thoroughly characterised of the NADH-ubiquinone oxidoreductases is the bovine mitochondrial complex I. Composed of at least 41 different subunits, this enzyme is one of the most complex protein structures yet discovered. The 41 subunits contain more than 7 724 amino acids of unique sequence and this compares in complexity to the *E. coli* ribosome which contains 7 336 amino acids (Walker, 1992). Like other eukaryotic respiratory enzymes some of the subunits from bovine mitochondrial complex I are the products of mitochondrial DNA. These are known as subunits ND_1-ND_6 and subunit ND_4L, and all seven are hydrophobic intrinsic membrane proteins (See Table 1.1.) (Walker, 1992).

Table 1.1. Properties of subunits of bovine complex I that are encoded in mitochondrial DNA (From Walker 1992)

Subunit	Molecular weight (kDa)	Predicted tramsmembrane α-helices
ND_1	30	8
ND_2	30	8
ND_3	15	3
ND_4	39	12
ND_4L	10	3
ND_5	50	13-14
ND_6	n.d.	4-5

Mitochondrial DNA sequences have been determined in a wide range of eukaryotes including mammals, fish, insects, amphibians, protozoans, fungi and higher plants (Walker, 1992). For most of the examples studied so far homologues of some or all of the ND_1-ND_6 and ND_4L structural genes have been found. A notable exception is the yeast *Saccharomyces cerevisiae* which has no ND genes in its mitochondrial DNA.

The bovine mitochondrial complex I was originally purified by Hatefi *et al.* (1962). Complex I was solubilised from mitochondrial membranes using potassium deoxycholate and fractionated using ammonium acetate. More recently complex I was purified using ammonium sulphate fractionation, followed by ion exchange chromatography on a Mono Q column after solubilisation of mitochondrial membranes using the detergent lauryl maltoside (Finel *et al.*, 1990). Some impurities are still present in both of these preparations and controversy remains as to the exact subunit composition of complex I. With an enzyme this complicated, reconstitution of the native enzyme from individual subunits is difficult to achieve and the role of individual subunits is difficult to ascertain by gene inactivation. For other complex multisubunit mitochondrial enzymes it has been accepted that co-purification of subunits with the complex under different conditions of purification is evidence that they are part of the assembly (Walker, 1992).

Fragmentation of bovine complex I has been achieved by treating it with perchlorate. This produces soluble material and a precipitate. From the soluble fraction flavoprotein (FP) and iron-protein (IP) complexes have been isolated. The precipitate is known as hydrophobic protein (HP). The FP fraction consists of three subunits of 51, 24 and 10 kDa respectively, and contains one molecule of FMN per complex I and six atoms of bound iron. The NADH and NAD^+ binding kinetics are the same for FP as they are for the whole complex I, although electron transfer

characteristics have changed, with ferricyanide being able to act as an electron acceptor (Ragan *et al.*, 1982a).

Complex I has also been disrupted in the presence of N,N-dimethyldodecylamine N-oxide and β-mercaptoethanol; this yields two subcomplexes, Iα and Iβ, which represent largely extrinsic and intrinsic membrane domains respectively. Subcomplex Iα can transfer electrons from NADH to ferricyanide or ubiquinone-1 and appears to contain all the iron-sulphur clusters present in the intact enzyme (Finel *et al.*, 1992). Complex I from bovine heart mitochondria is sensitive to the inhibitors rotenone, capsaicin, piericidin A and DCCD.

Another well characterised complex I is found in *Neurospora crassa* mitochondrial membranes and this has also been purified. Complex I was solubilised from mitochondrial membranes using Triton X-100 and purified on DEAE-Sepharose and HPLC size exclusion columns. At least 21 different polypeptides have been identified (Ise *et al.*, 1985). *Neurospora crassa* complex I has been isolated in a monodisperse state and this has made possible molecular size and shape determination by solution techniques such as analytical ultracentrifugation and X-ray scattering. One structural model based on electron microscopy work has suggested that the N. crassa complex I is an L shaped membrane bound enzyme in which the extrinsic peripheral arm protrudes into the mitochondrial membrane (Walker, 1992). A minimum of 15 complex I genes from *Neurospora crassa* mitochondria are homologues of bovine complex I genes (Tuschen *et al.*, 1990). Complex I from *Neurospora crassa* is also sensitive to rotenone, capsaicin, piericidin A and DCCD.

The NDH-1 complex has been studied in a number of bacteria including *Paracoccus denitrificans*, *Thermus thermophilus*, *E. coli* and *S. typhimurium* (Yagi, 1986; Yagi *et al.*, 1988, Matsushita *et al.*, 1987, Archer *et al.*, 1993) The NDH-1 from *P. denitrificans* has been purified and was shown to be composed of at least 10

polypeptides (Yagi, 1986). The structural genes of the NDH-1 from *P. denitrificans* has been cloned and sequenced; 14 subunits genes have been identified and they form a cluster (Xu *et al.*, 1993). Homology has been shown between these subunits and the equivalent ones in mitochondrial complex I. Six additional open reading frames have been identified in the NDH-1 gene cluster and these code for as yet unidentified polypeptides. The role of these proteins, in the NADH-ubiquinone oxidoreductase complex is unknown because the intact complex has not been isolated (Xu *et al.*, 1993).

Like the mitochondrial enzyme, NDH-1 from *P. denitrificans* contains non-covalently bound FMN and posseses iron-sulphur clusters as prosthetic groups. It is also inhibited by rotenone, capsaicin, piericidin A and DCCD; all classical inhibitor of NADH-ubiquinone reductase in mitochondria. The NQO1 subunit has been identified as the NADH binding subunit (Yagi and Dinh, 1990), while the NQO9 gene product has been shown to possess two [4Fe-4S] clusters (Xu *et al.*, 1991) The subunit composition and the catalytic centres are summarised in Figure 1.4.

The NDH-1 from *E. coli* has also been purified and the structural genes have been cloned and sequenced (Hayashi *et al.*, 1989; Weidner *et al.*, 1993). It has been shown to be composed of 14 subunits and all of them are related to subunits found in mitochondrial complex I, including the ones associated with substrate binding, redox centres and the seven mitochondrial encoded subunits. The NUO 6 subunit has been identified as the NADH binding subunit. The subunit composition and the catalytic centres are summarised in Figure 1.4. It has been suggested that the bacterial NDH-1 is the minimal form of the proton translocating enzyme.

Sequence comparisons between subunits from bovine complex I and a hydrogenase from the bacterium *Alcaligenes eutrophus* have identified a common 900 amino acid sequence which may represent an important structural feature of complex I enzymes. Similarities have also been observed between this hydrogenase

sequence and bacterial NDH-1 sequences. The bacterial hydrogenase is composed of four subunits, α, β, γ and δ. The β and δ subunits are associated as a dimer which is involved in splitting hydrogen. The α and γ subunits form a second dimer which contains non-covalently bound FMN and acts as a NADH oxidoreductase. The genes for these four proteins are encoded at the *hoxS* locus in *A. eutrophus* and are arranged in the order *hoxF*(α), *hoxU*(γ), *hoxY*(δ) and *hoxH*(β) (Figure 1.5.).

It was observed that the α subunit of the hydrogenase is a fusion of sequences closely related to those of the 24 and 51 kDa subunits of complex I. It also appears that the γ subunit of the hydrogenase is closely related to the first 200 amino acids of the 75 kDa subunit of complex I (Figure 1.4.). Together these sequences provide the NADH and FMN binding sites, two [4Fe-4S] and at least one [2Fe-2S] clusters and so are likely to account for a major part of the electron pathway in complex I (Tran-Betcke *et al.*, 1990). Similarities also exist between the hydrogenase subunits and the 51 kDa subunit in *P. denitrificans* and the 75 and 51 kDa subunits in *N. crassa.* Photoaffinity labelling studies have shown that the NADH binding site is located on the 51 kDa subunit from the bovine complex I and on the homologous subunit from *P. denitrificans*, although the precise location of the binding site within the subunit has not been identified using this method (Deng *et al.*, 1990; Yagi and Dinh, 1990).

Figure 1.4. Corresponding subunits encoded by the proton-translocating NADH-ubiquinone oxidoreductase in *E. coli, P. denitrificans* and *B. taurus.*

The polypeptide subunits of *E. coli* and *P. denitrificans* are listed according to their gene order in the *nuo* or *nqo* clusters, which are highly conserved in both bacteria. ND 1 to ND 6 are encoded by the mitochondria in *B. taurus,* while the other subunits are nuclear-encoded.

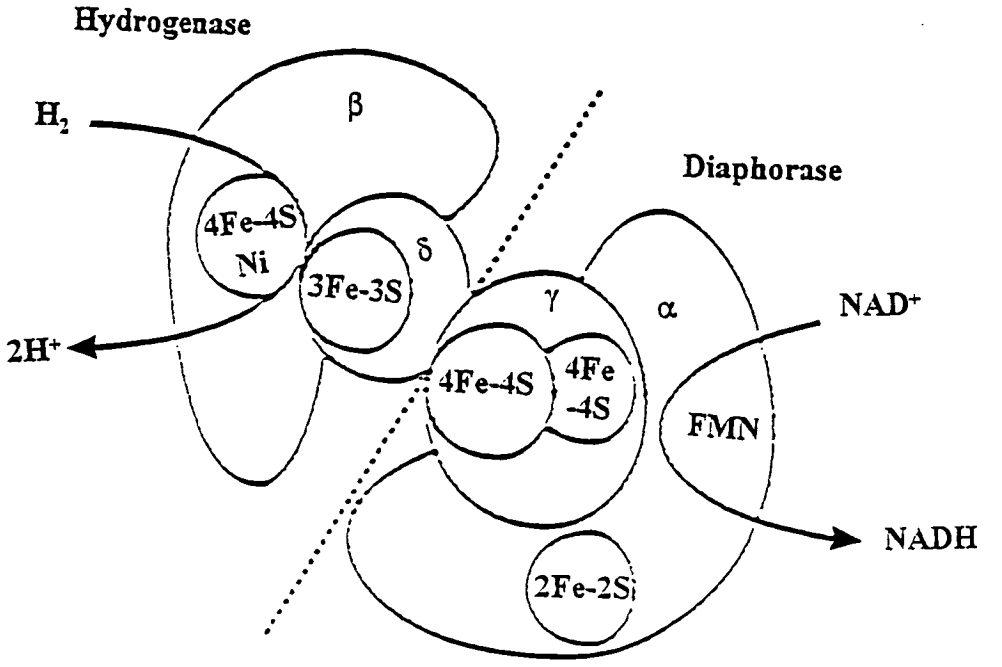
Escherichia coli	NUO 1	NUO 2	NUO 3	NUO 4	NUO 5	NUO 6	NUO 7	NUO 8	NUO 9	NUO 10	NUO 11	NUO 12	NUO 13	NUO 14
Paracoccus denitrificas	NQO 7	NQO 6	NQO 5	NQO 4	NQO 2	NQO 1	NQO 3	NQO 8	NQO 9	NQO 10	NQO 11	NQO 12	NQO 13	NQO 14
Bos taurus	ND 3	20 kDa	30 kDa	49 kDa	24 kDa	51 kDa	75 kDa	ND 1	23 kDa	ND6	ND 4L	ND 5	ND 4	ND 2
	HP	IP	IP	IP	FP	FP	IP	HP		HP	HP	HP	HP	HP
Catalytic centres		[FeS]			[FeS]	[FeS] NADH FMN	[FeS]	UB	[FeS]					

IP: Iron-sulphur protein

FP: Flavoprotein

HP: Hydrophobic protein

UB: Ubiquinone binding



(a)

α	γ	δ	β
hoxF	*hoxU*	*hoxY*	*hoxH*

(b)

24kDa	**51kDa**	**75kDa**

Figure 1.5. The NAD^+ reducing hydrogenase from *A. eutrophus*.

The enzyme has two domains made of a βδ and a αγ dimer containing its hydrogenase and diaphorase activities, respectively. The genes for the four subunits are arranged in the *hoxS* operon. Boxed regions denote (a) gene order and size for *A. eutrophus* b) corresponding polypeptide and subunit domains of the mitochondrial enzyme complex (Modified from Trans-Betcke *et al.*, 1990).

Regions exhibiting sequences known as Rossman folds are believed to form βαβ units which possess ADP-binding properties (Wierenga *et al.*, 1986) (Figure 1.6.). Protein sequences are compared with a core sequence of four invariant residues consisting of three glycines and an acidic amino acid which are conserved in NADH-binding sites of known structure. Seven additional positions have been examined within known NADH-binding sites and these are also conserved. The 51 kDa subunit of complex I from bovine and *N. crassa* mitochondria, the 51 kDa subunit from *P. denitrificans* and the α subunit from *A. eutrophus* hydrogenase all have a sequence containing three glycines which correspond to the essential glycines in the core.

Sequences have been scored by the number of amino acids that conform to the NADH-binding fingerprint. One point is assigned per residue that conforms to the fingerprint with a maximum score available of eleven (Wierenga *et al.*, 1986). The bovine sequence has a score of nine, the other three sequences score eight. No other regions on the NADH binding subunit managed to score as highly and so it has been concluded that these residues are involved in forming the ADP-binding pocket in the NADH binding site (Walker, 1992). In the two sites of deviation from the finger print in the bovine subunit (residues 76 and 79) small hydrophobic amino acids were expected, but glycine and tryptophan were found. The same substitutions are found in the NADH-binding site of dihydrolipoamide dehydrogenase in several species (Scrutton *et al.*, 1990).

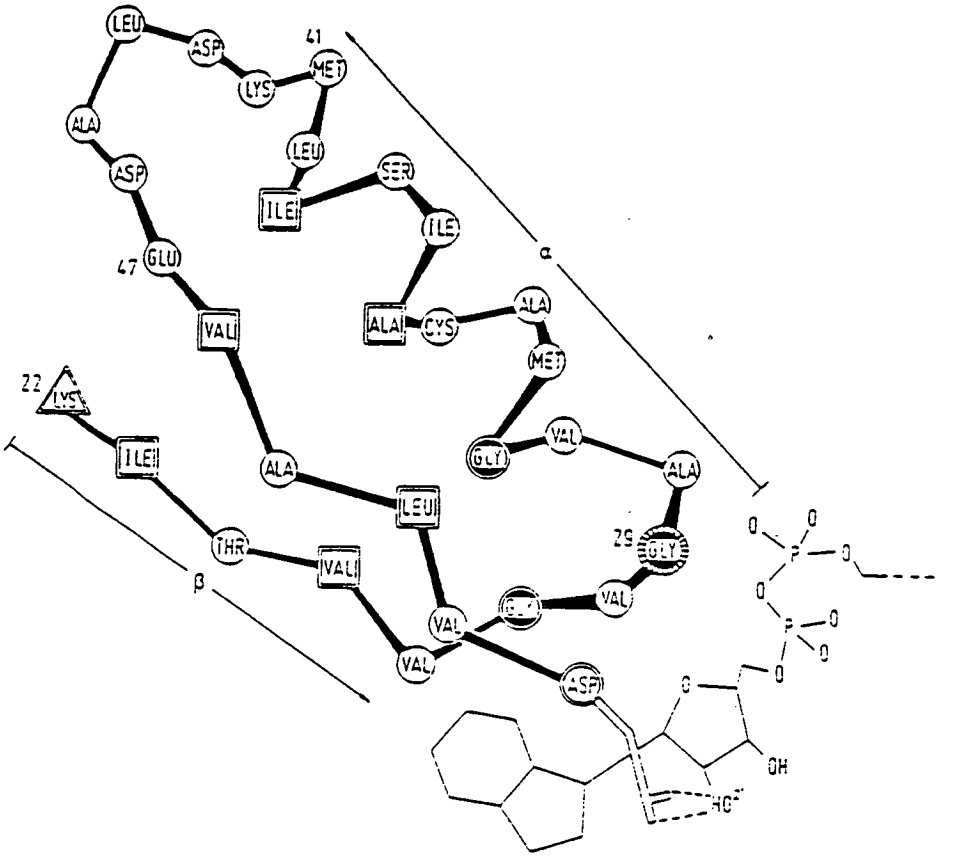


Figure 1.6. Schematic drawing of the ADP-binding βαβ-fold of spiny dogfish M-lactate dehydrogenase.

The residues at the finger print positions are indicated by the following symbols, Δ, basic or hydrophillic; □ small and hydrophobic. At these 11 positions the residue names are framed by two lines. Gly 29 allows for a close approach of the pyrophosphate to the N-terminus of the α-helix. Residues 43, 44, 45 and 46 form the loop between the α-helix and the second β-strand. The total length of this ADP-binding βαβ-fold is 31 residues (From Wierenga *et al.*, 1986).

FMN is generally considered to be the initial acceptor of electrons from NADH. Its standard reduction potential is intermediate between those of NADH and iron-sulphur clusters. FMN can accept electrons two at a time and release them individually to iron-sulphur clusters. Three different types of FMN-binding site have been described in flavoenzymes of known structure (Mathews, 1991). The first class is the $(\beta\alpha)_8$ flavoenzyme family, found in flavocytochrome b_2 (Xia and Mathews, 1990), glycolate oxidase (Lindqvist, 1989) and trimethylamine dehydrogenase (Lim *et al.*, 1986). In these examples the FMN lies at the end of an eight stranded α–β barrel; no similar structures were observed in complex I.

The flavodoxins contain a second class of FMN-binding site. They are small bacterial proteins which transfer electrons between redox proteins using FMN as their only prosthetic group. FMN is bound in a β–α–β fold similar to the ADP fold described previously, but without the characteristic pattern of amino acids (Smith *et al.*, 1983). No significant sequence homology has been observed between flavodoxins and complex I.

The third class is exemplified by phthalate dioxygenase reductase, which catalyses electron transfer from NADH to phthalate dioxygenase via FMN and a [2Fe-2S] cluster. Although the sequence of this protein is unknown there are some obvious similarities between it and complex I in terms of its electron transfer properties. The three-dimensional structure of the phthalate dioxygenase reductase has been resolved (Correll and Ludwig, 1991) and it appears that each of the redox centres is located in a separate domain. The NADH-binding domain is folded into a central five-stranded parallel β-sheet flanked on each side by an α-helix, and is similar to nucleotide-binding domains in dehydrogenases. FMN is bound to the edge of an antiparallel β barrel which is related to a structure in the FAD-binding flavoenzyme, ferredoxin reductase (Karplus *et al.*, 1990). From sequence comparisons with related proteins it has been proposed that the 51 kDa subunit from

bovine mitochondria complex I binds FMN and the region involved is the highly conserved glycine rich sequence from amino acids 180-234 (Walker, 1992). It also appears that the carboxyl-terminus of this protein binds a [4Fe-4S] cluster, so it would appear that the 51 kDa subunit contains at least three domains binding NADH, FMN, and an Fe-S cluster, arranged in that linear order from the N- to the C-terminus.

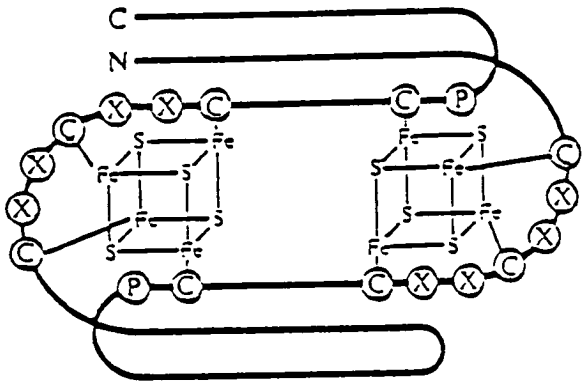
The bovine complex contains 22-24 atoms of iron per FMN, but the exact number and types of iron-sulphur clusters present is not firmly established (Ragan *et al.*, 1982b). From EPR studies it is generally accepted however that for mitochondrial complex I at least two [2Fe-2S] and three [4Fe-4S] iron-sulphur clusters exist. For *N. crassa* three [2Fe-2S] and one [4Fe-4S] cluster have been identified. In *P. denitrificans* the NDH-1 has the same iron-sulphur composition as the bovine enzyme while *T. thermophilus* has one [2Fe-2S] and two [4Fe-4S] clusters with the NDH-1 enzyme from *E. coli* having one of each (Yagi, 1991).

By looking at complex I and NDH-1 sequences it is now possible to identify potential amino acid ligands for Fe-S clusters; these are usually cysteine residues. The cysteine ligands in two [4Fe-4S] ferredoxins are usually arranged in the sequence motif CysXXCysXXCysXXXCysPro. The first three cysteines coordinate the same cluster and the fourth cysteine provides the fourth ligand for the other cluster (Figure 1.7.a). If the second cysteine has been replaced by another amino acid, then a [3Fe-4S] cluster may be found (Figure 1.7.b). If the ferredoxin has only a single [4Fe-4S] cluster the first three ligands are found in the same CysXXCysXXCys motif and the fourth ligand is often in a CysPro sequence elsewhere in the protein (Figure 1.7.c).

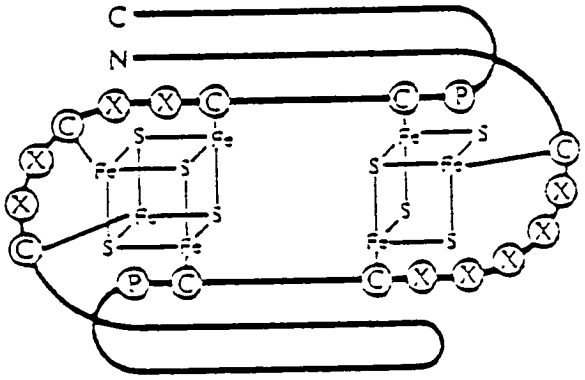
Figure 1.7. Folding of the polypeptide chain and location of cysteine ligands for [4Fe-4S] clusters in ferredoxins.

(a) A 2[4Fe-4S] ferredoxin; (b) omission of one of the cysteines leads to the formation of a [3Fe-4S] ferredoxin; (c) a [4Fe-4S] ferredoxin. Encircled C and P are cysteines and prolines, X is any other amino acid (From Walker, 1992).

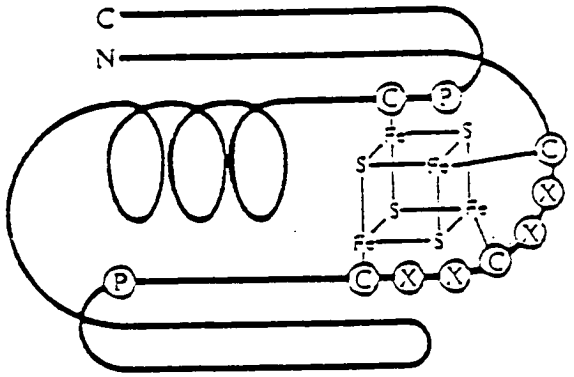
(*a*)



(*b*)



(*c*)



Subunit	Sequence
75 kDa (153-159)	**C** I Q **C** T R **C**
51 kDa (359-365)	**C** G Q **C** T P **C**
TYKY (23 kDa; 77-88)	**C** I A **C** K L **C** E A V **C** P
TYKY (23 kDa; 116-127)	**C** I Y **C** G F **C** Q E A **C** P

Figure 1.8. Sequence motifs for [4Fe-4S] clusters in subunits of bovine complex I. Cysteine residues are in bold (Sequences from Walker, 1992)

Sequence analysis of the bovine mitochondria complex I has identified four [4Fe-4S] clusters; one in both the the 51 kDa and 75 kDa subunits and two in subunit TYKY (See Figure 1.8.) (Walker, 1992). This is one more than was identified using EPR spectroscopy. The [4Fe-4S] sequence motif CysXXCysXXCys at residues 359-365 in the 51 kDa subunit is conserved in homologous proteins in other species and in the α subunit of *A. eutrophus* hydrogenase. The identity of the fourth cysteine is less certain. There is a CysPro sequence at residues 218-219, but the cysteine is not conserved in the HoxF protein. Three other cysteine residues in the 51 kDa subunit are conserved (residues 186, 312 and 405) and it is possible that one of these may provide the fourth ligand. The subunits of bovine complex I that may contain [Fe-S] clusters is summarised in Table 1.2.

The characteristic [4Fe-4S] sequence motif in the 75 kDa subunit is conserved in other species of complex I and in HoxU. Again there are several candidates for the fourth cysteine ligand; CysPro (203-204) is the most likely candidate, however seven other cysteines are also conserved and so any one may be the fourth ligand. The TYKY subunit is a 23 kDa polypeptide observed in the IP fraction (Matsui *et al.*, 1991). Its sequences contain no extensive hydrophobic regions and it appears to be a globular protein. It resembles a two [4Fe-4S] ferredoxin, and is likely to have the usual arrangement of cysteine ligands (Figure 1.8.) in which one cluster is liganded by cysteines 77, 80, 83, and 126 and the second one by cysteines 116, 119, 122 and 87.

Table 1.3 Subunits of bovine complex I that may contain Fe-S clusters (Modified from Walker, 1992)

Subunit	Number of conserved cysteines	Proposed cluster types from sequence	Proposed cluster types from EPR	Conclusion
75 kDa (IP)	11	1 x [4Fe-4S] 1 x [2Fe-2S]	1 x [2Fe-2S]	N-2 or N-4
51 kDa (FP)	4	1 x [4Fe-4S]	1 x [4Fe-4S]	N-3
24 kDa (FP)	3	1 x [2Fe-2S]	1 x [2Fe-2S]	N-Ib
TYKY (23kDa)	8	2 x [4Fe-4S]	-	N-2 or N-4
PSST (20 kDa)	3	1 x [4Fe-4S]	-	N-2 or N-4
PGIV (19 kDa)	8	Unknown	-	

The quinone binding site has been proposed to be located on the mitochondrially encoded 33 kDa ND_1 hydrophobic membrane subunit (Friedrich *et al.*, 1990). This is consistent with it interacting with ubiquinone which is lipophilic in nature. Rotenone has been used as an inhibitor of electron flow from Fe-S clusters to ubiquinone. It binds close to ubiquinone, but the inhibition is non-competitive with ubiquinone and is considered to arise from steric hindrance or from a conformational change in the protein which prevents the transfer of electrons to ubiquinone (Walker, 1992). In photoaffinity labelling of complex I, labelling was confined to the 33 kDa subunit. In the *N. crassa* complex I the transfer of electrons to ubiquinone is not affected by rotenone and it has been suggested that more than one ubiquinone binding site may exist for complex I (Friedrich *et al.*, 1989).

The carboxyl modifying reagent DCCD is an inhibitor of proton translocation and has been shown to inhibit the NADH-ubiquinone oxidoreductase activity of complex I (Yagi, 1987). DCCD has been used to label subunits which may be involved in proton translocation, however data from experiments so far is inconsistent. Yagi and Hatefi (1988) found that a 29 kDa and 49 kDa subunit of complex I were labelled, but only modification of the former (identified as hydrophobic subunit ND1) affected electron transfer. In other experiments using sub-mitochondrial particles, subunits of 14 and 21 kDa were modified, while up to six subunits are modified when purified complex I is treated (Vuokila and Hassinen, 1988, 1989). The 21 kDa subunit has been identified as subunit ASHI. Its sequence indicates that it has one transmembrane α-helix and the proposed helical region contains a single glutamic acid residue in a region which is related to a sequence that contains the site of modification by DCCD of cytochrome oxidase in subunit III (Walker, 1992).

The structural model of the *N. crassa* complex I based on electron microscopy work has suggested an L shaped membrane bound enzyme in which the extrinsic peripheral arm protrudes into the mitochondrial matrix and can also be applied to the bovine enzyme which can now be visualised as a complex made up of Iα and Iβ, with all the redox centres being located outside the lipid bilayer (Figure 1.9.) (Walker, 1992)

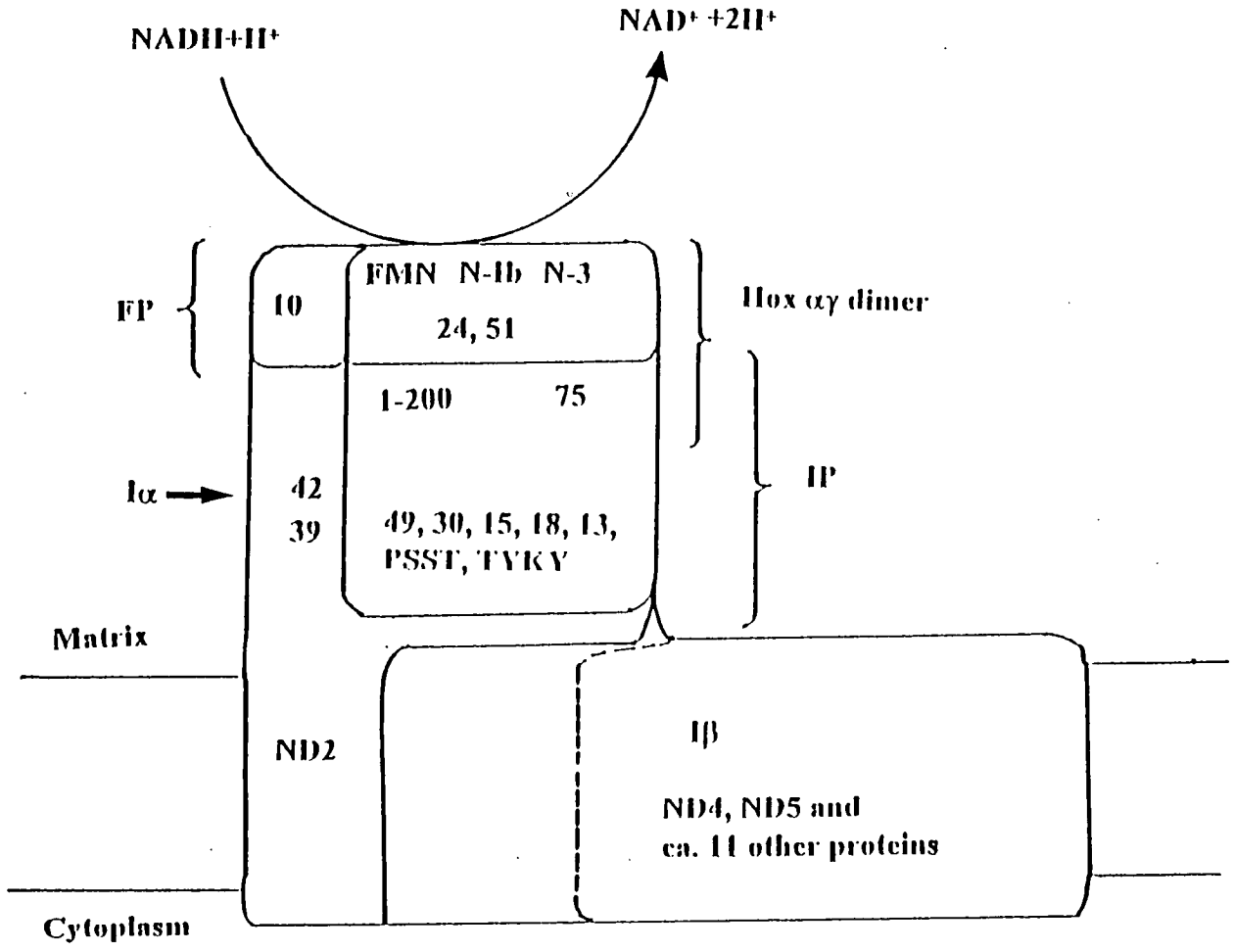
Bovine subcomplex Iβ is an assembly of 13 different polypeptides and includes the hydrophobic mitochondrial gene products ND_4 and ND_5, and 11 nuclear coded proteins. The hydrophobic profiles of ND4 and ND5 suggest that they have approximately 12 and 13-14 hydrophobic transmembrane spans respectively. All seven of the nuclear coded components appear to have one such span in their sequences. These predictions agree with the idea of Iβ being a mainly hydrophobic membrane complex. The location of four of the hydrophobic proteins (ND_1, ND_3, ND_4L and ND_6) and subunit KYYI are as yet unknown (Walker, 1992).

Many different estimates have been made of the value of the coupling ratio; that is the number of protons pumped by complex I per electron pair transferred from NADH to ubiquinone. The value may be different for various complex I enzymes but the consensus value of $4H^+/2e$ is widely quoted. It can not be explained by a simple loop mechanism (Mitchell, 1966) (Figure 1.10.a.) in which protons are extruded as a consequence of transfer of electrons from a proton and electron carrier (FMN) to an electron carrier (iron-sulphur cluster) since this predicts a $2H^+/2e$ stoichiometry.

The electron pathway and the mechanism of coupling electron transfer to proton transport have still to be elucidated. Intramolecular electron transfer has not been resolved by EPR and no suitable inhibitors that block the pathway at specific intermediate sites are available. It is not yet known if electron transfer in complex I is accompanied by any conformational changes in the enzyme.

Figure 1.9. Structural model of complex I from *N. crassa*.

Arrangement of some of some of the subunits from complex I, based upon the polypeptide compositions of subcomplexes Iα and Iβ, of the FP and IP fragments, and the relationship between the αγ dimer of the hydrogenase from *A. eutrophus* and the 24, 51 and residues 1-200 of the 75 kDa subunit of bovine complex I. All of the known redox centres are in subcomplex Iα (From Walker, 1992).


NADH+H+
NAD+ +2H+
FP
10
FMN N-1b N-3
24, 51
1-200
75
Hox αγ dimer
IP
Iα
42
39
49, 30, 15, 18, 13,
PSST, TYKY
Matrix
ND2
Iβ
ND4, ND5 and
ca. 11 other proteins
Cytoplasm

The simplest proposals for the arrangement of the Fe-S clusters are based primarily on their mid-point potentials. In one such scheme, FMN, acting as a transformer, receives two electrons from NADH and the resulting $FMNH_2$ transfers them in two single steps to the [2Fe-2S] cluster N-Ib. It is thought that clusters N-Ib, together with clusters N-3 and N-4, form an isopotential pool for electrons. This is supported by observations that activation of electron transfer requires several turnover cycles to reduce the Fe-S clusters (Burbaev *et al.*, 1989).

Cluster N-4 is placed on a side pathway in this pool where it has a buffering function; evidence for its location is that its EPR signal can be removed with treatment with N-bromosuccinimide without any inhibition of electron transfer (Krishnamoorthy and Hinkle, 1988). Finally it has been proposed that this pool donates electrons to the high potential cluster N-2 which then reduces ubiquinone. Electron transfer is likely to take place by single electron transfers via a membrane bound ubisemiquinone (Burbaev *et al.*, 1989). An internal ubiquinone has been suggested to act as an electronic connector between the isopotential clusters and cluster N-2 (Weiss *et al.*, 1991).

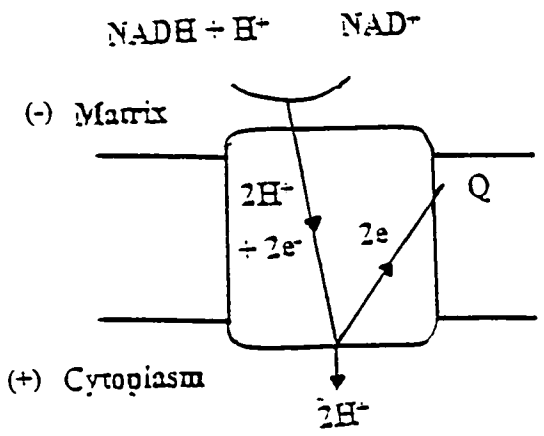
The redox potential differences in the electron pathway suggest that energy transduction could take place first of all between FMN and the isopotential clusters (N-I, N-3 and N-4), with FMN participating in a proton translocating flavin cycle (Krishnamoorthy and Hinkle, 1988). Different forms of flavin cycles have been described in explanation of the various $H^+/2e$ stoichiometries (Figure 1.10.b.) (Ragan, 1990). The cycles require a flavin reduction site at the negative side of the inner membrane and a flavin oxidation site at the positive cytoplasmic side of the membrane, this implies that the reoxidation of $FMNH_2$ is linked to the translocation of two protons to the cytoplasmic side by a proton channel (Krishnamoorthy and Hinkle, 1988; Weiss *et al.*, 1991).

In another scheme NADH transfers electrons one at a time to the low potential Fe-S clusters, and they reduce $FMNH_2$ to FMN perhaps involving an internal Q-cycle (Weiss *et al.*, 1991). A second possible site of enegy transduction is between the isopotential clusters and cluster N-2 perhaps involving an internal Q-cycle, analogous to the Q-cycle operating in the cytochrome bc_1 complex (Trumpower, 1990). A third possible energy transducing site has been proposed to exist between centre N-2 and the quinone pool, this mechanism involves protonated ubisemiquinones (Figure 1.10.c.) (Kotlyar *et al.*, 1990).

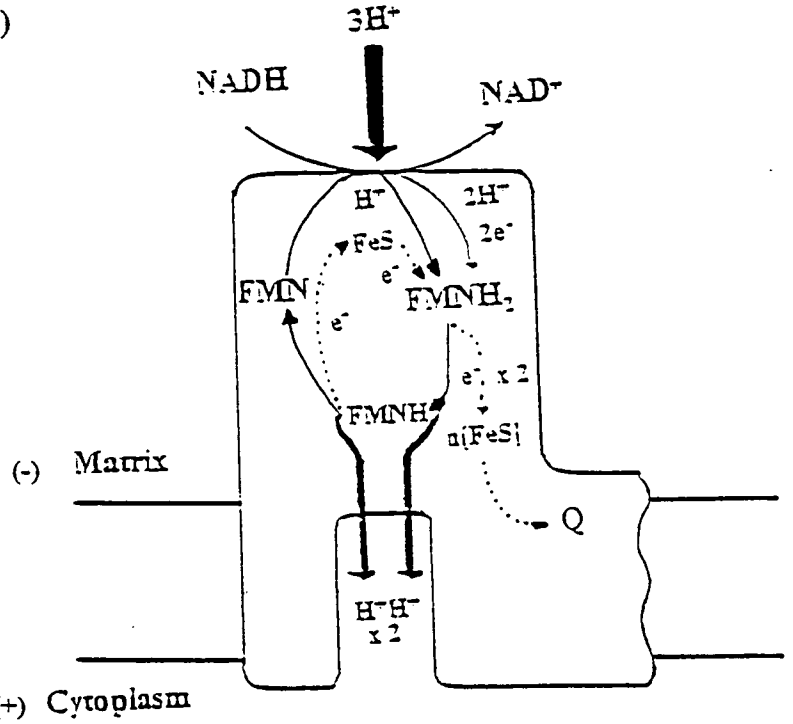
Figure 1.10. Models for energy transduction coupled to proton translocation in complex I.

(a) Loop mechanism (Mitchell, 1966); (b) A possible mechanism of the redox-linked proton translocation between NADH and FMN drawn as an analogue of the b cycle in cytochrome bc_1 to give a $3H^+/2e$ ratio in this part of complex I. The FMN is shown as communicating with the cytoplasm via a proton channel (Krishnamoorthy and Hinkle, 1988; Ragan, 1990); (c) Proton translocation between Fe-S cluster N-2, and the ubiquinone pool. Two electrons are transferred one at a time from N-2, and two protons are taken up from the matrix to generate two bound protonated ubisemiquinone molecules, from two molecules of bound ubiquinone (Kotlyar *et al.*, 1990).

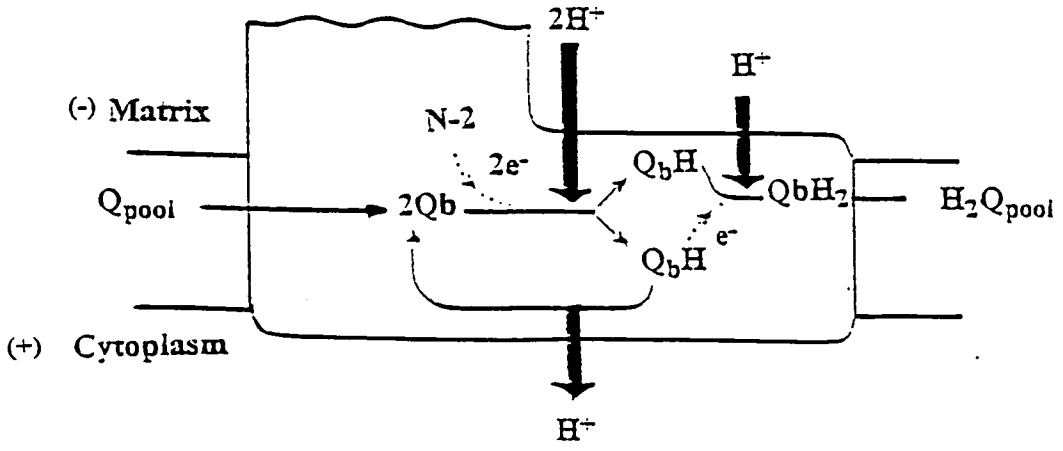
(a)


NADH + H+
NAD+
(-) Matrix
2H+
+ 2e-
2e
Q
(+) Cytoplasm
2H+

(b)


3H+
NADH
NAD+
H+
2H+
2e-
FeS
e-
FMN
FMNH2
e-
e-, x 2
FMNH2
n[FeS]
(-) Matrix
Q
H+H+
x 2
(+) Cytoplasm

(c)


2H+
H+
(-) Matrix
N-2
2e-
QbH
QbH2
H2Qpool
Qpool
2Qb
QbH
e-
(+) Cytoplasm
H+

Common features of NDH-2 family of enzymes include the fact that this type of enzyme is composed of a single polypeptide chain which contains non-covalently bound FAD, but no iron-sulphur clusters. They do not pump protons and are unaffected by inhibitors of complex I such as rotenone and piericidin. They are widely distributed among bacterial species and appear to be unrelated to NDH-1 (Yagi, 1991).

The gene for NDH-2 in *E. coli* has been cloned by complementation of a wild-type library with a NDH-2 mutant strain. The mutant was isolated as a strain unable to grow on mannitol as a sole carbon source. Under these conditions the only source of reducing energy available is NADH which is generated when mannitol-1-phosphate is converted to fructose-6-phosphate; a functional NADH-ubiquinone is therefore necessary for aerobic growth (Young *et al.*, 1981).

The gene for the NDH-2 from bacillus YN-1 (Xu *et al.*, 1991) has also been cloned, and sequence comparisons with the NDH-2 gene from *E. coli* identified three regions of homology. Two of the regions exhibit Rossman fold sequences which are believed to form a βαβ unit with ADP-binding properties (Wierenga *et al.*, 1986) (See Figure 1.5.). It has been suggested that these two regions may be the binding sites for FAD and NADH respectively, and that the third region of homology may be a conserved quinone binding domain. The only organisms for which both NDH-1 and NDH-2 have been sequenced are *E. coli* and *T. thermophilus*.

1.3. Sodium based energetics.

Although protons are considered to be the central coupling ion in bacterial energy metabolism it also appears that the cycling of sodium ions plays a crucial role in energy conservation and transduction in bacterial membranes. In fact sodium ions are essential for the growth of many marine, halophilic and certain alkalophillic species (Dimroth, 1987).

Sodium ions have a number of different functions in bacterial systems. These include solute cotransport, energy conservation and energy transduction, activation of enzymes, pH homeostasis and osmoregulation. It is clear that for these bacteria the movement of sodium ions across the bacterial membrane is an essential physiological function, and that import and export systems must exist which will balance the fluxes of sodium ions.

Two types of primary sodium pumps have been identified in a number of species, these are the sodium ion transport decarboxylases (Dimroth, 1980; Hilpert and Dimroth, 1983) and sodium-dependent NADH-ubiquinone oxidoreductases (Tokuda and Unemoto, 1981, 1983; Tsuchiya and Shinoda, 1985; Ken-Dror *et al.*, 1986). It appears that sodium/proton antiporters are widely distributed (Krulwich, 1983) and these are described as secondary sodium pumps, which exchange proton gradients for sodium gradients. Sodium motive forces generated by primary and secondary pumps are utilised by bacteria in the same way as proton motive forces; to transport solutes, synthesise ATP and drive flagellar motors. Systems performing energy coupling by sodium circulating are summarised in Figure 1.11.

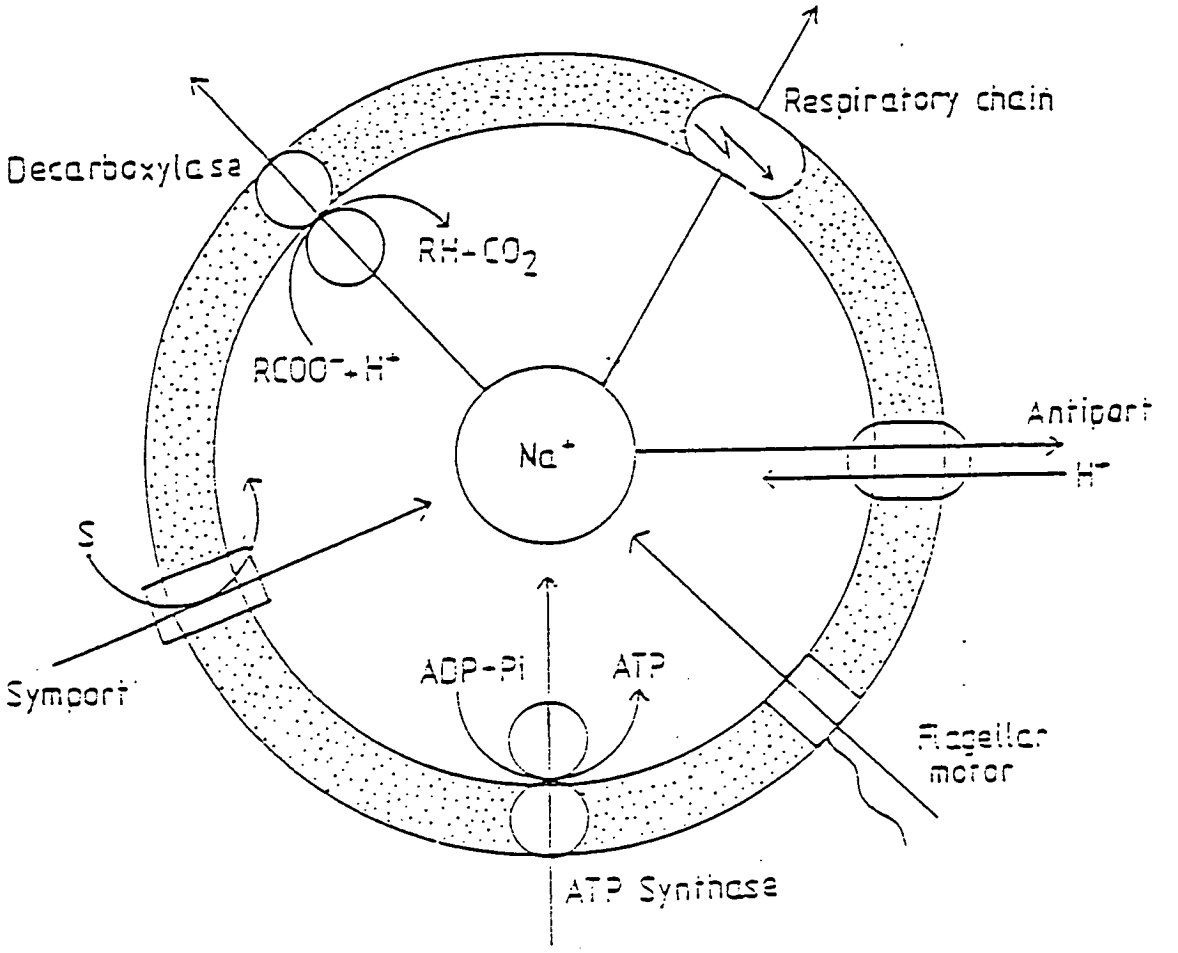


Figure 1.11. Summary of systems performing energy coupling by sodium circulation in bacteria.

Respiratory sodium pumps occur in marine organisms, sodium driven flagellar motors have been found in marine and alkalophillic species, sodium transport decarboxylases and ATP synthases exist in anaerobic bacteria and sodium/proton antiport and sodium symport systems are widely distributed (Dimroth 1987).

1.4. Sodium-dependent NADH-ubiquinone oxidoreductases.

The marine bacterium *Vibrio alginolyticus* has been shown to produce an electrochemical sodium gradient as a result of respiration at alkaline pH (Tokuda and Unemoto, 1981, 1982). The enzyme responsible, which couples sodium transport to electron transfer, has been identified as a NADH-ubiquinone oxidoreductase has been designated NQR1 (Tokuda and Unemoto, 1984). NQR1 requires sodium for maximal activity, but the K_m is very high at around 50mM (Figure 1.12). However other cations such as K^+, Li^+, Mg^{2+} and Rb^+ are unable to stimulate the enzyme as effectively. This enzyme can be conveniently assayed measuring menadione-dependent NADH or dNADH oxidation. The NADH analogue, dNADH is specific for the NDH-1 complex. The approximate K_m for dNADH is 23 μM compared to around 9 μM for NADH (Bourne and Rich, 1992).

The sodium-dependent NADH-ubiquinone reductase allows bacterial cells to grow at alkaline pH in the presence of proton uncouplers such as CCCP by generating sodium gradients, while mutants which lack this enzyme cannot (Tokuda, H., 1983). Studies of such sodium pump-deficient mutants (Nap 1 and Nap 2) have revealed that two distinct types of NADH-ubiquinone reductase exist in *V. alginolyticus*. The first type is the NQR1; the second type is the NQR2 enzyme which is a NADH-ubiquinone oxidoreductase that does not couple ion transport to electron transfer and cannot utilise dNADH. It is responsible for the residual NADH oxidase activity observed in the sodium pump mutants (Tokuda, 1986) (Figure 1.13.).

The subunit composition of NQR1 from *V. alginolyticus* has been determined and was found to be composed of equimolar amounts of three subunits, α, β and γ with apparent Mr of 52, 46 and 32 kDa respectively (Hayashi and Unemoto, 1987; Bourne and Rich, 1992). Purification of the sodium dependent NADH-ubiquinone

oxidoreductase has been hampered by the the fact that the enzyme complex disassociates during purification. It is thought that the NQR1 membrane complex has an approximate Mr of 254 kDa and may exist as a dimer of $\alpha\beta\gamma$ (Hayashi and Unemoto, 1987).

Isolated α subunit contains FMN but has no NADH dehydrogenase activity. The β subunit contains one non-covalently bound FAD and possesses NADH dehydrogenase activity. The γ subunit has no catalytic activity on its own although it is thought to increase the affinity of the β subunit for quinones. No iron-sulphur clusters have been detected in any of the NQR1 subunits. All three subunits are required for NADH-ubiquinone oxidoreductase activity and an electron transfer pathway from NADH to ubiquinone has been formulated (Hayashi and Unemoto, 1987). The FAD containing β subunit accepts electrons from NADH and is thought to reduce ubiquinone by a one electron transfer reaction to produce ubisemiquinone. In the absence of the α subunit, the ubisemiquinone radical is auto-oxidised by molecular oxygen, resulting in a oxidation-reduction cycle (Figure 1.14.). This corresponds to the NADH dehydrogenase activity found in the β subunit. In the presence of the α and γ subunits ubisemiquinone is rapidly converted to ubiquinol (Hayashi and Unemoto, 1984).

The inhibitor specificity of *V. alginolyticus* NQR1 suggests that major differences exist between it and the proton-translocating NADH-ubiquinone oxidoreductase complexes (Bourne and Rich, 1992) (Table 1.3.). Menadione-mediated NADH dehydrogenase activity of NQR1 is strongly inhibited by Ag^{+}. The inhibition by Ag^{+} is irreversible but can be prevented by prior incubation of the enzyme with a reducing agent such as β-mercaptoethanol. Cu^{2+} and Cd^{+} are irreversible inhibitors of this enzyme. Inhibition by these two metals appears to be dependent on the turnover of the enzyme. Enzyme preoxidised by menadione is not inhibited by Cu^{2+} or Cd^{2+}, provided that 0.5 mM EDTA is added to the reaction

mixture before turnover is started by the addition of NADH. Zn^{2+} and Pb^{2+} cause reversible inhibition of the enzyme activity. Inhibition can be prevented by including 0.5 mM EDTA in the assay buffer. NQR1 is resistant to most conventional quinone-binding site inhibitors, only HQNO and NQNO cause strong inhibition of quinone reductase activity at submicromolar concentrations. The mechanism of coupling electron transfer to sodium transport is not yet fully understood although it is thought that sodium translocation is associated with transfer of electrons either from FAD to FMN or from FMN to quinone.

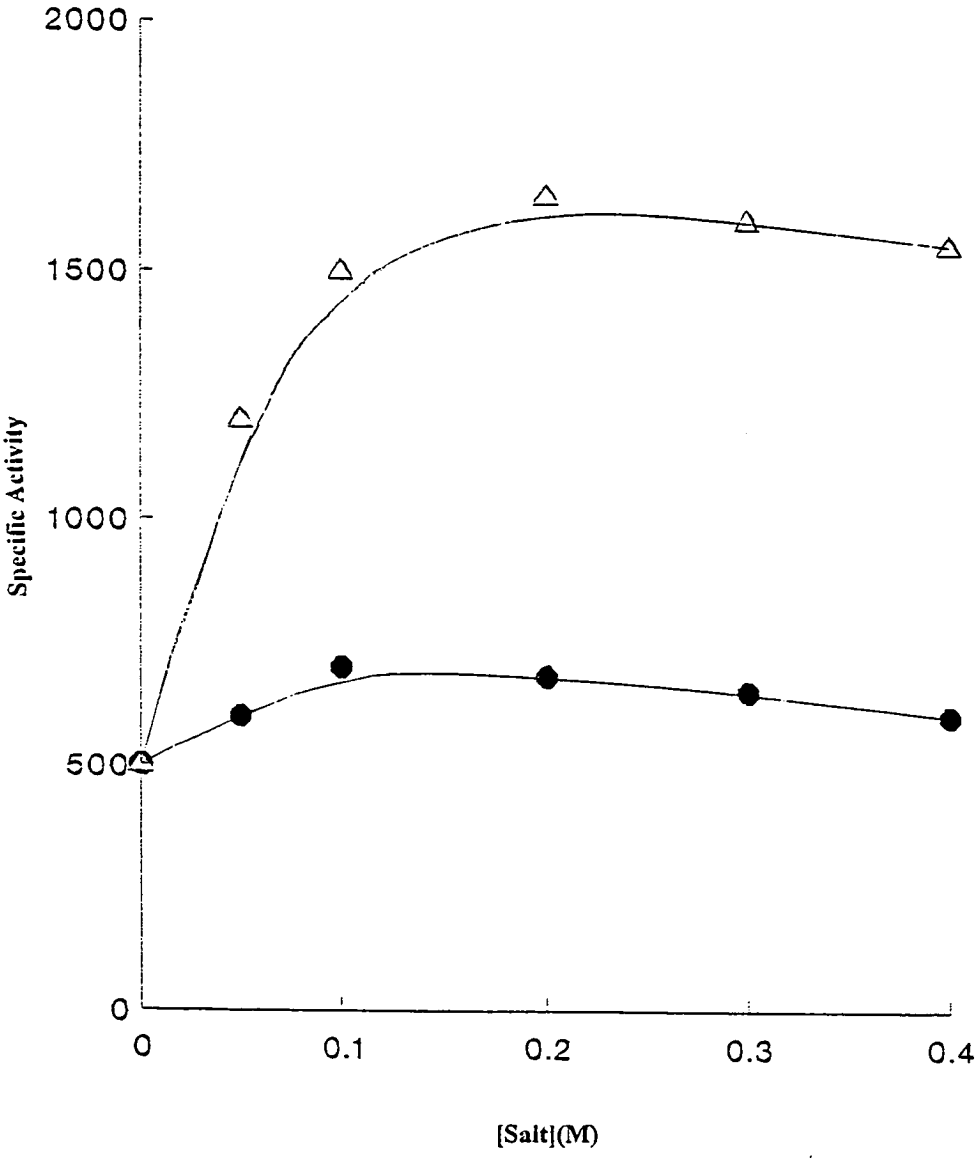


Figure 1.12. Salt dependent NADH oxidase activity from membranes of the marine bacterium *V. alginolyticus*.

The assay mixture contained in 1 ml contained 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and various concentrations of KCl (●), or NaCl (Δ). The assay at 30 °C was started by the addition of membrane suspensions containing 30 μg protein. Specific activities are expressed as nmol NADH oxidised/min/mg protein (Modified from Tokuda, 1986).

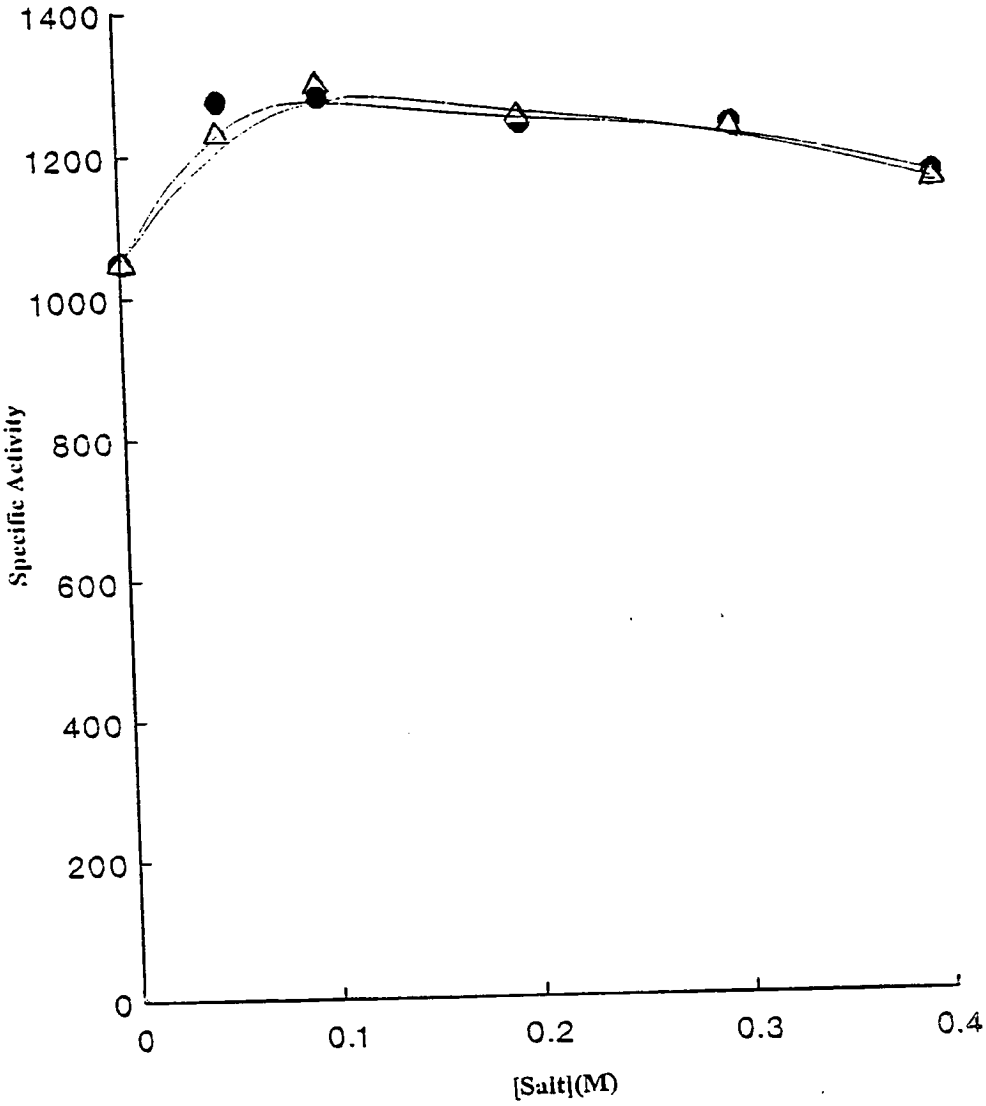


Figure 1.13. Salt dependent NADH oxidase activity from membranes of the sodium-pump defective mutants of *V. alginolyticus*, Nap 2.

The assay mixture contained in 1 ml contained 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and various concentrations of KCl (●), or NaCl (Δ). The assay at 30 °C was started by the addition of membrane suspensions containing 30 μg protein. Specific activities are expressed as nmol NADH oxidised/min/mg protein (Modified from Tokuda, 1986).

Figure 1.14. The relationship of the α and β subunit in the sodium-dependent NADH-ubiquinone oxidoreductase and the proposed electron transfer pathway from NADH to ubiquinone.

Q_1H_2 formation by the enzyme proceeds via the ubisemiquinone radical (Q_1^-) as an intermediate. Ag^+ and HQNO inhibit reactions before and after the intermediate, respectively. Sodium is required and is presumably translocated at the site after the intermediate. The γ subunit although not shown is required for NADH-ubiquinone oxidoreductase activity and is thought to increase the affinity of the β subunit for quinones (Modified from Tokuda and Kogure, 1989)

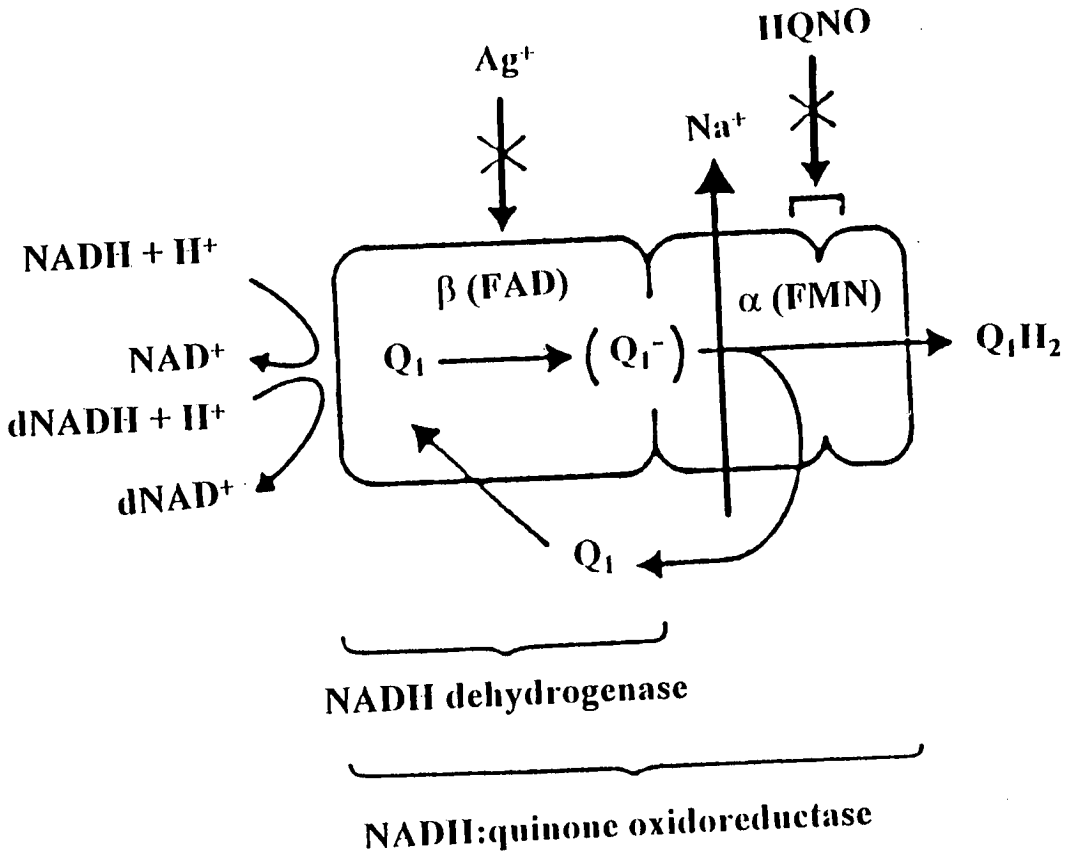

HQNO
Ag+
Na+
NADH + H+
β (FAD)
α (FMN)
NAD+
Q1
(Q1-)
Q1H2
dNADH + H+
dNAD+
Q1
NADH dehydrogenase
NADH:quinone oxidoreductase

Table 1.3. Comparison of NADH-ubiquinone oxidoreductases. (Modified from Bourne and Rich, 1992)

Type	**NDH-1**	**NDH-2**	**Na^+-NDH**	**Complex I**
Source	Bacterial	Bacterial, mitochondrial	Bacterial	Mitochondrial
Coupling ion	H^+	Not coupled	Na^+	H^+
Redox centres	FMN, FeS	FAD	FAD, FMN	FMN, FeS
No. of protein subunits	~14	1	3	25-41
Oxidation of dNADH	Yes	Variable	Yes	Yes
Inhibition by:				
Ag^+	No	No	Yes	No
Rotenone	Yes	No	No	Yes
HQNO/ NQNO	Yes	No	Yes	Yes
Capsaicin	Yes	No	No	Yes
Flavone	Yes	No	No	Yes
Piericidin A	Yes	No	No	Yes

1.5. Sodium ion transport decarboxylases.

During citrate fermentation in *Klebsiella pneumoniae* and *Salmonella typhimurium* oxaloacetate produced by citrate-lyase is decarboxylated to pyruvate. It was subsequently demonstrated that the oxaloacetate decarboxylase acted as a primary sodium pump in inverted vesicles. This enzyme requires sodium for maximal activity and is inhibited by avidin. This suggests that biotin is involved in catalysis (Dimroth, 1980).

A sodium-translocating methylmalonyl-CoA decarboxylase was discovered later in the strictly anaerobic bacterium *Veillonella alcalescens*; this enzyme was also sodium-dependent and avidin sensitive (Hilpert and Dimroth, 1983). A similar enzyme has since been discovered in the strict anaerobe *Propionigenium modestum* (Dimroth, 1982). In contrast to *V. alcalescens*, *K. pneumoniae* and *S. typhimurium*, energy metabolism in *P. modestum* involves neither electron transfer or substrate level phosphorylation and is entirely dependent on the sodium gradient generated by decarboxylation of methylmalonyl-CoA (Hilpert *et al.*, 1984). In *Acidaminococcus fermentans* and *Peptostreptococcus asaccharolyticus* an avidin-sensitive glutaconyl-CoA decarboxylase has been discovered (Buckel and Semmler, 1983; Wohlfarth and Buckel, 1985).

Sodium transport decarboxylases have been purified using avidin affinity chromatography columns. The oxaloacetate, methylmalonyl-CoA and glutaconyl-CoA decarboxylases all contain three different subunits α, β and γ which have molecular weights in the range 60-65 kDa, 33-35 kDa and 10-15 kDa respectively. A marked difference amongst the various decarboxylases is the attachment site of the prosthetic group biotin (Dimroth, 1987).

The overall reaction for decarboxylation has been proposed to be;

$R\text{-}COO^- + H^+ \rightarrow RH + CO_2$.

It has been shown that during this reaction the biotin prosthetic group of the enzyme becomes carboxylated. This reaction is sensitive to avidin but is not dependent on sodium. The sodium-dependent step is the decarboxylation of the carboxybiotin enzyme intermediate (Dimroth, 1982). It appears that the α subunit has carboxyltransferase activity and that the β subunit is responsible for the decarboxylation of the carboxybiotin complex.

Investigations using electron microscopy have revealed a cleft in the α-subunit, with the prosthetic biotin group located at its bottom in close proximity to the β and γ subunits. For the oxaloacetate decarboxylase the existence of two different domains in the α chain has been shown by limited proteolysis as well as from sequencing studies (Dimroth, 1990). Trypsin cleaves the α chain into an N-terminal 53 kDa and a C-terminal biotin domain of 12 kDa. The N-terminal domain is homologous to the 5S subunit of transcarboxylase from *Propionibacterium shermanii*, that catalyses the same reaction as the α chain of oxaloacetate decarboxylase. The sequence of the C-terminal domain was homologous to the 1.2S biotin subunit of transcarboxylase and other biotin containing peptides. A sequence that is unique to the α chain of the oxaloacetate decarboxylase is found upstream of what has been identified as the biotin binding lysine residue. The sequence contains a serine, 14 alanine and 7 proline residues. Such an extended area of alanine and proline residues may provide a point of flexibility within the protein structure to allow the flip-flop movement of the biotin group between the two catalytic centres on the α and β subunits, respectively. A similar sequence has been observed in the dihydrolipoamide acetyltransferase subunit of the pyruvate dehydrogenase from *E. coli*, and it has been shown by nuclear magnetic resonance spectroscopy that this region is highly mobile (Radford *et al.*, 1987).

Reversible dissociation of the enzyme complex has been carried out and it has been shown that the dissociation and reconstitution is dependent on pH. Acidic pH

promotes dissociation while a more alkaline pH promotes reconstitution. The sharp decrease in dissociation and increase in reconstitution above pH 6.5 implies that a single ionizable group is involved in these processes (Dimroth, 1990). The uncharged form of this residue appears to be required for the reconstitution of the decarboxylase from its subunits.

Reconstituted enzyme had full decarboxylase activity. Mixtures of the β and γ subunits allow sodium translocation in proteoliposomes only when α subunits are added and so the the β and γ subunits together do not constitute an open ion conducting channel analogous to the F_0 portion of F_1F_0 ATPases (Dimroth, 1990). A hypothetical model for the arrangement of sodium transport decarboxylases subunits suggests that the β and γ subunit are located in the membrane forming a sodium channel, and that the α subunit is associated with them on the cytoplasmic side and provides the biotin binding site (Figure 1.15.) (Dimroth, 1990).

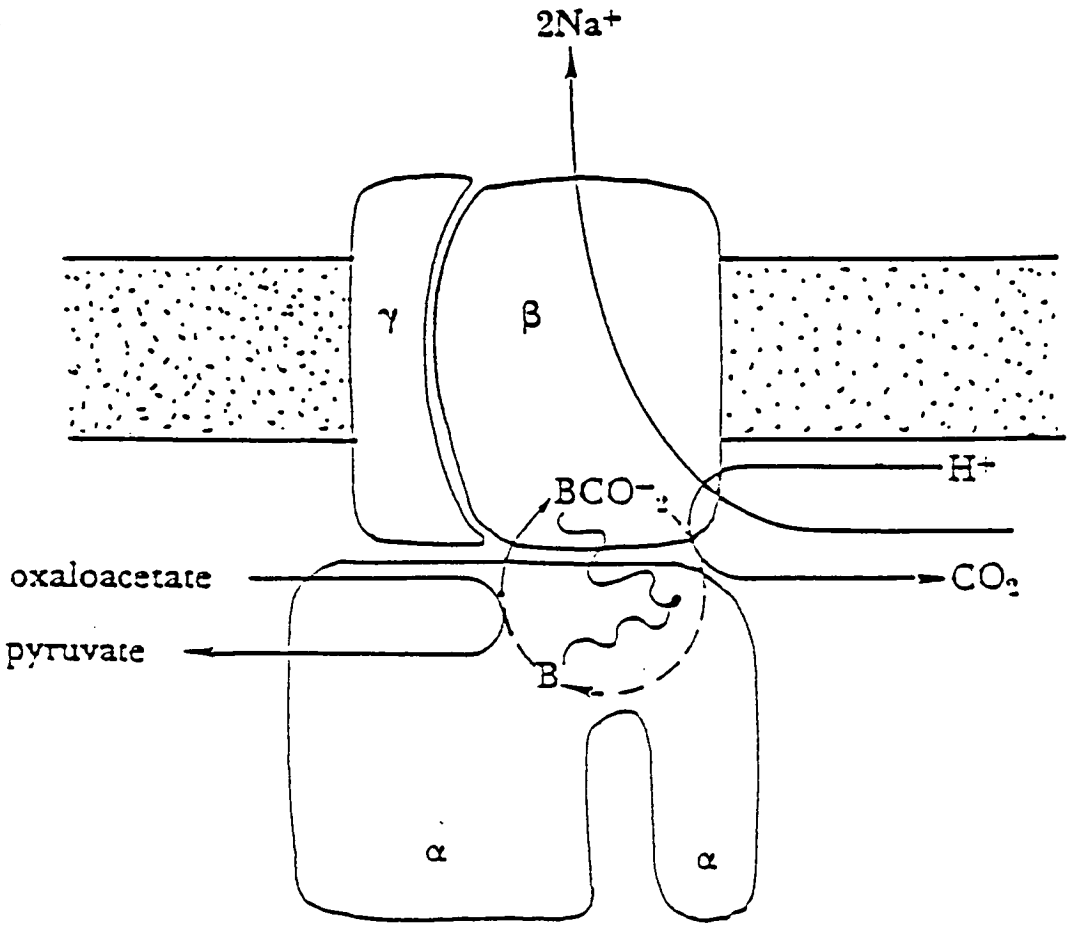


Figure 1.15. Hypothetical model linking structure and function of the oxaloacetate decarboxylase subunits (From Dimroth, 1990)

1.6. ATPases.

ATP synthases are the central enzymes in energy conservation in mitochondria, chloroplasts, and bacteria. They use ion motive forces generated across the membrane to drive the synthesis of ATP from ADP and inorganic phosphate. They are also involved in the regulation of intracellular ionic and pH balance, muscle relaxation, receptor recycling, hormone storage and food storage. As most cell types are impermeable to ATP, it must be recycled in each cell, so ATPases are of critical importance to cell function. It has been calculated that for a 70 kg adult, while at rest the equivalent of around 50% of body weight in ATP is turned over per day. This value may increase to almost 800 kg under working conditions (Erecinska and Wilson, 1978). At least three different classes of ATPase have been identified, and these are the P-type (phosphorylation), V-type (vacuolar) and F-type (F_1F_0) ATPases.

Examples of P-type ATPases include the Na^+/K^+, Ca^{2+} and H^+ transporting ATPases of eukaryotic cells, the Ca^{2+} transporting ATPases of the sarcoplasmic and endoplasmic reticulum and the K^+ transporting ATPases of *E. coli* and *Streptococcus faecalis*. The reactions of the P-type ATPases are characterised by the covalent phosphorylated enzyme intermediate formed when the γ-phosphate of ATP reacts with a single aspartic acid residue (Dane and Scarborough, 1981). This type of ATPase is inhibited by vanadate, a transition state analogue of phosphate. Structurally, these ATPases all consist of an α peptide of 70-100 kDa which contains the phosphorylation and ATP binding site. The Na^+/K^+ ATPase, unlike other known P-types, also has a β peptide of approximately 55 kDa the function of which is unknown.

Several of the P-type ATPases have been sequenced; significant sequence homology exists among them, and the regions involved in phosphorylation and ATP binding appear to be conserved. This seems to suggest that the P-type ATPases may

share common reaction mechanisms (Pedersen and Carafoli, 1987). The P-type ATPases exist in two different conformational states (E1 and E2) and hydropathy plots have suggested that a large portion of the ATPase molecules protrude from the membrane into the aqueous cytoplasm. Several groups believe that the ATPases aggregate into dimers in the membrane, although the enzyme does function normally in the monomeric state (Pedersen and Carafoli, 1987).

Despite similarities between different P-type ATPases, there are critically important differences which reflect their specific ion translocating properties. Na^+/K^+ ATPases exchange Na^+ and K^+ across a biological membrane while other P-type ATPases move either H^+, K^+ or Ca^{2+}. Clearly the amino acids comprising the ion binding or channels must vary depending on the ion being translocated.

The Ca^{2+} ATPases of heart muscle contain a rather unusual cluster of glutamic acid residues in a series of α-helical segments which form the stalk connecting the intramembrane sector to the hydrophilic extramembrane domains. This cluster of amino acids may represent specific Ca^{2+} binding domains (MacLennan *et al.*, 1985). P-type ATPases also demonstrate differences in their inhibitor sensitivities. Na^+/K^+ ATPases are inhibited by the cardiac glycoside ouabain, while plasma membrane H^+ ATPases are inhibited by DCCD and DES.

F_1F_0 type enzymes are the most thoroughly characterised of the ATPases. The structural genes from many bacterial and eukaryotic species have now been cloned and sequenced (Futai *et al.*, 1989). Additionally the enzyme has been purified and the F_1 and F_0 have been reconstituted (Futai and Kanazawa, 1983; Walker *et al.*, 1984). F_1F_0 ATPases from different sources have essentially the same structure; however in eukaryotes there are a number of extra polypeptides and some of the genes are coded for by mitochondrial DNA (Hashimoto *et al.*, 1983, 1984).

The catalytic portion of the enzyme, F_1, consists of five subunits, α, β, γ, δ, and ε, and F_1 can be detached from the membrane as a soluble ATPase by treatment

with EDTA. The F_1 domain is an approximate sphere and contains the catalytic binding sites for the substrates ADP and inorganic phosphate. F_1 is linked to the integral membrane component, F_0, by a slender stalk. F_0 functions as a proton pathway in ATP synthesis and hydrolysis (Abrahams *et al.*, 1994). Energy released by proton flux through F_0 is relayed to the catalytic site in the F_1 domain, probably by conformational changes through the stalk region (Figure 1.16.). Approximately three protons flow through the membrane per ATP synthesised (Abrahams *et al.*, 1994). When F_1 is removed from the complex F_0 becomes a passive proton pathway (Futai, *et al.*, 1989). In *E. coli* F_0 has three subunits a, b and c. In chloroplasts the F_0 has four subunits while in mitochondria there are ten (Abrahams *et al.*, 1994).

The β subunits have the most conserved primary structure with 42% of the amino acids being identical. The amino acid sequences from α subunits show 30% identity when aligned to obtain maximal homology. Three regions are conserved to an even higher degree, suggesting that they are important in catalysis or subunit interaction. The γ subunits have only 33 identical residues from a total of 286, however the C-terminal regions from eight species are highly conserved and seem to be essential for catalysis and assembly. The γ subunit of chloroplast has the highest homology with the same subunit from cyanobacteria (55%) and lower homology with the γ subunit from *E. coli* (33%) (Futai, *et al.*, 1989). Evidence suggests a close evolutional relationship between chloroplasts and cyanobacteria and this is supported by the similarities in the gene organisation of the ATPase subunits and homologies in other subunits. An extra domain of 40 amino acids is found in the γ subunit from chloroplasts and cyanobacteria which is not present in the γ subunit of other species (Futai *et al.*, 1989). The δ subunits show limited homologies, mainly in their C-termal regions and the ε subunits show homologies in their N-terminal regions (Futai *et al.*, 1989).

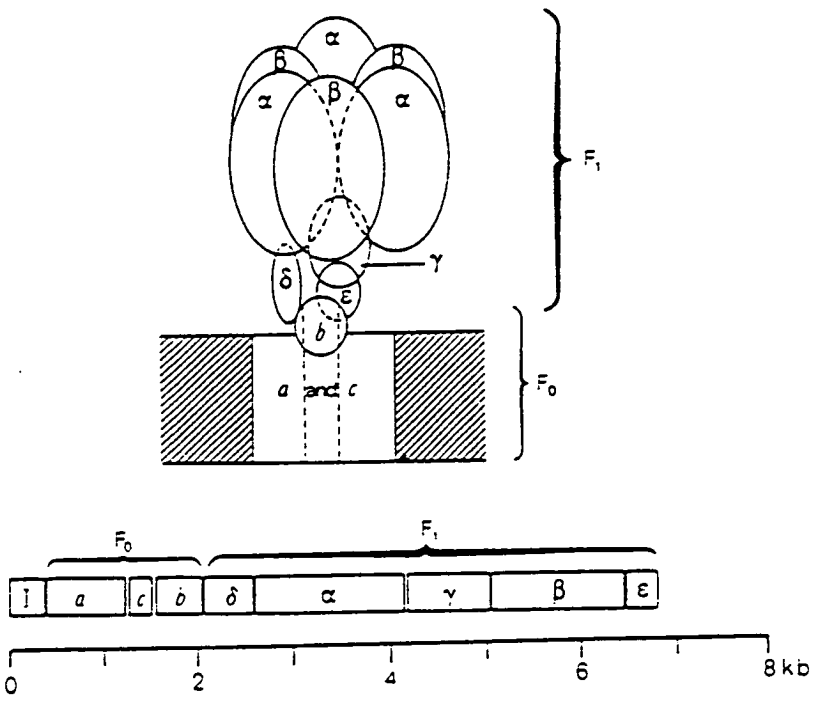


Figure 1.16. Arrangement of subunits in *E. coli* F_1F_0 ATP synthase and of the corresponding genes in the *unc* operon (From Walker *et al.*, 1990)

The homologies of the a subunits of different species are low, although their hydropathy plots are similar and conserved polar residues in the C-terminal region have been identified. For *E. coli* it has been predicted that the a subunit has between five and seven transmembrane segments (Figure 1.17.) (Schneider and Altendorf, 1987; Walker *et al.*, 1990). In *E. coli* the b subunit is a hydrophilic protein although it does have a hydrophobic stretch of 23 residues near its N-terminus (Figure 1.17.). The c subunit is an extremely hydrophobic protein which binds the F_0F_1 inhibitor DCCD by a Glu or Asp residue located in the centre of the second hydrophobic stretch from the N-terminus. Two hydrophobic stretches of the subunit, possibly α-helices, traverse the membrane in an antiparallel manner and a central polar region is exposed to the mitochondrial matrix or bacterial cytoplasm (Figure 1.17.) (Futai *et al.*, 1989).

The subunit stoichiometry for F_1 has been established as $3\alpha:3\beta:1\gamma:1\delta:1\varepsilon$ and crystal structure studies suggest that the three α and three β subunits are arranged alternately like the segments of an orange around a central α helix formed by the carboxyl-terminal region of the γ subunit (aa 209-272) (Abrahams *et al.*, 1994). Complexes of α, β and γ subunits reconstituted from isolated individual subunits show ATPase activity. It appears that the γ subunit is required for reconstitution of ATPase activity, even though the catalytic sites are on the β subunit or the interface of the α and β subunits (Futai, 1977). ATPase activity can be reconstituted by mixing α, β and γ subunits from different species (Futai *et al.*, 1980). Single amino acid substitutions in the α or β subunit of *E. coli* can result in the loss of normal F_1F_0 activity in membranes. Generally two types of mutations were observed, identifying residues important in either catalysis or assembly.

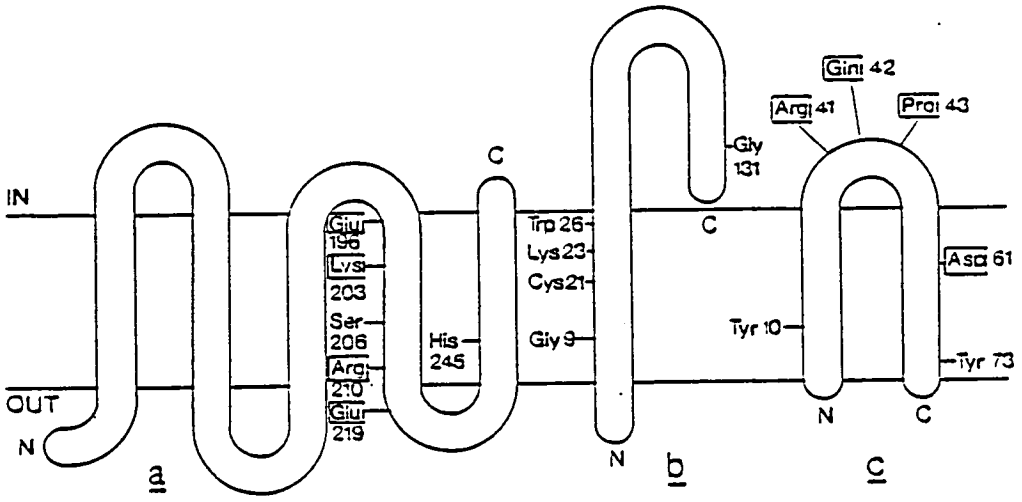


Figure 1.17. Subunits a, b and c from *E. coli* F_0 and their proposed orientation in the membrane. Conserved amino acid residues are boxed (From Schneider and Altendorf, 1987)

Three mutations in the α subunit, Gly-29 to Asp, Glu-299 to Lys and Ala-285 to Val, decreased the amount of membrane bound F_1 and made the membranes permeable to protons, suggesting that these residues may be in the region of the subunit that is important for the interaction between F_1 and F_0 (Maggio *et al.*; 1987, 1988, Soga *et al.*, 1989).

The αβγ complex of *E. coli* cannot bind to F_0 unless the δ and ε subunits are added. In agreement with this observation a mutant of the *unc*C gene (ε subunit) had F_1-ATPase activity only in the cytoplasmic fraction. Mutant strains with plasmids carrying truncated *unc*C genes indicated that the amino-terminal portion (78-80 residues) of the ε subunit was sufficient for forming active F_1 capable of binding to F_0 (Dunn and Futai, 1980). The effects of mutations to Pro-47 and Gly-48 in this region suggest that these residues are important in the movement of protons through F_1 (Cox *et al.*, 1987; Kuki *et al.*, 1988). Supporting this theory chloroplast F_1 lacking ε binds to F_0 but does not block passive proton conduction (Richter *et al.*, 1984). The *E. coli* ε subunit binds tightly to the γ subunit and can be cross-linked to the carboxyl-terminal of the β subunit using carbodiimide (Tozer and Dunn, 1987).

All three of the a, b and c subunits are required for a functional F_0 complex. Subunit stoichiometry for F_0 has been established as ab_2c_{6-10} (Futai *et al.*, 1989). Five to seven hydrophobic segments, possibly α helices, which span the membrane, were predicted for the a subunit in *E. coli* (Figure 1.17). The portions connecting the α helices may also be embedded in the membrane since they are not accessible to proteolytic enzymes (Eya *et al.*, 1988).

The hydrophobic amino-terminal region of the *E. coli* b subunit may be embedded in the membrane as a helical domain, and the two hydrophilic helical domains may be located in the cytoplasm. Consistent with this model is the fact that treatment with proteases has no affect on the proton permeability of the F_0 portion but abolished its functional binding of F_1 (Figure 1.17) (Hoppe *et al.*, 1983).

Confirmation of the embedded first segment came from studies using hydrophobic photoreactive labelling of amino acid residues. Residues from the N-terminal region of the b subunit were labelled (Hoppe and Sebald, 1984). Studies on membranes of pseudorevertants of a nonsense mutant (Try-26 to end) showed impaired proton permeability on the removal of F_1, suggesting that Trp-26, located near the surface of the lipid bilayer, stabilises the proton pathway. Mutations in the C-terminal region abolished F_1 binding as well as proton translocation (Takeyama *et al.*, 1988).

The c subunit is hydrophobic and can aggregate into a twelve subunit cluster even in the presence of detergents as shown by polyacrylamide gel electrophoresis. It has been proposed that two helices span the membrane (residues 15-40 and 51-76). The 41-50 region contains two Pro residues, which are well known helix breakers. This is consistent with a centrally exposed hydrophilic sequence which could interact with F_1 (Figure 1.17). The close interaction of the three F_0 subunits has been established. Hydrophobic cross-linking reagents have been shown to link the a and b subunits. The a subunit also appears to associate strongly with the c subunit, since an a-c complex has been isolated; furthermore liposomes embedded with various F_0 subunits, labelled using photoactivatible carbene-generating reagents has suggested that the conformation of c is mainly determined by interactions with the a subunit (Futai *et al.*, 1989). Consistent with results of reconstitution experiments, subunits of F_1 could be synthesised and assembled into an active ATPase in the absence of F_0. Similarly active F_0 could be assembled from the a, b and c subunits without the presence of F_1 subunits. Analysis of mutants however has suggested that the a and b subunits are essential for insertion of the c subunits into membranes and thus for the assembly of F_0 (Futai *et al.*, 1989).

There appear to be six nucleotide binding sites in the F_1 complex, which are located at the interfaces between the α and β subunits. There appear to be two groups of binding sites; three catalytic and three non-catalytic, according to their abilities to

exchange bound nucleotide rapidly during ATP hydrolysis (Kironde and Cross, 1986). The roles of the non catalytic sites are currently unknown, although they may play structural roles and may be essential for proper subunit assembly. The catalytic sites are predominantly in the β subunits with some contributions from side chains in the α subunit. The opposite is true for the non catalytic sites (Abrahams *et al.*, 1994).

Most experimental evidence supports the binding-change mechanism of ATP synthesis in F_1F_0 ATPases (Boyer, 1980). According to this mechanism, tight binding of ATP occurs at the catalytic site, and the electrochemical gradient of protons decreases the affinity for ATP and increases the affinities for ADP and inorganic phosphate. This mechanism requires co-operation between the three catalytic sites and it implies that the three sites are always different, with each passing through a cycle of open, loose and tight states. Such a mechanism suggests that the F_1F_0 ATPase is an inherently asymmetric structure and this has been supported by crystal structure study work. Additionally it has been proposed that the transition between the different catalytic states could be achieved by rotation of the catalytic subunits relative to the rest of the ATP synthase (Abrahams *et al.*, 1994). The binding-change mechanism is distinct from the one associated with P-type ATPases which involves the direct phosphorylation of an aspartate residue as discussed previously.

The V-type ATPases play a key role in the acidification of endomembrane-bordered compartments of all eukaryotic cells. In mammalian cells V-ATPases are involved in the acidification of Golgi vesicles and lysosomes (Harvey, 1992). In yeast and other fungi such as Neurospora, V-ATPases acidify vacuoles used for the storage of amino acids, Ca^{2+}, carbohydrates, phosphates and hydrolases. V-ATPases energise accumulation of neurotransmitter amines by synaptic vesicles and chromaffin granules and in renal plasma membranes V-ATPases energise the acidification of urine and the reabsorption of bicarbonate by blood which are

essential for pH buffering Harvey, 1992). V-ATPases are also present in a number of archaebacterial and eubacterial cell membranes in addition to F_1F_0-ATPases (Heefner and Harold, 1982). The V-ATPases exhibit a number of structural and functional similarities to the F_1F_0-ATPases. However whereas F_1F_0-ATPases can act in proton pumping and ATP formation, the V-ATPases of eukaryotes function exclusively as ATP-dependent proton pumps. F_1F_0-ATPases and V-ATPases from archaebacteria are composed of a water soluble catalytic portion and an integral membrane ion translocating portion. The catalytic and membrane domains of the eukaryotic V-ATPases cannot act separately. This property is correlated with the presence of a large proteolipid that spans the membrane four times (Nelson, 1992) The proteolipid in plant vacuoles has been shown to have a stoichiometry of six proteolipids per enzyme molecule. The proteolipids of eukaryotic V-ATPases have evolved by gene duplication and fusion into a double-size proteolipid. The archaebacterial proteolipid may have diverged from the F_1F_0-ATPases prior to the gene duplication event that led to the present eukaryotic V-ATPase (Nelson, 1992). In the catalytic portions of V-ATPases at least four subunits have been identified; A, B, C and E which have a stoichiometry of 3A:3B:1C:1E (Nelson, 1992). Sequence comparisons have confirmed that homology exists between the main subunits (α and B, β and A) of the F_1F_0-ATPases and V-ATPases. No sequence homology has been observed between the C or E subunits of the V-ATPases and the γ, δ or ε subunits of the F_1F_0-ATPase (Nelson, 1992). The amino acid composition of the V-ATPases suggests a higher sensitivity to oxygen than the F_1F_0-ATPases. There are several tryptophan and cysteine residues in the catalytic sites of V-ATPases. In particular a cysteine residue inside the P-loop structure of the A subunit is implicated in the binding of ATP. The cysteine is sensitive to oxidation and modification by NEM inactivates the enzyme. The catalytic portion of V-ATPases are also sensitive to nitrate while the membrane

segments like the equivalent in the F_1F_0-ATPases are sensitive to DCCD and DES (Pedersen and Carafoli, 1987).

1.7. Sodium driven ATPases.

The first discovery of sodium coupled ATP synthesis in a bacterium was in the strict anaerobe *P. modestum* (Hilpert *et al.*, 1984). When grown on succinate as a sole carbon source the decarboxylation of methylmalonyl-CoA results in the generation of a sodium gradient across the membrane. Under these conditions the sodium gradient is the only source of energy available to generate ATP. It has been shown that membranes of *P. modestum* contain large amounts of an ATPase which is specifically activated by sodium ions. This enzyme has a typical F_1F_0 structure, with the catalytic portion of the enzyme, F_1, consisting of five subunits, α, β, γ, δ, and ε. Purified F_1 ATPase was not stimulated by sodium ions in contrast to the reconstituted F_1F_0 which was. This implies that F_0 is responsible for sodium translocation. The F_0 component has been shown to be composed of three subunits, a, b and c (Dimroth, 1990).

There are similarities between the subunits of this enzyme and the proton-translocating F_1F_0-ATPase in *E. coli*. Antibodies raised against the b subunit of *E. coli* cross-reacted with the b subunit of *P. modestum*. Also amino acid sequence homology exists between the subunits from the two ATPases. For the β subunits 69% homology was found while 21% homology existed for the 41 amino-terminal residues of the c subunit. At sodium concentrations of <1mM, the ATPase of *P. modestum* has been shown to pump protons; however the rate of proton pumping in the absence of sodium does not exceed 10% of the maximal sodium transport rate at neutral pH. Proton pumping falls to zero as the sodium concentration increases from 0 to 1 mM, while the rate of sodium translocation increases from 0 to 80% of its maximal value over the same concentration change. It can be concluded that under physiological conditions the F_1F_0 ATPase of *P. modestum* is driven by sodium ions, although it is capable of pumping protons below 1mM Na^+ (Dimroth, 1990).

In *V. alginolyticus* a DCCD sensitive F_1F_0 type ATPase has been discovered which has been shown to pump sodium ions (Dibrov *et al.*, 1988). The structural genes for this enzyme were subsequently cloned and sequenced in 1990 by Krumholz, *et al.* From their work only one F_1F_0 ATPase in *V. alginolyticus* was identified and there appeared to be significant amino acid sequence homology between the subunits of F_1F_0 ATPase from *V. alginolyticus* and the subunits of F_1F_0 ATPase from *E. coli* (Table 1.4.). In addition the organisation of genes in the *V. alginolyticus unc* operon is identical to that in the *unc* operon from *E. coli*. In 1991 Capozza *et al.*, proposed that the F_1F_0 ATPase from *V. alginolyticus* could pump protons and sodium ions, but unlike the enzyme from *P. modestum* was highly active even in the absence of sodium. However Krumholz *et al.* (1990) were unable to show any sodium pumping activity from the F_1F_0 ATPase in *V. alginolyticus* and concluded that this F_1F_0 ATPase functioned exclusively as a proton pump. Therefore there remains some controversy as to the fate of sodium gradient generated by *V. alginolyticus* and whether or not there is a sodium-translocating ATPase.

A sodium-translocating ATPase has been discovered in the facultative anaerobic bacterium *Enterococcus hirae* (formerly *Streptococcus faecalis*) (Heefner and Harold, 1982). This enzyme unlike the sodium translocating F_1F_0 ATPase from *P. modestum* is insensitive to DCCD but is highly sensitive to other V-type ATPase inhibitors such as nitrate, N-ethylmaleimide and NBD-Cl. The presence of a V-type ATPase was confirmed when the A and B subunit genes were cloned and sequenced. Amino acid sequence homology with other members of this group was observed. It appears that in *E. hirae* cells a V-type Na^+-ATPase and a F_1F_0 H^+-ATPase are both present (Takase *et al.*, 1993).

Recent work in our laboratory has suggested that in the marine bacterium *V. harveyi* there may be two distinct forms of ATPase, a DCCD sensitive F_1F_0-ATPase and a nitrate sensitive V-type ATPase (Clark, 1994). Although further work needs to

be done to confirm these preliminary findings, the presence of a sodium translocating V-ATPase coexisting in a bacterial cell with a proton translocating F_1F_0-ATPase would resolve the controversy regarding the fate of sodium gradients generated by NADH-ubiquinone reductases complex.

Table 1.4. Amino acid similarities between the different subunits of the F_1F_0 ATPases of *V. alginolyticus* with *E. coli* (Modified from Krumholz *et al.*, 1989)

Subunit	*E. coli* (% Identity)
α	83
β	80
γ	66
δ	61.2
ε	73.8
a	62.2
b	72.6
c	48.1

1.8. Sodium proton antiporters.

Sodium/proton antiporters allow the exchange of protons for sodium ions and vice-versa. They are secondary transporters and can be used by bacteria which do not have a primary sodium pump to generate a sodium gradient. This type of antiporter is of importance in cytosolic pH regulation. For the acidification of the cytosol the rate of proton translocation inward must be greater than the rate of proton translocation outwards via respiration or the ATPase. At the same time the sodium/proton antiporter exchanges an electrochemical gradient of protons ($\Delta\mu_{H+}$) for an an electrochemical gradient of sodium ($\Delta\mu_{Na+}$) (Figure 1.18.). It has been shown that acidification of the cytoplasm by some alkalophilic bacteria at alkaline pH is sodium-dependent (Kitada *et al.*; 1982, McLaggan *et al.*, 1984). The most dramatic example of cytosolic pH regulation is in an alkalophilic Bacillus species which maintain an internal pH at or below pH 9.5 while growing at pH 12 (Krulwich, 1983).

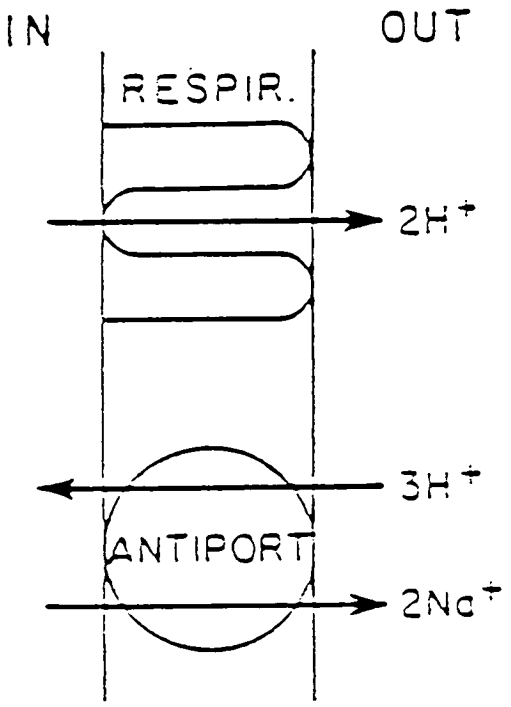
It appears that sodium/proton antiporters can utilise either sodium or lithium ions in exchange for protons. In prokaryotes, some antiport activities are unaffected by changes in $\Delta\psi$ and have therefore been characterised as electroneutral, functioning much like the artificial antiporters such as monensin. Others require a $\Delta\psi$, at least at some external pH values, and have been presumed to be electrogenic and perhaps gated. An electrogenic antiporter which, for example, catalysed the inward translocation of $3H^+$ in exchange for $2Na^+$ could be energised by a $\Delta\psi$ (outside positive) while an electroneutral antiporter would presumably not be affected by such a $\Delta\psi$ and would depend solely on a chemical gradient of the relevant ion (Krulwich, 1983). The gating of an antiporter could represent a conformational change that occurs above some threshold $\Delta\psi$ which is required for the activity of the porter. Electrogenic mechanisms in bacteria may be of particular advantage at alkaline pH where ΔpH is zero or negative and an uphill extrusion of sodium is only possible by

coupling to $\Delta\psi$. In eukaryotic cells the sodium/proton antiport activities appear to be electroneutral and have generally been found to be sensitive to the diuretic amiloride.

In *E. coli* expulsion of sodium ions is driven by proton flux via at least two distinct sodium/proton antiporters which are known as NhaA and NhaB. When the *nhaA* gene is deleted, cells become sensitive to high salinity, alkaline pH and toxicity of Li^+. The *nhaA* gene has been cloned by complementation, restoring the wild-type phenotypes of resistance to high salinity, Li^+ and high pH. The deduced molecular weight of NhaA is 41 kDa and hydropathy plots suggest that it possesses 11 membrane spanning α helices (Taglicht *et al.*, 1991). Expression of NhaA is increased by high Na^+ concentrations and increases in pH and it has been predicted that NhaA has a stoichiometry of two protons exchanged for each sodium ion (Taglicht *et al.*, 1993). NhaA has been overexpressed, purified and functionally reconstituted into membrane vesicles.

The gene for NhaB has also been cloned and sequenced. The molecular weight of the deduced protein is 47 kDa and hydropathy plots suggest that 12 transmembrane helices exist. Very limited homology was observed between *nhaA* and *nhaB*; however in one common region 43% identity was observed. NhaB also confers resistance to the toxic effects of Li^+ and Na^+ (Pinner *et al.*, 1992). For NhaB the Km for Li^+ is 0.06 mM, which is ten times the value obtained for NhaA. NhaB supports the growth of of strains deleted of *nhaA* under conditions of Na^+ concentrations of up to 0.7 M at low pH and up to 0.1 M at higher pH values up to pH 8.5. It appears that NhaB has a higher affinity for sodium ions and it has been suggested that the role of NhaB-mediated sodium/proton antiporter activity is important under conditions where sodium ion concentrations and pH are lower, in contrast to NhaA which has a more central role under conditions of increased sodium ion concentrations and increased pH (Pinner *et al.*, 1992).

V. alginolyticus also has at least one sodium proton antiporter which is important for the acidification of the cytoplasm when the external pH is alkaline (Nakamura, *et al.*, 1992). The structural gene for this sodium proton antiporter has been cloned and sequenced. The protein has a molecular weight of 40.4 kDa and it has been predicted to contain 11 membrane spanning domains. The *V. alginolyticus* sodium proton antiporter has 58% DNA sequence homology with NhaA from *E. coli*, which implies that there will be functional similarities between the two proteins (Nakamura *et al.*, 1994)



Net translocation

1 H^+ in (ΔpH)

1 positive charge out ($\Delta\psi$ positive outside)

2 Na^+ out ($\Delta\mu_{Na+}$)

Figure 1.18. Example of how operation of an electrogenic Na^+/H^+ antiporter together with primary H^+ pumping might produce a $\Delta\psi$ (outside positive), a $\Delta\mu_{Na+}$ and net acidification of the interior of a bacterial cell (Modified from Krulwich, 1983)

1.9. Sodium symports.

A number of sodium-driven solute symports have been identified in bacteria. *Klebsiella pneumoniae* has been shown to possess a sodium-dependent citrate transport system (Schwarz and Oesterhelt, 1985). In *E. coli* a sodium-dependent glutamate transport system has been reported (Frank and Hopkins, 1969). For both these systems it has been proposed that sodium and protons are symported with the solute simultaneously. Multiple citrate transport systems with different sodium and potassium requirements exist in *S. typhimurium*. In at least one of these a periplasmic binding protein has been identified (Kay and Cameron, 1978). Systems for the uptake of proline and in *E. coli* and *S. typhimurium* are also coupled to cotransport with sodium ions (Cairney *et al.*, 1984; Chen and Wilson, 1986).

The melibiose carrier of *E. coli* has unique cation coupling properties. This system is capable of using protons, sodium or lithium as coupling cations depending on the particular sugar being transported and ionic environment (Niiya *et al.*, 1982). It has been shown that sodium or lithium ions selectively increase the apparent affinity of the transporter for galactosides while protons inhibit. It has been established in kinetic studies of sugar/sodium cotransport by the melibiose permease that the electrical membrane potential generated during substrate oxidation enhances the initial rate of sugar flux. Detailed examination of the relation between initial rate of melibiose influx and magnitude of the electrical potential in membrane vesicles indicates that the profile of sugar influx activation by $\Delta\psi$ varies according to the coupling cation. It was initially observed that when the carrier acts as a sodium symport, the maximal rate of influx increases exponentially as a function of $\Delta\psi$. The stimulating effect of $\Delta\psi$ is much less marked with lithium than with sodium. Proton-coupled melibiose transport is already high in de-energised membranes and is only slightly enhanced by $\Delta\psi$.

The melibiose carrier has a molecular weight of 52 kDa and is coded for by the *melB* gene. Very little homology between the primary structures of the melibiose and lactose transport proteins of *E. coli* has been observed, even although the two transporters share several sugars as substrates (melibiose, *p*-nitrophenyl-α-D-galactoside and methyl-1-thio-β D-galactopyranoside). A secondary structure model has suggested that the melibiose carrier protein possesses 12 α helical membrane spanning segments (Figure 1.19.) (Poucher *et al.*, 1990). In agreement with observations on other bacterial membrane proteins (Von Heijne, 1986), the predicted cytoplasmic loops contain a high density of positively charged arginine and lysine residues. In a number of lactose permeases, a histidine residue plays a crucial role in carrier function (Kaback, 1988). In the melibiose transporter site directed mutagenesis experiments swapping each of the seven histidine residues in turn for an arginine, identified His 94 to be crucial for proper transport functions. Mutation of this residue to Arg drastically reduces the transport of the sugar analogue *p*-nitrophenyl-α-D-galactoside (Pourcher *et al.*, 1990).

It is apparent that bacteria from sodium rich enviroments use symport with sodium ions as their predominant or exclusive mechanism for amino acid uptake (Lanyi, 1979). Sodium dependent transport of a number of amino acids has been demonstrated in the marine bacteria *Alteromonas haloplactis* (Niven and MacLeod, 1980) and in *V. alginolyticus* (Tokuda and Unemoto, 1982; Tokuda *et al.*, 1982). In *V. alginolyticus* a sodium sucrose symporter has also been identified (Kakinuma and Unemoto, 1985).

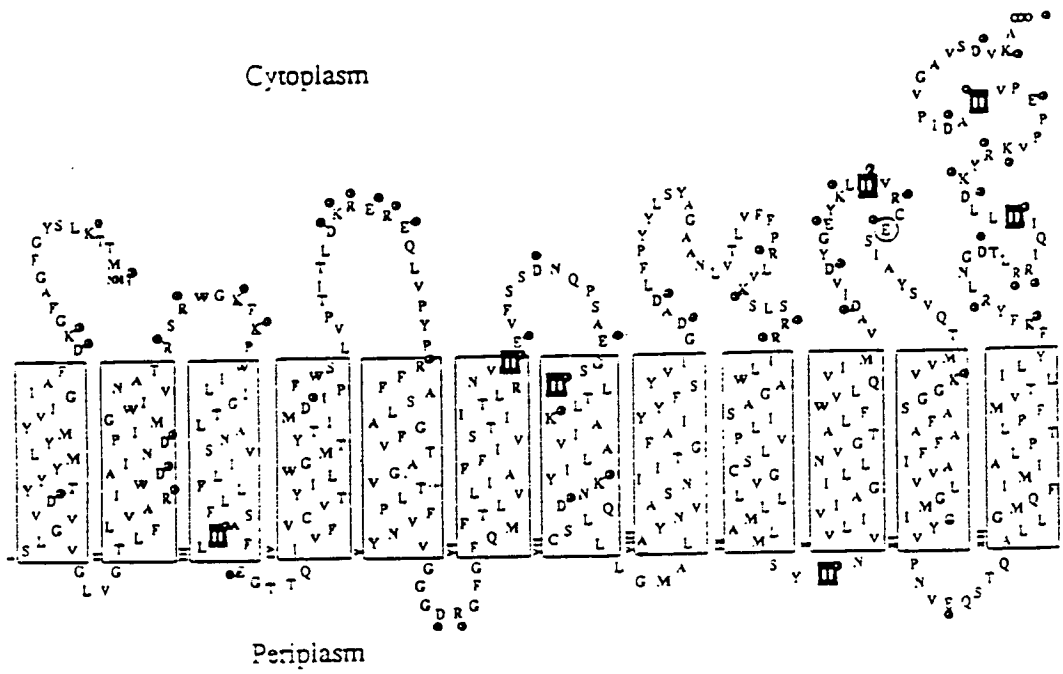


Figure 1.19. Secondary structure model of the melibiose permease of *E. coli*

α-helical transmembrane domains are shown in boxes; N- and C-termini are both located on the cytoplasmic surface of the membrane. Black squares indicate the location of the seven histidine residues which were mutated to identify any which are crucial to transporter function (Modified from Pourcher *et al.*, 1990).

1.10. Scope of the project.

This project has been set up to characterise the sodium-dependent NADH-ubiquinone oxidoreductase from the marine bacterium *Vibrio harveyi*. Although many bacterial and mitochondrial proton translocating NADH-ubiquinone oxidoreductases have been characterised, the mechanisms by which protons are translocated have still to be elucidated (Walker, 1992). Sodium-dependent NADH-ubiquinone oxidoreductases translocate sodium ions as an alternative to protons. This allows bacteria to maintain a constant intracellular pH irrespective of external pH conditions. Sodium gradients can be used in the same ways that proton gradients are utilised (Tokuda, *et al.*, 1982).

One of the aims of this project was to isolate transposon mutants of the sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi*. Isolation of such mutants could then facilitate the cloning and sequencing of the structural genes. The sequence of the NADH-ubiquinone oxidoreductase genes would allow studies to be carried out on the structure and function of the individual subunits and of the whole complex. The number and positions of transmembrane helices could be predicted and the regions involved in substrate and inhibitor specificity could be investigated. Additionally the gene for the NADH-ubiquinone oxidoreductase from *E. coli* (*ndh*) (Young, *et al.*, 1981) has been used as a probe to see if any homologous genes can be identified in *Vibrio* species.

A parallel objective was to purify the sodium-dependent NADH-ubiquinone oxidoreductase from membranes of *V. harveyi* using a number of chromatography techniques after solubilisation of the complex using a non-ionic detergent. Purified sodium-dependent NADH-ubiquinone oxidoreductase would be useful for characterising the enzyme with respect to its inhibitors, subunit composition, FAD and FMN content and would provide N-terminal sequence of the individual subunits.

N-terminal sequence could then be used to design probes for screening DNA libraries. The availability of the full DNA sequences of the subunits genes would then permit future studies into the structure and function of the enzyme complex and its individual subunits.

Chapter 2

Materials and methods.

2.1. Growth of bacterial cells.

Preparation of bacterial growth media.

General.

All media was prepared in distilled H_2O and sterilised before use in an autoclave for 20 min at 122 °C. For plates 1.5% (w/v) agar (Difco) was added prior to autoclaving. All chemicals were supplied by BDH unless otherwise stated. For growth of *V. harveyi* BB7 and *V. alginolyticus* 138-2 at pH 6 and pH 8.5 either KH_2PO_4 or Tris-HCl was added to the media to a final concentration of 50 mM. The pH was adjusted with 5 M NaOH or concentrated HCl before autoclaving as appropriate.

Luria broth (LB).

Luria broth was used for the growth of *E. coli* strains and contained the following in distilled H_2O:

tryptone (Oxoid)	10 g/l
yeast extract (Oxoid)	5 g/l
sodium chloride	5 g/l

LM broth.

LM broth was used for the growth of *V. harveyi* strains and contained the following in distilled H_2O:

tryptone	10 g/l
yeast extract	5 g/l
sodium chloride	20 g/l

Vibrio complex medium (VCM).

VCM broth was used for the growth of *V. alginolyticus* strains and contained the following:

bacteriological peptone (Oxoid)	5 g/l
yeast extract	5 g/l
sodium chloride	30 g/l

***Vibrio* minimal medium.**

Vibrio minimal medium was used for the growth of mutant *V. harveyi* strains. The following components were autoclaved separately and allowed to cool. Solution (1) (200 ml) was added to solution (2) (200 ml) and then mixed thoroughly with 2 ml of solution (3) and (4) and 6.5 ml of solution (5). Glucose or mannitol was added as a carbon source from a presterilised stock solution to give a final concentration of 30 mM.

Solution (1)	Na_2HPO_4	12.2 g/l
	$(NH)_2SO_4$	4 g/l
	sodium chloride	40 g/l
	KH_2PO_4	6 g/l
(2)	agar (Oxoid)	30 g/l
(3)	$FeSO_4$ 0.05% (w/v) in 0.5 N H_2SO_4	
(4)	$Ca(N0_3)_2$	1% (w/v)
(5)	$MgSO_4$	0.5 M

Bacterial strains and vectors used.

Lists of the bacterial strains and vectors used during this project are detailed in Tables 2.1 and 2.2.

Bacterial growth conditions.

All strains were grown aerobically with shaking in a Gallenkamp orbital shaker at 300 rpm. For small scale growth of bacterial cells a single bacterial colony was inoculated into either 5 ml of broth in a McCartney bottle or 50 ml of broth in a 250 ml flask. For large scale growth 5 ml of an overnight culture from a small scale growth was inoculated into 500 ml of broth in a 2 l flask. *V. harveyi* and *V. alginolyticus* strains were grown at 30 °C. *E. coli* strains were grown at 37 °C unless otherwise stated.

Bacterial cell harvesting.

Bacterial cells were harvested at 12 000 g for 10 min in a Sorvall RC-5B centrifuge at 4 °C. Cells were washed in a solution containing 0.3 M NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA and stored at -80 °C in the same solution containing 10% (v/v) glycerol unless otherwise stated.

Table 2.1. Bacterial strains used and their sources.

Strain.	**Reference.**
E.coli SM10(λpir). (*thi*, *thr*, *leu*, *ton*A, *lac*Y, *sup*E, *rec*A$^-$)	Lorenzo,*et al*,, 1990; Miller *et al.*, 1986
E.coli S17-1 (*thi*, *pro*, *hsd*R$^-$, *rec*A$^-$)	Fellay *et al.*,1989, Simon *et al.*, 1986
E.coli C600. (*thr*, *leu*, *thi*, *sup*E, *lac*Y, *ton*A)	Ely, 1985., Young *et al.*, 1981
E.coli MC4100 (F$^-$, *ara*D, *rps*L, mini-mu[Tet^r](P1*clr*100CM).	Belas *et al.*, 1984
V. harveyi BB7	"
V. harveyi BB7 Rif^r.	"
V. alginolyticus 138-2.	Tokuda, H., 1983.
V. alginolyticus Nap 2.	"

Table 2.2. Vectors used and their sources.

Vector	Specifications	Reference
pRK2013	12.5 kb, Tn*10*, Mob$^+$, Col E1 origin	Ely, 1985.
pRK2013	13.2 kb, Tn*5-132*, Mob$^+$, Col E1 origin	"
pJFF350	5.3 kb, Tn*5*, Mob$^+$, Col E1 origin	Fellay *et al.*, 1989.
pSUP1021	11.7 kb, Tn*5*, Mob$^+$, p 15A origin	Simon *et al.*, 1986.
pSUP2021	13.2 kb, Tn*5*, Mob$^+$, Col E1 origin	"
pSUP10141	11.7 kb, Tn*5*, Mob$^+$, Col E1 origin	"
pUT	7.4 kb, miniTn*5*, Mob$^+$, modified R6K origin	Lorenzo *et al.*, 1990.
pRKT733	12.6 kb, Tn*Pho*A, Mob$^+$, modified R6K	Miller *et al.*, 1986.
pIY1	12 kb, Col E1 origin	Young *et al.*, 1981.

2.2. Preparation of buffers and solutions.

General.

Buffers and solutions were prepared in distilled H_2O. All pH measurements were carried out using an Orion model pH meter which had been calibrated using two standards: one at pH 7, the other at pH 4 or pH 9 as appropriate.

Tris-HCl buffer.

The 1 M Tris-HCl buffer was prepared by dissolving 121.1 g of Tris in 500 ml of distilled H_2O. The pH was adjusted by adding concentrated HCl and the volume of solution was made up to 1 l.

KH_2PO_4 buffer.

The 0.5 M KH_2PO_4 buffer was prepared by dissolving 68.05 g KH_2PO_4 (BDH) in 500 ml of distilled H_2O. The pH was adjusted by adding 1 M NaOH and the volume of solution was made up to 1 l.

Glycine buffer.

The 0.5 M glycine buffer was prepared by dissolving 37.8 g of Glycine (Biorad) in 500 ml of distilled H_2O. The pH was adjusted by adding 1 M NaOH and the volume of solution was made up to 1 l.

TEN buffer.

TEN buffer was composed of 10 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA in distilled H_2O.

EDTA.

A 0.5M stock solution of EDTA was prepared by mixing 186.1 g of disodium EDTA (BDH) in 800 ml of distilled H_2O. The pH was adjusted with 5 M NaOH to pH 8 and the volume made up to 1 l.

TBE.

A 10 x TBE stock solution was prepared by dissolving 108 g of Tris, 55 g of Boric acid and 40 ml of 0.5 M EDTA in a final volume of 1 l distilled H_20. The buffer was diluted 1 in 10 prior to use.

Denhardt's solution.

The 5 x Denhardt's solution contained 1% (w/v) ficoll (Sigma), 1% (w/v) polyvinylpyrrolidone (Sigma) and 1% (w/v) BSA (Sigma) in distilled H_2O.

SSC.

A 20 x SSC stock solution was prepared by dissolving 87.7 g of NaCl and 88.2g of Sodium Citrate (Sigma) in 500 ml of distilled H_2O and then adjusting the volume to 1 l.

DNA loading buffer.

DNA loading buffer was 10 x TBE, 100% (w/v) sucrose (BDH) and 0.01% (w/v) bromophenol blue (BDH). DNA samples were mixed with equal volumes of loading buffer prior to electrophoresis.

Antibiotics.

All antibiotics were prepared as 10 mg/ml stock solutions. Ampicillin and kanamycin were prepared in distilled H_2O, tetracycline and chloramphenicol were prepared in ethanol and rifampicin was prepared in DMSO. All antibiotics excluding rifampicin were filter sterilised using a 0.45 μm Millipore filter.

CCCP.

CCCP was prepared as a 10 mM stock solution in ethanol. It was filter sterilised using a 0.45 μm Millipore filter.

Detergents.

All detergents were prepared as stock solutions of concentration 10% (w/v) in distilled H_2O. Detergents were dissolved with stirring and heating where required.

2.3. Transposon mutagenesis.

2.3.1. Conjugation.

E. coli donor strains were grown overnight aerobically at 37 °C in LB which was supplemented with the appropriate antibiotic. *V. harveyi* BB7, the recipient strain was grown overnight aerobically at 30 °C in LM broth. Both cultures were diluted with 10 volumes of their respective growth media without antibiotics and grown to an A_{660} of 0.4. Equal volumes of donor and recipient (5 ml of approximately 2 x 10^8 cells per ml) were mixed and then filtered through a 25 mm diameter 0.45 μm Millipore filter. The filter was then placed aseptically on a LM plate and incubated for 24 h at 30 °C. The filter was then placed in 5 ml of LM broth and the bacteria resuspended. Serial dilutions were then made and 100 μl aliquots plated out on LM agar containing the appropriate antibiotic to select for transconjugants. Rifampicin at 20 μg/ml was included to select for *V. harveyi* BB7 Rif^r recipients only. Plates were incubated at 30 °C for 24 h and observed for colonies. Donor only and recipient only plates were included as controls.

2.3.2. Bacteriophage P1 lysate production.

Bacteriophage P1 lysates were prepared by temperature induction (Silhavy, *et al.*, 1982). A 5 ml culture of *E. coli* MC4100 (mini-mu[Tet^r](P1*clr*100CM) was grown at 30 °C with aeration to an A_{600} of 0.2. The culture was then transferred to a water bath at 45 °C for 20 min followed by an incubation at 42 °C for 1-2 h with aeration until the bacterial cells had lysed. Afterwards 200 μl of chloroform was added and the lysate centrifuged at 3 500 g for 20 min to pellet any debris. The lysate was filter sterilised through a 0.45 μm Millipore filter and $CaCl_2$ and $MgSO_4$ added to give a final concentration of 5 mM.

2.3.3. Bacteriophage mediated P1 transduction.

V. harveyi BB7 was grown aerobically in LM broth at 30 °C to an A_{660} of 0.8 and $CaCl_2$ was added to a final concentration of 25 mM. Bacteriophage P1 were added to a multiplicity of 0.1. Cells were incubated at 30 °C without shaking for 30 min to allow phage adsorption. The cell suspension was then diluted with 5 volumes of LM broth and incubated for 2 h at 30 °C with shaking to permit expression of the tetracycline resistance phenotype. To select tetracycline resistant transductants 100 µl of this suspension was plated out on LM agar containing 10 µg/ml of tetracycline. Plates were incubated for 48 h at 30 °C. P1 only and recipient only plates were included as controls.

2.3.4. Screening for auxotrophic mutants.

Auxotrophic mutants were screened for by their inability to grow on *Vibrio* minimal media containing 30 mM glucose. Strains were replica plated on *Vibrio* minimal media and LM plates. For strains growing on LM but not on Vibrio minimal media, colonies were picked from the LM plates and purified by plating.

2.3.5. Screening for CCCP sensitive mutants.

CCCP sensitive mutants were screened for by replica plating on LM plates at pH 6 and on LM plates at pH 8.5 containing 10 µM CCCP.

2.4. Manipulation of bacterial DNA.

2.4.1. Chromosomal DNA preparation.

Chromosomal DNA was prepared from *V. harveyi* BB7 and *V. alginolyticus* 138-2 using the method of Silhavy *et al.*, 1984. A 500 ml flask containing 100 ml of either LM or VCM was inoculated with a single bacterial colony and incubated overnight at 30 °C with aeration. Cells were harvested by centrifugation at 10 000 g in a Sorvall RC-5B centrifuge at 4 °C. Cells were resuspended in 5 ml of TE (50 mM Tris-HCl, pH 8 and 50 mM EDTA) and the suspension frozen at -20 °C. The cells were thawed with gentle mixing in a room temperature water bath and 0.5 ml of a freshly prepared lysozyme solution (10 mg/ml in 0.25 M Tris-HCl, pH 8) was added. The preparation was put on ice for 45 min. Next, 1 ml of STEP (0.5% SDS, 50 mM Tris-HCl, pH 7.5, 0.4 M EDTA and 1 mg/ml Proteinase K (Sigma)) was added, mixed well and the solution incubated at 50 °C for 1 h with occasional gentle mixing. To the solution 6 ml of Tris-buffered phenol was added, mixed gently to emulsify and centrifuged at 3 000 g for 15 min in a bench top MSE Centaur 1 centrifuge to separate the layers. The top aqueous layer was transferred to a clean tube where the DNA was precipitated. A 0.1 volume of 3M sodium acetate (3 M sodium acetate with 11.5% acetic acid) was added and mixed gently. Then 2 volumes of ethanol were added and the tube was inverted several times. DNA, which precipitated as a glob, was spooled out using a sterile glass rod and redissolved in 5 ml of TE buffer containing 20 µg/ml RNAase (Sigma) and incubated overnight at 4 °C. An equal volume of chloroform was added, gently mixed to emulsify and centrifuged at 3 000 g for 15 min in a bench top MSE Centaur 1 centrifuge to separate the layers. The top aqueous layer was transferred to a clean tube where the DNA was precipitated as before. DNA which precipitated as long threads was again spooled out using a sterile glass rod and redissolved in TE buffer. Chromosomal DNA was stored at -20 °C.

2.4.2. Plasmid DNA preparation.

Plasmid DNA was prepared by the alkaline lysis method (Sambrook *et al.*, 1989). A M^{c} Cartney bottle containing 5 ml of either LB or LM was inoculated with a single bacterial colony and incubated overnight with aeration at either 30 °C for *V. harveyi* or 37 °C for *E. coli* strains. Cells were harvested by centrifuging 1.5 ml volumes of cultures in a Sorvall Micro spin 12 at 12 000 g for 30 s. Each pellet was resuspended in 100 µl of ice-cold Solution I (50 mM glucose, 20 mM Tris-HCl, pH 8 and 10 mM EDTA) by vortexing vigorously. Next 200 µl of freshly prepared Solution II (0.2 N NaOH and 1% SDS) was added and mixed by inverting the tubes rapidly 5 times. Then 150 µl of ice-cold Solution III (3 M potassium acetate and 11.5% (w/v) acetic acid) was added and mixed by inverting rapidly. The solution was stored on ice for 5 min and then centrifuged for 5 min at 12 000 g. The supernatant was transferred to a fresh tube and mixed with an equal volume of phenol:chloroform (1:1). After centrifuging for 2 min at 12 000 g the supernatant was transferred to a fresh tube. The double stranded DNA was precipitated with 2 volumes of ethanol at -20 °C for 15 min. After centrifuging for 2 min at 12 000 g the supernatant was removed carefully and the nucleic acid pellet resuspended in 50 µl of TE buffer containing 20 µg/ml RNAase. Plasmid DNA was stored at -20 °C.

2.4.3. Quantification of DNA.

The amount of DNA in each preparation was determined spectrophotometrically by measuring the absorbance of a sample at both 260 and 280 nm. Pure DNA gives an A_{260}/A_{280} ratio of around 1.8 and an A_{260} value of 1 corresponds to a DNA concentration of approximately 50 μg/ml (Sambrook *et al.*, 1989). The typical yield from a chromosomal DNA preparation was approximately 1 μg of DNA per ml of starting culture. For plasmid DNA preparations the typical yield was approximately 3 μg of DNA per ml of starting culture.

2.4.4. Restriction of chromosomal and plasmid DNA.

Chromosomal DNA from *V. harveyi* BB7 and *V. alginolyticus* 138-2 (approximately 1 μg DNA) was cut with the restriction enzymes *Eco*RI, *Bam*HI and *Hind*III (Boehringer Mannheim) for 4 h at 37 °C in 1 x B or H restriction buffer as supplied. Plasmid pIY1 (approximately 1 μg DNA) was restricted for 4 h at 37 °C with *Eco*RI in 1 x restriction buffer H.

2.4.5. Electrophoresis and staining of DNA.

Agarose gels were prepared by adding 0.8% (w/v) agarose (Sigma) to 50 ml of 1 x TBE. Agarose was dissolved by heating at 90 °C in a hot water bath. After it had cooled sufficiently the agarose was poured into a 80 mm x 65 mm casting chamber and allowed to set. The gel was then placed in an electrophoresis tank and covered with 500 ml of 1 x TBE. DNA samples containing approximately 1 μg of DNA were loaded into the wells and electrophoresed at 75 mA for 2 h. Gels were then stained for 20 min in a solution of 1 μg/ml ethidium bromide (Sigma) and examined using a UV transilluminator.

2.4.6. Purification and labelling of probes.

The 2.4kb *ndh* gene fragment obtained by *Eco*RI restriction of plasmid pIY1 was purified using the Gene Clean Kit (Stratatech) according to the manufacturer's instructions. The probe (approximately 100 ng of DNA) was then radioactively labelled using a Random Primer DNA Labelling System (Gibco BRL).

2.4.7. Southern blotting and hybridisation.

Southern Blotting was carried out using the protocol in Membrane Transfer and Detection Methods (Amersham). Chromosomal DNA from *V. harveyi* BB7 and *V. alginolyticus* 138-2 and plasmid pIY1 as a control were electrophoresed as described previously. The DNA was transferred from the agarose gel to a nitro-cellulose filter (Amersham). The filter was sealed in a bag with 20 ml of prehybridisation solution (which contained 6 x SSC, 5 x Denhardt's solution and 100-500 μg/ml denatured salmon sperm DNA) and incubated with gentle shaking for 2 h at 42 °C with gentle shaking. The radioactively labelled probe was boiled and then added to the filter in the prehybridisation mixture. Hybridisation proceeded for 18 h at 42 °C with gentle shaking. Afterwards the hybridisation mixture was poured off and the filter washed twice for 30 min with 50 ml of 4 x SSC. The filter was then wrapped in cling film and exposed to X-ray film for 4 h at -80 °C.

2.5. Bacterial membrane preparation.

V. harveyi BB7 and *V. alginolyticus* strains were grown aerobically at pH 8.5 to logarithmic phase and harvested at 12 000 g in a Sorvall RC-5B centrifuge for 20 min at 4 °C. The cells were then washed and the centrifugation repeated. The pellet was then resuspended in TEN buffer containing 1 mg/ml lysozyme and incubated at 30 °C for 30 min. The cells were cooled on ice and sonicated for 15 min using 1 min bursts. Throughout sonication the temperature of the preparation was monitored to ensure that it did not exceed 10 °C. The preparation was then centrifuged at 12 000 g for 20 min to remove unbroken cells. The membrane fraction was pelleted by ultra centrifugation at 100 000 g for 1 h in a Sorvall OT50B ultracentrifuge. The membrane fraction was then resuspended in lysis buffer containing 10% (w/v) glycerol and stored at -80 °C.

2.6. Protein determination.

Protein determination was done using a modified Lowry method (Peterson, *et al.*, 1977). Samples to be assayed were diluted in distilled H_2O to give a protein concentration of approximately 50-200 μg/ml in a final volume of 200 μl in 1 ml eppendorf tubes. Samples containing 50, 100, 150 and 200 μg/ml of BSA were also prepared and assayed to establish a standard curve. Samples were mixed with 50 μl of 0.15% (w/v) Deoxycholate (BDH) and incubated at room temperature for 10 min. An aliquot of 50 μl of 72% TCA (BDH) was then added, samples were mixed and centrifuged for at 12 000 g for 15 min in a Sorvall Micro spin 12 microfuge. The supernatant was removed and the pellet resuspended in 200 μl of distilled H_2O. Next 600 μl of a freshly prepared solution containing 2% (w/v) Na_2CO_3, 1% (w/v) SDS, 0.16% (w/v) Na tartrate, 0.4% (w/v) NaOH and 0.04% (w/v) $CuSO_4$ was added to each sample and mixed thoroughly. Samples were incubated at room temperature for 30 min. Samples were then mixed with 100 μl of a 50% (v/v) solution of Folin Ciocalteu's phenol reagent (BDH) and incubated at room temperature for 45 min. The A_{660} of the samples were measured and the protein concentrations were determined from the BSA standard curve plot of A_{660} v mg/ml protein.

2.7. Enzyme assays.

2.7.1. NADH and dNADH oxidase assays.

NADH and dNADH oxidase activity was measured at room temperature from the decrease in absorbance of NADH or dNADH at 340 nm. The standard assay buffer contained in 1 ml: 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and various concentrations of salt as specified in the text. For standard assays 0.4 M NaCl was used. The assay was started by the addition of between 30-50 μg of membrane protein.

2.7.2. NADH and dNADH-menadione oxidase assays.

Samples to be assayed were preincubated for 10 min with 20 mM KCN. The standard assay buffer which contained 20 mM Tris-HCl, pH 7.5, 2 mM NADH or dNADH and 0.4 M NaCl was added (1 ml), and the assay was started by the addition of 100 μM menadione.

2.8. PAGE.

Polyacrylamide gel electrophoresis was carried out using the Mini-Protean Dual Slab Cell (Biorad). A discontinuous buffer system was employed. The PAGE components are detailed in Table 2.3.

2.8.1. Gel preparation.

Gels were prepared by mixing the components shown in Table 2.4. For SDS-PAGE, SDS was added and for native PAGE, Triton X-100 was added to the gel mixtures as shown.

Table 2.3. Polyacrylamide gel electrophoresis reagents.

Resolving buffer	1.5 M Tris-HCl, pH 8.8
Stacking buffer	0.5 M Tris-HCl, pH 6.8
Running buffer	0.025M Tris base
	0.19 M glycine
	0.1% (w/v) Triton X-100 or SDS
Acrylamide stock	30% (w/v) acrylamide
	0.8% (w/v) bisacrylamide
SDS sample buffer	0.125 M Tris-HCl, pH 6.8
	2% (w/v) SDS (Sigma)
	10% (w/v) glycerol (Fisons)
	10% (w/v) β-mercaptoethanol (BDH)
	0.01% (w/v) bromophenol blue
Native sample buffer	0.125 M Tris-HCl, pH 6.8
	0.1% (w/v) Triton X-100 (Biorad)
	10% (w/v) glycerol (Fisons)
	0.01% (w/v) bromophenol blue

Table 2.4. Preparation of polyacrylamide gels.

Stacking gel (4%):	distilled H_2O	6.1 ml
	0.5M Tris-HCl, pH 6.8	2.5 ml
	10% (w/v) SDS or Triton X-100	100 µl
	acrylamide stock	1.3 ml
	10% (w/v) APS	60 µl
	TEMED	12 µl
Resolving gel (10%):	distilled H_2O	4 ml
	1.5 M Tris-HCl, pH 8.8	2.5 ml
	10% (w/v) SDS or Triton X-100	100 µl
	acrylamide stock	3.33 ml
	10% (w/v) APS	50 µl
	TEMED	5 µl

2.8.2. Sample preparation and electrophoresis.

For SDS PAGE, samples were mixed with an equal volume of SDS sample buffer and then boiled for 5 min. For native PAGE samples were mixed with an equal volume of native sample buffer. Samples were loaded onto the gel using a Hamilton syringe and electrophoresed at a constant voltage of 150 V for 1 h.

2.8.3. Silver staining.

Silver staining was carried out using the reagent 'Silver stain plus' (Biorad) and following the instructions provided.

2.8.4. Zymogram staining.

NADH or dNADH oxidase activity was visualised in native gels using a zymogram stain. In the presence of NADH or dNADH, the NADH-ubiquinone oxidoreductase can reduce dinitrotetrazolium blue from yellow to a deep blue/purple colour. After electrophoresis gels were incubated in 20 ml of a solution containing 50 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 6 mg/ml NADH or dNADH and 7 mg/ml dinitrotetrazolium blue at 30 °C. When colour had developed sufficiently the gel was then transferred to 0.5% acetic acid to prevent any further reaction.

2.9. Protein purification.

General.

All chromatography was done using Pharmacia columns at 4 °C with a Pharmacia P-1 peristaltic pump to achieve the required flow rates. To create gradients a Pharmacia GM-1 gradient mixer was employed. After chromatography each fraction was assayed for NADH and dNADH-menadione oxidase activity to detect the fractions in which the sodium dependent NADH-ubiquinone oxidoreductase was being eluted. Protein assays were carried out on active fractions and both specific and total activities recovered were determined.

Buffer exchange and desalting.

Buffer exchange and desalting was performed using PD-10 desalting columns prepacked with Sephadex G-25 (Pharmacia). The columns were equilibrated with 2 bed volumes of the appropriate buffer under gravity flow (approximately 90 ml/h). Up to 2.5 ml of sample could be applied to the columns and 1 ml fractions were collected.

Sample concentration.

Sample concentration was performed in micro concentrators which had a 3 kDa molecular weight cut off (Centricon). Up to 2 ml of sample per micro concentrator was spun at 3000 g in a Sorvall RC-5B centrifuge. Concentrated protein remained in the retentate cup while excess buffer passed through the filter unit.

2.9.1. Membrane solubilisation.

Membrane preparations were adjusted to 5-10 mg/ml protein with TEN buffer and then treated with detergent, to give a final detergent concentration of 1% (w/v). Samples were incubated on ice for 30 min and then centrifuged at 100 000 g for 1 h. Pellets were resuspended in their original volume in TEN buffer and both pellet and supernatant fractions assayed for protein and NADH oxidase activity. The supernatant represented the solubilised fractions. Solubilisation efficiencies were calculated for both protein concentration and NADH oxidase activity as a percentage of the totals.

2.9.2. Gel filtration chromatography.

Sephacryl 300HR.

The Sephacryl 300HR (Pharmacia) was packed to a bed volume of 100 cm x 2.5 cm according to the manufacturers instructions. For chromatography a flow rate of 30 ml/h was used and the column was equilibrated with a solution containing 50 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM EDTA and 0.1% (w/v) Triton X-100. The sample volume used was 5 ml and 5 ml fractions were taken. The column was calibrated using a calibration kit (Sigma) containing urease, BSA and ovalbumin.

Superdex 200.

Superdex 200 (Pharmacia) was provided as a prepacked column. A flow rate of 20 ml/h was used and the column was equilibrated with 20 mM Tris-HCl, pH 8, 5 mM EDTA, 100 mM NaCl and 0.1% (w/v) lauryl sulfobetaine (Aldrich Chemicals). A 1 ml sample was applied to the column and 2 ml fractions were taken. All buffers and samples were filtered through a 0.2 μm filter before chromatography.

2.9.3. Ion exchange chromatography.

DEAE Sepharose fast flow.

DEAE Sepharose fast flow (Pharmacia) was used in a 20 cm x 2.5 cm bed volume. Chromatography was carried out with a flow rate of 240 ml/h, although faster rates were used for packing, equilibration, washing and sample loading. DEAE Sepharose fast flow was equilibrated with a solution containing 50 mM Tris-HCl, pH 8, 5 mM EDTA and 0.1% (w/v) Triton X-100. For chromatography 5 bed volumes of an increasing linear gradient from 0 to 1 M NaCl was employed to elute proteins from DEAE Sepharose fast flow in 5 ml fractions. After initial experiments the gradient was modified to an increasing linear gradient from 0 to 0.3 M NaCl.

Mono Q.

Mono Q HR 5/5 (Pharmacia) was provided as a prepacked FPLC column. Chromatography was carried out at a flow rate of 60 ml/h. The column was equilibrated with a solution containing 20 mM Tris-HCl, pH 8 and 0.1% (w/v) Triton X-100. The applied sample volume was 1 ml and 1 ml fractions were taken. For chromatography 5 bed volumes of an increasing gradient from 0 to 1 M NaCl in the appropriate buffer condition was used. All buffers and samples were filtered through a 0.2 μm filter before chromatography. Various buffer conditions were employed to maximise enzyme recovery as described in the results section.

2.9.4. Hydroxylapatite chromatography.

Hydroxylapatite (Bio-Gel HTP (Biorad)) was used in a bed volume of 15 cm x 2.5 cm at a flow rate of 50 ml/h. Hydroxylapatite was equilibrated with a solution containing 20 mM KH_2PO_4, pH 7.2 and 0.1% (w/v) Triton X-100. For chromatography 5 bed volumes of an increasing linear gradient from 20 mM to 500 mM KH_2PO_4, pH 7.2 was used to elute proteins in 5 ml fractions. Later this was modified to a linear gradient of 20 mM to 120 mM KH_2PO_4, pH 7.2, using various conditions to improve recovery of active enzyme as described in the results section.

2.9.5. Ammonium sulphate fractionation.

$(NH_2)_4SO_4$ fractionation was carried out on 10 ml of a membrane sample partially purified on columns of S-300HR and DEAE Sepharose. The desired $(NH_2)_4SO_4$ saturation was achieved in two different experiments by the addition of either solid $(NH_2)_4SO_4$ or a saturated solution of $(NH_2)_4SO_4$ followed by equilibration using gentle stirring at 4 °C for 1 h. A saturated $(NH_2)_4SO_4$ solution was prepared by dissolving 756 g in 1 l of distilled H_2O. The pH was adjusted to 7 by the addition 5 N NH_3.When the desired $(NH_2)_4SO_4$ saturation was attained a 1 ml sample was removed and centrifuged for 15 min at 30 000 g in a Sorvall RC-5B centrifuge. The supernatant was removed and the pellet resuspended in 1 ml of a solution containing 50 mM Tris-HCl, pH 8 and 0.1% (w/v) Triton X-100. Both pellet and supernatant samples were desalted on PD-10 desalting column (Pharmacia). The samples were then assayed for NADH and dNADH oxidase activity and the protein concentration determined.

2.9.6. Hydrophobic interaction chromatography.

Phenyl Sepharose (Pharmacia) was used in a 10 cm x 2.5 cm bed volume with a flow rate of 30 ml/h. The column was equilibrated with a solution containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 0.1% (w/v) Tween 80. For chromatography 5 bed volumes of an increasing linear gradient of 0 to 1% (w/v) Triton X-100 were used.

2.9.7. Affinity chromatography.

Metal chelate chromatography.

Chelating Sepharose (Pharmacia) was used at a flow rate of 120 ml/h. A column bed of 5 cm x 2.5 cm was washed with 2 bed volumes of distilled H_2O. A single bed volume of 0.2 M $ZnSO_4$ was applied followed by 5 bed volumes of distilled H_2O. The column was then equilibrated with a solution containing 20 mM KH_2PO_4, pH 7.2, 0.5 M NaCl and 0.1% (w/v) Triton X-100.

Covalent chromatography.

Thiopropyl-Sepharose 6B was used at a flow rate of 30 ml/h with a column bed volume of 5 cm x 2.5 cm. The matrix was equilibrated with a solution of 20 mM KH_2PO_4, pH 7 and 0.1% (w/v) Triton X-100.

Dye affinity chromatography.

A Mimetic screening kit (PIKSI) (Affinity Chromatography Ltd.) was used to test the suitability of mimetic triazine dyes for use in affinity chromatography. Chromatography was performed using gravity flow. Columns were equilibrated with a solution containing 20 mM KH_2PO_4, pH 7 and 0.1% (w/v) lauryl sulfobetaine. Conditions were altered to improve binding as described in the results section. Where binding was observed samples were eluted using an increasing linear gradient of 0 to 0.25 M NaCl.

2.9.8. Denaturation and renaturation.

V. harveyi BB7 membranes were extracted in 2.5% (w/v) Tween 20 (BDH) and in 0.5% (w/v) Brij 35. For each extract 800 ml was mixed with 100 µl of 0.1 M SDS. After all activity had been lost, Brij 35 was added to a final concentration of 7% w/v. The samples were mixed regularly and incubated at room temperature for 3 days. Assays were carried out every hour for the first 8 h and then every 12 h.

2.9. Preparative electrophoresis.

Polyacrylamide gel electrophoresis.

Native polyacrylamide gel electrophoresis was carried out as described previously at 4 °C on a 6% polyacrylamide gel. After electrophoresis the gel was zymogram stained using dNADH. The deep blue/purple activity band was excised from the gel carefully using a clean razor blade. The gel fragments were electroeluted using a Biorad Model 422 electroeluter. Electro elution was carried out at a constant

current of 8 mA for 6 h in PAGE buffer containing 0.1% (w/v) Triton X-100. Eluted protein samples were collected from the electrophoresis caps, tested for NADH and dNADH oxidase activity and assayed for protein.

Agarose electrophoresis.

The electrode buffer solution for agarose electrophoresis contained 5.6 g diethylbarbituric acid (BDH), 11.1 g Tris base and 0.1% (w/v) Triton X-100 per litre. Agarose gels were prepared by adding 1% (w/v) agarose to 12ml of electrode buffer. The agarose was dissolved by heating it at 90 °C in a hot water bath. After it had cooled sufficiently the gel was poured onto a 84 mm x 94 mm glass plate and allowed to set. When the gel had set it was transferred to the cooling plate of the LKB multiphor. The cooling plate was attached to a cold water supply and 3 MM Whatman filter paper electrode wicks were wet in electrode buffer (500 ml of buffer was used for each electrode chamber) and then applied to opposite ends of the gel. Samples were mixed with an equal volume of loading buffer (10 mg/ml of bromophenol blue in electrode buffer) and applied to the gel surface on 2 mm wide pieces of filter paper next to the cathode wick. Gels were electrophoresed for 3 h with a constant voltage of 20 V/cm. After electrophoresis the gel was zymogram stained using dNADH. The deep blue/purple activity band was excised from the gel carefully using a clean razor blade. Agarose slices were treated for 5 min at 40 °C in 3 M sodium iodide to melt the agarose. Protein was recovered by filtering samples through a 0.2 μm filter and then passing them down a PD-10 desalting column. Samples were tested for NADH and dNADH oxidase activity and assayed for protein.

Starch electrophoresis.

The electrode buffer solution for starch electrophoresis was 0.05 M Tris-HCl, pH 8.6 containing 0.1% (w/v) Triton X-100. Starch gels were prepared by adding 13% (w/v) starch (BDH) to 50 ml of 0.03 M Tris-HCl, pH 8.6 containing 0.1% (w/v) Triton X-100. The starch was dissolved by heating it in a round bottom flask using a bunsen burner with constant mixing. After it had cooled sufficiently the gel was poured onto a 84 mm x 94 mm glass plate and allowed to set. When the gel had set it was transferred to the cooling plate of the LKB multiphor. The cooling plate was attached to a cold water supply and 3 MM Whatman filter paper electrode wicks were wet in electrode buffer (500 ml of buffer was used for each electrode chamber) and then applied to opposite ends of the gel. Samples were mixed with an equal volume of loading buffer (10 mg/ml of bromophenol blue in 0.03 M Tris-HCl, pH 8.6 containing 0.1% (w/v) Triton X-100) and applied to the gel surface on 2 mm wide pieces of filter paper next to the cathode wick. Gels were electrophoresed for 5 h with a constant voltage of 20 V/cm. After electrophoresis the gel was zymogram stained using dNADH. The deep blue/purple activity band was excised from the gel carefully using a clean razor blade. Starch slices were homogenised in 2 ml of electrode buffer and protein was recovered by filtering samples through a 0.2 μm filter. Samples were tested for NADH and dNADH oxidase activity and assayed for protein.

Chapter 3

Results

Initial characterisation of the NADH-ubiquinone oxidoreductase.

Initial characterisation of the NADH-ubiquinone oxidoreductase from *V. harveyi* BB7 was carried out using whole membrane fractions in NADH and dNADH oxidase assays. It has been reported previously that the substrate dNADH can be utilised by the sodium translocating NADH-ubiquinone oxidoreductase but not by the corresponding non-coupling enzyme (Bourne, and Rich, 1992). For stability studies 1% (w/v) Triton X-100 solubilised membranes were used in NADH and dNADH-menadione oxidase assays.

3.1. Salt dependence.

Membranes from *V. harveyi* BB7 demonstrated dNADH oxidase activity which required Na^+ for maximal activity. This was optimal at a concentration of 0.4 M with no other salt being able to substitute for NaCl. The ratio of Na^+ to K^+ dependent NADH oxidase activity was 3.4 (Figure 3.1). These results are comparable with the results obtained for *V. alginolyticus* 138-2 (Figure 3.2).

3.2. NADH and dNADH specificity.

Membranes from *V. harveyi* BB7 were capable of using either NADH or dNADH as a substrate for NADH oxidase. The ratio of NADH to dNADH oxidase activity was 1.8 (Figure 3.1) which is comparable to parallel results obtained with *V. alginolyticus* 138-2 membranes (Figure 3.2). The dNADH oxidase activity is due exclusively to the sodium-dependent species of NADH-ubiquinone oxidoreductase. For the sodium-pump defective mutant, Nap 2, the Na^+ specificty of NADH oxidase is lost and the ability to oxidised dNADH is severely reduced (Figure 3.3).

Figure 3.1. Salt dependence and substrate specificity of NADH oxidase activity in *V. harveyi* membranes.

The assay mixture in 1 ml contained 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH (Δ,) or dNADH (□) and various concentrations of KCl (●) or NaCl (Δ, □). The assay was started by the addition of about 30 μg of membrane protein. For each data set the experiment was repeated four times, the average values are shown. Specific activity was measured in nmol NADH oxidised/min/mg protein.

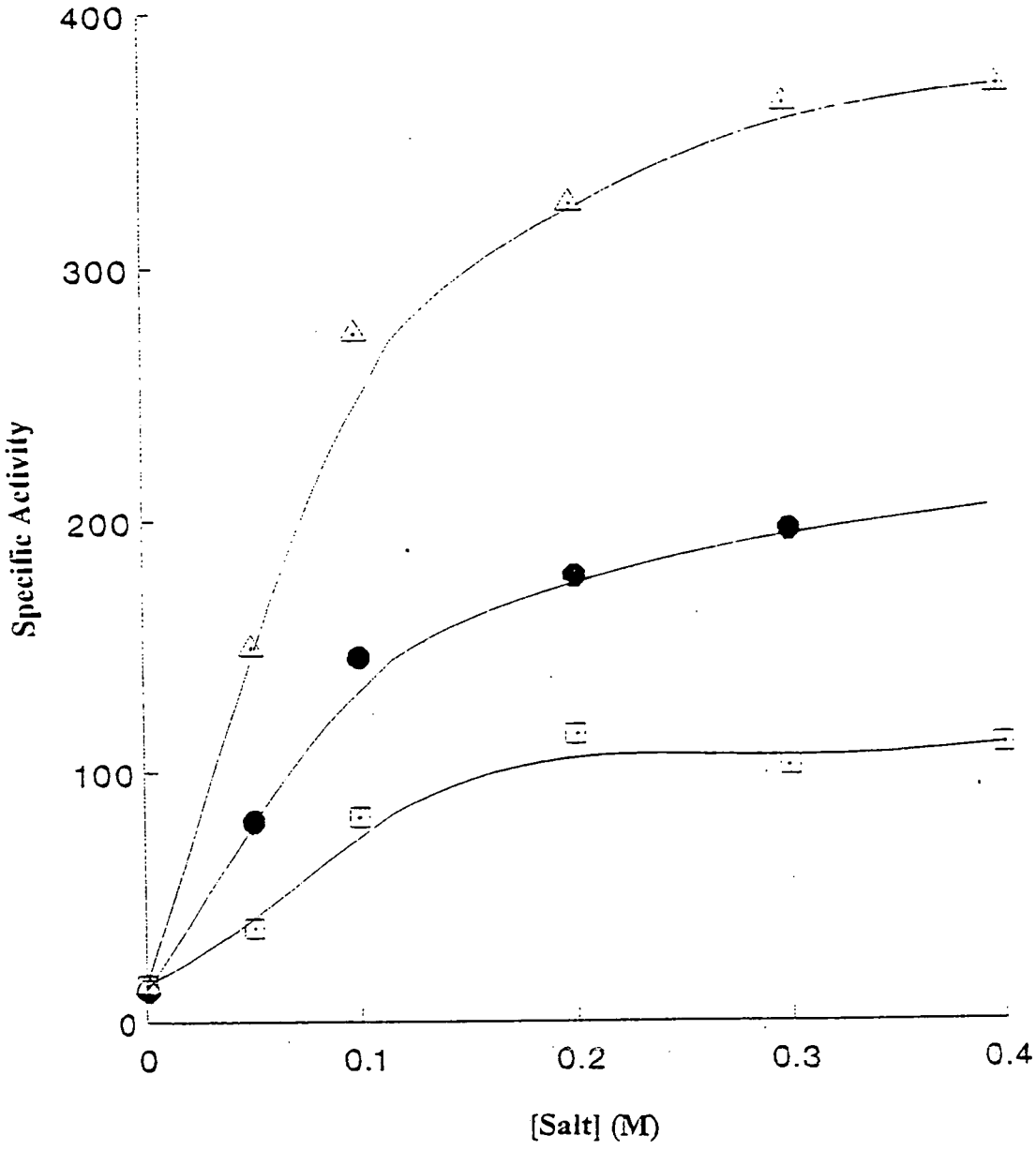
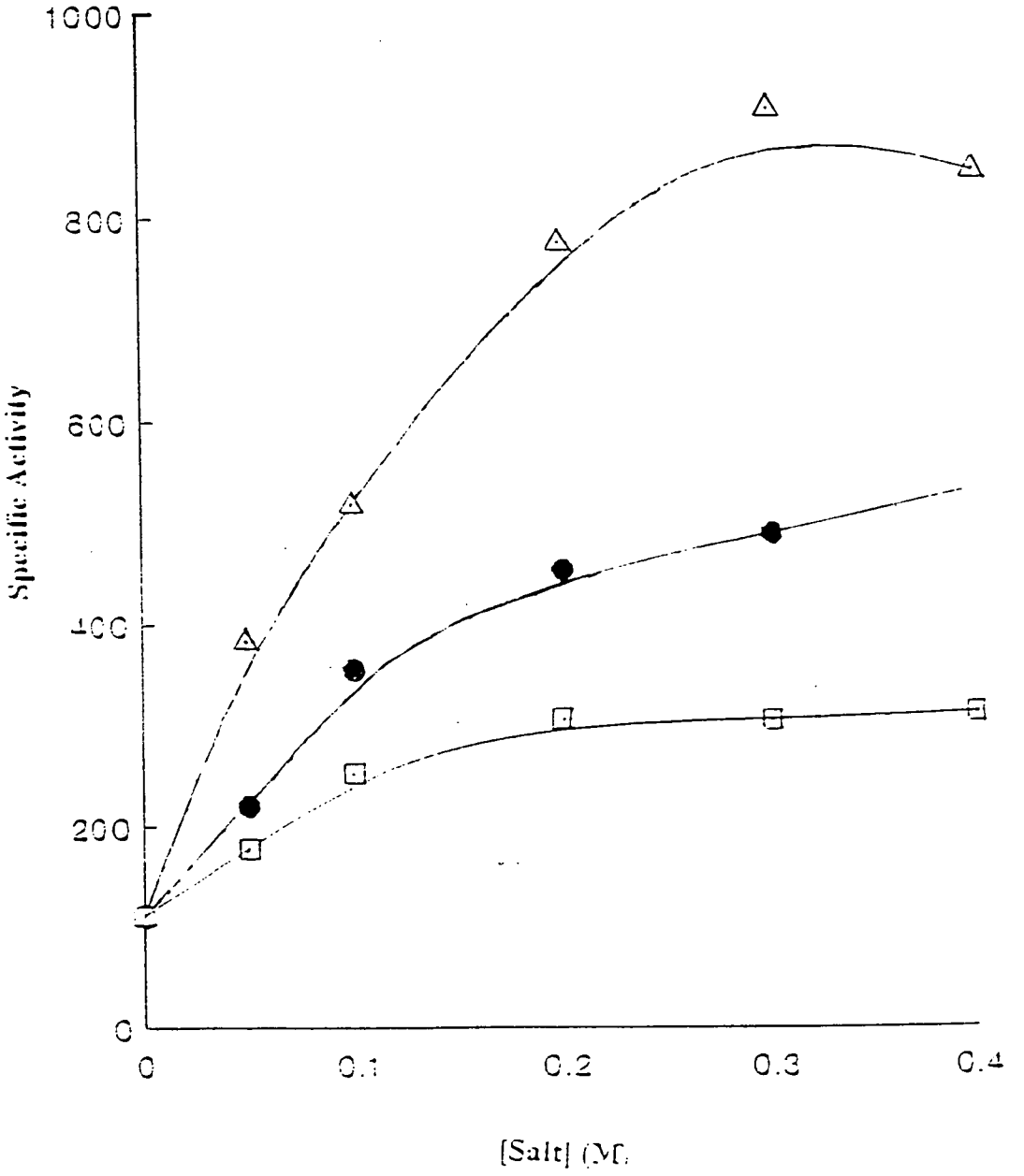

400
300
200
100
0
Specific Activity
0
0.1
0.2
0.3
0.4
[Salt] (M)

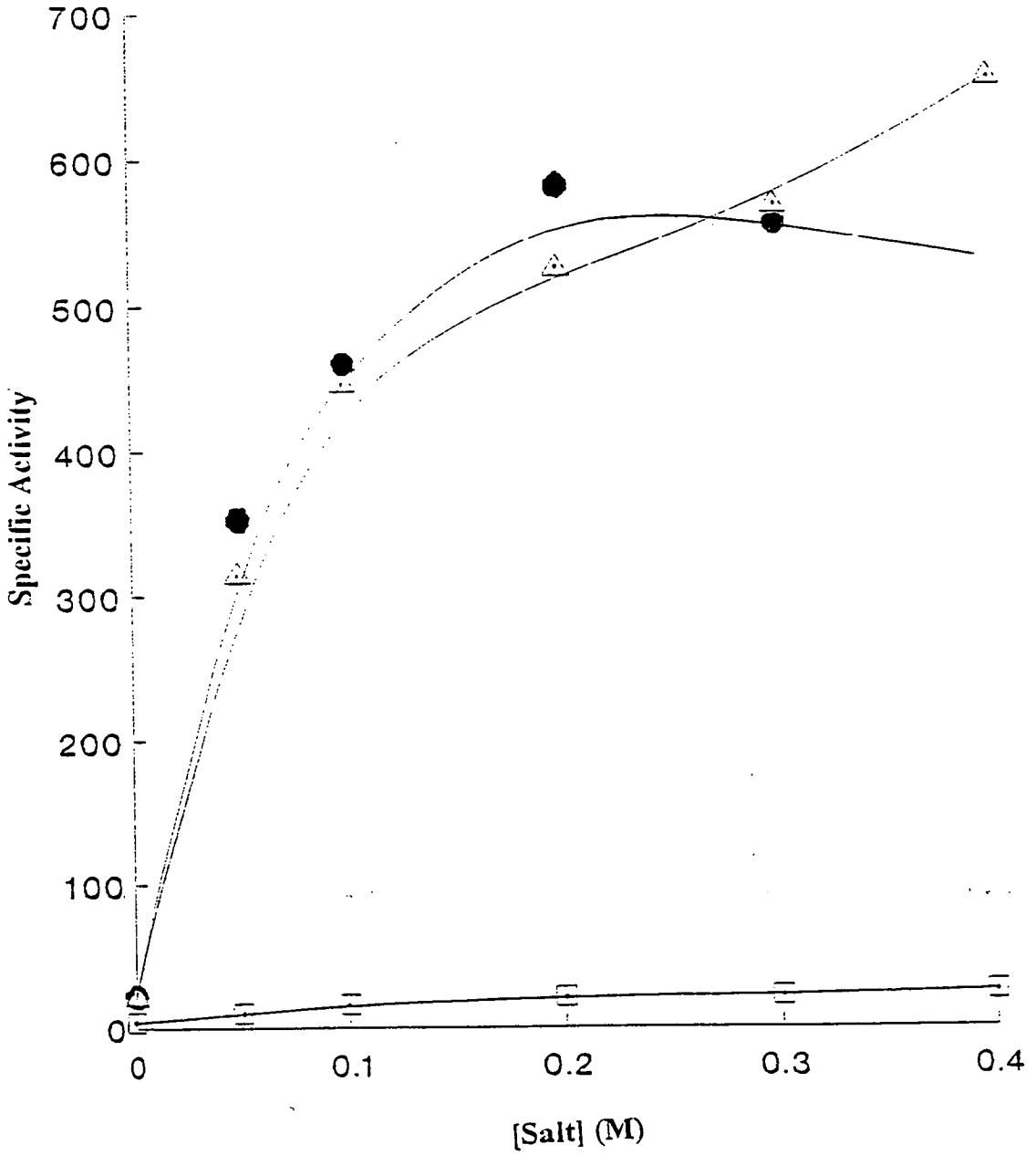
Figure 3.3. Salt dependence and substrate specificity of NADH oxidase activity in membranes of the sodium-pump defective mutant *V. alginolyticus* Nap 2.

The assay mixture in 1 ml contained 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH (Δ,) or dNADH (□) and various concentrations of KCl (●) or NaCl (Δ, □). The assay was started by the addition of about 30 μg of membrane protein. For each data set the experiment was repeated four times, the average values are shown.

Figure 3.2. Salt dependence and substrate specificity of NADH oxidase activity in *V. alginolyticus* membranes.

The assay mixture in 1 ml contained 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH (Δ, ●) or dNADH (□) and various concentrations of KCl (●) or NaCl (Δ, □). The assay was started by the addition of about 30 μg of membrane protein. Specific activity was measured in nmol NADH oxidised/min/mg protein. For each data set the experiment was repeated four times, the average values are shown.


1000
800
600
400
200
0
Specific Activity
0
0.1
0.2
0.3
0.4
[Salt] (M)


700
600
500
400
300
200
100
0
Specific Activity
0
0.1
0.2
0.3
0.4
[Salt] (M)

3.3. Molecular weight determination of the sodium-dependent NADH-ubiquinone oxidoreductase complex.

The approximate molecular weight of the sodium-dependent NADH-ubiquinone oxidoreductase complex was determined by running a 1% (w/v) Triton X-100 solubilised membrane sample on a 7.5% and 10% native PAGE gel. After electrophoresis the gel was zymogram stained using NADH and dNADH as the electron donor. The approximate molecular weight of the single purple band (due to the sodium-dependent NADH-ubiquinone oxidoreductase) could be determined by calculating the Rf and comparing it with the calibration curve obtained for the native gel using molecular weight markers. The molecular weight of the complex was calculated to be approximately 254 kD.

Stability studies.

Experiments were set up to determine the stability of the sodium-dependent NADH-ubiquinone oxidoreductase over time under varying conditions. Samples were assayed for NADH and dNADH oxidase activity before and after exposure to the different conditions. All the experiments were carried out at 4 °C and pH 8 unless otherwise stated.

3.4. Thermostability.

Membrane samples were incubated for 24 h at -80 °C, -20 °C, 4 °C and 20 ° C. The results show that 91% of the total dNADH oxidase activity was lost after 24 h at 20 °C. This implies that the sodium-dependent NADH-ubiquinone oxidoreductase is highly thermolabile in crude extracts. At 4 °C 19% of total activity was lost while at -20 °C and -80 °C only 11% and 2% respectively was lost (Figure 3.4). From these results it became apparent that manipulation of enzyme samples should be done at 4 °C where possible with all long term storage taking place at -80 °C.

3.5. pH stability.

The pH stability of the sodium-dependent NADH-ubiquinone oxidoreductase was tested after 5 h and 24 h over the range pH 5-11. The results show that the enzyme was unstable outside the range pH 6-10 with 100% losses in total activity after 24 h at pH 5 and pH 11. The enzyme was most stable between pH 8 and pH 9 (Figures 3.5).

3.6. Stability in the presence of salt.

The stability of the sodium-dependent NADH-ubiquinone oxidoreductase in the presence of NaCl was tested over the range 0-200 mM for 24 h. The results show that in the absence of NaCl 34% of the total activity was lost after 24 h. The enzyme was most stable over the 50-150 mM NaCl range with a 20% in loss in total activity after 24 h (Figure 3.6). These results might be expected since the 50-150 mM NaCl range most closely mimics physiological saline conditions.

3.7. Stability in the presence of protease inhibitors.

The protease inhibitors PMSF, Pepstatin A and EDTA were all tested to see if they would improve the stability of the sodium-dependent NADH-ubiquinone oxidoreductase in crude extracts. The inclusion of 1 mM EDTA improved the stability of the enzyme by 5% when compared to the control after 24 h. The inclusion PMSF and Pepstatin A did not improve stability, for both a small decrease in activity was observed when compared to the control sample (Figure 3.7).

Figure 3.4. Thermostability of dNADH oxidase activity in 1% (w/v) Triton X-100 extracted *V. harveyi* membranes.

Samples were stored at -80 °C, -20 °C, 4 °C and 20 °C for 24 h. Assays for dNADH oxidase activity were carried out before and after storage. About 30 μg of membrane protein was incubated with 20 mM KCN. Assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl was added and the assay started by the addition of 100 μM menadione. The total activities recovered (nmol dNADH oxidised/min) were calculated as a percentage of the starting total. For each data set the experiment was repeated three times, the average values are shown.

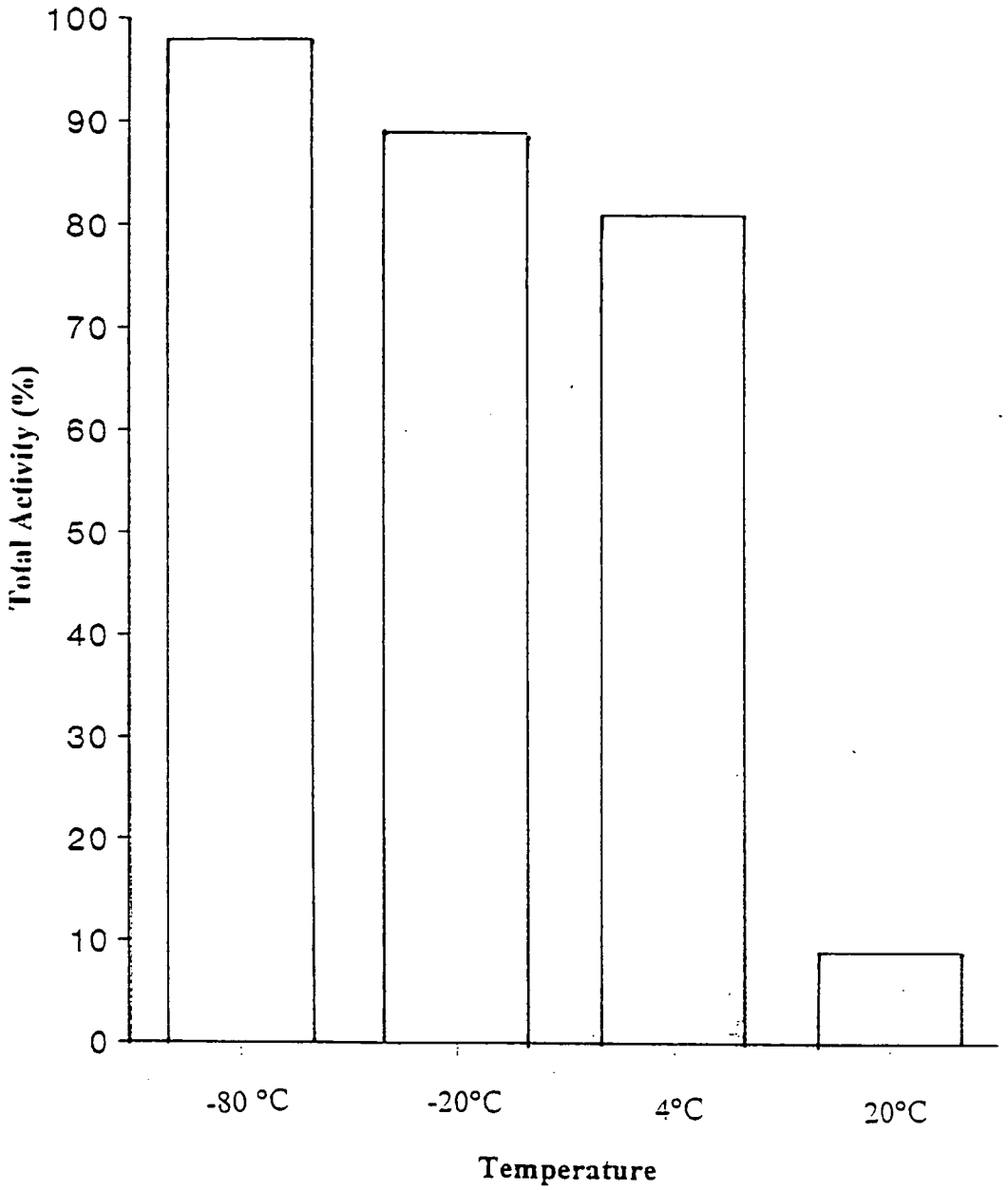
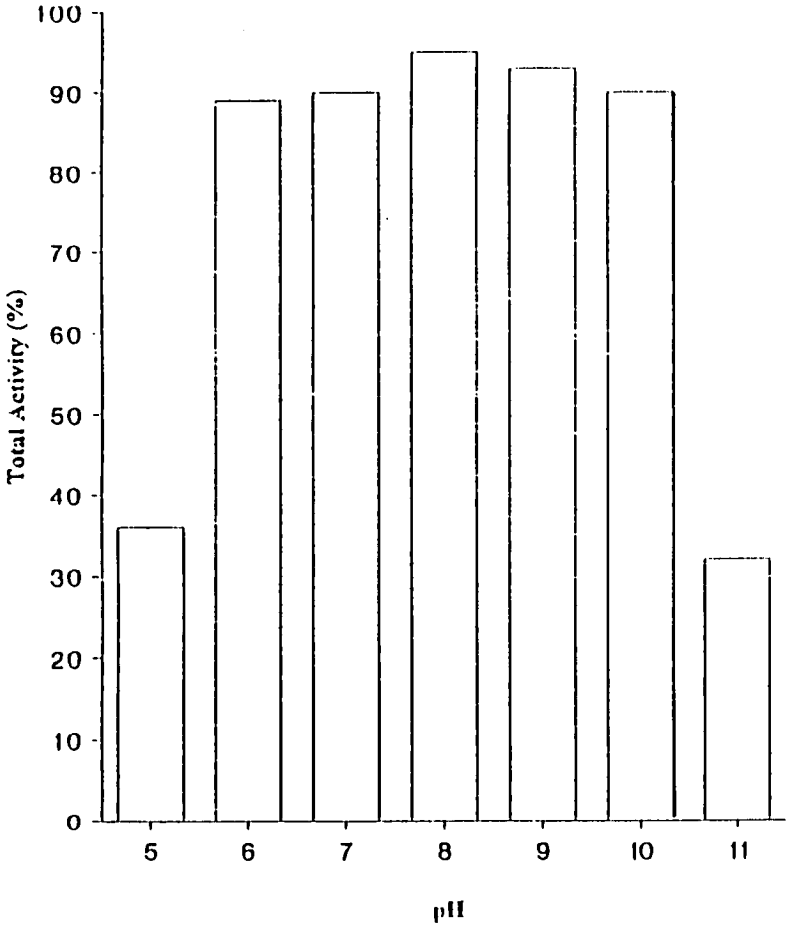

100
90
80
70
60
50
40
30
20
10
0
Total Activity (%)
-80 °C
-20°C
4°C
20°C
Temperature

Figure 3.5. The pH stability of dNADH oxidase activity in 1% (w/v) Triton X-100 extracted *V. harveyi* membranes.

Samples were incubated over the pH range pH 5 to pH 11 for a) 5 h and b) 24 h at 4 ° C. Assays for dNADH oxidase activity were carried out before and after incubation. About 30 μg of membrane protein was incubated with 20 mM KCN. Assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl was added and the assay started by the addition of 100 μM menadione. The total activities recovered (nmol dNADH oxidised/min) were calculated as a percentage of the starting totals. For each data set the experiment was repeated three times, the average values are shown.

a)


Total Activity (%)
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
10
11
pH

b)

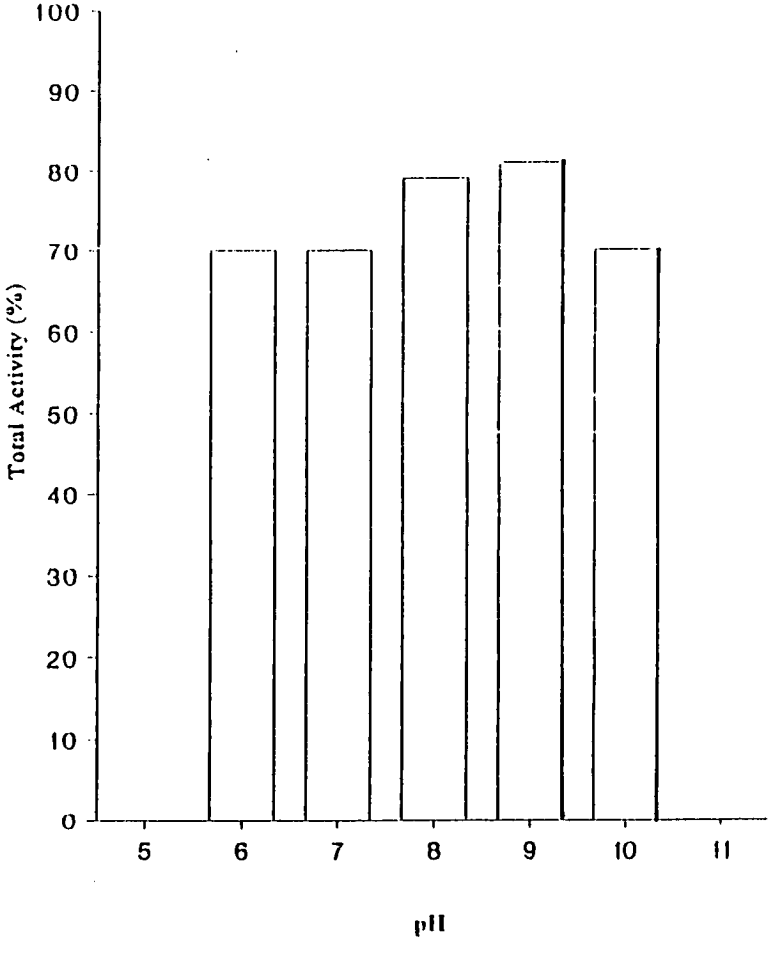

Total Activity (%)
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
10
11
pH

Figure 3.6. The stability of dNADH oxidase activity in 1% (w/v) Triton X-100 extracted *V. harveyi* membranes was determined in the presence of various NaCl concentrations.

Samples were incubate in 50 mM, 100 mM, 150 mM and 200 mM NaCl at 4°C for 24 h. Assays for dNADH oxidase activity were carried out before and after incubation. About 30 μg of membrane protein was incubated with 20 mM KCN. Assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl was added and the assay started by the addition of 100 μM menadione. The total activities recovered (nmol dNADH oxidised/min) were calculated as a percentage of the starting total. For each data set the experiment was repeated three times, the average values are shown.

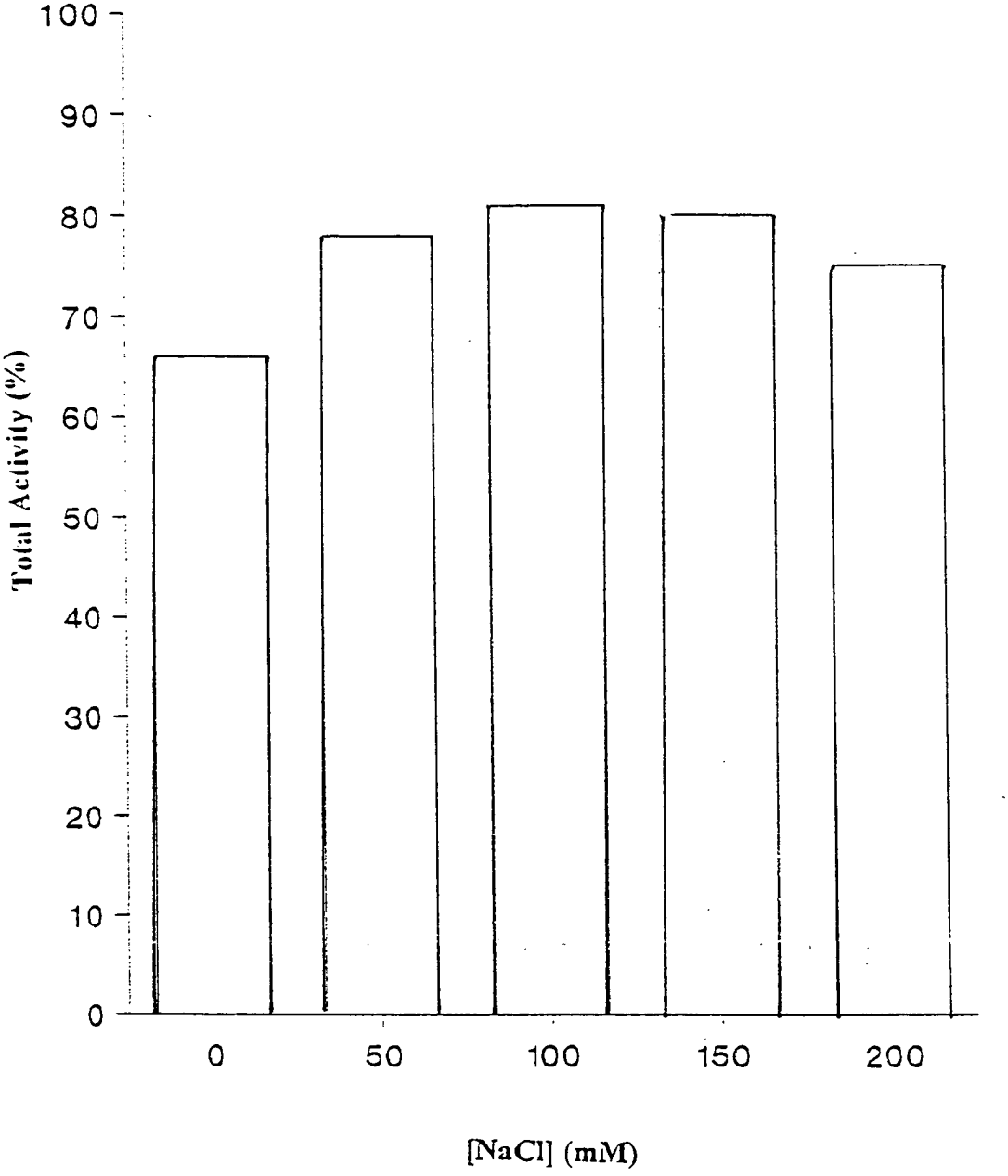
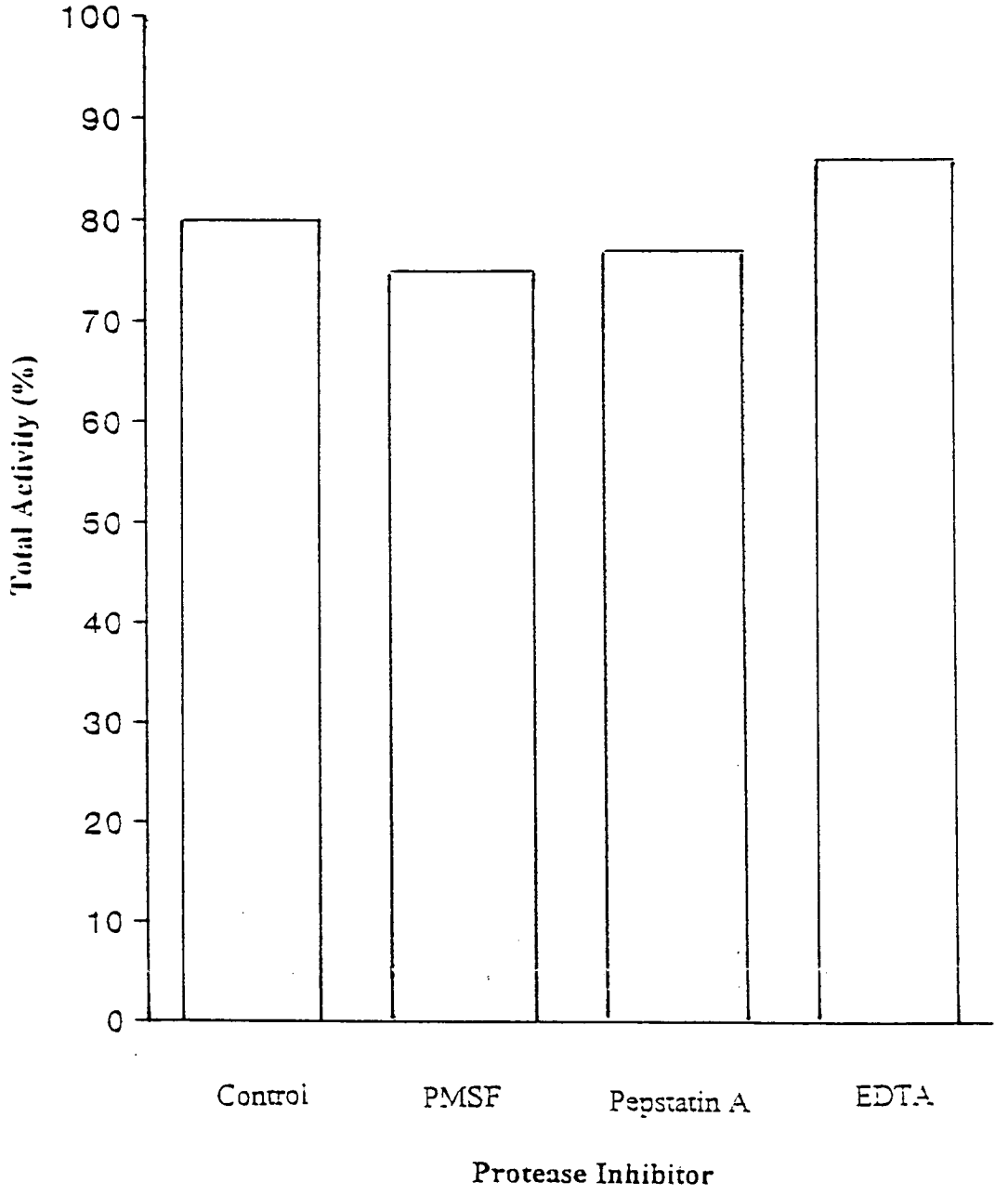

100
90
80
70
60
50
40
30
20
10
0
Total Activity (%)
0
50
100
150
200
[NaCl] (mM)

Figure 3.7. The stability of dNADH oxidase activity in 1% (w/v) Triton X-100 extracted *V. harveyi* membranes was determined in the presence of various protease inhibitors.

Samples were stored in the protease inhibitors at 4°C for 24 h. Assays for dNADH oxidase activity were carried out before and after storage. About 30 μg of membrane protein was incubated with 20 mM KCN. Assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl was added and the assay started by the addition of 100 μM menadione. The total activities recovered (nmol dNADH oxidised/min) were calculated as a percentage of the starting total. For each data set the experiment was repeated three times, the average values are shown.


100
90
80
70
60
50
40
30
20
10
0
Total Activity (%)
Control
PMSF
Pepstatin A
EDTA
Protease Inhibitor

3.8. Stability in the presence of reducing agents.

The stability of the sodium-dependent NADH-ubiquinone oxidoreductase was tested in the presence of the reducing agents DTT and β-mercaptoethanol. DTT and β-mercaptoethanol both helped stabilise the enzyme with only 15 and 9% losses in total activities respectively compared to a 23% loss in the control sample (Figure 3.8). These agents may be effective in promoting stability by mimicking the reducing environment found in the bacterial cell.

3.9. Inhibitors specificity.

A number of cations were tested to see if they were capable of inhibiting the sodium-dependent NADH-ubiquinone oxidoreductase and to see if inhibition was reversible. The results show that both Zn^{2+} and Pb^{2+} are capable of reversibly inhibiting this enzyme. Ag^{+}, Cu^{2+} and Cd^{2+} were also able to inhibit activity but their effects were irreversible (Table 3.1). These results correspond to the ones obtained for the NADH-ubiquinone oxidoreductase from *V. alginolyticus* (Bourne and Rich, 1992).

Figure 3.8. The stability of dNADH oxidase activity in 1% (w/v) Triton X-100 extracted *V. harveyi* membranes was determined in the presence of reducing agents .

Samples were stored in either DTT or βme at 4°C for 24 h. Assays for dNADH oxidase activity were carried out before and after storage. About 30 μg of membrane protein was incubated with 20 mM KCN. Assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl was added and the assay started by the addition of 100 μM menadione. The total activities recovered (nmol dNADH oxidised/min) were calculated as a percentage of the starting total. For each data set the experiment was repeated three times, the average values are shown.

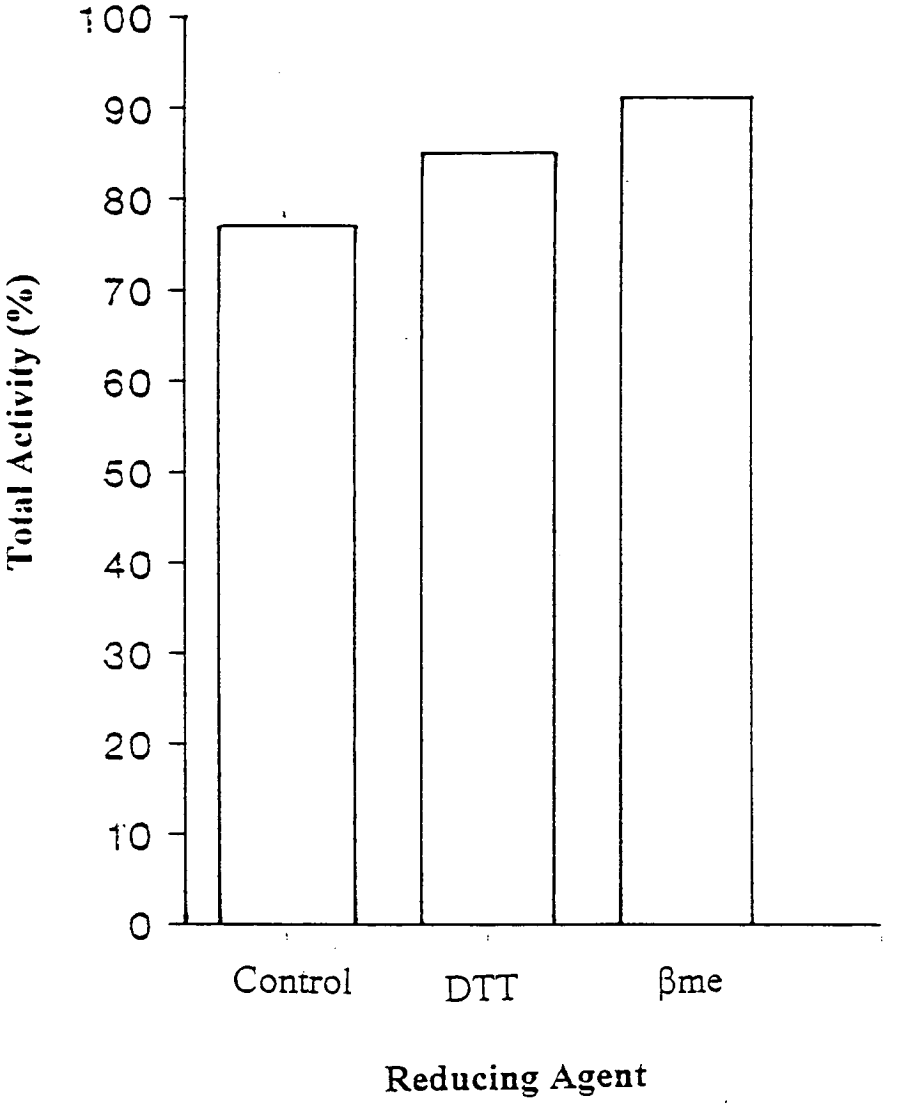

100
90
80
70
60
50
40
30
20
10
0
Total Activity (%)
Control
DTT
βme
Reducing Agent

Table 3.1. Cation inhibitors of the dNADH oxidase activity from *V.harveyi* membranes.

Inhibitor tested	Inhibition(%)	Recovery of Activity(%)
Zn^{2+}	>98	85
Pb^{2+}	>98	81
Ag^{+}	>99	0
Cu^{2+}	>99	0
Cd^{2+}	>99	0

Samples of 1% (w/v) Triton X-100 extracted *V. harveyi* membranes were incubated with 10 μM of the appropriate cation and then assayed for dNADH oxidase activity. Assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl was added and the assay started by the addition of 100 μM menadione. The amount of inactivation was calculated as a percentage of the starting total activity (nmol dNADH oxidised/min). The reversibility of the inhibition was determined by adding 5 mM EDTA to the reaction mixture, and detecting the amount of activity recovered after a 5 min incubation. For each data set the experiment was repeated four times, the average values are shown.

3.10. Conclusions.

Initial characterisation of the sodium-dependent NADH-ubiquinone from *V. harveyi* has revealed an enzyme which is very similar to the NQR1 from *V. alginolyticus* described by Tokuda and Unemoto (1981). The salt dependence and substrate specificities are comparable and the active complexes have approximately the same molecular weights. Another similarity between these enzymes is the fact that both are inhibited in the same way by a number of metal cations which suggests that they have similar catalytic sites (Bourne and Rich, 1992).

These findings are not suprising in that it appears that sodium-dependent NADH-ubiquinone oxidoreductases are distributed not only among *Vibrio*s but in a number of different bacteria including *Bacillus* species and *Klebsiella pneumoniae* (Vagina *et al.*, 1991; Dimroth and Thomer, 1989). There is also the strong possibility that sodium-dependent NADH-ubiquinone oxidoreductases like their H^+ translocating equivalents will be conserved. One striking difference between the two sodium-dependent NADH-ubiquinone oxidoreductases is the specific activities for NADH and dNADH oxidase. In *V. alginolyticus* the specific activity is almost double the value obtained for *V. harveyi*. This may explain why *V. alginolyticus* grows optimally at Na^+ concentrations of 3% while *V. harveyi* grows optimally at a concentration of 2% Na^+ (Hayashi and Unemoto, 1984; Belas *et al.*,1984).

The sodium-dependent NADH-ubiquinone oxidoreductases from *V. harveyi* is particularly unstable in crude extracts at room temperature and proteases may be responsible for the large losses in activity. Inclusion of the protease inhibitors PMSF and Pepstatin A does not improve recoveries in activity; however addition of EDTA and storage at 4 °C or below does help to stabilise the enzyme. The enyme is most stable in the pH range pH 7 to pH 9, in 100 mM NaCl and in the presence of

reducing agents. These conditions mimic physiological conditions and will be an important consideration when manipulating and purifying the enzyme complex.

Chapter 4

Transposon mutagenesis.

Transposon mutagenesis offers several advantages as an approach to cloning. Insertion of a transposon into a gene leads to a complete loss of function of that gene. Additionally the defect is physically linked to a selectable marker. Restriction digests from mutants will contain fragments possessing disrupted genes truncated by a drug resistance insert. These fragments can then be cloned and the flanking regions either sequenced directly or used as probes to pick out full length clones from a wild type library.

Transposons were introduced into *V. harveyi* BB7 using either of two delivery systems. These were conjugally transfered using a plasmid vector and bacteriophage-mediated P1 transduction.

4.1. Conjugal transfer.

Transposon vectors were conjugated into *V. harveyi* BB7 from *E. coli* donor strains using the filter mating method. Vectors which would be unlikely to replicate in *V. harveyi* were employed. In this way drug resistant exconjugants should represent transposon insertion events and not merely cells maintaining the plasmid vector.

Two groups of vectors were tested for their suitability to mobilise transposons into *V. harveyi*; the first group were narrow host range vectors which had ColE1 or p15A origins of replication (Ely, 1985, Simon *et al.*; 1986; Fellay *et al.*, 1988). This group would be unlikely to replicate outwith *E. coli* or closely related organisms. The second group were *pir* dependent vectors which contain a modified R6K origin of replication (Miller *et al.*, 1986; Lorenzo *et al.*, 1990). These vectors can only

replicate in strains which provide the R6K *pir* protein and should therefore not replicate in *V. harveyi*.

The conjugation frequencies from each of the matings experiments was calculated per donor. Maintenance of vectors was determined by making plasmid DNA from ten exconjugants for each of the mating. The narrow host range vectors gave high frequencies of conjugation, however all exconjugants appeared to maintain the plasmids and therefore were not transposon mutants. No exconjugants were observed for the *pir* dependent vectors. None of the vectors tested were suitable for transposon mutagenesis in *V. harveyi* BB7. The results for the conjugation experiments are summarised in Table 4.1.

4.2. Bacteriophage mediated P1 transduction.

Bacteriophage P1 was used to deliver the transposon mini-Mu *lac* into *V. harveyi* BB7 (Martin, *et al.*, 1989). Tetracycline resistant colonies were screened on minimal media. Auxotrophic mutants were observed at a frequency of 1%, indicating that tetracycline resistance colonies were in fact transposon mutants. Colonies were screened on plates containing CCCP at pH 8.5 to screen for mutants of the sodium-pump. In the presence of CCCP proton gradients are dissipated. Under these conditions sodium-pump mutants would be unable to use a sodium gradient as an alternative and would therefore be unable to grow (See introduction section 1.4). Of the 10,000 drug resistant colonies screened 16 were sensitive to 10 μM CCCP at pH 8.5.

Table 4.1. Conjugation of narrow host range and *pir* dependent vectors into *V. harveyi* BB7 Rif^r.

Vector/Transposon	Conjugation Frequency	Maintained
pRK2013 Tn 10[1]	3.4×10^{-3}	+
pRK2013 Tn5-132[1]	4.6×10^{-2}	+
pJFF350 Tn5[1]	1.1×10^{-2}	+
pSUP1021 Tn5[2]	2.3×10^{-2}	+
pSUP2021 Tn5[1]	1.7×10^{-2}	+
pSUP10141 Tn5[1]	2.1×10^{-2}	+
pUT mini Tn 5[3]	$<3.5 \times 10^{-8}$	?
pRKT733 Tn Pho A[3]	$<3.5 \times 10^{-8}$	?

Conjugation was carried out using the filter mating method described in the Materials and Methods section. Exconjugants were selected on antibiotic plates using the drug resistance marker of the transposon and the conjugation frequency was determined. Plasmid preparations were carried out on exconjugants to see if the vectors were being maintained ([1] ColE1 replicon, [2] p15A replicon and [3] *pir* dependent modified R6K replicon). For each data set the experiment was repeated twice, the average values are shown.

4.3. Characterisation of transposon mutants.

The 16 CCCP sensitive strains (CSM 1-16) were characterised further in terms of their NADH oxidase activity in the presence of Na^+ and K^+, their ability to utilise dNADH as a substrate and the presence of a 254 kD band detected by zymogram staining of native PAGE gels using dNADH as an electron donor. The CSM mutants are compared with wild type Vibrio strains (Figures 3.1 and 3.2) and the sodium pump mutant Nap 2 (Figure 3.3), in terms of the ratio of Na^{+-} to K^{+-} dependent NADH oxidase activity and the ratio of NADH to dNADH oxidase activity in the presence of Na^+. A sodium pump defective mutants would be expected to have a NADH-ubiquinone oxidoreductase which is unable to utilise dNADH as a substrate and which has lost its sodium dependence. The results are summarised in Table 4.2. It would appear however that none of the mutants isolated were found to be defective in the sodium-dependent NADH-ubiquinone oxidoreductase activity.

Table 4.2. Enzymatic characterisation of the CCCP sensitive mutants and comparison with wild type Vibrio strains.

Membranes isolated from the various strains were extracted with 1% (w/v) Triton X-100. About 30 μg of membrane protein was added to 1 ml of assay buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and 0.4 M NaCl or 0.4 M KCl and the assays started by the addition of 100 μM menadione. Protein determinations were also carried out. The ratio of Na^+ to K^+ dependent NADH oxidase activity (nmol NADH oxidised/min/mg protein) and the ratio of NADH to dNADH oxidase activity (nmol NADH or dNADH oxidised/min/mg protein) in the presence of 0.4 M Na^+ was measured. Solubilised membrane samples from each strain were electrophoresed on a 10% native PAGE at 150 V for 1 h. After electrophoresis PAGE gels were zymogram stained using dNADH as an electron donor. Sodium-dependent NADH-ubiquinone oxidoreductase activity was detected as a deep blue/purple band and from Rf values the approximate molecular weights of the enzyme species from the different strains could be determined. For each data set the experiment was repeated three times, the average values are shown.

Strain	**Na^+/ K^+**	**NADH/ dNADH**	**254 kD Band**
V. alginolyticus 138-2	2.7	1.6	+
Nap 2	1.3	27.4	-
V. harveyi BB7	3.4	1.8	+
CSM 1	5.6	2.9	+
CSM 2	5.7	1.6	+
CSM 3	3.7	1.1	+
CSM 4	3.9	3.4	+
CSM 5	5.7	1.4	+
CSM 6	4.4	2.8	+
CSM 7	3.1	2.2	+
CSM 8	3.7	1.6	+
CSM 9	3.7	1.6	+
CSM 10	5.7	1.4	+
CSM 11	3.4	1.3	+
CSM 12	4.7	1.8	+
CSM 13	3.3	1.3	+
CSM 14	5.1	1.6	+
CSM 15	4.8	1.2	+
CSM 16	4.2	1.5	+

4.4. Conclusions.

Transposon mutagenesis using narrow host range vectors was unsuccessful in *V. harveyi*. The *pir* dependent group of plasmids have been used successfully to deliver transposons to a number of Gram negative bacteria including *Vibrio cholerae* (Lorenzo et al., 1990). No conjugation was observed for the *pir* group of transposon vectors tested in *V. harveyi*. Initially host restriction systems were suspected as a problem although this now seems unlikely since other vectors were able to conjugate into *V. harveyi* at high frequencies. ColE1 based plasmids have also been used succesfully to perform transposon mutagenesis in a number of gram-negative soil and water bacteria including *Pseudomonas putida*, *Rhizobium leguminosarum* and *Paracoccus denitrificans* (Fellay *et al.*, 1989) and so were tested in *V. harveyi* for their suitability as transposon delivery systems. In *V. harveyi* ColE1 replicons were maintained stably so although drug resistant exconjugants were observed at a high frequency none of these strains were transposon mutants. From the results on ColE1 replicon maintenance it appears that *V. harveyi* and *E. coli* are more closely related than was first thought.

Bacteriophage mediated P1 transduction was used succesfully to deliver the transposon mini-Mu *lac* into *V. harveyi*. The frequency of auxotrophic mutations observed amongst the drug resistant strains was approximately 1% and this indicated that every drug resistant strain represented a transposon mutant and not merely a bacterial strain maintaining P1 DNA. When tested for enzymatic activity none of the 16 CCCP sensitive strains were found to be defective in the sodium-dependent NADH-ubiquinone oxidoreductase activity.

A possible explanation for the CCCP sensitive phenotype observed in the transposon mutants is that the genes inactivated by transposon insertion may have essential roles in cellular metabolism and may code for components of other primary

or secondary transporters which use sodium as a coupling ion, such as sodium ATPases or sodium/proton antiporters. These membrane transporters have important functions in energy coupling and in maintaining balances of solute concentration and pH. It is therefore feasible that mutations to the structural or regulatory genes from these system may be deleterious to growth at alkaline pH in the presence of the proton gradient uncoupler CCCP.

Recently the presence of a gene cluster coding for the sodium-dependent NADH-ubiquinone oxidoreductase in *V. alginolyticus* has been discovered (Beattie, *et al.*, 1994). It has been concluded that more than the three subunits as first proposed by Hayashi and Unemoto (1987) are involved in the sodium-dependent NADH-ubiquinone oxidoreductase activity from *V. alginolyticus*. Some of the CCCP sensitive mutants may therefore be affected in subunits not originally known to be associated with the NADH-ubiquinone oxidoreductase complex but which do not affect enzyme activity *in vitro*.

Another explanation as to why no sodium-dependent NADH-ubiquinone oxidoreductase defective strains were observed in *V. harveyi* could be that such a mutation may be lethal or sublethal. This is backed up by observations during growth of the sodium-pump defective mutant *V. alginolyticus* Nap2. A small colony phenotype was observed when comparing this strain to the wild type on standard complex medium. The absence of the-wild type sodium-dependent NADH-ubiquinone oxidoreductase enzyme has a serious effect on the aerobic growth of this strain. The sodium-dependent NADH-ubiquinone oxidoreductase plays a crucial role in aerobic respiration and a defect in this enzyme may well affect growth rates. Alternatively the accumulation of sodium-dependent NADH-ubiquinone oxidoreductase which is unable to assemble properly in the membranes may be toxic to this strain

4.5. Probing Vibrio chromosomal DNA with the *ndh* gene from *E. coli.*

The NDH 2 gene from *E. coli* has been cloned by Young *et al.*, (1981) and has been discussed in the introduction chapter section 1.2. This gene was used as a probe to see if any homologous genes could be identified in either *V. harveyi* or *V. alginolyticus* by southern blotting and DNA hybridisation. If hybridisation occurred then the *ndh* probe would be a useful tool in identifying NADH-ubiquinone oxidoreductase genes from *Vibrio* species. However under low stringency conditions no hybridisation was observed between the probe and either *V. harveyi* or *V. alginolyticus* chromosomal digests. This implies that there is no significant homology between the *ndh* gene from *E. coli* and any of the NADH-ubiquinone oxidoreductase genes from either *V. harveyi* or *V. alginolyticus*.

Chapter 5

Purification of the sodium-dependent NADH-ubiquinone oxidoreductase.

The availability of purified NADH-ubiquinone oxidoreductase would be useful for the further characterisation of the enzyme with respect to its inhibitor, subunit composition, FAD and FMN content and the N-terminal sequence of individual subunits.

Techniques which were adopted for preparative purification had to achieve purification while maintaining enzymatic activity. Generally methods which allowed the rapid processing of large amounts of material were preferred. All purification steps were carried out at 4 °C and for long term storage the enzyme samples were kept in 10% (w/v) glycerol at -80 °C.

5.1. Membrane protein solubilisation.

The first stage in the purification of any membrane protein is to select a suitable detergent for solubilising the protein from the membrane. Ideally a detergent would give selective solubilisation of the protein of interest while allowing maximal recovery of total activity. The detergent should be compatible with subsequent purification steps and would be economic to use. Several detergents were tested for their ability to solubilise the NADH-ubiquinone oxidoreductase from membranes of *V. harveyi*. The results are summarised in Table 5.1. Triton X-100 gave the best results for solubilisation in terms of enzyme activity extracted with little loss in the total activity, in fact 99% of the total activity was extracted together with 96% of the total protein. None of the detergents gave selective solubilisation and as Triton X-100 was economic to use it became the detergent of choice both for solubilising NADH-ubiquinone oxidoreductase from membranes and as a buffer additive to keep the enzyme in solution during subsequent processing.

Table 5.1. Extraction of sodium-dependent NADH-ubiquinone oxidoreductase from membranes of *V.harveyi* using different detergents.

Detergent	Enzyme Extracted (%)	Protein Extracted (%)
Triton X-100	99	96
Brij 36	91	80
Deoxycholate	75	58
Laurylsarcosine	98	95
CHAPS	87	70
Mega 8	73	65
Mega 9	99	95
Mega 10	99	96
Noxamine	96	93
Octyl-β–D-glucopyranoside	98	95
N-dodecylsucrose	99	95

Vibrio harveyi BB7 membranes were solubilised using a number of detergents. Membrane samples (1 ml of 5-10 mg/ml protein) were extracted in 1% (w/v) of detergent at 4°C for 30 min. Samples were then centrifuged at 100 000 g for 1h to remove unsolubilised material. The supernatant samples were pre-incubated in 20 mM KCN for 10 min, then assayed for NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assay was started by the addition of 100 μM menadione. Protein determinations were also carried out. The amount of total NADH oxidase activity (nmol NADH oxidised/min) and protein extracted was determined as a percentage of the totals before extraction. The average results from three extractions are shown.

5.2. Gel filtration chromatography.

Gel filtration separates molecules according to differences in molecular weight. The matrix used is normally uncharged and contains small pores the size of which is controlled by the degree of polymer cross linking. Molecules larger than the pores will only move between the particles and so travel through the bed fastest. Smaller molecules which can enter the gel pores are retarded more and so are eluted later. Molecules are therefore eluted in order of decreasing molecular weight (Reiland, 1971).

Sephacryl 300HR Chromatography.

The gel filtration S-300HR column was calibrated using the following molecular weight protein standards: urease, β-amylase, alcohol dehydrogenase, bovine serum albumin and carbonic anhydrase. The void volume for this column as determined using blue dextran was 200 ml.

A 1 % (w/v) Triton X-100 extracted *V. harveyi* membrane sample (5 ml containing 10.3 mg/ml protein) was applied to the S-300HR column. Fractions containing NADH and dNADH oxidase activity were those eluting between 240 and 265ml. A 2.5 fold increase in specific activity was observed with 95% of the starting total activity being recovered. These results are summarised in Table 5.2.

Sephacryl proved a useful matrix for the first stage in purifying the sodium-dependent NADH-ubiquinone oxidoreductase. The elution profile (Figure 5.1.) shows only one rather broad peak of many hydrophobic proteins suggesting that some non-specific aggregation may be occurring. However it did give some purification and was successful in removing proteases along with other low molecular weight species.

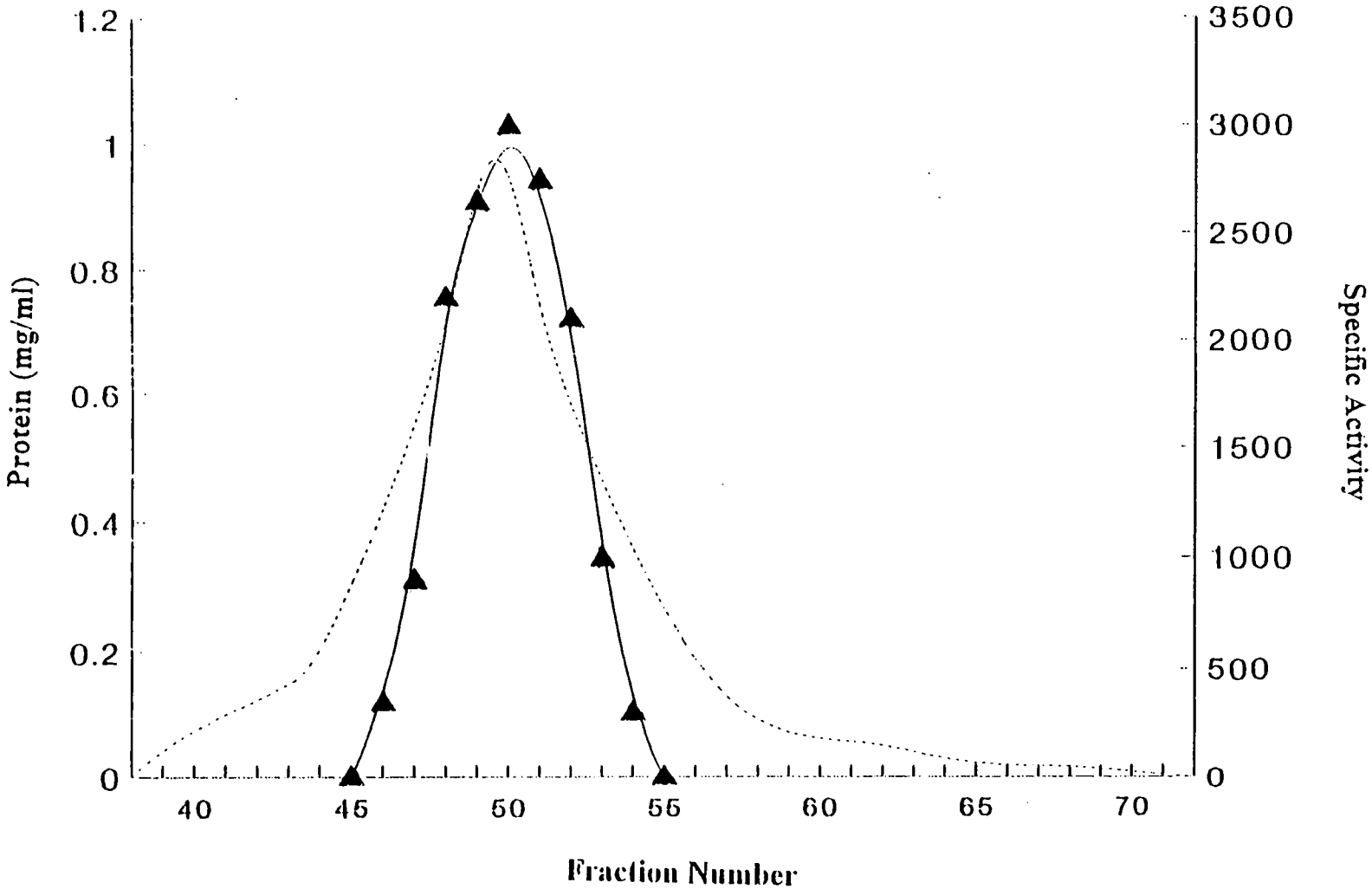
Table 5.2. Gel filtration chromatography of the sodium-dependent NADH-ubiquinone oxidoreductase from solubilised *V. harveyi* membranes on an S-300HR column.

Sample	Specific Activity (U/mg protein)	Total Activity (U)	Total Protein (mg)
Applied Sample	298	15 645	53
Eluted Sample	743	14 860	20

A 1% (w/v) Triton X-100 solubilised membrane sample (5 ml of 10.3 mg/ml protein) was applied to a S-300HR gel filtration column. Eluted fractions were pre incubated with 20 mM KCN for 10 min and then assayed for NADH and d NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined for before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

Figure 5.1. The elution profile of 1% (w/v) Triton X-100 extracted *V. harveyi* membranes chromatographed on an S-300HR gel filtration column.

An S-300HR gel filtration column was equilibrated with a solution containing 50 mM Tris-HCl, pH 8, 100mM NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100 at a flow rate of 30 ml/h. A 1% (w/v) Triton X-100 solubilised *V. harveyi* membrane sample (5 ml of 10.3 mg/ml protein) was applied onto the column and 5 ml fractions were collected. Eluted fractions were assayed for NADH and dNADH oxidase activity and specific activities were calculated (—▲—) (nmol NADH oxidised/min/mg protein). Protein concentrations were also determined (mg/ml) (-----).


1.2
1
0.8
0.6
0.4
0.2
0
Protein (mg/ml)
3500
3000
2500
2000
1500
1000
500
0
Specific Activity
40
45
50
55
60
65
70
Fraction Number

Superdex chromatography.

Superdex 200 is a high performance gel filtration column and was examined as a final purification step to remove the high molecular weight material which had co-purified with the sodium-dependent NADH-ubiquinone oxidoreductase after chromatography on columns of S-300HR, DEAE Sepharose, hydroxylapatite and Mono Q. The high molecular weight contaminants were greater than 400 kD as determined by native PAGE (Figure 5.3). A membrane sample (1 ml containing 0.3 mg/ml protein) which had been partially purified on columns of S-300HR, DEAE Sepharose, hydroxylapatite and Mono Q was applied to a prepacked Superdex 200 column. However after chromatography only one broad peak was detected on the elution profile (Figure 5.2), no increase in specific activity was observed (Table 5.3) and the high molecular weight contaminants remained (Figure 5.3). After zymogram staining using dNADH as the electron acceptor an activity band corresponding to the 250 kDa shown on the silver stained gel was observed.

The Superdex column failed to separate the high molecular weight contaminants from the sodium-dependent NADH-ubiquinone oxidoreductase. This suggests that non-specific aggregation of proteins may be occurring on the column. A reducing agent (10 mM β-mercaptoethanol) was included in the buffers to see if the interaction of sulphydryl groups on different proteins was the reason for poor resolution on this column. However no improvement in separation was observed when these conditions were adopted (Table 5.3).

Figure 5.2. The elution profile of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes chromatographed on a Superdex 200 gel filtration column.

A Superdex 200 gel filtration column was equilibrated with a solution containing 20 mM Tris-HCl, pH 8, 100mM NaCl, 5 mM EDTA, 0.1% (w/v) laurylsulfobetaine at a flow rate of 20 ml/h. A sample of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes (1 ml of 0.3 mg/ml protein) partially purified on columns of S-300HR, DEAE Sepharose, hydroxylapatite and Mono Q was applied onto the column and 2 ml fractions were collected. Eluted fractions were assayed for NADH and dNADH oxidase activity and measured for A_{280} (-----). Specific activities were calculated (—▲—) (nmol NADH oxidised/min/mg protein) and protein concentrations determined.

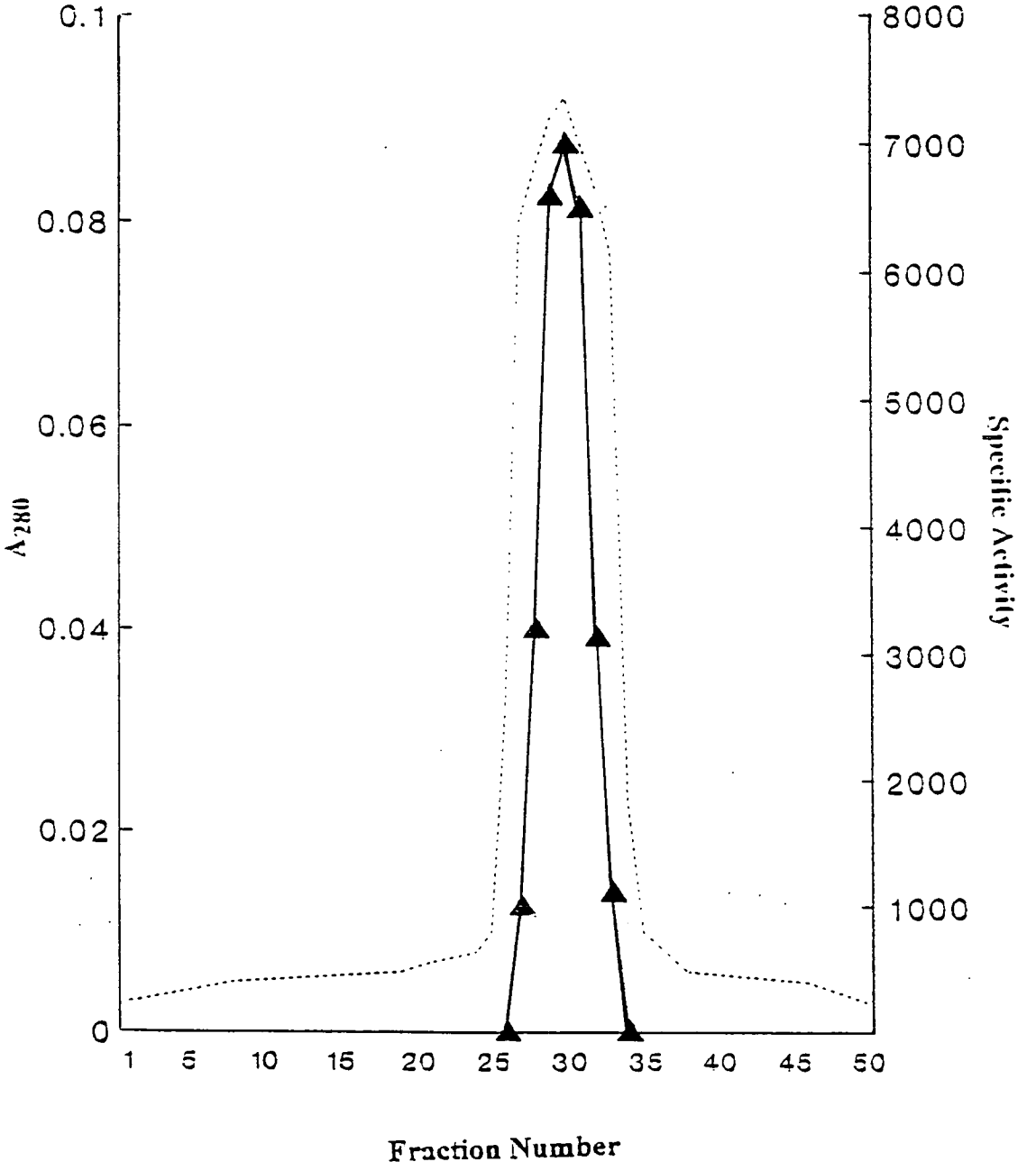

0.1
0.08
0.06
0.04
0.02
0
A_{280}
8000
7000
6000
5000
4000
3000
2000
1000
Specific Activity
1
5
10
15
20
25
30
35
40
45
50
Fraction Number

Figure 5.3. Native polyacrylamide gel of sodium-dependent NADH-ubiquinone oxidoreductase samples at various stages of purification.

Lane 1; molecular weight markers, lane 2; membrane proteins from *V. harveyi* unpurified, lanes 3, 4 and 5; sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes purified on columns of Sephacryl 300HR, DEAE Sepharose fast flow, Hydroxylapatite and Mono Q. Lanes 6, 7 and 8; sodium-dependent NADH-ubiquinone oxidoreductase partially purified on the previous columns, after chromatography on Superdex 200 gel filtration. Samples were run under native conditions on a 10% polyacrylamide gel. The gel was run at 150 V for 1 h and then silver stained.

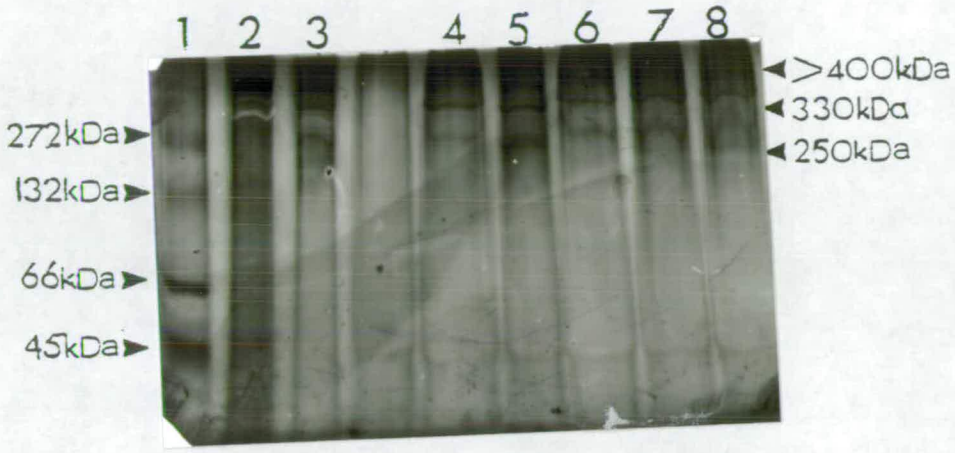

1 2 3 4 5 6 7 8
272kDa
132kDa
66kDa
45kDa
>400kDa
330kDa
250kDa

Table 5.3. Gel filtration chromatography of the partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes on a Superdex 200 column.

Buffer	Specific Activity (U/mg protein)	Total Activity (U)	Total Protein (mg)
Applied Sample	22 105	6 631	0.3
+10 mM β–me	21 860	6 120	0.28
-10 mM β–me	21 871	5 976	0.27

A membrane sample (1 ml of 0.3 mg/ml protein) which had been partially purified on columns of S-300HR, DEAE Sepharose, hydroxylapatite and Mono Q was applied to a prepacked Superdex 200 column. Chromatography was carried out with and without 10 mM β-mercaptoethanol (β-me) in the buffers. Eluted fractions were pre incubated with 20 mM KCN for 10 min and then assayed for NADH and d NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined for before and after chromatography. For each data set the experiment was repeated twice, the average values are shown.

5.3. Ion exchange chromatography.

Ion Exchangers are cross-linked polymer matrices with covalently attached ionised or ionisable groups and are used in chromatography to separate molecules according to charge. Charged molecules can reversibly adsorb to ion exchangers and can be bound or eluted by changing the ionic strength or pH of the buffering environment (Himmelhoch, 1971). Separation based on differences in ionic charge is a technique which is often chosen as an adjunct to gel filtration.

DEAE Sepharose fast flow.

DEAE Sepharose Fast Flow is an anion exchanger which has a very high capacity and excellent flow rates allow rapid processing of material. In binding experiments NADH and dNADH oxidase activity from 1% (w/v) Triton X-100 solubilised membranes bound to the matrix in 50 mM Tris-HCl, pH 8. Activity was eluted with an increasing linear gradient from 0 to 1 M NaCl. Three main chromatographic peaks were observed (Figure 5.4) with NADH and dNADH oxidase being eluted in the second peak at a NaCl concentration of 0.2 M NaCl.

For preparative chromatography a sample (25 ml containing 0.8 mg/ml protein) which had been partially purified on a column of S-300HR was bound onto a DEAE Sepharose column and then eluted with an increasing linear gradient from 0.1 M to 0.3 M NaCl. A five fold increase in specific activity was observed and 75% of the starting total activity was recovered (Table 5.4).

Figure 5.4. The elution profile of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes chromatographed on a DEAE Sepharose fast flow column.

A DEAE sepharose fast flow column was equilibrated with a solution containing 50 mM Tris-HCl, pH 8, 5 mM EDTA, 0.1% (w/v) Triton X-100 at a flow rate of 240 ml/h. A sample of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes (25 ml containing 0.8 mg/ml protein) partially purified on a column of S-300HR was loaded onto the column. Proteins were eluted with an increasing linear gradient from 0 to 1 M NaCl (-----) and 5 ml fractions were collected. Eluted fractions were assayed for NADH and dNADH oxidase activity. Specific activities were calculated (—▲—) (nmol NADH oxidised/min/mg protein) and protein concentrations determined (——) (mg/ml) .

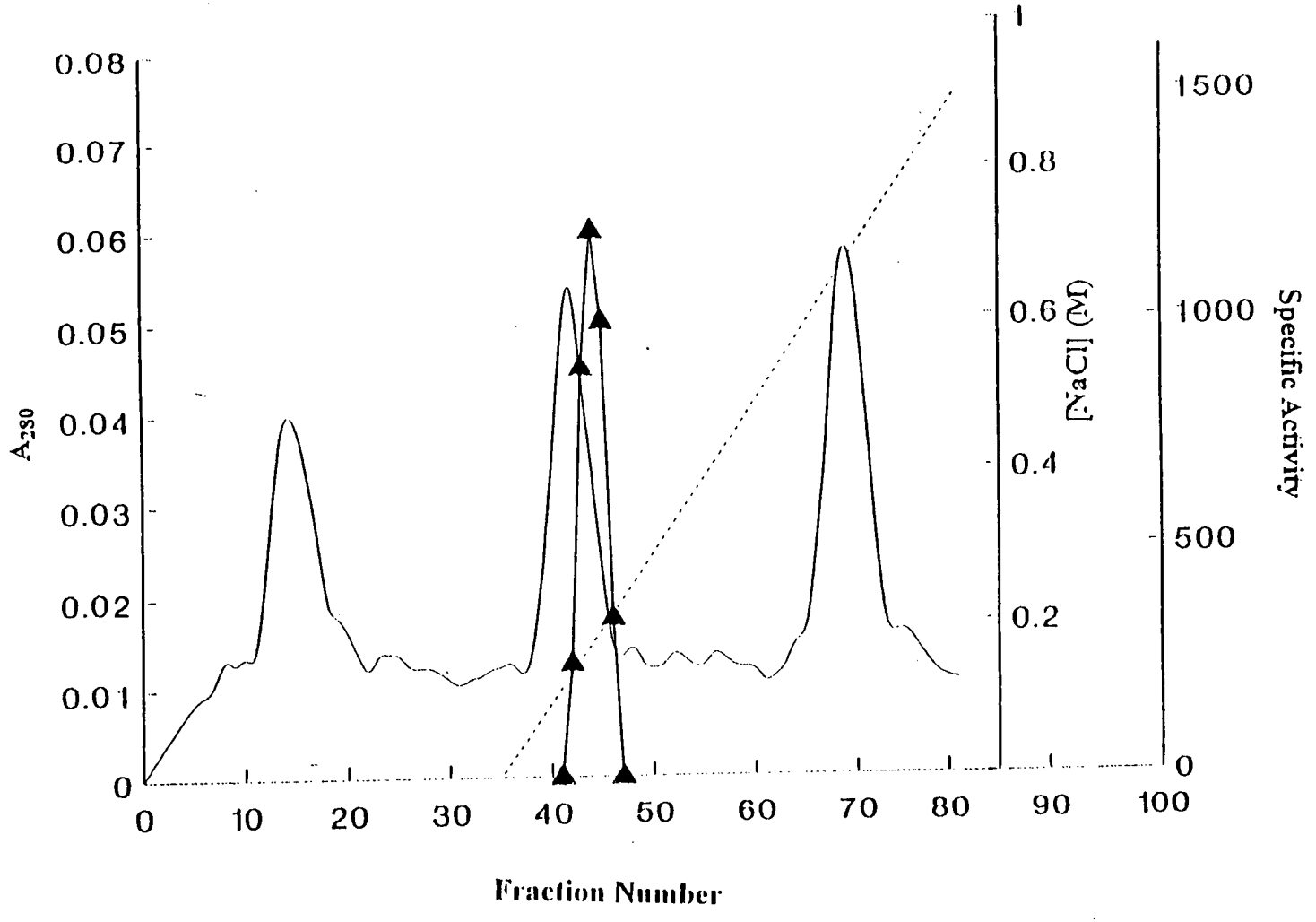

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
A280
0
10
20
30
40
50
60
70
80
90
100
Fraction Number
1
0.8
0.6
0.4
0.2
0
[NaCl] (M)
1500
1000
500
0
Specific Activity

Table 5.4. Chromatography of the partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes on a DEAE Sepharose fast flow matrix.

Sample	Specific Activity (U/mg protein)	Total Activity (U)	Total Protein (mg)
Applied	743	14 860	20
Eluted	3 715	11 733	3.2

A membrane sample (25 ml containing 0.8 mg/ml protein) partially purified on a column of S-300HR was bound onto a DEAE Sepharose column and then eluted with an increasing linear gradient from 0.1 M to 0.3 M NaCl. Eluted fractions were pre incubated with 20 mM KCN for 10 min and then assayed for NADH and d NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

Mono Q.

Mono Q is a prepacked FPLC column. It is also an anion exchanger and was tested to see if it could be used to complement the separation obtained on DEAE Sepharose. In binding experiments NADH and dNADH oxidase activity, from samples partially purified on columns of S-300HR and DEAE Sepharose, bound to the matrix in 20 mM Tris-HCl, pH 8. Proteins were eluted with an increasing linear gradient from 0 to 1 M NaCl. The dNADH and NADH oxidase activity was eluted at 0.4 M NaCl. The initial experiments gave poor results in terms of recovery of enzyme activity, only 39% of the total activity was recovered.

After chromatography the column was washed with 0.5 M NaOH and on application of a second gradient, material which eluted at the same NaCl concentration as the dNADH oxidase peak was observed. It was concluded that strong non-specific binding of some membrane sample components onto the Mono Q column was occurring. It may be that the high losses in activity experienced on Mono Q could be due to this effect. Buffer conditions were modified to minimise non-specific hydrophobic interactions between protein and the column. The results for these experiments are summarised in Table 5.5.

The 20 mM Tris-HCl pH 8 buffer containing 10% (w/v) ethylene glycol and 1% (w/v) Triton X-100 gave the best results with 70% of the total activity being recovered. These conditions were adopted for preparative chromatography of material which had been partially purified on columns of S-300HR, DEAE Sepharose and hydroxylapatite (2 ml containing 0.65 mg/ml protein was loaded). The elution profile (Figure 5.5.) shows two sharp peaks, with NADH and dNADH activity being eluted in the second peak at 0.4M NaCl. A 3 fold increase in specific activity was achieved with 40% of the starting total activity being recovered (Table 5.6).

Table 5.5. Chromatography of partially purified sodium-dependent NADH-ubiquinone oxidoreductase on Mono Q using different buffer additives.

Buffer Additive	Activity Recovered(%)
0.1% (w/v) Berol 185	8
0.1% (w/v) Triton X-100	39
1% (w/v) Berol 185	15
1% (w/v) Triton X-100	55
1% (w/v) Berol 185, 10% (w/v) ethylene glycol	30
1% (w/v) Triton X-100, 10% (w/v) ethylene glycol	70
1% (w/v) Berol 185, 20% (w/v) ethylene glycol	30
1% (w/v) Triton X-100, 10% (w/v) ethylene glycol	69

Membrane samples (5 ml containing 0.72 mg/ml protein) partially purified on columns of S-300HR and DEAE Sepharose were bound onto a Mono Q column and then eluted with an increasing linear gradient from 0 to 1 M NaCl. Chromatography was carried out using different buffer additives. Eluted fractions were assayed for dNADH oxidase and the total activity (nmol dNADH oxidised/min) recovered determined as a percentage of the starting total. For each data set the experiment was repeated three times, the average values are shown.

Figure 5.5. The elution profile of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes chromatographed on a Mono Q column.

A Mono Q column was equilibrated with a solution containing 20 mM Tris-HCl, pH 8, 1% (w/v) Triton X-100, 10% ethylene glycol at a flow rate of 60 ml/h. A sample of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membrane (2 ml containing 0.65 mg/ml protein) partially purified on a column of S-300HR, DEAE Sepharose and hydroxylapatite was loaded onto the column. Proteins were eluted with an increasing linear gradient from 0 to 1 M NaCl (——) and 1 ml fractions were collected. Eluted fractions were assayed for NADH and dNADH oxidase activity and monitored for A_{280} (----). Specific activities were calculated (—▲—) (nmol NADH oxidised/min/mg protein) and protein concentrations determined.

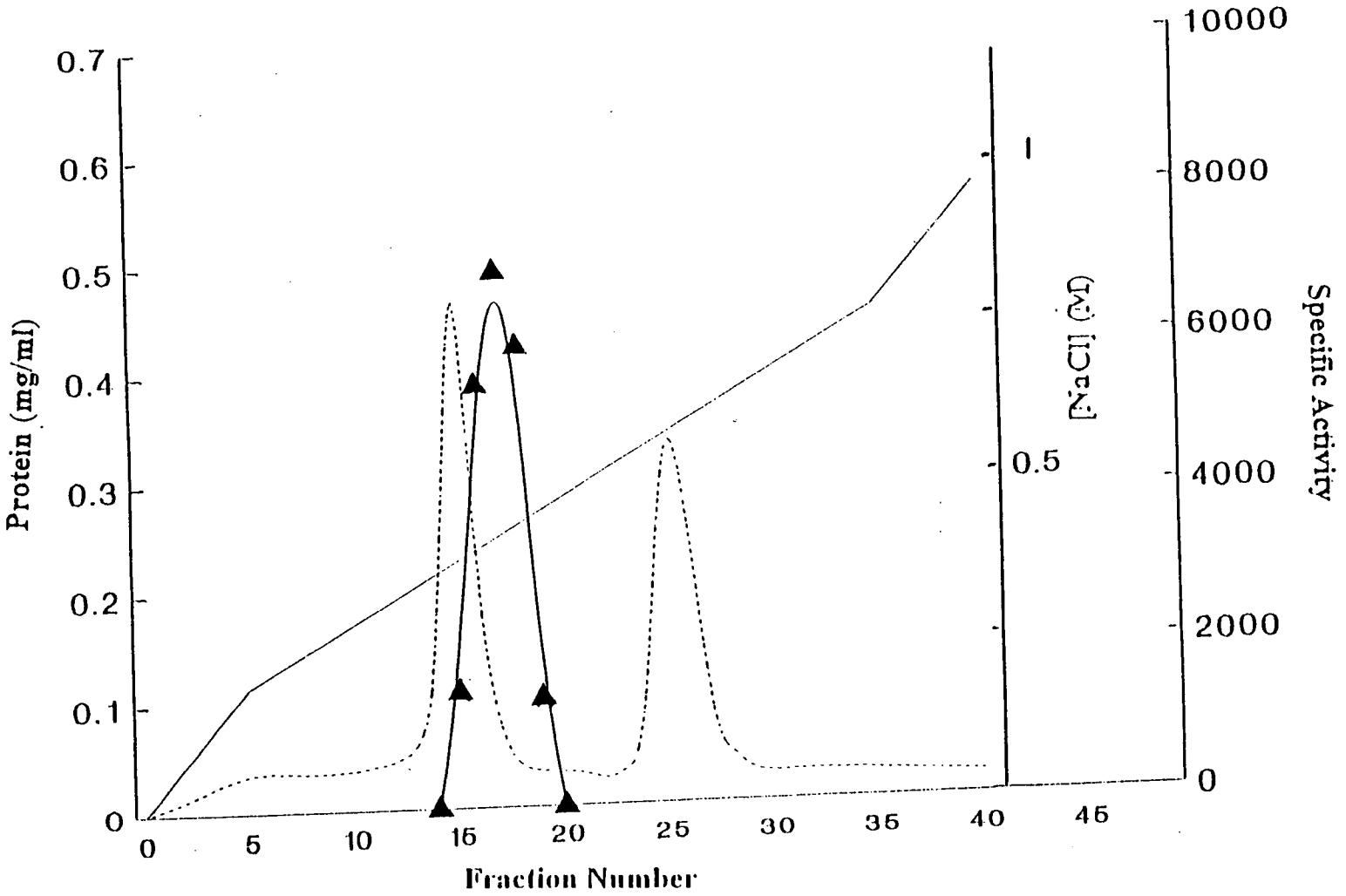

Protein (mg/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
Fraction Number
[NaCl] (M)
1
0.5
Specific Activity
10000
8000
6000
4000
2000
0

Table 5.6. Chromatography of the partially purified sodium-dependent NADH-ubiquinone oxidoreductase on Mono Q.

Sample	Specific Activity (U/mg protein)	Total Activity (U)	Total Protein (mg)
Applied Sample.	7 300	9 386	1.3
Eluted Sample.	21 900	6 476	0.3

A membrane sample (2 ml containing 0.65 mg/ml) partially purified on columns of S-300HR, DEAE Sepharose and hydroxylapatite was bound onto a Mono Q column and then eluted with an increasing linear gradient from 0 M to 1 M NaCl. Eluted fractions were pre incubated with 20 mM KCN for 10 min and then assayed for NADH and dNADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined for before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

5.4. Hydroxylapatite chromatography.

Hydroxylapatite is a crystalline form of calcium phosphate. Molecules can bind to hydroxylapatite via localised negatively or positively charged groups. Molecules are generally eluted using increasing concentrations of phosphate ions (Bernardi, 1971). This is a technique which is complimentary to gel filtration and ion exchange chromatography.

From initial experiments on hydroxylapatite columns (Bio-Gel HTP) 43% of the total activity was recovered. Buffer conditions were altered to improve recovery. The results for these experiments are shown in Table 5.7. The inclusion of either 10 mM FMN or FAD in the buffers did not improve recovery of activity. A KH_2PO_4 buffer with 100 mM NaCl, 10% (w/v) ethylene glycol and 1% (w/v) Triton X-100 was adopted for the chromatography of a sample which had been partially purified on columns of S-300HR and DEAE Sepharose. Using these buffer conditions 81% of the total activity was recovered. The elution profile is shown in Figure 5.6. NADH and dNADH oxidase activity was eluted at a concentration of 60 mM phosphate. The increase in specific activity was almost 2 fold and 60% of the starting total activity was recovered (Table 5.8).

Table 5.7. Chromatography of partially purified sodium-dependent NADH-ubiquinone oxidoreductase on hydroxylapatite using different buffer additives.

Buffer Additive	Activity Recovered(%)
0.1% (w/v) Triton X-100	41
0.1% (w/v) Deoxycholate	39
1% (w/v) Triton X-100	48
1% (w/v) Triton X-100, 10% (w/v) Ethylene Glycol	56
0.1% (w/v) Triton X-100, 100 mM NaCl	65
1% (w/v) Triton X-100, 10% (w/v) Ethylene Glycol, 100 mM NaCl	81
0.1% (w/v) Triton X-100, 10 μm FAD	40
0.1% (w/v) Triton X-100, 10 μm FMN	42

A membrane sample (5 ml containing 0.85 mg/ml) partially purified on columns of S-300HR and DEAE Sepharose was bound onto a hydroxylapatite column and then eluted with an increasing linear gradient from 0 to 120 mM KH_2PO_4, pH 7.2. Eluted fractions were pre incubated with 20 mM KCN for 10 min and then assayed for dNADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM dNADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

Figure 5.6. The elution profile of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes chromatographed on a hydroxylapatite column.

A hydroxylapatite column was equilibrated with a solution containing 20 mM KH_2PO_4, pH 7.2, 100 mM NaCl, 1% (w/v) Triton X-100, 1% (w/v) ethylene glycol at a flow rate of 50 ml/h. A sample of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes (10 ml containing 0.32 mg/ml protein) partially purified on a columns of S-300HR and DEAE Sepharose fast flow was loaded onto the column. Proteins were eluted with an increasing linear gradient from 20 mM to 120 mM KH_2PO_4 (---) and 5 ml fractions were collected. Eluted fractions were assayed for NADH and dNADH oxidase activity. Specific activities were calculated (—▲—) (nmol NADH oxidised/min/mg protein) and protein concentrations determined (—) (mg/ml) .

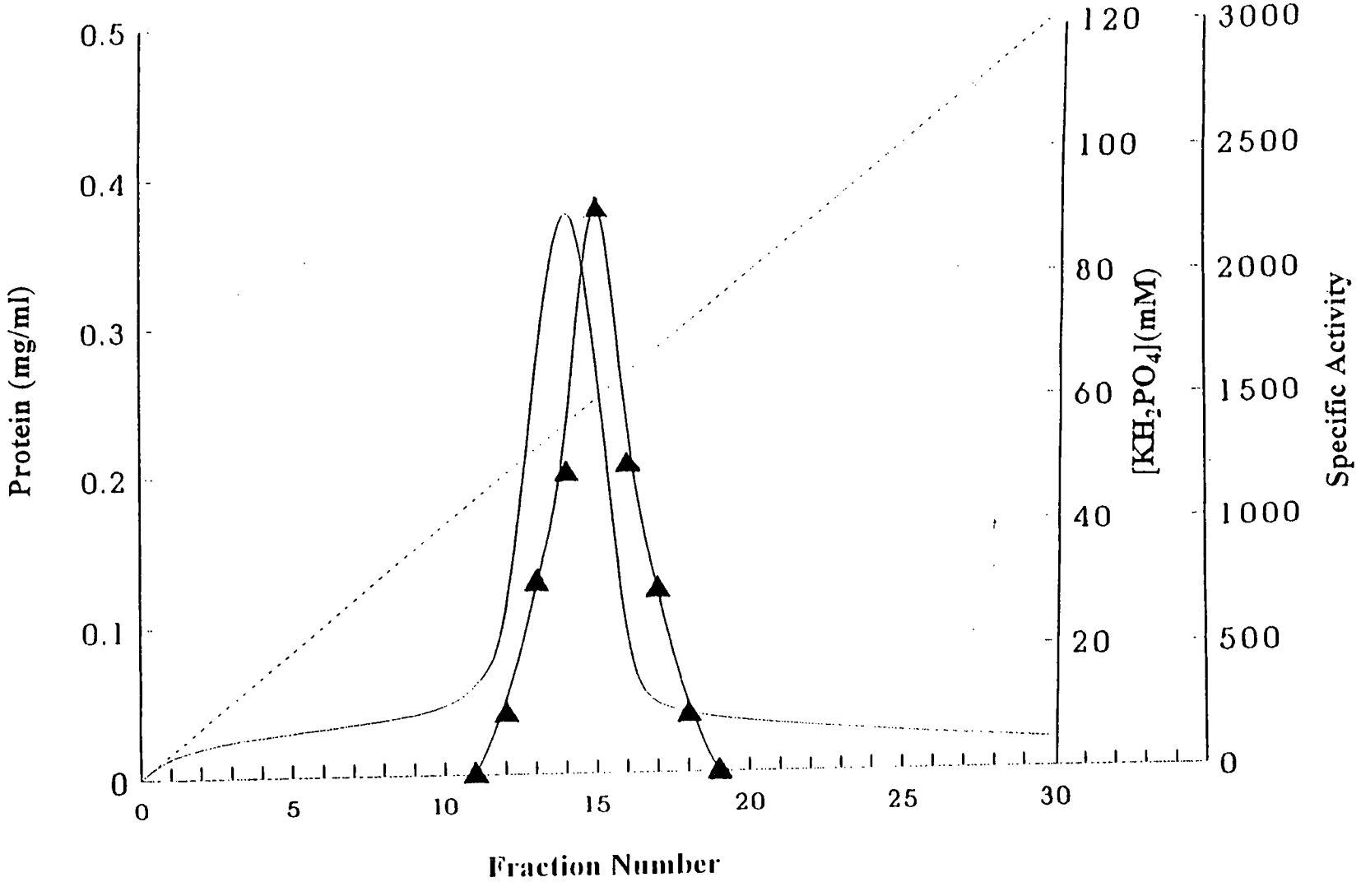

Protein (mg/ml)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Fraction Number
120
100
80
60
40
20
0
[KH2PO4](mM)
3000
2500
2000
1500
1000
500
0
Specific Activity

Table 5.8. Chromatography of the partially purified sodium-dependent NADH-ubiquinone oxidoreductase on Hydroxylapatite.

Sample	Specific Activity (U/mg protein)	Total Activity (U)	Total Protein (mg)
Applied Sample.	3 715	11 733	3.2
Eluted Sample.	7 300	9 386	1.3

A membrane sample (10 ml containing 0.32 mg/ml protein) partially purified on columns of S-300HR and DEAE Sepharose was bound onto a hydroxylapatite column and then eluted with an increasing linear gradient from 0 to 120 mM KH_2PO_4. Eluted fractions were pre-incubated with 20 mM KCN for 10 min and then assayed for NADH and dNADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

5.5. Ammonium sulphate precipitation.

The salting out of proteins using increasing concentrations of $(NH_4)_2SO_4$ has been widely used in enzyme purification. As $(NH_4)_2SO_4$ is solvated water molecules are removed from the hydrophobic patches on proteins and bind to the salt molecules. Highly hydrophobic proteins aggregate sooner than moderately hydrophobic proteins which only will precipitate at higher salt concentrations, thus proteins with differing hydrophobicities can be separated (Harris and Angal, 1989).

$(NH_4)_2SO_4$ precipitation was carried out at 30, 50 and 70% (w/v) saturation by adding solid $(NH_4)_2SO_4$ to a membrane sample partially purified on columns of S-300HR and DEAE Sepharose. Equilibration proceeded with gentle stirring at 4 °C Material was recovered by centrifugation at 30 000 g. NADH and dNADH oxidase activity was observed in the 30 and 50% pellets. There was a loss of 52% in total NADH oxidase activity and no increase in specific activity was observed. The results for total activities, specific activities for NADH oxidase and protein concentrations for the various fractions are shown in Figures 5.7.

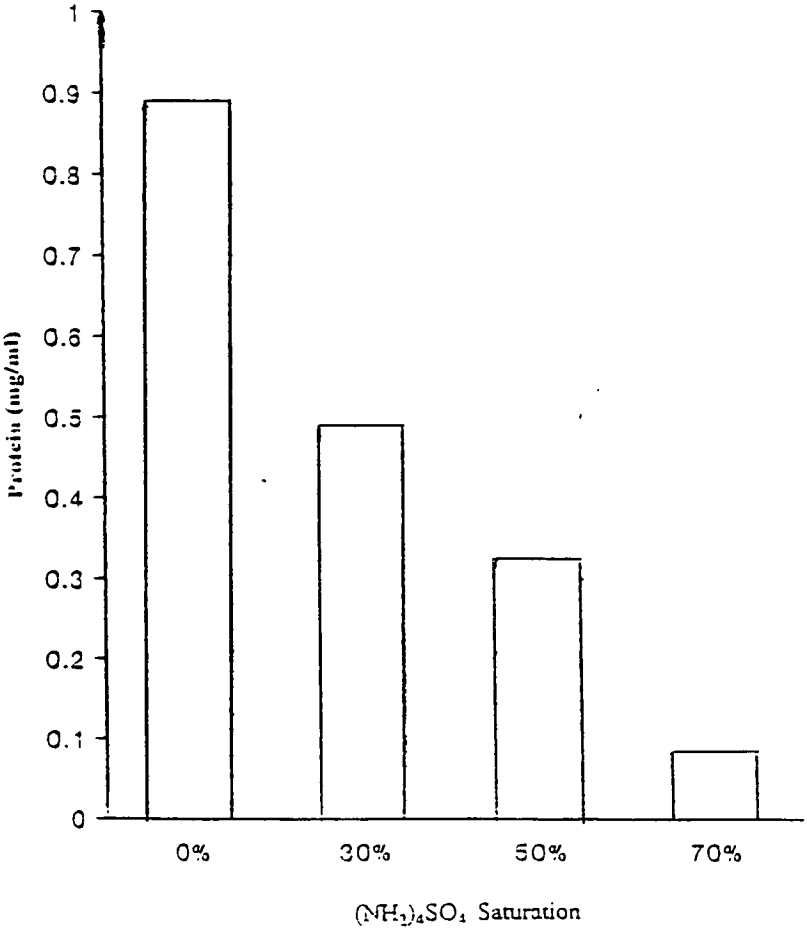
$(NH_4)_2SO_4$ precipitation was also carried out at 10, 20, 30, 40, 50 and 70% (w/v) saturation by adding volumes of neutralised solution of saturated $(NH_4)_2SO_4$. Lower initial $(NH_4)_2SO_4$ saturations were tested since it appeared from the first experiment that because of its hydrophobic nature the NADH-ubiquinone oxidoreductase was being precipitated at relatively low $(NH_4)_2SO_4$ concentrations. A neutralised solution of saturated $(NH_4)_2SO_4$ was used in this experiment to minimise any pH fluctuations and therefore increase recovery of activity. NADH and dNADH oxidase activity was observed mainly in the 10 and 20% pellets, with 25 and 11% of the total activity respectively. There was a 45% loss in total activity and for the 10% pellet a 2 fold increase in specific activity was observed. However when fractions were run on a 10% native PAGE gel and zymogram stained using dNADH only a

smear of activity was observed, with no discrete bands. A smear was also observed on the silver stain. The results are summarised in Figure 5.8.

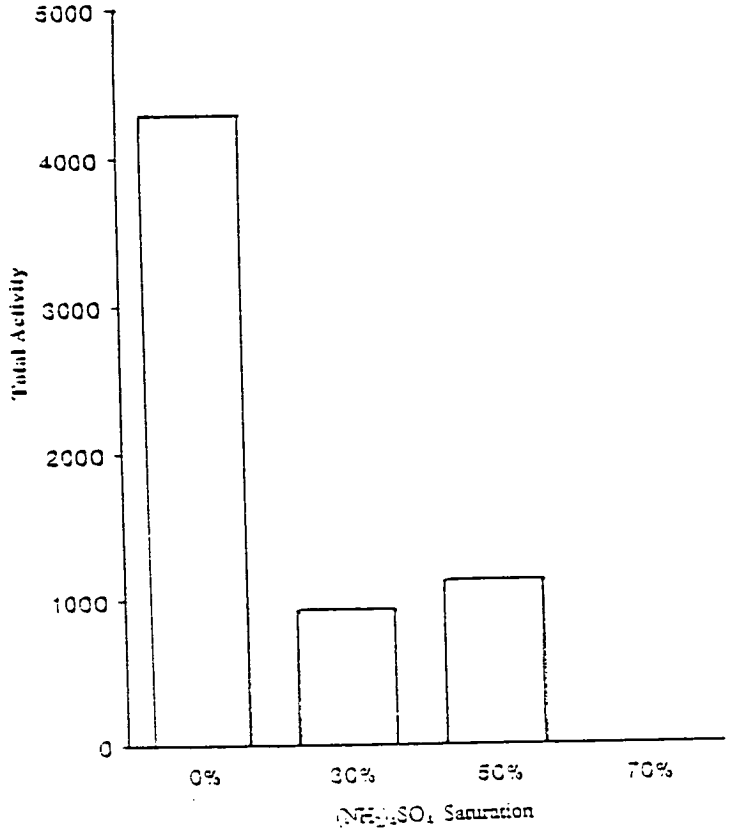
Figure 5.7. $(NH_4)_2SO_4$ fractionation of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes.

$(NH_4)_2SO_4$ fractionation of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes partially purified on columns of S-300HR and DEAE Sepharose fast flow was carried by adding solid $(NH_4)_2SO_4$, successively to give final saturations of 30%, 50% and 70% (w/v). Protein was precipitated by centrifugation at 30 000 g for 15 min. Pellets were re-suspended in buffer containing 50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1% (w/v) Triton X-100 and samples were desalted and then assayed for NADH and dNADH oxidase activity. Protein concentrations were determined (a) as were the total activities (b) (nmol NADH oxidised/min) and specific activities (c) (nmol NADH oxidised/min/mg protein) for NADH oxidase for before and after fractionation. For each data set the experiment was repeated twice, the average values are shown.

a)



b)


5000
4000
3000
2000
1000
0
Total Activity
0%
30%
50%
70%
$(NH_4)_2SO_4$ Saturation

c)

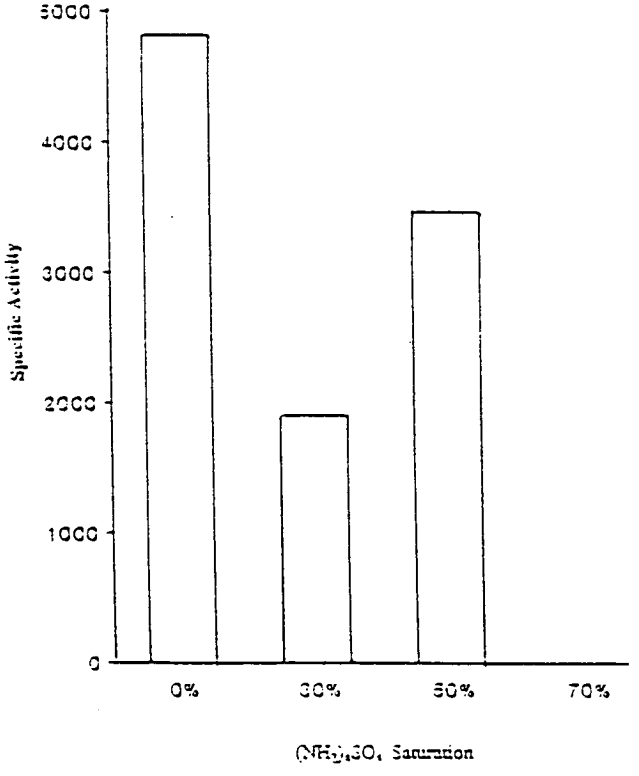
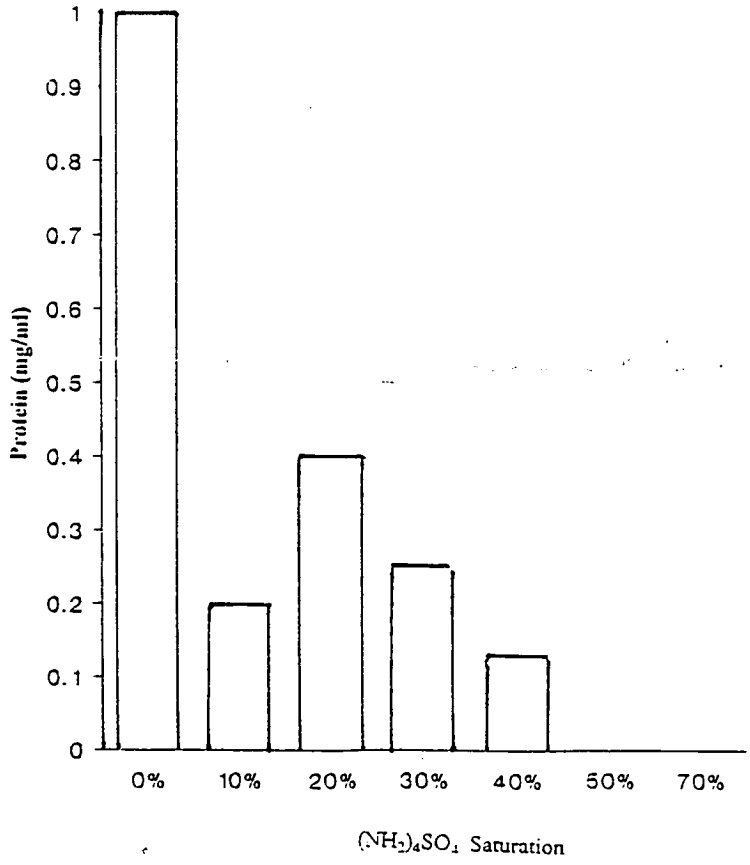

5000
4000
3000
2000
1000
0
Specific Activity
0%
30%
50%
70%
$(NH_4)_2SO_4$ Saturation

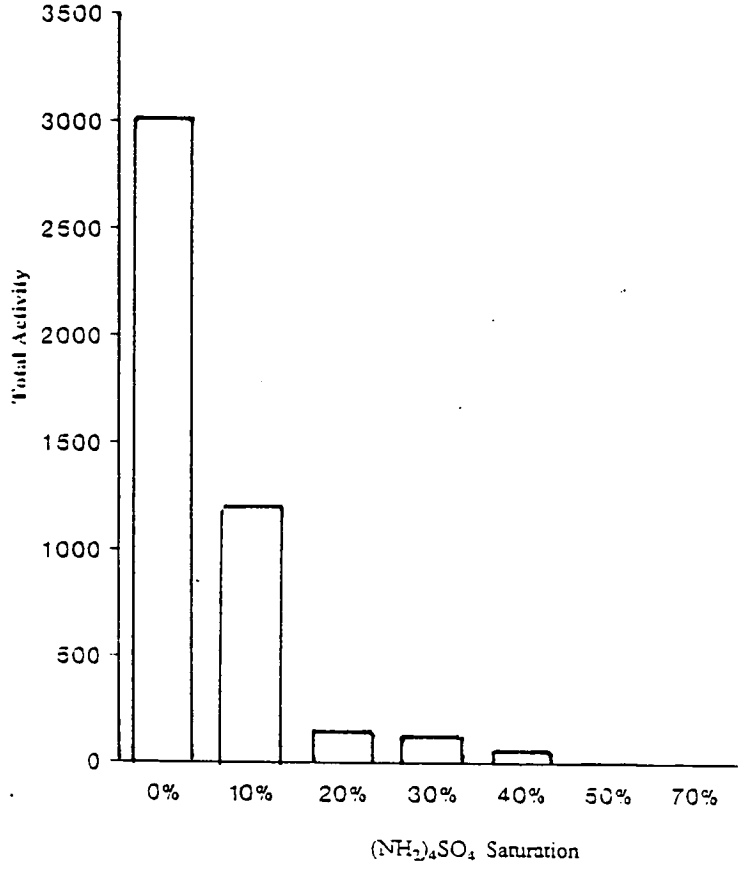
Figure 5.8. $(NH_4)_2SO_4$ fractionation of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes.

$(NH_4)_2SO_4$ fractionation of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes partially purified on columns of S-300HR and DEAE Sepharose fast flow was carried out by adding a neutralised saturated $(NH_4)_2SO_4$ solution successively to give final saturations of 10%, 20%, 30%, 40%, 50%, 60% and 70% (w/v). Protein was precipitated by centrifugation at 30 000 g for 15 min. Pellets were re-suspended in buffer containing 50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1% (w/v) Triton X-100 and samples were desalted and then assayed for NADH and dNADH oxidase activity. Protein concentrations were determined (a) as were the total activities (b) (nmol NADH oxidised/min) and specific activities (c) (nmol NADH oxidised/min/mg protein) for NADH oxidase for before and after fractionation. For each data set the experiment was repeated twice, the average values are shown.

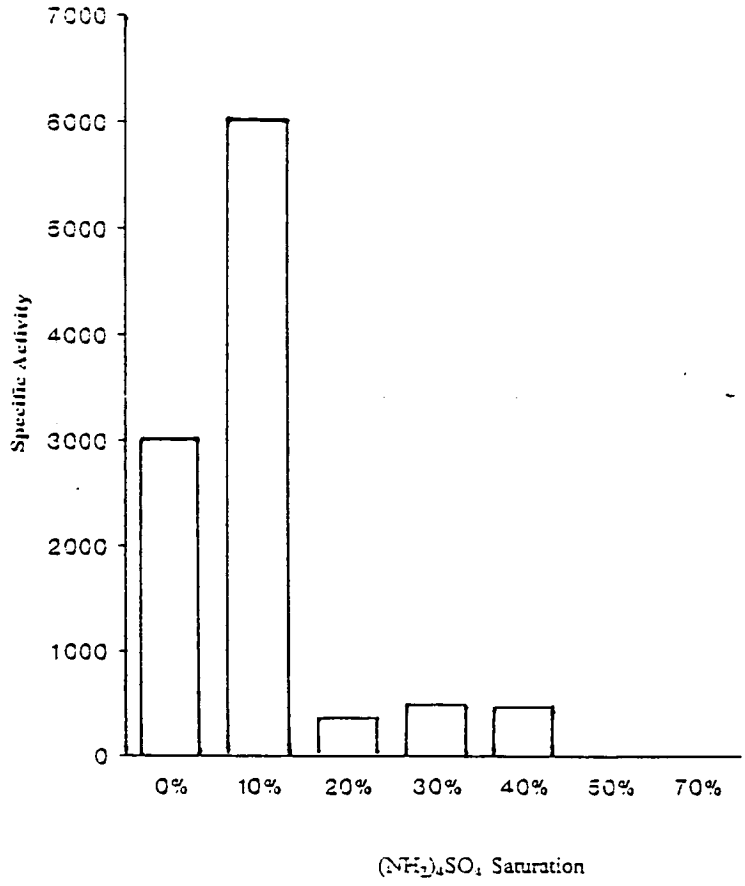
a)



b)


3500
3000
2500
2000
1500
1000
500
0
Total Activity
0%
10%
20%
30%
40%
50%
70%
$(NH_2)_4SO_4$ Saturation

(c)


7000
6000
5000
4000
3000
2000
1000
0
Specific Activity
0%
10%
20%
30%
40%
50%
70%
$(NH_2)_4SO_4$ Saturation

5.6. Hydrophobic Interaction Chromatography.

Hydrophobic interaction chromatography separates proteins on the basis of the differing strengths of their hydrophobic interactions with an uncharged bed material which contains hydrophobic groups. Many proteins have hydrophobic sites exposed on their surfaces and have been purified using this method (Shaltiel, 1974). Generally proteins bind onto the matrix at high ionic strength and are eluted with a decreasing ionic strength gradient. For some membrane proteins elution has been achieved by increasing the detergent concentration in the buffer (Rosen, 1978).

The NADH and dNADH oxidase activity bound strongly to Phenyl Sepharose in the absence of salt. Elution was achieved using an increasing linear gradient of 0 to 1% Triton X-100. Proteins were eluted from Phenyl Sepharose as a single peak (Figure 5.9) and no increase in specific activity was observed (Table 5.9). It was concluded that binding was primarily through the detergent of the detergent-protein complex and this was why all proteins were eluted from the matrix under the same conditions.

Figure 5.9. The elution profile of partially purified sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes chromatographed on a Phenyl Sepharose column.

A Phenyl Sepharose column was equilibrated with a solution containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% (w/v) Tween 80 at a flow rate of 30 ml/h. A sample of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes (10 ml containing 0.33 mg/ml protein) partially purified on columns of S-300HR and DEAE sepharose fast flow was loaded onto the column. Proteins were eluted with an increasing linear gradient from 0 to 1 % (w/v) Triton X-100 (----) and 5 ml fractions were collected. Eluted fractions were assayed for NADH and dNADH oxidase activity. Specific activities were calculated (—●—) (nmol NADH oxidised/min/mg protein) and protein concentrations determined (——) (mg/ml) .

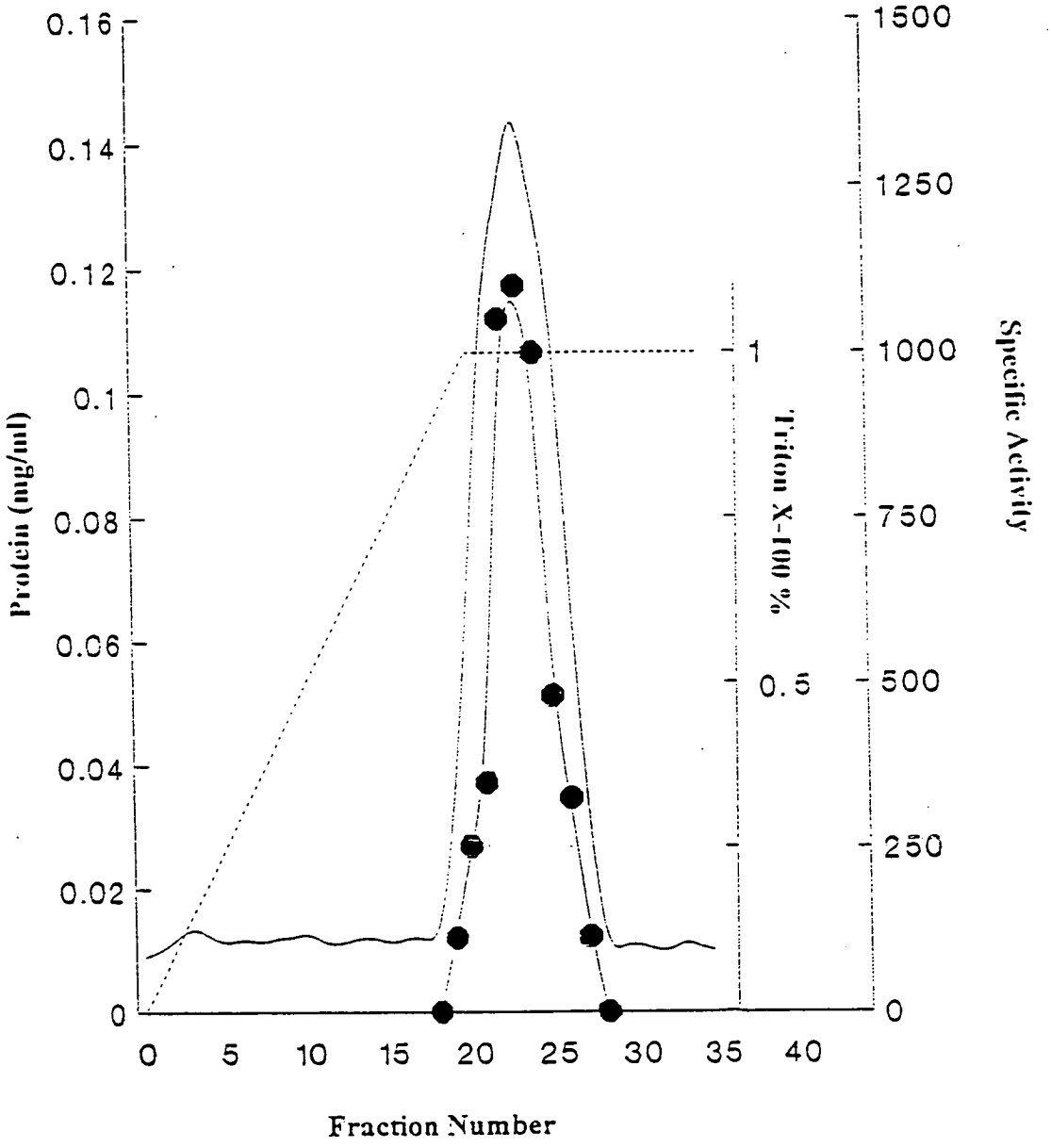

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Protein (mg/ml)
1500
1250
1000
750
500
250
0
Specific Activity
1
0.5
Triton X-100 %
0 5 10 15 20 25 30 35 40
Fraction Number

Table 5.9. Chromatography of the partially purified sodium-dependent NADH-ubiquinone oxidoreductase on Phenyl Sepharose.

Sample	Specific Activity (U/mg protein)	Total Activity (U)	Total Protein (mg)
Applied Sample	3 715	11 733	3.3
Eluted Sample	3 685	11 282	3.2

A membrane sample (10 ml containing 0.33 mg/ml) partially purified on columns of S-300HR and DEAE Sepharose was bound onto a Phenyl Sepharose column and then eluted with an increasing linear gradient from 0 to 1% (w/v) Triton X-100. Eluted fractions were pre-incubated with 20 mM KCN for 10 min and then assayed for NADH and d NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

5.7. Affinity chromatography.

A purification step to compliment gel filtration, ion exchange and hydroxylapatite chromatography was sought and a number of matrices were tested. It was hoped that these matrices would be able to take advantage of some of the biological properties of the NADH-ubiquinone oxidoreductase and would prove useful as a steps which could give rapid purification .

Metal chelate chromatography.

Metal chelate chromatography has been used to purify proteins which have the amino acids histidine, tryptophan or cysteine accessible on their surfaces. These amino acids are capable of reversible binding with transition metals Cu^{2+} and Zn^{2+} (Porath, *et al.*, 1975). Chelating Sepharose is composed of iminodiacetic acid linked to an agarose gel and can be charged with metal ions by washing it with a solution of the appropriate metal salt. Protein samples can then be tested for their ability to bind to the immobilised metal. Proteins can be displaced from the matrix by lowering the pH or by the use of stronger complexing agents such as imidazole or glycine.

Since both NADH and dNADH oxidase activity from *V. harveyi* membrane fractions were reversibly inhibited by Zn^{2+}, it suggested that a histidine, tryptophan or cysteine residue of the sodium-dependent NADH-ubiquinone oxidoreductase may be solvent accessible and that Zn^{2+} chelate chromatography could be useful as a purification method. A membrane sample partially purified on a S-300HR column was applied to a chelating Sepharose column. All the NADH and dNADH oxidase activity was found in the wash fractions, none had bound to the column and no increase in specific activity was observed. It may be that the Zn^{2+} binding region on the NADH-ubiquinone oxidoreductase is not fully exposed on the surface of the molecule and steric occlusion by the spacer arm preventing it binding Zn^{2+} ions.

Covalent chromatography.

Covalent chromatography is highly specific for the purification of proteins bearing accessible thiol groups. Such proteins can form a mixed disulphide with the matrix and are therefore covalently attached to the stationary phase. The proteins can subsequently be eluted by the addition of a reducing agent such as cysteine or β-mercaptoethanol (Kitson, 1982).

Since it appears that the sodium-dependent NADH-ubiquinone oxidoreductase contains at least one thiol group (Bourne and Rich, 1992) then covalent chromatography may could be useful in purifying this type of enzyme. A membrane sample partially purified on a column of S-300HR was applied to a Thiopropyl Sepharose 6B column. All the NADH and dNADH oxidase activity was found in the wash fractions, none had bound to the column and no increase in specific activity was observed.

Dye ligand chromatography.

Reactive triazine textile dyes immobilised on agarose have been effective in purifying a number of proteins including pyridine nucleotide-dependent oxidoreductases (Lowe and Pearson, 1984). It has been suggested that triazine dyes mimic the overall shape, size and charge distribution of naturally occurring substrate rather than acting as highly specific analogues.

Mimetic dye ligands were screened for their ability to bind the sodium-dependent NADH-ubiquinone oxidoreductase. A membrane sample partially purified on a column of S-300HR was applied. Initial conditions for binding were 20 mM KH_2PO_4, pH 7 and it was found that dNADH oxidase activity bound to the Mimetic blue 2 A6XL, Mimetic green 1 A6XL and Mimetic red 2 A6XL matrices. No binding was observed on any of the other columns (Table 5.10). Enzyme activity was eluted using a linear gradient of 0 to 0.25 M NaCl. Buffer conditions were altered to improve binding onto Mimetic blue 2 A6XL (Table 5.11) however after elution no increase in specific activity was observed.

Table 5.10. Binding of partially purified sodium-dependent NADH-ubiquinone oxidoreductase onto Mimetic dye ligand columns.

Mimetic Ligand	Activity Bound(%)
Mimetic blue 2 A6XL	44
Mimetic green 1 A6XL	31
Mimetic red 2 A6XL	11
Mimetic red 1 A6XL	0
Mimetic orange 1 A6XL	0
Mimetic orange 2 A6XL	0
Mimetic orange 3 A6XL	0
Mimetic yellow 1 A6XL	0
Mimetic yellow 2 A6XL	0
Mimetic blue 1 A6XL	0

Membrane samples (1 ml containing 1 mg/ml for each column) partially purified on a column of S-300HR were applied to the different Mimetic ligands columns. Wash and elution fractions were pre-incubated with 20 mM KCN for 10 min and then assayed for NADH and d NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

Table 5.11. Chromatography of the partially purified sodium-dependent NADH-ubiquinone oxidoreductase on a Mimetic blue 2 A6XL column.

Buffer Condition	Detergent Condition	Total Activity Bound(%)
KH_2PO_4, pH 7	0.1% (w/v) lauryl sulfobetaine	44
KH_2PO_4, pH 6.5	0.1% (w/v) lauryl sulfobetaine	92
KH_2PO_4, pH 6.5	0.05% (w/v) lauryl sulfobetaine	95
KH_2PO_4, pH 6.5	0.01% (w/v) lauryl sulfobetaine	17.5

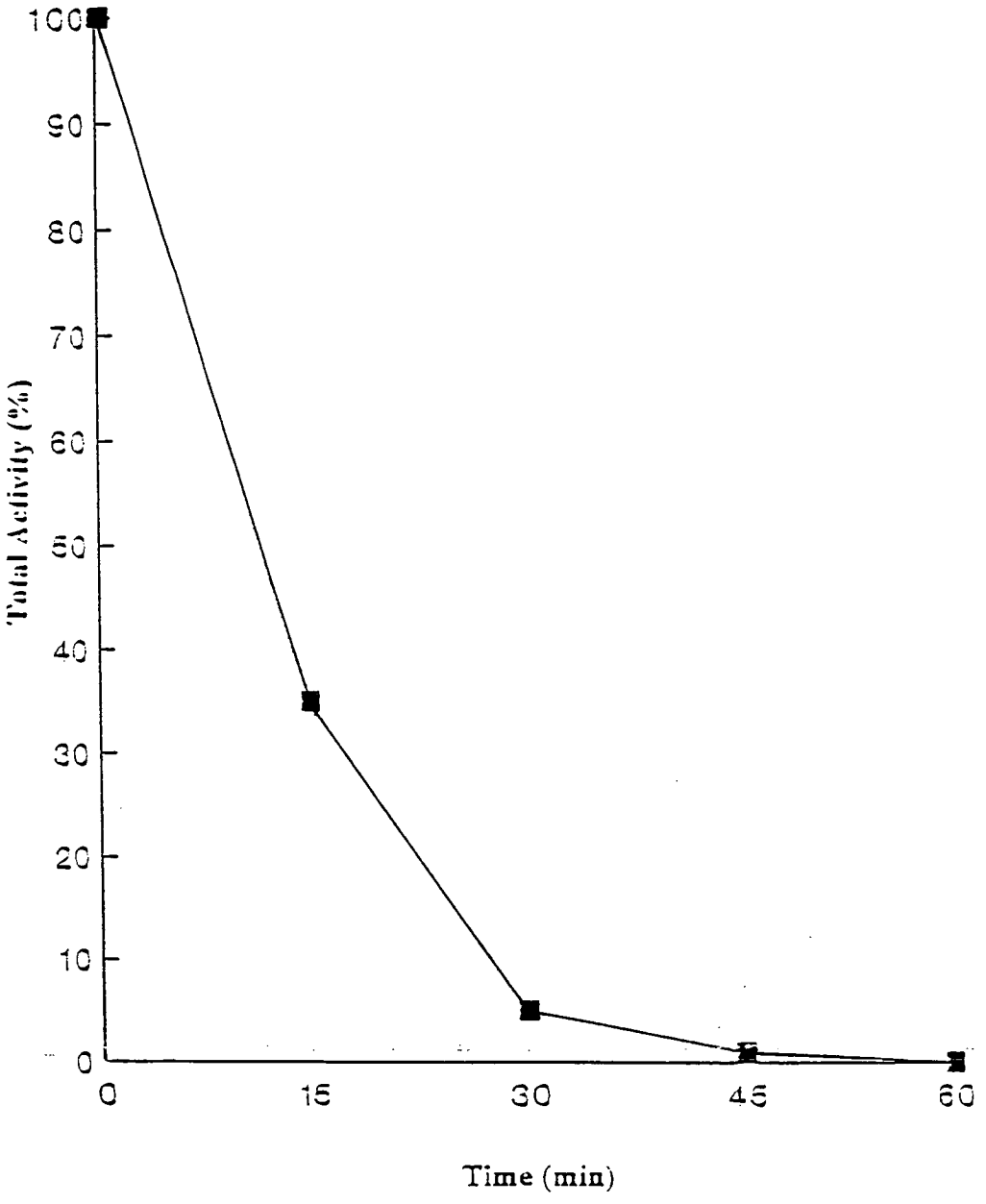
A membrane sample (1 ml containing 1 mg/ml of protein) partially purified on an a column of S-300HR was bound onto the Mimetic blue 2 A6XL column and then eluted with 0.25 M NaCl. Eluted fractions were pre-incubated with 20 mM KCN for 10 min and then assayed for NADH and d NADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography. For each data set the experiment was repeated three times, the average values are shown.

5.8. Denaturation and renaturation of membrane proteins.

It has been shown that it is possible to improve the separation of membrane proteins by preventing interactions of the proteins during chromatography. This has been achieved by denaturation of proteins followed by renaturation after chromatography. Denaturation has been achieved by treating proteins with SDS which has a highly dissociating and solubilising ability. Renaturation was achieved by incubating proteins samples with excess Brij 35. The hydrophobic part of the detergent mimics the hydrophobic tail of the predominant membrane lipid and is capable of displacing SDS. Brij 35 will form mixed micelles with SDS. The micelles associated with the protein will then dissociate rapidly allowing renaturation (Hjerten *et al.*, 1988). The NADH-ubiquinone oxidoreductase was rapidly denatured by 0.01 M SDS (Figure 5.10), however no renaturation of this protein was observed even after a 72 h incubation with 7% Brij 35. This approach of denaturation followed by renaturation after chromatography was therefore not suitable for use in purifying the sodium-dependent NADH-ubiquinone oxidoreductase.

Figure 5.10. SDS denaturation of the sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes.

V. harveyi membranes extracted with 0.5% (w/v) Brij 35 was treated with 0.01 M SDS. The samples were taken at various times and pre incubated with 20 mM KCN for 10 min and then assayed for NADH and dNADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The total activities (nmol NADH oxidised/min) for NADH oxidase were determined during denaturation. For each data set the experiment was repeated three times, the average values are shown.


100
90
80
70
60
50
40
30
20
10
0
Total Activity (%)
0
15
30
45
60
Time (min)

5.10. Preparative chromatography.

The sodium-dependent NADH-ubiquinone reductase from *V. harveyi* was successively purified using the columns and conditions which gave the best results in terms of purification and recovery of enzyme in the preliminary experiments. The columns used were the ion exchangers DEAE Sepharose fast flow and Mono Q, the gel filtration matrix Sephacryl 300HR and hydroxylapatite Bio-Gel HTP.

V. harveyi membranes were solubilised in 1% (w/v) Triton X-100 and applied onto the S-300HR coumn. Eluted fractions containing the sodium-dependent NADH-ubiquinone oxidoreductase were pooled and chromatographed on the next column. This was repeated on succesive columns of DEAE Sepharose fast flow, hydroxylapatite Bio-Gel HTP and Mono Q, with storage of samples being avoided to minimise losses in activity. When desalting or buffer exchange was required samples were passed down PD-10 columns which had been equilibrated with the appropriate buffer. Eluted fractions were pre-incubated with 20 mM KCN for 10 min and then assayed for NADH and dNADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined before and after chromatography on each column. After each column, fractions containing activity were run on 10% native polyacrylamide gels and zymogram stained using dNADH as the electron donor. This was to determine that the 254 kDa NADH-ubiquinone oxidoreductase complex was still intact.

A 75 fold purification was obtained with a loss of 51% total activity (nmol NADH oxidised/min) (Table 5.12). The 254 kDa sodium-dependent NADH-ubiquinone oxidoreductase was not purified to homogeneity as determined by native

and SDS polyacrylamide gel electrophoresis and silver staining. The reproducibility of the silver staining, particular on native polyacrylamide gels was poor. As yet unidentified high molecular weight contaminants (>300 kDa) were observed on 10% native polyacrylamide gels (Figure 5.2). On SDS polyacrylamide gels at least 20 polypeptide bands were observed (Figure 5.11) The molecular weights of some of the major polypeptide bands were determined (Table 5.13) and a number are in the correct size range to be considered as possible NADH-ubiquinone oxidoreductase subunits.

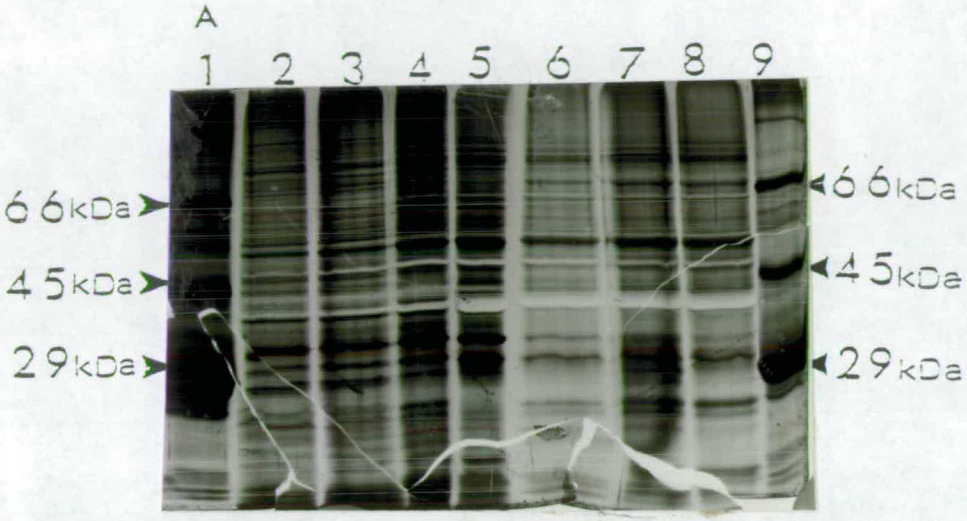
Table 5.12. Summary of the steps used in purifying the sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* BB7.

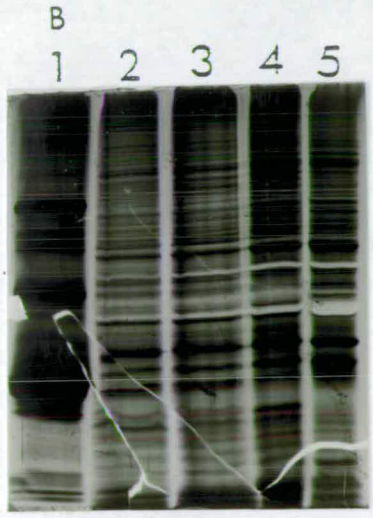
Sample	Specific activity (U/mg protein)	Total activity (U)	Protein concentration (mg)	Purification	Yield(%)
Solubilised membranes	298	15 645	53	*	*
S-300 gel filtration	743	14 860	20	2.5	95
DEAE Sepharose	3 715	11 733	3.2	12.5	75
Hydroxylapatite	7 300	9 386	1.3	25	60
Mono Q	21 900	6 476	0.3	75	41

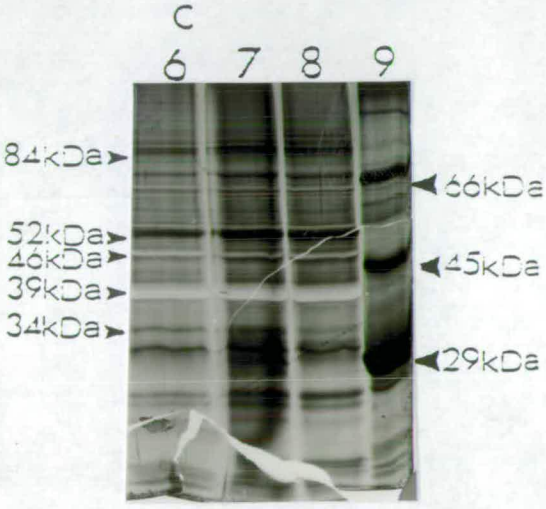
A 1% (w/v) Triton X-100 solubilised membrane sample (5 ml containing 10.3 mg/ml protein) was purified on an S-300 gel filtration column. Eluted fractions containing the sodium-dependent NADH-ubiquinone oxidoreductase were pooled and chromatographed on the next column. This was repeated for all the columns. Eluted fractions were pre-incubated with 20 mM KCN for 10 min and then assayed for NADH and dNADH oxidase activity in buffer (1 ml) containing 20 mM Tris-HCl, pH 7.5, 0.2 mM NADH or dNADH and 0.4 M NaCl. The assays were started by the addition of 100 μM menadione. Protein determinations were also carried out. The specific activities (nmol NADH oxidised/min/mg protein) and total activities (nmol NADH oxidised/min) for NADH oxidase were determined for before and after chromatography. The amount of purification obtained and the percentage of total activity recovered has been calculated. For each data set the experiment was repeated three times.

Figure 5.11. SDS polyacrylamide gel electrophoresis of sodium-dependent NADH-ubiquinone oxidoreductase at various stages of purification.

A. Lane 1 and 9, molecular weight markers; 2, unpurified *V.harveyi* membrane sample; lane 3, NADH-ubiquinone oxidoreductase purified on S-300HR; lane 4, NADH-ubiquinone oxidoreductase purified on S-300HR and DEAE Sepharose fast flow; lane 5, NADH-ubiquinone oxidoreductase purified on S-300HR and DEAE Sepharose fast flow and hydroxylapatite; lanes 6-8, NADH-ubiquinone oxidoreductase purified on S-300HR and DEAE Sepharose fast flow; hydroxylapatite and Mono Q. B, Lanes 1-5 from gel A. C, Lanes 6-9 from gel A with molecular weights of some of the major polypeptides (See Table 5.13).


A
1 2 3 4 5 6 7 8 9
66kDa
45kDa
29kDa
66kDa
45kDa
29kDa


B
1 2 3 4 5


C
6 7 8 9
84kDa
52kDa
46kDa
39kDa
34kDa
66kDa
45kDa
29kDa

5.9.2. Preparative electrophoresis.

Preparative electrophoresis was used to purify the sodium-dependent NADH:ubiquinone oxidoreductase from the high molecular weight contaminants which could not be separated from the enzyme on gel filtration columns. In these experiments membrane samples partially purified on columns of S-300HR, DEAE Sepharose, hydroxylapatite and Mono Q were electrophoresed under native conditions. The NADH-ubiquinone oxidoreductase was located in the gel after electrophoresis by using a zymogram stain with dNADH as the electron donor. Protein samples were then removed from the deep blue/purple section of the gels.

Polyacrylamide gel electrophoresis.

Membrane samples containing 0.5 mg of protein were run on native PAGE and gave neat, narrow bands of activity as shown by dNADH zymogram stains. The excision and electroelution of these activity bands from gels was straightforward. About 100 μg of protein and 10% of the total activity was recovered. The purity of the samples from two separate preparative electrophoresis experiments and the approximate molecular weights of the major polypeptide bands recovered was determined on SDS PAGE gels (Figure 5.12, Table 5.13). At least 10 polypeptides have been identified in the samples of NADH-ubiquinone reductase purified using preparative electrophoresis in PAGE gels and although it appeared that many contaminating polypeptides had been removed, very little protein was recovered. However a number of the polypeptides are in the correct size range to be considered as possible NADH-ubiquinone oxidoreductase subunits candidates. When higher concentrations of protein were run on PAGE gels the zymogram staining pattern observed became smeared, this made the precise location of NADH-ubiquinone reductase activity difficult to determine and subsequently more contaminants were

observed. Preparative electrophoresis therefore has potential for the purification of the sodium-dependent NADH-ubiquinone if conditions for electrophoresis and elution could be optimised.

Agarose electrophoresis.

Preparative agarose electrophoresis was also tried to see if the recovery of material after separation could be improved. Membrane samples containing 0.5 mg of protein were electrophoresed on native agarose gels. After electrophoresis the zymogram staining pattern obtained were smeared and indistinct. The amount of protein recovered from the gel was 80 μg which was less than was recovered from polyacrylamide gels.

Starch electrophoresis.

Preparative starch electrophoresis was also tried to see if the recovery of material after separation could be improved. Membrane samples containing 0.5 mg of protein were run on native starch gels. After electrophoresis the zymogram staining pattern obtained were smeared and indistinct. The amount of protein recovered from the gel was 70 μg which was less than was recovered from polyacrylamide and agarose gels. Additionally the starch gels were very fragile and difficult to handle.

Figure 5.12. SDS polyacrylamide gel electrophoresis of sodium-dependent NADH-ubiquinone oxidoreductase purified by preparative electrophoresis.

Lanes 1 and 4; molecular weight markers, lanes 2 and 3 sodium-dependent NADH-ubiquinone oxidoreductase purified by preparative electrophoresis. Partially purified NADH-ubiquinone oxidoreductase was run on native polyacrylamide gels and zymogram stained using dNADH as the electron donor. Activity bands were excised and protein samples electroeluted. Samples were then run on a 10% SDS polyacrylamide gel at 150 V for 1h and silver stained.

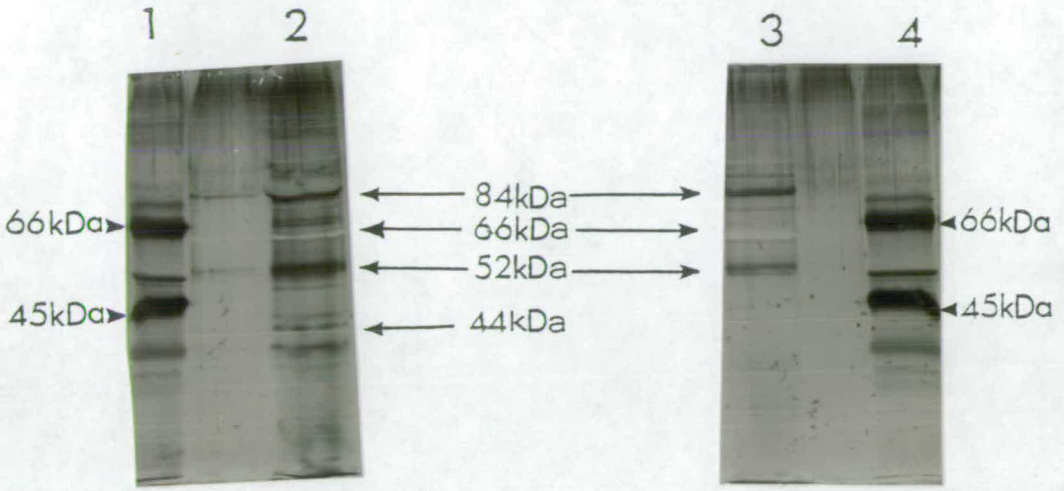

1
2
3
4
66kDa
45kDa
84kDa
66kDa
52kDa
44kDa
66kDa
45kDa

Table 5.13. Summary of the molecular weights of major polypeptides in *V. harveyi* membrane samples after preparative chromatography and preparative polyacrylamide gel electrophoresis of the sodium-dependent NADH-ubiquinone oxidoreductase.

Preparative Chromatography	Preparative PAGE gel electrophoresis	Preparative PAGE gel electrophoresis
84 kDa	84 kDa	84 kDa
66 kDa (ghost band)	76 kDa	76 kDa
62 kDa	66 kDa (ghost band)	66 kDa (ghost band)
52 kDa (doublet)	62 kDa	64 kDa
46 kDa (ghost band)	52 kDa (doublet)	52 kDa (doublet)
39 kDa (ghost band)		48 kDa
34 kDa		44 kDa
31 kDa		40 kDa
29 kDa		36 kDa
24 kDa		

After preparative chromatography (succesive purification on S-300HR, DEAE Sepharose fast flow, hydroxylapatite and Mono Q) or preparative electrophoresis samples containing dNADH oxidase activity were run on SDS PAGE gels for 1 h at 150 V. Gels were silver stained and the Rf values for the major polypeptide bands measured. Approximate molecular weights were determined by comparing Rf values with a standard curve obtained using molecular weight standards.

5.11. Conclusions.

The columns which gave the best results for the purification of the sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes were Sephacryl 300HR gel filtration, DEAE Sepharose fast flow, Mono Q and hydroxylapatite. A 75 fold increase in specific activity was observed with a recovery of 40% of the total enzyme activity.

Sephacryl 300HR gel filtration was used as a first step in the purification of sodium-dependent NADH-ubiquinone oxidoreductase. This was most useful in removing low molecular weight proteases from the higher molecular weight NADH-ubiquinone oxidoreductase. It gave some purification but was of limited use as the proteins tended to elute as a broad peak; this suggested that because of their hydrophobic nature the proteins may be interacting with each other or with the matrix during chromatography. A similar phenomenon was observed when Superdex 200 gel filtration was used as a later purification step to remove high molecular weight contaminants. Again a broad peak was observed but this time no purification was achieved.

Charged based separation was more succesful in purifying the sodium-dependent NADH-ubiquinone oxidoreductase; in particular the anion exchange matrices DEAE Sepharose fast flow and Mono Q. Three and two chromatographic peaks respectively were observed for these columns when sodium-dependent NADH-ubiquinone oxidoreductase was purified on them. This compared to only one elution peak when material was chromatographed on the other columns tested. Unfortunately the pH sensitivity of the NADH-ubiquinone oxidoreductase complex precluded the use of cation exchangers to purify it further using ion exchange.

It appears ammonium sulphate fractionation is an unsuitable method for purifying the sodium-dependent NADH-ubiquinone oxidoreductase; although an

increase in specific activity was observed, losses in activity and complete disasociation of the enzyme complex occurred. Consistent with the sodium-dependent NADH-ubiquinone oxidoreductase being large and very hydrophobic nature most of the enzyme activity was fractionated by 10% (w/v) ammonium sulphate.

Of the other columns that were unsuccessful in purifying the sodium-dependent NADH-ubiquinone oxidoreuctase two general problems were observed; either the complex bound to the matrix non-specifically or no binding was observed at all. For the hydrophobic interaction column it appeared that proteins were interacting with the matrix via the detergent and proteins were eluted as a single peak when excess detergent was added. For the dye ligand columns binding appeared to be occurring as a consequence of electrostatic interaction. With the metal chelate and covalent chromatography matrixes no binding was observed and it may be that the nature of the spacers used may affect the ability of the ligand to bind the target protein.

Preparative electrophoresis using polyacrylamide gels was useful for obtaining relativly pure material although the amounts of protein recovered from polyacrylamide gels was low (an average of 100 μg of protein per run) and the enzyme complex tended to dissociate. Preparative electrophoresis as a method for separating proteins has proved useful in purifying the sodium-dependent NADH-ubiquinone oxidoreductase from *V. alginolyticus* in other studies (Beattie *et al.*, 1994) so it may be possible to obtain purified enzyme from *V. harveyi* by optimising electrophoresis and elution conditions.

Although the sodium-dependent NADH-ubiquinone oxidoreductase samples were not purified to homogeneity a number of polypeptides were identified which appear to be in the correct size range to be considered as potential NADH-ubiquinone oxidoreductase subunits, in particular polypeptides with the following molecular

weights, 66 kDa (ghost band), 62 kDa, 52 kDa (doublet), 46 kDa, 36 kDa and 34kDa as determined on SDS-PAGE after preparative chromatography and preparative electrophoresis.

Chapter 6.

Discussion.

Initial characterisation of the sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* has revealed an enzyme which is very similar to NQR1 from *V. alginolyticus* (Tokuda and Unemoto, 1981; Bourne and Rich, 1992). Both require sodium for maximum activity (although the K_m values for sodium are high) and can utilise NADH and dNADH as substrates. The active complexes of these enzymes have similar molecular weights and both are inhibited reversibly and irreversibly by the same group of metal cations, which suggests that the two enzymes may well share common catalytic sites. One major difference between the two sodium-dependent NADH-ubiquinone oxidoreductases is their specific activities for NADH and dNADH. The values for *V. alginolyticus* are almost twice those obtained for *V. harveyi*. This may be explained by the slightly higher salt tolerance observed for *V. alginolyticus* when compared to *V. harveyi*.

Reports seem to support the idea that primary sodium pumps and in particular sodium-dependent NADH-ubiquinone oxidoreductases are widely distributed in bacteria (Dimroth and Thomer, 1989; Tokuda and Kogure, 1989; Vagina *et al.*, 1991) and there is the strong possibility that like their proton translocating equivalents sodium-dependent NADH-ubiquinone oxidoreductases from different species may have structural and functional similarities. As more sodium-dependent NADH-ubiquinone oxidoreductases are characterised using biochemical and genetic approaches the degrees of similarity and conservation among this group of enzymes will be become more apparent.

The sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* is particularly unstable in crude extracts at room temperature and protease activities were suspected for losses in activity and the breakdown of the enzyme complex, as detected by enzyme assays and zymogram staining of membrane samples run on native polyacrylamide gels. The addition of EDTA to samples helped improve the recovery of activity and this suggested that metalloproteases may be responsible for at least some of the proteolytic activity. Storage of samples at 4 °C or below, also helped to stabilise the enzyme and chromatography on a gel filtration column was useful for removing proteases which generally tend to be of lower molecular weights. This method of removing proteases is generally applicable when dealing with high molecular weight proteins or protein complexes and avoids the inclusion in sample buffers of a mixture of protease inhibitors which are not only very toxic but are also expensive to use.

The sodium-dependent NADH-ubiquinone oxidoreductase was most stable in conditions of pH and NaCl concentrations which most closely mimic physiological conditions. Another important factor in stabilising sodium-dependent NADH-ubiquinone oxidoreductase activity was the inclusion of reducing agents in sample buffers. Within cells various reducing compounds, notably glutathione, prevent protein oxidation. Once the cell has been disrupted, care must be taken to counteract the effects due to increased contact with oxygen and the dilution of naturally occurring reducing agents. Under these conditions many proteins become oxidised and lose activity. β-mercaptoethanol is a useful reducing agent and can be used at a working concentration of between 5-20 mM. After 24 h however β-mercaptoethanol becomes oxidised and then may in fact accelerate protein inactivation. DTT is another reducing agent but when it is oxidised a stable intramolecular disulfide is formed and this does not affect sulfhydryl groups. So β-mercaptoethanol is suitable

protection from oxidation during the short term but DTT is the compound of choice for long term storage of material.

Conditions which help to minimise losses of enzyme activity are an important consideration for purification of the enzyme complex to ensure that enough material is recovered after each purification step. Maintaining the biological integrity of the enzyme complex is also important regarding meaningful characterisation of purified material during subsequent biochemical and functional studies. This includes the important issues of determining the exact subunit composition of the sodium-dependent NADH-ubiquinone oxidoreductase complex and reconstituting the complex into liposomes to assay for sodium translocating ability.

Transposon mutagenesis using narrow host range vectors was unsuccesful in *V. harveyi*. In conjugation experiments using *pir* dependent transposon vectors no exconjugants were observed. Since other vectors were capable of conjugating into *V. harveyi* at high frequencies and were stably maintained, it therefore seems unlikely that host restriction systems were responsible for the failure of the *pir* dependent vectors to be conjugated from *E. coli* donor strains into *V. harveyi*. The *pir*-dependent type vectors have been used succesfully for transposon mutagenesis in a number of gram-negative species including *Vibrio cholerae* (Miller *et al.*, 1986).

In other conjugation experiments using *E. coli* as the donor strain it was observed that ColE1 replicons were introduced into *V. harveyi* at a high frequency and were stably maintained; so although drug resistant colonies were observed no transposon mutants were isolated. It would appear that ColE1 based vectors are therefore unsuitable for transposon mutagenesis in *V. harveyi*. ColE1 based vectors have been used succesfully in other studies to perform transposon mutagenesis in a number of Gram-negative soil and water bacteria including *Pseudomonas putida*, *Rhizobium leguminosarum* and *Paracoccus denitrificans* (Fellay *et al.*, 1989). So far

no conjugally mobilisable suicide transposon vector systems have been established for either *V. harveyi* or *V. alginolyticus*.

Bacteriophage mediated P1 transduction was used succesfully to deliver the transposon mini-Mu *lac* into V. *harveyi*. The frequency of auxotrophic mutants observed was around 1% and this implied that every drug resistant strain represented a transposon mutant and not merely a bacterium harbouring P1 DNA. Of 10 000 transposon mutants screened, 16 strains were identified which were sensitive to 10 μM CCCP at pH 8.5. None of these strains were defective in the sodium-dependent NADH-ubiquinone oxidoreuctase as determined by NADH and dNADH oxidase assays in the presence of NaCl or KCl, or by running solubilised membrane samples on native polyacrylamide gels and zymogram staining using dNADH as an electron donor. In all cases the enzyme behaved similarly to the wild type species.

The identity of genes insertionally inactivated by transposons and the reason for the absence of any sodium pump defective mutants among the CCCP sensitive strains is unclear. It is possible that the inactivated genes may have essential roles in cellular metabolism and may code for components of secondary transporters which use sodium as a coupling ion, such as ATPases, antiporters or solute symporters. Mutation of sodium/proton antiporter NhaA from *E. coli* gives a strain which is very sensitive to increased sodium concentrations and to lithium (Pinner *et al.*, 1992). Determining the growth phenotypes of CCCP sensitive strains under varying conditions of sodium and lithium ions may give insights into whether or not sodium/ proton antiporter genes have been affected.

More recent evidence in studies on the CCCP resistant growth of *E. coli* and *S. faecalis* at alkaline pH concluded that the maintenance of a cytoplasmic pH near neutrality is not essential for growth (Mugikura *et al.*, 1990). Strains grew in the presence of 100 μM CCCP at pH values above pH 8. In all cases the pH values obtained for the cytoplasm corresponded to the pH measurements of the growth media. This study suggest that in the presence of CCCP, pH regulation is seriously affected although it appears that residual proton gradients are present across bacterial membranes during aerobic growth. Under conditions where sub optimal levels of proton gradients are present, transporters may still be able to function at a reduced level to ensure homeostasis in the presence of CCCP, however a knock out mutation in one of these proteins may be enough to unbalance ion balances and seriously affect growth.

Sodium transporters may also have important roles in regulating cellular sodium levels. In the same way that proton translocating ATPase mutants are unable to regulate pH (Harvey, 1992), mutants of a sodium translocating ATPase may be unable to maintain optimal sodium balances. In addition such mutants would be unable to utilise sodium gradients in the synthesis of ATP. Another alternative is that the mutation may be in a gene which co regulates the various components of sodium metabolism.

Other CCCP mutants have been characterised. In a bacillus species CCCP sensitivity has been shown to be due to a change in lipid content. Mutations in the fatty acid desaturase system resulted in these membrane changes. The overall fatty acid desaturase activity was greatly increased in these mutants and this results in an increase in the fatty acid content of lipid from 7% to 16%. This is accompanied by a secondary change in the branching pattern of the branch-chain fatty acids (Krulwich *et al.*, 1990). Gram-negative species are in general more resistant to uncouplers than Gram-positives and resistance properties appear to depend on properties of the outer

cell layer (Krulwich *et al.*, 1990). In one *E. coli* mutant which lacks the outer membrane protein 3a, sensitivity to CCCP was greatly increased and it has been suggested that this protein may be involved in binding CCCP.

One of the benefits of using transposon mutagenesis is that the inactivated genes are now physically linked to a drug resistance marker. So one possible way to identify the transposon tagged genes of the CCCP sensitive strains would be by cloning DNA fragments containing the transposon and sequencing sections of DNA adjacent to the transposon. Sequence data could then be compared with genes of known sequence.

During experiments using the *V. alginolyticus* sodium-pump defective mutant Nap 2 it was observed that this strain grew poorly when compared to the wild type strain. Marine vibrios have become specialised to their particular environment and generally require sodium for growth, so it could be foreseen that knocking out an enzyme which is central to sodium metabolism ie. the sodium-dependent NADH-ubiquinone oxidoreductase, could be extremely deleterious to a bacterial cell and its growth. It could also be possible that in such a mutant the accumulation of NADH-ubiquinone oxidoreductase complex which is unable to assemble properly into the membrane may be toxic to the cell. *V. harveyi* transposon mutant strains defective in the sodium-dependent NADH-ubiquinone oxidoreductase in *V. harveyi* may therefore also have grown poorly when compared to the wild type and such strains may have formed drug resistant colonies which were very small and so were never picked for screening on CCCP.

During protein purification experiments a number of observations regarding the sodium-dependent NADH-ubiquinone oxidoreductase in *V. harveyi* were made. When detergent solubilised membranes were run on native polyacrylamide gels and zymogram stained using NADH or dNADH as an electron donor, only one high molecular weight activity band was observed (excluding breakdown products). Additionally during chromatography only one activity peak which could utilise both NADH and dNADH was ever observed. These finding suggest that analogous to the situation in mitochondria only one NADH-ubiquinone oxidoreductase has been found so far in *V. harveyi* and it could be foreseen that a mutation in this enzyme could be deleterious to growth. The reason for the presence of two distinct NADH-ubiquinone oxidoreductase in bacterial membranes is unclear although the non-translocating enzyme may be important for turning over NADH under conditions where the cell wants to limit the number of protons being exported.

Recently the presence of a gene cluster coding for the sodium-dependent NADH-ubiquinone oxidoreductase in *V. alginolyticus* has been discovered (Beattie, *et al.*, 1994). It has been concluded that more than the three subunits as proposed by Hayashi and Unemoto (1987) are involved in sodium-dependent NADH-ubiquinone oxidoreductase activity in *V. alginolyticus*. Oligonucleotide probes based on amino acid sequences of the NqrA and NqrC subunits have been used to clone genes for the sodium-dependent NADH-ubiquinone oxidoreductase from *V. alginolyticus*. Four consecutive open reading frames have been identified so far, encoding subunit proteins of 48.6, 46.8, 27.7 and 22.6 kDa respectively (NqrA-D). So far no NADH, [Fe-S] or FAD binding sites have been identified. So far no membrane spanning helices have been predicted for NqrA. NqrB however is very hydrophobic and 6-12 membrane spanning helices have been predicted. NqrC has a hydrophobic N-terminal region and possesses 1 predicted membrane spanning helice in this area. NqrD is also very hydrophobic and this polypeptide has 4-6 predicted membrane

spanning helices. NqrB and NqrD also have weak homologies with a number of hydrophobic Complex I subunits and sodium channels. How significant these homologies are, remains to be seen. Since it now appears that NQR 1 posses greater than 3 different subunits it may be possible that the 254 kDa band observed on zymogram stains is only a portion of the NDH-1 complex and that the CCCP sensitive strains from *V. harveyi* represent mutants of other subunits involved in sodium-dependent NADH-ubiquinone oxidoreductase activity.

The columns which gave the best results for the purification of sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi* membranes were; Sephacryl 300HR gel filtration, DEAE Sepharose fast flow, Mono Q and hydroxylapatite. A 75 fold increase in specific activity was observed with a recovery of 40% of the total activity.

The gel filtration column Sephacryl 300HR was used as a first step in the purification of the sodium-dependent NADH-ubiquinone oxidoreductase from *V. harveyi*. Gel filtration is often been used at later stages of protein purification and requires concentrated starting sample as material becomes diluted during chromatography (Harris and Angal, 1989). However concentration of membrane proteins is more difficult than for soluble proteins and so gel filtration was used at this early stage with detergent extracted membranes being the most concentrated source of starting material available. This column was most useful in removing low molecular weight proteases which had a deleterious effect on sodium-dependent NADH-ubiquinone oxidoreuctase activity in crude extracts and caused the subunits of the complex to disassociate. It gave some purification but was of limited use in that proteins tended to elute as a broad peak which suggested that proteins may be interacting with each other or with the matrix during chromatography. A similar phenomenon was observed when Superdex 200 gel filtration was used as a later

purification step to remove high molecular weight contaminants. Again a broad peak was observed but this time no purification was achieved.

Charged based separation was more succesful in purifying the sodium-dependent NADH-ubiquinone oxidoreductase; in particular the hydroxylapatite and the anion exchange matrices DEAE Sepharose fast flow and Mono Q. Three and two chromatographic peaks respectively were observed for the ion exchange columns when sodium-dependent NADH-ubiquinone oxidoreductase was purified on them. This compared to only one elution peak when material was chromatographed on the other columns tested. Unfortunately the pH sensitivity of the NADH-ubiquinone oxidoreductase complex precluded the use of cation exchangers to purify it further using ion exchange.

Although ammonium sulphate fractionation has been used successfully in the purification of complex I from mitochondrial membranes in other studies (Walker, 1992), it appears that this method is unsuitable for purifying the sodium-dependent NADH-ubiquinone oxidoreuctase since losses in activity were observed and it caused the subunits of the complex to disassociate. Consistent with the sodium-dependent NADH-ubiquinone oxidoreductase being both large and of a very hydrophobic nature, most of the enzyme activity was fractionated by 10% (w/v) ammonium sulphate. This is contrast to other membrane proteins which require up to 50% (w/v) ammonium sulphate to precipitate them (Rosen, 1978). Pursuing ammonium sulphate fractionation as a step for purifying the the sodium-dependent NADH-ubiquinone oxidoreductase may still be worthwhile though. Trying 2.5% (w/v), 5% (w/v) and 7.5% (w/v) ammonium sulphate cuts may give greater increase in specific activity. It would be particularly useful if the contaminants are more hydrophobic, this way they will fractionate in the earlier cuts and so precipitating the sodium-dependent NADH-ubiquinone oxidoreductase can be avoided.

Of the other columns that were unsuccessful in purifying the sodium-dependent NADH-ubiquinone oxidoreductase two general problems were observed; either the complex bound to the matrix non-specifically or no binding was observed at all. For the hydrophobic interaction column it appeared that proteins were interacting with the matrix very strongly and were eluted as a single peak when excess detergent was added. For the dye ligand columns binding appeared to be occurring as a consequence of electrostatic interaction. With the metal chelate and covalent chromatography matrixes no binding was observed and it may be that the nature of the spacers used may affect the ability of the ligand to bind the target protein.

Another important factor which may affect protein-matrix interactions, protein-protein interactions and protein stability is the nature of the detergent selected for solubilising proteins from the membrane and used as a buffers additive. Detergents are amphiphilic molecules with high solubility in water. The hydrophobic portion of the molecule usually consists of a linear or branched hydrocarbon tail, while the hydrophilic head group may vary in chemical structure.

An important property of detergents is the formation of micelles, which are clusters of detergent molecules in which the hydrophilic head portions face outwards. Solubilised membrane proteins form mixed micelles with detergent. The hydrophobic domain of the protein is shielded from contact from the aqueous buffer by detergent molecules. It has been proposed that the ordered structure of the protein-detergent mixed micelles may mimic the protein-membrane interaction found in cells and certain detergents, including Triton X-100, are involved in stabilising membrane proteins (Harris and Angal, 1990). The stripping of lipid from membrane proteins has been implicated in loss of membrane protein activity and often during purification of such protein exogenous lipid has been added to sample buffers to promote stability (Harris and Angal, 1990).

It could be envisaged that protein-detergent mixed micelle formation provides a protective or stabilising effect on the sodium-dependent NADH-ubiquinone oxidoreductase. This ordered structure may be severely disrupted by precipitation of the protein and this may explain the instability of the sodium-dependent NADH-ubiquinone oxidoreductase during ammonium sulphate fractionation. Measurement of protein/lipid ratios in the membrane and the control of protein/detergent ratios during solubilisation and subsequent processing may be important factors in promoting the stability of the sodium-dependent NADH-ubiquinone oxidoreductase complex.

The critical micelle concentration (cmc) is the lowest detergent concentration at which micelles form. The concentration of detergent used in experiments is important for protein solubilisation, keeping the protein in solution during chromatography and preventing non-specific interaction between protein and matrix. The micelle molecular weight may also be an important factor to consider for experiments involving dialysis of samples, gel filtration chromatography and electrophoresis under non-denaturing conditions and factors which will affect the critical micelle concentration include temperature, pH and ionic strength (Bollag and Edelstein, 1991).

Three broad groups of detergent exist; these are ionic, non-ionic and zwitterionic detergents. Ionic detergents possess head groups which are positvely or negatively charged. Ionic detergents are useful for disrupting protein-protein interactions, but have a tendancy to denature proteins. This type of detergent is not suitable for use with ion exchange chromatography or isoelectric focussing. In contrast non-ionic detergents have uncharged head groups and as a result are less likely to disrupt protein-protein interactions. They are however less denaturing than ionic detergents and are particularly useful for isolating functional protein complexes. Zwitterionic detergents are detergents whose head groups possess both

positive and negative charges and are more efficient than non-ionic detergents at overcoming protein-protein interactions, while causing less denaturation than ionic detergents. One such detergent, CHAPS, has been shown not to interfere with ion exchange chromatography or isoelectric focussing (Bollag and Edelstein, 1991). Adopting a zwitterionic or an ionic detergents during purification may be worthwhile to try and avoid some of the protein-protein interactions and this could improve further the separation achieved on gel filtration, ion exchange and hydroxylapatite columns. This idea of using a detergent which will minimise protein-protein interactions was the rationale behind treating the sodium-dependent NADH-ubiquinone oxidoreductase with SDS, and seeing if it would be possible to renature the enzyme afterwards. Unfortunately in this case SDS was too harsh; however other less denaturing ionic detergents are available and these may be worth further investigation (Bollag and Edelstein, 1991).

Another important aspect which affects the purification of the sodium-dependent NADH-ubiquinone oxidoreductase and must be considered is the non-specific interactions between proteins and the matrices tested. Already it has been mentioned that the inclusion of 10%(w/v) ethylene glycol, and 1%(w/v) Triton X-100 in buffers on Mono Q and hydroxylapatite columns helped reduce non-specific hydrophobic interactions between membrane proteins and chromatographic matrices and resulted in increased recovery of enzyme activity.

Another important factor which affects hydrophobic interactions is ionic strength. Generally higher salt concentrations promote hydrophobic interactions and this principle has been used in hydrophobic interaction chromatography to separate proteins with different hydrophobicities using a decreasing salt gradient (Harris and Angal, 1989). On the Mono Q column, proteins with different isoelectric points are separated by eluting proteins using an increasing gradient of NaCl (Himmelhoch,1971). It can be envisaged that an increasing salt gradient will also

promote hydrophobic interactions so separation on this column may be due to a combination of ion exchange and hydrophobic interaction effects. This would explain why proteins which appear to have similar isoelectric points (they were eluted at the same salt concentration from DEAE fast flow) are separated on a second ion exchange column.

Hydrophobic interaction and ion exchange effects may be of even greater significance during gel filtration chromatography. Chromatographic conditions including 200 mM NaCl are often adopted to avoid any ion exchange effects on the column, however increasing the salt concentration will also increase hydrophobic interactions. This dilemma may be why gel filtration chromatography is of limited use for purifying the sodium-dependent NADH-ubiquinone oxidoreductase. Trying to optimise these conditions using an ionic or zwitterionic detergent may be worthwhile to see if any further purification can be attained using gel filtration..

Reasonable amounts of purification were obtained after chromatography on columns of Sephacryl 300HR gel filtration, DEAE Sepharose fast flow, Mono Q and hydroxylapatite, however the individual subunits of the sodium-dependent NADH-ubiquinone oxidoreductase could not be identified unequivocally and many contaminating polypeptide bands were still observed on SDS PAGE. The major contaminants as observed on native PAGE had molecular weights greater than 400 kDa. This material co-purified with sodium-dependent NADH-ubiquinone oxidoreductase activity on a number of columns including a final stage gel filtration chromatography, and it would appear that these proteins interact in some way during chromatography.

Preparative electrophoresis on polyacrylamide gels proved successful as a method for obtaining samples of sodium-dependent NADH-ubiquinone oxidoreductase which had fewer contaminants. Unfortunately the amounts of protein recovered from these experiments were low and optimisation of protein recovery would be required to obtain enough pure material for N-terminal sequencing of individual polypeptides. Purified sodium-dependent NADH-ubiquinone oxidoreductase samples from preparative electrophoresis and preparative chromatography still had a number of polypeptide bands present as determined by SDS PAGE, however some of these bands were considered as potential sodium-dependent NADH-ubiquinone oxidoreductase subunits. In particular the 66 kDa (ghost band), 62 kDa, 52 kDa (doublet), 46 kDa, 36 kDa and 34 kDa polypeptides were all in the correct size range as described in previous studies (Hayashi and Unemoto, 1987; Bourne and Rich, 1992).

Since it now appears that the sodium-dependent NADH-ubiquinone oxidoreductase is a complex composed of more than three different subunits (Beattie *et al.*, 1994), some of the so called contaminants may in fact turn out to be part of the enzyme complex. In other studies of NADH-ubiquinone oxidoreductases the very fact that these enzymes are complex in nature and tend to disassociate when extracted from membrane have made their purification and subunit compositions tricky (Berks and Ferguson, 1991). In fact even for the most thoroughly studied of the NADH-ubiquinone oxidoreductases, genetic methods have been essential for characterising and determining their exact subunit composition. Preparative electrophoresis techniques may therefore be very useful in separating these polypeptides and resolving the subunit composition of sodium-dependent NADH-ubiquinone oxidoreductases by identifying the N-terminal sequences of individual polypeptides and comparing these with known sequences.

Another possible approach to further purifying the sodium-dependent NADH-ubiquinone oxidoreductase would be to cut partially purified material from a zymogram stained native gel and use that material to raise antibodies to the complex. Affinity purification using the antibody would then be a possibility. The strength and specificity of the antibodies binding ability are crucial to successful affinity purification. Routinely between 1000 and 10 000 fold purifications have been reported using a suitable antibody as a ligand (Harlow and Lane, 1988). Alternatively antibodies could be used to screen expression libraries or to identify subunits on SDS polyacrylamide gels by western blotting.

Once all the NQR 1 genes have been cloned and sequenced it may be possible to overexpress the individual subunits; this would provide large amounts of material for subsequent structural and functional studies. Unfortunately problems can be experienced when membrane proteins are overexpressed and sometimes the gene products are toxic to the cell. Subsequent purification of the individual subunits may also be difficult. Some overexpression systems have been developed which tag the target protein with histidine residues and this or some other affinity purification, such as an antibody approach would be extremely useful for facilitating the rapid purification of the subunits. Problems may also occur when reconstitution of enzyme from individual subunits is attempted due to the complicated nature and inherent instability of the complex.

If sufficient amounts of stable, functionally active complex can be isolated then a number of powerful tools may be applied to dissect its structure and the functions of the individual subunits. Reporter gene systems such as alkaline phosphatase can be used to tag a selected region of the membrane protein being studied. If enzyme activity is detected from whole cells expressing such fusion proteins then it can be concluded that the tagged region of the polypeptide is located outwith the membrane. Immunological techniques may also be applicable to determine membrane topology. Antibodies raised to peptide fragments of the protein being studied will only crossreact with regions of the polypeptide which are located outwith the membrane. These sorts of techniques will allow confirmation of models of numbers and positions of membrane spanning helices which are based on predictions using sequence data. Site directed mutagenesis is a technique which could be used to dissect the function of individual subunits and to identify important residues, such as those involved in substrate binding or those at catalytic sites which confer inhibitor specificity and make this group of NADH-ubiquinone oxidoreductases unique.

As has been found with other multisubunit membrane structures such as the F_1F_0 ATPases, mitochondrial complex I and the NDH-1 enzymes from *E. coli* and *P. denitrificans* it now appears that the best way to determine the exact subunit composition of the sodium-dependent NADH-ubiquinone oxidoreductases will be through a combination of biochemical and genetic approaches (Futai, 1983; Walker, 1992; Weidner, 1993; Xu *et al.*, 1992).

Some evolutionary considerations regarding complex I from mitochondria may be applicable to the sodium-dependent NADH-ubiquinone oxidoreductases. It has been suggested for Complex I that the electron pathways and the proton pumping activities may have evolved independently as separate structural modules which may have come together to make up the present day enzyme (Walker, 1992). An example of modular evolution of complex structures is provided by the F_1F_0ATPase. This can be regarded as two structural modules corresponding to the F_1 and F_0 domains which are often encoded by separate gene clusters (Walker, 1992). A structural module consisting of 900 amino acids is common to complex I and the NAD^+-reducing hydrogenase from *A. eutrophus* as described previously and biosynthetic studies of the *N. crassa* complex I indicate that the extrinsic and intrinsic membrane segments assemble independently (Walker, 1992).

One thing that still remains unclear about complex I is the reason for the large number of subunits that make up these assemblies when compared to the less complex bacterial NADH-ubiquinone oxidoreductases. It is likely that subunits involved in substrate and co-factor binding are likely to be present in the minimal form of the enzyme and it has been argued that some of the additonal subunits may be important in the assembly of the complex and that the complex as well as being a proton translocating NADH-ubiquinone oxidoreductase may have additional functions and enzyme activities (Walker, 1992). Whether any of these structural or evolutionary considerations can be applied to the sodium-dependent NADH-ubiquinone oxidoreductase remains to be seen. One thing is clear however, sequence comparisons between sodium-dependent NADH-ubiquinone oxidoreductase and their proton translocating equivalents in mitochondria or bacteria and analysis of their operon structures have shown that they are not closely related and must have diverged early in evolution (Beattie *et al.*, 1994).

References

Abrahams, V.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Structure at 2.8 Å resolution of the F_1-ATPase from bovine heart mitochondria. Nature **370**: 621-628.

Archer, C.D., Wang, X. and Elliott, T. (1993) Mutants defective in the energy-conserving NADH dehydrogenase of *Salmonella typhimurium* identified by a decrease in energy dependent proteolysis after carbon starvation. Proceedings of the National Academy of Science U.S.A. **90**: 9877-9881.

Beattie, P., Tan, K., Bourne, R., Leach, D., Rich, P. and Ward, F.B. (1994) Cloning and sequencing of four structural genes for the Na^+-translocating NADH-ubiquinone oxidoreductase of *Vibrio alginolyticus*. (In press)

Belas, R., Mileham, A., Simon, M. and Silverman, M. (1984) Transposon mutagenesis of marine Vibrio spp. Journal of Bacteriology **158**: 890-896.

Berks, B.C. and Ferguson, S.J. (1991) Simplicity and complexity in electron transfer between NADH and c-type cytochromes in bacteria. Biochemical Society Transactions **19**: 340-347.

Berman, M.L., Enquist, L.W. and Silhavy, T.J. (1982) Advanced bacterial genetics, P99. Cold Spring Harbor Laboratory Press.

Bernardi, G. (1971) Chromatography of proteins on hydroxyapatite. Methods in Enzymology **22**: 325-339.

Bollag, D.M. and Edelstein, S.J. (1991) Protein methods. Wiley-Liss Publications.

Bourne, R.M. and Rich, P.R. (1992) Characterization of a sodium motive NADH: ubiquinone oxidoreductase. Biochemical Society Transactions **20**: 577-582.

Boyer, P.D. (1980) The binding-change mechanism of ATP synthesis and its relation to proton translocation. European Bioenergetics Conference Report **1**: 133-134.

Buckel, W. and Semmler, R. (1983) Purification, chatacterization and reconstitution of glutaconyl-CoA decarboxylase, a biotin dependent sodium pump from anaerobic bacteria. European Journal of Biochemistry **136**: 427-434

Burbaev, D., Moroz, S.H., Kotlyar, I.A., Sled, V.D. and Vinogradov, A.D. (1989) Ubisemiquinone in the NADH-ubiquinone reductase region of the mitochondrial respiratory chain. FEBS Letters **254**: 47-51.

Capozza, G., Dmitriev, O.Y., Krasnoselskaya, I.A., Papa, S. and Skulachev, V.P. (1991) The effect of F_0 inhibitors on the *Vibrio alginolyticus* membrane ATPase. FEBS Letters **280**: 274-276.

Cairney, J., Higgins, C.F. and Booth, I.A. (1984) Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. Journal of Bacteriology **160**: 22-27.

Chen, C.C. and Wilson, T.H. (1986) Solubilisation and functional reconstitution of the proline transport system of *Escherichia coli.* Journal of Biological Chemistry **261**: 2599-2604.

Clark, J. (1994) Sodium-specific ATPase: search for a V-type ATPase in *Vibrio harveyi.* Microbiology Honours Thesis, University of Edinburgh.

Cox, G.B., Hatch, L., Webb, D., Fimmel, A.L., Lin, Z.H., Senior, A.E. and Gibson, F. (1987) Amino acid substitutions in the kappa-subunit of the F_1F_0 ATPase of *Eschericia coli.* Biochimica et Biophysica Acta **890** (2): 195-204.

Dane, J.B and Scarborough, G.A. (1981) Identification of the phosphorylated intermediate of the *Neurospora* plasma membrane H^+-ATPase as β-aspartyl phosphate. Journal of Biological Chemistry **256**: 10724-10730

Deng, P.S.K., Hatefi, Y. and Chen, S. (1990) *N*-aryl-β-alanyl-NAD^+, a new NAD^+ photoaffinity analogue. Synthesis and labelling of mitochondrial NADH dehydrogenase. Biochemistry **29**: 1094-1098.

Dibrov, P.A., Skulachev, V.P., Sokolov, M.V. and Verkhovskaya, M.L. (1988) The ATP-driven primary Na^+ pump in subcellular vesicles of *Vibrio alginolyticus*. FEBS Letters **233**: 355-358.

Dimroth, P. (1980) A new sodium transport system energized by the decarboxylation of oxaloacetate. FEBS Letters **122**: 234-236.

Dimroth, P. (1982) The role of biotin and sodium in the decarboxylation of oxaloacetate by the membrane-bound oxaloacetate decarboxylase from *Klebsiella aerogenes*. European Journal of Biochemistry **121**: 435-441.

Dimroth, P. (1987) Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. Microbiological Reviews **51** (3): 320-340.

Dimroth, P. (1990) Mechanisms of sodium transport in bacteria. Philosophical Transactions of the Royal Society of London B **326**: 465-477.

Dimroth, P. and Thomer, A. (1983) Subunit composition of oxaloacetate decarboxylase and characterization of the α-chain as carboxyltransferase. European Journal of Biochemistry **137**: 107-112.

Dimroth, P. and Thomer, A. (1989) A primary respiratory Na^+ pump of an anaerobic bacterium: the Na^+-dependent NADH-ubiquinone oxidoreductase of *Klebsiella pneumoniae*. Archives of Microbiology **151**: 439-444.

Ely, B. (1985) Vectors for transposon mutagenesis of non-enteric bacteria. Molecular and General Genetics **200**: 302-304.

Erecinska, M. and Wilson, D.F. (1978) Homeostatic regulation of cellular energy metabolism. Trends in Biochemical Sciences **3**: 219-222.

Eya, S., Noumi, T., Maeda, M. and Futai, M. (1988) Intrinsic membrane sector (F_0) of H^+-ATPase (F_1F_0) from *Escherichia coli*. Journal of Biological Chemistry **263** (21): 10056-10062.

Fellay, F., Krisch, H., Prentki, P. and Frey, J. (1989) Omegon-Km: a transposable element designed for *in vivo* insertional mutagenesis and cloning of genes in Gram-negative bacteria. Gene **76**: 215-226.

Finel, M., Skehel, J.M., Albracht, S.P.J., Fearnley, I.M. and Walker, J.E. (1992) Resolution of NADH: ubiquinone oxidoreductase from bovine heart mitochondria into two subcomplexes one of which contains the redox centres of the enzyme. Biochemistry **31**: 11425-11434.

Frank, L. and Hopkins, I. (1969) Sodium-stimulated transport of glutamate in *Escherichia coli.* Journal of Bacteriology **100**: 329-336.

Friedrich, T., Hofhaus, G., Ise, W., Nehls, U. Schmitz, B. and Weiss, H. (1989) A small isoform of NADH: ubiquinone oxidoreductase (complex I) without mitochondrially synthesized subunits is made in chloramphenicol treated *Neurospora crassa.* European Journal of Biochemistry **180**: 173-180.

Friedrich, T., Strohdeicher, M., Hofhaus, G.,Preis, D., Sahm, H. and Weiss, H. (1990) The same domain motif for ubiquinone reduction in mitochondrial or chloroplast NADH dehydrogenase and bacterial glucose dehydrogenase. FEBS Letters **265**: 37-40.

Futai, M. and Kanazawa, H. (1983) Structure and function of proton-translocating adenosine-triphosphate (F_1F_0): biochemical and molecular biological approaches. Microbiological Reviews **47**: 285-312.

Futai, M. and Kanazawa, H. (1989) ATP Synthase (H^+-ATPase): Results by combined biochemical and molecular biological approaches. Annual Reviews in Biochemistry **58**: 111-136.

Harlow, E. and Lane, D. (1988) Antibodies: A laboratory manual. Cold Spring Harbor Press.

Harris, E.L.V. and Angal, S. (1989) Protein purification methods: A practical approach. IRL Press.

Harris, E.L.V. and Angal, S. (1990) Protein purification applications: A practical approach. IRL Press.

Harvey, W.R., (1992) Physiology of V-ATPases. Journal of Experimental Biology **172**:1-17.

Hashimoto, T., Yoshida, Y. and Tagawa, K. (1983) Binding properties of an intrinsic ATP-ase inhibitor and occurrence in yeast mitochondria of a protein factor which stabilises and facilitates the binding of the inhibitor to F_1F_0-ATPase. Journal of Biochemistry **94**: 715-720.

Hashimoto, T., Yoshida, Y. and Tagawa, K. (1984) Purification and properties of factors in yeast mitochodria stabilising F_1F_0-ATPase-inhibitor complex. Journal of Biochemistry **95**: 131-136.

Hatefi, Y. Haavik, A.G. and Griffiths, D.E. (1962) Studies on the electron transfer system. XL. Preparation and properties of mitochondrial DPNH-coenzyme Q reductase. Journal of Biological Chemistry **237**: 1676-1680.

Hayashi, M. and Unemoto, T. (1984) Characterization of the Na^+-dependent respiratory chain NADH:quinone oxidoreductase of the marine bacterium *Vibrio alginolyticus*. Biochimica et Biophysica Acta **767**: 470-478.

Hayashi, M. and Unemoto, T. (1987) Subunit component and their roles in the sodium-transport NADH-quinone oxidoreductase of a marine bacterium, *Vibrio alginolyticus*. Biochimica et Biophysica Acta **890**: 47-54.

Hayashi, M., Miyoshi, T, Takashina, S. and Unemoto, T. (1989) Purification of NADH-ferricyanide dehydrogenase and NADH-quinone reductase from *Eschericia coli* membranes and their roles in the respiratory chain. Biochimica et Biophysica Acta **977**: 62-69.

Heefner, D.L. and Harold, F.M. (1982) ATP-linked sodium transport in *Streptococcus faecalis.* Proceedings of the National Academy of Sciences, U.S.A. **79**: 2798-2802.

Hilpert, W. and Dimroth, P. (1983) Purification and characterisation of a new sodium transport decarboxylase from *Veillonella alcalescens*. European Journal of Biochemistry **132**: 579-587.

Hilpert, W., Schink, B. and Dimroth, P. (1984) Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as a coupling ion. The EMBO Journal **3**: 1665-1670.

Himmelhoch, S.R. (1971) Chromatography of proteins on ion-exchange adsorbents. Methods in Enzymology **22**: 273-286.

Hjerten, S., Sparrman, M. and Liao, J. (1988) Purification of membrane proteins in SDS and subsequent renaturation. Biochimica et Biophysica Acta **939**: 476-484.

Hoppe, J., Friedl, P., Schairer, H.U., Sebald, W. and Vonmeyenburg, K. (1983) The topology of the proton translocating F_0 component of the ATP synthase from *Escherichia coli* K12, studies with proteases. The EMBO Journal **2** (1): 105-110.

Hoppe, J. and Sebald, W. (1984) The proton conducting F_0 part of the bacterial ATP synthases. Biochimica et Biophysica Acta **768** (1): 1-27.

Ise, W., Haiker, H. and Weiss, H. (1985) Mitochondrial translation of subunits of the rotenone-sensitive NADH:ubiquinone reductase in *Neurospora crassa*. The EMBO Journal **4**: 2075-2080.

Kaback, H.R. (1988) Site-directed mutagenesis and ion-gradient driven active transport. Annual Reviews of Physiology **50**: 234-256.

Kakinuma, Y. and Unemoto, T. (1985) Sucrose uptake is driven by the Na^+ electrochemical potential in the marine bacterium *Vibrio alginolyticus*. Journal of Bacteriology **163**: 1293-1295.

Karplus, P.A, Daniels, M.J. and Herriott, J.R. (1990) Ferredoxin $NADP^+$ reductase: prototype for a structurally novel flavoprotein family. Science **251**: 60-66.

Kay, W.W. and Cameron, M. (1978) Citrate transport in *Salmonella typhimurium*. Archives of Biochemistry and Biophysics **190**: 270-280.

Ken-Dror, S., Lanyi, J.K., Schobert, B., Silver, B. and Avi-Dor, Y. (1986) An NADH-quinone oxidoreductase of the halotolerant bacterium Ba_1 is specifically dependent on sodium ions. Archives of Biochemistry and Biophysics **244**: 766-772.

Kitada, M., Guffanti, A.A. and Krulwich, T.A. (1982) Bioenergetic properties and viability of alkalophilic *Bacillus firmus* RAB as a function of pH and Na^+ contents in the incubation medium. Journal of Bacteriology **152**: 1096-1104.

Kitson, T.M. (1982) Purification of cytoplasmic aldehyde dehydrogenase by covalent chromatography on reduced Thiopropyl-Sepharose 6B. Journal of Chromatography **234**: 181-186.

Kironde, F.A.S. and Cross, R.L. (1986) Adenine nucleotide-binding sites on beef heart F_1-ATPase. Conditions that affect occupancy of catalytic and noncatalytic sites. Journal of Biological Chemistry **261** (27): 2544-2549.

Kotlyar, A.B., Sled, V.D., Burbaev, D., Moroz, I.A. and Vinogradov, A.D. (1990) Coupling site I and the rotenone-sensitive ubisemiquinone in tightly coupled in submitochondrial particles. FEBS Letters **264**: 17-20.

Krishnamoorthy, G. and Hinkle, P.C. (1988) Studies on the electron transfer pathway, topography of iron-sulphur centres, and site coupling in NADH-Q oxidoreductase. Journal of Biological Chemistry **263**: 17566-17575.

Krulwich, T.A. (1983) Na^+/H^+ antiporters. Biochimica et Biophysica Acta **726**: 245-264.

Krulwich, T.A., Quirk, P.G. and Guffanti, A.A. (1990) Uncoupler-resistant mutants of bacteria. Microbiological Reviews **54**: 52-65.

Krumholz, L.R., Esser, U. and Simoni, R.D. (1990) Characterization of the H^+-pumping F_1F_0 ATPase of *Vibrio alginolyticus*. Journal of Bacteriology **172** (12): 6809-6817.

Kuki, M., Noumi, T., Maeda, M., Amemura, A. and M. Futai. (1988) Functional domains of ε-subunit of *Escherichia coli* H^+ ATPase (F_1F_0). Journal of Biological Chemistry **263**: 17437-17442.

Lanyi, J.K. (1979) The role of Na^+ in transport processes of bacterial membranes. Biochimica et Biophysica Acta **559**: 377-397.

Lim, L.W., Shamala, N., Mathews, F.S., Steenkamp, D.J., Hamlin, R. and Xuong, N.H. (1986) Three-dimensional structure of the iron-sulfur flavoprotein trimethylamine dehydrogenase at 2.4 Å resolution. Journal of Biological Chemistry **261**: 15140-15146.

Lindqvist, Y. (1989) Refined structure of spinach glycolate oxidase at 2.4 Å resolution. Journal of Molecular Biology **209**: 151-166.

Lorenzo, V., Herrero, M., Jakubzk, U. and Timmis, K.N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram-negative Eubacteria. Journal of Bacteriology **172** (11): 6569-6572.

Lowe, C.R. and Pearson, J.C. (1984) Affinity chromatography on immobilized dyes. Methods In Enzymology **104**: 97-113.

MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Amino-acid sequence of Ca^{2+} + Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature **316**: 696-700.

Maggio, M.B., Pagan, J., Parsonage, D., Hatch, L. and Senior, A.E. (1987) The defective proton-ATPase of *uncA* mutants of *Escherichia coli.* Journal of Biological Chemistry **262**: 8981-8984.

Maggio, M.B., Parsonage, D. and Senior, A.E. (1988) A mutation in the alpha-subunit of F_1-ATPase from *Escherichia coli* affects the binding of F_1 to the membrane. Journal of Biological Chemistry **263**: 4619-4623.

Masui, R., Wakabayashi, S., Matsubara, H. and Hatefi, Y. (1991) The amino acid sequences of two 13 kDa polypeptides and partial amino acid sequence of 30 kDa polypeptide of complex I from bovine heart mitochondria: possible location of iron-sulfur clusters. Journal of Biochemistry **109**: 534-543.

Mathews, F.S. (1991) New flavoproteins. Current Opinion in Structural Biology **1**: 954-967.

McLaggan, D., Selwyn, M.J. and Dawson, A.P. (1984) Dependence on Na^+ of control of cytoplasmic pH in facultative alkalophile. FEBS Letters **165**: 254-258.

Miller, V. and Mekalanos, J. (1986) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *tox R*. Journal of Bacteriology **170** (6): 2575-2583.

Mitchell, P. (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biological Reviews **41**: 455-502.

Mugikura, S., Nishikawa, M., Igarashi, K. and Kobayashi, H. (1990) Maintenance of a neutral cytoplasmic pH is not obligatory for growth of *Escherichia coli* and *Streptococcus faecalis* at an alkaline pH. Journal of Biochemistry **108**: 86-91.

Nakamura, T., Kawasaki, S. and Unemoto, T. (1992) Role of K^+ and Na^+ in pH homeostasis and growth of the marine bacterium *Vibrio alginolyticus*. Journal of General Microbiology **138**: 1271-1276.

Nakamura, T., Komano, Y., Itaya, E., Tsukamoto, K., Tsuchiya, T. and Unemoto, T. (1994) Cloning and sequencing of an Na^+/H^+ antiporter gene from the marine bacterium *Vibrio alginolyticus*. Biochimica et Biophysica Acta **1190**: 465-468.

Nelson, N. (1992) The vacuolar H^+-ATPase- one of the most fundamental ion pumps in nature. Journal of Experimental Biology **172**: 19-27.

Niiya, S., Yamasaki, K., Wilson, T.H. and Tsuchiya, T. (1982) Altered cation coupling to melibiose transport in mutants of *Escherichia coli*. Journal of Biological Chemistry **257**: 8902-8906.

Niven, D.F. and MacLeod, R.A. (1980) Sodium ion substrate symport in a marine bacterium. Journal of Bacteriology **142**: 603-607.

Pedersen, P. and Carafoli, E. (1987) Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. Trends in Biochemical Science **12**: 146-150.

Peterson, G.L. (1977) A simplication of the protein assay method of Lowry *et al.* which is more generally applicable. Analytical Biochemistrty **83**: 346-356.

Pinner, E., Padan, E. and Schuldiner, S. (1992) Cloning, sequencing and expression of the *nhaB* gene, encoding a Na^+/H^+ antiporter in *Escherichia coli*. The Journal of Biochemistry **267**: 11064-11068.

Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. Nature **258**: 598-599.

Pourcher, T., Bassilana, M., Sarkar, H.K. Kaback, H.R. and Leblanc, G. (1990) The melibiose/Na^+ symporter of *Escherichia coli*: kinetic and molecular properties. Philosophical Transactions of the Royal Society London B **326**: 411-423.

Radford, S.E., Laue, E.D., Perham, R.N., Miles, J.S. and Guest, J.R. (1987) Domains in pyruvate dehydrogenase complex. Biochemical Journal **247**: 641-649.

Ragan, C.I., Galante, Y.M., Hatefi, Y. and Ohnishi, T. (1982a) Resolution of a mitochondrial NADH dehydrogenase and the isolation of two iron-sulphur proteins. Biochemistry **21**: 590-594.

Ragan, C.I., Galante, Y.M. and Hatefi, Y. (1982b) Purification of three iron-sulphur proteins from the iron-protein fragment of mitochondrial NADH-ubiquinone reductase. Biochemistry **21**: 2518-2524.

Ragan, C.I. (1990) Structure and function of an archetypal respiratory chain complex: NADH-ubiquinone reductase. Biochemical Society Transactions **18**: 515-516.

Richter, M.L., Patrie, W.J. and McCarty, R.E. (1984) Preparation of the ε-subunit deficient chloroplast coupling factor I in reconstitutively active forms. Journal of Biological Chemistry **259** (12): 7371-7373.

Rosen, S. (1978) Purification of beef heart cytochrome c oxidase by hydrophobic interaction chromatography on Octyl-Sepharose CL-4B. Biochimica et Biophysica Acta **523**: 314-320.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.

Schneider, E. and Altendorf, K. (1987) Bacterial adenosine 5'-triphosphate synthase (F_1F_0): Purification and reconstitution of F_0 complexes and biochemical and functional characterization of their subunits. Microbiological Reviews **51** (4): 477-497.

Schwarz, E. and Oesterhelt, E. (1985) Cloning and expression of *Klebsiella pneumoniae* genes coding for citrate transport and fermentation. The EMBO Journal **4**: 1499-1603.

Scrutton, N.S., Berry, A. and Perham, R.N. (1990) Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature **147**: 147-149.

Shaltiel, S. (1974) Hydrophobic chromatography. Methods In Enzymology **34**: 126-140.

Simon, R., O'Connell, M., Labes, M. and Puhler, A. (1986) Plasmid vectors for the genetic analysis and manipulation of Rhizobia and other gram-negative bacteria. Methods In Enzymology **118**: 640-659.

Smith, W.W., Pattridge, K.A. and Ludwig, M.L. (1983) Structure of oxidised flavodoxin from *Anacystis nidulans*. Journal of Molecular Biology **165**: 737-755.

Soga, S., Noumi, T., Takeyama, M., Maeda, M. and Futai, M. (1989) Mutational replacements of conserved amino acids residues in the α-subunit change the catalytic properties of *Escherichia coli* F_1-ATPase. Archives of Biochemistry and Biophysics **268**: 643-648.

Suzuki, H. and Ozawa, T. (1986) An ubiquinone-binding protein in mitochondrial NADH-ubiquinone reductase (complex I). Biochemical and Biophysical Research Communications **138**: 1237-1242.

Taglicht, D., Padan, E. and Schuldiner, S. (1991) Overproduction and purification of a functional Na^+/H^+ antiporter coded by *nhaA* (*ant*) from *Eschericia coli*. Journal of Biological Chemistry **266** (8): 11289-11294.

Taglicht, D., Padan, E. and Schuldiner, S. (1993) Proton-sodium stoichiometry of NhaA, an electrogenic antiporter from *Eschericia coli*. Journal of Biological Chemistry **268**: 5382-5387.

Takase, K., Yamato, I. and Kakinuma, Y. (1993) Cloning and sequencing of the genes coding for the A and B subunits of the vacuolar-type Na^+-ATPase from *Enterococcus hirae*. Journal of Biological Chemistry **268** (16): 11610-11616.

Takeyama, M., Noumi, T., Maeda, M. and Futai, M. (1988) F_0 portion of the *Escherichia coli* H^+-ATPase: carboxyl-terminal region of the b-subunit is essential for the assembly of functional F_0. Journal of Biological Chemistry **263** (31):16106-16112.

Tokuda, H. (1983) Isolation of *Vibrio alginolyticus* mutants defective in the respiration-coupled Na^+ pump. Biochemical and Biophysical Research Communications **144**: 113-118.

Tokuda, H. (1986) Sodium translocation by NADH oxidase of *Vibrio alginolyticus*: isolation and characterization of sodium pump defective-mutants. Methods in Enzymology **125**: 520-530.

Tokuda, H. and Unemoto, T. (1981) A respiration-dependent primary sodium extrusion system functioning at alkaline pH in the marine bacterium *Vibrio alginolyticus*. Biochemical and Biophysical Research Communications **102**: 265-271.

Tokuda, H. and Unemoto, T. (1982) Characterization of the respiration-dependent Na^+ pump from the marine bacterium *Vibrio alginolyticus*. Journal of Biological Chemistry **257**: 10007-10014.

Tokuda, H. and Unemoto, T. (1983) Growth of a marine *Vibrio alginolyticus* and moderately halopholic *V. costicola* becomes uncoupler resistant when the respiration-dependent Na^+ pump functions. Journal of Bacteriology **156**: 636-643.

Tokuda, H. and Unemoto, T. (1984) Na^+ is translocated at NADH:quinone oxidoreductase segment of the respiratory chain of *Vibrio alginolyticus*. Journal of Biological Chemistry **259**: 7785-7790.

Tokuda, H., Sugusawa, M. and Unemoto, T. (1982) Roles of Na^+ and K^+ in α-aminoisobutyric acid transport by the marine bacterium *Vibrio alginolyticus*. Journal of Biological Chemistry **257**: 788-794.

Tokuda, H., and Kogure, K. (1989) Generalized distribution and common properties of Na^+-dependent NADH:quinone oxidoreductases in gram-negative marine bacteria. Journal of General Microbiology **135**: 703-709.

Tozer, G. and Dunn, S.D. (1987) The e subunit and inhibitory monoclonal antibodies interact with the carboxy-terminal region of the b subunit of the *Eschericia coli* F_1-ATPase. Journal of Biological Chemistry **262** (22): 10706-10711.

Tran-Betcke, A., Warnecke, U., Bocker, C., Zaborosch, C. and Friedrich, B. (1990) Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. Journal of Bacteriology **172**: 2920-2929.

Trumpower, B.L. (1990) The proton motive Q-cycle. Journal of Biological Chemistry **265**: 11409-11412.

Trumpower, B.L. and Gennis, R.B. (1994) Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. Annual Review of Biochemistry, **63**: 675-716.

Tsuchiya, T. and Shinoda, S. (1985) Respiration-driven Na^+ pump and Na^+ circulation in *Vibrio parahaemolyticus*. Journal of Bacteriology **162**: 794-798.

Tuschen, G., Sackmann, U., Nehls, U., Haiker, H., Buse, G. and Weiss, H. (1990) Assembly of NADH: ubiquinone reductase (complex I) in *Neurospora* mitochondria: independent pathways of nuclear encoded and mitochondrially encoded subunits. Journal of Molecular Biology **213**: 845-857.

Vagina, M.L., Skulachev, V.P. and Smirnova, I.A. 1991 Sodium and proton pumps in the respiratory chain of an alkalotolerant Bacillus. Biochemistry-USSR **56**: 499-509.

Von Heijne, G. (1986) The distribution of positvely charged residues in bacterial inner membrane proteins correlates with transmembrane topology. The EMBO Journal **5:** 3021-3027.

Vuokila, P.T. and Hassinen, I.E. (1988) N,N'-dicyclohexyylcarbodiimide sensitivity of bovine heart mitochondrial NADH:ubiquinone oxidoreductase. Biochemical Journal **249**: 339-344.

Vuokila, P.T. and Hassinen, I.E. (1989) DCCD sensitivity of electron and proton transfer by NADH:ubiquinone oxidoreductase in bovine heart submitochondrial particles, a thermodynamic approach. Biochimica et Biophysica Acta **974**: 219-222.

Walker, J.E., Saraste, M., and Gay, N.J. (1984) The *unc* operon, nucleotide sequence, regulation and structure of ATP-synthase. Biochimica et Biophysica Acta **768**: 164-200.

Walker, J.E., Fearnley, I.M., Lutter, R., Todd, R.J. and Runswick, M.J. (1990) Structural aspects of proton-pumping ATPases. Philosophical Transactions of the Royal Society London B **326**: 367-378.

Walker, J. E. (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. Quarterly Reviews of Biophysics **25** (3): 253-324.

Weidner, U., Geier, S, Ptock, A., Friedrich, T., Leif, H. and Weiss, H. (1993) The gene locus of the proton-translocating NADH:ubiquinone oxidoreductase in *Escherichia coli*. Journal of Molecular Biology **233**: 109-122.

Weiss, H., Friedrich, T., Hofhaus, G. and Pries, D. (1991) The respiratory chain NADH dehydrogenase (complex I) of mitochondria. European Journal of Biochemistry **197**:563-576.

Wierenga, R.K., Terpstra, P. and Hol, W.G.J. (1986) Prediction of the occurence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. Journal of Molecular Biology **187**: 101-107.

Wohlfarth, G and Buckel, W. (1985) A sodium ion gradient as energy source for *Peptostreptococcus asaccharolyticus*. Archives of Microbiology **142**: 128-135.

Xu, X., Koyama, N., Cui, M., Yamagishi, A., Nosoh, Y. and Oshima, T. (1991) Nucleotide sequence of the gene encoding NADH dehydrogenase from an alkalophile, Bacillus sp. strain YN-1. Journal of Biochemistry **109** (5): 678-683.

Xu, X., Matsuno-Yagi, A. and Yagi, T. (1993) DNA sequencing of the seven remaining structural genes of the gene cluster encoding the energy-transducing NADH-quinone oxidoreductase of *Paracoccus denitrificans*. Biochemistry **32**: 968-981.

Yagi, T. (1986) Purification and characterization of NADH dehydrogenase complex from *Paracoccus denitrificans*. Archives of Biochemistry and Biophysics **250**: 302-311.

Yagi, T. (1987) Inhibition of NADH:ubiquinone oxidoreductase activity by N,N'-dicyclohexyylcarbodiimide and correlation of this inhibition with the occurence of energy-coupling site I in various organisms. Archives of Biochemistry **26**: 2822-2828.

Yagi, T. (1991) Bacterial NADH-quinone oxidoreductases. Journal of Bioenergetics and Biomembranes **23** (2): 211-225.

Yagi, T. and Dinh., T.M. (1990) Identification of the NADH-binding subunit of NADH-ubiquinone oxidoreductase of *Paracoccus denitrificans.* Biochemistry **29**: 5515-5520.

Yagi, T. and Hatefi, Y. (1988) Identification of the dicyclohexyylcarbodiimide binding subunit of NADH:ubiquinone oxidoreductase (complex I). Journal of Biological Chemistry **263**: 16150-16155.

Yagi, T., Hon-nami, K. and Ohnishi, T. (1988) Purification and characterisation of two types of NADH-quinone reductase from *Thermus thermophilus* HB-8. Biochemistry **27**: 2008-2013.

Young, I.G., Rogers, B.L., Campbell, H.D., Jaworowski, A. and Shaw, D.C. (1981) Nucleotide sequence coding for the Respiratory NADH Dehydrogenase of *Escherichia coli.* European Journal of Biochemistry **116**: 165-170.