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Purification and characterisation of the plasma membrane NADH:oxidoreductase

A thesis presented for the degree of

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By

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Summary

In this thesis the molecular identification of those proteins involved in forming the PMOR was undertaken. Only one example (the NADPH-oxidase of neutrophils) of a molecularly characterised transplasma membrane electron transport system within mammalian cells existed when this thesis was started. In chapter 3, I report on the process of identifying a transmembraneous NADH:ferricyanide-reductase from Namalwa plasma membranes. A 35 kDa protein was identified as a likely NADH:ferricyanide-reductase, based on its co-elution with enzyme activity from a Blue Sepharose affinity column. In chapter 4, I report on the identification of that 35 kDa protein as VDAC1. Using immunoprecipitation and purification of VDAC1, I demonstrate that this protein is able to act as an NADH-ferricyanide-reductase *in vitro*.

VDAC1 was shown by FACS analysis to be present on the plasma membrane of Namalwa cells. I used both monoclonal and polyclonal antibodies to detect the presence of the protein at the level of the plasma membrane.

To demonstrate that the NADH:ferricyanide-reductase activity of VDAC1 was not an *in vitro* artefact, COS7 cells were transfected with pl-VDAC1-GFP. Cells transfected with this vector demonstrated at least a 40-fold increase in whole cell ferricyanide-reduction when compared to the mock transfected cells.

Chapter 5 shows the partial purification and characterisation of the NADH-oxidase from Namalwa plasma membranes. Dihydrocapsiate, a member of the caspsiate family of chemicals found naturally in sweet chilli peppers, was shown to be an inhibitor of the plasma membrane NADH-oxidase. Dihydrocapsiate represents

a new class of non-pungent vanilloids, that inhibits the PMOR, but not the VR1 receptor. Vanillylamine, a further vanilloid, was shown to also inhibit the NADH-oxidase. The inhibitory properties of the vanilloid family of compounds were used in an attempt to purify the plasma membrane NADH-oxidase. Vanillylamine was linked to an agarose column and used to partially purify the NADH-oxidase. Elution of the column with inhibitors of the NADH-oxidase activity (capsaicin and vanillylamine) allowed no further purification of the enzyme. However when I eluted the column with stimulators of the oxidase (CoQ), an active enzyme preparation was eluted from the vanilloid column.

Declaration

This thesis contains no material that has been presented or accepted for the award of any other degree or diploma in this or any other university. Except where indicated in the text, the data presented herein are the results of the author, and to the best of my knowledge have not been previously published by any other person.

Mark A. Baker

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Publications

Journal Publications - published

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Macho, A., Lucena, C. Tron, G.C., Minassi, A., **Baker, M.A.**, Lawen, A. Appendion, G. and Muñoz. M. (2001) Chemopreventive and antitumor activity of non pungent capsaicinoids from sweet pepper.

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Baker, M.A. and Lawen, A. (2000) Partial purification and characterisation of a plasma membrane NADH:ferricyanide-reductase from human lymphoblastoid Namalwa cells. Fifth International Conference on Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease, 26th-29th March (Hamburg, Germany). http://rrz.uni-hamburg.de/biologie/ialb/redox2000/abstracts/P7.htm

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Oral presentations

VDAC1 is an NADH:ferricyanide-reductase. Department of Biological Science, Newcastle University, NSW, Australia 4th April, 2001.

Partial purification and characterisation of a plasma membrane NADH:ferricyanide-reductase from human lymphoblastoid Namalwa cells. Fifth International Conference on Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease, 27th March, 2000 (Hamburg, Germany).

Partial purification and characterisation of a plasma membrane NADH oxidoreductase (PMOR) from human lymphoblastoid Namalwa cells. Forschungsstelle 'Enzymologie der Proteinfaltung' der Max-Planck-Gesellschaft, Martin-Luther-Universitat Halle, Germany, 31st March, 2000.

Purification and characterisation of the plasma membrane NADH:ferricyanide-reductase. Department of Biochemistry and Molecular Biology 4th annual PhD Conference 16th July 1998.

Partial purification of the plasma membrane NADH-oxidase from Namalwa cells. Department of Biochemistry and Molecular Biology, Melbourne Australia. 18th September, 1998.

List of abbreviations

AFR	Ascorbate free radical
AIF	Apoptosis inducing factor
ANT	Adenosine nucleotide transporter
BCA	Bichinchoninic acid
CoQ	Coenzyme Q-*10*
DCCD	Dicyclohexylcarbodiimide
DCIP	2,6-Dichloroindophenol
DEAE	Diethylaminoethyl-Sephacel
DHA	Dehydroascorbate
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethlenediaminetetracetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FRE1	Ferric reductase enzyme 1
FRE2	Ferric reductase enzyme 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HTP	Hydroxylapatite
NEM	*N*-ethylmaleimide
NP-40	Nonidet 40
NTSB	2-Nitro-5-thiosulfobenzoate
PBS	Phosphate buffered saline
*p*CMBS	*p*-chloromercuriphenylsulfonic acid
PMOR	Plasma membrane NADH:oxidoreductase

PTP	Permeability transition pore
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N'N'-tetramethylethylenediamine
tNOX	NADH-oxidase of transformed cells
TPA	12-*O*-tetradecamoylphorbal-13-acetate
VDAC	Voltage dependent anion channel
VR1	Vanilloid Receptor 1
WST-1	2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt
YVDAC	Yeast voltage dependent anion channel

CHAPTER 1 - Introduction

1.1 The plasma membrane NADH:oxidoreductase

1.1.1 Introduction

The inner mitochondrial and the thylakoid membranes contain the best-characterised transmembrane electron transport systems to date, namely the respiratory and photosynthetic electron transport chains. However, within every membrane there appears to exist similar electron transport systems and within every plasma membrane studied so far there exists at least one transmembrane electron transport system. namely the plasma membrane NADH:oxidoreductase or PMOR. The initial observations of redox regulation at the plasma membrane level were described by Voegtlin and co-workers[1] who showed that dyes, which were later shown to be impermeant to cells[2,3], were reduced by tissue slices and *in vivo*. Further evidence pointing to an electron transport system located in the plasma membrane was then reported by Brooks[4], who described plasma membrane-impermeant redox dyes being capable of activating the growth of sea urchin eggs whilst being reduced. Theories explaining the presence of such enzymes in the plasma membrane then surfaced, according to which, components of the endoplasmic reticulum would presumably be transported through the Golgi, onto the plasma membrane taking along with it, components of a transmembrane electron transport system from the endoplasmic reticulum[5]. A plasma membrane electron transport system was later evidenced by Barnes and Sato[6] who defined ferrisulphate as a growth factor as it could replace transferrin and maintain cell survival. Later on, it was shown by Ellem and Kay[7] that ferrisulphate can be replaced by ferricyanide. Since ferricyanide is anionic, large in structure and hydrophilic in nature, it is believed not to permeate

the plasma membranes of cells[2,3]. This finding established the plasma membrane ferricyanide-reductase as a vital communicator from the outside world to intracellular signalling. Many oxidoreductases have since been found to be present on the plasma membrane from protozoa to man[8].

Erythrocyte membrane preparations (which lack any contamination with intracellular membranes) were shown to contain ferricyanide-reductase activity which is specific for NADH as a co-factor[9,10]. Plasma membrane preparations from other mammalian sources have also been shown to contain such an activity, which is not inhibitable by the mitochondrial inhibitors cyanide, 2-heptyl-4-hydroxyquinoline-*N*-oxide, rotenone and antimycin A[11,12], thus clearly distinguishing it from mitochondrial oxidoreductases. On the other hand atebrin has been described as a specific inhibitor of the NADH:ferricyanide-reductase activity[11,12], thereby distinguishing it from the atebrin-insensitive corresponding enzymes from the endoplasmic reticulum.

Transplasma membrane NADH-oxidoreductase activities have been linked to several vital cellular functions, including growth control[13], iron uptake[14], apoptosis[15-17], bioenergetics[11,18] and hormone responses[19]. This diversity of functions may be due to the privileged position in which such enzymes are located in, being the sensory switch between the external environment and the intracellular signalling cascades.

1.1.2 Introduction into nomenclature of plasma membrane redox enzymes

Proteins that bind to, or associate with, the plasma membrane, can be classified by structural and functional characteristics. Structurally, ecto, extrinsic or peripheral type proteins are defined as those proteins that associate with the surface of the plasma membrane[20]. In contrast, intrinsic or transmembrane type proteins

span the plasma membrane, having access to both the extracellular matrix and the cytoplasm. Functionally, there are a number of different enzymes associated with the plasma membrane, including the family of oxidoreductases. Oxidoreductases catalyse oxido-reduction reactions. The substrate which is oxidised is referred to as the hydrogen or electron donor. In contrast, the substrate which is reduced is referred to as the hydrogen or electron acceptor. The recommended name given to enzymes involved in oxido-reduction reactions are dehydrogenases or reductases[21]. Oxidase is [illegible] cases where O_2 is the electron acceptor.

1.1.3 Characteristics of mammalian plasma membrane NADH:ferricyanide-reductases

The analysis of the NADH:ferricyanide-reductase from Ehrlich ascites tumour cells has revealed that the enzyme may be a glycoprotein and that the glycosidic moiety may be required for full activity since enzyme activity is sensitive to treatment with various glycosidases[22]. Furthermore, NADH:ferricyanide-reductase activity can be strongly activated by the bivalent cations Ca^{2+}, Mn^{2+}, Sr^{2+} and Mg^{2+} at micromolar concentrations[22]. An enzyme partially purified from rat liver has been described to be NADH specific. Exchange of NADH by NADPH in the *in vitro* incubation medium leads to abolishment of the activity[23]. The enzyme can be stimulated by addition of co-factors such as co-enzyme Q-*10* (CoQ) and inhibited by covalent binding of the thiol compound *p*-chloromercuriphenylsulfonic acid (*p*CMBS)[23,24], suggesting an active thiol group either in its active site or very close to it. There are at least two lines of experimental evidence to suggest transmembraneous localisation of the enzyme:

1) it has been shown that a dehydrogenase capable of oxidising NADH must have both sides of the plasma membrane available for maximal activity[10];

2) upon modification with diazobenzenesulfonate (a plasma membrane impermeant agent which can bind to exposed functional groups of membrane proteins and thus can react with both transmembraneous and external proteins but not with internal proteins) cells lose some of their ferricyanide-reductase activity[10,11].

In HeLa cells, biphasic ferricyanide-reduction kinetics have been observed[11]. Recently, evidence has been presented for two different ferricyanide-reductases in HL-60 cells[25], making the measurement of data on *in vivo* ferricyanide-reduction rather difficult to interpret. Therefore the major aim of the field obviously has to be the molecular characterisation of the enzymes involved in the transplasma membrane complex.

The plasma membrane NADH:ferricyanide-reductase is believed to be in tight association with another plasma membrane redox enzyme called the plasma membrane NADH-oxidase[13]. The oxidase also uses NADH as a co-factor and presumably oxygen as a final electron acceptor[13]. The NADH-oxidase may be functionally linked to the NADH:ferricyanide-reductase via CoQ[26].

1.1.4 Ascorbate regulation

Although the NADH:ferricyanide-reductase is commonly analysed using ferricyanide as a substrate, clearly ferricyanide is not the physiological substrate of the enzyme. To date many redox compounds have been found in, and isolated from, the plasma membrane. They include thiols, iron/copper cytochromes (including b_5,

P-420 and P-450) and CoQ[27-29]. These all constitute potential physiological substrates for the NADH:ferricyanide-reductase. An additional potential physiological substrate for the NADH:ferricyanide-reductase is ascorbate. Ascorbate, like ferricyanide, can increase the rate of growth of tumour cells when added to the media[30]. 0.2 mM ascorbate stimulates A-204 rhabdomyosarcoma and HL-60 cell growth[30], however the same concentration inhibits growth of U-20s osteosarcoma, Y-79 retinoblastoma and several neuroblastoma cell lines[31,32]. There appears to be a connection between ascorbate oxidation and ferricyanide-reduction. There is some evidence to suggest that ascorbate may be able to act as another intracellular donor for the NADH:ferricyanide-reductase *in vivo*. Cell membranes loaded with ascorbate show a higher rate of external ferricyanide-reduction than those loaded with NADH[33]. However, the suggestion of ascorbate being an electron donor for the reductase has not yet been examined *in vitro*, with purified enzyme preparations. Furthermore, evidence presented by Van Duijn *et al* [25] indicates that the ferricyanide-reduction that is stimulated by ascorbate is not due to the plasma membrane NADH:ferricyanide-reductase. Addition of extracellular ascorbate has been shown to increase the reduction of ferricyanide at the plasma membrane[34]. One mechanism that has been proposed to explain the increase of ferricyanide-reduction by external ascorbate, is that upon addition of extracellular ascorbate, ferricyanide reacts directly, and non-enzymatically forms ferrocyanide and ascorbate free radical (AFR)[34] (Fig. 1.1). The ascorbate free radical then becomes reduced into ascorbate by a second enzyme distinct from the NADH:ferricyanide-reductase, namely the AFR-reductase. However, since addition of excess ferricyanide to a solution of ascorbate yields dehydroascorbate (DHA) in an extremely quick manner, this mechanism appears to be unlikely[25]. Nevertheless the AFR-reductase may still play

a role in ascorbate homeostasis. It has been shown that ascorbate, at 37°C, in the presence of oxygen, spontaneously undergoes autoxidation to form the free radical[35]. However in the presence of cells, both the rate of ascorbate autoxidation decreases and the amount of ascorbate in the media significantly increases[36]. Such a stabilisation in external ascorbate may be due to the AFR-reductase. Increasing the intracellular concentration of ascorbate has also been shown to result in an increase in ferricyanide-reduction in whole red blood cells[37]. The mechanism proposed for this increase is a more complicated one. The stimulation of ferricyanide-reduction involves the uptake of the two-electron oxidised form of ascorbate, DHA into the cytosol of cells. DHA and extracellular ferricyanide are utilised by transplasma membrane ascorbate:ferricyanide-reductase forming ascorbate and ferrocyanide[38].

The uptake of DHA has been shown to occur through the Glut 1 transporter[39]. Once taken into the cell, DHA is converted back to ascorbate (Fig. 1.1). The mechanism of this conversion remains controversial. The reduction of DHA to ascorbate inside the cells has been linked to a second plasma membrane electron transport system, not involving the NADH:ferricyanide-reductase[25]. The evidence for this comes from experiments which show that *p*CMBS, normally a potent inhibitor of the NADH:ferricyanide-reductase, has little effect on the reduction of DHA to ascorbate[25]. This second plasma membrane ascorbate:ferricyanide-reductase may be capable of converting DHA back into ascorbate, using extracellular ferricyanide. This would explain the increased rate of ferricyanide-reduction upon addition of ascorbate[25]. Interestingly, upon blocking the Glut 1 receptor before addition of ascorbate, no further ascorbate-dependent ferricyanide-reduction occurs. However, blocking the Glut 1 receptor after addition of ascorbate does not inhibit ascorbate-dependent ferricyanide-reduction[25]. One

candidate for the ascorbate:ferricyanide-reductase could be the selenoenzyme thioredoxin reductase. The evidence to suggest this comes from experiments in which selenium deficient rats show a decreased generation of ascorbate in rat liver cytosolic fractions when compared to normal rats[40]. Also, gold containing compounds have been shown to inhibit selenoenzymes[41]. Upon addition of gold thioglucose (aurothioglucose) to purified rat liver cytosol, a decrease of 90% is seen in ascorbate regeneration[40]. This suggests that the NADPH-dependent thioredoxin reductase may be the second electron transport system. An additional route of DHA uptake was evidenced by nuclear magnetic resonance studies in whole red blood cells. Extracellulary added ^{13}C-DHA was shown to enter the cells. During this process, DHA became rapidly reduced. This uptake and the concomitant reduction are believed to occur through a novel transport mechanism, a process that appears to be linked to NADH oxidation within cells[37].

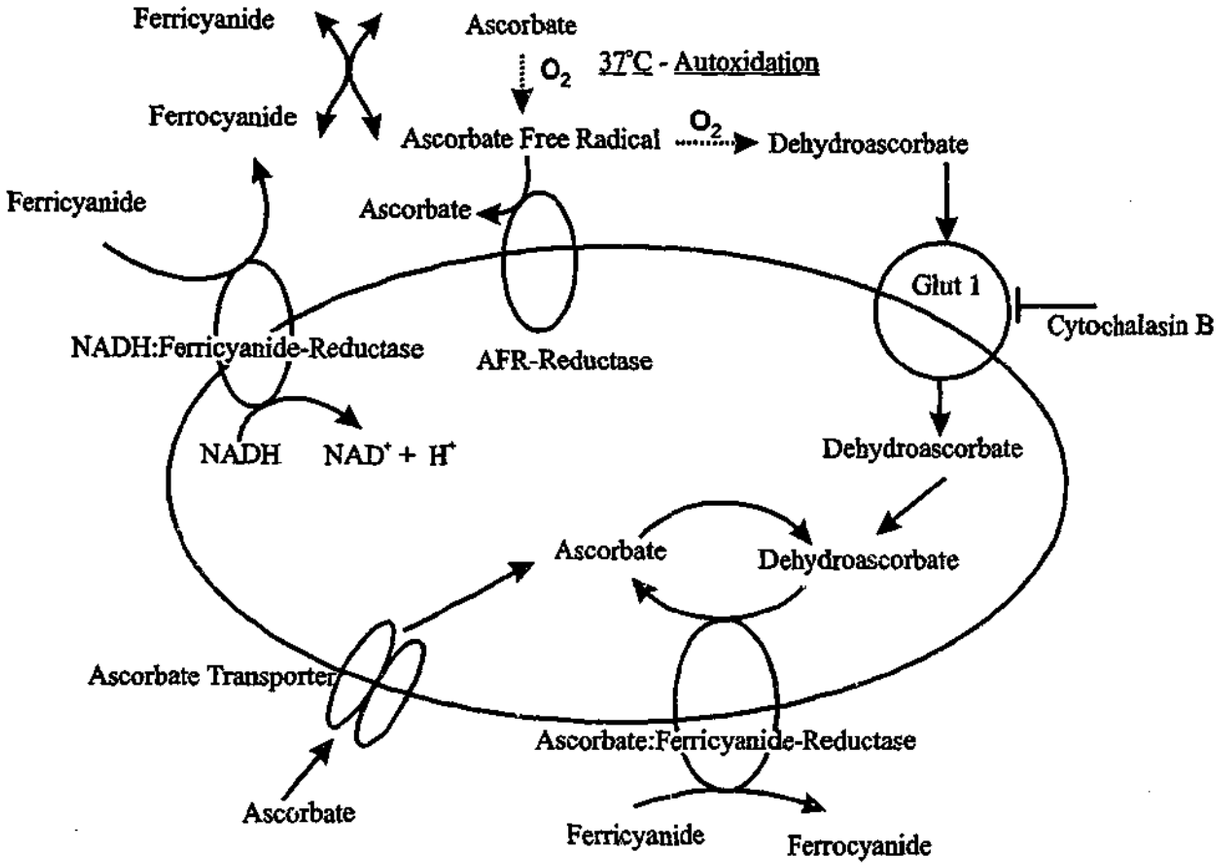

Fig. 1.1 Ascorbate regulation within cells

1.1.5 Iron uptake

It is commonly believed that iron uptake occurs by endocytosis of transferrin and its receptor followed by the release of iron into an endosome at low pH[42]. However, there is evidence to suggest that this is not the only mechanism for iron uptake in mammalian cells, as cells from which the transferrin receptor has been removed are still capable of iron uptake. This suggests that there exists a non-transferrin iron uptake pathway[43]. The first steps involved in the non-transferrin iron uptake into cells include the specific binding of iron to the plasma membrane[44]. It is unknown to which receptor the iron binds on the plasma membrane, however there is evidence to suggest that it may be the plasma membrane NADH:ferricyanide-reductase. A yeast plasma membrane reductase has been identified, which is responsible for the transport of ferrous iron across the cell surface[45]. A similar enzyme may exist in mammalian cells. Studies based mainly upon the unique properties of K562 cells have demonstrated that a ferricyanide-reductase, may be responsible for transferrin-independent iron transport[14].

K562 cells have maintained a unique ferricyanide-reductase that is not involved in growth, nor is it responsive to insulin. This NADH:ferricyanide-reductase isozyme appears to be tightly coupled to the transferrin-independent iron pathway[14,46]. This was demonstrated by the following experiments:

1) Ferricyanide competing with the iron binding site in the ferricyanide-reductase completely inhibits iron uptake of ^{55}Fe nitriloacetic acid[46];

2) The alkylating agent *N*-ethylmaleimide (NEM) completely inactivates the ferricyanide-reductase[14]. NEM also inhibits transferrin-independent iron transport in K562 cells[14];

3) Cadmium inhibits both the ferricyanide-reduction and the transferrin-independent iron transport. This suggests that the ferricyanide-reductase and the iron transport are one and the same, or alternatively they both contain the same Cd^{2+} binding site[14].

Robinson *et al.*[47] have identified an FRO2 gene from *Arabidopsis,* an iron deficient plant, which is homologous to that of ferric-chelate reductase. The latter is required in plants to acquire soluble iron. FRO2 is a member of the superfamily of flavocytochromes that transport electrons across membranes and that have binding sites for heme and cytoplasmic nucleotide binding co-factors.

Yeast have a similar set of genes, which appear to play a major role in iron reduction. The first gene isolated is known as FRE1 (ferric reductase enzyme 1). There now exist nine open reading frames which, based on amino acid similarity, fall into the category of iron/copper reductase-related genes[48]. Disruption of both the FRE1 and the related gene FRE2 completely abolished ferric reductase activity in yeast[49]. These genes encode regions with sequence homology to the gp91-phox protein, a component of the human phagocyte NADPH oxidoreductase[50], suggesting that a reductase may play an important role in iron uptake. Also, hydropathic analysis points to proteins with five membrane spanning helices. Both genes are induced by iron deprivation[49].

The plasma membrane reductase has been shown to be capable of reducing iron bound to transferrin[13], thus the diferric receptor site may be identical with, or very close to the transmembrane electron-transfer complex[13].

1.1.6 Growth control signalling

The first evidence for the involvement of the transplasma membrane NADH:ferricyanide-reductase in growth control was given when Ellem and Kay[7] demonstrated that the addition of external ferricyanide can stimulate the growth of melanoma cells, in the absence of sufficient growth factors. Concentrations between 0.01-0.1 mM ferricyanide are needed to stimulate growth of cells. Ferrocyanide, on the other hand, does not stimulate or inhibit cell growth[7]. Ferricyanide, being impermeant to cells, must be working on the plasma membrane level. However, Löw *et al.*[12] later demonstrated that ferricyanide cannot activate growth on its own, but rather maintains an already established signal. Supporting this idea, Crane *et al.*[51] have established that the reductase activity of *S. cerevisiae* is dependent on the growth phase of yeast cells which stop reducing extracellulary added electron acceptors once they enter the stationary phase.

The exact mechanism by which ferricyanide simulates or maintains cell growth still needs to be established. A likely explanation, however, is a stimulation of the plasma membrane NADH:ferricyanide-reductase, which leads to electron transfer, causing an increase in cytoplasmic pH (cf Section 1.1.7), mobilisation of calcium ions, turnover of phosphatidyl inositol, changes in ratios of cyclic nucleotides or redox state of pyridine nucleotides, all signals finally able to lead to growth stimulation of a cell.

1.1.7 pH changes and mitogenic activity

It was noted in the early days of research on plasma membrane redox proteins, that addition of ferricyanide to the external medium causes a proton release from erythrocytes, adipocytes and HeLa cells[52]. The protons released from cells lead to acidification of the media and alkalisation of the cell[12]. Such alkalisation of the cytosol has been proposed to lead to growth of the cell, since mitosis has been related to an increase in cytosolic pH[53]. Upon addition of ferricyanide to the medium, the NADH:ferricyanide-reductase begins to oxidise cytosolic NADH. The free protons formed upon oxidation are then believed to be pumped from the cytosol, out of the cell by a Na^+/H^+ antiporter[54]. Under steady state conditions, proton influx and efflux would remain the same and no difference in plasma membrane potential would occur. However, the plasma membrane potential increases upon accumulation of protons in the cytosol (hyperpolarisation). Alternatively, a decrease in the plasma membrane potential occurs when the majority of cytosolic protons are "pumped out" into the media or extracellular matrix (depolarisation). Upon addition of 0.5 mM ferricyanide to bean root cells, a depolarisation (20-40 mV) of the plasma membrane is observed[55], suggesting that the protons generated by the NADH:ferricyanide-reductase are exported to the other side of the plasma membrane. Such a depolarisation is consistent with the observation of cytosolic alkalisation. Three minutes after the addition of ferricyanide, the cell becomes slightly repolarised[55]. The short-term electrochemical gradient produced by the addition of external ferricyanide may aid in the uptake of amino acids through a Na^+-dependent amino

acid transport system. The cell would then use these amino acids during the mitogenic process[56].

Contrary to these findings, Thomas *et al.*[57] reported that the addition of 100 μM ferricyanide to serum starved PC12 cells causes a cytosolic acidification and a drop in pH from 7.52 to 7.25. This pH drop has been proposed to play a major role in signalling, since DNA synthesis and ATP production increases[57]. It is still unclear as to why ferricyanide causes a decrease in PC12 cells. The authors suggest that the addition of physiological concentrations of external bicarbonate causes the activation of one or more bicarbonate-dependent acid extrusion mechanisms. Such a mechanism aids in the acidification of the cytosol when ferricyanide is added. There seems to be no doubt that stimulation of the plasma membrane NADH:ferricyanide-reductase leads, in the first instance, to cytosolic acidification and in the second instance to growth stimulation. Whether the initial acidification itself serves as a growth signal, or has to be converted into an alkalisation, still remains to be finally established.

1.1.8 Second messengers

The signalling pathway originating from the transplasma membrane oxidoreductase remains unclear. In addition to proton movement in the cytosol, G proteins may also play a role in the transduction pathway[58]. Rodrigúez-Aguilera *et al.*[59] have shown that the transplasma membrane dehydrogenase may work through a signalling cascade involving the second messenger cAMP. Additionally, increasing PMOR activity increases the cytosolic Ca^{2+} concentration, which then leads to activation of protein kinase C (PKC). PKC itself has also been found to stimulate

ferricyanide-reductase activity[60], which may suggest the existence of a self amplification loop.

The importance of a transplasma membrane protein system in growth control is also evidenced by the use of common anticancer drugs. Such drugs are believed to induce cell arrest and to finally cause the cells to undergo apoptosis. Azaserine, acivicin[61], daunomycin, AD32[62], cisplatin[63], mitoxantrone[64], adriamycin[65], bleomycin[66], *cis*-diaminedichloroplatinum (II) and actinomycin D[13] all have been shown to inhibit the plasma membrane NADH-reductase. Once thought to disrupt the internal environments of cells, many of the aforementioned drugs may actually be acting at least additionally at the level of the plasma membrane. Triton and Yee[65] immobilised adriamycin on agarose beads, making it totally impermeant to cell membranes, and found that the compound had improved cytotoxicity. Inhibition of the reductase with drugs at concentrations, which inhibit growth, also points to the vital role the reductase may play within a cell.

1.1.9 PMOR activity changes during transformation and differentiation of cells

The plasma membrane NADH:ferricyanide-reductase has been ubiquitously found in cells ranging from protozoa to man. Nevertheless, the function of such an enzyme has remained inconclusive over the past several decades. Some knowledge has been gained whilst observing the relationship between the NADH:ferricyanide-reductase and the transformation of cells. Such transformation was achieved using two oncoproteins, N-*myc* and *Ha-Ras*[67,68]. When observing the level of N-*myc* and the plasma membrane reductase, a positive correlation between N-*myc* expression and a decrease in ascorbate oxidation in three neuroblastoma cell lines was found[67].

Ascorbate (as discussed in Section 1.1.4) may be a natural electron acceptor for the transplasma membrane NADH-oxidoreductase. A decrease in ascorbate oxidation suggests that a reductase may be up-regulated to maintain ascorbate in its reduced form. This enzyme may be the NADH:ferricyanide-reductase. Similarly, the NADH:ferricyanide-reductase activity increased 5-fold during the transfection of the *Ha-ras* gene into C3H 10T1/2 mouse embryo fibroblast cells[68]. However, since reduction of externally added cytochrome *c* also increased under these conditions, that increase may be attributed to cytochrome b_5 reductase activity. Another explanation may be an increase in the internal supply of NADH[13]. This would fit with the hypothesis that the NADH:ferricyanide-reductase is involved in increased in cell growth.

The up-regulation of the transplasma membrane NADH:ferricyanide-reductase is not restricted to transformation of cells only. A change in the NADH:ferricyanide-reductase activity was shown during differentiation of HL-60 cells after a period of 24 h with 12-*O*-tetradecamoylphorbal-13-acetate (TPA)[69]. TPA causes promyelocytic HL-60 cells to undergo a monocytic differentiation. These cells lose their proliferative capacity and acquire maturation features of macrophage-like cells[70]. During the differentiation, a 1.4-fold increase in ferricyanide-reduction occurs. This may indicate a stimulation of the NADH:ferricyanide-reductase during TPA-induced differentiation, again supporting the hypothesis that the enzyme may be generally involved in cell signalling and growth control. TPA-induced stimulation of ferricyanide-reduction provides a specific example for a potential involvement of the NADH:ferricyanide-reductase in the regulation of cell differentiation. Such small changes however, could also be caused by the secretion of a substrate for the reductase, because stimulations in that

order have also been described upon ascorbate addition to the medium[37]. The alteration of the PMOR during transformation of different cell lines is summarised in Table 1.1.

TABLE 1:1 Alteration of the PMOR during transformation of different cell lines.

Cell line	Cell treatment	Reductase	Oxidase	Comments	References
Neuroblastoma	Transfection with N-*myc*	16%-72%	n/d*	Tumour vs. normal cells, faster growth = greater PMOR activity	67
Mouse embryonic fibroblasts	Transfection with *Ha-ras*	5-fold increase	n/d*	1) Increase in amount of enzyme on plasma membrane 2) Increase in internal [NADH]	68
HL-60	TPA+	1.4-fold increase	n/d*	Internal [NADH] and [NAD^+]	71
HL-60/ Namalwa	Ethidium bromide	3-fold increase	4-fold increase	An increase in "hypoxic"-like conditions, leads to up-regulation of PMOR	18; Malik, S., Marzuki, S. and Lawen, A. unpublished data
Human fibroblasts	Ethidium bromide	decrease	decrease		Malik, S., Marzuki, S. and Lawen, A. unpublished data
Swiss 3T3 mouse embryo fibroblast cells	Transformation with SV-40	4-fold decrease	n/d*		72
K562 human erythroleukemia	Mitochondrial inhibitors	increase	n/d*	Decrease in cellular ATP concentration	72

*** Not determined**

+ 12-*O*-tetradecamoylphorbal-13-acetate

1.1.10 Bioenergetics

It is still unclear whether the transplasma membrane NADH:ferricyanide-reductase causes a direct change in the nucleotide concentration within cells, or if a change in the nucleotide ratio modulates the enzyme. It has been shown that lactate as a direct source of cytosolic NADH enhances ferricyanide activity in whole cells[74]. This suggests that an increase in the NADH:NAD^+ ratio may regulate the NADH:ferricyanide-reductase. One role the PMOR may play is that of a cellular redox sensor. Supporting this view, we have shown in our laboratory that during the generation of Namalwa and HL-60 ρ^0 cells, in which NADH is no longer oxidised by the respiratory chain, a 4-fold increase in the rate of NADH:ferricyanide-reduction occurs[18]. The ρ^0 cells appear to respond to the increased NADH:NAD^+ ratio by increasing the activity of the NADH:ferricyanide-reductase. Supporting this view, the addition of mitochondrial inhibitors such as potassium cyanide can stimulate ferricyanide-reduction[73]. Impairment of the respiratory chain has also been implicated during ageing[75]. Increased NADH, in response to the nucleotide pool may enhance survival in such energy deficient cells. Similarly, the increase of the NADH:ferricyanide-activity may explain the survival of tumour cells, in which the natural environment of the cell is believed to become hypoxic. Since normal mitochondrial respiration may be impaired during tumour progression, levels of NADH may be maintained by up-regulation of the enzyme.

However the phenomena described appear to be dependent on the cell line used. Löw *et al.*[72] found that transformation of Swiss 3T3 fibroblasts with the simian virus 40 (SV 40) resulted in a decrease in ferricyanide-reduction. This was accompanied by an increase in the number of transferrin receptors. A down-regulation of both NADH:ferricyanide-reductase and NADH-oxidase activity during prolonged treatment with ethidium bromide in fibroblast cell lines has also been observed (Malik *et al.* unpublished results) (Table 1.1).

1.1.11 Apoptosis

The plasma membrane NADH-oxidoreductase (PMOR) system is believed to consist of at least two enzyme activities, the plasma membrane NADH:ferricyanide-reductase and the plasma membrane NADH-oxidase[13]. The purification and characterisation of the plasma membrane NADH-oxidase has shown that a 32 kDa ectoenzyme exists on the outer surface of the plasma membrane. *In vitro*, this enzyme uses NADH and presumably oxygen as a final electron acceptor[13]. Evidence is accumulating, that in transformed cells, an isoform of the NADH-oxidase exists. This isoform may be shed into the sera of media and cancer patients[76]. It has been reported that the NADH-oxidase can be stimulated with the addition of CoQ and its analogues[17,18,23,77]. These analogues were also shown to stimulate activity of the plasma membrane NADH:ferricyanide-reductase of whole cells[18]. The ability of the CoQ to stimulate both the NADH-oxidase and the NADH:ferricyanide-reductase suggests a functional link between the two moieties. A compound

related in structure to CoQ is capsaicin. Capsaicin is the pungent ingredient found in a wide variety of red peppers. It has been demonstrated that capsaicin and its analogues, dehydrocapsaicin and resiniferatoxin (all three CoQ analogues belonging to the class of vanilloids), are potent inhibitors of the NADH-oxidase[17,23,78]. The inhibition of the NADH-oxidase by capsaicin has been shown to induce rapid apoptosis in leukemic cell lines[17]. Apoptosis is a form of programmed cell death, whereby cells undergo morphological changes including blebbing of the plasma membrane, after which *in vivo*, the cell is engulfed by macrophages[79]. The induction of apoptosis in many systems can be inhibited by the classical anti-apoptotic oncogene product, Bcl-2[80], however it is unclear how Bcl-2 exerts its anti-apoptotic effect[80]. Upon addition of capsaicin to Daudi, FDCP-1 or Namalwa cells, apoptosis occurs in 8-12 h. However after transfection of these cells with Bcl-2, capsaicin no longer exerts its apoptosis-inducing effect, suggesting that this apoptotic pathway is sensitive to Bcl-2[17]. The mechanism by which inhibition of the NADH-oxidase leads to apoptosis has been suggested to be due to an imbalance in the cellular NADH:NAD^+ ratio[15]. Such an imbalance can be considered as oxidative stress (in an electron sense rather than an oxygen sense). One means of overcoming such oxidative stress would be to up-regulate the NADH:ferricyanide-reductase. This can be achieved by the stimulation of the reductase through addition of ferricyanide to the cells. Preliminary data from our laboratory have shown that co-incubation with ferricyanide and a vanilloid leads to a decrease in the amount of apoptosis in

HL-60 and Daudi cells when compared with cells treated with the vanilloid alone[15,81]. One interpretation of these data is that by inhibiting the oxidase, capsaicin causes an increase in the NADH:NAD^+ ratio. However upon addition of ferricyanide the NADH:ferricyanide-reductase is up-regulated[18]. This leads to the oxidation of excess NADH and a lowering of the nucleotide ratio, thus maintaining cell survival[82]. Alternatively the PMOR may be regulating anti-oxidants such as ascorbate (see section 1.1.4) and CoQ which protect cells from undergoing mild oxidative stress by mediating electron transport within the PMOR. It is for this reason that CoQ has been described as the 'plasma membrane-equivalent of Bcl-2'[83].

1.2 The voltage dependent anion channel 1

1.2.1 VDAC1, a general and historical introduction

The ability of proteins to regulate ion flow, was demonstrated in the outer membrane of gram negative bacteria[84]. Eukaryotic systems were later observed to contain "leaky" mitochondria which appeared to have preference for anionic over cationic conductance[85]. Mitochondrial fractionation studies later showed that the outer mitochondrial membrane was not simply leaky, but contained anionic conducting pore-forming proteins, which may be the predominant proteins in that location[86]. A protein, with single-channel conductance, voltage dependence and anionic selectivity (for Cl^- ions) was termed the "voltage dependent anion channel" or "VDAC". Although the existence of VDAC isoforms was established in the mid-1990's, earlier studies on VDAC did not discriminate between any variants. However, most of the work done on VDAC before the knowledge of specific isoforms, would be on the specific isoform VDAC1, since it is the major protein expressed in mitochondrial outer membranes[86].

VDAC has been purified from a number of sources including rat liver cells[87] where a concanavalin A column was used, suggesting the presence of (a) glycosidic residue(s) bound to the protein[87]. Purified VDAC can be reconstituted in artificial membranes, such as liposomes, in order to measure the ion conducting properties of the protein *in vitro*. Conductance measurements have demonstrated that VDAC exists in multiple conformation

states. By applying a triangular potential difference across the membrane (from –80 mV to +80 mV) and measuring the conductance of VDAC, a bell shaped curve is produced (Fig. 1.2)[88]. These data led to a model in which VDAC exists in an open (between –20 mV to +20 mV) or closed (<–20 mV or > +20 mV) state[89]. However, *in vitro* ion conductance analysis of VDAC in reconstituted liposomes may not reflect VDAC's physiological activity. *In situ* measurements on the ion conductivity of VDAC in liver mitochondria, using patch clamp techniques, produce different conductance curves[89]. At positive membrane potentials, mitochondrial liver membranes follow the normal bell shaped curve. However as the potential becomes negative the conductance of mitochondrial membrane VDAC continues to increase, even beyond –20 mV[90]. One explanation for this behaviour, is that at positive membrane potentials (+80 to +1 mV) VDAC may exist as a single channel. However as the potential becomes increasingly negative (-1 to -80 mV), VDAC molecules may polymerise forming (a) higher conducting complex(es)[90,91]. Alternatively, mitochondrial membranes may contain an unidentified protein, contributing to the enhanced conductance at negative potentials. The protein may be either a novel VDAC like protein[92], or may stimulate the activity of VDAC[93]. In either case, the use of planar lipid systems to measure VDAC conductance does not take into effect the potential influence of other proteins.

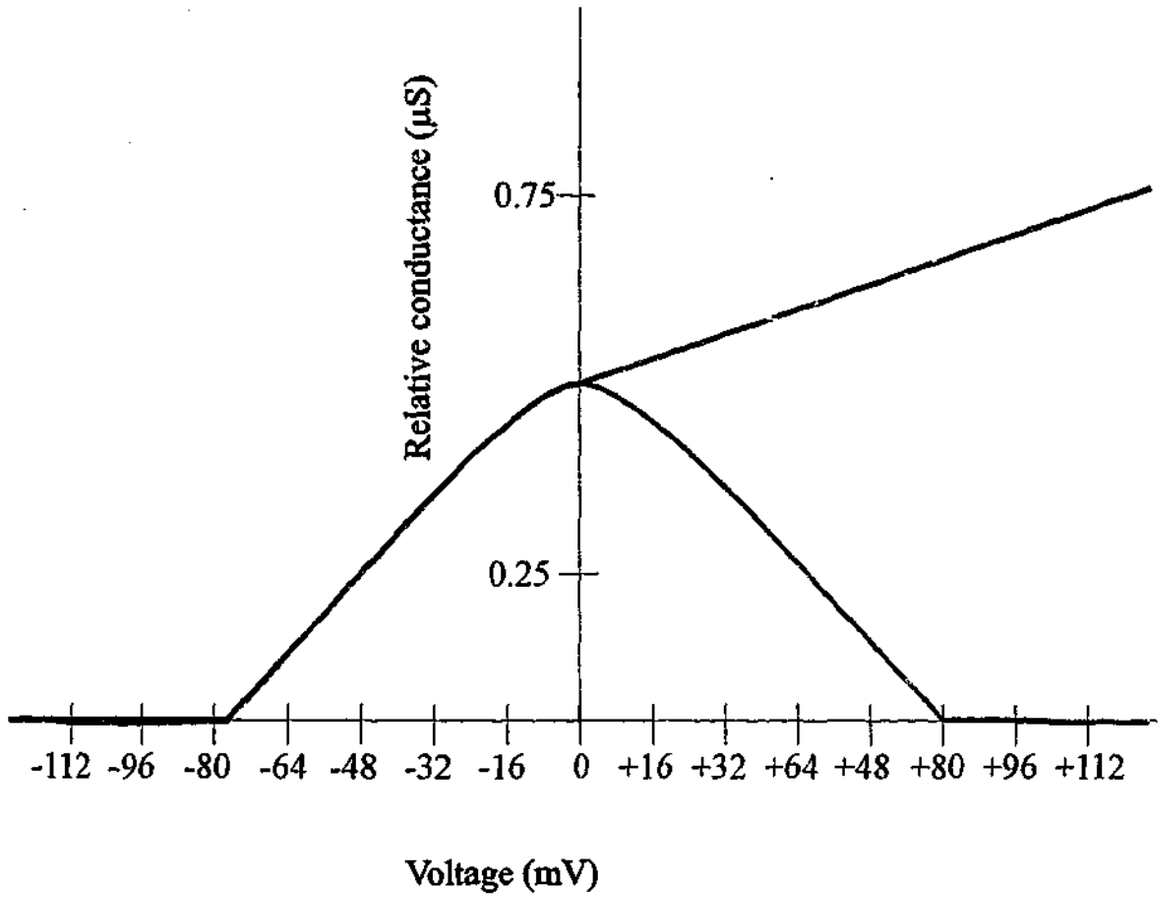

Fig. 1.2 Conductance of VDAC1.

Purified VDAC1 (orange line) demonstrates typical "bell shaped" conductance properties. This differs from *in situ* conductance measurements on the outer mitochondrial membrane (purple line).

The pore diameter of a single VDAC molecule in the open state is approximately 4 nm[94] which allows that passage of compounds 6-8 kDa in size[95]. The closed state VDAC has a pore diameter of approximately 1.8 nm[96]. The shift from open to closed state, upon a change in the potential, may occur in one of two ways. VDAC may close the entrance of the pore, thereby restricting those molecules gaining access to the rest of the tunnel. Alternatively, VDAC may close the entire tunnel, causing a general restriction of ions passing through the entire length of the pore. A decision of the mechanism used can be made by measuring the amount of water dispersed whilst VDAC undergoes a transition from the open to the closed state and it is now generally accepted that the latter of the two options occurs during VDAC's conformational change[97].

VDAC, as the name suggests, has a voltage gating mechanism, which, *in vitro,* demonstrates a preference for anionic conductance. Such selectivity may be due primarily to a set of positive charges lining the channel[98]. This has been demonstrated in a number of ways. Compounds capable of inhibiting the voltage dependent conductance of VDAC all possess similar characteristics. Dextran sulphate[99] and the synthetic polyanion (Königs polyanion)[100] are compounds that contain negative charges and they appear to interact electrostatically with VDAC, causing inhibition of ion permeation. Secondly, upon addition of succinic anhydride (a compound that replaces the positive charged amino groups with negatively charged carboxyl groups), VDAC loses its voltage selectivity for anions[98]. Furthermore, as the

concentration of succinic anhydride (and consequent succinylation) increases and the majority of positively charged amino acids become negatively charged, VDAC becomes cation selective[101]. The amino acids responsible for the positive charges appear to have been identified. Increasing the pH of the buffered solution, causes the eventual inhibition of VDAC's voltage dependence[102]. Based on the experimental *p*K value of 10.6 obtained, the authors suggested that lysine residues were responsible for the positive charges lining VDAC's tunnel[102]. Conformation of this was obtained by mutation analysis of the positively charged lysines to negatively charged glutamic acid residues. This resulted in the reversal of VDAC's ion selectivity, now having cationic, instead of anionic, conductance[103].

With the ability for anionic conductance and its location in the outer mitochondrial membrane, it is generally assumed that VDAC functions primarily as a pathway for metabolite flux between the cytoplasm and the mitochondria[104,105]. *In vitro* analysis, using planar bilayer experiments would argue in favour of this hypothesis. *In vivo,* VDAC may be primarily regulating the flow of adenine nucleotides, since it has been demonstrated that one VDAC molecule is capable of transporting 2-5 x 10^9 ATP molecules per minute[106]. In this experiment, VDAC was permeable to ATP, only in the open state. Furthermore, the addition of Königs polyanion inhibited the uptake of adenine nucleotides in isolated mitochondria[105]. However non-specific effects of the polyanion may have also contributed to the regulation of adenine nucleotides[86].

In addition to functioning as a major metabolic pathway regulator of adenine nucleotides, VDAC may play a secondary role in other outer-mitochondrial functions. In 1983, it was shown that 65% of the cell's hexokinase bound to the outer mitochondrial membrane in Novikoff ascites tumor cells[107]. Pretreatment of the membrane with dicyclohexylcarbodiimide (DCCD) which covalently labels the C-terminus of the VDAC protein, without any effect on the VDAC channel-forming activity, inhibited the binding of hexokinase to the outer membrane[108]. This suggests that VDAC may form part of a hexokinase receptor and hexokinase binding to VDAC, may result in preferential access of newly formed ATP from the mitochondria[109].

1.2.2 Structural studies

X-ray crystal structure analysis on bacterial *Rhodobacter capsulatus* VDAC (commonly known in bacteria as porin which is found in the outer membrane) has demonstrated that the tertiary structure of the protein is a β-barrel[110]. *Rhodobacter capsulatus* porin consists of 16 membrane spanning β–strands (Fig 1.3)[110].

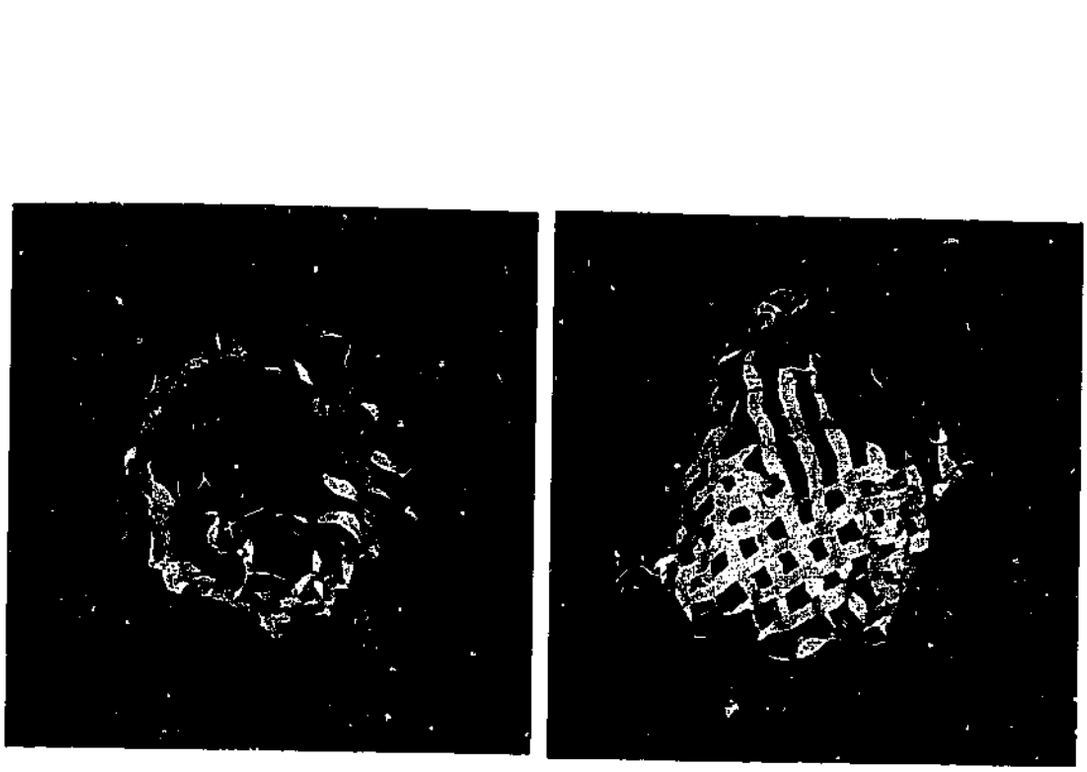

Fig. 1.3 Crystal structure of *Rhodobacter capsulatus*.[110]

The tertiiary structure of *Rhodobacter capsulatus* VDAC (porin) is a β-barrel, consisting of 16 transmembrane spanning strands.

To date there is no published X-ray crystal structure of VDAC from higher organisms such a yeast or humans. However, both appear to have a similar structure as their bacterial counterpart. CD analysis of yeast *Neurospora crassa* VDAC1 showed the presence of 81% β–sheet content in purified VDAC[111]. The function of VDAC is dependent on the tertiary structure, since treatment of VDAC with sodium dodecyl sulphate (SDS) causes an irreversible conformational change of VDAC, with reduced β-sheet content and loss of pore forming activity[112]. The number of membrane spanning strands in yeast has given rise to much controversy. Blachy-Dyson *et al.*[113] have mutated the YVDAC gene in 29 positions, all of which differed in charge from the wild type sequence. Replacement of some of the positive charges lining the wall of the pore should reduce or even reverse VDAC's ion selectivity. However, a change in charge of molecules located outside the tunnel should have no effect on ion selectivity[113]. The vectors containing the mutant genes were expressed in yeast, the genetic products were purified and introduced into a planar phospholipid bilayer for conductance measurements[113]. Based on their data, the authors have suggested that YVDAC consists of 12 membrane spanning strands[113]. However, this model may be a little simplistic, given the criteria it is based upon. Kleene *et al.*[90] purified the cDNA of YVDAC1 from *Neurospora crassa* and determined its nucleotide sequence. Based on this sequence, the amino acid residues of YVDAC1 were established[90]. Hydrophobicity plots were then used to profile

YVDAC1, which suggested the protein contained 15 membrane spanning strands[90].

A final study into the molecular architecture of YVDAC1 was performed using computer analysis. A computer algorithm was used to generate a model of this protein from the amino acid sequence of YVDAC1. Based on known crystal structures of other proteins, this method predicts which portions of the protein are α helices or β sheets, and which molecules are buried inside the protein or lipid, or exposed to the water phase. From this analysis, 19 membrane spanning strands have been predicted for YVDAC1[114].

Structural studies into human VDAC1 have also led to inconsistencies between each of the different models. By exposing human VDAC1 to different proteolytic enzymes and antibodies raised against the N-terminus de Pinto and Palmieri[115] have suggested the protein contains 16 membrane spanning strands. In contrast, by comparing the folding patterns of bacterial and YVDAC1 to human VDAC1, Song and Colombini[116] have predicted that human VDAC1 contains only 13 membrane spanning strands. To demonstrate the validity of their model, they mutated lysine 112 and 114 (located on the outer surface of the tunnel in their model) to glutamine. The mutants displayed normal VDAC1 like conductance. Conversely mutating lysine 118 (located very close to the opening of the tunnel) to glutamine changed the selectivity of VDAC1. This model is also consistent with proteolytic data reported by de Pinto *et al.*[117]. Residues 108-119 would be accessible to trypsin cleavage[116], whilst residues 117 and 172 are accessible to chymotrypsin

cleavage[116]. In the future, crystal structure analysis of human VDAC1 will finally have to demonstrate which model is correct.

Topological studies into mammalian VDAC1 have been shown to be less controversial. Using antibodies directed to the N-terminus of bovine VDAC1, it has been demonstrated, by enzyme linked immunosorbent assay, that this antibody reacts with intact mitochondria. This suggests that the N-terminus of VDAC is exposed to the cytoplasm. In contrast, carboxypeptidase failed to cleave VDAC embedded in intact mitochondria, suggesting that the C-terminal is not exposed to the buffer[117].

1.2.3 Genetic analysis of VDAC

At least four isoforms of the VDAC have been found in humans[118]. VDAC1, the first of the isoforms, has been mapped to position Xq13-q21.1 on the X-chromosome[118]. VDAC2, the second of the isoforms, has been mapped to chromosome 21[118]. During the process of mapping these genes to the relevant chromosomes, a further two isoforms of VDAC have been identified. VDAC3 was shown to map to chromosome 12 and VDAC4 to chromosome 1[118]. Following the finding of the isoforms in humans, further experiments in the yeast *Saccharomyces cerevisiae* demonstrated the presence of YVDAC isoforms. Yeast knockout YVDAC1 (deficient in POR1 gene) cells are capable of growing in non-fermentable carbon sources at 30°C but not at 37°C, suggesting that the normal oxidative phosphorylation pathway remains intact[119]. A screening of compensatory proteins in these yeast

mutants led to the finding of a second yeast isoform with VDAC like activity, designated YVDAC2, which is encoded by the POR2 gene[119]. The up-regulation of YVDAC2 to the same level as wild type YVDAC1 led to the restoration of the yeast to grow on glycerol at 37°C, demonstrating that the protein must act in a similar manner to that of YVDAC1[119].

1.2.4 Regulation of VDAC1

Upon the reconstitution of VDAC1 into planar phospholipid membranes, addition of micromolar concentrations of NADH doubled the voltage dependence of the mitochondrial channel[120]. Interestingly, the single channel conductance was not changed. This implies, that *in vivo,* the opening of VDAC1 in the presence of NADH is more sensitive to changes in the mitochondrial membrane potential and that therefore the same level of NADH in the cytoplasm could either favour the opening or closing of the channel[120]. Since NADH is critical for the regulation of glycolysis and oxidative phosphorylation, this may be the mechanism by which glycolysis can suppress oxidative phosphorylation[120].

A protein named "VDAC-modulator", which is found in the mitochondrial matrix, has also been documented[93,121]. Partial purification of the VDAC-modulator protein has revealed a molecular mass of 54 kDa in SDS-PAGE [122] and of 100 kDa in gel filtration columns. Addition of the VDAC modulator protein to purified, reconstituted VDAC1 has been shown

to cause an increase in VDAC's voltage dependence, which leads to consequent closing of the pore.[122].

1.2.5 VDAC and apoptosis

As previously mentioned, apoptosis is a form of regulated cell death, which has been well characterised physically and functionally. Various apoptotic signals eventually converge and activate a family of cysteine proteases ("caspases") leading to the initiation of cell death. Mitochondria appear to play an essential role in apoptosis by releasing apoptogenic molecules, which include cytochrome *c*[123,124] and apoptosis inducing factor (AIF)[125]. Once in the cytosol, cytochrome *c*, together with Apaf-1 and ATP can activate a major apical caspase, caspase 9[126]. Caspase 9 subsequently activates other caspases, such as the effector caspase, caspase 3. Several competing models have been proposed to explain how cytochrome *c* is released during apoptosis. The first model postulates the involvement of a permeability transition pore (PTP) which spans the outer and inner mitochondrial membranes. The permeability transition pore is made up of at least three major contributors, including VDAC, the adenine nucleotide transporter (ANT) and cyclophilin D[127-129]. PTP may release cytochrome *c* in one of two ways. PTP openers such as the proapoptotic protein Bax, may bind to the PTP leading to consequent permeablisation and entry of water and solutes into the mitochondria[130]. This may cause rupture of the outer membrane and consequent release of cytochrome *c*. However this model does not explain why, under certain conditions, such as quinacrine-induced apoptosis in human cervical carcinoma cells, a drop in membrane potential or change in mitochondrial morphology appear to be late events[131].

A second model also predicts the cooperative effects between Bcl-2 family members and the PTP. It is believed that the PTP can exist in three states, including a closed (reversible) low-conductance and high (irreversible) conductance state[132]. In its low conductance state, the PTP is permeable to molecules < 300 Da[132]. However in the high conductance state, the PTP has been shown to be permeable to molecules > 1500 Da[132]. Regulation of the low to high conductance of the PTP may occur through members of the Bcl-2 family[133]. Bax and Bak, two proapoptotic members of the Bcl-2 family, have been shown to bind to and open VDAC1[133]. Conversely Bcl-x_L, an antiapoptotic protein, has been shown to induce a closed state of VDAC1[133]. In reconstituted liposomes, with recombinant expression of VDAC, both Bax and Bak allow the passage of cytochrome *c*. Bcl-x_L and Bcl-2 (a further anti-apoptotic protein) both inhibit cytochrome *c* release under these conditions[132,133]. Therefore it appears that upon binding to VDAC1, Bcl-2 family members regulate a high or low conducting state of the PTP. However the involvement of Bcl-2 family members and the PTP has been questioned[134]. Using a yeast model, it has been demonstrated that VDAC was not involved in Bax-induced release of cytochrome *c*, or in the prevention of this release by Bcl-x_L[134].

Other models predict the release of cytochrome *c* through the formation of a different pore. Bax is capable of forming novel pores in lipid planar channels[135]. The addition of Bax directly to isolated mitochondria

triggers the release of cytochrome *c* which is insensitive to PTP blockers[136], suggesting Bax may form a pore, allowing cytochrome *c* to be released.

In this thesis I show that VDAC1 is identical with the plasma membrane NADH:ferricyanide-reductase. VDAC1 was shown to be present on the plasma membrane of human Namalwa cells. The ability of VDAC1 to function as a redox enzyme may lead to a deeper understanding of how this important enzyme functions. It also may hold the key in to the understanding of how Bcl-2 exerts its anti-apoptotic effect. VDAC1 represents the first established mammalian transplasma membrane NADH:ferricyanide-reductase reported to date. As such, VDAC1 may be a vital subunit of the PMOR.

CHAPTER 2 - Materials and Methods

2.1 Materials and suppliers

Tween 20, Chaps, Triton X-100, Blue Sepharose affinity beads, DEAE-Sephacel, poly-L-ascorbic acid (sodium salt), polyethylene glycol 3350, mannitol, β-mercaptoethanol, bromophenol blue, Coomassie blue, Bactotryptone, potassium ferricyanide, NADH, NADPH, *p*-nitrophenol phosphate, *p*-nitrophenol acetate, cytochrome *c*, rotenone, poly-L-ascorbic acid, polyethyleneglycol 3350, pyruvate, propidium iodide, sodium dodecyl sulphate (SDS), acrylamide, bis-acrylamide, anti-mouse antibodies, ammonium persulphate, silver nitrate and Celite-545 were purchased from Sigma chemicals (Castle Hill, NSW Australia). Hepes was purchased from ICN (Aurora, OH, USA). Penicillin, streptomycin, RPMI and foetal calf serum were purchased from Trace Biosciences (Clayton, VIC, Australia). Nitrocellulose membrane and ECL-detection kit were purchased from Amersham (Castle Hill, NSW, Australia). Ethylenediaminetetracetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), sodium chloride, sodium thiosulphate, sodium carbonate, absolute ethanol, methanol, sucrose, MOPS, urea, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and acetic acid were purchased from BDH chemicals Australia Pty Ltd. (Kilsyth, VIC, Australia). Nonidet 40 (NP-40), tris-hydroxymethyl-methylamine, formaldehyde, sucrose was purchased from Ajax chemicals (Castle Hill, NSW, Australia). Dextran T500 was purchased

from Pharmacia Biotech (Castle Hill, NSW, Australia). Goat anti-rabbit IgG-FITC was purchased from Molecular probes (Eugene, OR, USA). Pre-stained and other molecular mass markers (broad range, low range and kaleidoscope), hydroxylapatite and skim milk powder were purchased from Bio-Rad (Regents Park, NSW, Australia).

2.2 List of buffers and solutions

Buffer A	50 mM Tris, pH 8.0
Buffer B	50 mM Tris, pH 8.0; 3% Triton X-100
Lysis buffer 1	210 mM mannitol; 70 mM sucrose; 5 mM Hepes, pH 7.2; 1 mM EGTA
Lysis buffer 2	225 mM mannitol; 25 mM sucrose; 2mM MOPS, pH 7.4; 1 mM EGTA
SDS-running buffer	25 mM Tris, pH 8.3; 192 mM glycine; 0.1% (v/v) SDS
2 x loading dye	180 mM Tris, pH 6.8; 8 M urea; 30% (v/v) glycerol; 6% (w/v) SDS; 7% (v/v) β-mercaptoethanol; 0.004% Bromophenol blue
Transfer buffer	SDS running buffer + 10% (v/v) methanol
Tris buffered saline (TBS)	20 mM Tris, pH 7.6; 137 mM NaCl
Tris-EDTA (TE)	10 mM Tris, pH 8.0; 1 mM EDTA
Phosphate buffered saline	8.2 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 138 mM

(PBS)	NaCl, 2.7 mM KCl, pH 7.4
Staining solution	0.1% Coomassie R-250; 10% acetic acid (v/v); 10% methanol (v/v)
First destaining solution	50% methanol (v/v); 10% acetic acid (v/v)
Second destaining solution	10% methanol (v/v); 10% acetic acid (v/v)
Luria broth (LB)	1% Bacto-tryptone; 0.5% Bacto yeast extract; 1% NaCl
LB medium	15 g of agar added per 100 ml LB
P1	50 mM Tris, pH 8.0; 10 mM EDTA; 100 µg / ml RNAse A
P2	200 mM NaOH; 1% SDS
QBT	50 mM MOPS, pH 7.0; 750 mM NaCl, 15% isopropanol; 0.15% Triton X-100
QC	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol
QF	1.25 M NaCl; 50 mM Tris, pH 8.5; 15% isopropanol

2.3 Cell culture

Human Namalwa cells were maintained in RPMI 1640 media supplemented with 10% (v/v) foetal calf serum, 200 μM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were incubated at 37°C in a humidified incubator under 5% CO_2. Cell concentrations were determined by trypan blue exclusion, which involved diluting the cells 1:1 in sterile trypan blue. Cells were then loaded onto and counted on a Neubauer improved haemocytometer.

2.4 Preparation of plasma membranes from Namalwa cells

Unless indicated otherwise, all steps were performed at 4°C. 6×10^8 cells were pelleted and washed twice with ice cold PBS. Following the second wash, the supernatant was removed and the cells were adjusted to 2×10^8 cells/ml using ice cold lysis buffer 1 and 1.4 mg per 10^9 cells of digitonin was added and the cells were lysed on ice using 72 strokes in a glass homogeniser (Sigma, Castle Hill, NSW, Australia). After lysis, the homogenate was centrifuged at 2,000 x g for 5 min. The supernatant was removed and kept for later analysis. The pellet was washed twice with buffer A then resuspended in buffer A to 4×10^8 cells/ml. The resuspended pellet was then loaded onto a two-phase system constituted on a weight basis. The two phase system contained 6.4% (w/w) dextran T-500, 6.4% (w/w) polyethylene glycol 3350

and 0.1 M sucrose. The homogenate (2 g) was added to the two-phase system and the weight of the system was brought to 16 g with distilled water. The tubes were inverted vigorously 40 times in the cold (4°C). The phases were separated by centrifugation at 150 x g in an IEC Centra-7R refrigerated centrifuge. The upper phase was carefully withdrawn, together with the interface, and transferred into 10 ml centrifuge tubes. The sample was then centrifuged at 5,000 x g for 30 min. The fluffy pellet resulting from the spin constituted the plasma membrane enriched fraction.

2.5 Determination of protein concentrations

Protein concentrations were determined by using the bicinchoninic acid (BCA) assay[137]. Briefly, 10 µl of each standard (0-200 µg/ml) and unknown sample were aliquoted into appropriately labelled tubes. 50 parts BCA reagent A were mixed with 1 part BCA reagent B forming a green turbid mixture. 100 µl of the mixture were then incubated with the standards and unknown samples. After 30 min of incubation, the absorbance was measured at 540 nm.

2.6 Spectrophotometric assays for the PMOR

2.6.1 NADH:ferricyanide-reductase

NADH:ferricyanide-reductase activity was measured in an Aminco DW-2a spectrophotometer. A quartz cuvette containing 10 μg of membrane, 2 μM rotenone, 2 mM KCN and 250 μM ferricyanide was adjusted to 1 ml with buffer A and allowed to pre-incubate for 5-10 min. The reaction was initiated by the addition of 50 μM NADH and the oxidation of NADH was followed at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) against a 600 nm reference for 10 min at 37°C. Rates of NADH oxidation are expressed in pmol of NADH oxidised per second per mg protein.

2.6.2 Whole cell ferricyanide-reduction

Whole cell ferricyanide-reduction was measured in Aminco DW-2a spectrophotometer. 1×10^6 cells were placed in a quartz cuvette and the volume was adjusted to 1 ml with PBS containing 1 mM pyruvate and 10 mM glucose. 250 μM ferricyanide was added and the reduction was followed at 420 nm ($\varepsilon = 1{,}000\ M^{-1}\ cm^{-1}$).

2.6.3 Whole cell DCIP-reduction

Whole cell DCIP-reduction was measured in Aminco DW-2a spectrophotometer. 1×10^6 cells were placed in a quartz cuvette and the

volume was adjusted to 1 ml with PBS containing 1 mM pyruvate and 10 mM glucose. 10 μM DCIP was added and the reduction was followed at 600 nm ($\varepsilon = 5{,}500\ M^{-1}\ cm^{-1}$).

2.6.4 NADH-oxidase

NADH-oxidase activity was measured in a quartz cuvette containing 150 μg of protein, which was added to 1 ml buffer A containing 2 μM rotenone and 2 mM KCN. Following 5 min pre-incubation, 150 μM NADH was added and the oxidation of NADH was measured at 340 nm ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

2.7 Enzyme markers assays

All the enzyme marker analyses were performed using a Beckman DU 7500 spectrophotometer. For every assay, a baseline rate (usually without protein) was established before the addition of the protein to begin the assay.

2.7.1 Alkaline phosphatase

Alkaline phosphatase (plasma membrane fraction) was determined using an adaptation of a previously described method[138]. Briefly, 10-20 μg protein were added to 1 ml, 50 mM Tris, pH 10.5. The reaction was started with the addition of 10 μM *p*-nitrophenol phosphate. The increase in absorbance was measured at 37°C for 1 h.

2.7.2 Succinate-cytochrome *c* reductase activity

Succinate-cytochrome *c* reductase activity (mitochondria) was determined by adapting a previously described method[139] where 1 ml assay mixture contained 20 μM oxidised cytochrome *c*, 20 μM sodium succinate and 1 mM KCN in 25 mM potassium phosphate buffer, pH 7.5. The reaction was started by the addition of 10-20 μg protein and the reduction of cytochrome *c* was followed spectrophotometrically at 550 nm (ε = 18.7 mM^{-1} cm^{-1}) at room temperature for 20 min.

2.7.3 NADPH-cytochrome *c* reductase activity

NADPH-cytochrome *c* reductase activity (membrane bound endoplasmic reticulum) was determined as described[23]. The 1 ml incubation mixture containing 20 μM oxidised cytochrome *c* and 10-20 μg protein in 25 mM potassium phosphate buffer, pH 7.5, was preincubated with 1 mM rotenone for 10 min at room temperature. The reaction was started by addition of 150 μM NADPH and the reduction of cytochrome *c* was followed as described (Section 2.7.2).

2.7.4 Esterase activity

Esterase activity (soluble endoplasmic reticulum) was determined by a modification of a previously described method[140]. The 1 ml reaction mixture

contained 20 mM potassium phosphate, pH 6.6, 1 mM EDTA, 0.1% (w/v) Triton X-100 and 10-20 µg protein. The reaction was started by addition of 3 mM *p*-nitrophenyl acetate dissolved in ice cold methanol. The production of *p*-nitrophenol was measured spectrophotometrically at 405 nm at 37°C.

2.7.5 Lactate dehydrogenase activity

Lactate dehydrogenase activity (cytosol) was determined by a modification of a previously described method[23]. The 1 ml reaction mixture contained 20 mM potassium phosphate, pH 7.4, 1 mM KCN, 50 µM NADH and 10-20 µg protein. The reaction was started by addition of 100 µM pyruvate and the decrease in the absorbance was followed spectrophotometrically at 340 nm at room temperature for 15 min.

2.8 SDS-PAGE

Approximately 1-15 µg of protein was added per lane. SDS-polyacrylamide gel electrophoresis was carried out using standard 12% to 15% resolving gels, with each gel consisting of the appropriate amount of acrylamide, 0.4% N'N'-bis-methylene-acrylamide, 375 mM Tris, pH 8.8, 0.1% ammonium persulphate, 0.0004% N,N,N'N'-tetramethylethylenediamine (TEMED). A stacking gel consisting of 4.9% acrylamide, 0.1% N'N-bismethylene-acrylamide, 125 mM Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 0.04% TEMED was used for all

gels. Proteins were resolved at 20 mA, for 1 h. The SDS running buffer consisted of 25 mM Tris, 192 mM glycine and 0.1% SDS.

2.9 Staining procedures

2.9.1 Silver stain

Gels were stained according to the method of Nesterenko *et al.*[141]. Briefly, proteins were fixed in 50% methanol (v/v), 10% acetic acid (v/v) for 30 min. Following fixation, the gel was washed twice in 10% methanol for 10 min and once in distilled H_2O. 0.03% (w/v) sodium thiosulphate was added for 1 min under gentle agitation, after which the gel was washed four times in dH_2O for a total of 6 min. 0.2% silver nitrate (w/v) was added for 6 min, following which, the gel was washed with two changes of dH_2O. Visualisation of the bands was achieved with addition of developing solution consisting of 3% (w/v) sodium carbonate, 0.03% (w/v) sodium thiosulphate and 0.0001% formaldehyde.

2.9.1 Coomassie blue stain

SDS polyacrylamide gels were carefully transferred to a plastic container and staining solution was added. After 30 min of constant agitation, the staining solution was removed and replaced with first destaining solution. After 30 min constant agitation, first destaining solution was decanted and replaced with successive rounds of second destaining solution until protein bands were clearly visible.

2.10 Western transfer

Nitrocellulose membrane (Hybond C-extra) was presoaked for 5 min in transfer buffer before use. 50 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes on a semi-dry type transfer apparatus in a sandwich of eight sheets of Whatman 3 MM paper also presoaked in transfer buffer. Gels used for the determination of VDAC1 and GFP expression were transferred for 1 h at 1 mA/cm^2 of nitrocellulose paper.

2.11 Western blot

Following transfer of proteins to nitrocellulose, the membrane was blocked in 5% skim milk powder (BioRad, Regents Park, NSW, Australia) in TBS for 1 h at 37°C. Following three washes with washing buffer (0.05% Tween 20 in TBS) for 20 min the membrane was hybridised overnight at 4°C with primary antibody at 1/1,000 dilution. The membrane was then washed three times with TBS and incubated a 1/1,000 dilution of horseradish-peroxidase labeled secondary antibody (Amersham, Castle Hill, NSW, Australia) for 1 h at 37°C. Following three washes, ECL detection kit (Amersham, Castle Hill, NSW, Australia) was used according to the manufacturer's instructions to detect protein expression. The signal was recorded on Lumi-Film chemiluminescent detection film (Boehringer-Mannheim, Nunawading, VIC, Australia)

2.12 Immunoprecipitation

1-2 μg of rabbit anti-porin IgG antibody was added to the 1 ml purified fraction and allowed to agitate at 4°C overnight. 50 μl of a 50% protein G bead slurry were added and allowed to agitate for a further 2 h. The sample was spun at 10,000 x g for 15 s and the supernatant containing the unbound material was removed and kept for later analyses. The protein G-antibody-antigen complex was further washed three times with buffer A + 0.5 M NaCl and once in buffer B. The immunopellet was then resuspended in 1 ml buffer B.

2.13 Precipitation of protein from detergents

Proteins were precipitated according to the method of Wessel and Flügge[142]. Briefly, 1 volume of sample was added to 4 volumes of methanol, 2 volumes of chloroform and 3 volumes of water. After vigorous vortexing, the sample was spun at 2,000 x g for 30 s and the upper phase was removed. A further 3 volumes of methanol was added and the sample spun at 10,000 x g for 10 min. The supernatant was aspirated and the pellet was left to air dry for at least 10 min before re-suspending in 10% SDS to analyse on SDS-PAGE.

2.14 Purification of the NADH:ferricyanide-reductase

The chromatographic steps of DEAE-Sephacel and Blue Sepharose affinity column were carried out using conventional stepwise elution and gradient elution, respectively.

Solubilised proteins from the enriched plasma membrane pellet were applied to the DEAE-Sephacel column (5 ml bed volume) equilibrated with buffer B. The column was washed three times with 20 ml buffer B. The bound material on the column was eluted by 10 ml buffer B containing 50 mM NaCl. Active fractions eluting from the column were pooled and applied to the Blue Sepharose affinity column (5 ml bed volume) which had been equilibrated with buffer B. The column was washed twice with 10 ml buffer B, then eluted with a linear 10 ml gradient (0-10 μM) of NADH in buffer B. 1 ml fractions were collected and assayed for NADH:ferricyanide-reductase activity.

2.15 FACS analysis

Approximately 1 x 10^7 cells were harvested, pelleted (800 x g, 5 min) and washed twice in 1% FCS in PBS. The cells were suspended in 4 ml PBS and incubated for 40 min with Fc fragment (Bethyl laboratories, Montgomery, TX, USA). The cells were then washed and divided equally into four tubes. Tubes 2 and 4 were incubated for 40 min at 4°C with anti-VDAC1 antibody at

1/1,000 dilution. All tubes were pelleted (800 x g, 5 min) and the supernatant aspirated. The cells were then washed twice (1% FCS in PBS). Following the second wash, tubes 3 and 4 were incubated with FITC-conjugated anti-rabbit antibody for 40 min. FITC histogram was obtained by analysing 15,000 cells with the MODfit program (Becton-Dickinson, Wheelers Hill, VIC, Australia). The number of viable cells was simultaneously obtained by staining intracellular DNA with propidium iodide and the fluorescence was measured using the same program.

2.16 Bacterial cell manipulations

2.16.1 Growth and maintenance of *E. coli*

Transformed *E. coli* bearing the recombinant plasmids were grown in liquid Luria Broth (LB) supplemented with 50 μg/ml kanamycin. Cells were incubated at 37°C overnight for growth.

2.16.2 DNA plasmid amplification

pl-VDAC1-GFP was a kind gift from Professor Dermietzel. The DNA arrived on PVDF membrane and was excised. 100 μl TE buffer were added to the membrane containing the DNA. The sample was boiled for 2 min and 5 μl of supernatant were taken and used to inoculate 10^7 viable *E. coli* cells. The cells were grown on solid LB medium with 50 μg/ml kanamycin to select for inoculated cells. 5 ml LB medium containing kanamycin was added overnight

at 37°C. 10^7 bacteria were then resuspended in 4 ml of buffer P1. 4 ml of buffer P2 were added and the solution was mixed gently 4 times and left to incubate for 5 min at room temperature. 4 ml of chilled buffer P3 were added and the solution left to stand on ice for 15 min. The sample was then centrifuged (10,000 x g, 30 min) and the supernatant was taken. The supernatant was applied to a QIAGEN-Tip 100 which had been pre-equilibrated with 4 ml buffer QBT. The QIAGEN-Tip was washed with 2 x 10 ml buffer QC and finally eluted with 5 ml buffer QF. The DNA was precipitated with the addition of 0.7 volumes of isopropanol at room temperature. The sample was centrifuged (10,000 x g, 30 min) and the supernatant was decanted. The DNA pellet was washed with 2 ml 70% (v/v) ethanol and finally dissolved in TE buffer. As a control, the commercially available pEGFP vector was used (CLONTECH laboratories, East Meadow Circle, PA, USA).

2.16.2 Electroporation of COS7 cells

Cells to be electroporated were grown logarithmically (70% confluent). Each transfection required 2 x 10^6 cells to yield a reasonable number of transfectants. 1 ml of trypsin was added for 5 min per 10 cm dish and the cells were collected and harvested by centrifuging for 5 min at 1,200 rpm and then resuspending in 400 ml of ice-cold medium and placed in a 0.8 cm cuvette (BioRad, Castle Hill, NSW, Australia). The cuvette was placed in the electroporation apparatus (Gene – Pulsar apparatus, BioRad, Castle Hill,

NSW, Australia) and current was applied at a voltage of 200 V. The capacitor was set to 960 μF and the time constants obtained ranged from 15–45 ms. The cells were then transferred to growth medium and allowed to grow for at least 48 h.

2.17 Purification of rat liver mitochondrial VDAC

VDAC1 was purified according to de Pinto *et al.*[143] with a slight modification. Wister rats were decapitated, their livers excised and immediately washed twice in lysis buffer 2. Following the second wash, the livers were diced into 2 mm^3 pieces and diluted 1:3 (w/w) with ice cold lysis buffer 2. The diced livers were then homogenised in a 50 ml glass Teflon homogeniser (Sigma, Castle Hill, NSW, Australia) at 1,500 rpm, for five strokes. The homogenate was centrifuged at 600 x g for 5 min to remove nuclei and unbroken cells. The supernatant was decanted and a second centrifugation was performed at 600 x g for 5 min to remove any contaminating nuclei. Following the second spin, the supernatant was carefully taken so as not to disturb the pellet. The supernatant was then centrifuged at 4,500 x g for 30 min in order to pellet the mitochondria. The supernatant was removed and the pellet was resuspended in lysis buffer 2. A second centrifugation was performed at 4,500 x g for 30 min to wash the mitochondria. Following the second spin, the mitochondrial pellet was taken and diluted to 5 mg/ml in buffer A. After incubating in buffer A for 1 h, the sample was centrifuged at 4,500 x g for 30 min and the pellet, consisting

mainly of mitochondrial membranes was taken. The membranes were then solubilised in buffer A + 5% Triton X-100. Following constant stirring for 1 h, the sample was centrifuged at 100,000 x g, and the supernatant was taken. The supernatant was then applied onto a DEAE column, which had been pre-equilibrated with buffer B. The flow through was taken and applied to a dry 2:1 (w/w) hydroxylapatite:celite (HTP:celite) column. The flow through from the HTP:celite column was collected, which consisted of purified VDAC1.

2.18 Preparation of a vanillylamine-agarose affinity column

10 ml ester-activated agarose beads (BioRad, Castle Hill, NSW, Australia), 1.0 ml of triethylamine, 56 mg of 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide and 52 mg of 4-hydroxy-3 methoxybenzylamine hydrochloride were gently mixed overnight at 25°C. The resin was washed with 3 ml triethylamine to react with any non-reacted groups, followed by methanol and buffer B to remove unreacted materials prior to use.

CHAPTER 3 – Purification of a plasma membrane NADH:ferricyanide-reductase

3.1 Introduction

A number of electron transport systems have been shown to exist within cell membranes. The mitochondrial and endoplasmic reticulum electron transport chains are perhaps two of the best-characterised systems in mammalian cells. However, little interest has been shown for the identification and function of a plasma membrane redox system. Evidence to support the existence of a plasma membrane redox system, comes from experiments, in which two artificial impermeable electron acceptors (potassium ferricyanide and DCIP) become reduced by whole cells[23]. Reduction of ferricyanide causes concomitant oxidation of cytosolic NADH[82], suggesting the presence of an NADH:ferricyanide-reductase in the plasma membrane. However little headway has been made in the identification and characterisation of the proteins involved in plasma membrane redox function. NADH:cytochrome b_5 reductase flavoprotein has been suggested to be involved in plasma membrane electron transfer. Originally found in the endoplasmic reticulum, where it is involved in fatty acid desaturation[144], NADH:cytochrome b_5 reductase also exists in the plasma membrane of human erythrocytes[145]. Purification of the plasma membrane bound NADH:cytochrome b_5 reductase isoform from erythrocyte ghosts involved solubilising the membrane proteins in 1% Triton X-100, DEAE-Sepharose anion exchange, hydroxylapatite absorption and 5′-ADP-hexane-agarose

affinity chromatography. The 36 kDa enzyme was capable of reducing ferricyanide and cytochrome b_5[145,146] in the presence of NADH. The enzyme could also reduce cytochrome *c* in the presence of cytochrome b_5[146], suggesting that it has the same functional characteristics as its endoplasmic reticulum counterpart. NADH:cytochrome b_5 reductase has also been purified 2,200-fold from rat liver plasma membranes via hydroxylapatite chromatography, anion exchange chromatography and gel filtration[147]. The apparent molecular mass of the holoenzyme was 34 kDa and that of the apoenzyme was 32 kDa. Though in both cases, NADH:cytochrome b_5 reductase was purified by measuring reduction of ferricyanide in the presence of NADH at 420 nm, this enzyme, on its own, cannot be responsible for whole cell ferricyanide-reduction, since it is only found on the inner side of the plasma membrane[10]. Whole cell ferricyanide-reduction requires transmembraneous electron transport; evidence for the existence of a transmembraneous plasma membrane NADH:ferricyanide-reductase has been discussed earlier[81] (section 1.1.4).

A second plasma membrane redox protein was identified and purified, using DCIP (and not ferricyanide) as the artificial electron acceptor. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isozyme was identified as the functional redox protein responsible for DCIP-reduction[148]. The enzyme was purified by taking plasma membrane proteins, solubilised with 1% lubrol from either bovine or rat brains, or neuroblastoma NB41A3 cells and adding ammonium sulphate (30-70% saturation) followed by

superose 12 size exclusion and butyl-Sepharose chromatography. *In-situ* staining of native PAGE proteins detected the presence of the redox protein. Excision of the active band, enabled the identification of a membrane bound GAPDH isoform. The protein was in tight connection with three other proteins, namely 'turned on after cell division 64', enolase-γ and aldolose C. This is the first time a plasma membrane "redox complex" has been shown to exist. Confirmation of the presence of a plasma membrane bound GAPDH comes from the *Staphylococcal* transferrin receptor protein, Tpn, which was found to be a cell wall enzyme, with high homology to GAPDH[149]. However, much like NADH:cytochrome b_5 reductase, GAPDH cannot account on its own for whole cell DCIP-reduction, because it is only found associated with the inner side of the plasma membrane[10].

A third plasma membrane redox protein has been shown to exist in human erythrocytes[150]. Interestingly, ghost membranes were initially washed with 1 mM EDTA to remove extrinsic proteins such as GAPDH[151] followed by a solubilisation step in 5% Triton X-100. Further purification of the protein involved NAD^+ Sepharose 4B affinity chromatography, ammonium sulphate precipitation [0-25% (w/v)] and isoelectric focusing, upon which the enzyme focused at pH 6.6. The unidentified protein showed an apparent molecular mass of 40 kDa in SDS-PAGE. Divalent ions such as Ca^{2+} or Mg^{2+} caused slight inhibition of the NADH:ferricyanide-reductase activity and full inhibition of the enzyme could be seen with the addition of sulfhydryl reagents, suggesting the presence of at least one –SH functional group.

Carbohydrate and lipid analysis of the protein suggested the presence of a glycolipoprotein with fucose, galactose, mannose and glucosamine as the sugar components. The enzyme is suggested to be transmembraneous based on the first step of purification, where removal of peripheral protein was performed with addition of EDTA. However, purification of the peripheral enzyme NADH:cytochrome b_5 reductase was achieved even when this same step was used[147].

Here I show the purification of an NADH:ferricyanide-reductase from human Namalwa cells. The enzyme was purified 189-fold from Namalwa plasma membranes and found to co-elute with NADH:ferricyanide-reductase activity from a Blue Sepharose column. High enzyme activity was maintained in the presence of the non-ionic detergent Triton X-100, however enzyme activity was lost, when analysed after a number of concentration procedures, including ammonium sulphate precipitation and dialysis, which is indicative of a hydrophobic protein.

3.2 Results

3.2.1 Optimisation of detergent solubilisation conditions

Human Namalwa cells have been previously shown to possess a high amount of ferricyanide-reductase activity[18]. Therefore, they were chosen as suitable starting material for the purification of the enzyme. Newly developed procedures have allowed the use of homogenous cell line(s) for the purpose of extracting plasma membrane fractions in relatively large yields[31]. From 8 x 10^8 cells, 37 pkatal of enzyme activity could be obtained (Table 3.2 p 82).

Research carried out on the purification of a plasma membrane NADH:ferricyanide-reductase has, on the most part, resulted in the purification of peripheral proteins. Therefore in an attempt to remove external proteins, the plasma membrane enriched fraction was washed with 0.1 M EDTA[152,153]. After constant stirring for 1 h in the chelating reagent, the membranes were spun (10,000 x g). The resultant pellet contained 35 pkatal of enzyme activity and the protein was purified 1.2 fold higher than the original starting material (Table 3.2).

In order to solubilise the membrane proteins from the resultant pellet, a range of detergents was assessed for their ability to solubilise enzyme activity. Detergents were added to membrane fractions at concentrations ranging from 1-3%. Following constant stirring overnight, the preparations were centrifuged at 100,000 x g, and the supernatants were taken and used for enzyme analysis (Fig. 3.1).

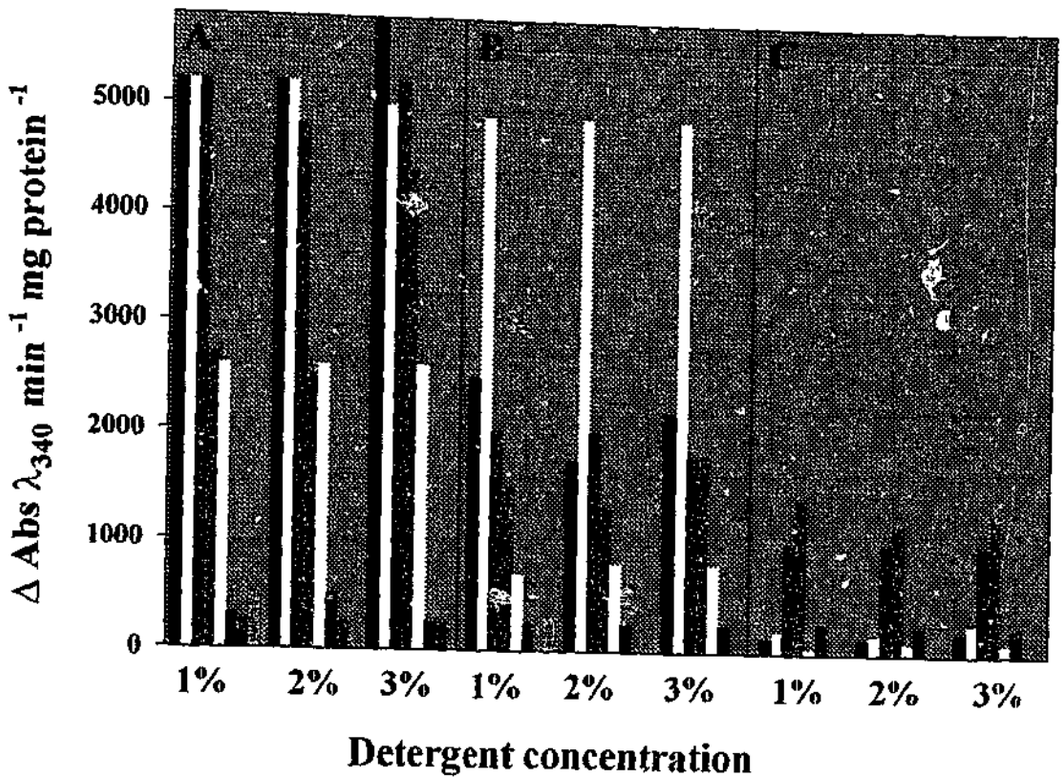

Fig. 3.1 Optimisation of detergent concentrations used to solubilise enzyme activity. Plasma membranes were obtained from Namalwa cells and diluted (A) 5 mg/ml (B) 2.5 mg/ml (C) 0.05 mg/ml. Various detergents (Triton X-100, black; NP-40, white; Tween 20, red; Chaps, green; Tween 80, yellow; sodium deoxycholate, blue; SDS, pink) were added to the final concentrations indicated, stirred overnight, then centrifuged (100,000 x g). The turquoise bars represent the detergent-free controls. The supernatant was assayed for NADH:ferricyanide-reductase activity.

Solubilisation of the proteins required detergent since without addition of detergent to the membrane pellet, no activity was detectable in the supernatant. Solubilisation in either 3% Triton X-100, NP-40 or Tween 20 yielded the highest enzyme activity when the protein concentration was 5 mg/ml. Since the former produces an extra 500 units of activity per mg protein, it was chosen as the choice detergent. When Triton X-100 was used as the detergent, dilution of the protein resulted in a loss of specific enzyme activity. The amount of detergent needed is 3-fold more than was reported by Kim *et al.*[147] (who purified NADH:cytochrome b_5 reductase) and suggests that integral membrane proteins were solubilised. Detergents unsuitable for enzyme purification included SDS and sodium deoxycholate, which yielded no enzyme activity in the supernatant (Fig. 3.1), probably due to a loss in secondary structure[111]. The enzyme itself appeared to be quite unstable, because loss of enzyme activity could be detected if the solubilised membrane preparation was left for more than 7 days at 4°C (data not shown). Furthermore, addition of more than 20% ammonium sulphate (w/v), freeze drying, Amicon (centricon) centrifuging or dialysing the enzyme resulted in a loss of enzyme activity (Fig. 3.2).

3.2.2 Purification of the NADH:ferricyanide-reductase

To further purify the enzyme, membrane proteins, solubilised in 3% Triton X-100, were applied to an anion exchange column, which had been pre-equilibrated with buffer B. The column was washed with 10 column

volumes of buffer A + 1% (w/w) Chaps and eluted with stepwise increments of 25 mM NaCl in buffer A + 1% Chaps (Fig. 3.3). The major active fractions were found to elute from the column with 25-50 mM NaCl. This produced a further 8-fold purification with 2 pkatal of enzyme activity (Table 3.2). 75 mM NaCl also eluted enzyme activity, however Brightmen *et al.*,[77] have demonstrated this fraction has NADH-oxidae activity present. Since this fraction contains relativley low amounts of NADH:ferricyanide-reductase activity, it was decided not to use it for further purificaiton.

In an attempt to ascertain the size of the enzyme, active DEAE eluant was applied to various size exclusion columns (see Table 3.1 for explanation) which had been pre-equilibrated with buffer A + 1% Chaps. As shown (Table 3.1) none of the columns yielded any enzyme activity in the fractions collected. This may have been due to non-specific interactions of the protein with the different column matrices. In an attempt to overcome such non-specific interactions, the concentration of salt and detergent present in the buffer were changed. However, none of the different buffer conditions used resulted in obtaining an active enzyme preparation (Table 3.1).

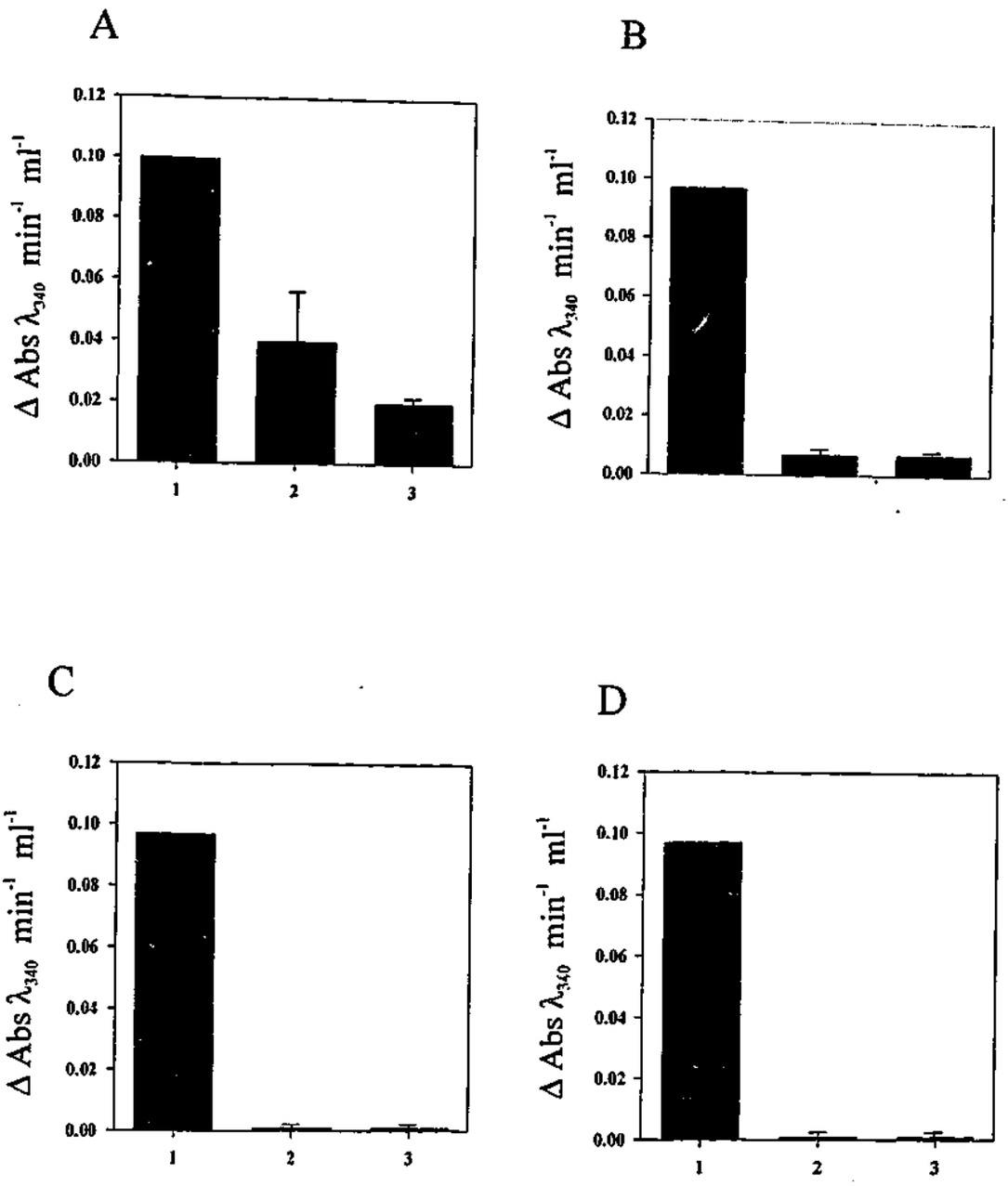

Fig. 3.2 Concentration of NADH:ferricyanide-reductase activity.

Solubilised plasma membrane proteins were analysed under various concentration procedures, including (A) ammonium sulphate precipitation [20% (w/v)] (B) freeze drying (C) dialysis (D) Amicon (centricon) centrifugation. Enzyme activity before the procedure (lane 1) was greatly reduced following the various concentration methods (lane 2). In most cases, the enzyme activity was not above background levels (lane 3).

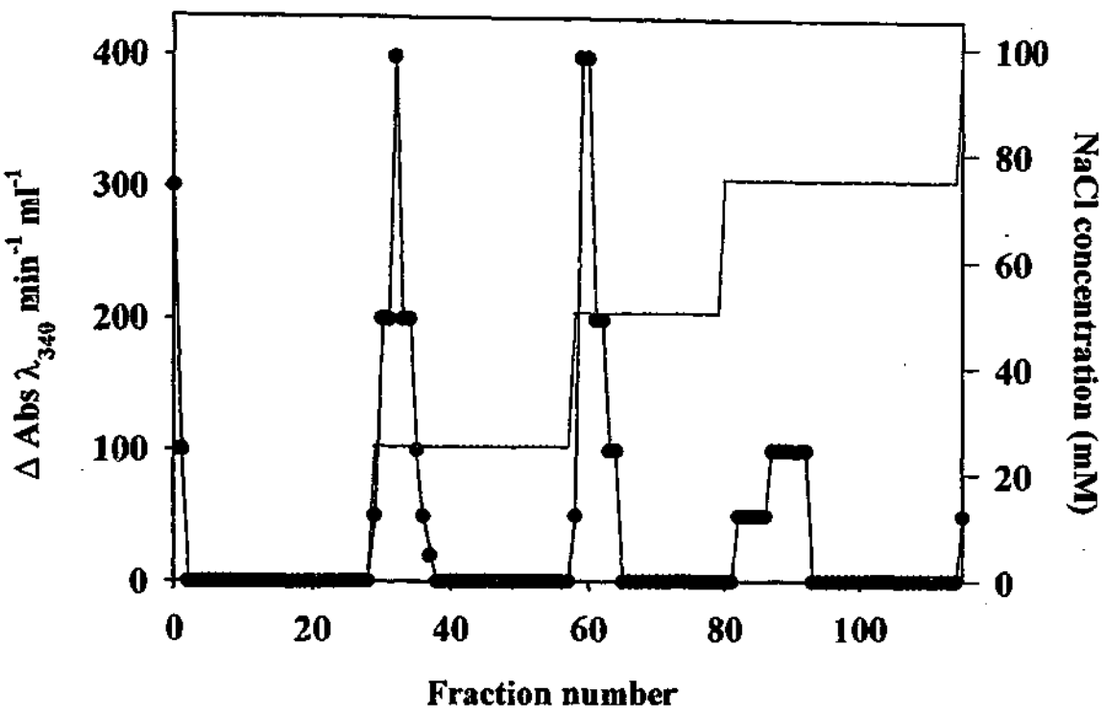

Fig. 3.3 Elution of NADH:ferricyanide-reductase activity from DEAE column.

Plasma membrane proteins, solubilised in 3% Triton X-100 were loaded onto the DEAE column (5 x 1.5 cm) which had been pre-equilibrated with buffer B. The column was washed with buffer B, and then elution was performed with stepwise increments of 25 mM NaCl in buffer B (red line). 1 ml fractions were collected and measured for enzyme activity (black line).

Column Used	Compostition of column	Buffer	Buffer Concentration	Detergent Concentration	Enzyme activity
Sephadex 75 (80 x 1 cm)	Cross linked dextran	50 mM Tris, pH 7.0 pH 8.0	No salt 50 mM NaCl 100 mM NaCl 1 M NaCl	0.5%-2.0% Chaps 0.5% Triton X-100	No No No No
Superdex 75 (45 x 1 cm)	Cross linked agarose and dextran	50 mM Tris, pH 7.0 pH 8.0	No salt 50 mM NaCl 100 mM NaCl 1 M NaCl	0.5%-2.0% Chaps	No No No No
Superose 12 (FPLC) (15 x 1 cm)	Cross linked agarose	50 mM Tris, pH 7.0 pH 8.0	No Salt 100 mM Nacl	0.5%-2.0% Chaps	No No
Tsk 3000 (HPLC) (5 x .25 cm)	Silica gel	50 mM Tris, pH 7.0	No Salt	0.5%-2.0% Chaps	No No

Table 3.1. Attempted purification of the NADH:ferricyanide-reductase using size exclusion chromatography.

Active DEAE eluant were loaded onto the columns, which were pre-equilibrated with 5 column volumes the indicated buffer. 1 ml fractions were taken and analysed for NADH:ferricyanide-reductase activity as described in Section 2.6.1.

3.2.3 Purification of the NADH:ferricyanide-reductase using affinity chromatography

Any NADH:ferricyanide-reductase should possess an NADH binding motif. Many columns are available for the purification of nucleotide binding proteins, including Blue Sepharose. Blue Sepharose affinity chromatography, has been used to purify dehydrogenases, including alcohol dehydrogenase and GAPDH[154]. Therefore, the active fractions eluting from the DEAE Sepharose column were applied to the Blue Sepharose affinity column, which had been pre-equilibrated with buffer B. The bound proteins were washed with 10 column volumes of buffer B and eluted with a single volume of 50 μM NADH in buffer B. The resulting fraction contained NADH:ferricyanide-reductase activity and the protein profile demonstrated the presence of a silver stained band at 35 kDa (Fig 3.4). To confirm that the 35 kDa band was an NADH:ferricyanide-reductase, the Blue Sepharose column was washed and re-loaded with fresh active DEAE eluate. The Blue Sepharose column was then eluted with a gradient of 0-50 μM NADH in buffer B. 1 ml fractions were collected and assayed for enzyme activity (Fig. 3.5). Those fractions with NADH:ferricyanide-reductase activity were then precipitated with methanol/chloroform, as described in section 2.13 and run in an SDS-PAGE (Fig. 3.5). A 35 kDa band was shown to correlate with enzyme activity suggesting it could be the NADH:ferricyanide-reductase. The protein had been purified 187-fold from the plasma membrane level (Table 3.2). The Blue

Sepharose affinity column proved to be the most powerful column for the purification of the protein.

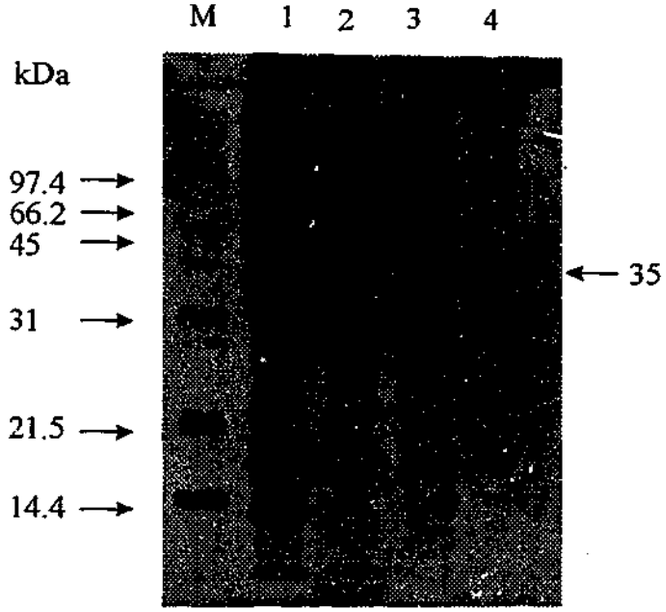

Fig. 3.4 Purification procedure for the plasma membrane NADH:ferricyanide-reductase as analysed by SDS-polyacrylamide gel electrophoresis.

Active enzyme preparations from 15 µg plasma membranes (lane 1), 13 µg 3% Triton X-100 solubilised proteins (lane 2), 7 µg DEAE eluant (lane 3) and 1 µg Blue Sepharose eluant (lane 4), were precipitated ($MeOH/CHCl_3$), resuspended in loading dye and subject to 15% polyacrylamide gel electrophoresis. The proteins were silver stained as described (Section 2.9.1). The positions of the molecular mass markers (M) are shown. The arrow indicates the 35 kDa protein eluting from the Blue Sepharose column.

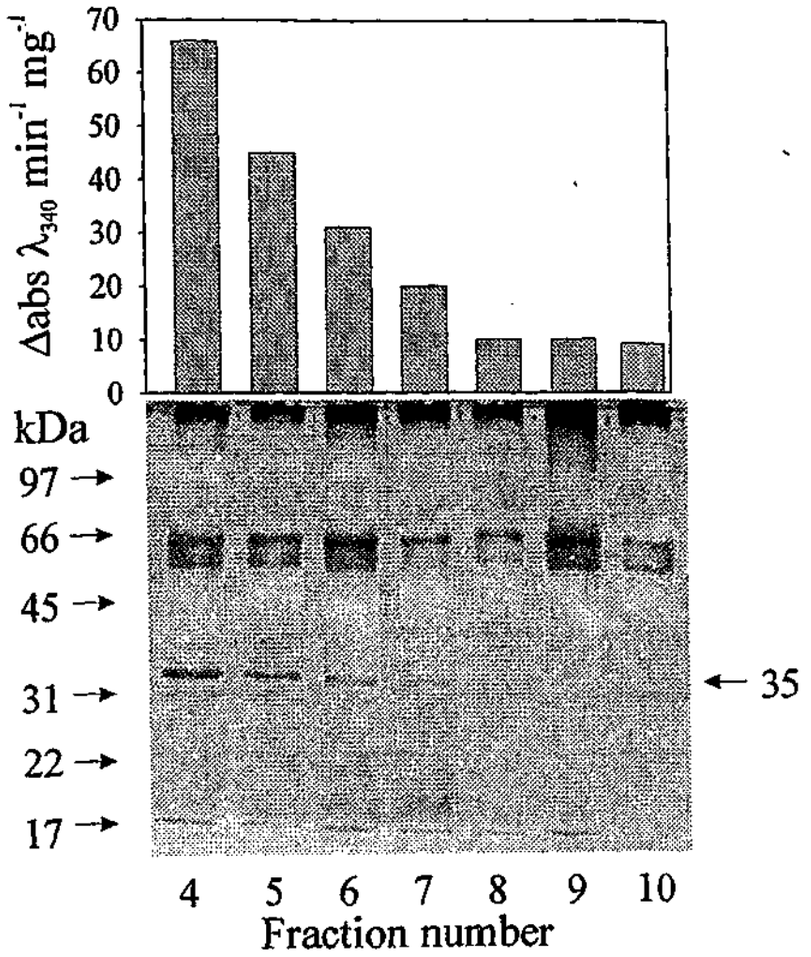

Fig. 3.5. Co-elution of the 35 kDa band with NADH:ferricyanide-reductase activity.

(TOP) Active DEAE eluant was applied to the Blue Sepharose affinity column, which was pre-equilibrated with buffer B. The column was washed with buffer B and eluted with a gradient of 0-50 μM NADH in buffer B. 1 ml fractions were collected and assayed for NADH:ferricyanide-reductase activity (grey bars)

(BOTTOM) Active enzyme fractions eluting from the Blue Sepharose column were precipitated (MeOH/$CHCl_3$), resuspended in loading dye, subjected to 12% polyacrylamide gel electrophoresis and silver stained as described in Section 2.9.1. The positions of the molecular mass markers (M) are shown. The arrow indicates the 35 kDa protein.

Step Performed	Total Protein concentration (mg)	Total Volume (ml)	Specific Activity (pmol $K^+[Fe(CN)_6]^{3-}$ $min^{-1}mg^{-1}$)	Purification factor
Plasma membrane	25	25	88.80	-
EDTA wash	20	25	105	1.2
Solubilisation	10	25	127.58	1.4
DEAE	0.16	3	759.37	8.6
Blue Sepharose	0.001	1	6368.00	186.9

Table 3.2 Purification table for the NADH:ferricyanide-reductase.

Factions of the individual purification procedures indicated were taken, and assayed for both NADH:ferricyanide-reductase activity and protein concentration. A molar extinction co-efficient at λ=340 nm, of 6.22 x 10^3 M^{-1} cm^{-1} was used for NADH in determining the specific activity.

3.3 Discussion

Two important observations have led to the conclusion that a transmembraneous ferricyanide-reductase is present in the plasma membrane of mammalian cells. Upon addition of extracellular ferricyanide to viable cells, a reduction of the compound is observed[23] with concomitant oxidation of cytosolic NADH[82]. Since ferricyanide is large, anionic and hydrophobic in nature, it does not cross the plasma membrane[3]. NADH on the other hand is found in the cytosol of cells. Therefore, an NADH:ferricyanide-reductase must be either membrane spanning, or a complex of proteins would be necessary for the redox reaction to occur. To date, two plasma membrane redox enzymes have been suggested to contribute to NADH:ferricyanide activity. However both NADH:cytochrome b_5 reductase and GAPDH are located on the cytosolic face of the plasma membrane, therefore they cannot account for transmembraneous ferricyanide-reduction on their own[147,148].

To purify a transmembraneous NADH:ferricyanide-reductase, plasma membrane enriched fractions were obtained from Namalwa cells. Procedures using cultured cells to obtain plasma membrane fractions have always had the inherent problem of obtaining enough starting material[155]. However, new subcellular fractionation procedures have made it possible to obtain a large amount of plasma membrane in relatively short time[31]. In order to remove extrinsic proteins from the plasma membranes, EDTA was added[152,153]. However a report in which the peripheral enzyme NADH:cytochrome b_5

reductase was purified also included the same procedure[147], therefore, it is unlikely that the remaining proteins are exclusively transmembraneous.

The choice of detergent for membrane solubilisation can be critical for the purification of an active enzyme. This cannot only lead to an increased yield, but also to a more stable enzyme preparation. Solubilisation in both SDS and sodium deoxycholate, resulted in the complete loss of enzyme activity, probably due to denaturation of secondary structure[111]. The highest amount of solubilised enzyme activity was yielded when Triton X-100, Tween 20 and NP-40 were used. This suggested that the NADH:ferricyanide-reductase maintains integrity in non-ionic detergents, which form large micelles. Interestingly, high concentrations (3%) of Triton X-100 orbv Tween 20 were required for maximal enzyme rates. During the purification of peripheral enzymes such as NADH:cytochrome b_5 reductase, much lower detergent concentrations (1%)[147] were reported, suggesting that in these preparations, most integral membrane proteins may not have been solubilised.

Before further purification of an NADH:ferricyanide-reductase proceeded, a number of methods for concentrating the enzyme was tested. Addition of ammonium sulphate, freeze-drying or Amicon centrifugation proved deleterious to the enzyme activity. Furthermore, the enzyme did not retain activity after being dialysed for 1 h, which is indicative of a hydrophobic enzyme.

The enzyme was further purified by the use of a DEAE anion exchange column. Enzyme activity could be eluted from the DEAE column

with stepwise increments of 25 mM NaCl at pH 8.0. Some NADH:ferricyanide-reductase activity could still be detected upon addition of 75 mM NaCl to the DEAE column (Fig. 3.3). Interestingly, NADH-cytochrome b_5 reductase can be eluted from a DEAE column using 75 mM NaCl at pH 7.4. A direct comparison of the conditions used to purify my NADH:ferricyanide-reductase and NADH:cytochrome b_5 reductase from a DEAE column demonstrates that the major difference is the pH of the buffer[147] (Table 3.3).

Table 3.3 Elution of NADH:reductases from DEAE-Sephacel

	NADH:ferricyanide-reductase	NADH:cytochrome b_5 reductase
Buffer concentration	50 mM	25 mM
NaCl concentration	75 mM	75 mM
pH of buffer	8.0	7.4

Under the conditions used to purify my NADH:ferricyanide-reductase, it appeared likely that the protein I was purifying was not NADH:cytochrome b_5 reductase.

Since Triton X-100 forms large micelles, it is unsuitable for the use in size exclusion chromatography. Therefore, during the anion exchange chromatography run, the detergent was changed from Triton X-100 to Chaps.

This procedure is often utilised during membrane protein purification[147]. Chaps has the additional advantage that a UV detector can be used to detect the protein during the column run. A number of size exclusion columns were used, in an attempt to further purify the enzyme. However, in no case was enzyme activity detectable in the fractions collected. This indicated that interaction of the protein with the column matrices may have been the cause of the loss in enzyme activity. In an attempt to prevent any non-specific interactions, the concentrations of detergent and salt present in the buffer were changed, however no further purification (due to a loss in enzyme activity) occurred under these conditions. Perhaps a combination of factors, such as protein denaturation in the high pressure columns (HPLC/FPLC) or the loss of an important substrate or co-factor during the column run, may have also contributed to the loss of NADH:ferricyanide-reductase activity. Therefore, for the further purification of the enzyme, a change in the purification procedure was implicated.

A number of columns have been identified that are capable of interacting with nucleotide binding proteins. One example is Blue Sepharose, which has been previously used to purify GAPDH and alcohol dehydrogenase[154], both of which use NADH as a co-factor. Therefore, the Blue Sepharose affinity column was used to further purify the NADH:ferricyanide-reductase. Following application and elution of the Blue Sepharose column, NADH:ferricyanide-reductase activity could be detected. The corresponding SDS-PAGE of the fraction revealed a 35 kDa band in a

silver stained gel. Confirmation that the 35 kDa band was the NADH:ferricyanide-reductase was obtained when the affinity column was eluted with a gradient of NADH. The 35 kDa band co-eluted with enzyme activity on the SDS-PAGE, indicating that this protein is an NADH:ferricyanide-reductase.

CHAPTER 4 – Identification of VDAC1 as the plasma membrane NADH:ferricyanide-reductase

4.1 Introduction

Since I had obtained strong evidence that the 35 kDa protein eluting from the Blue Sepharose column was an NADH:ferricyanide-reductase (see chapter 3), this band was excised and a matrix assisted laser desorption ionisation mass spectrometry was performed with a micromass TofSpec 2E time of flight mass spectrometer (MALDI-TOF) (see this chapter). Predicted sequences from four resulting peptides, identified the 35 kDa protein as VDAC1.

VDAC is a small, 30-35 kDa protein, predominantly found in the outer membrane of mitochondria[86]. Structural studies of human VDAC predict the protein to be a β-barrel, consisting of either 13[116], or 16[117] membrane spanning strands. Molecular mapping and cloning studies in human[156,157] and bovine cells[158] suggest the existence of genetic isoforms of VDAC[157]. The human isoforms have been characterised as VDAC1, VDAC2, VDAC3 and VDAC4. Research to date has focused primarily on VDAC1. Although VDAC1 is predominately expressed in the outer mitochondrial membrane, it has been demonstrated recently that VDAC1 can be expressed in the plasma membrane[159]. Consistent with the plasma membrane localisation of VDAC1, several patch clamp techniques have documented the presence of plasma membrane channels with physiological properties similar

to VDAC1[160,161]. Immunocytochemical studies using antibodies raised against the NH_2-terminal of VDAC1 block a high conductance anion channel found in the plasma membrane of bovine astrocytes[158]. These antibodies also appear to label specifically the plasma membrane in human B-lymphocytes[162-165]. VDAC1 has been reported to co-purify with plasmalemma-bound central type A γ-aminobutyric acid ($GABA_A$) receptor[166] and caveolae[167] (both of which are located in the plasma membrane). Finally, the use of impermeable NH-SS-biotin, to specifically label and pull down external plasma membrane proteins, followed by Western blotting with anti-VDAC1 antibodies, confirmed the presence of the protein in the plasma membrane[168,169]. *In vivo,* VDAC1 harbours two alternative first exons, which leads to the expression of VDAC1 with different N-terminal leader sequences. One of these leader sequences directs the protein via the Golgi apparatus into the secretory pathway[170]. The other, second leader sequence, directs VDAC1 to the mitochondria, where it is efficiently inserted into the outer membrane[170].

The presence of VDAC1 in the plasma membrane leads to the question of its role there. It has been suggested that like its mitochondrial counterpart, VDAC1 may also direct the passage of ions through the plasma membrane[171]. Indeed evidence has suggested that VDAC1 is involved in a plasma membrane chloride channel complex[171]. Here, it is established that VDAC1 can function as a redox enzyme, capable of reducing ferricyanide in the presence of NADH at the level of the plasma membrane. These results

suggest that VDAC1 functions as a transmembraneous redox protein, and in doing so, is likely to form part of the PMOR complex.

4.2 Results

4.2.1 MALDI-TOF analysis of 35 kDa band

The 35 kDa band co-eluting with NADH:ferricyanide-reductase activity from the Blue Sepharose column, was excised and following a 24 h in gel tryptic digest, a MALDI-TOF analysis of the resulting peptides was performed by the Australian proteome research laboratory (Fig. 4.1). Four peptides with molecular masses of 2176.4, 2600.3, 2103.5 and 2418.3 were used to search the BLAST and TREMBL database for sequence homology to other known proteins. This search revealed the 35 kDa band to be identical to VDAC1 (Fig. 4.2).

4.2.2 The amino acid sequence of VDAC1 is consistent with the function of an NADH-reductase

Co-elution of VDAC1 with enzyme activity from the Blue Sepharose column is strong evidence that VDAC1 is an NADH:ferricyanide-reductase. However because the eluant from the affinity column was not homogeneous, further evidence was sought to confirm that VDAC1 could function as a redox protein.

Upon inspection of the amino acid sequence of VDAC1, a putative NAD^+ binding motif on residues 143-152 was identified. The amino acid sequence VLGXXGXXXG on VDAC1 is very similar to that of the NADH binding domain in alcohol dehydrogenase (VLGXXGXXG)[172], with the

exception that VDAC1 has an extra amino acid substituted before the final glycine. The involvement of NADH in the regulation of VDAC has been well documented[120,173,174]. Upon addition of 5 μM β-NADH to isolated mitochondria, the permeability of the outer membrane decreased by a factor of 6[173]. This decrease was suggested to be due to VDAC1, since purified, reconstituted VDAC1, also demonstrated a decrease in permeability to ATP upon addition of NADH[174].

Further investigations into the amino acid sequence of VDAC1, identified the existence of two cysteine residues (Fig. 4.2). Cysteine residues appear to be essential for electron transfer in the NADH:ferricyanide-reductase, since upon addition of sulfhydryl binding reagents, inhibition of ferricyanide-reduction occurs in whole cells[23]. Two cysteines would be sufficient for electron transfer from intracellular NADH to extracellular ferricyanide. The identification of a putative NADH binding site and two cysteine residues within the amino acid sequence of VDAC1 is consistent with a function as an NADH-reductase.

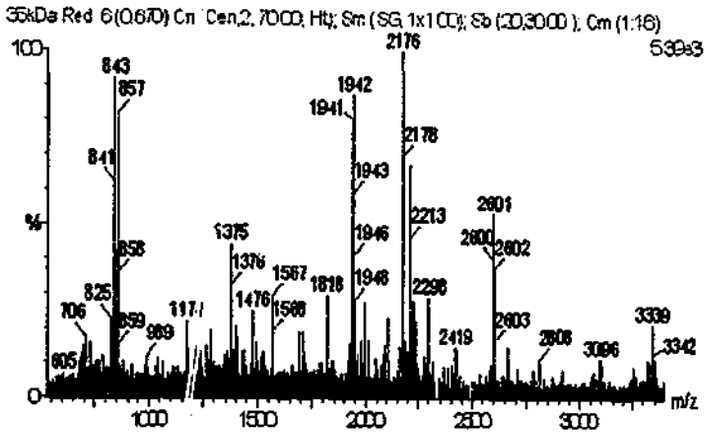

Fig. 4.1 MALDI-TOF analysis of 35 kDa band.

The 35 kDa band eluting from the Blue Sepharose affinity column was excised and following a 24 h in-gel tryptic digest, a MALDI-TOF analysis was performed.

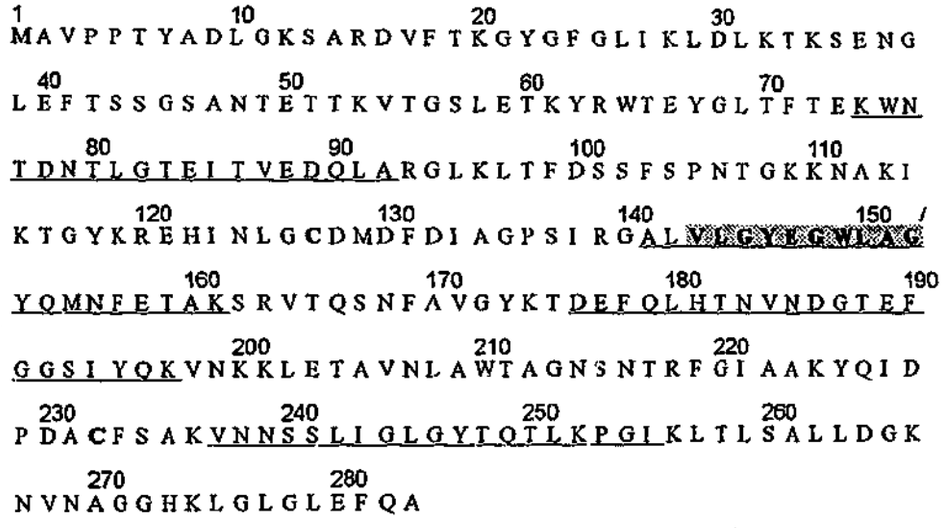

Fig. 4.2 Amino acid sequence of VDAC1.

The predicted amino acid sequence of 4 resulting peptides (underlined) from the MALDI-TOF analysis matched to VDAC1. The primary amino acid sequence of VDAC1 contains an hypothesised NADH binding domain (blue) and 2 cysteine residues (yellow) which is consistent with the function of an NADH:dehydrogenase.

4.2.3 Immunoprecipitation and purification of VDAC(1)

To confirm further that VDAC1 can function as an NADH:ferricyanide-reductase, two different antibodies were used to immunoprecipitate the protein. These antibodies included a polyclonal antiserum, raised against the entire VDAC protein and a monoclonal antibody, raised against the N-terminal portion of VDAC1. The specificity of these antibodies was established by Western blot analysis (Fig. 4.3). Both antibodies recognise a band in whole cell homogenates of approximately 35 kDa in size. To demonstrate that enzyme activity could be immunoprecipitated with both antibodies, rat liver mitochondrial VDAC1 was purified as described (Section 2.17) and 1 µg of either antibody was added to active fractions eluting from the Blue Sepharose column. Upon addition of the polyclonal (Fig. 4.4 panel A) or monoclonal (Fig. 4.4 panel B) antibody, no direct inhibition of the original activity was observed (lane 2). In both cases however, following immunoprecipitation, most of the NADH:ferricyanide-reductase activity was present in the immunopellet fraction (lane 4). Conversely, very little enzyme activity was left in the unbound supernatant fraction (lane 3). The addition of only protein G beads as a control, did not result in any enzyme activity being precipitated (lane 5). The ability of both antibodies to immunoprecipitate enzyme activity, suggests that VDAC1 can function as a redox protein. However, this result can still be explained by VDAC1 co-immunoprecipitating with another protein, which may be

responsible for the enzyme activity. To address this possibility, VDAC was purified from rat liver mitochondria, based on a known purification procedure[175] with a slight modification (see Section 2.17). The resultant purified fraction contained a single 32 kDa band (Fig. 4.5). This fraction also contained NADH:ferricyanide-reductase activity (Fig. 4.5) suggesting that VDAC1 alone can function as an NADH:ferricyanide-reductase and not activating another specific plasma membrane NADH:ferricyanide-reductase.

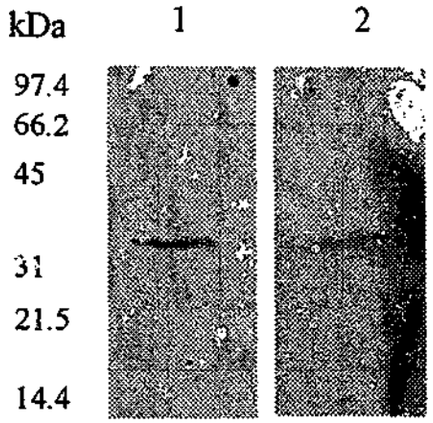

Fig. 4.3 Anti-VDAC antibodies specifically recognise a 35 kDa band in Western Blot.

Namalwa cells were homogenised and 50 µg of protein were resuspended in loading dye and run in a 15 % polyacrylamide gel. Protein expression of VDAC (1) via Western blot analysis was determined using either the polyclonal (lane 1) or monoclonal (lane 2) antibody as described (Section 2.8). The positions of the molecular mass markers are shown at the left hand side.

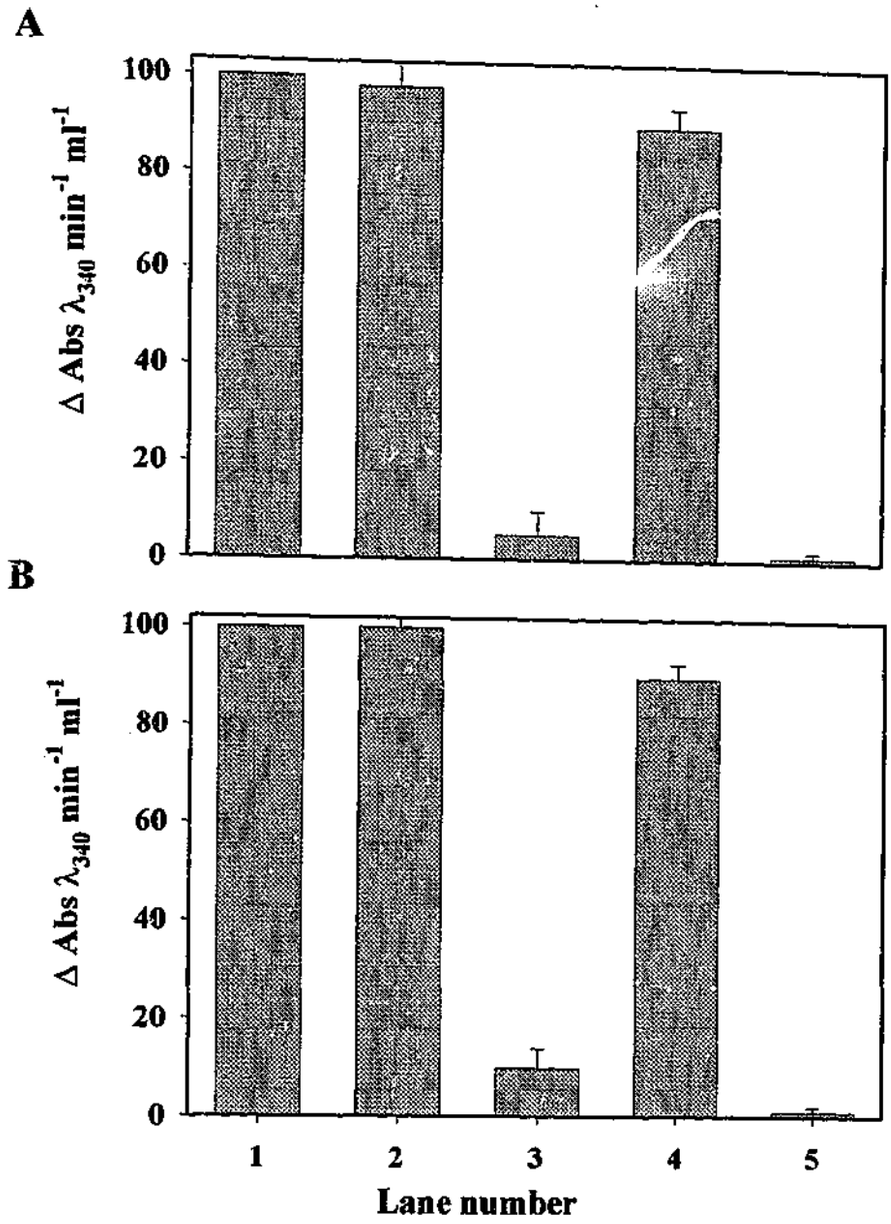

Fig. 4.4 Immunoprecipitation of enzyme activity of anti-VDAC polyclonal and monoclonal antibodies.

An active fraction eluting from the Blue Sepharose column (3 X 0.5 cm) was used as starting material for the immunoprecipitation (lane 1). 3 µg of monoclonal (panel A) or polyclonal (panel B) antibody were added (lane 2), following which, 60 µl of a 50% slurry of protein G, were added. The sample was centrifuged (30 s, 10,000 x g) and the unbound supernatant (lane 3) and immunopellet (lane 4) were measured for NADH:ferricyanide-reductase activity as described in section 2.2.1. The control shows the addition of protein G only (lane 5).

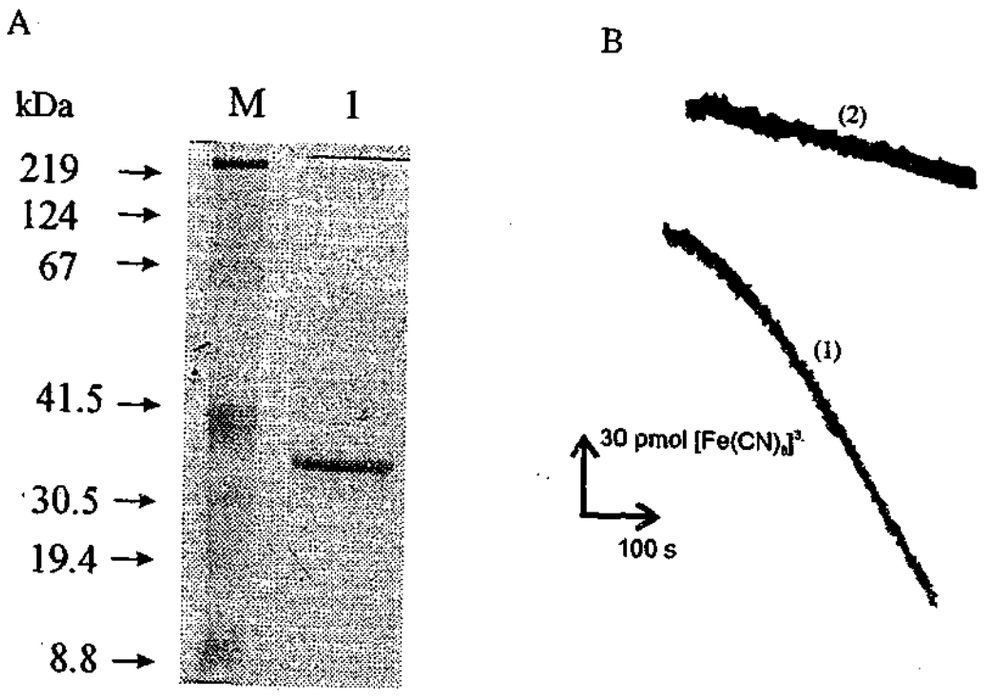

Fig. 4.5 Purification of rat liver mitochondria VDAC1 as an active NADH:ferricyanide-reductase.

(A) Rat liver mitochondria VDAC1 was purified according to Materials and Methods (section 2.17). 30 µl of the material eluting from the celite:HTP column were precipitated ($MeOH/CHCl_3$) and loaded onto a 12% SDS polyacrylamide gel (lane 1). After electrophoresis, the gel was stained with either Coomassie blue (shown) or silver as described (Section 2.9). The positions of the molecular mass markers (M) are shown at the left hand side.

(B) (1)30 µl eluting from the Celite:HTP column were tested for NADH:ferricyanide-reductase activity as described (section 2.6.1). (2) Background oxidation of NADH.

4.2.4 VDAC1 is present in the plasma membrane of Namalwa cells.

Using immunohistochemical and other approaches, VDAC1 has been documented to be present on the plasma membrane[160-165]. However, criticism of these experiments has arisen, based on the possibility that antibodies raised against VDAC1 can non-specifically interact with the plasma membrane[176,177]. To confirm the observation that VDAC1 is present in the plasma membrane, a set of FACS analyses was performed on Namalwa cells. To overcome any non-specific interactions of the antibody with the plasma membrane, a specific F_c fragment was used as a blocking agent on all cells, prior to the addition of any antibodies. Fig. 4.6 shows an increase in fluorescence in cells treated with both the primary anti-VDAC1 monoclonal and secondary FITC-conjugated antibodies, when compared to control cells with only the primary or secondary antibody. The significant shift in fluorescence with cells incubated in both primary and secondary antibody confirms the presence of VDAC1 in the plasma membrane of Namalwa cells. This shift in fluorescence cannot be accounted for by mitochondrial VDAC1, since neither fixation nor permeablisation of the cells prior to FACS analysis was performed. Furthermore the cells were viable as determined by propidium iodide staining, ensuring that no major quantities of antibody could cross the plasma membrane.

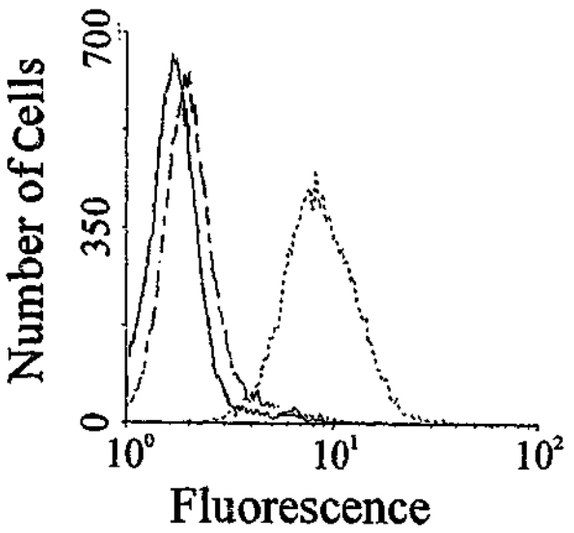

Fig. 4.6 FACS analysis of VDAC1 expression on the plasma membrane. 1 x 10^6 Namalwa cells were harvested, washed and blocked with anti-Fc for 1 h as described (Section 2.15). The cells were then incubated with either monoclonal mouse anti - VDAC (solid line), goat FITC conjugated anti - mouse (long dashed line) or both antibodies (short dashed line) After washing the cells, FITC expression was analysed by FACS analysis as described (Section 2.15).

4.2.5 VDAC1 can function as an NADH:ferricyanide-reductase *in vivo*

To demonstrate that the redox activity of VDAC1 was not an *in vitro* artefact, COS7 cells were transfected with a plasmid containing the pl-VDAC1-GFP gene. The genetic product contains an N-terminal leader sequence, directing the protein to the plasma membrane[170]. The plasmid was initially amplified by transforming *E. coli* cells, then purified as described (section 2.16.1). Based on the size of the excised inserted DNA, the plasmid was successfully amplified (Fig. 4.7).

COS7 cells (2-3 x 10^6) were then transfected with either 20 μg of pl-VDAC1-GFP, or the vector control (see section 2.16.1). 48 h post-transfection, the cells were harvested, washed and subjected to Western blot analysis as described (section 2.12). Mouse monoclonal anti-GFP antibodies were used to determine GFP expression in the transfected cells. In COS7 cells transfected with the pl-GFP-VDAC1 vector, the antibodies recognised a 52 kDa band (Fig. 4.8). This suggested that VDAC1 (35 kDa), fused to GFP (27 kDa), was being expressed in these cells. On the other hand, in COS7 cells transfected with the vector control only, the antibodies recognised a 27 kDa product, which is identical to the molecular mass of GFP alone (Fig 4.8).

To determine whether the pl-VDAC1-GFP product was being efficiently processed through the secretory pathway, confocal analysis was performed. COS7 cells transfected with pl-VDAC1-GFP were shown to have green fluorescence, confirming that GFP was being expressed (Fig. 4.9). In

these cells, the pattern of GFP expression closely resembled the teratocarcinoma cell line, PA-1 clone 9117 cells transfected with the same vector[170]. In PA-1 cells, such expression was demonstrated to be in the Golgi and secretory pathway[170]. This suggests that the pl-VDAC1-GFP genetic product was also expressed at the level of the plasma membrane in COS7 cells.

In contrast, COS7 cells transfected with the control vector demonstrated green fluorescence in the typical cytosolic localisation (Fig. 4.9).

To determine if the pl-VDAC1-GFP gene product could function as a redox protein *in vivo*, transfected COS7 cells were assayed for their ability to reduce ferricyanide. Those cells expressing pl-VDAC1-GFP demonstrated at least 40-fold stimulation in whole cell ferricyanide-reduction, compared to the mock transfected cells (Fig. 4.10). Wild type COS7 cells at either 2×10^5 or 4×10^5 showed no measurable whole cell ferricyanide reduction (Fig. 4.10) This is similar to other cell lines, which at these concentrations also do not exert any measurable ferricyanide reduction rates, under the conditions described (cf section 2.6.2).

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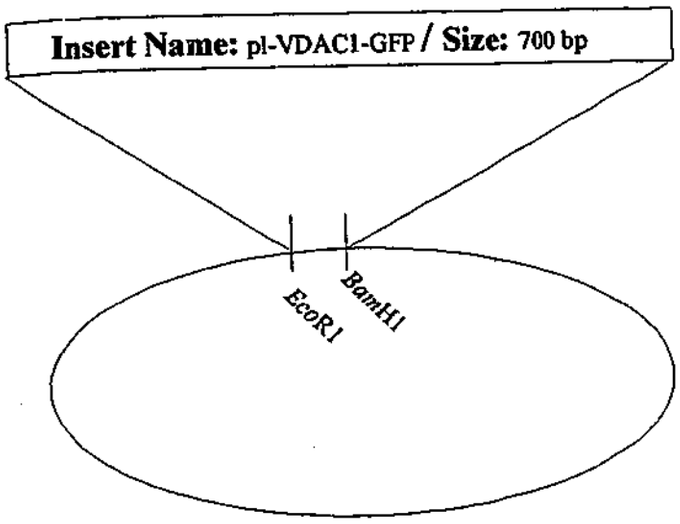
A



B

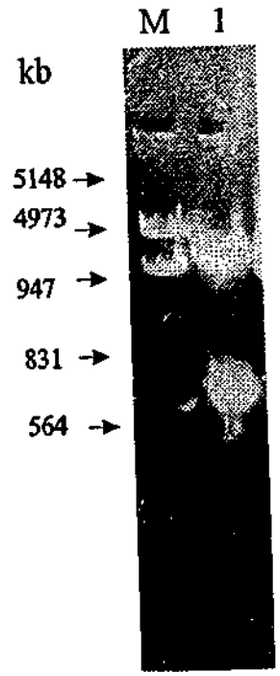


Fig. 4.7 Restriction enzyme digestion of pl-VDAC1-GFP plasmid.

Plasmid DNA was purified as described (2.16.1).

(A) The plasmid contained two restriction enzyme sites as shown.

(B) Following overnight digestion with *Eco*R1 and *Bam*H1 the product was run in a 1% agarose gel (lane 1). The position of the markers (M) are shown on the left hand side.

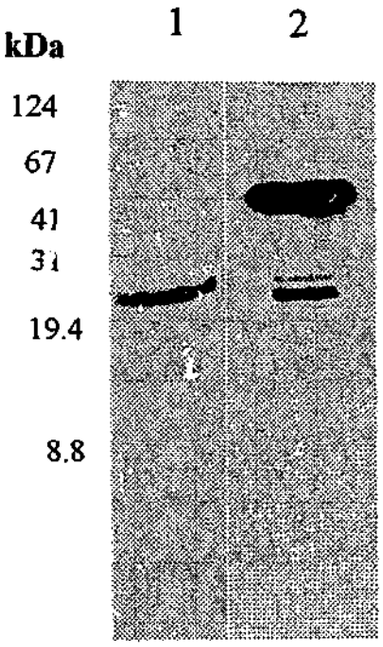


Fig. 4.8 Western blot analysis of transfected COS7 cells.

COS7 cells were transfected with pI-VDAC1-GFP (lane 2) or the vector control (lane 1) as described (Section 2.16.2). The presence of GFP was determined by Western blot analysis as described (Section 2.11). The positions of the pre-stained markers (M) are shown on the left hand side.

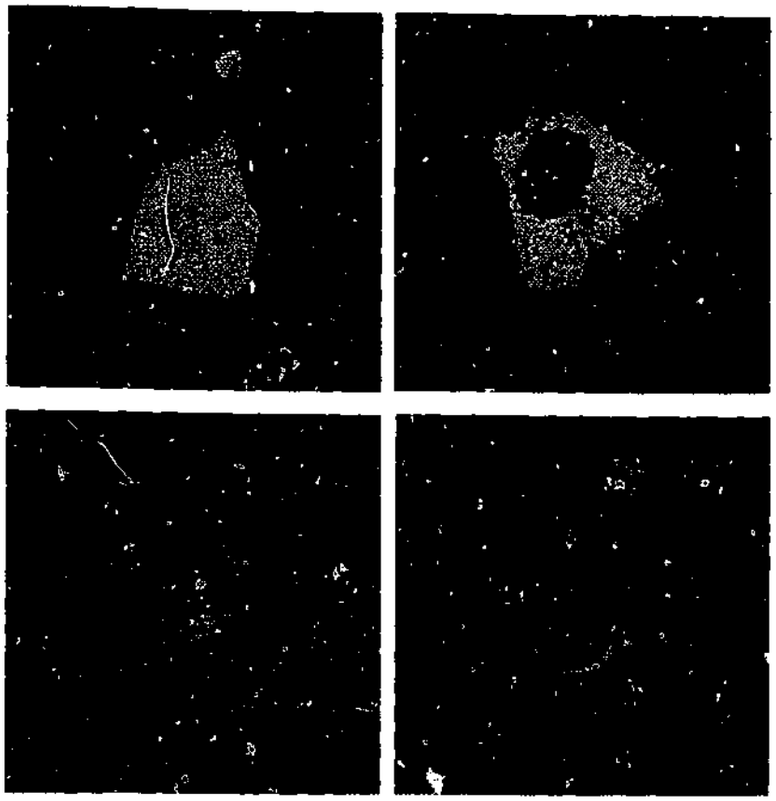


Fig. 4.9 GFP expression in transfected COS7 cells.

COS7 cells were harvested, pelleted and electroporated with the vector only (left hand side) or pl-VDAC1-GFP (right hand side) as described (Section 2.16.2). 48 h post transfection, the cells were visualised using confocal microscopy. Plasma membrane (yellow arrow - lower right hand quadrant) and secretory pathway (red arrow -upper right quadrant) are indicated.

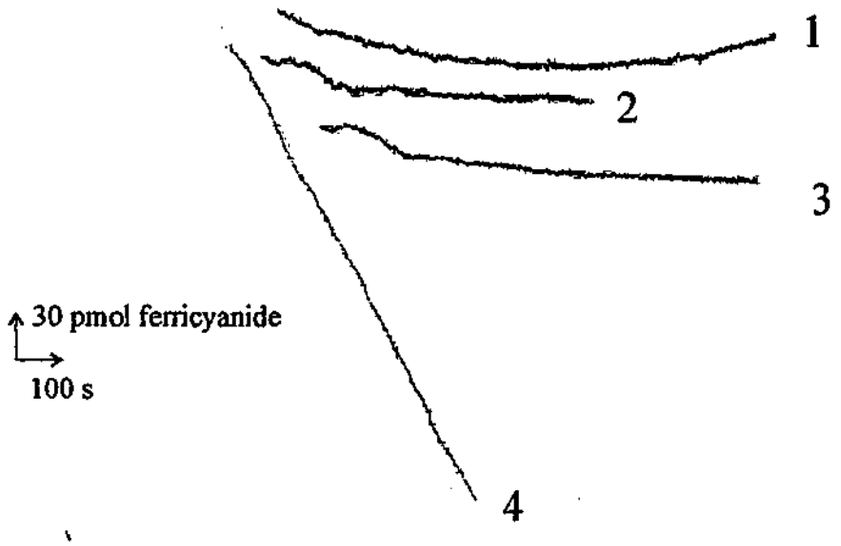


Fig. 4.10 Whole cell ferricyanide reduction in COS7 cells transiently transfected with pl-VDAC1-GFP.

COS7 cells were transfected with pl-VDAC1-GFP (line 4) or the vector control only (line 3). 48 h post transfection, 2 x 10^5 cells were assayed for whole cell ferricyanide reduction as described (Section 2.6.2). 2 x 10^5 (line 1) or 4 x 10^5 (line 2) wild type COS7 cells were used as background controls.

These results demonstrate that overexpression of VDAC1 in the plasma membrane, leads to an enhanced rate of whole cell ferricyanide-reduction. This suggests that VDAC1 functions *in vivo* as a redox protein at the level of the plasma membrane.

4.2.6 VDAC1 is not an NADH:DCIP-reductase *in vivo* or *in vitro*

Previously, our laboratory has demonstrated that there also exists a NADH:DCIP-reductase, in the plasma membrane of mammalian cells[23]. DCIP, much like ferricyanide is a cell membrane impermeant electron acceptor. To analyse whether VDAC1 was also responsible for DCIP-reduction, COS7 cells were again transfected with the plasmid containing the VDAC1 gene and whole cell DCIP activity was assayed for. No difference could be detected in the rate of DCIP-reduction, between the mock transfected and pl-VDAC1-GFP transfected cells (Fig. 4.11), suggesting that the DCIP-reductase is a different protein to VDAC1. To confirm that VDAC1 was incapable of NADH:DCIP-reduction, VDAC1 was immunopurified. NADH and DCIP were added to the immunopurified product. As shown in Table 4.1, no increase in NADH-oxidation above background level was observed, suggesting *in vitro*, VDAC1 cannot function as an NADH:DCIP-reductase. Buillard *et al.* have purified an NADH:DCIP-reductase on the plasma membrane of neuronal cells[148] and showed the protein responsible was a GAPDH isozyme. However GAPDH is found only on the cytosolic surface of the plasma membrane[148]. In a whole cell DCIP assay, GAPDH would not

have access to the electron acceptor and therefore cannot be responsible for transmembraneous DCIP-reduction on its own. The most likely explanation for whole cell DCIP-reduction is through the presence of another transmembraneous NADH:DCIP-reductase.

4.2.7 Natural electron carriers of VDAC1

In vitro analysis of VDAC1 shows that the enzyme cannot efficiently use NADPH as an electron donor (Fig. 4.12; Table 4.1). Therefore NADH is more likely to be the natural electron donor *in vivo*. Ferricyanide on the other hand cannot be the natural electron acceptor, since it is not a physiological compound. A number of natural electron acceptors are present within cells, including CoQ, α-tocopherol, glutathione and ascorbate[13]. To determine what may be the natural electron acceptor of VDAC1 the protein was immunopurified and some natural electron acceptors/donors were used to replace NADH or ferricyanide in the assay medium (Table 4.1). Upon addition of either glutathione and ascorbic acid to medium containing ferricyanide, reduction of ferricyanide occurred, non-enzymatically in an extremely quick manner. Therefore, it was not possible to measure whether these compounds could act as electron donors under these conditions. Neither CoQ nor cytochrome *c* were reduced in the presence of NADH and VDAC1 (Table 4.1), suggesting CoQ and cytochrome *c* are not the electron acceptors of VDAC1 *in vivo*.

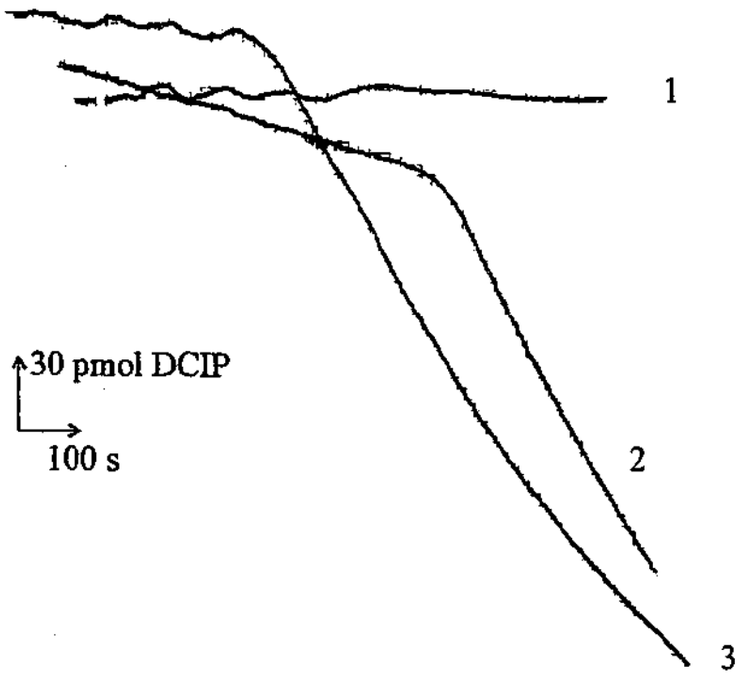


Fig. 4.11 Whole cell DCIP reduction in COS7 cells transiently transfected with pl-VDAC1-GFP.

COS7 cells were transfected with pl-VDAC1-GFP (line 3) or the vector control only (line 2). 48 h post transfection, 2 x 10^5 cells were assayed for whole cell DCIP reduction as described (Section 2.6.4). Auto reduction of DCIP was measured as the background control (line 1). This graph is demonstrative of an expermient which was repeated three times.

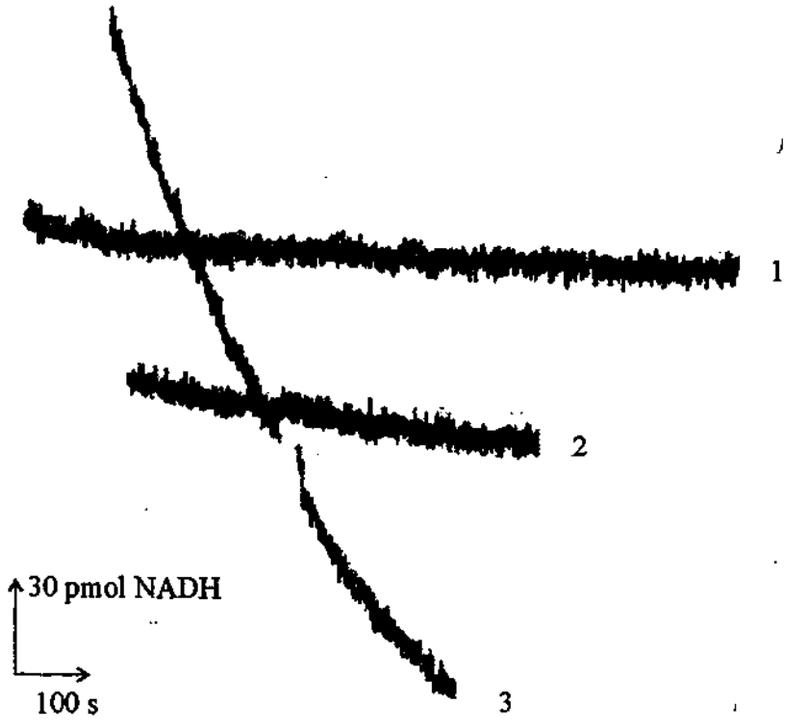


Fig. 4.12 VDAC1 is specific for NADH as a co-factor.

Active plasma membrane VDAC1 eluting from the Blue Sepharose column was incubated with NADH and NADPH as co-factors. The traces for VDAC1 assayed in the presence of ferricyanide with NADH (line 3) and NADPH (line 2) are shown. The background control demonstrates the auto-oxidation of NADH (line 1).

Compound	Rate [pmol nucleotide min^{-1} mg^{-1}]
Background (NADH:$[Fe(CN)_6]^{3-}$ only)	10± 2
NADH:$[Fe(CN)_6]^{3-}$	300 ± 7
NADH:cytochrome *c*	10 ± 2
NADH:CoQ	10 ± 1
NADPH:$[Fe(CN)_6]^{3-}$	30 ± 4
NADH:DCIP	10 ± 2
GSH: $[Fe(CN)_6]^{3-}$	*
Ascorbic Acid:$[Fe(CN)_6]^{3-}$	*

*These compounds auto-oxidise non-enzymatically, therefore cannot be assayed under these conditions

Table 4.1 Natural electron carriers of VDAC1.

VDAC1 was immunopurified as described (section 2.12). 50 μM of the electron donor was indicated was added and the baseline rate established. 50 μM of the electron acceptor was added and the rate was measured over 10 min. The data represent the mean of two experiments done in duplicate. The background rate was established by calculating change/inch/100 sec. For every 1.5 inch's, 30 pmol of NADH is consumed.

4.2.8 Inhibition of VDAC1 with known inhibitors of the PMOR and ion conducting proteins

Several chemicals, including adriamycin and taxol, have been demonstrated to cause inhibition of whole cell ferricyanide-reduction. Furthermore, there are two known potent inhibitors of VDAC1, namely DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonate) and Königs polyanion. Both compounds are thought to bind to positive charges lining the tunnel of VDAC1, and through a conformational change, to decrease the rate of ion permeation through VDAC1[99]. To test whether inhibition of VDAC1's redox activity could be achieved upon addition of these compounds, VDAC1 was again immunopurified. After establishing an enzymatic rate in the immunopurified product, different inhibitors were added (Table 4.2).

The compounds tested were unable to inhibit the rate of NADH oxidation. Since adriamycin has previously been demonstrated to inhibit the rate of ferricyanide-reduction in whole cells[178] this suggests that either a further adriamycin sensitive ferricyanide-reductase exists in cells or that adriamycin can only inhibit the NADH:ferricyanide-reductase activity of the PMOR complex.

DIDS and Königs polyanion have been shown to bind to the cytoplasmic entrance of VDAC1's tunnel[179] perhaps to positively charged lysine residues[108]. Although these compounds can inhibit the conductivity of VDAC1, neither was capable of inhibiting VDAC's redox activity. This implies that the lysine residues spanning the entrance of VDAC1's tunnel are

not involved in the proteins redox activity. Furthermore, it suggests that VDAC1 contains two important and distinct active sites in its structure. The front of the tunnel, may be necessary for the regulation of anion conductance. Within the pore of the protein, two cysteine residues, may form the bases for the proteins active redox activity.

Compound	Concentration	% Inhibition
Absolute ethanol	1 mM	2 ± 2
Königs Polyanion	400 mM	5 ± 4
DIDS	1 mM	4 ± 4
pCMBS	1 mM	98 ± 3
NEM	1mM	98 ± 3
Adriamycin	1 mM	2 ± 2
$CaCl_2$	100 µM	0 ± 1
$MgCl_2$	100 µM	0 ± 2
	200 µM	0 ± 1
	400 µM	0 ± 2
$MnCl_2$	100 µM	0 ± 2
	200 µM	0 ± 1
	400 µM	0 ± 1
KCl	100 µM	0 ± 1
	200 µM	0 ± 1
	400 µM	0 ± 1
$ZnCl_2$	50 µM	*

* Inhibitor reacted non-enzymaticaly with NADH, therefore could not be measured under these conditions.

Table 4.2 Action of known inhibitors of VDAC1 or the plasma membrane NADH:ferricyanide-reductase.

VDAC1 was immunopurified as described (Section 2.2). The compounds indicated were incubated with VDAC1 for 5 min. NADH and ferricyanide were then added as described (Section 2.6.1) and the enzyme rate was continuously measured for a further 10 min. The results show the average of two experiments done in duplicate.

4.3 Discussion

The outer membrane of mitochondria highly regulates the passage of solutes traveling between the cytosol and the intermembrane space[86,180]. One protein suggested to be involved in anionic regulation is the voltage dependent anion channel or VDAC[181]. A major role VDAC may play on the outer surface of the mitochondria is to regulate the flow of adenine nucleotides within cells[182], since one VDAC molecule is capable of transporting 2-5 x 10^6 ATP molecules per second[183]. Certain cellular kinases', such as hexokinase, may have preferential access to ATP, since they bind directly to VDAC[109]. VDAC has also been suggested to play a role in the process of cell death. Upon addition of various apoptotic stimuli, VDAC, together with ANT and cyclophilin D, form the basis of the PTP[129]. This megapore was suggested to be responsible for the release of cytochrome *c* from the mitochondria, which leads to cell death[129]. Recently, VDAC1 has been demonstrated to be present in the plasma membrane[159], leading to the question of its role in that location. Here I demonstrate that VDAC1 in the plasma membrane functions as an NADH:ferricyanide-reductase, suggesting plasma membrane localised VDAC1 may act as a redox sensor within cells.

Upon the excision of the 35 kDa band correlating with enzyme activity, a MALDI-TOF analysis followed by a BLAST data base search matched 4 tryptic peptides to VDAC1. VDAC1 is one of 4 isoforms known to exist in human cells. VDAC1 harbours two alternative first exons, which leads to its expression in either the mitochondria, or the secretory pathway[170].

The amino acid sequence of VDAC1 demonstrates characteristics of an NADH:reductase. On residues 143 to 152 a putative NAD^+ binding domain is suggested, with the sequence VLGXXGXXXG. This sequence is very similar to that of the yeast alcohol dehydrogenase NAD^+ binding domain (VLGXGXXXG)[172], only that VDAC1 contains an extra amino acid between the first and second glycine residue. Zizi *et al.* have previously reported another putative NAD^+ binding motif in human VDAC1, on residues 270-278[184]. This sequence (GXXXGXG) is also similar to that of the yeast alcohol dehydrogenase NAD^+ binding domain, but it is inverted. Since the motif identified in this thesis is non-inverted, it is most likely the one to represent the true NAD^+ binding site. However, only mutation of either of the two putative NAD^+ binding domains, followed by enzyme activity analysis of the mutated proteins, can conclusively demonstrate in the future which is the correct NAD^+ binding domain of VDAC1. However, in the absence of such mutation studies, it may be possible to look at the two models of human VDAC1 to gain more evidence which NAD^+ binding motif may be the correct one.

Table 4.3 Location of the NAD^+ binding domains in the two predicted structural models of human VDAC1

Model	Ref.	Location of residues 143-152	Location of residues 270-278
13 membrane spanning strands	116	Cytosol	Within plasma membrane
16 membrane spanning strands	117	Within plasma membrane	Within plasma membrane

As shown in table 4.3, aligning residues 143-152 (the non-inverted NAD^+ binding motif), to the 13 membrane spanning strand model of human VDAC1 places these residues in a cytosolic location. This would be consistent with an NAD^+ binding domain. In contrast, both the 13 and 16 membrane spanning strand models place residues 270-278 (inverted NAD^+ binding motif) within the plasma membrane, suggesting it would be inaccessible to cytosolic NADH.

Further inspection of the amino acid sequence of VDAC1 identified the presence of two cysteine residues. Addition of either *p*CMBS or NEM to purified VDAC1 resulted in a complete inhibition of its redox activity. Both *p*CMBS and NEM bind to free thiol groups, therefore, VDAC1 must have cysteine residues at or near its active site. The importance of cysteine residues in the PMOR has been well established, since upon the addition of *p*CMBS, a

thiol binding compound, inhibition of whole cell ferricyanide-reduction occurs[23].

Inspection of the predicted 13 membrane spanning strand model structure of human VDAC1 suggests the two cysteine residues are located at the boundary between the lipid phase and the water phase[116]. However, de Pinto *et al.*[185] have used Affi-Gel 501 (with a 1.75 nm long spacer), and Thiopropyl-Sepharose 6B (with a 0.51 nm spacer) to label both cysteine residues in bovine heart mitochondrial VDAC1. One of the cytseines bound to AffiGel 501, but not Thiopropyl-Sepharose 6B, suggesting that at least one of the cysteines is localised between 0.51 nm and 1.75 nm deep within the tunnel of bovine VDAC1. The cysteines in bovine VDAC1, based on the amino acid sequence and inhibitory studies, have been suggested to be similar to human VDAC1[185].

Strong evidence suggesting that VDAC1 is an NADH:ferricyanide-reductase is provided by the fact that the 35 kDa protein elutes with enzyme activity. To confirm that VDAC1 can function as an NADH:ferricyanide-reductase both monoclonal and polyclonal antibodies anti-VDAC(1) antibodies were used to immunoprecipitate the protein from plasma membrane preparations. The immunopellet was shown to have NADH-ferricyanide-reductase activity. However this experiment may also be interpreted as another protein co-immunoprecipitating with VDAC1. This second protein may be responsible for the enzyme activity. To ensure this was not the case, VDAC1 was purified form rat liver mitochondria, using the

method of de Pinto *et al.*[143]. Both a Commassie blue and sliver stained SDS-PAGE of the purified extract demonstrated that the sample eluting from the celite:HTP column was homogenous. Furthermore, the celite:HTP eluant contained NADH:ferricyanide-reductase activity, suggesting that VDAC1 is directly causing the reduction of ferricyanide in the presence of NADH.

The monoclonal antibody used in this study has previously been shown to inhibit anionic conductance of VDAC1[165]. This antibody however, did not inhibit the redox activity of VDAC1, suggesting the conductance pathway and reductase properties of this enzyme are independent of each other. There are two other established inhibitors of VDAC1's conductance, DIDS and Kŏnigs polyanion. Both of these compounds are thought to bind to the positively charged lysine residues found on the cytoplasmic entrance of the proteins tunnel[100,164]. Although the binding of these compounds has been shown to decrease the pore diameter of VDAC1[100], it is generally believed that the electrostatic interaction of the positively charged compounds, with the negatively charged lysine residues, results in the inhibition of VDAC1 conductance[111,186]. The inability of either compound to inhibit the redox activity of VDAC1 implies that the positively charged lysine residues spanning the entrance of VDAC1's tunnel are not involved in the proteins redox activity. Furthermore, closure of the pore does not seem to alter VDAC1's NADH:ferricyanide-reductase activity. These data suggest that VDAC1 contains two important and distinct active sites in its structure. The front of the tunnel, may be necessary for the regulation of anion conductance

through electrostatic charges or perhaps through closure of the tunnel. Within the pore of the protein however, two cysteine residues exists. In the latent state, theses amino acids may form a disulphide as suggested[185]. However, upon binding of NADH to VDAC1, The disulphide bond may be broken and electron transport may occur as shown in Fig. 4.13.

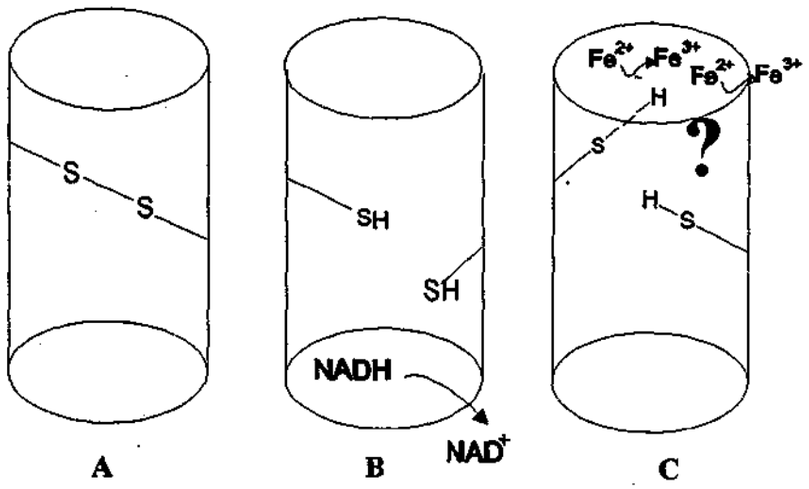


Fig. 4.13 Proposed model for the redox activity of VDAC1.

(A) VDAC1 begins with a disulfide bond between the two cysteine residues.
(B) NADH binds and donates two electrons toVDAC1. This leads to the consequent reduction of the disulfide bridge.
(C) Electron transfer through the plasma membrane occurs when two ferricyanide molecules accept an electron from the internal thiol groups.

Using a number of approaches, including immunohistochemical studies, VDAC1 has previously been shown to exist in the plasma membrane of cells[162-165]. However, criticism of these data has arisen based on the non-specific interaction of VDAC1 antibodies with the plasma membrane. The F_c fragment added to the cells in the FACS analysis experiments (Fig. 4.6), blocked the non-specific binding of anti-VDAC1 antibodies to the plasma membrane of Namalwa cells. The significant shift in fluorescence in those cells incubated with both primary anti-VDAC1 and secondary FITC antibodies compared to the control cells further supports the observation that VDAC1 is present in the plasma membrane of these cells.

If VDAC1 was directed to the plasma membrane and capable of redox function, then over expression of the protein should increase the rate of whole cell ferricyanide-reduction *in vivo*. To prove this principle, the plasmid gene pl-VDAC1-GFP was obtained[170]. The gene product, as discussed in section 4.2.5, is directed to the secretory pathway. COS7 cells transfected with that construct demonstrated green fluorescence, suggesting that the transfection was successful. Confirmation that the pl-VDAC1-GFP fusion protein was intact, was obtained via Western blot analysis. The shift in molecular mass in the pl-VDAC1-GFP transfected cells, compared to the mock transfected cells suggests pl-VDAC1-GFP was being expressed.

When transfected cells were measured for whole cell ferricyanide-reduction, the enhanced rate in pl-VDAC1-GFP cells compared to either the

mock transfected or wild type cells, suggests that VDAC1 can function *in vivo* as a ferricyanide-reductase at the level of the plasma membrane.

The finding by our laboratory that both a ferricyanide and DCIP-reductase exist in the plasma membrane of cells[23], led to the question of whether the enzyme responsible was one in the same, since both enzyme activities are up-regulated during the generation of ρ^0 cells[18]. The inability of VDAC1, both *in vivo* and *in vitro* to reduce DCIP suggests that a further enzyme is responsible for DCIP-reduction. Previously, Buillard and co-workers[148] have demonstrated a GAPDH isozyme can function as a NADH:DCIP-reductase. However these authors also conclude that GAPDH is located on the cytosolic surface of the plasma membrane. This being the case, GAPDH cannot be responsible on its own for whole cell DCIP-reduction, since it does not have access to extracellular DCIP.

VDAC1 is capable of oxidising NADH in the presence of ferricyanide, it is therefore likely that this is the natural electron donor *in vivo*. Nevertheless, ferricyanide, being an artificial compound, cannot be the natural electron acceptor. The natural electron acceptor of the reductase should be present or associate with the plasma membrane. Examples of membrane-bound electron acceptors include, α-tocopherol, retinol, cytochrome *c* and CoQ[13,187-190]. Two alternative receptors include ascorbate and glutathione. Our laboratory has previously demonstrated that CoQ can up-regulate both enzyme activities of the PMOR[18]. However, upon replacing ferricyanide with CoQ in the assay mixture, no reduction of the compound was evident,

suggesting a further CoQ-stimulated plasma membrane NADH:ferricyanide-reductase exists in cells. No reduction of cytochrome *c* was evident when this protein was used to replace ferricyanide in the assay mixture. These data suggest that neither CoQ nor cytochrome *c* are the electron acceptors of VDAC1 *in vivo*. Attempts to analyse whether glutathione or ascorbate could replace NADH as the natural electron donor failed, since both glutathione and ascorbate react non-enzymatically with ferricyanide, in very quick manner. One way of overcoming this, would be to use VDAC1 containing membrane vehicles which have been loaded with the electron donor. Addition of ferricyanide to these vesicles would give a clear indication of VDAC1's natural electron carriers.

The ability to inhibit VDAC1's enzymatic function on the plasma membrane, may have implications for cancer research. Previously adriamycin has been shown to inhibit NADH:ferricyanide-reductase activity in erythrocytes and mouse plasma membrane. 1 μM adriamycin was shown to inhibit 73% of the total ferricyanide-reductase activity[13], suggesting a large amount of ferricyanide-reduction is due to an adriamycin sensitive ferricyanide-reductase. The inability of adriamycin to inhibit VDAC1 suggests another unidentified adriamycin sensitive NADH:ferricyanide-reductase exists within cells.

CHAPTER 5 – Partial purification and characterisation of the plasma membrane NADH-oxidase

5.1 Introduction

Plasma membrane oxidoreductase systems are multienzyme complexes, transferring, electrons from cytosolic NADH to external electron acceptors. One major electron acceptor of these systems is oxygen[23], which suggests the presence of a terminal NADH-oxidase as part of the overall PMOR complex. An NADH-oxidase from rat liver plasma membrane has been described[191], which can be stimulated by various hormones and growth factors. The observed rate of NADH oxidation was reported to be insensitive to cyanide, azide and antimycin A; therefore it cannot be due to a contamination by the mitochondrial oxidase[191]. Furthermore, the plasma membrane NADH-oxidase was shown to be specific for NADH, suggesting that this enzyme is different from the NADPH-oxidase found in leukocytes[191]. Rat liver plasma membrane NADH-oxidase activity can be inhibited with the active compound of chilli pepper, capsaicin[17]. Capsaicin is part of the vanilloid family of compounds, which have previously been described as inhibitors of the vanilloid receptor 1 (VR1)[192]. VR1 belongs to the store-operated calcium channel family of proteins and is expressed in peripheral pain sensing neurons[192]. The inhibition of the plasma membrane NADH-oxidase by capsaicin represents the second biological activity of this compound.

Attempts to purify a plasma membrane NADH-oxidase have, in part, been successful. By immobilising a capsaicin analogue (vanillylamine) on an agarose bead, Wilkinson *et al.*[76] have managed to purify an NADH-oxidase, designated "tNOX" (NADH-oxidase of transformed cells). The 33.5 kDa protein was purified from media, conditioned with HeLa cells, and from the sera and urine of cancer patients[76,193,194]. The authors have suggested that the protein may be modified and cleaved in cancer cells, therefore would make the ultimate marker for cancer diagnosis[194-196].

It is unlikely that tNOX alone is responsible as the PMOR's terminal oxidase activity. The terminal oxidase of the PMOR uses cytosolic NADH as the electron donor, and reduces molecular oxygen to water[13,197]. For this to occur, an individual protein or a complex of proteins must have access to the cytosol. tNOX has been demonstrated to be on the outer surface of the plasma membrane[198,199], making it inaccessible to cytosolic NADH. Therefore, NADH is an unlikely physiological substrate for the enzyme[78]. Furthermore, enzyme activity (NADH-oxidation) of purified tNOX, is not inhibited in the presence of a nitrogen or argon atmosphere[200], suggesting oxygen may not be the final electron acceptor for tNOX. Although it has never been shown, it would be anticipated that the terminal NADH-oxidase of the PMOR would be inactive without the presence of oxygen.

In the search of other physiological substrates for tNOX, two further enzyme activities have been demonstrated for this protein. Partially purified tNOX from the surface of HeLa cells demonstrated "ubiquinase" activity,

being capable of reducing ubiquinone in the absence of NADH. The reduction of ubiquinone was sensitive to the tNOX inhibitors capsaicin and the sulphnylurea compound LY181984. This suggested that the ubiquinase not only had a similar active site to tNOX, but possibly both enzyme activities were due to the same protein[201].

A second study has identified a role for protein disulphides as potential electron acceptors for tNOX, which implies that tNOX has protein disulphide isomerase (PDI) activity. This activity was found by measuring the reduction of protein disulphides to free cysteine groups in the presence of NADH. A direct assay of HeLa cells to obtain the total amount of plasma membrane thiols is achieved using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)[198]. The total number of thiols plus disulphides can be calculated using 2-nitro-5-thiosulphobenzoate (NTSB)[198]. In the presence of NADH, HeLa plasma membranes demonstrate an increase in the level of thiol groups[198]. Inhibition of this increase can be achieved with the addition of LY181984 or *p*CMBS[202] suggesting tNOX may be the protein responsible for the increase in thiol groups at the level of the plasma membrane[198]. A stoichiometric relationship between the rate of NADH-oxidation and disulphide reduction is consistent with tNOX being able to function as a multi-disciplinary enzyme, having both NADH-oxidase and PDI activity[41,200]. The choice of which electron acceptor tNOX uses (oxygen or disulphides) may depend on extracellular factors. Hormones and growth factors may stimulate protein disulphide reduction over oxygen consumption[200].

Since oxygen has been suggested to be the final electron acceptor in the NADH-oxidoreductase system (and tNOX is only located at the cell surface) another plasma membrane NADH-oxidase appears to be responsible for this function. Supportive evidence that this assumption may hold true, comes from studies of plant plasma membrane redox systems, were at least two NADH-oxidases have been shown to be present in soybean plasma membranes[203]. In mammalian systems, two NADH-oxidase activities can be distinguished on the basis of the enzymes sensitivity toward different inhibitors. Our laboratory has previously reported NADH-oxidase activity present in rat liver plasma membranes which was shown to be insensitive to 20 μM *p*CMBS[23]. In contrast, the cell surface NADH-oxidase (tNOX) is almost completely inhibited by that concentration of *p*CMBS[16]. This is confirmed by Berridge and Tan[204,205], who measured cell surface and transplasma membrane NADH-oxidase activity in whole cells with the tetrazolium dye WST-1. They found that 20 μM *p*CMBS had little effect on transplasma membrane NADH-oxidase activity, however at the same concentration, *p*CMBS inhibited 59% of the cell surface oxidase[204,205]. Therefore, the purification of a *p*CMBS insensitive transplasma membrane NADH-oxidase remains outstanding. Here I report on the attempted purification of this enzyme.

5.2 Results

5.2.1 A *p*CMBS insensitive NADH-oxidase exists in the plasma membrane of Namalwa cells

Fractions enriched in plasma membrane were obtained from Namalwa cells as previously described (section 2.4).

These plasma membranes were solubilised in 3% Triton X-100. The solubilised proteins were then assayed for NADH-oxidase activity in order to ascertain whether a *p*CMBS insensitive plasma membrane NADH-oxidase existed in human Namalwa cells. NADH-oxidase activity was present in the Namalwa plasma membranes (Fig. 5.1). The enzyme activity was specific for NADH, as NADPH could not function as an electron donor (Fig. 5.1). Only approximately 20% of the enzyme activity was inhibited with 20 μM *p*CMBS (Fig. 5.2). Even though the total activity of the cell surface NADH-oxidase varies depending on the cell type[196] it appears, that the cell surface NADH-oxidase activity can be inhibited, in all cases, to a large extent by the addition of *p*CMBS. Therefore, the 20% of enzyme activity inhibited by the addition of *p*CMBS may be attributable to tNOX. The remaining 80% of NADH-oxidase activity is *p*CMBS insensitive, this activity was previously attributed by Berridge and Tan to a trans-plasma membrane NADH-oxidase[194].

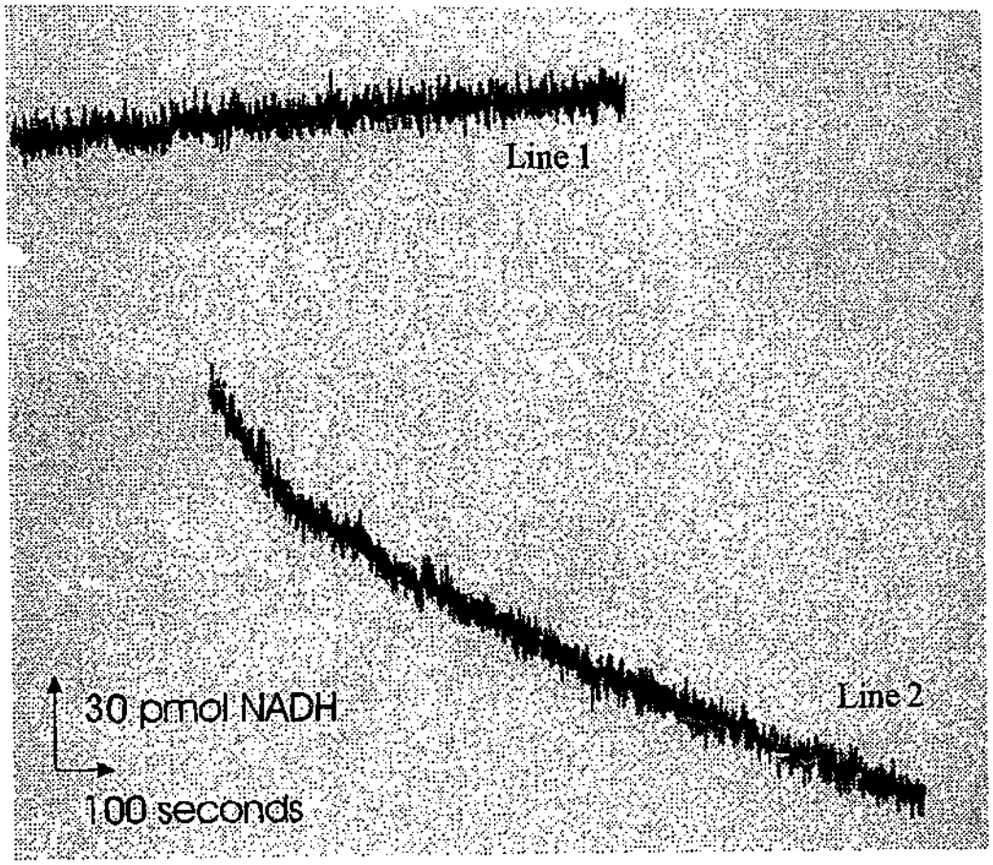


Fig. 1. An NADH specific oxidase exists in Namalwa cell plasma membranes Namalwa cell plasma membranes were purified as described in materials and methods. 100 ug of membrane was incubated with NADPH (line 1) of NADH (line 2). The change in absorbance at λ340 was monitored over 10 min.

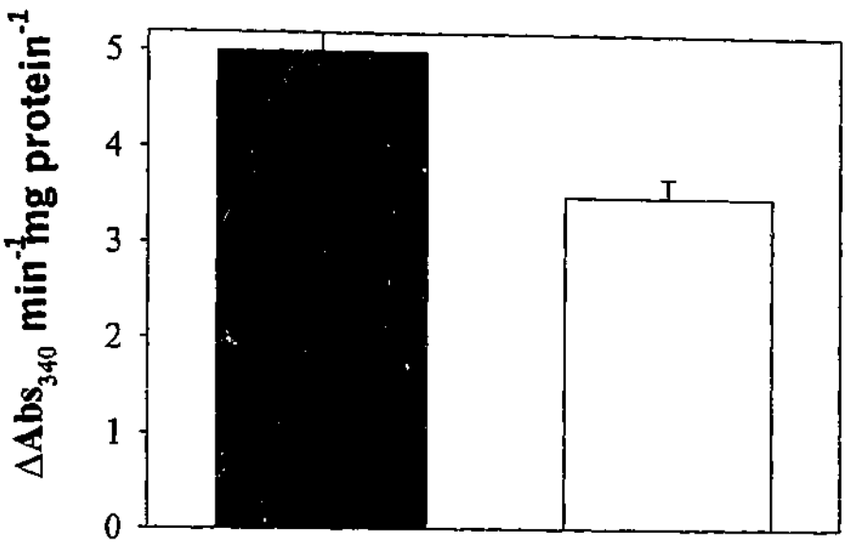


Fig. 5.2 Effect of *p*CMBS on the rate of NADH-oxidation

Namalwa plasma membranes were prepared as described (Section 2.4); 100 µg of plasma membrane protein were added to start the reaction (black). After 5 min, 20 µg of *p*CMBS was added and enzyme activity was monitored for a futher 5 min (white). The data represent the mean of three experiments and denote ± standard deviation.

5.2.2 Use of a DEAE-Sephacel for the attempted purification of the NADH-oxidase

To further purify the *p*CMBS insensitive NADH-oxidase activity, 10 ml of solubilised membrane proteins (5 mg/ml) were loaded onto a DEAE column (5 x 1 cm) which had been pre-equilibrated with buffer B. After washing the column with 10 column volumes of buffer B, elution was performed with a 10 ml gradient of 0-1 M NaCl in buffer B. 1 ml fractions were collected and measured for NADH-oxidase activity (Fig. 5.3).

Very little enzyme activity was recovered either in the flow through or in the eluted fractions from DEAE-Sephacel. Very low enzyme rates were observed in fractions 16-24, with the peak activity in fraction 21 (Fig. 5.3).

Upon application of these fractions to SDS page, a 67 kDa protein appeared to increase in intensity with enzyme activity and could therefore be a potential candidate for the NADH-oxidase (Fig. 5.4, arrow). However due to the irreproducibility in obtaining active enzyme preparations under these conditions, the identity of the 67 kDa band as an NADH-oxidase could not be confirmed. The reason behind the loss of NADH-oxidase activity during the column run is still unknown. Triton X-100 soluble enzyme activity was not inhibited by the addition of 1 M NaCl (data not shown). Therefore, loss of enzyme activity may be due to denaturation or loss of an essential co-factor during the column run. Due to the difficulties in obtaining active enriched plasma membrane NADH-oxidase preparations using anion exchange

chromatography, a different purification procedure was utilised in the following purification approaches.

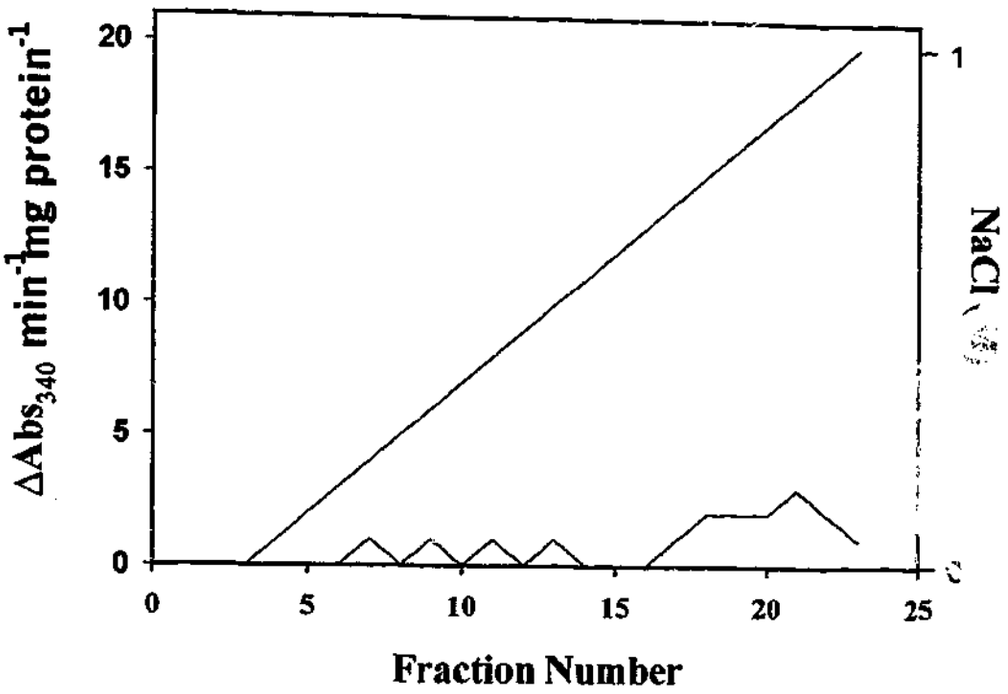


Fig. 5.3. Elution of NADH-oxidase activity from DEAE-sephacel column. Solubilzed Namalwa plasma membranes were loaded onto the ion-exchange column. Following washing of the column, elution took place using a 10 ml gradient of 0-1 M NaCl (green line) in buffer B. 1 ml fractions were collected and assayed for NADH-oxidase activity (red line) as described in Section 2.6.4.

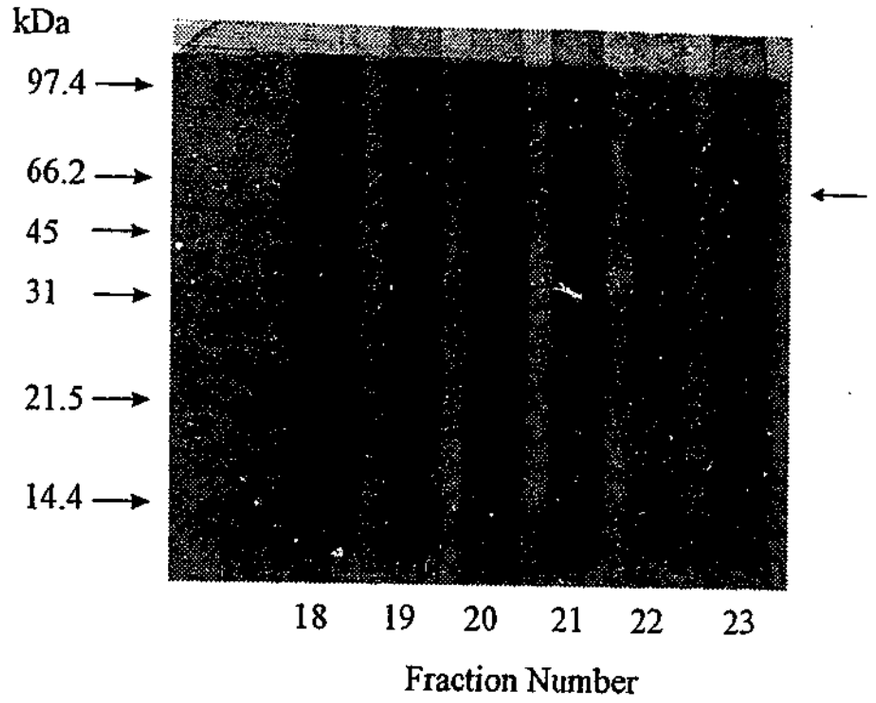


Fig. 5.4 SDS-PAGE of active fractions eluted from the DEAE-Sephacel column.

Samples eluting from the ion-exchange column (Fig. 5.3) were precipitated as described (Section 2.13)and subject to SDS-PAGE. Following silver staining (section 2.9.1) a 67 ka band was identified which increased and decreased in intensity in correlation to enzyme activity measured in the eluted fractions (right hand side arrow). The positions of the molecular mass markers are shown on the left hand side.

5.2.3 Inhibition of the NADH-oxidase activity with vanillylamine

Capsaicin has previously been reported to inhibit the activity of the NADH-oxidase[16,17,23,194]. Vanillylamine is an analogue of capsaicin, which contains a free functional group, allowing the compound to be coupled to an ester activated agarose bead (Fig. 5.5). Vanillylamine lacks the hydrophobic tail associated with capsaicin therefore, being different in structure, its inhibitory effect on the NADH-oxidase had to be ascertained. As demonstrated (Fig. 5.6), vanillylamine can inhibit the NADH-oxidase, albeit at a higher concentration than that required for capsaicin, suggesting that the compound has less affinity for the NADH-oxidase than capsaicin.

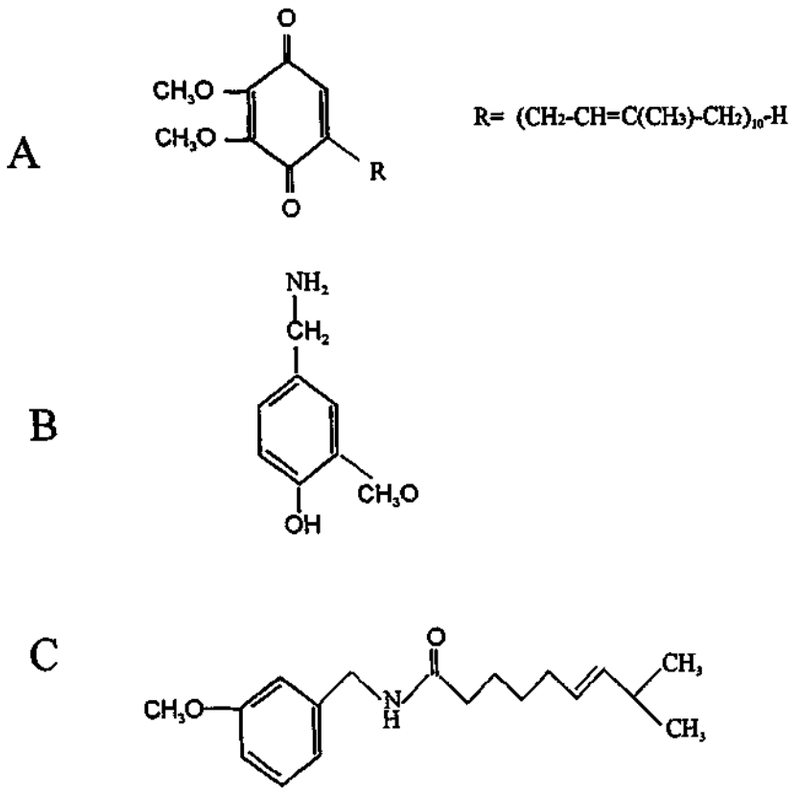


Fig. 9. Comparison of the family of vanilliods Both (A) co-enzyme Q and (B) Vanillylamine contain the aromatic head associated with (C) capsaicin. Capsaicin also contains a hydrophobic tail extension.

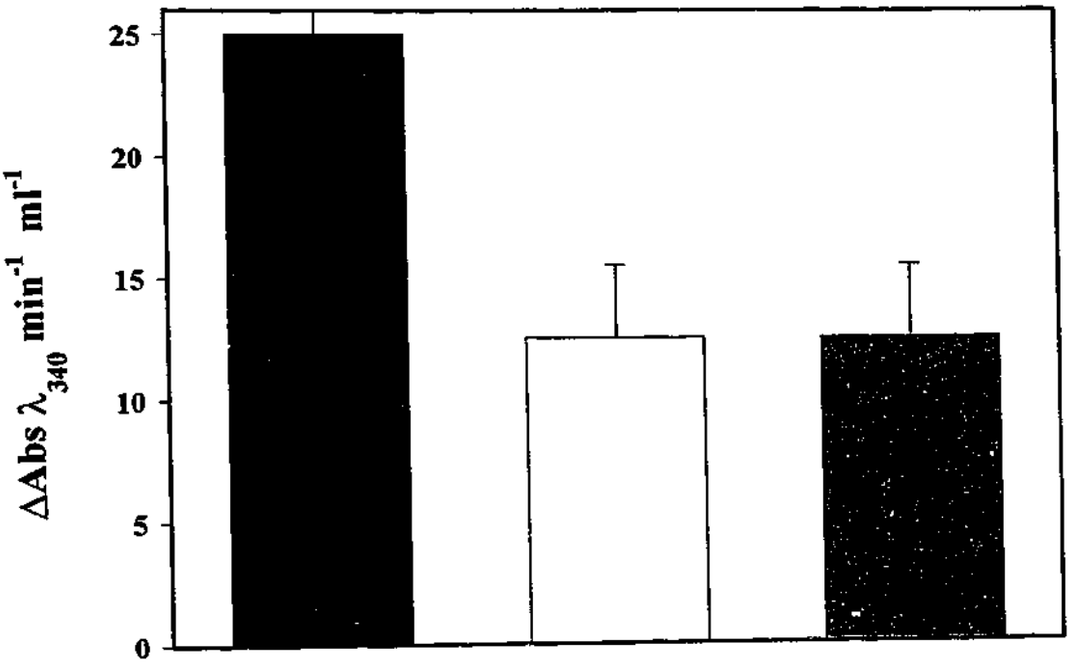


Fig. 5.6 Inhibition of NADH-oxidase acitivty by vanilloids.

150 µg plasma membrane proeins solubilised in 3% Triton X-100 were incubated with either 200 µM Capsaicin (white) 1mM Vanillylamine (red) or ethanol (black). The results show the average of three experiments done in duplicate.

5.2.4 Affinity purification of the NADH-oxidase

In an attempt to further purify the plasma membrane NADH-oxidase, vanillylamine was immobilised on an agarose bead, as described in section 2.18. Plasma membrane preparation solubilised in Triton X-100 was applied on the column. The unbound particulate was collected and the column was washed with a further 10 column volumes.

The unbound particulate contained 70% of the total NADH-oxidase activity originally applied to the affinity column (Fig. 5.7). This indicated that only 30% of the total NADH-oxidase activity can have bound to the column, or, if they did not, became denatured during the column run. The effect of chemical leakage causing the loss of activity could be ruled out since vanillylamine absorbs at 280 nm, and after washing the affinity column, no absorbance could be detected at that wavelength (data not shown). Upon elution of the affinity column with 10 mM vanillylamine and application of the eluant onto SDS-PAGE, a number of binding proteins were detected (Fig. 5.8).

Due to the number of vanillylamine binding proteins being eluted from the affinity column, it was not possible to judge which band may be the NADH-oxidase. In an attempt to reduce the number of proteins eluting from the affinity column, capsaicin was used as an eluant. Capsaicin binds and inhibits the NADH-oxidase more specifically when compared to vanillylamine (Fig. 5.6). Following application of the solubilised plasma

membrane proteins to the affinity column and subsequent washing of the column with buffer B, capsaicin (100 μM in buffer B) was used to elute the binding proteins. When compared to the vanillylamine eluate, the capsaicin eluate demonstrated only a few differences in the number and intensity of proteins being eluted from the column (Fig.5.9).

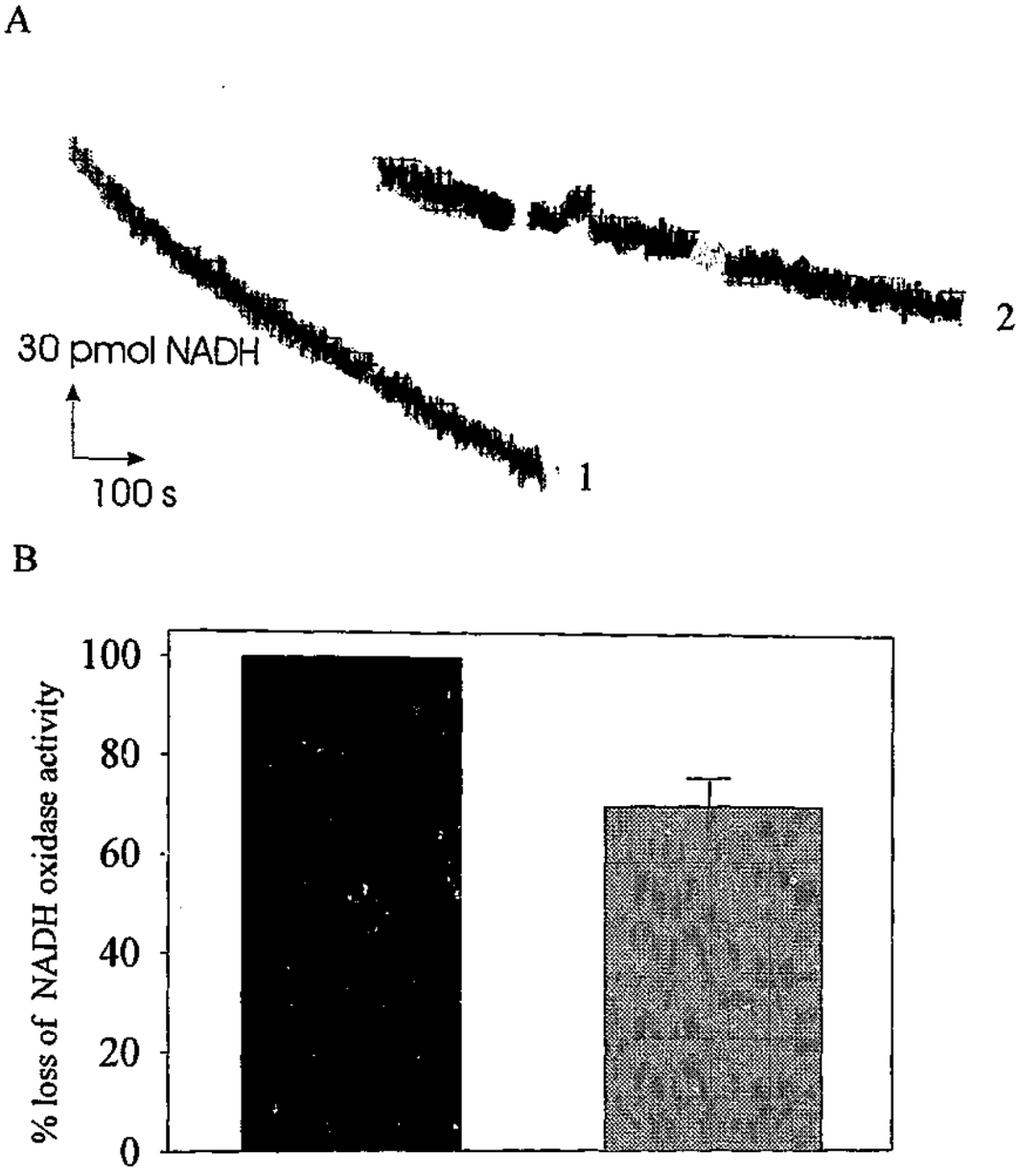


Fig. 5.7 Loss of activity through vanillylamine column.

(A) NADH-oxidase activity of 1 ml solubilized membrane proteins (line 1) was measured The proteins were then loaded onto the vanillylamine column and 1 ml fractions of the unbound particulate were collected and assayed for loss of NADH-oxidase activity. (Line 2).

(B) The total amount of NADH-oxidase activity in the solubilized membrane proteins were set to 100% (black bar). The loss in the unbound flow through (grey bar) were calculated. The data represent the average of three experiments.

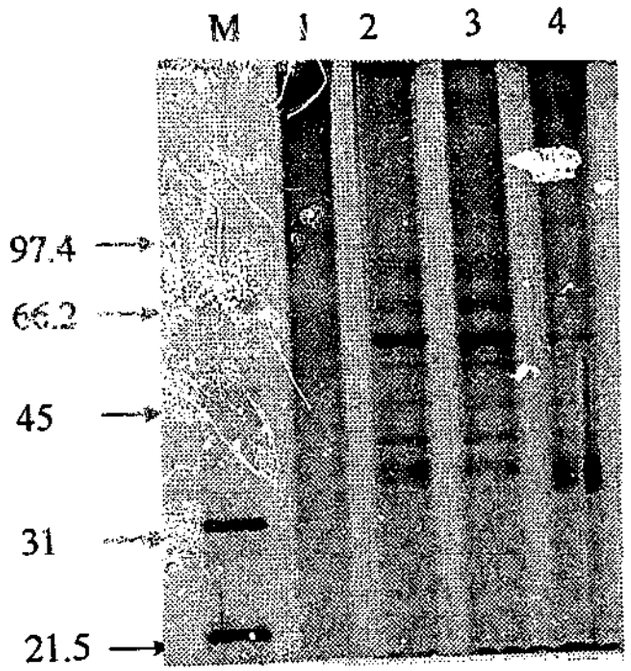


Fig. 5.8 Binding proteins the vanilyllamine column. 15 µg Namalwa plasma membranes (lane 1) were solubilised in 3% triton X-100 (lane 2). The solubilised extract was loaded onto the vanillylamine column. The unbound material (lane 3) was measured for a loss in NADH-oxidase activity. After washing the column, 1 mM vanillylamine was added to elute the binding proteins (lane 4). The size of the molecular weight markers (M) is shown on the left hand side.

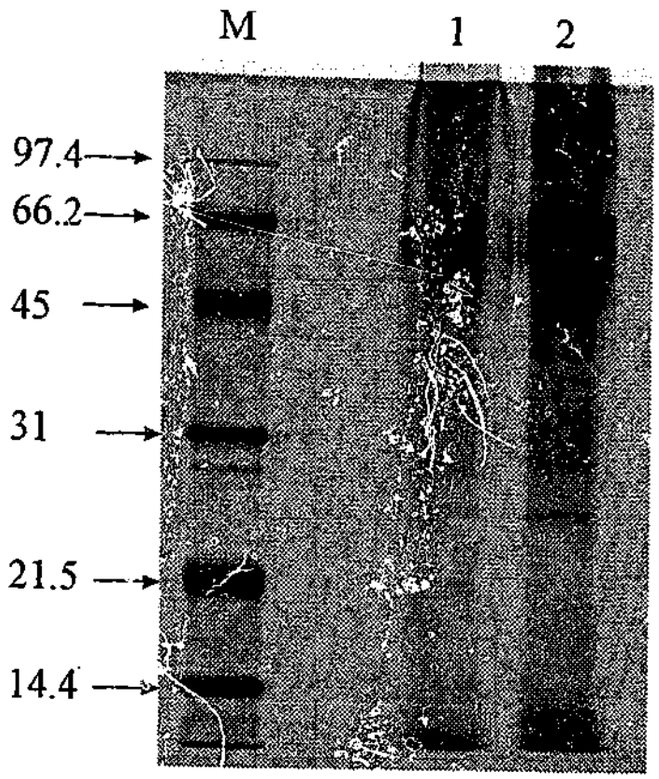
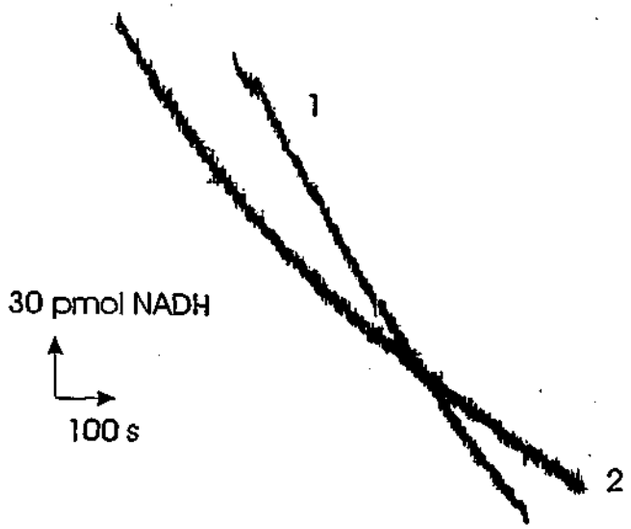


Fig. 5.9 Elution of the vanillylamine column with different vanilloids. Vanillylamine binding proteins were eluted with either Capsaicin (lane 1) or Vanillylamine (lane 2). The proteins were precipitated as described (Section 2.13), run on 15% SDS-PAGE and silver stained as described (Section 2.9.1). The position of the molecular weight standards (M) is shown on the left hand side.

No NADH-oxidase activity was detectable in either of the capsaicin or vanillylamine eluants, since both compounds are inhibitors of the NADH-oxidase. Therefore no further purification of the enzyme could be achieved following elution of the affinity column with either of these compounds. In order to elute an active enzyme preparation from the affinity column, CoQ was used as an eluant. CoQ has been previously shown to stimulate the NADH-oxidase activity[23]. CoQ has a structure similar to the vanilloids (Fig. 5.5), suggesting that all these compounds bind to a "quinone-like" binding domain found in the NADH-oxidase. Following the application of an active 100,000 x g supernatant from a plasma membrane fraction solubilised in Triton X-100 to the affinity column, the column was washed and eluted with 1 mM CoQ in buffer B. An active NADH-oxidase preparation was eluted from the vanillylamine affinity column (Fig. 5.10). Upon application of the active fractions to SDS-PAGE, several binding proteins were detected (Fig. 5.10). In an attempt to correlate a single protein band with the enzyme activity, the affinity column was again loaded with active solubilised plasma membrane proteins. A gradient of CoQ was then applied to the column. No significant level of enzyme activity was found in any of the eluting fractions(Fig. 5.11) suggesting that enzyme activity had been diluted so much that it was no longer detectable.

A



B

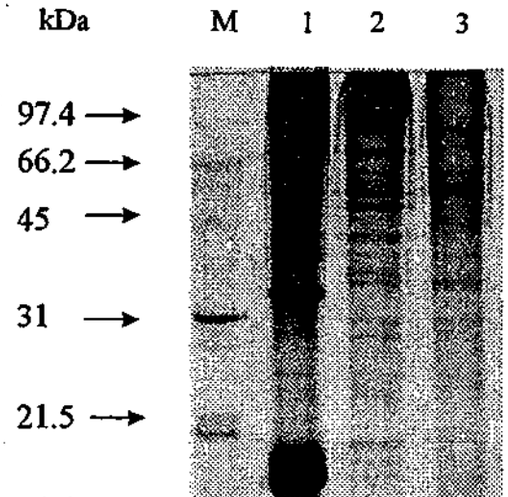


Fig. 5.10 NADH-oxidase activity following vanillylamine column. **Panel A:** Solubilized membrane proteins were loaded on the vanillylamine column and, following washing, were eluted with 1 mM CoQ.

3 ml fractions were colleted and measured for NADH-oxidase activity (line 1). Auto-oxidation of NADH was measured as a background control (line 2). **Panel B:** 15 μg plasma membranes (lane 1), 10 μg solubilzed membrane proteins (lane 2) and 5 μg CoQ eluate (lane 3) were precipitated as described (section 2.13) and run on a 12% SDS-PAGE. The positions of the molecular mass standards (M) is shown on the left hand side.

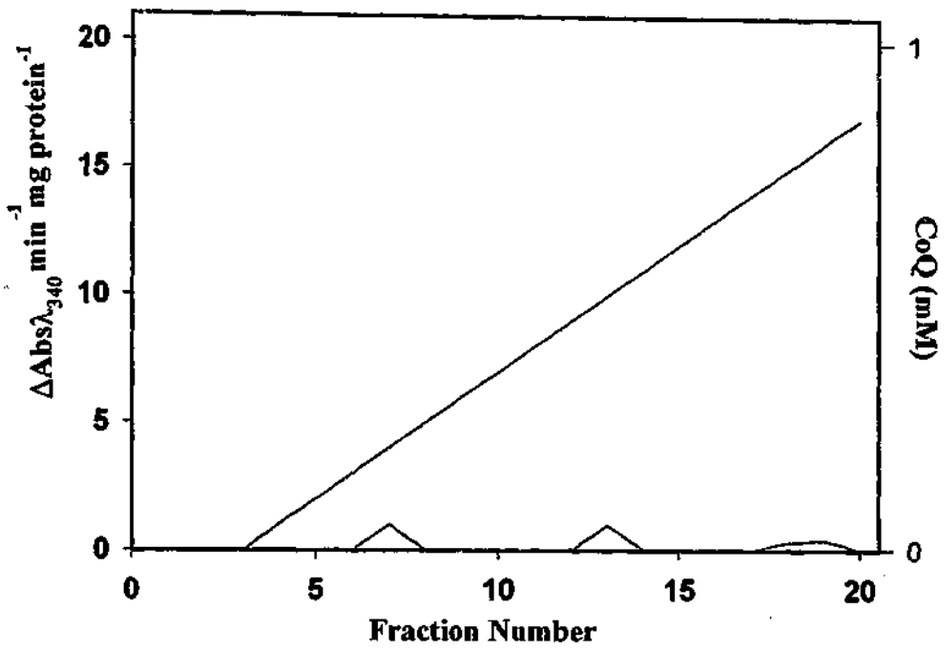


Fig. 5.11 Elution of NADH-oxidase activity from DEAE-Sephacel column.

Solubilzed Namalwa plasma membranes were loaded onto the DEAE column. Following washing of the column, elution took place using a 10 ml gradient 0-1 M NaCl (green line) in buffer B. 1 ml fractions were collected and measured for enzyme activity (red line).

5.2.1 Characterisation of dihydrocapsiate as an inhibitor of the NADH-oxidase

Capsaicin and vanillylamine belong to the family of vanilloids, whose biological activities were discovered by research into pain managment[192]. They bind to VR1 which is found in peripheral pain-sensing neurons[192]. These compounds also inhibit the plasma membrane NADH-oxidase, and by doing so induce apoptosis in human Daudi and Namalwa cells[17]. Since the plasma membrane NADH-oxidase may be constitutively active, inhibition of the enzyme may be of potential therapeutic value especially in tumour treatment. However, none of the described vanilloid inhibitors of the NADH-oxidase, would be suitable for use *in vivo* since they also target the VR1 receptor. However, non-pungent vanilloid derivatives exist in sweet chilli and one group of those are the capsiates. Capsiates differ from the 'classical' vanilloids in the way their vanillyl and acyl moieties are joined together; *via* an amide bond in hot pepper (capsaicin-type compounds) or *via* an ester bond in sweet pepper (capsiate-type compounds) (Fig. 5.5). Capsiates have been tested for their capability of binding to VR1. Our co-workers in Prof. Muñoz laboratory (Córdoba. Spain) have been able to show that these compounds do to not activate VR1. However, like capsaicin, they can induce apoptosis in cancer cells. To test whether the induction of apoptosis, like capsaicin, would be through inhibition of the plasma membrane NADH-oxidase, I tested one member of the family of capsiates, dihydrocapsiate (Fig 5.5). The inhibitory

capacity of dihydrocapsiate was tested on the NADH-oxidase activity of enriched plasma membrane preparations from Namalwa cells (Fig. 5.12).

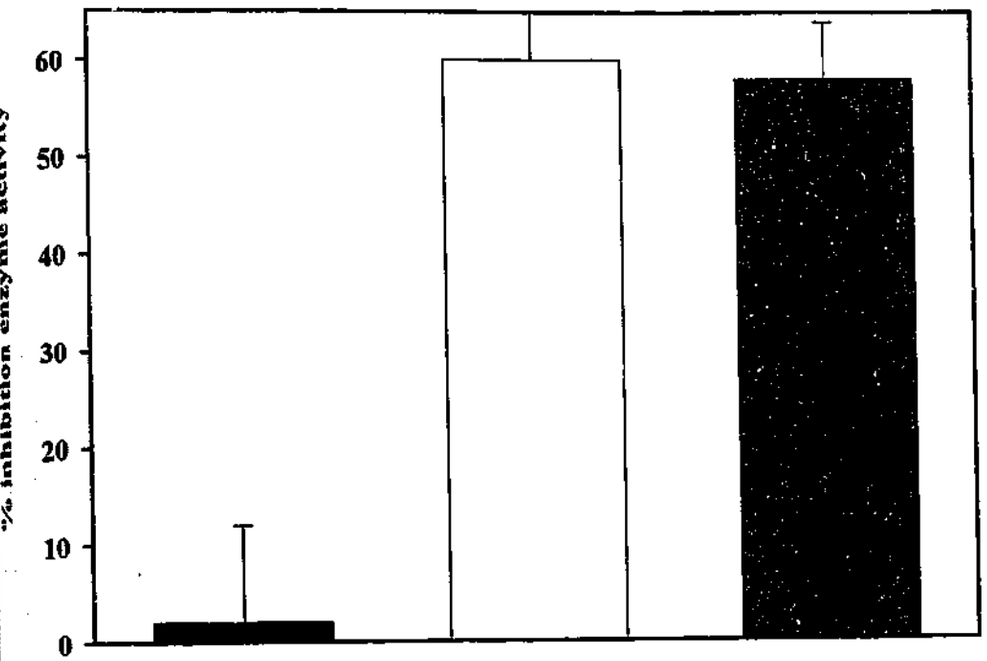


Fig. 4.12 Inhibitors of the plasma membrane NADH-oxidase

150 μg of plasma membrane proteins were solubilised in 3% Triton X-100 were incubated with either 200 μM Capsaicin (white) 1 mM dihydrocapsiate (red) or ethanol (black). The results show the average of three experiments done in duplicate.

The ability of dihydrocapsiate to inhibit the NADH-oxidase activity of enriched plasma membrane fractions, suggests this compound may target the NADH-oxidase. It also suggests this may be the mechanism of induction of apoptosis in Jurkat T cells (Macho *et al.,* manuscript in preparation). Furthermore, capsiates may be of potential benefit for *in vivo* treatment of tumours. In a two-stage mouse skin carcinogenesis test, mice treated with capsiates demonstrated a 50% decrease in tumor incidence, compared to control mice, treated with the vehicle only.

5.3 Discussion

5.3.1 Partial purification of the NADH-oxidase

Two functionally related enzyme activities have been documented in the plasma membrane of cells[13], namely the NADH:ferricyanide-reductase and the NADH-oxidase, which, similar to mitochondrial oxidases, may function as a transplasma membrane redox chain. The molecular characterisation of the NADH-oxidase is still in its preliminary stages. The laboratory of Morré, has purified, cloned and characterised a soluble tNOX[199,206]. This enzyme functionally resembles characteristics of the plasma membrane NADH-oxidase and is also sensitive to capsaicin[16]. However tNOX is not the final electron acceptor of the PMOR system, as tNOX is only present on the outer surface of the plasma membrane. The plasma membrane NADH-oxidase of the PMOR must, however, have access to cytosolic NADH[23]. This second NADH-oxidase enzyme activity can be distinguished from that of tNOX based on the susceptibility to *p*CMBS[194]. Upon addition of 20 µM *p*CMBS to 150 µg of Namalwa plasma membranes, only 20% of the enzyme activity is inhibited, which may be attributed to tNOX activity. The remaining enzyme activity may be due to a transmembraneous NADH-oxidase.

For further purification of the *p*CMBS insensitive NADH-oxidase, anion exchange chromatography was used as an initial starting column. The enzyme appeared to bind to the ion-exchange column, since no NADH-

oxidation was detectable in the unretarded fraction. After elution of the column with 0-1 M NaCl only 10% of the total NADH-oxidase activity applied to the column was recovered in the eluate. Those fractions with enzyme activity were subjected to SDS-PAGE. A 67 kDa band was detected to undergo a slight intensity change, which correlated with enzyme activity. However, the lack of reproducibility in obtaining an active enzyme preparation under these conditions did not permit following up on the identity of the 67 kDa protein. The reason behind the loss of enzymatic function has not been established. Enzyme activity was not affected with the addition of 1 M NaCl to solubilised plasma membrane extracts. Perhaps the loss of an essential co-factor such as CoQ or NADH, or denaturation of the protein through interaction with the column matrices may explain the loss of activity. In the future, the addition of essential co-factors, such as CoQ or NADH, in the running buffer may help to obtain an active enzyme preparation. Alternatively, the loss of NADH-oxidase activity may be due to the 'uncoupling' of essential subunits, making up the NADH-oxidase[23]. However, pooling the different eluting and flow through fractions from the anion exchange column, did not result in restoration of enzyme activity (data not shown).

The purification of the NADH-oxidase from solubilised plasma membranes was further attempted using affinity chromatography. Vanilloids, including capsaicin, have been shown to inhibit the NADH-oxidase. This inhibition presumably occurs through the same quinone binding site in which

CoQ stimulates the enzymes activity. Capsaicin itself cannot be linked to an ester activated agarose bead due to a lack of a functional group. Therefore a related compound, vanillylamine, was used. Vanillylamine looks similar to capsaicin, however it lacks the hydrophobic tail. To ensure that vanillylamine would act in the same manner as its related compound capsaicin, inhibition studies were carried out on the NADH-oxidase activity. Vanillylamine was shown to inhibit enzyme activity at a slightly higher concentration than capsaicin suggesting it has lower affinity for the protein. However, since vanillylamine clearly inhibited enzyme activity, the compound was immobilised to ester activated agarose beads. Upon application of the soluble plasma membrane proteins to the affinity column, 70% of the initial NADH-oxidase activity was detected in the unretarded material. This suggested that 30% of the enzyme activity had bound to the column. Alternatively 30% of the protein may have become denatured in the column run. Competitive elution of the column with vanillylamine, revealed a number of binding proteins, any of which could be the NADH-oxidase. One way of demonstrating which silver stained band could potentially be the NADH-oxidase is to analyse vanillylamine and capsaicin eluates for bands that have the same apparent molecualr weights. Capsaicin has a slightly higher affinity for the NADH-oxidase, therefore, could hope to obtain a higher enrichment of the oxidase in the corresponding eluant. Therefore I tested to see whether an up-regulation in any protein could be detected upon elution with the same concentration of capsaicin and vanillylamine. However upon comparison of

the eluted protein profiles no band could be identified as a putative NADH-oxidase.

The major problem with the affinity column, is that no enzyme activity was detectable in the eluates therefore no further purification of the enzyme could be achieved. Previously, Wilkinson *et al.* has reported binding tNOX to a vanillylamine affinity column[70]. Upon eluting the enzyme with capsaicin and addition of H_2O_2, the enzyme activity was restored. However, upon addition of H_2O_2 to either of the vanillylamine or capsaicin eluates, no restoration of the enzyme activity was observed (data not shown). In an attempt to obtain an active enzyme preparation from the affinity column, the elution buffer was changed and CoQ was used in place of the inhibitory vanilloids. It has previously been shown that CoQ can up-regulate the NADH-oxidase[23]. Therefore CoQ was expected to elute the active enzyme. Indeed, upon elution with CoQ, active enzyme was obtained. However, again the number of protein bands present in the silver stained SDS-PAGE did not allow identificaiton of the NADH-oxidase. Nevertheless, the ability of CoQ to elute active enzyme demonstrates that NADH-oxidase(s) was binds to the affinity column. In an attempt to correlate enzyme activity with a protein band, a gradient of CoQ was used to elute the column. However, no activity was detected in any of the corresponding fractions, most likely due to dilution of the enzyme during the column run.

The difficulties in purifying active NADH-oxidase from the plasma membrane, and the time factors involved did not allow for the complete

purification of this protein. Nevertheless, some initial characterisation of the protein has been accomplished and in future, should cut down the time required for the establishment of a purification protocol for this important enzyme.

5.3.2 Characterisation of dihydrocapsiate as a novel inhibitor of the plasma membrane NADH-oxidase.

The biological activity of hot tasting chilli peppers has been attributed to the pungent vanilloid capsaicin. The activity of capsaicin is due the activation of VR1, a pain receptor found in peripheral pain-sensing neurons[192]. Capsaicin, along with other vanilloids, has previously been described to inhibit the plasma membrane NADH-oxidase[17]. Since both NADH and oxygen are present under normoxic conditions, it would be logical to conclude that this enzyme is constitutively active. Inhibition of this enzyme results in apoptosis in human and mouse myeloid and human B cells, suggesting potential therapeutic value for vanilloids. However, since capsaicin and other pungent vanilloids bind to the VR1 receptor, they could not be used for therapeutic treatment *in vivo*. Non-pungent vanilloid derivatives exist in sweet chilli peppers. Collaborative work in Prof. Muñoz' laboratory, has demonstrated that capsiates do not bind to, nor inhibit the VR1 receptor. Furthermore, non-pungent capsiates induce apoptosis in Jurkat T cells (Macho *et al.* Manuscript in preparation). One naturally occurring non-pungent capsiate is dihydrocapsiate. Dihydrocapsiate was shown to inhibit the plasma membrane NADH-oxidase from human Namalwa cell (Fig. 5.12).

This suggests that dihydrocapsiate induces apoptosis, like capsaicin, through inhibition of the plasma membrane NADH-oxidase. Non-pungent capsiates may therefore have potential benefit *in vivo.* Our co-workers in Prof. Muñoz laboratory have shown that capsiates have anti-tumorigenic properties. Using DC-1 mice, a single topical application to the dorsal region was made of 200 μM capsiate, or the vehicle control (acetone). One hour later, the carcinogen phorbol 12-myristate 13-acetate was applied. In mice treated with capsiate, a 50% reduction in tumor development compared to control mice was observed. This finding further confirms the importance of the plasma membrane NADH-oxidase and the need to characterise this enzyme at the molecular level.

CHAPTER 6 – The importance and future of the NADH:oxidoreductase

6.1 Discussion

A plasma membrane redox system has been shown to exist in all cell types from protozoa to man. The enzyme complex consists of at least two proteins including an NADH:ferricyanide-reductase and an NADH-oxidase[13]. Electron transfer between these two proteins may occur through the common intermediate, CoQ[23]. The importance of the PMOR system for cell survival, has been documented. ρ^{o} cells (which lack a functional mitochondrial respiratory chain) were shown to up-regulate the ferricyanide-reductase activity up to 4-fold[18]. These cells remain viable, as long as pyruvate or ferricyanide is added to the cell medium[18]. Both of these compounds are believed to maintain cell viability, through the restoration of the $NADH/NAD^{+}$ ratio[207]. Pyruvate may accomplish this through the pyruvate-lactate couple. Ferricyanide on the other hand, has been suggested to maintain the $NADH/NAD^{+}$ ratio through the PMOR system, in particular the NADH:ferricyanide-reductase[207].

The establishment of an *in vitro* assay system allowed the purification and identification of a transmembraneous plasma membrane NADH:ferricyanide-reductase in this thesis. By MALDI-TOF analysis this protein was identified as VDAC1. VDAC1 is the major protein of the outer mitochondrial membrane[181] but has also been reported to be present in the plasma membrane[162-165]. The plasma membrane localisation of VDAC1 was

confirmed in the work presented in this thesis by FACS analysis. Four important experimental observations demonstrate that VDAC1 can function as an NADH:ferricyanide-reductase. Firstly, VDAC1 was identified as the protein co-eluting with enzyme activity from the Blue Sepharose column. Secondly, NADH:ferricyanide-reductase activity could be immunoprecipitated with anti-VDAC(1) antibodies. Thirdly, rat liver mitochondrial VDAC1 purified to apparent homogeneity displayed NADH:ferricyanide-reductase activity. Finally, overexpression of recombinant VDAC1 led to a 40-fold increase in whole cell ferricyanide-reduction in COS7 cells.

Consistent with the role of VDAC1 as an NADH:dehydrogenase, its amino acid sequence contains a putative NAD^+ binding domain. This is not the first time NADH has been shown to be interacting with VDAC1. NADH has been previously reported to double the voltage dependence of VDAC1 at micromolar concentrations[120]. NAD^+ on the other hand has no effect[120]. The regulation of VDAC1 by NADH, suggests that the protein may be acting as a redox sensor, opening and closing depending on the concentration of NADH present[179]. The amino acid sequence of VDAC1 on residues 145-152 VLGXXGXXXG is very similar to that of the NADH binding domain in alcohol dehydrogenase (VLGXGXXXG) with the exception that VDAC1 has an extra amino acid substituted between the first and second glycine. Another 'inverted' NAD^+ binding motif has been suggested on residues 270-278[184] of human VDAC1. The sequence GXXXGXG is similar to yeast alcohol

dehydrogenase, only inverted. Ultimately, mutation of either of the two NAD^+ binding domains would determine which is responsible for electron transport. In the absence of such studies, the alignment of each of these motifs to predicted model structures may give some insight into the correct NAD^+ binding site. Two predicted model structures exist for human VDAC1, which consist of either a 16[115] or 13[116] membrane spanning strands. Aligning residues 145-152 to the 13 transmembrane domain model places the NAD^+ binding domain near the cytosolic side of the plasma membrane, which is consistent with this function. In contrast, the inverted NAD^+ binding site (residues 272-278) is found closer to the plasma membrane/extracellular matrix border in either the 13 or 16 transmembrane domain models. Therefore this sequence reported by Zizi *et al* [120], as the NAD^+ motif, appears unlikely. The amino acid sequence of human VDAC1 contains two cysteine residues. Upon addition of either *p*CMBS or NEM to purified VDAC1, the redox activity is inhibited. This suggests that cysteine residues are responsible for electron transport. Based on these observations, a model for the transport of electrons through VDAC1 has been proposed (cf Fig. 4.13, p 121). In this model, NADH binds to the cytosolic binding motif of VDAC1 acting as an electron donor. A hydrogen atom is then passed through two cysteine residues, onto ferricyanide.

VDAC1 represents an exciting platform from which future (unknown) NADH:ferricyanide-reductase(s) may be identified. Once the NAD^+ binding motif has been established via mutation analysis, it would be or particular

interest to determine which residues are necessary for binding of ferricyanide. If found, then a screen on the protein database bank can be made, to look for potential NADH:ferricyanide-reductases. This may shed light into new roles of previously characterised proteins.

Increasing importance is being placed on role or oxidoreductases and redox homeostasis. In response to Fas/Apo-1/CD95, cross-linking or stimulation with cell-permeant ceramide, Jurkat T cells demonstrate a major, early decrease in blue autofluorescence[208], before undergoing apoptosis. Blue autofluorescence within cells has been demonstrated to be due to the presence of oxidised nucleotides. This suggests that oxidation of NADH and/or NADPH is an early step in the onset of apoptosis[208]. Consistent with this hypothesis, superoxide production, which occurs *in vivo* under ischemic[209] and normal respiratory conditions[210] can result in cell death. Production of superoxide *in vivo,* can be mimicked *in vitro,* with the addition of *t*-butyl hydroperoxide. Upon addition of *t*-butyl hydroperoxide to hepatocytes, oxidation of GSH and pyridine nucleotides[211] precede the opening of the PTP and cell death. Therefore, the authors of this report have suggested that PTP activation may be mediated by oxidation of GSH, NADPH and NADH[211]. Interestingly, the PTP consists of cyclophilin D, the adenosine nucleotide transporter and VDAC. The ability of the pore to open, upon a change of redox status within the cell, suggests a complementary relationship between VDAC's function as a pore, and a redox enzyme.

Reports have suggested a relationship between the PMOR, the redox status within cells and apoptosis[15,81]. Upon addition of NADH-oxidase inhibitors such as capsaicin to human B and mouse-myeloid cell lines, apoptosis occurs[17]. Since the NADH-oxidase may be constitutively active, inhibition of the enzyme, has been suggested to alter the $NADH:NAD^+$ ratio, which induces apoptosis[207]. Interestingly the addition of ferricyanide can prevent capsaicin-induced apoptosis in Namalwa and Daudi cells. Ferricyanide, acting through VDAC1 may enable the restoration of the $NADH:NAD^+$ ratio, thereby maintaining cell viability.

The ability of VDAC1 to function as a redox enzyme, helps to explain some reported observations which thus far remained unexplainable. Most of the research done to date on VDAC1, has focused on the anionic conductivity behaviour of the protein. However, recently it has been suggested that VDAC's role as an ion-regulator, is not necessary for cell survival. Yeast contains at least 4 VDAC isoforms (YVDACs), which include YVDAC1, YVDAC2, YVDAC3 and YVDAC4[119]. YVDAC1 knockout cells are unable to grow on non-fermentable carbon sources at 37°C suggesting that the oxidative phosphorylation pathway is compromised. Expression of YVDAC2 in the same cells (to the level YVDAC1 is normally expressed at), restores the normal growth of the yeast. Therefore, YVDAC2 can compensate for the loss of YVDAC1. Interestingly, YVDAC2, unlike YVDAC1, is unable to form pores in planar bilayers and transport anions across membranes. Therefore, the ion regulatory behaviour of YVDAC2 does not appear to be the basis of

the restoration of YVDAC1 yeast knockout cells[119]. Confirmation that VDAC pore formation is not necessary to restore yeast YVDAC1 knockout cells was provided when the *Drosophila melanogaster* VDAC1 (DVDAC1), was overexpressed in yeast VDAC1 knockout cells. Although capable of forming pores in planar bilayers, the DVDAC1 protein could not restore growth of the yeast knockout cells.

Upon inspection of the YVDAC1, YVDAC2 and DVDAC1 amino acid sequences, different characteristics can be identified. Two cysteine residues (necessary for electron transport) are present in the YVDAC1 and YVDAC2 (and notably human VDAC1) but not in the DVDAC1 protein (Table 6.1). These data suggest that DVDAC1, which is capable of forming pores, but unable to restore the growth of YVDAC1 knockout cells, cannot function as a ferricyanide-reductase. However YVDAC2 contains two cysteine residues and the non-inverted NAD^+ binding motif, which would be sufficient for transplasma membrane electron transport. Therefore, the restoration of YVDAC1 yeast knockout cells to 37°C on non-fermentable carbon sources, may be due to the redox activity of the VDAC proteins. Interestingly, YVDAC2 does not contain the inverted NAD+ binding domain reported by Zizi *et al.*[120]. An important glycine residue at position 253 (position 252 on human VDAC1) has been substituted with isoleucine (Table 6.1). If the restoration of YVDAC1 yeast knockout cells was due to the redox activity of YVDAC2, this would suggest that the inverted sequence is not the NADH biding site used by VDAC. The necessity for functional redox activity

for the restoration of YVDAC1 deficient cells was further demonstrated using potato VDAC isoforms. POM34 and POM36 are two VDAC isoforms isolated from the outer mitochondrial membrane of potato[212]. Expression of either of these genes in yeast YVDAC1 knockout cells[213], cannot complement these cells, therefore they fail to grow on non-fermentable carbon sources[212]. Failure to complement the YVDAC1 knockout cells was not due to a difference in conductance properties, since POM34 displayed single channel conductance, similar to that of YVDAC1. Therefore, the authors suggested that the phylogenic distance between potato and yeast was too great for POM34 and POM36 to complement the yeast YVDAC1 knockout cells[212]. Upon inspection of the amino acid sequence of these potato isoforms, the presence of only one cysteine residue in POM36 is apparent, suggesting this protein would not have transplasma membrane redox activity. This may explain why POM36 is unable to complement the yeast YVDAC1 mutants. Nevertheless, POM34 possess two cysteine residues, which suggests that POM34 has maintained the necessary functional groups for electron transport. However comparison of the NADH binding domain of HVDAC1, YVDAC1, YVDAC2 (GXXGXXXG) and POM36 (GXXGXXXXXXG) identifies 3 extra amino acids present in POM36 before the final glycine residue. These extra amino acids may prevent the binding of NADH, leading to a consequent loss of redox function in that protein. Such a loss in redox function may explain why POM36 is unable to restore the respiratory defect in yeast

YVDAC1 knockout cells. A summary of the relevant amino acid data and ability to form pores and regulate growth is given in Table 6.1.

Organism (Reference)	Non-inverted NAD+ binding domain (amino acid positions)	Inverted NAD+ binding domain (amino acid positions)	Cysteine residues	Ability to form pores	Growth restoring capacity
Human VDAC1 (214)	GXXGXXXG (168-172)	GXXXGXG (272-278)	127,232	Yes	Yes
YVDAC1 (103)	GXXGXXXG (254-260)	GXXXGXG (254-260)	130,110	Yes	Yes
YVDAC2 (119)	GXXGXXXG (242-248)	XXXXGXG (252-258)	161,187	No	Yes
D. melanogaster (215)	GXXGXXXG (244-251)	GXXGXG (270-277)	-	Yes	No
POM36 (212)	GXXGXXXXXXG (233-243)	XXXXXXXXG (251-259)	158	Yes	No
POM34 (212)	GXXGXXXXXXG (226-233)	XXXXGXXXG (251-259)	118,158	Yes	No

Table 6.1 A comparison of VDAC amino acid sequence from different organisms and the ability of those proteins to restore yeast YVDAC1 knockout cells to the wild type situation.

V

YVDAC2 could potentially use the two cysteine residues and the non-inverted NAD^+ binding domain, to function as an NADH:ferricyanide-reductase. However this would not be true if YVDAC2 uses the inverted NAD^+ binding domain, since that sequence lacks a vital glycine residue at position 252. Although unable to form pores, YVDAC2 can restore yeast YVDAC1 knockout cells back to the wild type situation.

In addition to the characteristics of VDAC1 being a reductase, observations made on the PMOR are consistent with VDAC1 being part of that system. Upon addition of 0.5 mM potassium ferricyanide to bean root cells, a depolarisation (20-30 mV) of the plasma membrane occurs[216]. Such a depolarisation may be explained by the following process: VDAC1 passes a hydrogen ion from cytosolic NADH to ferricyanide. As a result the cytosol is depleted of hydrogen ions, leading to a consequent depolarisation of the plasma membrane. This hypothesis is supported by the observation that the anionic regulation of VDAC1 directly affects the membrane potential of mitochondria[217,218]. The passage of hydrogen ions from NADH to ferricyanide via VDAC1, may also explain why the cytosol of erythrocytes, adipocytes and HeLa cells demonstrate an increase in pH following addition of ferricyanide to cells[12].

It remains to be elucidated how, upon addition of ferricyanide, a depolarisation of the plasma membrane followed by a cytosolic drop in pH maintains cell survival. This may be a complex process, possibly involving other subunits of the PMOR, including the NADH-oxidase.

Time restrictions did not make it possible to purify a *p*CMBS insensitive NADH-oxidase from Namalwa cell plasma membranes to homogeneity. The low level of enzyme activity from these preparations, suggests a number of alternative routes should be considered for further purification. Innovative molecular biology techniques may be used to screen

for another oxidase. Such a screening process can be accomplished with the use of degenerate primers. Nevertheless if the primers are based upon the sequence of tNOX such a strategy may be of little benefit, since tNOX is an ectoenzyme. Therefore, traditional protein purification procedures for the further purification of the transplasma membrane NADH-oxidase may need to be employed. The main problem in purifying the NADH-oxidase from any source, is the low level of enzyme activity. Therefore, large amounts of plasma membrane starting material need to be used and further purification using affinity columns, such as vanillylamine-agarose may be employed. However the expense both in money and time to attempt this procedure, would be a consideration. Overcoming this barrier, work from the laboratory of Berridge, has attempted to design a novel way to measure NADH-oxidase activity. A cell impermeable, sulphonated tetrazolium salt WST-1 (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, mono monosodium salt) is reduced by superoxide to form a stable water soluble formazan with high molar absorptivity[204,219]. WST-1 has been used to measure superoxide production by the NADPH-oxidase[219] and the plasma membrane NADH-oxidase of proliferating cells[205]. However, using the commercial form of the dye for the measurement in biochemical assays with NADH has its disadvantages, because with the presence of the intermediate electron acceptor, 1-methoxy phenazine methosulfate, WST-1 is rapidly reduced by NADH[220]. Nevertheless, this rapid reduction of WST-1 by NADH may be useful for the measurement of multiple fractions during a purification

process. If, during a column run, more than 50+ fractions need to be assayed, using the conventional spectrophotometer this can be only achieved one fraction at a time. However, if all the fractions are placed in a microplate (96 well plate), NADH can be added and left for 30 min. Following this, the reduction of WST-1 can be measured. If a change in the NADH concentration is achieved this may be demonstrated in the absorbance reading of WST-1.

Now the molecular identification of VDAC1 as an NADH:ferricyanide-reductase has been achieved, further characterisation of VDAC1 as part of the overall PMOR complex needs to take place. Molecular identification of the binding sites, including the NADH and ferricyanide binding motif would be beneficial. This can be achieved via mutation analysis of VDAC1 followed by enzymatic analysis of the mutated proteins. The finding of the necessary amino acids would have several beneficial effects. Firstly, the amino acid sequence(s) can be used to screen the protein database bank, to gain insight into which other proteins may possess NADH:ferricyanide-reductase activity. Secondly, liver or blood cells, for example, such as patients with chronic fatigue syndrome, can be investigated to see if mutation of VDAC1 has lead to the phenotype. A mutation of VDAC1 may lead to an increase in the NADH:NAD^+ ratio, resulting in down regulation of ATP production.

The involvement of VDAC1 as a redox enzyme in apoptosis can now be studied. Bcl-2 family members have been shown to interact with VDAC1[133,221]. How this interaction affects the redox capability of VDAC1

can be explored. Bcl-2-agarose beads are available commercially. The addition of these beads to pure VDAC1 should establish whether Bcl-2 exerts a regulatory effect on VDAC1.

Bcl-2 and VDAC1 have been shown to co-imunoprecipitate, suggesting that VDAC1 interacts with other proteins. If VDAC1 were part of the PMOR complex, then the isolation of that complex may be attempted. The NADH-oxidase suggested to form part of the PMOR, may be purified by immunoprecipitating VDAC1. To establish whether the oxidase co-immunoprecipitated with VDAC1, NADH-oxidase activity could be measured in the immunopellet. If enzyme activity was present, it may be possible to identify the NADH-oxidase by running a 2D SDS-PAGE of the immunopellet and identifying, by MALDI-TOF analysis, any binding proteins to VDAC1.Alternatively, if no enzyme activity could be detected, native gel electrophoresis of plasma membrane enriched fractions could be run. Following transfer to a nitrocellulose membrane, Western blot analysis using anti-VDAC1 antibodies would detect VDAC1 bound to other proteins. Excision of the relevant bands, followed by 2D SDS-PAGE and MALDI TOF analysis, would identify VDAC1 binding proteins and perhaps the NADH-oxidase.

The ability of ferricyanide to maintain cell growth, under otherwise fatal circumstances represents an important biological role for PMOR. The establishment of VDAC1 as part of this process may be possible. Purification of VDAC1 binding proteins can also be achieved by linking homogeneous

VDAC1 to a column. Binding proteins to VDAC1 may be found by applying subcellular extracts over the column. The column can then be washed and eluted. Confirmation of any proteins binding to VDAC1 may be achieved via immunoprecipitation and/or Western blot analysis. Having isolated proteins that bind to VDAC1, a change in either phosphorylation or molecular mass of those proteins upon addition of ferricyanide to cells, may indicate plasma membrane VDAC1's involvement in cell growth.

Finally, to establish what role VDAC1 plays on the plasma membrane, inhibitors of its redox activity need to be found. Already, from this thesis, it has been established that neither DIDs, Königs polyanion n or the mouse monoclonal anti-VDAC1 antibody used, inhibited the redox activity of VDAC1. However all of these compounds have been shown to inhibit the conductivity of this protein. The PMOR inhibitors *p*CMBS and NEM are too non-specific to be used for clinical applications, as they appear to inhibit many enzymes[222,223]. The finding of a specific inhibitor of VDAC1 would demonstrate what percentage of whole cell ferricyanide reduction this enzyme contributes toward.

Now VDAC1 is established as a transplasma membrane NADH:ferricyanide-reductase, it opens up countless possibilities for future research on the PMOR. Those studying the PMOR can only hope, that like the mitochondrial electron transport chain, the plasma membrane electron transport chain becomes recognised as a vital regulator of cell redox homeostasis.

Chapter 7-References

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