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Plasma Membrane NADH-Oxidoreductase System: A Critical Review of the Structural and Functional Data

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ABSTRACT

The observation in the early 1970s that ferricyanide can replace transferrin as a growth factor highlighted the major role plasma membrane proteins can play within a mammalian cell. Ferricyanide, being impermeant to the cell, was assumed to act at the level of the plasma membrane. Since that time, several enzymes isolated from the plasma membrane have been described, which, using NADH as the intracellular electron donor, are capable of reducing ferricyanide. However, their exact modes of action, and their physiological substrates and functions have not been solved to date. Numerous hypotheses have been proposed for the role of such redox enzymes within the plasma membrane. Examples include the regulation of cell signaling, cell growth, apoptosis, proton pumping, and ion channels. All of these roles may be a result of the function of these enzymes as cellular redox sensors. The emergence of many diverse roles for ferricyanide utilizing redox enzymes present in the plasma membrane might also, in part, be due to the numerous redox enzymes present within the membrane; the poor molecular characterization of the enzymes may be the reason for some of the diverging results reported in the literature as various researchers may be working on different enzymes. Here we review the diverse proposals given for structure and function to the plasma membrane NADH-oxidoreductase system(s) with a specific focus on those enzyme activities which can couple ferricyanide and NADH. Although they are still ill-defined enzymes, evidence is rising that they are of utmost significance for cellular regulation. Antiox. Redox Signal. 2,197–212.

INTRODUCTION

TO DATE THE INNER MITOCHONDRIAL and the thylakoid membranes contain the best-characterized electron transmembrane transport systems, 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 (PMOR). The initial observations of redox regulation at the plasma membrane level were described by Voegtlin and co-workers (Voegtlin *et al.*, 1925) when they showed that dyes, which were later shown to be impermeant to cells (Keilin and Hertree, 1946; Székely *et al.*, 1952), were still reduced by tissue slices and *in vivo*. Further evidence pointing to an electron transport system located in the plasma membrane was then formed by Brooks (1947), who described plasma membrane-impermeant redox dyes being capable of activating the growth of sea urchin eggs while being reduced. Theories explaining the presence of such enzymes in the plasma membrane then surfaced, according to

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which, components of the endoplasmic reticulum would be transported through the Golgi, onto the plasma membrane taking along with them components of a transmembrane electron transport system from the endoplasmic reticulum (Morré *et al.*, 1979). A plasma membrane electron transport system was later evidenced by Barnes and Sato (1980), who defined ferrisulfate as a growth factor as it could replace transferrin and maintain cell survival. Later on it was shown by Ellem and Kay (1983) that ferrisulfate can be replaced by ferricyanide. Because ferricyanide is an anion, large in structure and hydrophilic in nature, it is believed not to permeate the plasma membranes of cells (Keilin and Hertree, 1946; Székely *et al.*, 1952; Mishra and Passow, 1969). This finding therefore linked the plasma membrane ferricyanide-reductase as a vital communicator from the outside world, to intracellular signaling. Many oxidoreductases have since been found to be present on the plasma membrane, from protozoa to humans. Two major families can be distinguished, the NAD(P)H acceptor oxidoreductases and the NADH acceptor oxidoreductases, the latter of which will be the focus of this review.

Erythrocyte membrane preparations (which lack any contamination by intracellular membranes) were shown to contain ferricyanide reductase activity which is specific for NADH as a cofactor (Löw and Werner, 1976; Grebing *et al.*, 1984). 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 (Sun *et al.*, 1984; Löw and Crane, 1995), thus clearly distinguishing it from mitochondrial oxidoreductases. On the other hand, these authors describe atebrin as a specific inhibitor of the activity, 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 (Crane *et al.*, 1985), iron uptake (Inman and Wessling-Resnick, 1993), apoptosis (Lawen *et al.*, 1994; Morré, *et al.*, 1995a; Wolvetang *et al.*, 1996), bioenergetics (Sun *et al.*, 1984; Larm *et al.*, 1994), transformation (Morré *et al.*, 1997; Morré and Reust, 1997; Chueh *et al.*, 1997), and hormone responses (Pershadsingh and McDonald, 1979). The diversity of such functions may be due to the privileged position in which these enzymes are located, potentially making them the (redox) sensory switch between the external environment and the intracellular signaling cascades.

CHARACTERISTICS OF MAMMALIAN NADH:FERRICYANIDE-REDUCTASES

The investigation of the NADH:ferricyanide-reductase from Ehrlich ascites tumor cells has revealed that the enzyme may be a glycoprotein, and that the glycosidic moiety appears to be required for full activity because the enzyme is sensitive to treatment with various glycosidases (del Castillo-Olivares *et al.*, 1994). Furthermore the NADH:ferricyanide-reductase activity can be strongly activated by the bivalent cations Ca^{2+}, Mn^{2+}, Sr^{2+} and Mg^{2+} at micromolar concentrations (del Castillo-Olivares *et al.*, 1994). The enzyme is NADH specific, because exchange of NADH by NADPH in the *in vitro* incubation leads to abolishment of the activity of partially purified enzyme preparations (Vaillant *et al.*, 1996). The enzyme, partially purified from rat liver plasma membranes, can be stimulated by addition of co-factors such as coenzyme Q (CoQ), and inhibited by covalent binding of the thiol compound *p*-chloromercuriphenylsulfonic acid (Sun *et al.*, 1984; Vaillant *et al.*, 1996), 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 localization of the enzyme: (1) it has been shown that a dehydrogenase capable of oxidizing NADH must have both sides of the plasma membrane available for maximal activity (Grebing *et al.*, 1984); (2) upon modification with diazobenzenesulfonate (a plasma membrane impermeant agent that can bind to exposed functional groups of membrane proteins and thus react with both transmembraneous and external proteins but not with internal proteins), cells lose some of their ferricyanide reductase activity (Sun *et al.*, 1984; Grebing *et al.*, 1984).

In HeLa cells, biphasic ferricyanide reduction kinetics have been observed (Sun *et al.*, 1984). Recently, evidence has been presented for two different ferricyanide reductases in HL-60 cells (Van Duijn *et al.*, 1998), 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 characterization of the enzymes involved in the transplasma membrane NADH-oxidoreductase complex.

The plasma membrane NADH:ferricyanide-reductase is believed to be in tight association with a second plasma membrane redox enzyme called the plasma membrane NADH-oxidase (Crane *et al.*, 1985). The oxidase also uses NADH as a cofactor, and presumably oxygen as a final electron acceptor (Crane *et al.*, 1985). The NADH-oxidase may be linked to the NADH:ferricyanide-reductase via CoQ (Sun *et al.*, 1992) to form the bigger PMOR complex.

CANDIDATES FOR THE PLASMA MEMBRANE NADH:FERRICYANIDE-REDUCTASE

To date various plasma membrane oxidoreductases have been described and partially characterized from many different sources. Potentially several of these enzymes could fulfil the role as the key player(s) in redox regulation at the plasma membrane level (see Medina *et al.*, 1997 for a list of enzymes). Here we shall focus on the plasma membrane NADH:ferricyanide-reductase enzyme(s).

Dehydrogenases from the plasma membrane of erythrocytes and human lymphoblastoid cells lines have been purified and include the NADH methemoglobin reductase. This enzyme can function as an NADH:ferricyanide-reductase and has been found to be antigenically undistinguishable from NADH: cytochrome b_5-reductase (Goto-Tamura *et al.*, 1976). However NADH:cytochrome b_5-reductase is not transmembraneous because inside-out erythrocyte ghosts retain 100% of cytochrome *c* reducing activity in the presence of NADH (Grebing *et al.*, 1984). However, right-side-out ghosts do not reduce cytochrome *c* at all, suggesting that NADH:cytochrome b_5-reductase is associated with the inner side of the plasma membrane only (Grebing *et al.*, 1984). These same authors showed that exposure to both sides of the membrane was required for at least 50% of the total erythrocyte NADH:ferricyanide-reductase activity. Therefore, the presence of another NADH:ferricyanide-reductase was postulated. This second plasma membrane NADH:ferricyanide-reductase was suggested to be the transplasma membrane transferrin reductase, or part of the transferrin receptor itself (Crane *et al.*, 1985). Indeed, a recent publication demonstrated that the staphylococcal transferrin receptor protein, Tpn, is a multifunctional cell wall enzyme with high homology to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Modun and Williams, 1999). GAPDH is capable of reducing 2,6-dichloroindophenol (DCIP), a substance commonly used instead of ferricyanide to assay plasma membrane NADH-dehydrogenases (Buillard *et al.*, 1997). However the NADH:ferricyanide-reductase itself is most probably not part of the transferrin receptor because erythrocytes with very good ferricyanide reduction have little transferrin receptor (Octave *et al.*, 1983). Furthermore, affinity-purified transferrin receptors from human placental trophoblast plasma membrane preparations do not exhibit any measurable NADH:ferricyanide-reductase, whereas the membrane preparations retain measurable NADH:ferricyanide-reductase activity (Berczi and Faulk, 1992).

Another promising plasma membrane candidate protein that has been purified and that would qualify for the NADH:ferricyanide-reductase is a still ill-defined 40-kDa protein isolated by Wang and Alaupovic (1978). This enzyme can be inhibited by divalent ions and sulfhydryl agents. Furthermore, it is specific for NADH, as NADPH is ineffective as a reducing agent when added together with ferricyanide, DCIP or cytochrome *c*. This enzyme is a glycoprotein and it is likely to be a transmembraneous protein (Wang and Alaupovic, 1978).

Yet another dehydrogenase suggested to be part of the PMOR complex was found by Bulliard and co-workers (1997). In their report, the authors describe the isolation of a complex of four proteins from rat brain using NADH and

DCIP in the enzyme assays. The isolated complex comprised of a GAPDH isozyme, a bovine homologue of the rat 64-kDa protein "turned on after division" (TOAD)-64 (Minturn *et al.*, 1995), enolase-γ, and aldose C. No NADH-oxidase activity, commonly believed to be in association with the NADH:ferricyanide-reductase, was found in the isolated complex (Buillard *et al.*, 1997).

ASCORBATE REGULATION

Although the NADH:ferricyanide-reductase is most commonly analyzed using ferricyanide as a substrate, this clearly 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, P420, and P450), and CoQ (Bruder *et al.*, 1980; Cunningham *et al.*, 1982; Loeper *et al.*, 1993). 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 growth rate of tumor cells when added to the media (Alcaín *et al.*, 1990). Ascorbate at 0.2 m*M* stimulates growth of A-204 rhabdomyosarcoma and HL-60 cells (Alcaín *et al.*, 1990), however the same concentration inhibits growth of U-20s osteosarcoma, Y-79 retinoblastoma, and several neuroblastoma cell lines (Medina *et al.*, 1994; Garcia de Veas *et al.*, 1995). There appears to be a connection between ascorbate oxidation and ferricyanide reduction.

As discussed in more detail in a recent review by May (1999), there is some evidence to suggest that ascorbate may be able to act as another intracellular electron 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 (May *et al.*, 1998). However, to the authors' knowledge, 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.* (1998), 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 level (Schweinzer and Goldenberg, 1993). 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 nonenzymatically forms ferrocyanide and ascorbate free radical (AFR) (Schweinzer and Goldenberg, 1993). The ascorbate free radical then becomes reduced into ascorbate by a second enzyme, distinct to the NADH:ferricyanide reductase, namely the AFR-reductase. However, because addition of excess ferricyanide to a solution of ascorbate yields dehydroascorbate in an extremely quick manner, this mechanism appears to be unlikely (Van Duijn *et al.*, 1998). 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 (Winkler, 1987). However in the presence of cells, both the rate of ascorbate autoxidation decreases, and the amount of ascorbate in the media increases significantly (Alcaín *et al.*, 1991). Such a stabilization in external ascorbate may be due to the AFR-reductase.

An additional route of dehydroascorbate (DHA) uptake was evidenced by nuclear magnetic resonance studies in whole red blood cells. Extracellularly added, ^{13}C-labeled 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 (Himmelreich *et al.*, 1998).

Increasing the intracellular concentration of ascorbate has also been shown to result in an increase in ferricyanide reduction in whole red blood cells (Himmelreich and Kuchel, 1997). The mechanism proposed for this increase is a more complicated one. The stimulation of ferricyanide reduction involves the uptake of the two-electron oxidized form of ascorbate, DHA, into the cytosol of cells. DHA and extracellu-

lar ferricyanide are utilized by a transplasma membrane ascorbate:ferricyanide-reductase, forming ascorbate and ferrocyanide.

The uptake of DHA has been shown to occur through the glucose transporter GLUT-1 (Guaiquil *et al.*, 1997). Once taken into the cell, DHA is converted back to ascorbate. 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 (Himmelreich *et al.*, 1998; Van Duijn *et al.*, 1998). The authors reached that conclusion because pCMBS, normally a potent inhibitor of the NADH:ferricyanide-reductase, has little effect on the reduction of DHA to ascorbate (Van Duijn *et al.*, 1998). This second plasma membrane ascorbate:ferricyanide-reductase may be capable of converting dehydroascorbate back into ascorbate, using extracellular ferricyanide. This would explain the increased rate of ferricyanide reduction upon addition of ascorbate (Van Duijn *et al.*, 1998). Interestingly, blocking the GLUT-1 receptor before addition of ascorbate totally abolishes ascorbate-dependent ferricyanide reduction. However blocking the GLUT-1 receptor after addition of ascorbate, does not inhibit ascorbate-dependent ferricyanide reduction (Himmelreich *et al.*, 1998; Van Duijn *et al.*, 1998). One candidate for the ascorbate:ferricyanide-reductase could be the selenoenzyme thioredoxin reductase, as liver cytosolic fractions from selenium-deficient rats show a decreased generation of ascorbate, when compared to cytosolic fractions from normal rats (May *et al.*, 1997). Also, gold-containing compounds have been shown to inhibit selenoenzymes (Chaudiere and Tappel, 1984). Upon addition of gold thioglucose (aurothioglucose) to purified rat liver cytosol, a 90% decrease in ascorbate regeneration is seen (May *et al.*, 1997). This suggests that the NADPH-dependent thioredoxin reductase may be the second electron transport system involved in ascorbate-dependent ferricyanide reduction. In accordance with that hypothesis, oxidation of NADPH and glutathione (GSH) has been reported to occur when ferricyanide was added to ascorbic acid-loaded red blood cells (Himmelreich *et al.*, 1998). Although an ascorbate transporter has been described to be present on some nucleated cells (Rose, 1988), ascorbate itself may not directly be taken into the cell, since intracellular ascorbate only accumulates when DHA is present in the medium (Van Duijn *et al.*, 1998). A summary of the putative pathways and enzymes involved in ascorbate-dependent redox reactions is given in Fig. 1.

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 (Baldwin *et al.*, 1982). But there is evidence to suggest that this is not the only mechanism for iron uptake in mammalian cells: cells from which the transferrin receptor has been removed, are still capable of taking up iron. This suggests that there exists a transferrin-independent iron uptake pathway (Berczi and Faulk, 1992). The first steps involved in the nontransferrin iron uptake into cells, include the specific binding of iron to the plasma membrane (Musílková *et al.*, 1998). It is unknown to which receptor the iron binds on the plasma membrane, however there is evidence from studies with yeast to suggest that it may be the plasma membrane NADH:ferricyanide-reductase. A yeast plasma membrane NADH-reductase has been identified, which is responsible for the transport of ferrous iron across the cell surface (Dancis *et al.*, 1990). A similar enzyme may exist in mammalian cells. Studies based mainly upon the unique properties of K562 cells have demonstrated that a ferricyanide NADH-reductase may be responsible for transferrin-independent iron transport in these cells (Inman and Wessling-Ressnick, 1993).

K562 cells have maintained a unique ferricyanide reductase that is not involved in growth. This NADH:ferricyanide-reductase isozyme appears to be tightly coupled to the transferrin-independent iron uptake pathway (Inman and Wessling-Resnick, 1993; Inman *et al.*, 1994). This was demonstrated by the following experiments: (1) Ferricyanide competing with the iron binding site in the ferricyanide reductase completely inhibits iron

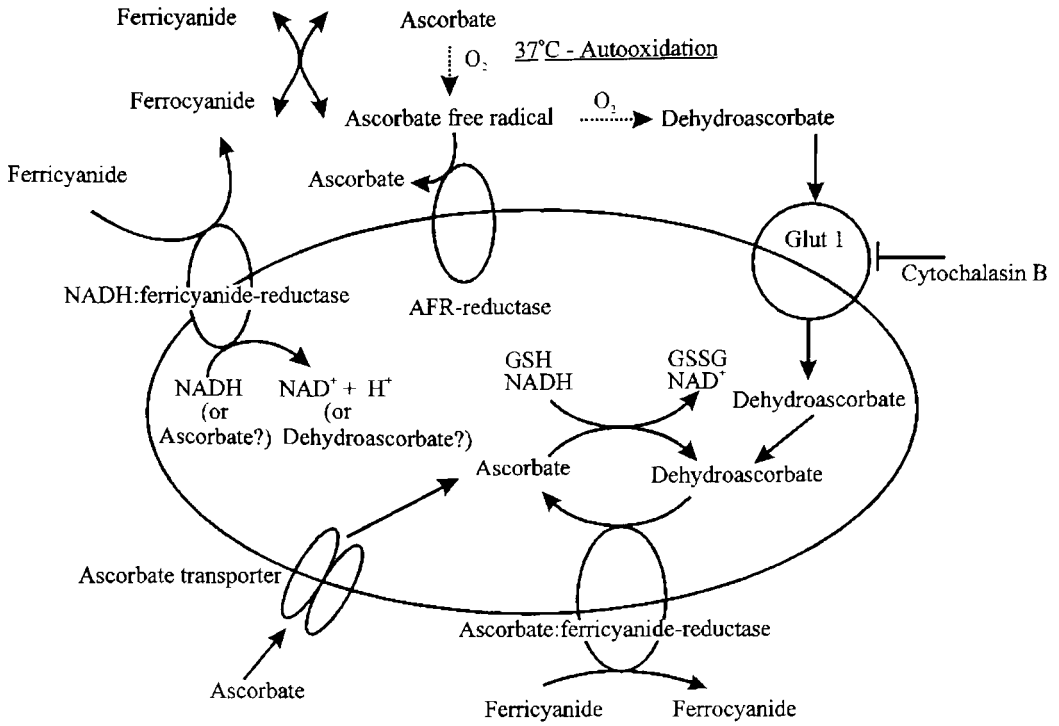

FIG. 1. Pathways and enzymes involved in ascorbate-dependent-ferricyanide reduction.

uptake of ^{55}Fe nitriloacetic acid (Inman *et al.*, 1994); (2) the alkylating agent *N*-ethylmaleimide (NEM) completely inactivates the ferricyanide reductase (Inman *et al.*, 1994). NEM also inhibits transferrin-independent iron transport in K562 cells (Inman and Wessling-Resnick, 1993); (3) cadmium inhibits both the ferricyanide reduction and the transferrin-independent iron transport. This suggests that ferricyanide reductase and the iron transport are one and the same, or alternatively they both contain the same Cd^{2+} binding site (Inman *et al.*, 1994).

Robinson *et al.* (1999) have identified an FRO2 gene from *Arabidopsis*, an iron-deficient plant, that 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 has 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 (Georgatsou and Alexandraki, 1999). Disruption of both the FRE1 and the related FRE2 gene completely abolishes ferric reductase activity in yeast (Georgatsou and Alexandraki, 1994). These genes encode regions with sequence homology to the gp91-phox protein, a component of the human phagocyte NADPH oxidoreductase (Roman *et al.*, 1993), 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 have been shown to be induced by iron deprivation (Georgatsou and Alexandraki, 1994).

The plasma membrane NADH:ferricyanide-reductase is capable of reducing iron in transferrin (Crane *et al.*, 1985). Thus, the diferric receptor site may be identical with or very close to the transmembrane electron-transfer complex (Crane *et al.*, 1985).

GROWTH CONTROL SIGNALING

The first evidence for the involvement of the transplasma membrane NADH:ferricyanide-reductase in growth control came when Ellem and Kay (1983) showed that the addition of external ferricyanide can stimulate the growth of melanoma cells, in the absence of sufficient growth factors. Concentrations between 0.01 and 0.1 m*M* ferricyanide are needed to stimulate growth of cells. Ferrocyanide, on the other hand, does not stimulate nor does it inhibit growth (Ellem and Kay, 1983). Ferricyanide, being impermeant to cells, must be working on the plasma membrane level. Löw and Crane (1995) later demonstrated that ferricyanide cannot activate growth on its own, but rather maintains an already established signal. Supporting this idea, Crane *et al.* (1982) have established that the reductase activity of *Saccharomyces cerevisiae* is dependent on the growth phase of yeast cells, which stop to reduce extracellularly added electron acceptors once they enter the stationary phase.

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

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 (Dormandy and Zarday, 1965). The protons released from cells cause acidification of the medium and alkalization of the cytosol (Löw and Crane, 1995). Such alkalization has been proposed to lead to growth of the cells, since mitosis has been related to an increase in cytosolic pH (Gerson *et al.*, 1982). Upon addition of ferricyanide to the medium, the NADH:ferricyanide-reductase begins to oxidize 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 (Sun *et al.*, 1988). Under steady-state conditions, a balance of proton influx and efflux would remain the same and no difference in plasma membrane potential would occur. However, the plasma membrane potential increases when accumulation of protons in the cytosol occurs (hyperpolarization). Alternatively, a decrease in the plasma membrane potential occurs when the majority of cytosolic protons are "pumped out" in cell culture into the medium, or *in vivo* into the extracellular matrix (depolarization). Upon addition of 0.5 m*M* ferricyanide to bean root cells, a depolarization (20–40 mV) of the plasma membrane is seen (Sijmons *et al.*, 1984), suggesting that the protons generated by the NADH:ferricyanide-reductase are exported to the other side of the plasma membrane. Such a depolarization is accompanied by a cytosolic alkalization. Three minutes following addition of ferricyanide, the cell becomes slightly repolarized (Sijmons *et al.*, 1984). 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. These amino acids would then be used by the cell, during the mitogenic process (Valdeolmillos *et al.*, 1986).

Contrary to these findings, Thomas *et al.* (1996) reported that the addition of 100 μM ferricyanide to serum-starved PC12 cells causes a cytosolic acidification, a drop of pH from 7.52 to 7.25 was measured. This pH drop has also been proposed to play a major role in mitogenic signaling, because DNA synthesis and ATP production increase (Thomas *et al.*, 1996). It is still unclear as to why ferricyanide causes a pH decrease in PC12 cells. The authors suggest that the addition of physiological concentrations of bicarbonate to their culture media causes the activation of one or more bicarbonate-dependent acid extrusion mechanism(s). 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 first converted into an alkalization still remains to be finally established.

SECOND MESSENGERS

The signaling pathway originating from the transplasma membrane dehydrogenase, also remains unclear. Apart from proton movement in the cytosol, G proteins may play a role in the transduction of the signal (del Castillo-Olivares *et al.*, 1995). Rodriguez-Aguilera *et al.* (1993) have shown that the transplasma membrane dehydrogenase may work through a signaling cascade involving the second messenger cAMP. Additionally, increasing PMOR activity increases the cytosolic Ca^{2+} concentration, which then leads to activation of PKC. PKC itself has also been found to stimulate ferricyanide reductase activity (del Castillo-Olivares *et al.*, 1996), which may suggest the existence of a self-amplification loop.

The importance of the transplasma membrane NADH:ferricyanide-reductase in growth control is also evidenced by the effects of common anticancer drugs. Such drugs are believed to induce cell arrest and finally to cause the cells to undergo apoptosis. Azaserine, acivicin (Medina *et al.*, 1992b), adriamycin, daunomycin, AD32 (Vyskocil *et al.*, 1983), cisplatin (Sun and Crane, 1984), mitoxantrone (Medina *et al.*, 1991), adriamycin (Tritton and Yee, 1982), bleomycin (Sun and Crane, 1985), cis-diaminedichloroplatinum (II), and actinomycin D (Crane *et al.*, 1985) all are strong inhibitors of the plasma membrane NADH:ferricyanide-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. Tritton and Yee (1982) immobilized adriamycin on agarose beads, making it totally impermeant to cell membranes and found that the compound had an improved cytotoxicity when compared to soluble compound. Inhibition of the reductase with drugs at concentrations that inhibit growth also points to the vital role the plasma membrane NADH:ferricyanide-reductase may play in the regulation of growth.

PMOR ACTIVITY CHANGES DURING TRANSFORMATION AND DIFFERENTIATION OF CELLS

The plasma membrane NADH:ferricyanide-reductase has been found ubiquitously in cells, ranging from protozoa to humans. Nevertheless, the role of the mammalian enzyme has remained inconclusive over the past several decades. Some knowledge has been gained while observing the relationship between the NADH:ferricyanide-reductase and the transformation of cells, which was achieved using two oncogenes, N-*myc* and Ha-*Ras* (Medina *et al.*, 1992a; Crowe *et al.*, 1993). A reverse relationship between N-*myc* expression and ascorbate oxidation in three neuroblastoma cell lines was found (Medina *et al.*, 1992a). Ascorbate (as discussed above) may be a natural electron acceptor for the transplasma membrane NADH:ferricyanide-reductase. 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 five-fold during the transfection of the Ha-*ras* gene into CBH 10T1/2 mouse embryo fibroblast cells (Crowe *et al.*, 1993). However, because reduction of externally added cytochrome *c* also increased under these conditions, the increase may be attributed to an increase in cytochrome b_5 reductase activity. Yet another explanation may be an increase in the internal supply of NADH (Crane *et al.*, 1985). The increase of plasma membrane NADH:ferricyanide-reductase activity upon transformation (perhaps upon increase in intracellular NADH concentration) would fit with the hypothesis that the NADH:ferricyanide-reductase is involved in up-regulation of cell growth and correlates with cell proliferation.

The up-regulation of the transplasma membrane NADH:ferricyanide-reductase is not restricted to cell transformation. A change in the NADH:ferricyanide-reductase activity was

shown during differentiation of HL-60 cells after a period of 24 hr with 12-*O*-tetradecamoylphorbal-13-acetate (TPA) (Burón *et al.*, 1993). TPA causes the promyelocytic HL-60 cells to undergo a monocytic differentiation. These cells lose their proliferative capacity and acquire maturation features of macrophage-like cells (Rovera *et al.*, 1979). However, 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 signaling 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 (Himmelreich and Kuchel, 1997).

BIOENERGETICS

It is still unclear whether the transplasma membrane NADH:ferricyanide-reductase causes a direct change in the nucleotide concentration of cells, or whether a change in the nucleotide ratio modulates the enzyme activity. Lactate as a direct source of cytosolic NADH enhances ferricyanide reductase activity in whole cells (Medina *et al.*, 1988), suggesting that an increase in the $NADH:NAD^+$ ratio may stimulate 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 ρ^0 cells, in which NADH is no longer oxidized by the respiratory chain, a more than three-fold increase in the rate of NADH:ferricyanide-reduction occurs. The ρ^0 cells appear to respond to the increased $NADH:NAD^+$ ratio as a result of an nonfunctional electron transport chain by up-regulating the activity of the NADH:ferricyanide-reductase. Similarly, the addition of mitochondrial inhibitors such as potassium cyanide can stimulate ferricyanide reduction (Gutierrez *et al.*, 1998). Impairment of the respiratory chain has also been implicated during ageing (Linnane *et al.*, 1990). The up-regulation of the NADH, in response to the nucleotide pool may enhance survival in such energy-deficient cells (Lawen *et al.*, 1994). Similarly, the up-regulation of the NADH:ferricyanide-activity may explain the survival of tumor cells, in which the natural environment of the cell is believed to become hypoxic. Because normal mitochondrial respiration may be impaired during tumor 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.* (1991) found that transformation of Swiss 3T3 fibroblasts with the simian virus 40 (SV40) caused a decrease in ferricyanide reduction. This was accompanied by an increase in the number of transferrin receptors. Similarly, we have observed a down-regulation of both NADH:ferricyanide-reductase and NADH-oxidase activity during prolonged treatment with ethidium bromide in fibroblast cell lines (Malik *et al.*, unpublished results). A summary of the available data on the regulation of the two PMOR activities depending on the various cellular states is given in Table 1.

APOPTOSIS

The PMOR system is believed to consist of at least two enzyme activities, the plasma membrane NADH:ferricyanide-reductase and the plasma membrane NADH-oxidase (Crane *et al.*, 1985). In an attempt to purify and characterize the plasma membrane NADH-oxidase a 32-kDa ectoenzyme has been shown on the outer surface of the plasma membrane (Morré *et al*, 1995b). *In vitro*, this enzyme uses NADH and presumably oxygen as a final electron acceptor (Crane *et al.*, 1985). Evidence is accumulating, that in transformed cells, an isoform of the NADH-oxidase exists. This isoform may be shed into the medium or sera of cancer patients (Wilkinson *et al.*, 1996). It has been reported by us and others that the NADH-oxidase can be stimulated by the addition of CoQ and its analogues (Brightman *et al.*, 1992; Larm

TABLE 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%	nd[a]	Tumor vs. normal cells, faster growth = greater PMOR activity	Medina *et al.*, 1992
Mouse embryonic fibroblasts	Transfection with Ha-*ras*	5-Fold increase	nd[a]	(1) Increase in amount of enzyme on plasma membrane (2) Increase in internal [NADH]	Crowe *et al.*, 1993
human myeloid HL-60	TPA[b]	1.4-Fold increase	nd[a]	Internal [NADH] and [NAD^+]	Burón *et al.*, 1993
human myeloid HL-60	Ethidium bromide	3-Fold increase	4-Fold increase	An increase in "hypoxic"-like conditions, leads to up-regulation of PMOR	Larm *et al.*, 1994; S. Malik, S. Marzuki, and A. Lawen, unpublished data
human lymphoblastoma Namalwa					
Human fibroblasts	Ethidium bromide	Decrease	Decrease		S. Malik, S. Marzuki, and A. Lawen, unpublished data
Swiss 3T3 mouse embryo fibroblast cells	Transformation with SV-40	4-Fold decrease	nd[a]		Löw *et al.*, 1991
K562 human erythroleukemia	Mitochondrial inhibitors	Increase	nd[a]	Decrease in cellular ATP concentration	Gutierrez *et al.*, 1998

[a]nd, not determined
[b]TPA, 12-*O*-tetradecamoylphorbal-13-acetate.

et al., 1994; Vaillant *et al.*, 1996; Wolvetang *et al.*, 1996). These analogues were also shown to stimulate activity of the plasma membrane NADH:ferricyanide-reductase of whole cells (Larm *et al.*, 1994). The ability of the CoQ to stimulate both the NADH-oxidase and the NADH:ferricyanide-reductase may suggest 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. We and others have demonstrated that capsaicin and its analogues dihydrocapsaicin and resiniferatoxin (all three CoQ analogues belonging to the class of vanilloids) are potent inhibitors of the NADH-oxidase (Morré *et al.*, 1995a; Vaillant *et al.*,1996; Wolvetang *et al.*, 1996). The inhibition of the NADH-oxidase by vanilloids has been shown to induce rapid apoptosis in leukemic (Lawen *et al.*, 1994; Wolvetang *et al.*, 1996) and HeLa cells (Morré *et al.*, 1995a). Apoptosis is a physiological form of cell death, whereby cells undergo morphological changes, including a characteristic blebbing of the plasma membrane, and after which, *in vivo*, the cell is engulfed by macrophages (Schwartzman and Cidlowski, 1993). The induction of apoptosis in many systems can be inhibited by the classical anti-apoptotic oncogene product, Bcl-2, however it is unclear how Bcl-2 exerts its anti-apoptotic effect. Upon addition of capsaicin to Daudi, FDCP-1, or HL-60 cells, apoptosis occurs within 6–12 hr. The induction of apoptosis by the vanilloids can be prevented by the PMOR substrate CoQ (Wolvetang *et al.*, 1996). After transfection of the cell lines with Bcl-2, capsaicin no longer exerts its apoptosis-inducing effect. (Wolvetang *et al.*, 1996). The mechanism by which inhibition of the NADH-oxidase leads to apoptosis has been suggested by us to be the generation of an imbalance in the cellular NADH/NAD^+ ratio (Lawen *et al.*, 1998). 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.

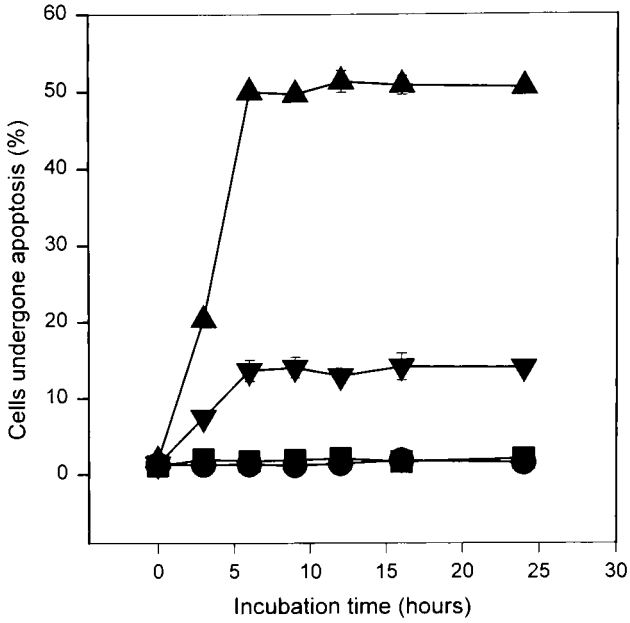

FIG. 2. Prevention of plasma membrane NADH-oxidase inhibition-induced apoptosis by stimulation of the plasma membrane NADH:ferricyanide-reductase. Daudi cells were incubated with 10 m*M* ferricyanide (■), 10 m*M* resiniferatoxin (▲) or both (▼). Cells were harvested at the indicated times, cytospun, and stained with DAPI (Grubb *et al.*, 1995) to assess apoptosis. (●) Vehicle-treated control 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 (Fig. 2; Lawen *et al.*, 1998; Benning, Miglic, and Lawen, unpublished results).

Our interpretation of these data is that by inhibiting the oxidase, capsaicin causes increases of the NADH/NAD^+ ratio. However upon addition of ferricyanide an up-regulation of the NADH:ferricyanide-reductase occurs (Navas *et al.*, 1986). This leads to the oxidation of excess NADH and a lowering of the nucleotide ratio, thus maintaining cell survival (Fig. 3; Lawen *et al.*, 1998).

Alternatively, the PMOR may be regulating antioxidants such as ascorbate (see above) 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' (Barroso *et al.*, 1997).

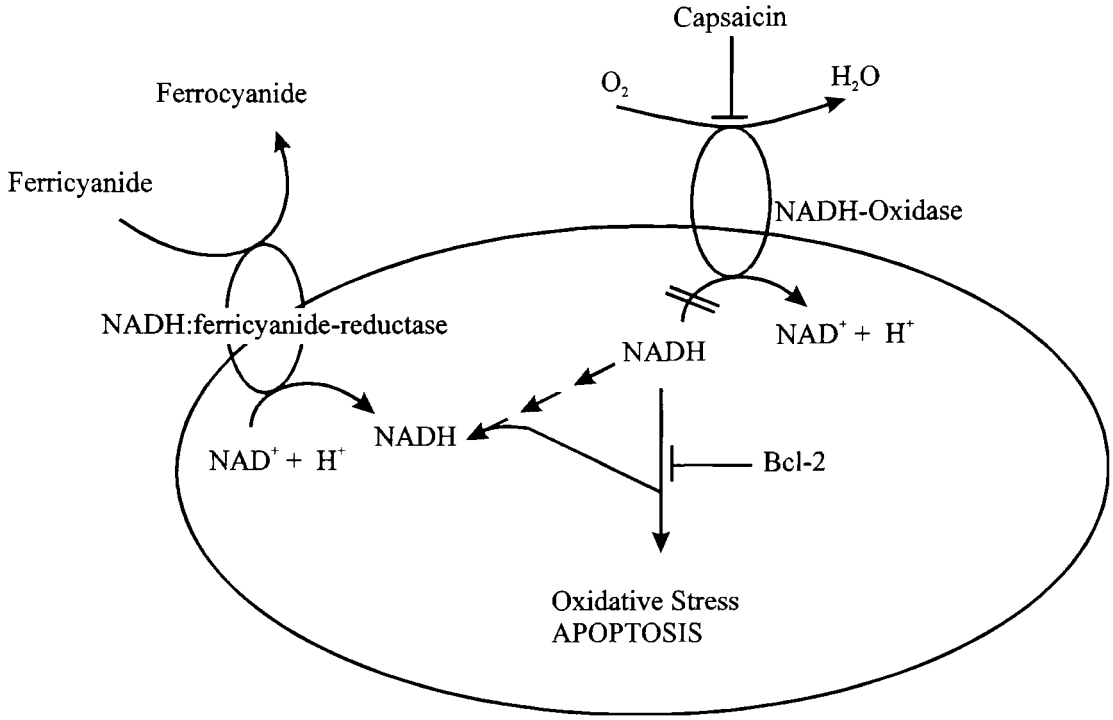

FIG. 3. Schematic representation of the involvement of the PMOR in redox homeostasis and cell death.

CONCLUSION

The plasma membrane contains a vast number of unique enzymes waiting to be uncovered. The NADH:ferricyanide-reductase is just one of those still ill-defined proteins that are enigmatic as to their function in mammalian cells. By the use of pharmacological assays, the NADH:ferricyanide-reductase has been implicated in a host of functions. Growth or death of a cell may be regulated by the transplasma membrane NADH:ferricyanide-reductase in response to changes in the cellular redox potential. Ascorbate stabilization is seen to increase proportionally, with NADH:ferricyanide-reductase activity. The enzyme may play a role in protection from oxidative stress and in the uptake of iron. Obviously, the molecular identification of the major NADH:ferricyanide-reductase(s) involved in all these functions needs to be established. Whether it be only one dehydrogenase or a host of such enzymes found in the plasma membrane working together needs to be clarified. Whether the enzyme forms a complex with other redox enzymes, the transferrin receptor, and/or growth hormone receptors or not also needs to be established. Furthermore, following the molecular characterization, the potential use of knockout mice would be of considerable interest for the analysis of the role(s) of this enzyme. Our understanding of these unusual multifunctional plasma membrane redox enzymes is still in its infancy. Their role in apoptosis and drug responsiveness may, in the future, warrant such enzymes to be explored for cancer therapies.

ABBREVIATIONS

AFR, Ascorbate free radical; CoQ, co-enzyme Q; DCIP, 2,6-dichloroindophenol; DHA, dehydroascorbate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; NEM, *N*-ethylmaleimide; PMOR, plasma membrane NADH-oxidoreductase; SV40, simian virus 40; TOAD-64, turned on after division, 64 kDa; TPA, 12-*O*-tetradecamoylphorbal-13-acetate.

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