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Chapter 5

PLASMA MEMBRANE COQ, PORIN, AND REDOX CONTROL OF AUTISM

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ABSTRACT

Autism is a neurological condition starting in childhood that is characterized by behavioral and intellectual problems. Its occurrence is increasing and although there are some treatments, they are of limited effect or have undesirable side effects. A recent study showed that autistic children had increased serum levels of auto-antibodies to Voltage Dependent Anion Channel (VDAC). Interestingly, in addition to the membrane transport function of VDAC a second function was recently described by A. Lawen's group at Monash University in Melbourne. This group showed that VDAC was also a trans-PM NADH dehydrogenase.

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The VDAC autoantibody detected in autistic children inhibits the dual transport and dehydrogenase functionality of VDAC. In this report we implicate Coenzyme Q as an important co-factor for redox control of PM pores including VDAC. We show that the PM redox function is dependent on Coenzyme Q and propose that this novel function for CoQ has therapeutic implications for treatment of autism disorders. More broadly, the Coenzyme Q requirement for the PM redox function of porin in diverse species including bacteria, plants, and mammals suggests a mechanistically conserved feature of pore redox control.

Keywords: Coenzyme Q10, NADH-Ferricyanide reductase, Redox, Autism, VDAC

Abbreviations: Coenzyme Q10 (CoQ10), PM (PM), Ferricyanide ($FeCN_6$), Voltage Dependent Anion Channel (VDAC), Uncoupler Protein (UCP), Inner Mitochondrial Membrane (IMM), para-dichloromercury benzoic acid (pCMB).

INTRODUCTION

Diverse Role of Q10 in Biological Membranes

Coenzyme Q_{10} (CoQ_{10}) is well recognized for energy coupling and the function of the electron transport chain in the inner mitochondrial membrane (IMM). CoQ_{10} is also present in other biological membranes [1, 2] where it functions in diverse roles. For example, electron transport by CoQ_{10} is thought to be important for acidification and maintenance of the lysosomal pH gradient required for proper function of lysosomal enzymes [3]. This proton pumping in the lysosome has been shown to be inhibited by chloroquine which may reflect an inhibition of coenzyme Q function in the lysosome [4]. In this review we will focus on the role of CoQ_{10} in the Plasma membrane (PM) and provide evidence for a role between the CoQ_{10}- directed trans-PM electron transport system and the function of VDAC. We also discuss the implications for the emerging biomedical implications of CoQ_{10} in relation to the function of VDAC and autism spectrum disorders. These new insights point to a more general role of CoQ_{10} in redox control of many types of PM pores, which could warrant CoQ_{10} supplementation for therapy or treatment of other neurological conditions.

CoQ_{10} exists in two pools within the cell, in lipid bilayers CoQ_{10} is thought to freely diffuse within the membrane where the reduced form, $CoQ_{10}H_2$, participates in free radical scavenging. CoQ_{10} can also be bound to proteins within the cell, these proteins include CoQ_{10} transport proteins and membrane bound proteins such as pores. CoQ_{10} has been shown to directly bind to a rapidly expanding list of pore proteins, suggesting a direct yet unknown mechanism for CoQ control of pore redox states. For example CoQ binding has been demonstrated in *V. cholera* where a Gly-Gly amino acid pair in the NADH:quinone oxidoreductase is proposed to participate in CoQ binding and required for Na^+ pumping of this pore [5]. Following the original recognition of CoQ in mitochondria, CoQ has been found in all membranes in amounts sufficient to have significant function in membrane structure or redox activity. CoQ has also been shown to be important for the function of the uncoupler protein in the inner mitochondrial membrane. The uncoupler proteins (UCP) facilitate diffusion of H^+ ions into the mitochondrial matrix thus bypassing the ATPase and effectively uncoupling electron transport from oxidative phosphorylation. The importance of CoQ for electron transport chain uncoupling was shown [6-8], where addition of CoQ_{10} was necessary for the holoenzyme function of recombinant UCP. Given the emerging function of PM CoQ_{10} in whole cell redox control and the expanding list of CoQ_{10} binding proteins we provide evidence that suggests the CoQ_{10} directed PM redox system is required for the proper function of VDAC.

THE SENTINELS OF PLASMA MEMBRANE REDOX CONTROL

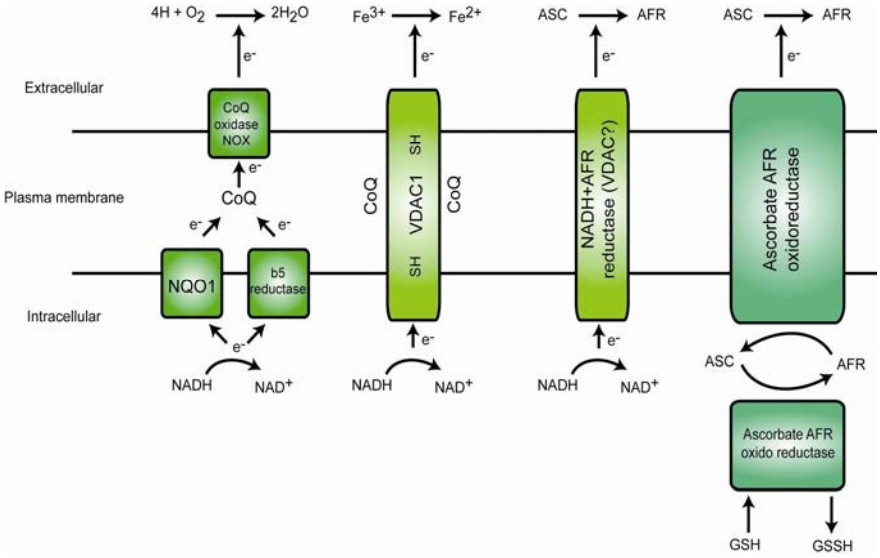
Most cells have two systems for transfer of electrons from cytosolic NADH through the plasma membrane to the external surface. Each of these systems involves interaction with coenzyme Q (Figure 1). The first system to be discovered was an NADH-oxidase which was shown to be subject to hormonal and growth factor control and involved in proliferation of transformed cells [9, 10]. The NADH oxidase in the plasma membrane involves two possible dehydrogenases, NADH cyotochrome b5 reductase [11] or DT-diaphorase encoded by the *NQO1* gene located on the cytosolic side of the membrane. Each dehydrogenase acts as a NADH coenzyme Q reductase to reduce the coenzyme Q within the plane of the lipid bilayer. The reduced QH_2 is then oxidized by a reduced coenzyme Q oxidase on the extracellular side of

the membrane plasma. High levels of *NQO1* activity cause an increase in the $CoQ_{10}H_2$ pools and is condisered protective due to heightened free radical scavenging within the cell. On the contrary, mutations or lowered expression of NQO1 is detrimental to cells [12].

The second system discovered was an NADH-ferricyanide reductase, which transfers electrons to reduce ferricyanide outside the cell. This later system was recently identified as the protein polymer VDAC [13, 14]. The VDAC has no oxidase activity but it transfers electrons from inside the cell to the external oxidant ferricyanide [13, 14]. In VDAC the only known groups for electron transport across the membrane are two cysteine residues (-SH) on the inside of the channel [15]. Both of these systems oxidize cytosolic NADH to NAD^+ and consequently increase cytosolic NAD^+ concentrations (Figure 1) [16]. Principally, VDAC was shown to selectively reduce ferricyanide, and is therefore not classified as an NADH-oxidase [17]. Currently, ferricyanide is the only oxidant that works as an external electron acceptor for VDAC, a likely natural electron acceptor would be semi-dihydroascorbate (discussed below) [18] especially in neural tissue where ascorbate concentration is found to be high [19]. VDAC also reduces coenzyme Q, but this reaction may be based on a functional requirement for coenzyme Q in the function of this enzyme. In other words, coenzyme Q would act as part of the electron chain as in the mitochondria or as part of the VDAC holoenzyme rather than as a substrate for VDAC. Therefore the natural and final electron acceptor for VDAC remains elusive and it is tempting to speculate that a natural redox function for VDAC is in keeping coenzyme Q reduced in the plasma membrane (Figure 1).

A third system for trans-PM electron transport, which involves ascorbate recycling, has been proposed [20, 21]. Although no ascorbate is used with isolated plasma membrane in this study, ascorbate recycling is a biologically relevant process that should be noted in trans-plasma membrane electron transport. In particular, an ascorbic oxidase could provide a good supply of ascorbate free fradicals (AFR) [22-24] for the VDAC system given the high concentrations of naturally occurring ascorbate in the brain [19, 22-25].

Experimentally the requirement for coenzyme Q involvement in these systems is often demonstrated by depletion or inhibition of coenzyme Q followed by a rescue effect after coenzyme Q reintroduction. For example, extraction of coenzyme Q from membrane preparations decreases NADH-oxidase activity, which would be restored by re-addition of coenzyme Q. This experimental concept will be further demonstrated in the context of VDAC [4].



Teske, B.F. et al.

Figure 1. Trans-plasma membrane electron transport. (Left to right): 1. NADH-oxygen oxidoreductase (NADH Oxidase), 2. NADH-ferricyanide reductase, (VDAC), 3. NADH-ascorbate free radical reductase, 4. Ascorbate/Ascorbic free radical oxidoreductase.

REQUIREMENT OF Q IN PM MEDIATED REDUCTION OF FECN6

When it was found that the Plasma Membrane (PM) had NADH-ferricyanide reductase activity it was assumed to be based on the microsomal NADH dehydrogenase (NADH cytochrome reductase). Later when the PM NADH-oxidase was found, ferricyanide reduction was assumed to be a side reaction from the primary dehydrogenase. When we studied the ferricyanide reductase at Monash University, Lawen and Wolvetang found a connection between the PM oxidase and apoptosis [26]. In the course of further study Baker and Lawen took up purification of the NADH-ferricyanide reductase from liver preparations. When it was purified they discovered that it was the protein which was contained in VDAC found in many PMs . This was surprising because at first the only apparent electron carriers in VDAC were two internally located cysteine residues. However, in 1992 during a study of

coenzyme Q function we found that coenzyme Q was necessary for the erythrocyte NADH-ferricyanide reductase activity in the erythrocyte PM. At the time we did this study the ferricyanide reductase activity of the PM was attributed to residual activity from a broken down oxidase. Ten years later, Lawens group purified the reductase and identified it as VDAC.

PLASMA MEMBRANE COENZYME Q IS REQUIRED FOR FULL REDOX FUNCTION OF VDAC

The evidence for coenzyme Q involvement with VDAC is exemplified with plasma membrane preparations and E coli deficient for porin. Direct testing with isolated recombinant VDAC enzyme will solidify the key observations provided in this study.

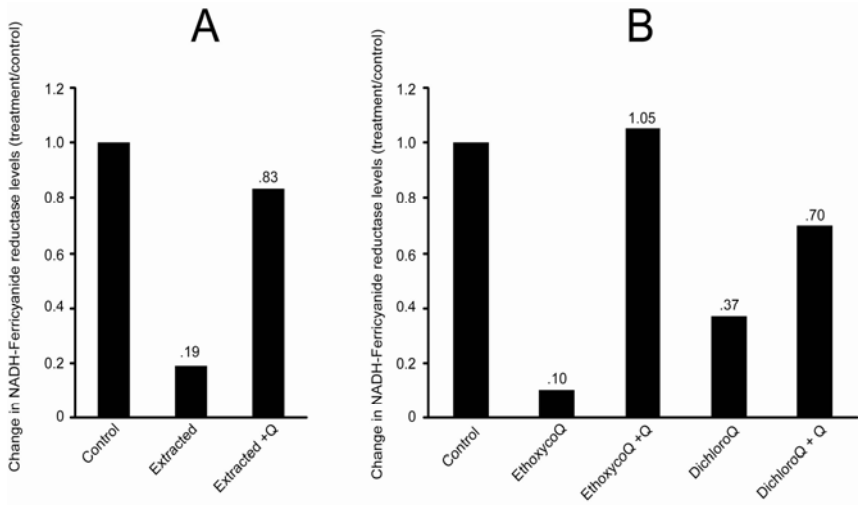
Table 1. Inhibition of plasma membrane electron transport by PCMB.

PCMB[a] concentration (μM)	Membrane preparation	Inhibition (%)
1.0	erythrocyte	100%
2.0	erythrocyte	100%
10	erythrocyte	100%
5	Rat Liver	60%
100	Ehrlich Ascites	89%
100	HeLa	55%

[a] para-dichloromercury benzoic acid

The properties of the erythrocyte plasma membrane are unusual in that there is no detectable NADH oxidase activity despite a high activity for ferricyanide reduction. The presence of an NADH-oxidase in erythrocyte plasma membranes would be futile and compete with the oxygen loading of hemoglobin in this cell type. This means the erythrocyte plasma membrane system used in this study has potential to uncouple the competing plasma membrane redox systems found in other cells and provide a means to attribute the NADH-ferricyanide reductase capacity of erythrocyte plasma membranes to VDAC. An important distinguishing characteristic of electron transport in VDAC is the extreme sensitivity to thiol inhibitors such as mercurials or lead [27]. Thus the electron transport by ferricyanide reductase of erythrocytes is 100% inhibited by micromolar levels of (1.0 μM) PCMB, whereas rat liver plasma membrane ferricyanide reduction is only partially inhibited (15%) by a PCMB concentration 100 times greater (100 μM) (Table 1). These larger 100

μM PCMB concentrations were also needed to inhibit ferricyanide reduction in preparations of HeLa cell plasma membranes where a 55% inhibition was noted. These results highlight the unique sensitivity of the erythrocyte plasma membrane system used in this study when compared to plasma membrane preparations of other cell types. The contrasting sensitivities for mercurial compounds between the cell lines tested indicates that erythrocyte membranes have key differences, likely attributed to VDAC exclusively when compared with other plasma membranes which have a ancillary ferricyanide reductase capacity and are therefore less sensitive to mercurial inhibition (Table 1).



Teske, B.F. et al.

Figure 2. Coenzyme Q is required for NADH-Ferricyanide reduction. (A) Ferricyanide reductase activity was measured for erythrocyte PM preparations (control). Membranes that were lipid depleted with heptane extraction (extracted) or rescued with 10μM Coenzyme Q (Extracted + Q) were also measured for Ferricyanide reduction. (B) NADH-Ferricyanide reductase activity was measured in the presence of CoQ analogs or in the presence the CoQ analog with Q rescue as indicated. Data is represented as the mean values of three independent experiments.

Further evidence for coenzyme Q function in VDAC is shown by two additional lines of investigation. The first experiment in this series exhibited a requirement of CoQ_{10} for NADH-ferricyanide reduction demonstrated after the depletion of CoQ_{10} by heptane extraction of erythrocyte membrane preparations (Figure 2A). This lipid depletion caused an 81% decrease in ferricyanide reduction, an activity that was restored by the addition of CoQ_{10}.

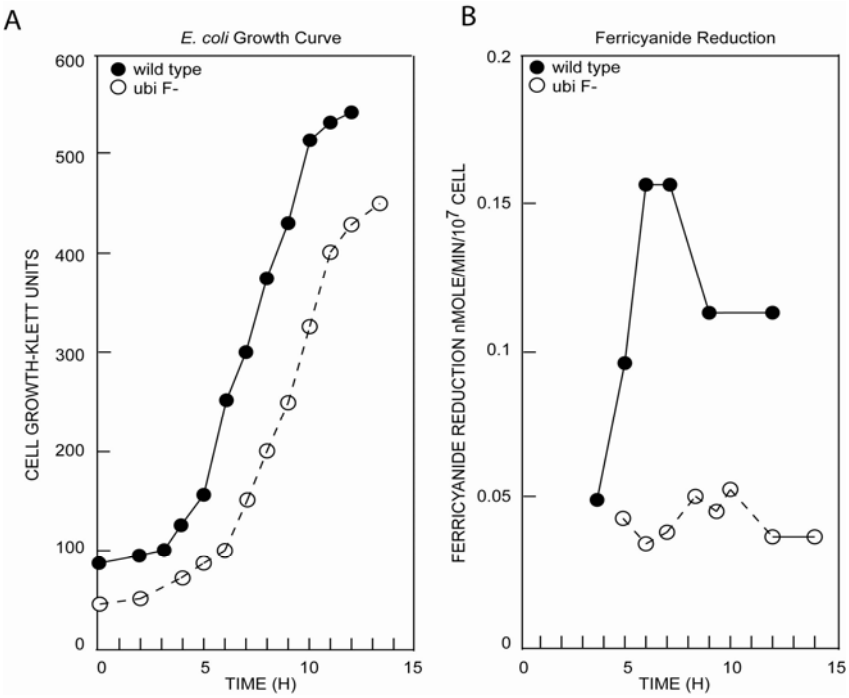
Thus it appeared that coenzyme Q was required for the NADH-ferricyanide reductase activity of VDAC as proposed [28]. The second experiment featured a more specific line of experimentation by the addition of the Coenzyme Q analogs, such as EthoxycoQ or DichloroQ, to erythrocyte membrane preparations. EthoxycoQ caused a 90% inhibition of NADH-ferricyanide reductase activity which was fully rescued with the addition of CoQ_{10} (Figure 2B). A more modest, 63%, decrease in ferricyanide reduction was noted in DichloroQ treated samples. This inhibition was similarly rescued with the addition of Coenzyme Q further indicating a direct contribution of coenzyme Q to the activity of NADH-ferricyanide reductase [29]. Taken together these experiments suggested that CoQ_{10} was required for VDAC mediated NADH-ferricyanide reductase activity of human erythrocytes PMs as proposed [28].

The requirement of coenzyme Q for the function of a pore protein, such as VDAC, is not without precedence, for example binding of coenzyme Q toUCP in the mitochondria is required for optimal function [7, 8]. Mechanistically, the requisite binding of coenzyme Q to VDAC may lower the effective redox potential of coenzyme Q from 100 mV to a value closer to the redox potential of thiol groups at approximately -225 mV. It is noted that there is also evidence for residual, chelator-sensitive iron in the plasma membrane, which may bridge any remaining redox potential gap [30, 31].

CoQ_{10} IS REQUIRED FOR NADH-FERRICYANIDE REDUCTION IN DIVERSE SPECIES

The CoQ_{10} requirement of NADH-ferricyanide reductase also extends to *E.coli*. In this line of experimentation both Wild type *E. coli* and *E. coli* deficient for CoQ (*ubi F-)* were analyzed. Figure 3A depicts a growth comparison between Wild type and ubi F- mutants. Here, the *E. coli* strain deficient for coenzyme Q displayed a less robust growth phenotype when compared with the wild type strain. E coli were also tested for NADH-ferricyanide reductase activity. The CoQ deficient *ubi F- E. coli* had a diminished ability to reduce ferricyanide with the largest 3-fold difference detected during the mid-log growth phase between 6 and 8 hours. To determine if the defect in ferricyanide reduction was due specifically to CoQ deficiency the Q deficient *ubi F-* strain was supplemented with either CoQ_1 or CoQ_8 or no treatment (Figure 4). In this experiment the ferricyanide reductase activity was measured with increasing concentrations of ferricyanide substrate

present 1-10 μM. Importantly, the addition of CoQ_1 and to a lesser extent CoQ_8 was able to rescue the NADH-Ferricyanide reductase activity to near wildtype levels. Ferricyanide reductase activity was also determined for the *fepA- E. coli* strain. This strain has a mutation in the outmembrane porin OmpF and exhibited a 5-fold decrease in ferricyanide reductase activity compared to WT control (Table 2). Collectively this data suggests that CoQ and the PM electron transport system are required for the NADH-ferricyanide reductase activity of VDAC in a diversity of species. This evolutionary conservation suggests that the CoQ and VDAC connection is a key tenant for PM electron transport and ferricyanide reduction and proposed to be a general feature of other membrane pore proteins.

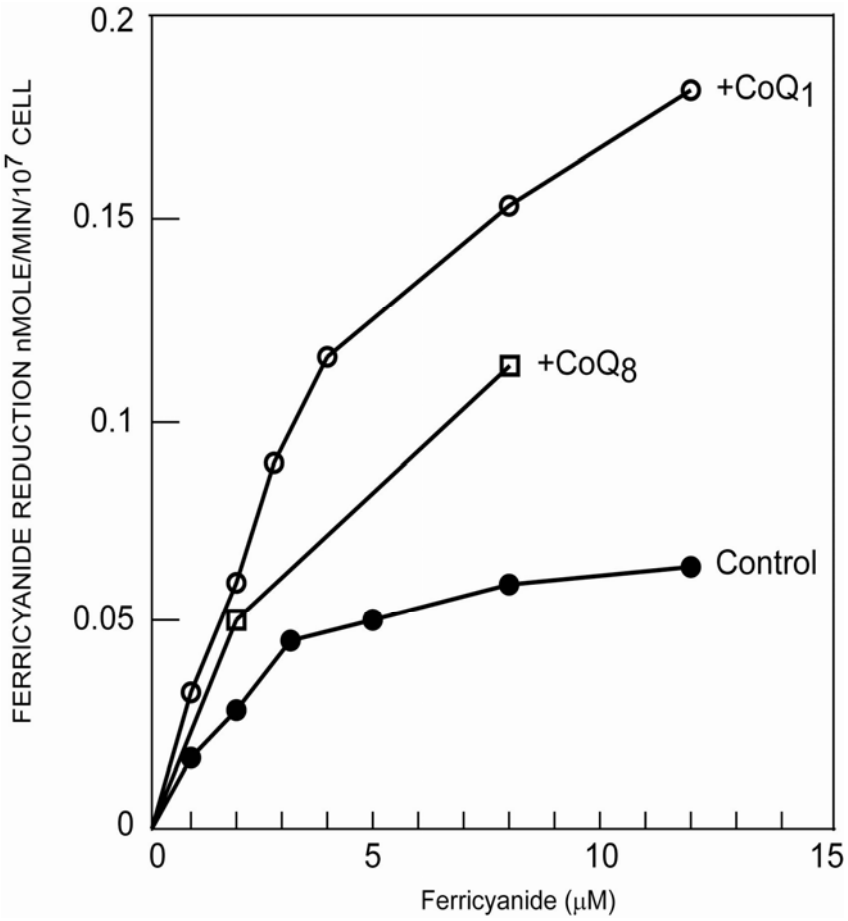


Teske, BF et al.

Figure 3. Coenzyme Q deficiency causes a reduced capacity for growth and $FeCN_6$ reduction in *E. Coli.* (A) Wildtype *E.coli* AN704 (wt) and *E. coli* deficient for Coenzyme Q AN761 (ubi F^-) were grown on complete media supplemented with 2,3 dihydrobenzoate and delta-aminolevulinic acid, growth was monitored at time points indicated. (B) Wildtype *E. coli* and *E. coli* deficient for Coenzyme Q (ubi F-) were assayed for ferricyanide reductase activity at the time points indicated.

Table 2. Ferricyanide reduction in *E.coli.*

Strain	growth (klet units)	Ferricyanide reduction (nmole/min/10^7 cells)
WT	300	0.16
fepA-	100	0.03
ubi-	225	0.05



Teske, BF et al.

Figure 4. CoQ supplementation improves $Fe(CN)_6$ reduction in Q deficient *E. coli.* Addition of coenzyme Q rescues ferricyanide reduction in E. coli deficient for coenzyme Q. E. Coli AN761 deficient for coenzyme Q was incubated with CoQ_1 (0.06mM), CoQ_8 (0.04 mM) or untreated. Ferricyanide reductase activity was measured with increasing levels of $K_3Fe(CN)_6$ as indicated.

VDAC AND REDOX CONTROL OF PORIN IN AUTISM

Experimental evidence is rapidly emerging linking VDAC dysregulation to autism spectrum disorders and other neurological conditions [32]. With this in mind the requirement of CoQ_{10} for the function of VDAC may provide an avenue of therapeutic potential for the treatment of these debilitating conditions. The preliminary studies in this report provide an important connection between CoQ, VDAC, and electron transport of porin. One of the foundational studies linking VDAC to autism was a study by Gozalez-Gronow [33]. In this study an increase in VDAC autoantibodies in autistic patients was discovered. Other immunological links from Sun, I.L., et al. showed VDAC electron transport is inhibited by cytokines [34]. The cytokines TNFa and IL2 that inhibit plasma membrane ferricyanide reduction [34] are increased in autism [35, 36]. Similar to VDAC dysregulation, deficiencies and alterations in other PM redox systems have been implicated in autistic patients. It has been reported that in triiodothyronine (T3) has properties in stimulating the plasma membrane oxidase which could represent an important link between thyroid hormone deficiencies and reduced plasma membrane redox function [37]. Deficiencies in T3 and other thyroid hormones, which are necessary for neuronal migration and fetal brain development, have been noted in autistic patients [38, 39]. Glutathione is low in autism, which may decrease electron transport through VDAC. There are also documented positive effect of hyperbaric oxygen on autism [40] which can be based on stimulation of the plasma membrane oxidase. Serum profiles of autistic patients also indicate that the levels of ceruloplasmin are diminished [41]. Ceruloplasmin can act as an ascorbate oxidase and acts to stimulate the reductase cycling of the ascorbic free radical oxidoreductase by increasing the availability of substrate. Although this last point is still under investigation it should be mentioned that ascorbic acid supplementation has been the subject of preliminary trials as a therapy for autism. Since ascorbate is also involved in transmembrane oxidation systems it should be considered along with CoQ for combinatorial treatment of autism.

CONCLUDING REMARKS

Attack on the VDAC pore is proposed to be the underlying basis for a specific disease such as autism whereas chronic oxidation through oxygen

radicals would be suggested to contribute to a more pleiotropic range of neurological conditions such as diseases of aging. Therefore, attempts to relate the antioxidant affects to damage of a specific enzyme is not very successful and in its most unspecific form would tend to generate clusters of diseases. Broader age related diseases would more likely be base on oxidative damage to a specific functional system in this case the effect could be from selective damage to a key component or deficiency of many components. Therefore inhibition of electron transport increases apoptosis and maintenance of electron flux through the PM transport system would encourage cell survival [42].

METHODS

E. Coli Growth and NADH-FeCN6 Reduction

The Escherichia coli K12 strains (*ilv C⁻, arg H⁻, ent A⁻, hem A⁻ and leu⁻)* were provided by F. Gibson and G. B. Cox (Australian National University). Strains were grown on media with growth supplements as described (cox gb (1977) BBA 462 113-120). Cells were grown in a 37 degree C shaker and 1 ml aliquots were taken at hourly intervals. Growth was determined using a Klett colorimeter. For ferricyanide reduction experiments cell samples were centrifuged in the cold room and the supernatant was discarded. Samples were resuspended to a total volume of 3.0 ml in 0.1 M potassium phosphate buffer pH 7.0. $FeCN_6$ reduction was measured using an Aminco DW-2a spectrophotometer in the dual wavelength mode subtracting absorbance at 500 nm from absorbance at 420 nm. The change in rate of absorbtion was determined for two to five minutes before addition of FeCN6 to a final concentration of 5 μM. Rates were measured at 20 degrees C. For CoQ rescue experiments CoQ was added in ethanol.

Erythrocyte PM Preparation

Human erythrocyte PMs were prepared from blood bank erythrocytes with final separation on dextran gradients as described [43]. PM preparations were subject to heptane extraction in the dark for 4-6 hours at room temperature. CoQ_{10} was added to the CoQ extracted membranes in heptane, the heptane was removed by evaporation prior to analysis of NADH-$FeCN_6$ activity.

NADH-$FeCH_6$ activity was determined spectrophotometrically by following the decrease in absorbance at 420 nm. The assay was carried out in sodium phosphate buffer (100 mM, pH 7.0) containing 0.17 mM NADH and 0.35 mM $K_3Fe(CN)_6$ and the reactions were carried out at room temperature. Coenzyme Q and analogs were added from a stock solution prepared in ethanol.

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