SURFACE POTENTIAL CHANGES ON ENERGIZATION OF THE MITOCHONDRIAL INNER MEMBRANE

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1. Introduction

One of the postulates of the chemiosmotic hypothesis for oxidative phosphorylation is the requirement for an electrochemical proton gradient across the inner mitochondrial membrane [1]. This gradient is made up of two contributions: a transmembrane potential and a pH difference [2]. In mitochondria, the pH difference is always small (estimated to be $\leqslant 1$ pH unit) whereas the transmembrane potential can be as high as 180 mV [3–5].

pH and ionic strength influence the surface potential of charged phospholipid membranes [6]. Previous reports of Schäfer and Rowohl-Quisthoudt [7] and Mehlhorn and Packer [8] show the influence of modification of surface potentials on the mitochondrial proton pump; in particular, the H^+ pump [7] and respiration [8] are inhibited when the surface charge is shifted more positive. A change in the transmembrane potential may also modify the surface potential of the inner mitochondrial membrane.

The partition of impermeable charged paramagnetic amphiphiles between phospholipid membranes and their aqueous environment strongly depends on the electrostatic surface potential of the membranes [9,10]; this technique was used to show that the surface potential of charged phospholipid vesicles depends on the ionic aqueous concentration [10] in a way similar to that predicted by the Gouy equation [11].

In this report we have used impermeable charged paramagnetic amphiphiles to show how energization of the inner mitochondrial membrane can affect its outer surface potential.

2. Materials and methods

2.1. *Preparations and assays*

Rat liver mitochondria were prepared in a 0.25 M sucrose, 1 mM Tris base and 1 mM EDTA medium, at pH 7.6, as previously described [12]. Mitoplasts (mitochondria depleted of outer membrane) were then prepared as described [13] and suspended in a 0.25 M sucrose and 1 mM Tris base (pH 7.6) medium at 20 mg protein/ml. Two different incubation media were used: (a) 0.24 M sucrose and 10 mM Tris base (pH 7.6) and (b) 0.14 M KCl and 10 mM Tris base (pH 7.6). Energization of the mitoplasts followed the addition of ATP and de-energized mitoplasts were obtained by the addition of oligomycin. Oxidizable substrates were not used to energize the mitoplasts because electron transport reduces the spin label [14]. Analogues of ATP such as GTP, ITP and cyclic-AMP were also used. The effect of pH was studied for both media (a) and (b) in the range of pH 5–9. Spin label and fluorescent probes were used at concentrations (~14 nmol/mg protein) where no effect on the rate of respiration by succinate, the state of coupling or swelling at 620 nm of the mitoplasts could be detected.

2.2. *Surface potentials*

Changes in the partition between the membrane and the aqueous environment of the positively-charged spin-labeled detergent 4-(dodecyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT_{12}) (fig.1) and of the uncharged spin label 2,2-dimethyl-5,5-methylnonyl-*N*-oxazolidinyloxyl (2N11) (fig.2) synthesized by Dr R. J. Mehlhorn of

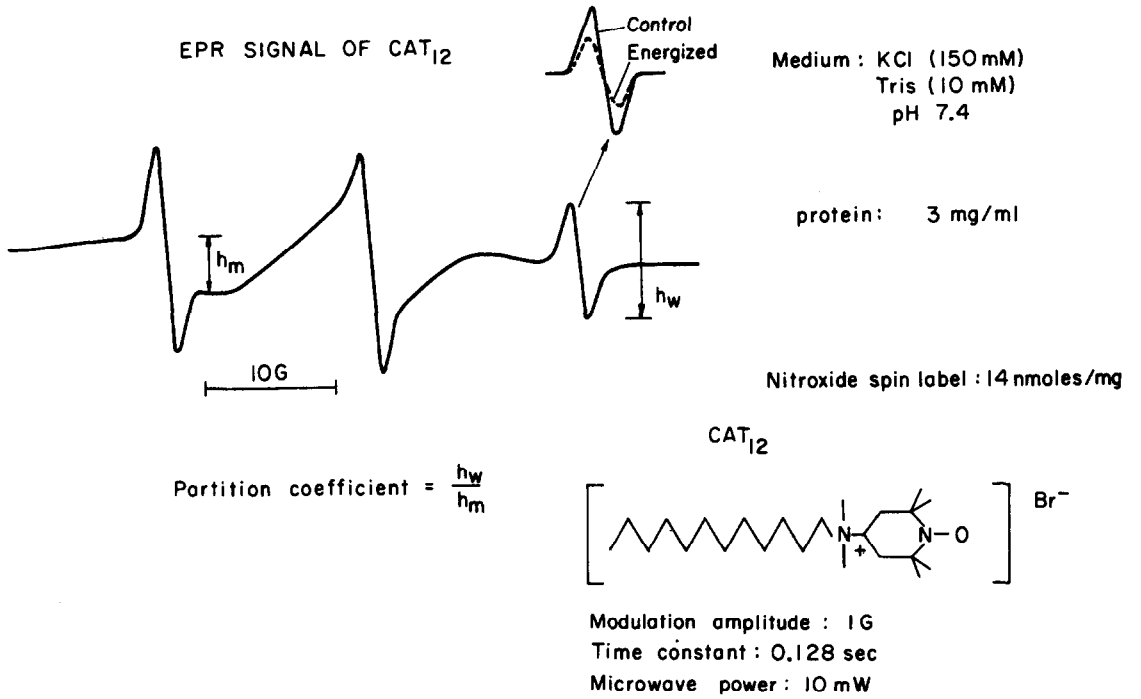

Fig.1. Structure and spectrum of the CAT_{12} spin label. This spin label has a localized positive charge and is impermeable to the inner membrane. Changes are due to energization with ATP as shown (see insert).

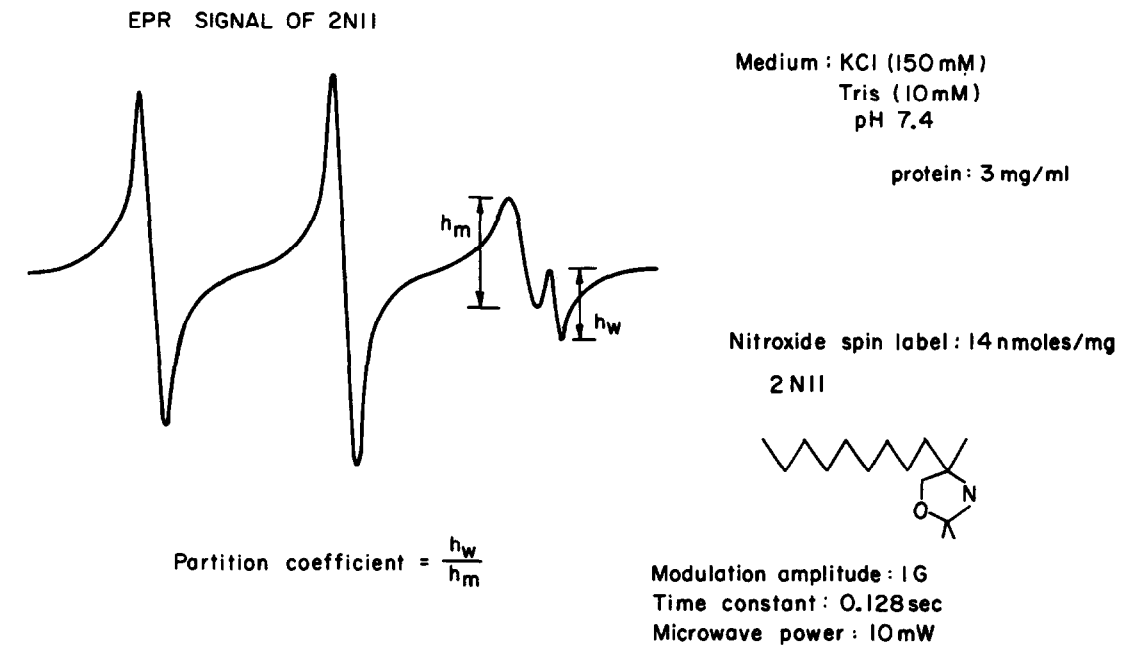

Fig.2. Structure and spectrum of the 2N11 spin label. This spin label is uncharged and does not respond to changes in membrane charge distributions.

our laboratory, were recorded in a Varian E109 spectrometer at room temperature. Assays were carried out in media (a) and (b) at mitoplast protein concentrations of 3 mg/ml and spin label concentrations of 14 nmol/mg protein.

The partition P is usually defined as the ratio of spin label concentration in the aqueous medium to the concentration on the membrane. However, since the partition of the spin labels is such that more than 90% is always on the membrane, we will consider the membrane concentration of the spin labels to be essentially constant and take P simply as the ratio of the water to the membrane signals (figs 1 and 2) provided the line width of the aqueous component remains constant.

To determine the changes in surface potential $\Delta\psi_s$ in mV as a function of changes in partition P we follow the procedure described in [9], viz.

$$\Delta\psi_s = \frac{RT}{ZF} \ln \frac{P_1}{P_2}, \quad (1)$$

where P_1 is the partition in say state 1, P_2 the partition in state 2, Z is the charge on the spin label which is equal to 1 in the case of CAT_{12}, F is the Faraday constant, R the universal gas constant and T the absolute temperature.

2.3. *Fluorescence quenching of cyanine dyes*

Changes in relative fluorescence intensity of the cyanine dye 3,3′-dipropylthiodicarbocyanine, denoted diS-C_3-(5), kindly supplied by Dr M. Miyahara (Tokyo University) were recorded in a Perkin-Elmer MPF44A spectrofluorometer at room temperature [15]. Media used were (a) and (b) above and assaying conditions were: 3 ml incubation medium, 0.25 mg mitoplast protein/ml and 10 μl dye (0.5 mg/ml ethanol). Higher concentrations of mitoplast protein were also used and the results obtained found to be qualitatively similar but the fluorescence changes were much smaller. Excitation was at 620 nm and measurements at 670 nm.

3. Results

The effect of energization with ATP and de-energization in the presence of oligomycin on the partition coefficient P of CAT_{12} (14 μmol/mg protein) in a sucrose (0.25 M) and a KCl (0.15 M) medium is shown in table 1. GTP and ITP change the partition only slightly and the changes are oligomycin insensitive. Identical results are obtained in non-ionic or ionic buffered media.

Table 1
Dependence of CAT_{12} spin label partitioning on adenine nucleotides

Additions	Partition coefficient (P)	
	Sucrose medium	KCl medium
None	2.0	6.0
ATP	0.7	2.8
ATP + Oligomycin	1.5	4.6
GTP or ITP	1.5	4.5

Adenine nucleotides (1.33 mM) and oligomycin (3 μg/mg mitochondrial protein) were tested in 0.25 M sucrose or 0.15 M KCl medium.

The positively-charged detergent, cetyl trimethyl ammonium bromide ($CTAB^+$), at concentrations of 10 nmol/mg protein, is known to partition exclusively into the membrane [14]; it increases P in either the sucrose or the KCl media, whether in the energized or de-energized states of the mitoplasts. P can also be increased by the addition of Mg^{2+}, Mn^{2+} (20 nmol/mg protein) or decreasing the pH of the medium (fig.3)

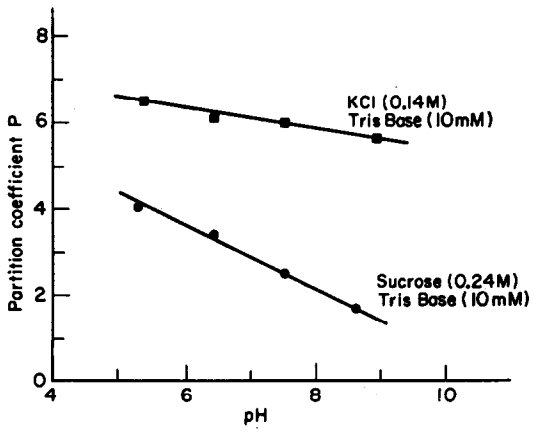

Fig.3. The pH-dependence of the partition of CAT_{12}. Protein concentration was 3 mg/ml, media as indicated.

but the increase is greater in the non-ionic sucrose medium in accordance with predictions of the Gouy theory [11]. However, the addition of equivalent amounts of the cyanine dye or even higher amounts of the uncharged detergents Triton-X or digitonin (which also partition mainly on the membrane) do not change P appreciably in either media.

When the values of the partition coefficient for the energized (+ ATP) and de-energized (+ ATP + oligomycin) states (table 1) are used in eq. (1), the surface potential of the outer surface of the mitoplasts is found to be 20 mV more negative when energized in sucrose, whereas in KCl medium the corresponding value is 17 mV. Control studies indicate that:

(a) The changes in partitioning upon addition of GTP, ITP or cyclic-AMP (not shown) are oligomycin insensitive and that therefore part of the partition change on addition of ATP is probably due to binding of CAT_{12} to ATP; this is also observed in the absence of mitoplasts.

(b) The partition of the uncharged spin label 2N11 is not altered by energization, de-energization or the addition of the strongly membrane partitioning positively-charged $CTAB^+$.

The cyanine dye we use has been shown [16] to be a useful qualitative indicator of changes in transmembrane potential in mitochondria energized with ATP. The percentage of cyanine dye bound to energized and non-energized mitoplasts is at least 90% and 80% respectively, in good agreement with other results in red blood cells [17]. Figure 4 shows the changes in fluorescence of the cyanine dye upon energization with ATP and de-energization by oligomycin in the KCl medium (the same is observed in the sucrose medium); also indicated are the changes in fluorescence produced by the addition of CAT_{12} (10 nmol/mg protein). Qualitatively similar results can be obtained upon addition of Mg^{2+} and Mn^{2+} at 20 nmol/mg protein but the effects are much smaller in the KCl medium. In control studies it is found that:

(a) The addition of up to 40 nmoles/mg protein of either the uncharged detergent Triton-X or the spin label 2N11 has no effect on the fluorescence of the cyanine dye.

(b) The addition of equivalent amounts of GTP, ITP or cyclic-AMP decrease the fluorescence of the cyanine dye only slightly, yet the effect is oligomycin insensitive.

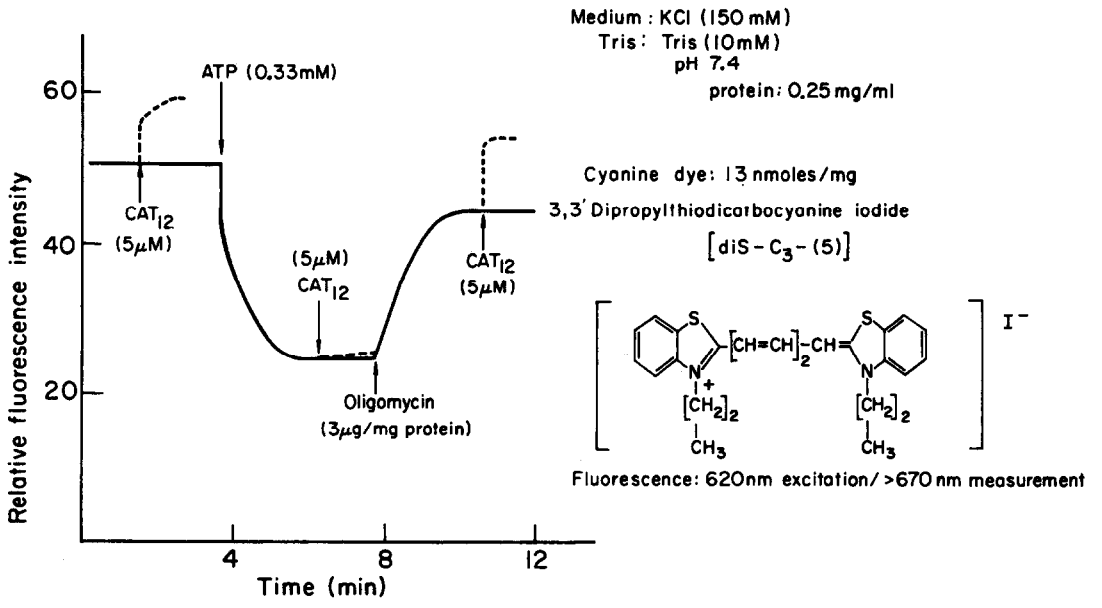

Fig.4. Structure and fluorescence response to energization and de-energization of the cyanine dye diS-C_3-(5). Changes due to addition of CAT_{12} are also shown.

4. Discussion

Two main findings have emerged from this investigation. First, that the positively-charged amphipathic spin label probe CAT_{12}, which partitions between membrane and aqueous domains, is a useful indicator of changes in surface charge in mitoplasts; and second that upon energization negative charges appear at the outer surface of the inner membrane.

With respect to the first finding, the evidence for CAT_{12} serving as a new and useful probe for measuring surface potentials may be briefly summarized as follows:

(a) $CTAB^+$ increases the partition P of CAT_{12} whether in the sucrose or the KCl medium in both the energized and de-energized states while Mg^{2+} and Mn^{2+} increase P of CAT_{12} appreciably only in the sucrose medium.

(b) CAT_{12}, like Mg^{2+} and Mn^{2+}, does not have any effect on the fluorescence of the cyanine dye once the mitoplast is energized; we conclude that in the above state CAT_{12} and the cyanine dyes are on opposite sides of the membrane and also that, if CAT_{12} 'flip-flops', this process is negligible in the time scale required to measure its partition coefficient P (~1 min). Moreover, the positively-charged amphipathic spin label probe CAT_9, which has a shorter hydrocarbon chain than CAT_{12}, has already been shown by Castle and Hubble [10] to be a useful indicator of changes in surface charge and to 'flip-flop' very slowly in liposomes. The reason why the cyanine dye is permeable to membranes and both CAT_9 and CAT_{12} are not, may be that positive charges in the latter are localized whereas in the former they are delocalized.

With regard to the second finding that more negative groups appear on the outer surface of the inner mitochondrial membrane, the question arises as to their origin and significance. Although transmembrane potentials as high as 180 mV can be established across the inner membrane the change in the outer surface potential of this membrane is small, of the order of 17–20 mV (more negative in the energized state). Since changes in surface potential are small in relation to the transmembrane potential, it follows that only slight changes in surface charge will allow for regulation of the electrochemical gradient across the membrane. This would explain previous findings [8,7] showing inactivation of mitochondrial respiration and respiration linked proton translocation caused by adding very small quantities of impermeable amphipathic charged molecules.

Acknowledgements

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