Biochimica et Biophysica Acta, 764 (1984) 55–62
Elsevier

BBA 41434

MEMBRANE POTENTIAL AND SURFACE POTENTIAL IN MITOCHONDRIA

BINDING OF A CATIONIC SPIN PROBE

KEN HASHIMOTO, PAUL ANGIOLILLO and HAGAI ROTTENBERG

Department of Pathology, Hahnemann University School of Medicine, 230 North Broad Street, Philadelphia, PA 19102 (U.S.A.)

(Received July 13th, 1983)

Key words: Membrane potential; Surface potential; Mitochondrial membrane; Spin probe; Oxidative phosphorylation

The interaction of the cationic spin probe 4-(*N*,*N*-dimethyl-*N*-dodecyl)-ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (Cat_{12}) with intact mitochondria and submitochondrial particles was investigated as a function of salt concentration, pH and energization by ATP. In the presence of 1 mM $Fe(CN)_6^{-3}$, which inhibits the probe reduction by the mitochondria, the probe signal is stable and shows both bound and free forms. The partition of the probe into mitochondrial membranes is decreased by various salts depending on the cation valency, indicating that the membrane is negatively charged (−10 to −15 mV at pH 7.0). The surface potential increases with pH from −3 mV at pH 5.0 to −18 mV at pH 8.0. Energization of intact mitochondria by ATP reduces the magnitude of both bound and free signals by more than 50%; the signal of the bound form slowly disappears on further incubation. The ATP effect is inhibited and also reversed by either oligomycin or CCCP. Similar effects of ATP were observed in mitoplasts but not in submitochondrial particles. In submitochondrial particles ATP has no effect on the probe signal or binding. These results suggest that the formation of membrane potential in mitochondria induces uptake and internal binding of the probe which results in broadening of the EPR signal of the internally bound probe. It is concluded that Cat_{12} is not a suitable probe for measurement of surface potential in energized mitochondria.

Introduction

Substrate oxidation or ATP hydrolysis by intact mitochondria generates a large negative membrane potential (for review see Ref. 1). In mitochondria the membrane potential is the major component of the proton electrochemical potential which is believed to be the driving force for ATP synthesis [2,3]. The accurate estimation of the potential is crucial for the elucidation of the mechanism and energetics of oxidative phosphorylation [1–4]. Recently, it was suggested that energization of intact mitochondria significantly increases the negative surface charge on the cytosolic surface of the inner mitochondrial membrane [5–9]. This conclusion, if valid, has important implications concerning the mechanism of oxidative phosphorylation and also in relation to the validity of the estimation of membrane potential in mitochondria. Many of the probes used for estimation of membrane potential bind to the membrane and their response to $\Delta\Psi$ would be affected by changes of surface charge [10,11]. However, recent studies in our laboratory do not support the conclusion that energization of mitochondria significantly increases the negative surface charge. We have rigorously tested the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Cat_{12}, 4-(*N*,*N*-dimethyl-*N*-dodecyl)-ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl); Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

suggestion that the quenching of ANS fluorescence in energized mitochondria is due to an increase in the negative surface charge [5–7] and have concluded that this is not the case [12]. We have also studied the binding and phosphorescence of Tb^{3+}, as a measure of the surface potential, and could not detect a significant increase in the membrane surface charge [14].

In this study we employ the positively charged spin probe 4-(*N*,*N*-dimethyl-*N*-dodecyl)-ammonium-2,2,6,6-tetramethyl-piperidine-1-oxyl (Cat_{12}) to study the surface charge in mitochondria and submitochondrial particles. It was previously shown that the binding of positively charged spin probes to phospholipid membranes is determined by both the surface charge and the membrane potential [10,11]. However, those probes which do not permeate the membrane respond only to the external surface charge. Quintanilha and Packer [9] have studied the binding of Cat_{12} to mitoplasts and have concluded that energization by ATP increases the negative surface potential by −20 mV. This conclusion is based on the implicit assumption that the probe is impermeable. Our results do not support their conclusion. We show that, although Cat_{12} responds to the surface charge in non-energized mitochondria, the amplitude of the total probe signal is reduced after the addition of ATP, apparently as a result of potential driven transport into the mitochondrial matrix. Hence, the probe response is determined by the membrane potential and does not allow estimation of the surface potential.

Materials and Methods

Isolation of liver mitochondria from male Sprague-Dawley rats by differential centrifugation [13] and preparation of mitoplasts and submitochondrial particles by Yeda press disruption [14] were as previously described. Protein was determined by the biuret method [15]. All EPR spectra were obtained with a Varian E-9 spectrometer. In most experiments the probe concentration was 50 μM and the mitochondrial content 8–10 mg protein/ml. Experiments with non-energized mitochondria were carried out at a low temperature (4–7°C) to minimize probe reduction. Experiments with ATP were carried out at 25°C with 1 mM $K_3Fe(CN)_6$ to minimize probe reduction. Total signal intensity is calculated from the height of the midfield line and normalized for signal amplification. The ratio of bound-to-free probe is estimated from the heights of the upfield 'hump' and upfield line as described previously [16]. Cat_{12} was obtained from Molecular Probes. All other chemicals were of reagent grade.

Results

Incubation of the cationic spin probe Cat_{12} with mitochondria resulted in a progressive reduction of the probe and disappearance of the EPR signal [17]. The rate of reduction depends on the mitochondrial concentration and has a very high temperature coefficient, suggesting that an enzymatic activity of the mitochondria is responsible for the probe reduction. While we observed similar reduction with neutral probes such as doxyldecane (5N10) and anionic probes such as doxyl stearic acid, the rate of reduction of the cationic probes is

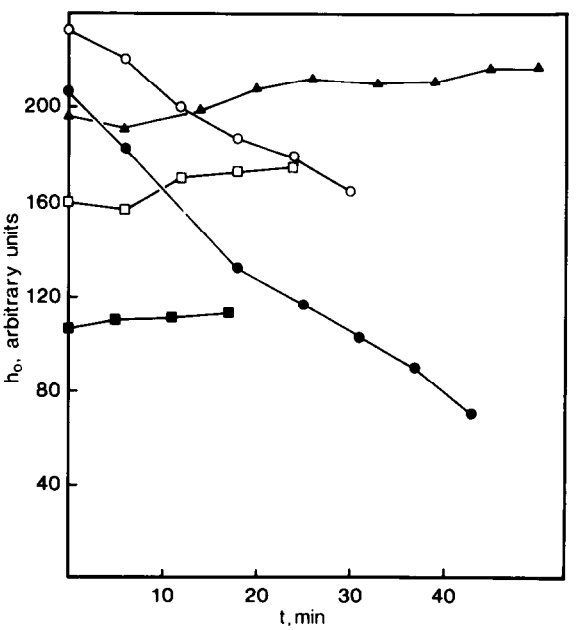

Fig. 1. The effect of $K_3Fe(CN)_6$ on the amplitude (h_0) and the rate of reduction of the EPR signal of Cat_{12} in mitochondrial suspensions. Mitochondria (10 mg/ml) were incubated in 0.25 M sucrose/10 mM Hepes (pH 7.0)/50 μM Cat_{12} at 25°C, with the following concentration of $K_3Fe(CN)_6$: zero (●); 100 μM (○): 1 mM (▲); 10 mM (□); and 50 mM (■).

much greater. At temperatures above 25°C and with mitochondrial content of 10 mg protein/ml, 50 nmol of probe will be reduced within a few minutes. This problem is even more pronounced in energized mitochondria. Added substrate oxidation leads to extremely rapid reduction of the probe [17]. Energization by ATP also enhances probe reduction. However, this reduction can be inhibited by oxidants. Ferricyanide effectively inhibits the rate of probe reduction, but also broadens the EPR signal of the free probe and therefore could not be used at high concentration. Fig. 1 shows the effect of increasing concentrations of $K_3Fe(CN)_6$ on the signal amplitude and rate of reduction of Cat_{12} by mitochondria. At a concentration of 1 mM and above the reduction was effectively inhibited. However, the signal was considerably broadened, as expressed in the lowering of the signal amplitude. At 1 mM $K_3Fe(CN)_6$, the amplitude was lowered by only 10–15%, while the signal remained stable for more than 1 h. The slight rise in amplitude with time may reflect the slow reduction of $Fe(CN)_6^{-3}$ itself. In all of the experiments with ATP-induced energization we have included 1 mM $Fe(CN)_6^{-3}$ to inhibit probe reduction.

The partitioning of the cationic spin-probe Cat_{12} into the mitochondrial membrane depends on the surface potential [10–12]. The surface potential can be screened by various salts in a characteristic manner as described by the Gouy-Chapman theory [18]. Fig. 2 shows the effect of various salts on the partitioning of the probe and the calculated change of surface potential due to the salt addition. In the upper panel we show the ratio of bound to free probe in the suspension as determined from the composite EPR spectra [11]. As can be observed, all salts are effective in reducing

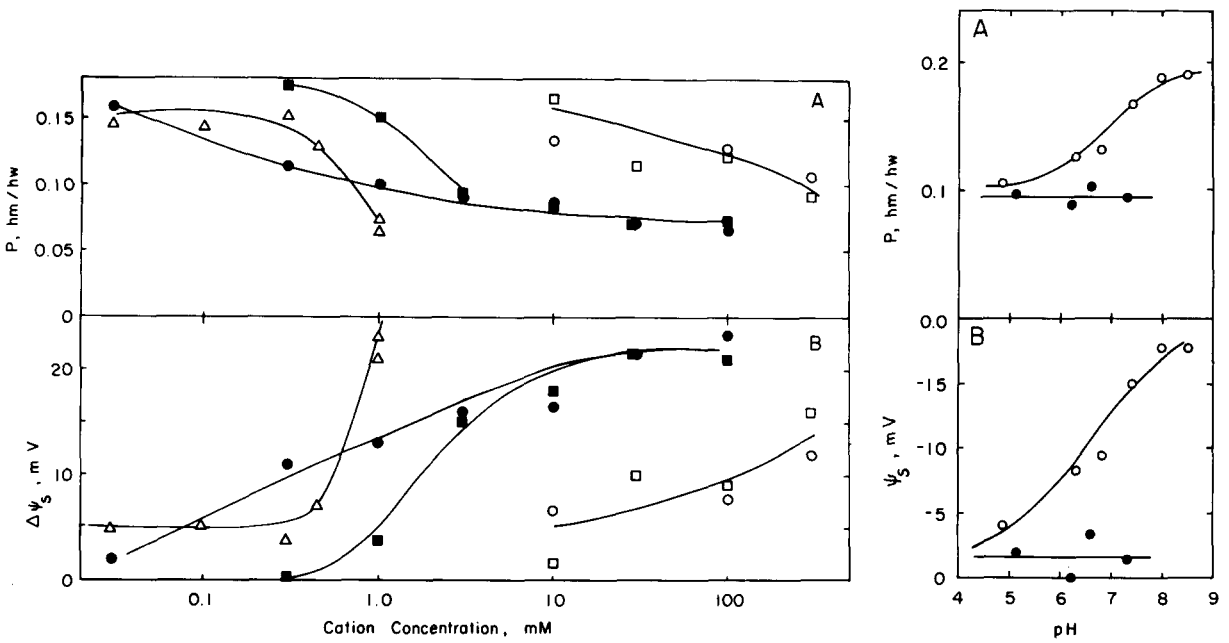

Fig. 2. The effect of salts on the partitioning of Cat_{12} in mitochondrial suspensions and the calculated effect on surface potential. Mitochondria, 8.1 mg/ml protein, were incubated in 0.25 M sucrose/10 mM Tris-Cl (pH 8.0)/4 μM rotenone/0.3 μg/ml antimycin. Probe concentration was 50 μM. Temperature 7°C. The following salts were added: NaCl (□); KCl (○); $CaCl_2$ (■); $MgCl_2$ (●); $LaCl_3$ (△) (A) Apparent partitioning (P) as calculated from the upfield amplitudes of the bound (hm) and free (hw) species. (B) Surface potential was calculated from the apparent partition with (P_2) and without salt (P_1) according to the relation $\Delta\Psi_s = (RT/F)\log(P_1/P_2)$.

Fig. 3. The effect of pH on the partition of Cat_{12} in mitochondrial suspension and the calculated surface potential. Mitochondria, 8.1 mg/ml protein, were suspended in 0.25 M sucrose/10 mM Mes or Hepes buffer at the indicated pH/5 μM rotenone/0.3 μg antimycin/ml. No other salt was added in one experiment (○), in a second experiment 5 mM $MgCl_2$ was included (●). Temperature 4°C. The partitioning (P) and the surface potential $\Delta\Psi_s$ are calculated as in Fig. 2.

the binding of Cat_{12}. The trivalent cation La^{+3} appears to be most effective followed by the divalent cations Mg^{2+} and Ca^{2+}, while K^+ and Na^+ are the least effective. Qualitatively, this dependence on valency is expected from the Gouy-Chapman theory, and it is therefore reasonable to assume that the salt effect is due to the screening of the surface potential. The change in surface potential can be calculated from the change in the partition coefficient according to the relationship $\Delta\Psi_s = (RT/ZF)\ln(P_1/P_2)$. The results of these calculations are shown in the lower panel. If the effect of the cations was entirely due to screening, all salt titrations should approach, at high concentration, the same limiting value. This is clearly not the case. This discrepancy is probably due to the binding of multivalent cations to the mitochondrial membrane. La^{3+} is known to bind to the mitochondrial membrane with high affinity and may change the net charge of the membrane to positive values. Ca^{2+} and Mg^{2+} also bind to the membrane, with lower affinity and may also change the net charge of the membrane [12]. Hence, the change in surface potential produced by multivalent cation is due both to screening and binding. From the change induced by monovalent salts, the surface potential estimated for these conditions is about -10 to -15 mV, in fair agreement with previous determination [5–9,12,14].

Fig. 3 shows the pH dependence of the partitioning and the calculated surface charge in the presence and absence of salt. Increasing the pH increases the surface potential of mitochondria. However, the potential is screened by the addition of salt. These results are in good agreement with the results of Quintanilha and Packer with Cat_{12} [9] and our previous results with ANS [12] and Tb^{3+} [14]. Quintanilha and Packer [9] also reported that ATP increased the negative surface charge of mitoplasts. This conclusion was based on the observation that the amplitude of the signal of the free probe is reduced by ATP. Fig. 4 shows the EPR spectra of Cat_{12} in mitochondrial suspension without ATP (a), with ATP (b), and with ATP and oligomycin (c). It is observed that in the presence of ATP the height of the signal of the free form (sharp peak at upfield position) is diminished as reported [9]. But, the total signal (centerfield peak) is diminished to a larger extent and, most

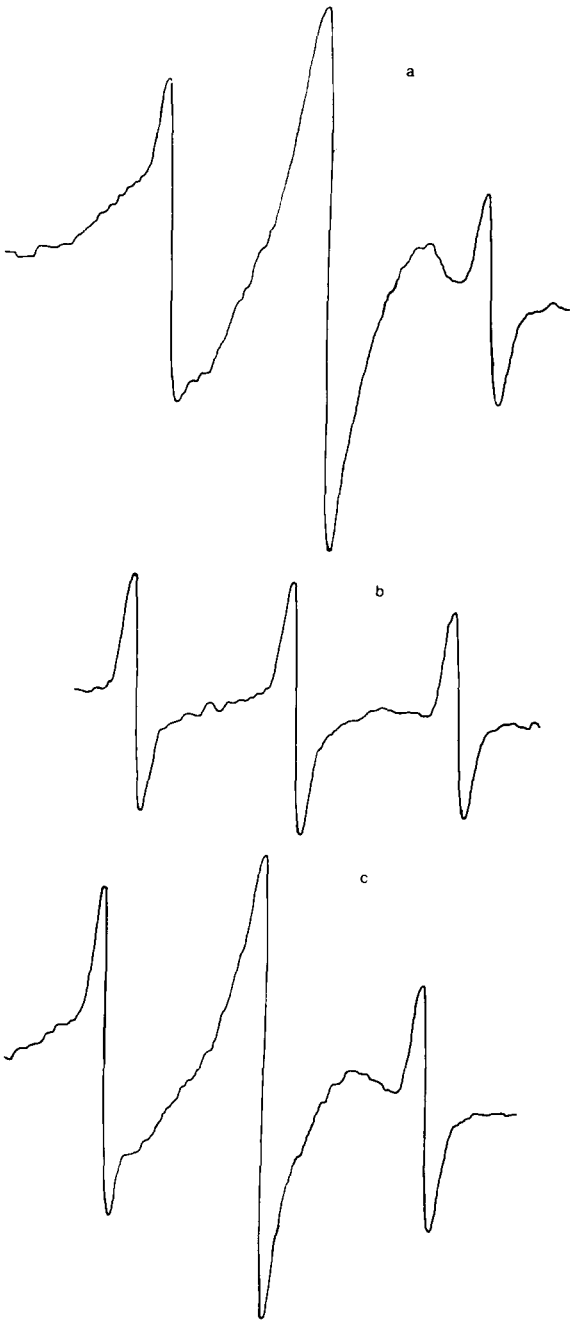

Fig. 4. The effect of ATP on the EPR spectrum of Cat_{12} in mitochondrial suspensions. The suspension comprised 10 mg/ml mitochondrial protein/2 μM rotenone/0.25 M sucrose/10 mM Hepes (pH 7.0)/1 mM $K_3Fe(CN)_6$/50 μM Cat_{12}. ATP (when added) was 10 mM and oligomycin 1 μg/mg protein. Temperature 25°C. Spectral scan, 100 G; microwave power, 5 mW; time constant, 0.128. Spectra shown are averaged of four scans. (a) No substrate; (b) +ATP; (c) +ATP+oligomycin.

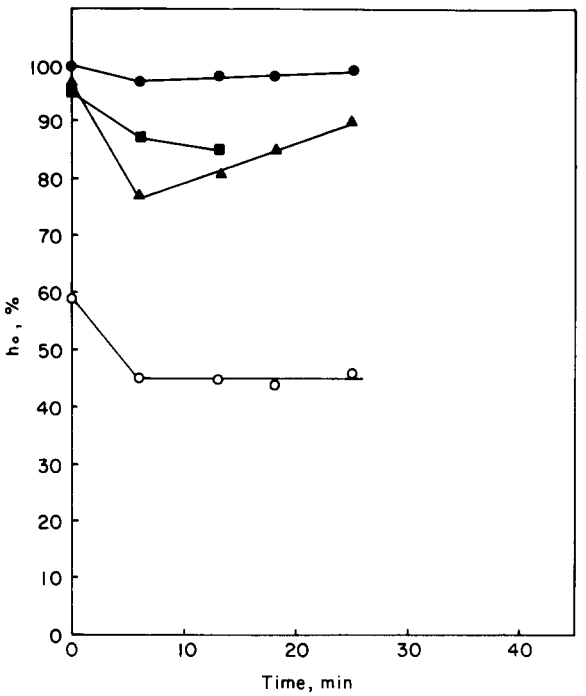

Fig. 5. Time-course of the effect of ATP on total probe signal in mitochondria. Conditions as in Fig. 4. CCCP (when added) was 1 μM. ●, Control; ■, ATP + oligomycin; ▲, ATP + CCCP; ○, ATP.

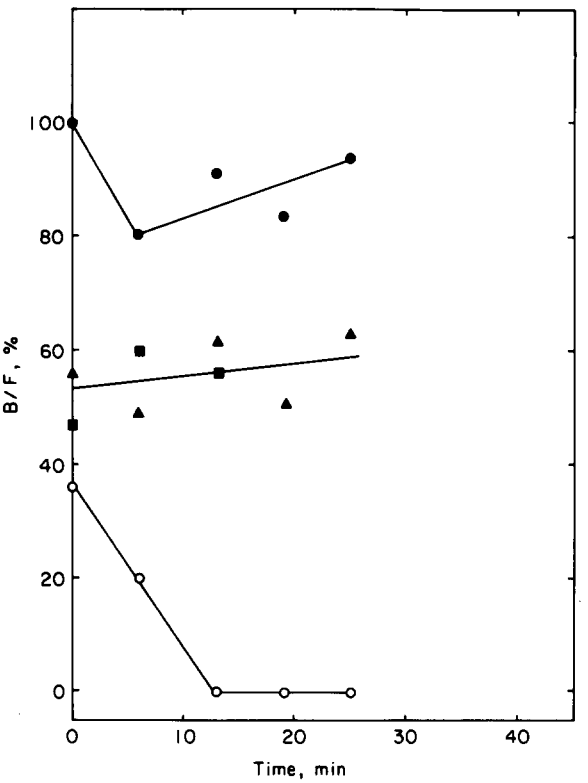

Fig. 6. Time-course of the effect of ATP on the bound/free ratio (B/F) of Cat_{12} mitochondria. The ratio was calculated as in Fig. 2. Results are from the same experiments shown in Fig. 5. Symbols as in Fig. 5.

importantly, the peak of the bound probe (hump at upfield position) disappears almost completely. Oligomycin partly prevents the ATP effect and can also reverse the effect if added after ATP (not shown). The time-course of the changes in the total signal intensity due to ATP addition is shown in Fig. 5. In the presence of 1 mM $K_3Fe(CN)_6$ the signal is stable over a 30 min period. Addition of ATP decreases the signal immediately by 40% and a further slow decrease of 15% is observed within the next 10 min. Oligomycin inhibits the ATP effect almost completely. CCCP markedly inhibits the effect, and after 10 min (probably due to depletion of ATP) the signal recovers further. Oligomycin and also CCCP reversed the signal decrease if added after ATP (not shown). The addition of salts does not affect the magnitude of the ATP effect; however, valinomycin (in the presence of K^+) abolishes the effect (not shown). These results indicate that the signal disappearance is due to energization and is reversible. Thus, it is not due to a chemical reduction of the probe. Most likely, the formation of a membrane potential, which may be prevented or reversed by inhibition of ATP hydrolysis or by uncoupling, leads to the broadening of the signal. Fig. 6 shows the ratio of bound to free probe as a function of time. ATP lowered the ratio immediately by more than 60% and further lowering took place slowly until no bound form could be detected. Both oligomycin and CCCP inhibited the effect of ATP on the ratio but to a lesser extent than the inhibition of the effect on the total signal.

We have also tested the ATP and salt effect on mitoplasts, and the results are essentially the same as in mitochondria (not shown); apparently the outer membrane does not contribute significantly to probe binding. To test the effect of membrane polarity (and the polarity of the membrane potential) on the partitioning of the probe we have studied the effect of ATP on probe partition in

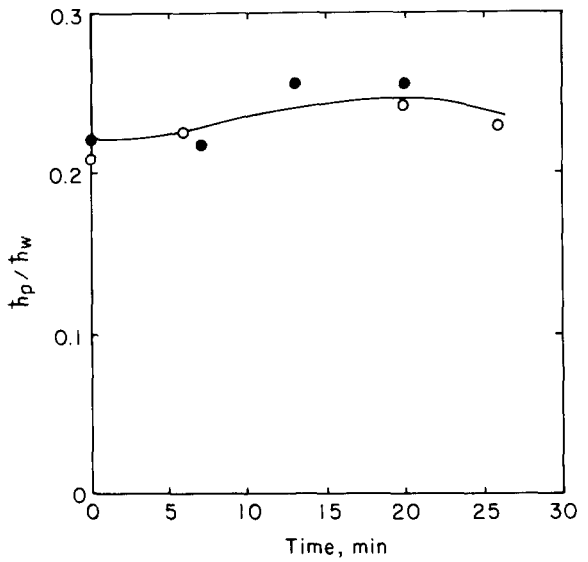

Fig. 7. The effect of ATP on Cat_{12} partition in submitochondrial particles. hp/hw ≡ B/F. Conditions are as in Fig. 4. ●, Control; ○, ATP.

submitochondrial particles. Fig. 7 shows the time-course of probe partitioning in the presence and absence of ATP in submitochondrial particles. No significant effect of ATP on probe partition is observed in this system. ATP also has no effect on the amplitude of the total signal in submitochondrial particles (not shown). In control experiments with these particles the addition of ATP generated a substantial membrane potential, as measured by the fluorescent probes ANS and oxanol (not shown). Since the membrane potential in submitochondrial particles is positive inside and the probe is positively charged, no uptake of probe is expected to result from energization in this system.

Discussion

Cat_{12} as a probe for surface potential measurements in mitochondria

The partitioning of charged molecules into lipids membranes is modulated by the membrane surface potential (for review see Ref. 18). In a suspension of vesicles, membrane impermeable amphiphiles will distribute, at equilibrium, between the suspending medium and the external surface of the vesicles. The apparent partition coefficient is a function of the surface potential and, since the surface potential may be screened by high concentration of salt, the ratio of the partition coefficient at high and low salt allows the estimation of the surface potential. The partition coefficient of charged spin probes can be estimated from their EPR spectra [10,19]. This method has been used to estimate the surface potential in liposomes [10], mitochondria [19] and purple membrane preparation [20]. Castle and Hubbell [10] have measured the rate of membrane translocation of a similar probe, dimethyl-nonyl-tempoylammonium, in phospholipid membrane and found a half-time for equilibration of approx. 10 h. Thus, in liposomes, measurement of probe partitioning after several minutes of incubation essentially yields the partition coefficient between the medium and the external surface. However, it is not possible to conclude that probes which are impermeable in liposomes are also impermeable in mitochondrial membranes. In general, biological membranes, and, in particular, mitochondria are much more permeable to ions than liposomes. Dilger et al. [21] have shown that this difference is due to the very low dielectric constant of phospholipid membrane as compared to mitochondria. The permeability of thiocyanate and perchlorate is up to 3000-fold greater in mitochondria than in pure phospholipid membranes. Thus, it is quite possible that Cat_{12} is transported across the mitochondrial membrane within the time scale of the EPR spectra collection. If the probe is equilibrating across the membrane within the time-scale of the experiment, the partition coefficient will reflect both the transmembrane potential and the surface charge on both sides of the membrane [11]. In non-energized mitochondria, where the transmembrane potential is low and the internal surface potential is mostly screened by the high concentration of internal salt, a major fraction of the probe is expected to be bound externally, even though the probe is free to equilibrate across the membranes. Thus, this fraction of the bound probe responds to the effect of external salt or external pH and appears to reflect correctly, at least qualitatively, the external surface charge. However, since the amount of internally bound probes depends on the membrane potential, which in non-energized mitochondria is largely

due to a Donnan potential, the value obtained by this method is not accurate.

The effect of energization on the EPR spectra of Cat_{12} in mitochondrial suspensions

It is not possible to study the effect of substrate induced energization on Cat_{12} binding, because substrate oxidation results in very rapid probe reduction [17]. ATP also substantially increases the rate of probe reduction. However, by using an electron transport inhibitor (rotenone) and an oxidant ($Fe(CN)_6^{-3}$), we were able to inhibit probe reduction and maintain a stable signal (at 25°) for more than 1 h even in the presence of ATP. We found that ATP greatly diminished the amplitude of the total probe signal and caused complete disappearance of the bound form. This change can be reversed by either oligomycin which inhibits the ATP hydrolysis or CCCP, which stimulates ATP hydrolysis but uncouples the mitochondria and collapses the membrane potential. The fact that the ATP effect is reversible indicates that there is no chemical reduction of the probe, but rather a change in probe binding and distribution which affects the amplitude of the signal. A reasonable explanation of the signal broading is as follows. Cat_{12} is a slowly permeable cation. When a membrane potential is generated by ATP hydrolysis, Cat_{12} is accumulated by the mitochondria, as are other permeable cations [1–4]. The signal from the internalized bound probe is broadened either by internal paramagnetic ions or by spin-spin interaction (see below). The magnitude of signal reduction (greater than 50%) is expected from the magnitude of the membrane potential and the amount of mitochondrial protein added. At 10 mg protein/ml the internal matrix volume is approx. 1% of the total suspension volume [1]. A potential of 120 mV would cause the probe to be 100-times more concentrated in the matrix (at equilibrium), resulting in accumulation of about 50% of the probe inside the matrix. Quintanilha and Packer [9] have concluded from the reduction of the free probe signal that there is increased probe binding due to an increased surface charge. However, if their interpretation is correct we expect to see an increase in the bound-probe signal and no change in total-probe signal. In fact, we found that the total-probe signal is reduced while the bound-probe signal disappears completely. The latter effect is apparently due to the similar transfer of the bound probe from the outer surface to the inner surface as a result of the establishment of the transmembrane potential. Since the internal and external surface area are about the same, most of the bound probe (over 99%) would be located internally which leads to disappearance of the signal. The internalization of the probe may also explain the increased rate of chemical reduction induced by ATP. If the probe is taken up, most of the internalized probe would be located on the surface of the membrane because of its high partition coefficient and very high surface to internal volume ratio. Since, in the presence of ATP, the external concentration of the probe is 25 μM, an accumulation factor of 100 and a partition coefficient of 30 would bring the internal surface concentration to 75 mM, which no doubt will result in signal broadening. Is it possible that the broadening and disappearance of the signal is due to binding of the probe to the external surface as a result of an increase in the surface potential? This interpretation [9] is rejected for the following reasons. (a) Salts which reduce the surface potential have no effect on the ATP induced change; and (b) the ATP effect does not depend strongly on the probe concentration. In most of our experiments with non-energized mitochondria, the signal of the bound form is readily detected even at a 3 nmol/mg protein of bound probe. When we reduce the added probe to a concentration of 20 μM the ATP effect was the same, even though the amount of probe signal that disappears corresponds to less than 1 nmol/mg protein. Hence, it appears that the transfer of probe across the membrane where it becomes highly concentrated is required for signal broadening. This interpretation of the disappearance of the probe signal is compatible with the absence of an ATP effect in submitochondrial particles. The generation of a membrane potential in these inverted vesicles does not result in cation accumulation.

Surface charge in mitochondria

In a recent investigation of surface charge in mitochondria [12], utilizing the negatively charged fluorescence probe ANS, we arrived at a similar conclusion, namely, that the probe is permeable

and hence distributes across the membrane. The existence of a transmembrane potential, in particular during energization, precludes an accurate determination of surface potential with ANS. As has already been pointed out [12], microelectrophoretic measurements [5,6,8] are also equivocal, since they may also respond to the generation of a transmembrane potential and may reflect changes in particle configuration and charge distribution. We have recently introduced the use of Tb^{3+} phosphorescence to estimate surface potential in mitochondria [14]. This probe does not permeate the mitochondrial membranes and it responds to salt and pH as expected for a surface potential probe. This probe does not indicate a significant change in surface potential of mitochondrial membranes on energization. In summary, the results of this study indicate that it is not possible to use the cationic spin probe, Cat_{12}, for the estimation of surface charge in energized mitochondria. The disappearance of the free signal is most likely the result of a transmembrane potential driven probe uptake which would mask any energy-dependent changes in the surface charge.

Acknowledgements

The authors thank Dr. J. Hoek for critical comments. This research was supported by grants GM28173 from the National Institute of General Medical Sciences, and AA3432 and AA5662 from the National Institute of Alcoholism and Alcohol Abuse.

References

1 Rottenberg, H. (1979) Methods Enzymol. 45, 547–569
2 Mitchell, P. (1966) Biol. Rev. 14, 445–502
3 Rottenberg, H. (1970) Eur. J. Biochem. 15, 22–28
4 Rottenberg, H. (1979) Biochem. Biophys. Acta 549, 225–253
5 Kamo, N., Murotsugu, M., Kurihara, K. and Kabatake, Y. (1976) FEBS Lett. 72, 247–250
6 Aiuchi, T., Kamo, N., Kurihara, K. and Kobatake, Y. (1977) Biochemistry 16, 1626–1630
7 Nalecz, M.J., Famulski, K.S., Dygas, A. and Vojtczak, L. (1982) 2nd EBEC short reports, LBTM-CNRS Edn., pp. 536–536
8 Archbald, G.P.R., Farrington, C.L., Lappin, S.A., McKay, A.M., Malpress, F.H. (1980) Biochem. Int. 1, 422–427
9 Quintanilha, A.T. and Packer, L. (1977) FEBS Lett. 78, 161–165
10 Castle, J.D. and Hubbell, W.L. (1976) Biochemistry 15, 4818–4831
11 Casifo, D.S. and Hubbell, W.L. (1981) Annu. Rev. Biophys. Bioeng. 10, 217–244
12 Robertson, D.E. and Rottenberg, H. (1983) J. Biol. Chem. 258, 11039–11048
13 Rottenberg, H., Robertson, D.E. and Rubin, E. (1980) Lab. Invest. 42, 318–326
14 Hashimoto, K. and Rottenberg, H. (1983) Biochemistry, in the press
15 Szarkowska, L. and Klingenberg, M. (1963) Biochem. Z. 338, 674–697
16 Waring, A.J., Rottenberg, H., Ohnishi, T. and Rubin, E. (1982) Arch. Biochem. Biophys. 216, 51–61
17 Quintanilha, A. and Packer, L. (1977) Proc. Natl. Acad. Sci. U.S.A. 71, 570–574
18 McLaughlin, S. (1977) Curr. Top. Membrane Trans. 9, 71–144
19 Melhorn, R.J. and Packer, L. (1976) Biophys. J. 16, 194a
20 Carmeli, C., Quintanilha, A.T. and Packer, L. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4707–4711
21 Dilger, J.P., McLaughlin, S.G.A., McIntosh, T.J. and Simon, S.A. (1979) Science 206, 1196–1198