J. Biochem. Mol. Biol. Vol. 30, No. 2, pp. 138~143 March 31, 1997

Characterization of the Stearic Acid-Induced Uncoupling of Mitochondrial Respiration

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(Received January 14, 1997)

Abstract : In order to assess controversial proposals concerning the fatty acid-induced uncoupling of mitochondrial oxidative phosphorylation, we investigated the interaction of stearic acid with key mitochondrial proteins and measured the effect of stearic acid on the respiration of cytochrome c oxidase vesicles. Electron paramagnetic resonance spectra of spin-labeled stearic acid clearly demonstrated that cytochrome c oxidase interacts strongly with stearic acid. However, the respiration of detergent-solubilized cytochrome c oxidase was not altered significantly by stearic acid. Surprisingly, adenine nucleotide carrier, which was assumed to bind and translocate fatty acid anions in the Skulachev model of uncoupling, did not bind stearic acid at all. The respiration rate of cytochrome c oxidase vesicles was increased by ~70% in the presence of 20 μM stearic acid and this uncoupling was attributed to a simple protonophoric effect of stearic acid.
Key words : cytochrome c oxidase, fatty acid, spin-label, uncoupling.

Mitochondrion is an energy transducing apparatus that converts the free energy obtained from NADH oxidation to a proton electrochemical potential gradient across the inner mitochondrial membrane. Protons flow back into the matrix via ATP synthase which utilizes the released free energy to phosphorylate ADP. As such the proton pumping of the electron transport proteins is coupled to the synthesis of ATP as long as the membrane is not permeable to protons.

Artificial uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and dinitrophenol dissipate the proton electrochemical potential gradient by transporting protons across the membranes. Heat is generated instead of ATP. In brown adipose tissue, uncoupling protein (also called thermogenin) functions as a natural uncoupler. Fatty acids interact with uncoupling protein to deliberately release heat that keeps a newborn animal from shivering. Recently Garlid *et al.* (1996) proposed a novel mechanism for the uncoupling protein: a neutral fatty acid picks up a proton from the intermembrane space, transverses the membrane, and releases the proton in the matrix. The resulting fatty acid anion is transported back to the cytosolic side by the uncoupling protein. According to this model, the uncoupling protein is a pure anion porter and does not transport protons. This is very similar to the Skulachev model for the fatty acid-induced mitochondrial uncoupling (see below).

Long-chain fatty acids are also known to uncouple mitochondria from other tissues that perform their normal energy transduction [see Wojtczak and Schfeld (1993) for a review]. Upon addition of fatty acid to mitochondria, the rate of State 4 respiration increases and that of ATP synthesis decreases. Fatty acid may act as a simple protonophore like a classical uncoupler and/or it can interfere with the proteins involved in oxidative phosphorylation to modify their activities. Protonated fatty acids and their derivatives are known to transverse phospholipid bilayer very rapidly with $t_{1/2} < 1$ s (Kamp and Hamilton, 1993). Fatty acid anions, however, exhibit relatively slow transbilayer movement which makes it doubtful that fatty acid by itself functions as a pure protonophore (Schönfeld, 1992). In this respect, measurements on cytochrome c oxidase vesicles (COV) do not lead to a clear-cut conclusion. Labonia *et al.* (1988) found that neither proton pumping nor proton permeability was affected by fatty acids. A significant uncoupling by fatty acids was observed in bovine heart COV but not in bovine liver COV (Thiel and Kadenbach, 1989). They also suggested that a threshold membrane potential of ~125 mV is required for the fatty acid-induced uncoupling (Köhnke *et al.*, 1993). Nagami *et al.* (1988) on the other hand argue that fatty acid is simply a modifier of the interaction between cytochrome c oxidase and membranes. Whether or not the protonophoric effect alone can fully account for the fatty acid-induced uncoupling remains

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controversial (Luvisetto *et al.*, 1987; Shinohara *et al.*, 1995).

Andreyev *et al.* (1989) found that skeletal muscle and liver mitochondria are uncoupled by 10~20 μM fatty acids. Interestingly, uncoupling was suppressed by the inhibitors of adenine nucleotide carrier (ANC), whose function is to exchange ATP and ADP. Therefore ANC in normal mitochondria may be a counterpart of the uncoupling protein in the mitochondria from brown adipose tissue. In fact, there is a significant sequence homology between ANC and the uncoupling protein (Runswick *et al.*, 1987). Based on these observations, Skulachev (1991) proposed a model for the fatty acid-induced uncoupling in which a fatty acid transverses the inner mitochondrial membrane and releases a proton in the matrix. The ionized form, whose transbilayer movement is otherwise slow, is transported by ANC to the cytosolic side of the membrane completing a protonophoric cycle. The model is supported by experimental evidence (Schfeld, 1990; Dedukhova *et al.*, 1991; Brustovetsky *et al.*, 1994; Vianello *et al.*, 1994).

In order to test the validity of the above models, we employed spin-label electron paramagnetic resonance (EPR) techniques to carefully examine the interaction of stearic acid with the two key enzymes in the respiration, cytochrome c oxidase and ANC. Since only cytochrome c oxidase was shown to interact with stearic acid, we determined the respiration rates of COV in the presence of stearic acid to estimate its protonophoric effect under various conditions.

Materials and Methods

Preparation of ferrocytochrome c

Cytochrome c from horse heart (type IV, Sigma, St. Louis, USA) in 20 mM K^+-phosphate buffer (pH 7.2) was reduced by 20-fold excess ascorbate in the presence of a small amount of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (Sigma, St. Louis, USA) and the unreacted reductants were removed by anaerobic gel filtration on a Sephadex G-15 (Sigma, St. Louis, USA) column. Almost 100% reduction was achieved (Park *et al.*, 1996). Concentration was determined by the extinction coefficient of ferrocytochrome c at 550 nm, ε_{550}(red)=27 $mM^{-1} \cdot cm^{-1}$.

Purification and reconstitution of cytochrome c oxidase

Cytochrome c oxidase was prepared from fresh bovine hearts according to Yonetani (1961). Concentration was estimated by $\varepsilon_{605\sim630}$(red-ox)=27 $mM^{-1} \cdot cm^{-1}$. The enzyme was reconstituted into asolectin (Fluka) vesicles by a cholate dialysis method (Gregory and Ferguson-Miller, 1989). Briefly, 200 mg of asolectin was stirred anaerobically in 5 ml of 75 mM K^+-Hepes (pH 7.4) containing 2% cholate. The cloudy suspension was sonicated under nitrogen with occasional cooling on ice until it became translucent. Cytochrome c oxidase was added to a final concentration of 5 μM and dialyzed for 4 h against 1 l of 75 mM K^+-Hepes, for 4 h against 1 l and finally for 12 h against 2 l of buffer containing 10 mM K^+-Hepes, 41 mM KCl, and 38 mM sucrose. COV with a high respiratory control ratio (larger than 6, see below) was used in this study.

Purification and solubilization of the adenine nucleotide carrier

ANC was purified by differential adsorption on hydroxyapatite (Bio-Rad) of mitochondrial proteins solubilized in Triton X-100 (Sigma, St. Louis, USA) as described by Krämer (1986). The Keilin-Hartree particles were used instead of intact mitochondria. As described in the literature, ANC was purified only partially to avoid the loss of activity. SDS PAGE identified a band at M_r=~32 K.

Spectrophotometric measurement of the respiration rate

10 nM of cytochrome c oxidase was dissolved in 20 mM K^+-Hepes buffer (pH 7.2) containing 0.5% Tween 80. Ferrocytochrome c (15 μM) was mixed in and the absorption at 550 nm was monitored to follow the oxidation of ferrocytochrome c by cytochrome c oxidase. For COV, the same measurement was done in the final dialysis buffer (coupled state). The respiration rate of the uncoupled state was measured in the presence of 0.2 μM valinomycin (Sigma, St. Louis, USA) and 0.6 μM CCCP. The ratio of the two rates (Vuncoupled/Vcoupled) was taken as the respiratory control ratio. To estimate the protonophoric effect of stearic acid, the reaction mixture was incubated for a few minutes with a desired concentration of stearic acid prior to the addition of ferrocytochrome c.

Spin-label EPR techniques

Doxyl stearic acid (Sigma, St. Louis, USA) labeled at the 5th or 16th carbon (see Fig. 1) was used to monitor its interaction with cytochrome c oxidase and ANC. EPR spectra were recorded at room temperature using a flat quartz cell on a Bruker ER-200D spectrometer operating at 9.76 GHz with 100 kHz field modulation. Other spectrocopic conditions are specified in the figure legends.

Results and Discussion

Uncoupling of oxidative phosphorylation can be induced by the interaction of fatty acids with mitochondrial proteins and/or with the inner mitochondrial membrane (Wojtczak and Schönfeld, 1993). The former includes alteration of the efficiency of the mitochondrial electron transport chain, interference with ATP synthesis, and facilitated transport of fatty acid anions by ANC. Fatty acid can also function as a protonophore in the membrane or modify the interaction of the mitochondrial proteins with the inner membrane. In order to assess these factors that affect the fatty acid-induced-uncoupling of mitochondrial respiration, we examined the interaction of stearic acid with two key mitochondrial proteins, cytochrome c oxidase and ANC, and its consequences. The protonophoric effect of stearic acid was also estimated under various conditions.

Interaction of stearic acid with cytochrome c oxidase and ANC

Fig. 1 shows the structures of the two spin-labeled stearic acids used in this study to monitor the interaction of fatty acid and the two mitochondrial proteins. In aqueous solution, both show isotropic EPR spectra reflecting a rapid rotational motion (data not shown). When dissolved in a buffer containing a detergent, the spin-labels are embedded in micelles and show a different motional freedom. As well documented in the literature, the EPR spectrum of 5-doxyl-stearic acid (Fig. 2a) exhibits a more restricted motion whereas that of 16-doxyl-stearic acid reflects a much faster rotational motion (Fig. 3a).

The EPR spectra of the spin labels were not altered by a large excess of cytochrome c, a peripheral mitochondrial protein which donates an electron to cytochrome c oxidase. Positively-charged cytochrome c does not appear to interact with fatty acids even though the latter are anionic at the neutral pH. However, when an equimolar concentration of cytochrome c oxidase (20 μM) was added to the micellar solution containing 5-doxyl-stearic acid (20 μM), a dramatic decrease in the peak height was observed (Fig. 2b). In addition a new set of signals appeared as evidenced in the difference spectrum (Fig. 2c), which was obtained by subtracting 40% of Fig. 2a from Fig. 2b. In other words, about 60 % of the stearic acid was bound to cytochrome c oxidase. Separation between the outermost lines (marked by arrows) increased from 46 G (Fig. 2a) to 63 G (Fig. 2c) upon binding to cytochrome c oxidase, indicating an unusually strong immobilization of the spin-label. The same measurements were repeated with 16-doxyl-stearic acid and the results are shown in Fig. 3a-c. Again there was a large change in the spec-

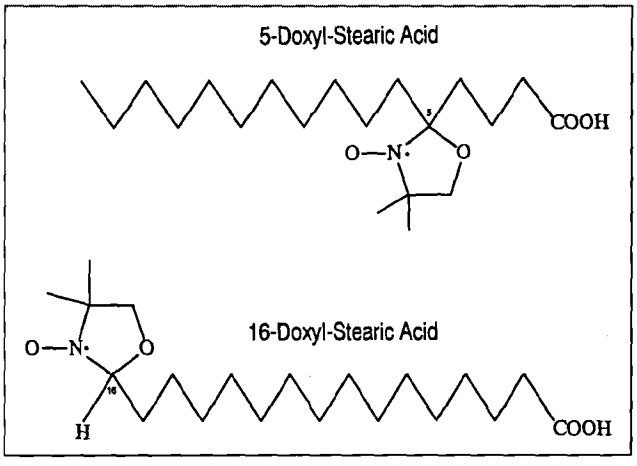

Fig. 1. Structures of doxyl-stearic acid spin-labels used in this study.

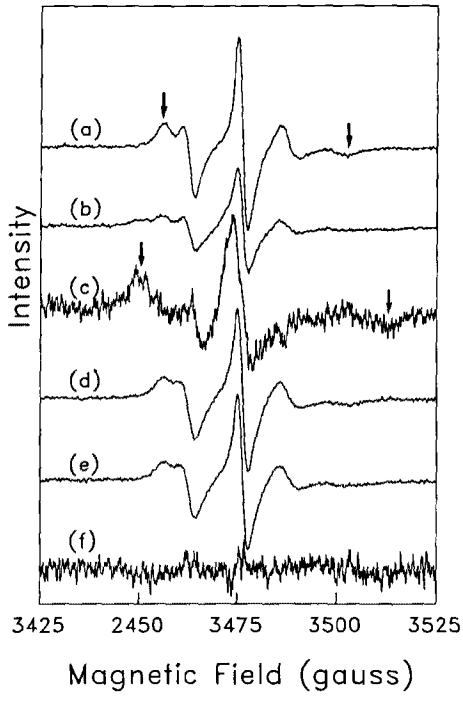

Fig. 2. Interaction of 5-doxyl-stearic acid with cytochrome c oxidase and adenine nucleotide carrier (ANC). (a) EPR spectrum of 20 μM 5-doxyl-stearic acid in 20 mM Na^+-phosphate buffer (pH 7.4) containing 0.5% Tween 80; (b) Cytochrome c oxidase was added to (a) to a final concentration of 20 μM; (c) A difference spectrum obtained by subtracting 0.4× (a) from spectrum (b); (d) EPR spectrum of 20 μM 5-doxyl-stearic acid in the solubilizing buffer (3 % Triton X-100, 150 mM Na_2SO_4, 0.5 mM EDTA, 20 mM Tricine-NaOH, pH 8.0) alone; (e) ANC was added to (d) to a final concentration of 0.3 mg/ml; (f) A difference spectrum obtained by subtracting (d) from spectrum (e). Spectra (c) and (f) are drawn in a 6-fold expanded scale. Arrows indicate the outermost lines that were used to estimate the degree of motional restriction.

tral shape and peak height when the spin-label was bound to cytochrome c oxidase. Unlike 5-doxyl-stearic acid, however, subtraction of Fig. 3a from Fig. 3b did not yield a clean single component. It seems that addition of cytochrome c oxidase causes a small change in the structure of the micelles, which was not evident in the spectrum of 5-doxyl stearic acid probably due to its low mobility.

In the Skulachev model for the fatty acid-induced uncoupling, ANC translocates fatty acid anions across the inner mitochondrial membrane just like the uncoupling protein in the brown fat mitochondria. Therefore the validity of the model relies on an experimental proof of the fatty acid binding to ANC. Recently, spin-label EPR techniques demonstrated the binding of 5-doxyl-stearic acid to the uncoupling protein (Jezek and Freisleben, 1994; Jezek *et al.*, 1995): upon addition of the uncoupling protein, the low-field line showed a slight broadening although the overall shape of the EPR spectrum was not altered. Further evidence was obtained from a photoaffinity labeling experiment (Woldegiorgis *et al.*, 1995; Ruzicka *et al.*, 1996). Spin-label EPR techniques also showed that phospholipids bind to ANC (Drees and Beyer, 1988; Horvath *et al.*, 1990). We applied the same techniques to the monitor binding of fatty acid to ANC. Addition of ANC, however, did not result in any changes in the EPR spectra of 5-doxyl- (Figs. 2d-f) or 16-doxyl-stearic acid (Figs. 3d-f). The results indicate that fatty acids interact more weakly with ANC than with Triton X-100, the detergent used to solubilize ANC.

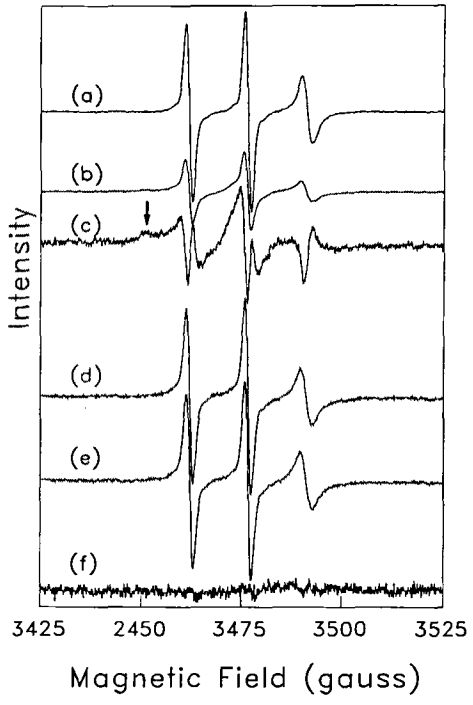

Fig. 3. Interaction of 16-doxyl-stearic acid with cytochrome c oxidase and ANC. (a) EPR spectrum of 20 μM 16-doxyl-stearic acid in 20 μM Na^+-phosphate buffer (pH 7.4) containing 0.5 % Tween 80; (b) Cytochrome c oxidase was added to (a) to a final concentration of 20 μM; (c) A difference spectrum obtained by subtracting 0.4(a) from spectrum (b); (d) EPR spectrum of 20 μM 5-doxyl-stearic acid in the solubilizing buffer alone; (e) ANC was added to (d) to a final concentration of 0.3 mg/ml; (f) A difference spectrum obtained by subtracting (d) from spectrum (e). Spectra (c) and (f) are drawn in a 6-fold expanded scale. Arrow indicates an immobilized component.

Uncoupling of COV by stearic acid

Effect of fatty acids on the respiration rate of COV was estimated by measuring the oxidation rate of ferrocytochrome c by cytochrome c oxidase in the presence of oxygen. Decay in the concentration of ferrocytochrome c was followed by absorption at 550 nm. As shown in Fig. 4, the respiration rate increased with the concentration of stearic acid. At 20 μM of stearic acid, the rate was 1.7 times as large as that without stearic acid. The result is similar to what Köhnke *et al.* (1993) obtained using a polarographic method. Addition of valinomycin to dissipate the membrane potential increased the respiration rates by ~70% in the absence of stearic acid. In the presence of 20 μM stearic acid and valinomycin, the rate becomes even larger.

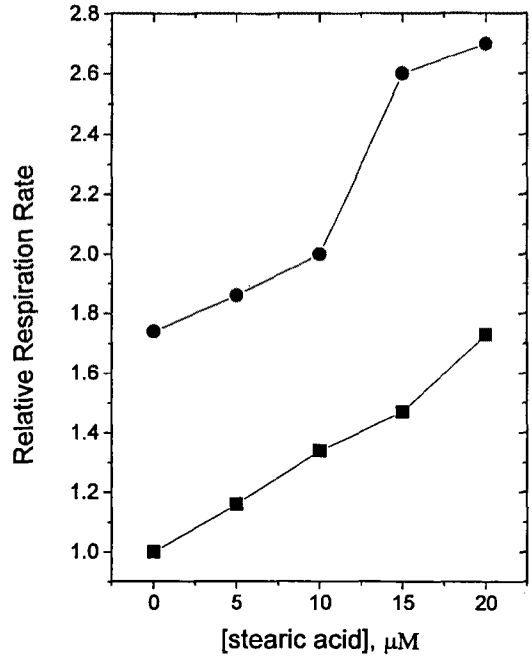

Fig. 4. Effect of stearic acid on the repiration rate of cytochrome c oxidase vesicles (COV). The rate of ferrocytochrome c oxidation by COV was determined in the absence (squares) and in the presence (circles) of valinomycin. The reaction was carried out in a medium having the same composition as that used in the last dialysis step. See Materials and Methods for details.

In the measurements, the concentration of COV was 10 nM, which corresponds to a concentration of 110 mM in phospholipids assuming an average M_r of phospholipids to be 740. Then the ratio [phospholipids]/[stearic acid] becomes ~11 which is very similar to that employed in the work of Rottenberg and Hashimoto (1986). They found a ~40% increase in State 4 respiration using rat liver submitochondrial particles. Although our COV has a different phospholipid composition and [protein]/[phospholipid] ratio, a 70% increase in the respiration rate observed in the present study is large enough to account for the fatty acid-induced uncoupling of the mitochondrial system.

As shown in Figs. 2 and 3, stearic acid obviously interacts with cytochrome c oxidase. The respiration rate of detergent-solubilized cytochrome c oxidase, however, decreased slightly in the presence of stearic acid (data not shown). Therefore the above mentioned uncoupling (increase in the respiration rate) was not due to the interaction of stearic acid and cytochrome c oxidase: it is a pure protonophoric effect of stearic acid on the membrane. This is contrary to what Nagami *et al.* (1988) proposed.

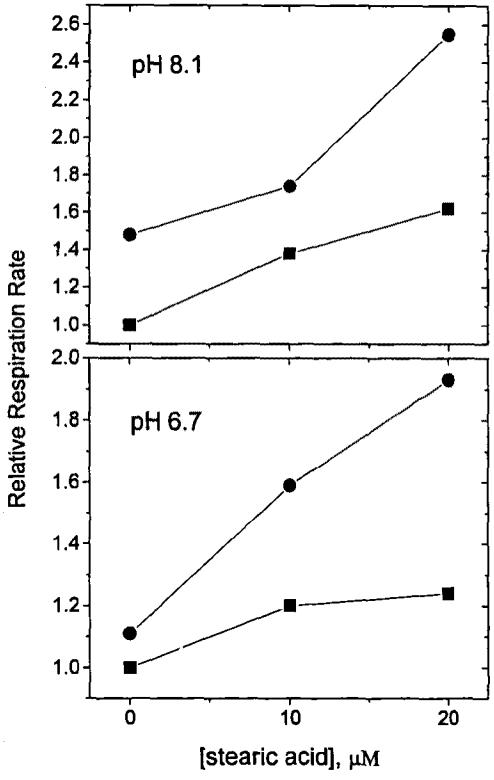

Fig. 5. Effect of the extravesicular pH on the stearic acid-induced uncoupling. The buffer used in Fig. 4 (pH 7.4) was titrated with KOH or HCl, respectively, to a final pH of 8.1 or 6.7. Relative rates of respiration were measured at pH 8.1 (upper panel) and pH 6.7 (lower panel) in the absence (squares) and in the presence (circles) of valinomycin.

The fatty acid-induced uncoupling was further characterized by measuring the respiration rates of the COV in a buffer at different pH. The pH in the lumen (pH_{in}) of COV was kept at 7.4 and the pH in the medium (pH_{out}) was either 8.1 or 6.7. Activity of cytochrome c oxidase increases as the pH_{out} decreases so that the rates in Fig. 5 were referenced to the value without stearic acid and valinomycin at each pH_{out}. The data at pH 8.1 were similar to those at pH 7.4. At pH 6.7, the degree of uncoupling was slightly less than that at pH 7.4. If translocation by stearic acid of protons from outer space to the lumen is a rate determining step, uncoupling would be larger at low pH_{out} since the proton concentration is higher. The overall trend, however, was nearly independent of the pH of the medium, suggesting that this step occurs very rapidly in the membrane. Kamp and Hamilton (1993) also reported a very rapid translocation of the protonated fatty acid across phospholipid membranes.

Fig. 6 illustrates the effect of membrane potential on the fatty acid-induced uncoupling. Concentration of K^+ was higher in the medium than in the lumen so that, in the presence of valinomycin, the membrane potential was dissipated by an inward movement of K^+, and thus respiration increased. However, there was no synergistic effect from the presence of fatty acid: the uncoupling activity of stearic acid alone was not enhanced by K^+/val-

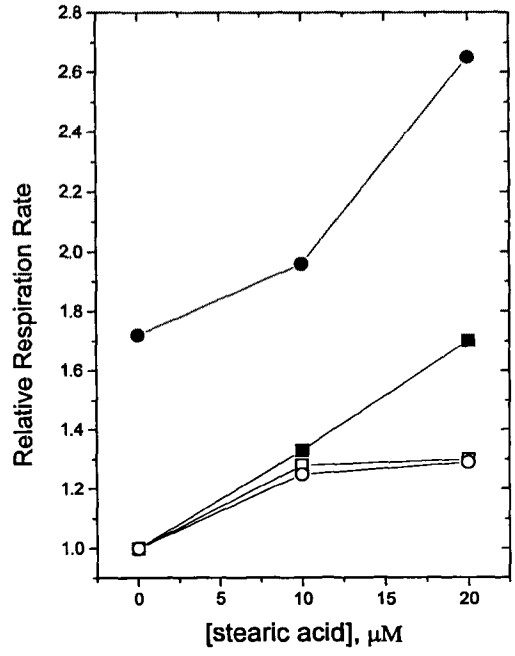

Fig. 6. Effect of the membrane potential on the stearic acid-induced uncoupling. Measurements of the repiration rate were repeated in the same medium as that in Fig. 4 (closed symbols) and in the medium in which K^+ was replaced by Na^+ (open symbols). Valinomycin was absent (squares) and present (circles) in the medium.

inomycin. The concentration gradient of K^+ can be reversed by replacing K^+ with Na^+ to make $[K^+]_{in} > [K^+]_{out} = 0$. As expected, addition of valinomycin can not dissipate the membrane potential, and the rate of respiration remains unchanged. Schfeld (1992) found that a lipophilic cation enhances the fatty acid-induced uncoupling by forming an ion-pair complex with the fatty acid anion. Kamp and Hamilton (1993) argue that the K^+-valinomycin complex as a lipophilic cation also forms an ion-pair with the fatty acid anion and facilitates its transmembrane movement. In both cases, an inward movement of protons is counterbalanced by an outward movement of lipophilic cations. Our result does not agree with this view since valinomycin did not enhance respiration of COV in the medium containing Na^+ where the outward flow of K^+-valinomycin can take place.

In summary, EPR spectra clearly demonstrate a strong interaction between stearic acid and cytochrome c oxidase but this interaction is not reponsible for the fatty acid-induced uncoupling of mitochondrial respiration. Fatty acid binding to ANC as proposed in the Skulachev model was not detected in the EPR spectra. Stearic acid at ~20 μM increased the repiration of COV by ~70% and the result is comparable to that observed in rat liver mitochondria. This uncoupling can be accounted for by a simple protonophoric effect of stearic acid, and the concept of ion-pairing between K^+-valinomycin and fatty acid anion appears to be unnecessary.

Acknowledgement

This work was supported by a grant (941-0300-019-2) from the Korea Science and Engineering Foundation.

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