Fatty acids as modulators of cytochrome *c* oxidase in proteoliposomes

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The control of cytochrome *c* oxidase turnover in proteoliposomes by membrane potential ($\Delta\Psi$) and by pH gradient (ΔpH) is probably kinetic in nature, and inhibition by valinomycin and stimulation by nigericin indicate that ΔpH exerts a greater influence than does an equivalent $\Delta\Psi$. Oleic acid at 100 μM removes all $\Delta\Psi$ and ΔpH control, whereas a similar concentration of palmitic acid increases turnover but does not completely abolish control. Valinomycin acts synergistically with both fatty acids, indicating that the latter can act as H^+/K^+ exchangers, but neither fatty acid alone markedly affects ΔpH , showing that they

cannot fully mimic nigericin. Oleate, but not palmitate, diminishes $\Delta\Psi$, and can move electrophoretically as oleate anion. Submicromolar palmitic acid concentrations partly stimulate turnover in $\Delta\Psi$ - and ΔpH -controlled proteoliposomes, as reported by Labonia, Muller and Azzi [(1988) *Biochem. J.* **254**, 130–145], which might represent a direct effect on cytochrome *c* oxidase. The ubiquity of fatty acids in biological membranes suggests that these substances might be responsible for limiting respiratory control and enzyme activity *in vivo*.

INTRODUCTION

The ability of free (non-esterified) fatty acids (FFA) to uncouple mitochondrial oxidative phosphorylation was shown by Pressman and Lardy [1]. In brown-fat mitochondria, FFA have a physiological role in stimulating the protonophoric action of thermogenin [2]. They also promote proton permeability in other mitochondria. Thus in isolated hepatic mitochondria up to 30% of proton influx can be due to FFA [3]. In addition to this direct action, fatty acids can 'decouple' oxidative phosphorylation, abolishing ATP production and increasing respiration rate, without altering the protonmotive force [3,4]. ATP synthesis in submitochondrial particles in the presence of an artificially imposed membrane potential was unaffected by FFA [5]. In contrast, Azzone and co-workers detected a decrease in membrane potential ($\Delta\Psi$) under conditions in which ATP synthesis was inhibited by fatty acids [6–8]. They proposed that FFA act both as general (membrane) protonophores and as intrinsic uncouplers of proton pumps such as cytochrome *c* oxidase ('slip' inducers).

Cytochrome *c* oxidase-containing phospholipid vesicles (COV) are a model for mitochondria, lacking the complexity of the native organelles. Using such proteoliposomes, Labonia and co-workers [9] reported that low levels of fatty acids (especially palmitate) hardly affect proton permeability of COV under conditions where oxidase turnover is markedly stimulated. Despite the loss of respiratory control induced by low concentrations of palmitate, there was no effect on the H^+/e^- ratio, in contrast with the results of Azzone and co-workers [8]. Other reports describe significant effects on all parameters tested at higher levels of fatty acids [10–12]. It is thus possible that some FFA can affect the oxidase directly as well as indirectly via modulation of membrane ion permeability.

The ionophoretic action of FFA in membrane systems, both in the presence of artificially induced cation gradients and in actively respiring COV, is therefore controversial. This might arise partly because of difficulty in separating direct and indirect effects of

fatty acids. The free enzyme might be implicated in some fatty acid actions ([9,13], and M. A. Sharpe, I. Perin, B. Tatttrie and P. Nicholls, unpublished work), whereas others might involve only the membrane in which it is located. In this paper we examine the effects of fatty acids on COV in controlled, partly controlled (plus either valinomycin or nigericin), and uncontrolled states. The paper also describes the effects of fatty acids on steady-state pH gradient (ΔpH) and $\Delta\Psi$ gradients. We conclude that both electroneutral and electrophoretic activities of FFA must be taken into account in analysing their effects on COV respiration, as well as possible direct effects on the membrane-bound enzyme. The physiological action of cytochrome *c* oxidase as proton pump and electron transfer system and its control in mitochondria might involve intrinsic fatty acids or fatty acid-like compounds in the membrane. Some of the results have been presented previously in abstract form [14].

MATERIALS AND METHODS

Bovine heart cytochrome *c* oxidase was purified as described in [15], with Tween-80 substituting for Emulsol in the final stages. The enzyme was stored at -80°C in 100 mM sodium phosphate buffer containing 0.25% Tween-80. Protein concentration was determined by the biuret method [16], and cytochrome *c* oxidase concentration was determined with an absorption coefficient of $27\text{ mM}^{-1}\cdot\text{cm}^{-1}$ for reduced minus oxidized enzyme (cytochrome *aa*₃) at 605–630 nm [17].

Sodium ascorbate, cytochrome *c* (horse heart type VI), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and valinomycin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) were products of Avanti Lipids (Alabaster, AL, U.S.A.). Nigericin was from Calbiochem Corporation (San Diego, CA, U.S.A.). Hepes was from Research Organics (Cleveland, OH, U.S.A.). 3,3'-dipropylthiadicarbocyanine [diS-C₃-(5)] from Molecular Probes (Portland, OR,

Abbreviations used: COV, cytochrome *c* oxidase-containing vesicles, or proteoliposomes; diS-C₃-(5), 3,3'-dipropylthiadicarbocyanine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; ΔpH , pH gradient; $\Delta\Psi$, membrane potential; FFA, free fatty acids; pyranine, 8-hydroxy-1,3,6-pyrenetrisulphonate; RCI, respiratory control index (the ratio of the respiration rate in the presence of ionophores to that in their absence); TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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U.S.A.) and trisodium 8-hydroxy-1,3,6-pyrene trisulphonate (pyranine) from Eastman Kodak Co. (Rochester, NY, U.S.A.).

COV were prepared as described by Wrigglesworth et al. [18], with either asolectin or DOPE/DOPC mixtures. DOPE (125 mg) and DOPC (125 mg) were dissolved in chloroform, the solvent was removed by rotary evaporation under nitrogen, and the thin film of lipid dispersed by mixing in the appropriate buffer (or, for ΔpH measurements, with 10 mM pyranine) with the addition of 100 mg of sodium cholate. The sample was vortex-mixed and sonicated, on ice under a nitrogen stream, for 8 min in the pulsed mode at 30% duty cycle (Heat Systems, Ultrasonics W-375 sonicator). To a 5 ml sample, cytochrome *c* oxidase was added to a final concentration of approx. 6 μM cytochrome *aa*₃. The mixture was sonicated for 30 s to disperse the oxidase into the lipid, centrifuged for 10 min at 20 000 *g* at 4 °C to remove undispersed lipid and titanium particles, and then dialysed twice against 100 vol. (including 10 mM pyranine) of 100 mM K⁺/Hepes, pH 7.0, and finally a further 200 vol. of pyranine-free buffer for 3 days at 4 °C. After dialysis, excess dye was removed from the liposomes by gel filtration on a Sephadex G-25 column equilibrated with 100 mM K⁺/Hepes, pH 7.0. The proportion of outwardly facing cytochrome *c* oxidase [18] was normally 65% or more.

For spectrophotometry, 1 ml samples of COV were used at a final cytochrome *aa*₃ concentration of 100 nM. Turnover was initiated by the addition of either 20 mM sodium ascorbate plus 300 μM TMPD, or 40 mM sodium ascorbate alone. After anaerobiosis a few grains of sodium dithionite were added to indicate the 100% reduction level of cytochrome *c*, and 1 μM nigericin and 1 μM valinomycin were added to the sample, which was then titrated against 1 M KOH.

Turnover of COV was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments). The electrode was placed in a thermostatically controlled, magnetically stirred, glass-jacketed reaction vessel, with a working volume of either 12 or 4 ml. The medium used was therefore either 12 or 4 ml of 100 mM K⁺/Hepes, pH 7.0, containing 30 μM EDTA, equilibrated at 30 °C. To this were added 10 mM ascorbate and 100 μM TMPD, and COV at a cytochrome *c* oxidase concentration of approx. 7 nM. Turnover was initiated by the addition of 10 μM cytochrome *c*. Ionophores were added as ethanolic solutions, up to an ethanol concentration no greater than 0.3% (v/v).

The absorbances of pyranine, diS-C₃-(5) and cytochrome *c* were recorded with a Beckman DU-7400 diode-array spectrophotometer. Redox changes of cytochrome *c* were measured with the 550/540 nm wavelength pair. The acid/base behaviour of entrapped pyranine was monitored with the 450/490 nm wavelength pair, corrected for the change in the overlying cytochrome *c* spectrum ($0.537 \times \Delta A_{550/540}$). Absorbance changes due to vesicular accumulation of the membrane potential probe diS-C₃-(5) were monitored at the 700/666 nm wavelength pair.

RESULTS

Figure 1 compares valinomycin and nigericin effects on charged ('asolectin') COV prepared as described above. In the absence of nigericin (Figure 1, upper panel), low concentrations of valinomycin slow turnover and increase the respiratory control index (RCI; defined as the ratio of the respiration rate in the presence of ionophores to that in their absence). Under these conditions, $\Delta\psi$ has collapsed in response to K⁺ influx, with a consequent decrease in H⁺ influx, increase in ΔpH , and inhibition [19,20]. At 10 nM nigericin, respiratory control is completely lost at valinomycin levels below 2 nM (one valinomycin molecule per three

Figure 1 Effects of ionophores on the respiratory control behaviour of charged proteoliposomes

Upper panel: effect of valinomycin on respiratory control. The medium used was 12 ml of 100 mM K⁺/Hepes, pH 7.0, containing 30 μM EDTA, equilibrated at 30 °C. To this were added 10 mM ascorbic acid and 100 μM TMPD, and 'asolectin' COV to a cytochrome *c* oxidase concentration of approx. 7 nM. Turnover was initiated by the addition of 10 μM cytochrome *c*. Symbols: Δ, responses in the absence of added reagents; ▲, responses with 10 nM nigericin present. Lower panel: effect of nigericin on respiratory control. Symbols: Δ, responses in the absence of added reagents; ▲, responses with 20 nM valinomycin (val) present; ○, responses with 800 nM oleic acid present. Other conditions are as in the upper panel.

vesicles). Higher levels of valinomycin act protonophorically, and at approximately five valinomycin molecules per vesicle the RCI returns to its starting level. At even higher concentrations valinomycin alone can fully release respiration (results not shown), but it is far less efficient than the combined nigericin-valinomycin couple and RCI values remain substantial up to micromolar valinomycin levels.

Figure 1 (lower panel) shows the action of nigericin alone and in the presence of valinomycin or a submicromolar amount of oleic acid. Nigericin alone (at 10 nM) induces a decline in the RCI from 10 to 3. There is a modest modulation of the effect of nigericin by small amounts of oleic acid. Increasing the nigericin concentration to 10 μM does not decrease the RCI further. Nigericin therefore cannot act as an electrophoretic ionophore. In the presence of valinomycin the RCI drops to 1.0 at very low concentrations of nigericin. ΔpH is abolished by nigericin, and in its presence the oxidase is controlled by $\Delta\psi$ alone.

Figure 2 shows the effects of oleic and palmitic acids on respiratory control in charged COV over a wide range of concentrations. Submicromolar levels of palmitic acid caused a decrease in RCI from approx. 11 to approx. 6, as reported by Labonia and co-workers [9]. Oleic acid is much less effective at such low concentrations but at 10 μM it decreases the RCI from approx. 11 to approx. 8. At higher levels (50 μM or more), oleic

Figure 2 Behaviour of charged proteoliposomes towards fatty acids in the presence and absence of valinomycin

The medium used was 12 ml of 100 mM K^+ /Hepes, pH 7.0, containing 30 μ M EDTA, equilibrated at 30 °C. To this were added 10 mM ascorbic acid and 100 μ M TMPD, and 'asolectin' COV to a cytochrome *c* oxidase concentration of approx. 7 nM. Turnover was initiated by the addition of 10 μ M cytochrome *c*. The broken line indicates the respiratory control in the absence of fatty acids or ionophores. The plot shows the RCI as a function of fatty acid concentration, plotted logarithmically: ○, oleate; □, palmitate; ●, oleate plus 20 nM valinomycin (val); ■, palmitate plus 20 nM valinomycin.

Figure 3 Behaviour of uncharged proteoliposomes towards the fatty acids oleate and palmitate

Upper panel: COV turnover plotted as a function of fatty acid concentration (turnover is given in electron equivalents/s per mole of cytochrome aa_3). The oxygen electrode chamber contained 4 ml of 100 mM K^+ /Hepes, pH 7.0, containing 30 μ M EDTA, equilibrated at 30 °C. To this were added 10 mM ascorbic acid and 100 μ M TMPD, and DOPC/DOPE COV to a cytochrome *c* oxidase concentration of approx. 7 nM. Turnover was initiated by the addition of 10 μ M cytochrome *c*. The broken line represents fully released turnover. Symbols: ○, oleic acid; □, palmitic acid. Lower panel: RCI as a function of fatty acid concentration: ○, oleic acid; □, palmitic acid. Data from Figure 3 (upper panel).

Figure 4 Effects of fatty acids on $\Delta\Psi$ in uncharged COV

Samples (1 ml) of COV were used in semi-microcuvettes at a final concentration of cytochrome aa_3 of 100 nM. Turnover was initiated by the addition of 20 mM sodium ascorbate or 40 mM ascorbate plus 300 μ M TMPD. Measurement of $\Delta\Psi$ with the probe diS-C₃(5) was performed spectrophotometrically as described in the Materials and methods section. After completion of steady-state measurements, 1 μ M nigericin plus 1 μ M valinomycin was added to the sample, to obtain a 'zero' position for the diS-C₃(5) response. The medium contained 100 mM K^+ /Hepes, pH 7.0, 30 μ M EDTA, equilibrated at 30 °C, and 10 μ M cytochrome *c*. Symbols: ○, oleic acid in the presence of ascorbate alone; □, palmitic acid in the presence of ascorbate alone; ●, oleic acid in the presence of ascorbate plus TMPD; ■, palmitic acid in the presence of ascorbate plus TMPD. After anaerobiosis a few grains of sodium dithionite were added to obtain the final reduction level of cytochrome *c*.

acid is able to abolish all respiratory control, even though this molar ratio of FFA to phospholipid is far too small to cause lysis [11,12]. Valinomycin potentiates the oleate action. Palmitic acid is a potent effector of oxidase turnover at low concentrations, but alone it is unable to decrease the RCI to 1.0. Valinomycin again acts synergistically, causing the RCI to fall further than in the presence of palmitate alone. The addition of oleic or palmitic acid to fully uncontrolled COV (plus 1 μ M nigericin and valinomycin) caused no further increase in turnover (results not shown).

These FFA also release respiration in uncharged (DOPE/DOPC) COV (Figure 3). Figure 3 (upper panel) shows COV turnover as a function of FFA concentration. Neither fatty acid is as potent in uncharged DOPE/DOPC lipid vesicles as in charged 'asolectin' vesicles. Oleic acid caused a progressive loss of control, the effect being 50 % complete at 40 μ M. Palmitic acid was less effective and did not fully release respiratory control at the highest concentrations employed. However, at low palmitate concentrations the partial release described above was again displayed. Figure 3 (lower panel) plots RCI values as a logarithmic function of fatty acid concentration. Palmitate is far less effective in uncharged COV than in charged COV. Thiel and Kadenbach [21] were also unable to observe polarographically any drop in RCI induced by low levels of FFA. Their FFA-to-phospholipid ratios were also lower than those used in [9] and in our work; they also added cardiolipin to their lipid mixture, which might 'stiffen' the bilayer and decrease the rate of transmembrane movement of fatty acids.

The action of fatty acids on the COV membrane potential ($\Delta\Psi$) during respiration in the presence of cytochrome *c*, ascorbate and TMPD is shown in Figure 4. These experiments employed a 7-fold higher COV concentration than that used in the experiments shown in Figures 1, 2 and 3. Oleic acid induced a fall in $\Delta\Psi$, with a decrease of 50 % at 50 μ M. The same effect required a palmitic acid concentration of 200 μ M. Over the usual 10–50 μ M concentration range, in both the absence (open symbols) and presence (filled symbols) of TMPD, oleic acid

Figure 5 Effects of fatty acids on ΔpH in uncharged COV

Samples (1 ml) of COV were used in semi-microcuvettes at a final concentration of cytochrome a_3 of 100 nM. Turnover was initiated by the addition of 20 mM sodium ascorbate or 40 mM ascorbate plus 300 μM TMPD. Measurement of ΔpH by using entrapped pyranine was performed spectrophotometrically as described in the Materials and methods section. After completion of steady-state measurements, 1 μM nigericin plus 1 μM valinomycin was added to the sample, which was then titrated against 1 M KOH to calibrate the pyranine response. The medium contained 100 mM K^+ /Hepes, pH 7.0, 30 μM EDTA, equilibrated at 30 °C, and 10 μM cytochrome c . Symbols: $\circ$, oleic acid in the presence of ascorbate alone; $\square$, palmitic acid in the presence of ascorbate alone; $\bullet$, oleic acid in the presence of ascorbate plus TMPD; $\blacksquare$, palmitic acid in the presence of ascorbate plus TMPD. After anaerobiosis a few grains of sodium dithionite were added to obtain the final reduction level of cytochrome c .

acted as an electrophoretic ionophore, whereas palmitic acid did not.

Palmitate was, however, as effective as oleate in decreasing the steady-state ΔpH , as shown in Figure 5. Neither fatty acid has a large effect on ΔpH over the 10–50 μM concentration range, in either the presence or absence of TMPD, and the collapse of this gradient requires higher concentrations of FFA. The two FFA thus modulate the pH gradient in similar ways.

DISCUSSION

Palmitic acid is not an effective protonophore. However, at low concentrations it causes a finite reduction in RCI that cannot be emulated by the normally more effective oleic acid. Oleic acid at higher concentrations causes a decrease in the steady-state ΔpH present in respiring COV, which will decrease the passive proton influx. This might increase the inhibitory steady-state ΔpH [19,20]. In liposomal experiments, when ΔpH is induced by an alkali or K^+ ion pulse, oleic acid at concentrations exceeding 10 μM is a much more efficient protonophore than palmitic acid [1] (compare Figures 2 and 3), whereas at concentrations below 5 μM palmitic acid is slightly more effective.

We are presented with something of a puzzle. Normal control of cytochrome c oxidase turnover by ΔpH and ΔpH is kinetic rather than thermodynamic [11,20,22,23], and ΔpH exerts a greater control than does an equivalent ΔpH . This is highlighted by the action of valinomycin as both inhibitor and activator, behaviour that was also seen in the present experiments. Conversion of the steady-state ΔpH into ΔpH increased the RCI from 10 to 16. Subsequent protonophoric action decreased the RCI again. In contrast, nigericin, which can only act electroneutrally as a K^+/H^+ exchange carrier, collapsed ΔpH and increased ΔpH , causing the RCI to decline from approx. 10 to 3.

Nigericin and valinomycin act synergistically, but small amounts of oleic acid do not potentiate the action of nigericin, indicating that the acid is not effective as an electrophoretic K^+

ionophore. Valinomycin potentiates the action of both palmitic and oleic acids, which suggests that they are effective as electro-neutral K^+/H^+ exchangers. By using the ΔpH and ΔpH indicators diS-C₃-(5) and pyranine, the effects of FFA on the controlling gradients could also be studied directly. Both acids are protonophores, diminishing the steady-state ΔpH . However, they also seem to act as electrophoretic ionophores, diminishing the steady-state ΔpH . High concentrations of oleate completely dissipated the electrochemical proton gradient produced by COV turnover, whereas palmitate was only partly effective.

Are the decreases in RCI values obtained with low concentrations of palmitic acid [9] evidence for a direct action of this fatty acid on cytochrome c oxidase? There might be such direct interactions between FFA and the oxidase ([13], and M. A. Sharpe, I. Perin, B. Tattre and P. Nicholls, unpublished work). However, the palmitate-specific drop in the RCI is seen only in COV that exhibit a large RCI. There is also no increase in the turnover of COV pretreated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (M. A. Sharpe, I. Perin and B. Tattre, unpublished work).

Kohnke and co-workers [24] recently proposed that FFA act electrophoretically only when the steady-state ΔpH exceeds approx. –120 mV. However, oleic acid is able to collapse ΔpH fully, and palmitic acid can also lower the steady-state ΔpH below –30 mV. There might be other reasons why some workers have been unable to see both electroneutral and electrophoretic actions of FFA. First, there is the choice of medium. FFA form complexes with and catalyse the transmembrane movement of a number of monovalent and divalent cations [12]. Na^+ in particular seems to form strong complexes, but crosses liposomal membranes only very slowly. The buffer used by Rottenberg and co-workers [4,5] contains 50 mM NaCl, which favours the formation of Na^+ soaps that exchange electroneutrally with H^+ slowly and promote electrophoretic proton transport by FFA.

Secondly, the bulk pH of the medium can have a large impact on the mobilities of FFA derivatives. Fatty acid pK_a values in membranes are close to neutrality [25], the pK_a of oleate in asolectin liposomes lying between pH 7.0 and 7.3 (M. A. Sharpe, unpublished work). At a starting bulk pH of 7.4 for mitochondria and COV [3,6,8,21], this will favour the presence of the anionic FFA, decreasing both cation/ H^+ exchange pathways and electrophoretic protonophore action. Although 7.4 may be the physiological pH of blood, the pH in the mitochondrial intramembrane space is the same as that of the cytosol, closer to pH 7.0 [26]. Thirdly, most workers who have examined the effects of FFA on mitochondria and COV have used very low FFA-to-lipid ratios. Although FFA can act as detergents, their addition to cholate-dialysed liposomes at FFA-to-phospholipid ratios up to 1:1 does not cause lysis. Stable pH gradients are created by the addition of potassium salts to fatty acid-treated liposomes [12].

Recently, models of proton pumping and scalar proton entry to the binuclear centre in cytochrome c oxidase have been proposed that rely on protons shuttling along lines of acidic residues gated by His-325 of subunit 1 [27,28]. There are numerous water- and phospholipid-containing volumes within the X-ray structure of the beef heart enzyme [29] where fatty acids could reside *in vivo* and play a role in modulating proton movements. A model in which the pK_a values of intrinsic FFA are altered by the redox/oxygen intermediate state of the binuclear centre is attractive, but a more likely non-specific but physiologically important role for FFA is for them to act as intra-enzymic or intramembraneous proton carriers, capable of moving protons electroneutrally from the inner aqueous phase to the binuclear centre and thereby modulating respiratory control in the ways that we have shown here.

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