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The effect of non-esterified fatty acids on the proton-pumping cytochrome *c* oxidase reconstituted into liposomes

Nestor LABONIA, Michele MÜLLER and Angelo AZZI*
Institut für Biochemie und Molekularbiologie der Universität Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland

Bovine heart cytochrome *c* oxidase was reconstituted in phospholipd vesicles, and the effect of different non-esterified fatty acids (NEFA) was studied on its proton pump and on the proton permeability of the vesicles. Neither parameter appeared to be affected by concentrations of NEFA known to uncouple oxidative phosphorylation (10 μM). Also the permeability for K^+ was not affected by them. The fatty acids caused an increase in the rate of electron transfer in the absence, but not in the presence, of uncoupler and/or valinomycin [diminution of the respiratory-control index (RCI)]. The RCI of 8.7–7.5 was decreased to about 4.5 in the presence of 0.27–10 μM-NEFA. Oleic acid was not effective at the above concentrations. Subunit III-depleted enzyme preparations gave vesicles with an RCI of about 5.5, which was decreased to 4.5 in the presence of NEFA. With both native and subunit III-depleted oxidase the RCI was never decreased to the value of 1 by NEFA, as happens with classical protonophores.

INTRODUCTION

It is general knowledge that non-esterified fatty acids (NEFA) are uncouplers of mitochondrial oxidative phosphorylation (Pressmann & Lardy, 1956; Borst *et al.*, 1962; Van den Berg, 1967). Since the latter reaction depends upon the coupling between the protons transported by redox pumps and an ATPase (F_0F_1), uncoupling is generally intended as dissipation of the proton electrochemical gradient caused by protonophores (Mitchell, 1985). The mechanism of NEFA-produced uncoupling appears to be, however, more complicated than that produced by protonophores, and the term 'decoupling' has been used to define the action of the former group of substances, which have been suggested to inhibit ATP synthesis by dissipating intramembranous protons without inhibiting ATP synthesis driven by the proton electrochemical gradient (Rottenberg & Hashimoto, 1986; Rottenberg & Steiner-Mordoch, 1986). The relevance of the phenomenon to the mechanism of coupling and its physiological implications are such that a clarification of the NEFA action at a molecular level would be of value. However, the complexity of mitochondrial membranes is so great that evidence for one or other mechanism of action of NEFA cannot easily be obtained. A simple model system has been therefore utilized here, which consists of isolated bovine heart cytochrome *c* oxidase incorporated into artificial lipid vesicles. The system has been shown to be able to perform redox-mediated proton transport (Azzi *et al.*, 1986) and is a suitable system to answer the following two questions: (1) is the inhibition of the ATP synthesis induced by NEFA the result of an increase in the proton conductance of the coupling phospholipid membrane?; (2) is the redox proton pump associated with cytochrome *c* oxidase decoupled by NEFA? We show that the answer to both questions is 'no', thereby leaving room for other possible mechanisms as the basis of the action of NEFA on cytochrome *c* oxidase, one of which will be proposed.

EXPERIMENTAL

Bovine heart cytochrome *c* oxidase was prepared by the method described by Yu *et al.* (1975). Haem *a* was determined by using an $\epsilon_{605-630}$ value of 13.5 $mM^{-1} \cdot cm^{-1}$. Subunit III-depleted cytochrome *c* oxidase was prepared as described by Malatesta *et al.* (1983) and Püttner *et al.* (1985). The chymotrypsin treatment was performed in octyl β-D-glucopyranoside. Cytochrome *c* oxidase-containing vesicles were prepared by cholate dialysis, essentially by the method described by Casey (1986), except that the last dialysis was carried out against a 3-fold larger buffer volume. The buffer was composed of 1 mM-Hepes/K^+, 44 mM-KCl, 56 mM-sucrose, pH 7.2, at 4 °C. Ferricytochrome *c* was reduced with excess dithionite, followed by gel filtration on Sephadex G-25 equilibrated with the last dialysis buffer (Casey, 1986). The respiratory-control index (RCI) was measured spectrophotometrically with an Aminco DW-2a spectrophotometer, cytochrome *c* oxidation at 550–540 nm being monitored as described by Müller *et al.* (1986). A portion of the dialysed vesicles was diluted 50 times with a medium having the same composition as that used in the last dialysis step. The diluted preparation was added to 2 ml of 5 μM-ferrocytochrome *c*/20 mM-Hepes/50 mM-KCl, pH 7.3, thermostatically maintained at 20 °C with stirring. The $\epsilon_{550-540}$ value used was 19.4 $mM^{-1} \cdot cm^{-1}$. Proton equilibration measurements were carried out potentiometrically as described by Thelen *et al.* (1985), with a Philips CA 14/02 electrode connected with a Radiometer PHM 64 pH-meter amplified by a custom-built five-step Butterworth amplifier. A portion of the vesicles was diluted into a 2 ml final

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NEFA, non-esterified fatty acid; RCI, respiratory-control index.

* To whom correspondence and reprint requests should be addressed.

volume of the last dialysis medium. Valinomycin (2.3 nmol/mg of lipid) and 3 nM-CCCP (final concn.) were added to the vesicles, which were incubated for 15–30 min before starting the measurements at 20 °C. The analysis of the pH-equilibration kinetics after the pulse addition of ferrocytochrome *c* was performed as described by Thelen *et al.* (1985). Potential-driven H^+ translocation was examined as described in the legend to Fig. 4 (below). Incubation with NEFA [1 mM stock solution in 20 mM-Tris/HCl, pH 8, or in ethanol; 15 min incubation time] was performed on ice for the RCI measurements and at room temperature for the potentiometric measurements. Synthesis of 12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoic acid and of 12-[*N*-(4-azido-2-nitro[3,5-^{3}H]phenyl)]aminododecanoic acid was performed as described by Bisson & Montecucco (1981).

Materials

Cholic acid was from Fluka A.G., Buchs, Switzerland, or from Sigma, St. Louis, MO, U.S.A.; it was dissolved in KOH solution and treated with granular activated charcoal, filtered through a layer of Celite 535 and precipitated by addition of HCl. The precipitate was recrystallized two to four times from acetone/water (4:1, v/v) at −20 °C. The recrystallized acid was neutralized with KOH or with choline (Sigma) and diluted to a final concentration of 20% (w/v). Asolectin was from Associated Concentrates, Woodside, NY, U.S.A., and treated as described by Kagawa & Racker (1971). NEFA were from Fluka. Chymotrypsin was from Serva, Heidelberg, Germany. 4-Fluoro-3-nitro[2,6-^{3}H]phenylazide was from Amersham International, Amersham, Bucks., U.K.

RESULTS

Effect of palmitic acid on the proton pump and proton permeability

The experiment shown in Fig. 1 is a potentiometric measurement of the rapid H^+ release from cytochrome *c* oxidase reconstituted in phospholipid vesicles upon addition of ferrocytochrome *c* (upper trace). When the added ferrocytochrome *c* had been oxidized, which occurred in a few seconds, the pH returned slowly to its original value and increased further to an equilibrium level. The higher pH value attained after the addition of ferrocytochrome was the consequence of the chemical reduction of oxygen to water which involved the utilization of four protons from the interior of the liposomes. The slow time course of the alkalinization is to be attributed to the low permeability of the phospholipid membrane to protons (an excess of valinomycin and K^+ were present to assure rapid charge compensation). In the presence of the protonophore CCCP, no acidification was seen, the outward-transported protons falling back immediately into the vesicle lumen. In the lower trace of Fig. 1 the same experiment is recorded, but it was carried out in the presence of 30 μM-palmitic acid and shows clearly the lack of NEFA effects on the proton pump and on the passive proton permeability of the liposome membrane. A quantitative comparison of the results reported in Fig. 1 is shown in Fig. 2. The logarithm of the extent of released protons in the presence and absence of 30 μM-palmitic acid was plotted as a function of the time

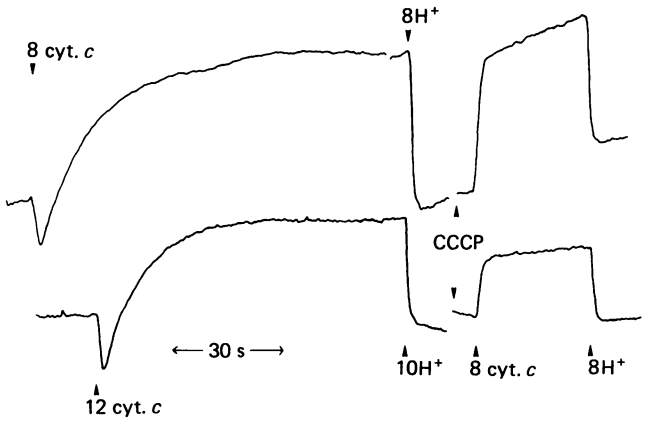

Fig. 1. Potentiometric measurements of the proton pump of cytochrome *c* oxidase reconstituted in liposomes

The experimental conditions were as follows: cytochrome *c* oxidase vesicles were diluted in the last dialysis medium to a final volume of 2 ml in the absence (upper trace) and in the presence (lower trace) of 30 μM-palmitic acid; upper trace: 360 pmol cytochrome *c* oxidase vesicles; the addition of 8 nmol of cytochrome *c* (cyt.*c*) corresponded to 5.6 mol of oxygen reduced per enzyme unit; lower trace: 500 pmol of cytochrome *c* oxidase vesicles; the addition of 12 nmol of cytochrome *c* corresponded to 6 mol of oxygen reduced per enzyme unit. The traces labelled 'CCCP' were obtained in the presence of 10 μM-CCCP; in both cases the calibration was made with 8 nmol of cytochrome *c* and 8 nmol of HCl. Acid additions were made with calibrated HCl (1 mM).

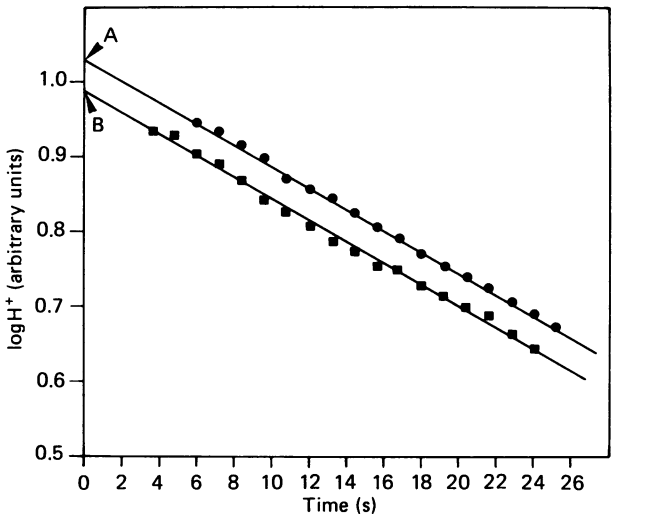

Fig. 2. Determination of the H^+/e^- ratio of the potentiometric measurements of Fig. 1.

The logarithm of the amount of extruded protons was plotted against time. ●, Untreated cytochrome *c* oxidase vesicles (upper trace of Fig. 1); ■, cytochrome *c* oxidase vesicles incubated with 30 μM-palmitic acid (lower trace of Fig. 1). The extrapolation to zero time of the control experiment (upper trace of Fig. 1), point A, gave a value of 1.03 corresponding to 0.6 H^+/e^- for 5.6 mol of oxygen reduced per enzyme unit. The value of point B (experiment in the presence of 30 μM-palmitic acid; lower trace of Fig. 1) was 0.99, corresponding to 0.59 H^+/e^- for 6 mol of oxygen reduced per enzyme unit. The slope of the two decays was almost identical, showing that there was no change in the proton conductance under the described conditions.

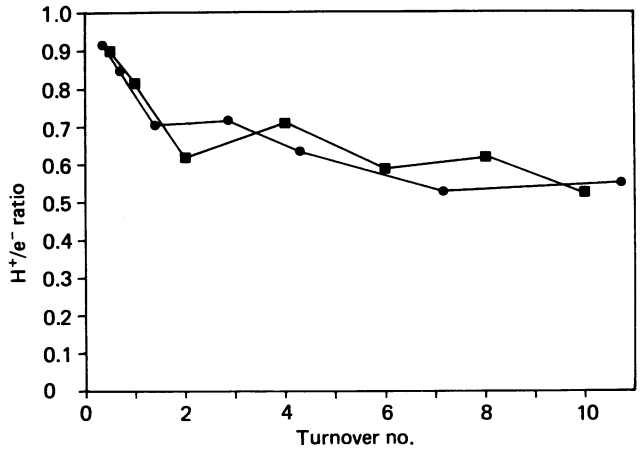

Fig. 3. Effect of multiple turnovers on the H^+/e^- ratio of cytochrome *c* oxidase vesicles

Measurements conditions were as described for Fig. 1. Determination of the H^+/e^- ratio was made as described in Fig. 2. ●, ■, Vesicles without (●) and with (■) 30 μM-palmitic acid. One enzyme turnover corresponded to the amount of 4 equiv. reductant (ferrocytochrome *c*) necessary to reduce an oxygen molecule.

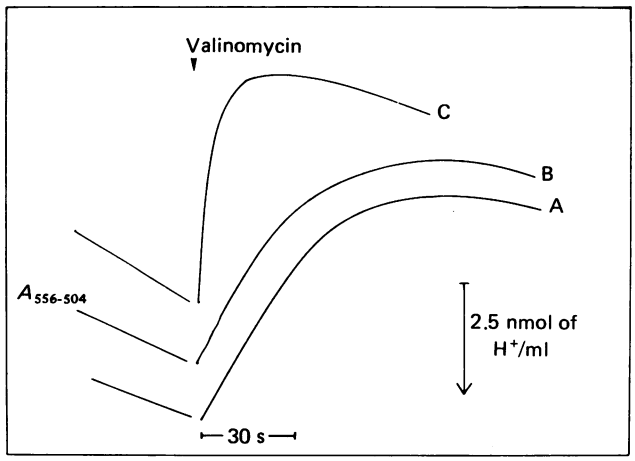

Fig. 4. Effect of the K^+ electrical diffusion potential on the proton re-entry in the presence and absence of palmitic acid

Cytochrome *c* oxidase was reconstituted as described in the Experimental section. The buffer of the last dialysis step was composed of 0.1 M-choline/HCl and 0.5 mM-Hepes, pH 7.2. A 20 μl portion of vesicles diluted 50-fold with the last dialysis buffer was added to 2 ml of the same buffer and the pH changes after valinomycin addition and the absorbance were monitored by 4 μM-Phenol Red at the 556–504 nm wavelength pair. Trace A, untreated control vesicles; trace B, vesicles in the presence of 1 μM-palmitic acid; trace C, vesicles in the presence of 10 μM-CCCP. The K^+-driven alkalinization was started by the addition of 2.5 μM-valinomycin. Additions of HCl were made from a 1 mM calibrated solution.

elapsed from the addition of ferrocytochrome *c*. The linear traces indicate that, in both cases, the proton re-entry occurred as a single exponential. The extrapolation to zero time and subsequent calculations gave values for both lines close to 0.6 at six turnovers, indicating no diminution of the efficiency of the pump by palmitic acid. The fact that the two decays, in the presence and absence of palmitic acid, had almost identical time constants suggests that the proton permeability of the vesicles was not modified by the addition of palmitic acid. This result was substantiated by exploring the proton-pump efficiency in the presence and absence of palmitic acid over a wide range of enzyme turnovers from 0.5 to 10 (Fig. 3). The presence of palmitic acid did not modify, within experimental error, the proton-pump efficiency of the native enzyme.

In the above-reported experiment the driving force for the H^+ re-entry into the vesicles, due to the high K^+ permeability caused by the presence of valinomycin, was most probably the pH difference across the membrane. A second experiment (Fig. 4) was thus carried out in which the effect of the NEFA was studied when the driving force for the H^+ re-entry was an electric potential difference across the membrane. Proteoliposomes, preloaded with 100 mM-KCl, were incubated in a medium containing Phenol Red as a pH indicator and 100 mM-choline/HCl. Valinomycin addition induced an alkalinization of the external medium, as expected from the creation of a K^+ diffusion potential (negative inside) acting as a driving force for the entry of protons. The reaction was monitored as a change in absorbance at 556–504 nm. At a concentration of 1 μM, NEFA did not exhibit any effect on the proton conductivity of the membrane as compared with the control experiment. In the presence of 10 μM-CCCP, as expected, the proton equilibration was very fast.

Effect of NEFA on the RCI of the reconstituted proteoliposomes

The effect of different NEFA on the RCI of the reconstituted proteoliposomes is shown in Fig. 5. A significant diminution of the RCI (rate of cytochrome *c* oxidation in the presence of valinomycin and CCCP divided by the rate in their absence) was constantly observed. For comparison, the effect of myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$) and the nitrophenylazido derivative of 12-aminomyristic acid ($C_{12:0}$) were assayed. Except for oleic acid, the others were fully active at a concentration of about 1 μM. The analysis of the reaction as a function of ferrocytochrome *c* concentration from 0.12 to 26 μM showed that the diminution in the RCI was caused by an increase of the rate in the absence of valinomycin and CCCP (results not shown), whereas the rate in the presence of valinomycin and CCCP remained constant. Since NEFA were found in the above experiment not to behave as protonophores, the possibility that they could facilitate K^+ transport through the membrane was examined by reconstituting cytochrome *c* oxidase in a medium in which KCl was replaced by choline/HCl. In such a medium the addition of 2.5 mM-KCl did not stimulate the rate of cytochrome *c* oxidation (result not shown).

Effect of palmitic acid on subunit III-depleted oxidase

Subunit III was removed from the native complex by chymotrypsin digestion by a published procedure (Malatesta *et al.*, 1983). It has been found that, functionally (Püttner *et al.*, 1985) and spectrally, such a preparation was not qualitatively different from the native enzyme. The only important difference relative to the native enzyme was found in the proton-pumping

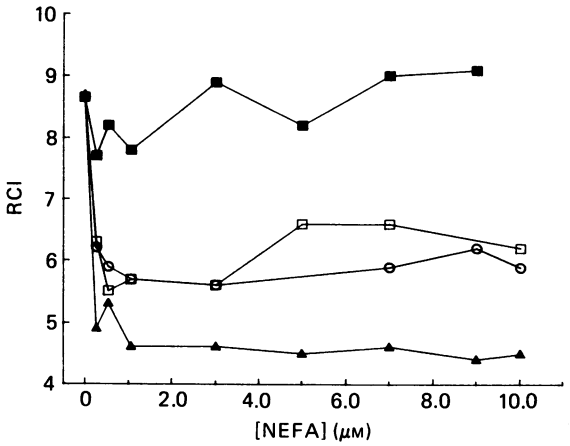

Fig. 5. Effect of different fatty acids on the RCI of cytochrome *c* oxidase vesicles

Reconstituted cytochrome *c* vesicles were incubated after a 50-fold dilution with the different NEFA at the given final concentrations. After 15 min on ice, a portion of the vesicles was assayed for the respiratory control as described in the Experimental section. ■, Oleic acid ($C_{18:1}$); ○, palmitic acid ($C_{16:0}$); □, myristic acid ($C_{14:0}$); ▲, 12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoic acid.

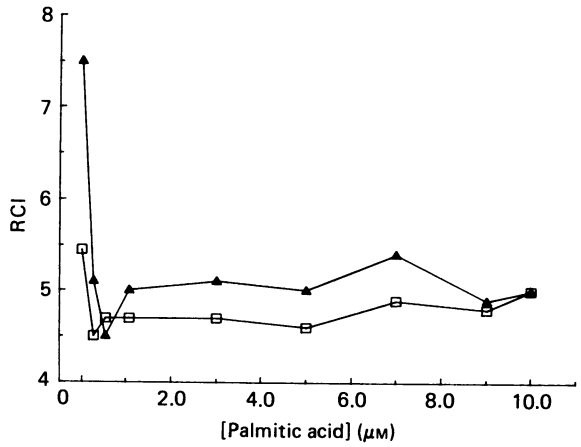

Fig. 6. Comparison of the effect of palmitic acid on the RCI in native and subunit III-depleted cytochrome *c* oxidase

Conditions were as described in the Experimental section. ▲, Native enzyme; □, subunit III-depleted enzyme.

efficiency of the chymotrypsin-treated enzyme, which has been reported to be about 50% of that of the native (Püttner *et al.*, 1985). The effect of palmitic acid on the respiratory control of the chymotrypsin-treated enzyme compared with the native enzyme is shown in Fig. 6. Although the subunit III-depleted enzyme showed a lower RCI value as compared with the native enzyme, this value (5.5) was high enough to guarantee an accurate measurement, as reported by Püttner *et al.* (1985) (RCI = 3–6) or by Prochaska & Reynolds (1986) (RCI = 3.1–9.3). The effect of palmitic acid was clearly present with the native enzyme, by analogy to what was shown in Fig. 5. On the other hand the effect of the fatty acid on the chymotrypsin-treated enzyme was much less evident. It is noteworthy that, even at high palmitic acid concentrations, the RCI was not abolished, remaining at a value of 4.5–5 for both enzyme preparations. The addition of a classical uncoupler such as CCCP changed the RCI of the above preparations to a value of 1.

Table 1 summarizes the conditions employed by Rottenberg & Hashimoto (1986) for mitochondrial inner membranes and our conditions with reconstituted cytochrome *c* oxidase proteoliposomes. The reconstituted system had a lipid/protein ratio 18 times larger than had the natural system. Owing to the preferential lipid solubilities of NEFA, their concentration should be referred, in order to compare the two situations, to the amount of lipid present. The RCI and the proton-pump measurements were performed with NEFA/lipid ratios much lower than those employed by Rottenberg & Hashimoto (1986). Only in the control experiment of the K^+-driven proton translocation were higher NEFA/lipid ratios deliberately employed (lipid/NEFA = 11:1). We can confirm that, under both Rottenberg & Hashimoto's (1986) conditions and ours, NEFA did not act as a protonophore. Only when the amount of NEFA in the K^+-driven proton equilibration experiment was increased 10-fold (lipid/NEFA = 1.1:1) did we observe an increase of the proton permeability in the case of both palmitic acid and oleic acid which never reached the values obtained with CCCP. Another point emerging from Table 1 is specificity of the NEFA–protein interaction, which can be inferred from the very low NEFA/protein ratios employed in particular in the RCI measurements (cf. also Fig. 5). In the light of this consideration, photoaffinity experiments were undertaken using the NEFA derivative 12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoic acid and reconstituted proteoliposomes. Although this compound had effects on the RCI similar to those produced by the other NEFA (Fig. 5), we were not able to detect its binding to the protein after photolysis and separation by SDS/polyacrylamide-gel electrophoresis.

DISCUSSION

Effects of NEFA on the membrane proton permeability

The inhibition by NEFA of electron-transport-driven ATP synthesis can be attributed to several factors. As classical uncouplers they may act by increasing the permeability of the coupling membrane to protons. However, it has been reported that, in both perfused liver and in isolated mitochondrial membranes, the experimental results do not fit a model in which NEFA solely affect the proton permeability of the coupling membrane. Alternatively, a decoupling in the functioning of the proton pumps of the mitochondrial membrane has been proposed, which can be ultimately reconducted to a diminution of the efficiency of the pump *per se*. Such lack of efficiency can reside either on the side of the redox-linked proton pump or on the side of the H^+-pumping ATPase. In the first case the redox pump will produce less H^+ per electron traversing the enzyme; in the second case the ATPase would function at a larger difference in proton electrochemical activity. Finally it has been suggested that NEFA uncouple by dissipating intramembraneous localized protons involved in ATP synthesis.

The model system which we have set up and exploited during the present study consists of cytochrome *c* oxidase reconstituted in phospholipid vesicles capable of acting

Table 1. Summary of the different conditions employed for studying the effect of NEFA on complex V (ATP synthase) and on complex IV (cytochrome *c* oxidase)

The complex V (F_0F_1-ATP synthase) content was taken from Schwerzmann *et al.* (1986) for rat liver mitochondria (222 pmol/mg of protein).

Source of information	Conditions			Molar ratio		
	Enzyme (pmol/ml)	Lipid† (nmol/ml)	NEFA (nmol/ml)	Lipid/protein	Lipid/NEFA	NEFA/protein
A. Fig. 2 of Rottenberg & Hashimoto (1986)	167*	243*	7.5–30.0	1455	8–32	45–180
B. Proton pump (Fig. 1 of the present study)	200	1621	30	27000	162–225	45–180
C. Respiratory control (Figs. 5 and 6 of the present study)	40	1080	0.257–10	27000	108–4202	6–250
D. K^+-induced pH changes (Fig. 4 of the present study)	0.4	10.8	1	27000	11	2500

* The lipid/protein (w/w) values were taken from Cheneval *et al.* (1985) for rat liver mitochondria (lipid/protein = 0.24) and submitochondrial particles (lipid/protein = 0.36). These values are calculated for the total protein content of mitochondria. The value reported in the Table is the specific value for complex V, without taking into account the other mitochondrial proteins. This calculation was made with the intention to show the possible NEFA–target relationship.

† For the calculations a lipid average M_r of 740 was assumed.

as a redox-coupled proton pump. The efficiency of the pump was high. The measured H^+/e^- ratio was greater than 0.5 and, after correction for the 'back leak' of protons during the time of extrusion, became nearly 1. It was, however, not affected by amounts of palmitic acid which were fully active with mitochondrial membranes.

The model system employed in the present study can thus provide a first answer, namely that the measured cytochrome *c* oxidase proton pump is not de-coupled by NEFA. The second question, which can be also answered on the basis of the results presented above, concerns the effect of NEFA on the proton permeability of the coupling membrane. From the inspection of the back flow of protons in Fig. 1 and the replot in semilogarithmic form in Fig. 2, it appears clear that the proton conductance is the same in the presence as in the absence of palmitic acid.

However, since the RCI measurements are determined under conditions in which the driving force for protons across the membrane is mainly represented by the electrical component of the total protonmotive force (absence of valinomycin), and in the proton-permeability studies of Figs. 1–3, the protonmotive force was attributable essentially to its pH component, the experiment of Fig. 4 was carried out, in which a diffusion potential was essentially responsible for the proton-driving force. Also in this case no effect of the fatty acid was visible. If protons responsible for the coupling reaction were produced by the pump activity in a localized domain, NEFA should discharge them and make them appear in the medium, where the bulk protons were measured. The fact that no difference was observed in the amount of extruded protons in the presence or absence of palmitic acid suggests either that the localized protons do not exist or that they are present in such small amounts that they could not be detected with our present system. Alternatively, and preferably, we believe that the system of the oxidase reconstituted in liposomes is coupled through a delocalized electrochemical proton gradient.

The diminution of the RCI of the vesicles incubated with palmitic acid did not correlate with a decrease of H^+/e^- ratio of the proton pump. Such a conclusion could be confirmed within a large number of cytochrome *c* oxidase reconstitutions, giving liposomes provided with an RCI varying from 3 to 9 and having a H^+/e^- ratio close to 1. It seems, therefore, that the RCI is not a parameter having a direct relationship with the efficiency of the proton pump.

Another interesting feature of the action of NEFA was its partial effect on the RCI; this parameter was affected by NEFA at low concentrations (below 1 μM, a diminution from 3.7–7.5 to 4.5–6.5 was typically observed) without any further effect when the concentration was increased.

The non-trivial nature of the effect of NEFA on the RCI alluded to above is indicated by the lack of action of oleic acid, this being attributed possibly to its unsaturated bond. It was observed, nevertheless, that when palmitic acid or oleic acid was present in high concentrations with respect to the lipid concentration (lipid/NEFA ratio = 1.1), they permeabilized the membranes to protons. This effect at high concentration is probably that described for a number of observations in mitochondria. It seems, however, that the effects of NEFA we have observed at low concentrations cannot

be explained by changes of the membrane proton permeability.

An important aspect which needs discussion is the effect of NEFA on the subunit III-depleted oxidase. Despite the lower RCI of the subunit III-depleted enzyme, a further diminution of the value to 1 can be easily obtained with CCCP, but it is practically insensitive to NEFA. Such a result suggests that subunit III may be associated with the binding of the fatty acid. To prove this point the photoactivatable compound 12-[*N*-(4-azido-2-nitrophenyl)]aminododecanoic acid was synthesized and proved to be effective (see Fig. 5) in diminishing the RCI of reconstituted cytochrome oxidase. The photolysis of the azido derivative did not result in binding to any subunit of the enzyme. Such a negative result does not exclude the possibility that the effect of FFA on the RCI can be related to its binding to subunit III, since it is not known whether the photoactive group interacts with the protein or with the lipid.

Possible mechanism of action of NEFA on cytochrome *c* oxidase

NEFA appear to play a control role at the level of the rate-limiting reaction of the oxidase: the internal electron transfer between the two centres of the enzyme. This reaction is sensitive to the transmembrane potential that causes a decrease in the overall rate of electron transfer across the enzyme, conventionally defined as 'respiratory control'. NEFA are able to increase the rate of the electron transfer only in reconstituted, and not in detergent-dispersed, enzyme, and only when the rate has been decreased by the presence of a membrane electrochemical potential (Brunori *et al.*, 1985; Sarti *et al.*, 1985). Such an effect of a potential can be realized molecularly at two levels: one by a thermodynamic modification (midpoint potential of the haem group) in such a way to oppose the flow of electrons from the outside to the inside portion of the membrane. A second control level may result from a potential-induced protein conformational change which would bring the enzyme into a less active state. According to published calculations (Blair *et al.*, 1986), a conformational change inducing a modification of the inter-haem distance of 0.2 nm would be sufficient to bring about a 100-fold decrease in the rate of electron transport. The partial NEFA-induced acceleration of the electron flow may be attributable to the membrane-potential induced conformational change being prevented. The consequence of the acceleration of the electron flow should be a small increase in the protonmotive force rather than the decrease expected from a change in proton permeability or in the degree of coupling.

In conclusion, although the mechanism of action of NEFA has not been entirely clarified in the present work, some proposed hypotheses have not been substantiated: NEFA do not increase the proton and the K^+ permeability of phospholipid membranes under conditions of apparent uncoupling, indicated by a diminution in RCI. They also do not affect the efficiency of the redox-driven cytochrome *c* oxidase proton pump. The following possibilities remain thus open, namely that NEFA act at the level of other pumps by decoupling them or that they act at the level of localized protons. In this case a model system should be selected such as to make subtle changes in this respect evident. Rottenberg & Hashimoto (1986) found that, in contrast with ionophoric uncoupling, the effect of NEFA is not associated with a significant decrease in $\Delta\mu_{H^+}$ (electrochemical activity) indicating another mechanism. Our finding that the proton pump of cytochrome *c* oxidase and the proton permeability of the associated membrane are not affected by NEFA is consistent with the above-mentioned hypothesis. The conclusion of Rottenberg & Hashimoto (1986), namely that the ATPase is the site of action of NEFA, is not contrary to our view favouring a model in which NEFA interact at multiple sites within mitochondria. Such a model would be also supported by the evidence (Andreev *et al.*, 1988) that NEFA act also at the level of the adenine-nucleotide carrier and of the 2-oxoglutarate carrier (D. Clayes & A. Azzi, unpublished work).

A possible physiological explanation for the effect of fatty acids of the level of cytochrome *c* oxidase cannot be ignored. Since the terminal oxidative enzyme exerts a partial control on the overall rate of electron flow in the mitochondrial respiratory chain (Tager *et al.*, 1983), it is important that the rate at this step can be modulated without a diminution in the transmembrane electrochemical gradient responsible for energy conservation. Our present interpretation of the effect of NEFA on cytochrome *c* oxidase is that they act at physiological concentrations on the enzyme by making it less sensitive to the potential established across the mitochondrial membrane by coupled electron flow.

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