Free fatty acids decouple oxidative phosphorylation by dissipating intramembranal protons without inhibiting ATP synthesis driven by the proton electrochemical gradient

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Free fatty acids (FFA) uncouple oxidative phosphorylation and reverse electron transport and inhibit ATP-P_i exchange in beef heart submitochondrial particles. In this, they resemble classical uncouplers and ionophores. However, in contrast to the latter agents, FFA do not collapse the substrate generated proton electrochemical potential and do not inhibit ATP synthesis when the latter is driven by artificially imposed $\Delta\tilde{\mu}H$. These results lend further support to the suggestion that oxidative phosphorylation depends, in part, on direct intramembranal proton transfer – a process which is specifically uncoupled by FFA and other membrane perturbing agents (e.g. general anesthetics).

Oxidative phosphorylation *Mitochondria* *Uncoupler* *Fatty acid* *Chemiosmosis* *Proton electrochemical difference*

1. INTRODUCTION

Oxidative phosphorylation and photophosphorylation depend on coupling of proton transport by redox H^+ pumps and an ATPase (F_0F_1) proton pump [1]. It is generally assumed that protons released at the membrane surface by the redox H^+ pumps quickly equilibrate with the bulk solvent phase and are taken up by the ATPase H^+ pump from this bulk phase in a process which is driven by the transmembrane proton electrochemical potential difference, $\Delta\tilde{\mu}H$ [1,2]. However, recent studies of the kinetics and energetics of oxidative phosphorylation and photophosphorylation, and in particular, studies of the relationships between the magnitude of $\Delta\tilde{\mu}H$, the kinetics and the free energy balance of these reactions raise serious doubts regarding the validity of this assumption of the chemiosmotic hypothesis (reviews [3–5]). We have previously suggested that in addition to the bulk mediated coupling, a more direct, intramembranal coupling pathway exists in mitochondria [6]. This model was named the parallel coupling model. Intramembranal coupling was suggested to be the result of direct interactions, due to collisions and/or aggregation of the redox H^+ pumps and the ATPase H^+ pumps [7,8]. More recently, we have found membrane perturbing agents that uncouple oxidative phosphorylation without collapsing $\Delta\tilde{\mu}H$ [9]. These agents, named decouplers, appear to uncouple specifically the intramembranal pathway. A particularly potent group of decouplers are the free fatty acids (FFA), which stimulate state 4 respiration and ATPase rates, while inhibiting oxidative phosphorylation and reverse electron transport without a significant effect on $\Delta\tilde{\mu}H$ within the uncoupling concentration range [10]. Because FFA moderately inhibit $\Delta\tilde{\mu}H$ generation by the ATPase pump, the question arises as to whether the uncoupling effect is due to an intrinsic uncoupling of the ATPase H^+ pump. This mechanism, however, does not explain the stimulation of state 4 respiration, which occurs without effect on $\Delta\tilde{\mu}H$, and which is not affected by the ATPase inhibitors, oligomycin and dicyclocarbodiimide (DCCD). Nevertheless, it was

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necessary to characterize in more detail the effect of FFA on the ATPase H^+ pump. Here, we show that palmitic acid partially inhibits oxidative phosphorylation and ATP-P_i exchange in beef heart submitochondrial particles and completely inhibits ATP-driven reverse electron transport while under the same conditions ATP synthesis, when driven by artificially imposed $\Delta\tilde{\mu}H$, is not affected. These results suggest that FFA do not impair the ability of the ATPase H^+ pump to couple proton flow to ATP synthesis but do impair the ability of F_0F_1 to receive and/or utilize protons directly from the redox H^+ pumps.

2. MATERIALS AND METHODS

Heavy beef heart mitochondria were prepared from hearts obtained at a local slaughterhouse by a conventional procedure [11]. Submitochondrial particles (ETP_H-Mg-Mn) were prepared from frozen mitochondria and used within 3–6 h of preparation [12]. Phosphorylation and P_i-ATP exchange were determined from ^{32}P incorporation into ATP or glucose 6-phosphate, extracted as in [13]. $\Delta\tilde{\mu}H$-driven ATP synthesis was assayed as in [14]. Reverse electron transport was assayed by following the fluorescence of NADH essentially as described [15]. ANS fluorescence was measured as in [19].

3. RESULTS

Fig.1 shows the uncoupling of oxidative phosphorylation by palmitic acid and FCCP in beef heart submitochondrial particles at 37°C. As was previously observed with rat liver submitochondrial particles, the uncoupling by FFA is not complete, in contrast to uncoupling in intact mitochondria. This was attributed to the lower phosphorylation potential against which phosphorylation takes place in submitochondrial particles [10]. Note that CCCP is approx. 10^3-fold more potent than palmitate and that the uncoupling is complete. As discussed above, the effect of palmitate on oxidative phosphorylation may be due to intrinsic uncoupling of the ATPase. If this is the case, we expect that palmitate will also inhibit ATP synthesis driven by an artificially imposed $\Delta\tilde{\mu}H$ [14,16]. Fig.2 shows the effect of palmitate and CCCP on ATP synthesis driven by an artificially

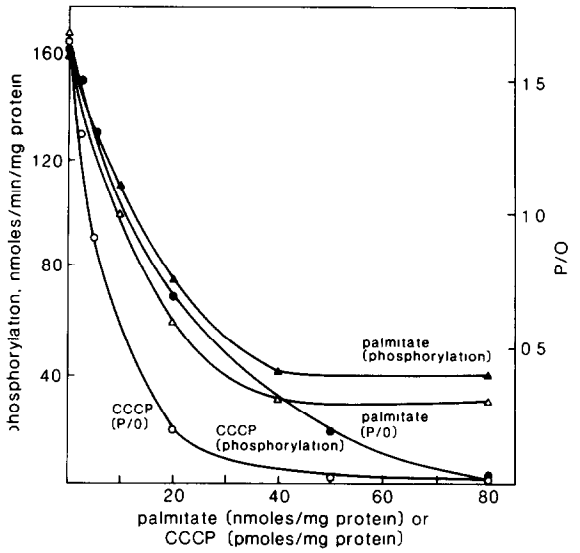

Fig.1. The effect of palmitate and CCCP on oxidative phosphorylation. Beef heart submitochondrial particles (0.5 mg/ml) were incubated in 1 ml of medium composed of: 0.25 M sucrose, 5 mM Na-Hepes (pH 7.5), 3 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM succinate and the indicated amount of uncoupler for 10 min at 37°C. Phosphorylation was started by the addition of 1 mM ADP and ^{32}P (10^6 cpm/ml). The reaction was terminated after 1 min by the addition of 0.2 ml of 50% trichloroacetic acid. Phosphorylation rates were estimated from the incorporation of ^{32}P into ATP, as described in section 2. Respiration rates were measured with the aid of an oxygen electrode. Full symbols indicate the phosphorylation rate (▲, palmitate; ●, CCCP). Empty symbols indicate the P/O ratio (△, palmitate; ○, CCCP).

imposed pH gradient combined with an artificially imposed potassium diffusion potential. Net ATP synthesis is observed only when both pH and valinomycin induced potassium diffusion potential are generated (not shown). The synthesis is very sensitive to CCCP. In sharp contrast, palmitate does not inhibit $\Delta\tilde{\mu}H$-driven synthesis at all. The experiment shown in fig.2 was done under optimal conditions for $\Delta\tilde{\mu}H$-induced ATP synthesis, which are considerably different from the conditions of fig.1 which are optimal for FFA uncoupling. Of particular importance is the difference in temperature since, unlike CCCP and other classical uncouplers, palmitate uncoupling strongly depends on temperature and is more pronounced at high temperatures [10]. Also, as discussed above, the

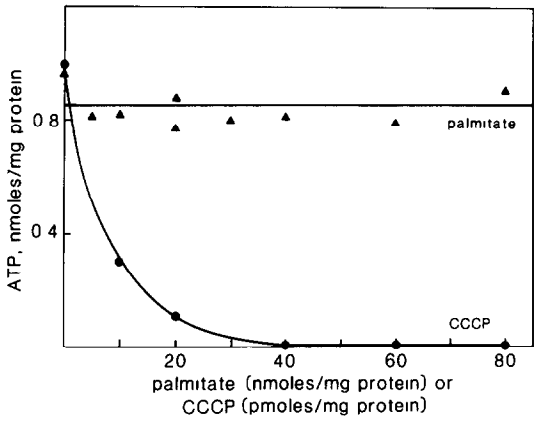

Fig.2. The effect of palmitate and CCCP on ATP synthesis driven by artificially imposed $\Delta\tilde{\mu}H$. Beef heart submitochondrial particles (1.5 mg) were incubated with Na^+ malonate (20 mM), NaCN (2.5 mM) and valinomycin (1.6 μM), pH 5.0, in a total volume of 0.2 ml, at room temperature. After 40 s of stirring 0.3 ml base stage solution was added to final concentration of 100 mM Na^+-Hepes, 100 mM KCl, 2 mM $MgCl_2$, 2 mM ADP, 5 mM $Na^{32}P_i$, 40 mM glucose and 80 U/ml hexokinase (final pH 7.5). The reaction was terminated after 20 s by the addition of 0.15 ml of 20% trichloroacetic acid and analysed for glucose 6-[^{32}P]phosphate content. (▲) Palmitate, (●) CCCP.

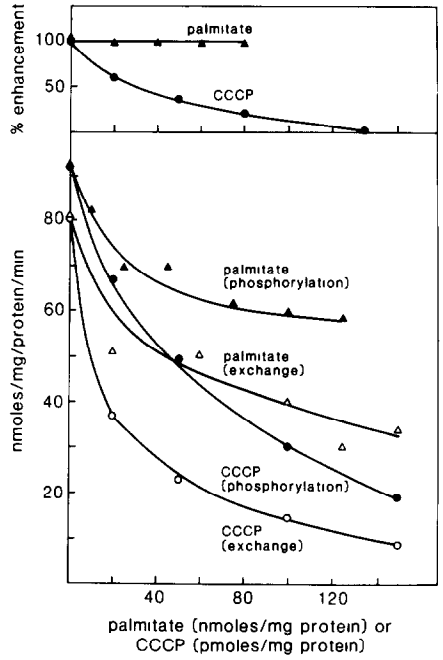

Fig.3. Effect of CCCP and palmitate on the rate of oxidative phosphorylation, $^{32}P_i$ exchange and membrane potential under conditions similar to the assay conditions of ATP synthesis driven by imposed $\Delta\tilde{\mu}H$. For measurement of $\Delta\psi$ (as indicated by the enhancement of ANS fluorescence), 15 μM ANS was added. Enhancement was calculated from the fluorescence before and after the addition of 1 mM CCCP [19] (top). For assay of oxidative phosphorylation beef heart submitochondrial particles (1.5 mg/ml) were incubated in a medium composed of: 0.25 M sucrose, 3 mM Na_2HPO_4, 5 mM Na-Hepes (pH 7.5), 1 mM $MgCl_2$, 5 mM succinate, 20 mM glucose and 30 U/ml hexokinase; T = 23°C. Other conditions as in fig.1. For assay of $^{32}P_i$ exchange beef heart submitochondrial particles (0.6 mg/ml) were incubated in a medium consisting of: 0.25 M sucrose, 5 mM Na-Hepes (pH 7.5), 3 mM ATP, 3 mM $MgCl_2$, 4 mM KCN; T = 23°C. Other conditions as in fig.1.

phosphate potential, against which phosphorylation proceeds, affects the efficiency of FFA uncoupling and also CCCP uncoupling [15]. Since $\Delta\tilde{\mu}H$-induced synthesis is assayed in the presence of an ATP trap (glucose + hexokinase) and at room temperature a lower sensitivity to FFA and CCCP might be expected. We therefore studied the uncoupling of oxidative phosphorylation by palmitate and CCCP, under conditions which closely match those of fig.2. As expected, under these conditions, oxidative phosphorylation is less sensitive to both palmitate and CCCP (fig.3, bottom). Nevertheless, a clear and highly significant inhibition of the rate of phosphorylation by palmitate is observed, resulting in up to 50% reduction of the P/O ratios. Hence, quite clearly, there is a real and significant difference between the effect of palmitate on oxidative phosphorylation, which is partially uncoupled, and artificially imposed $\Delta\tilde{\mu}H$-driven ATP synthesis, which is not. In contrast the protonophore CCCP is a more potent inhibitor of artificially imposed $\Delta\tilde{\mu}H$-driven ATP synthesis than of oxidative phosphorylation (figs 2,3). These findings rule out the explanation of uncoupling by FFA as an intrinsic uncoupling of the proton ATPase. In beef heart submitochondrial particles, FFA inhibit neither the succinate-induced enhancement of ANS fluorescence (fig.3, top), indicating no effect on membrane potential [19], nor the succinate-induced quenching of 9-aminoacridine (not shown), indicating no effect

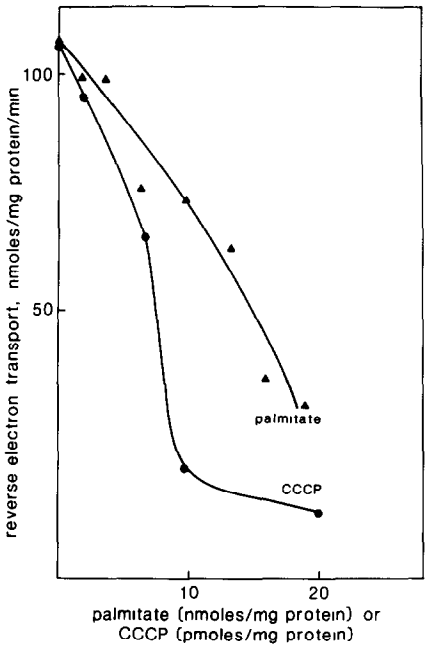

Fig.4. The effect of palmitate and CCCP on ATP driven reverse electron transport. Beef heart submitochondrial particles (0.3 mg/ml) were incubated in 0.2 M sucrose, 1 mM NAD, 50 mM Tris-acetate (pH 7.5), 5 mM $MgCl_2$, 5 mM succinate and 10 ng/ml antimycin. The reaction was started by the addition of 1 mM ATP. Initial rates of NADH formation were determined from the increased fluorescence of NADH [15].

on ΔpH. Therefore, in this system as well, uncoupling of oxidative phosphorylation does not depend on the collapse of $\Delta\tilde{\mu}$H. Fig.4 shows that ATP-driven electron transport from succinate to NAD^+ is strongly inhibited by both CCCP and palmitate.

4. DISCUSSION

The demonstration of an artificially imposed $\Delta\tilde{\mu}$H-driven ATP synthesis [14,16,17] is the best evidence for the chemiosmotic mechanism of energy conversion. Obviously, agents that collapse $\Delta\tilde{\mu}$H, such as protonophores or other suitable ionophores, inhibit this process. Since FFA do not inhibit this process, it is clear that FFA do not uncouple oxidative phosphorylation by collapsing $\Delta\tilde{\mu}$H. Moreover, the observation that FFA do not inhibit net ATP synthesis driven by an artificially imposed $\Delta\tilde{\mu}$H clearly indicates that their uncoupling of oxidative phosphorylation is not the result of intrinsic uncoupling of the ATPase. Hence, presently our suggestion that the uncoupling of energy conversion is due to impairment of intramembranal proton transfer and/or storage is the most plausible explanation for the effect of FFA (and other decouplers of oxidative phosphorylation). The fact that FFA inhibit ATP-P_i exchange probably results from their inhibition of $\Delta\tilde{\mu}$H when the latter is generated by ATP [10]. In this way they are similar to classical uncouplers and ionophores. Why should ATP-driven $\Delta\tilde{\mu}$H be sensitive to FFA while substrate-driven $\Delta\tilde{\mu}$H is not, if, as shown here, FFA are not intrinsic uncouplers of F_0F_1? It is difficult to provide a totally satisfactory answer for this question at present. However, the observation does point out the possibility that the intrinsic coupling in F_0F_1 is not totally symmetric. Consider the suggestion [3] that there is a capacity for F_0 to store a number of 'energized' protons (i.e. protons stored at an electrochemical potential higher than the proton electrochemical potential on the m-side of the membrane). This could be crucial for the generation of $\Delta\tilde{\mu}$H by the ATPase and for intramembrane coupling, but could be unessential for $\Delta\tilde{\mu}$H-driven ATP synthesis (since energized protons are stored in the bulk phase on the c-side of the membrane). Hence, FFA, which may release protons from these storage sites, would inhibit oxidative phosphorylation and ATP-generated $\Delta\tilde{\mu}$H, but not $\Delta\tilde{\mu}$H-driven ATP synthesis. A related observation was made in studies of oxidative phosphorylation in alkalophilic bacteria [18]. In these bacteria, oxidative phosphorylation by an F_0F_1 ATPase proceeds under conditions of very low $\Delta\tilde{\mu}$H. When an artificially induced $\Delta\tilde{\mu}$H of the same magnitude was imposed, no net ATP synthesis was observed, indicating that oxidative phosphorylation may depend on intramembranal proton transfer.

In summary, the results of our studies of the effect of FFA on energy conversion processes in beef heart submitochondrial particles strongly support our previous suggestion, based on studies with rat liver mitochondria, that FFA interfere with intramembranal proton transfer, most probably by dissipation of intramembranal pools of occluded protons.

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