EFFECTS OF FLUORESCAMINE MODIFICATION OF RAT LIVER MITOCHONDRIA ON THE ACTION OF UNCOUPLERS

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1. Introduction

According to the chemiosmotic hypothesis [1] uncouplers facilitate electrogenic proton movement across mitochondrial inner membrane, thus dissipating a H^+ gradient and membrane potential. A close relation between the action of uncouplers on mitochondria and the proton conductivity of various artificial membrane preparations has been observed [2,3]. However, controversial results were also reported [4,5]. The detailed kinetic study of uncoupler-mediated proton transport across bilayer membrane suggests that the greater dielectric constant of mitochondrial inner membrane may account for the greater efficiency of transporting protons by uncouplers [6].

The ability of uncoupling agents to transport protons into mitochondria, measured by passive swelling, has been compared with respiratory stimulation [7]. The result which shows a close correlation between respiratory stimulation and facilitated proton transport, is consistent with the suggestion that these two events are directly coupled [8].

In this report, fluorescamine was used to covalently modify the primary amino groups [9] of mitochondrial membrane. The efficiency of uncouplers to discharge proton gradient was compared with their ability to release respiration in rat liver mitochondria modified with various amounts of fluorescamine. The results show that modification by fluorescamine inhibits uncouplers' ability to stimulate respiration but not their proton transport efficiency. It is suggested that the proton movement is tightly but indirectly linked to respiration in intact rat liver mitochondria.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; DTFB, difluoromethyltetrachlorobenzimidazole; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1 piperazineethane sulfonate; NEM, *N*-ethylmaleimide, S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide

2. Materials and methods

Rat liver mitochondria were isolated in 0.25 M sucrose containing 3 mM Hepes at pH 7.4 as in [10] and assayed under the conditions in [11]. Mitochondria were modified with fluorescamine in 2.2 ml of a medium containing 120 mM LiCl, 10 mM KCl, 3 mM EGTA, and 3 mM Hepes (pH 7.4) by injecting small volumes of fluorescamine in acetone to the suspension under rapid stirring. After incubating for 1 min at 22°C, 40 nmol NEM/mg, 1 μg oligomycin/mg, 500 ng valinomycin (or 100 ng valinomycin/mg) and 3 μM rotenone were added to the suspension unless indicated otherwise in the figure legend. The suspension was then further incubated for 2–3 min. Reactions were started by the additions of 1 mM succinate (final concentration). Proton extrusion and oxygen consumption were measured simultaneously in a modified Gilson Medical oxygen chamber. Proton extrusion was followed by a Markson Model J-445 combined electrode connected to a Corning Model 112 pH meter and the change of H^+ concentration was recorded by a Varian A-25 recorder. The buffering capacity of the mitochondrial suspension was determined by injecting a series of 2 μl standard HCl

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before and after the experiment. The respiration rates were followed polarographically, using a Yellow Spring Oxygen Monitor (Model 53). Organic solvents (acetone and ethanol), at the amounts used (<15 μl), were determined to have no significant effect on either the proton movement or respiration.

Valinomycin, oligomycin, fluorescamine, Hepes, EGTA, rotenone, and sodium succinate were obtained from Sigma Co. CCCP and FCCP were from Pierce Chemical. NEM (Gold Label) was purchased from Aldrich. DNP of Fisher was recrystallized before use. S-13 and DTFB were gifts from Dr D. Durst of Bioenergetics Laboratory, SUNY at Buffalo and Dr S. McLaughlin of our University, respectively.

3. Results

3.1. *Effects of fluorescamine modification on respiration-dependent H^+ extrusion*

Upon energization with substrates, protons are extruded from mitochondria to the medium. The apparent H^+ gradient can be discharged by the addition of uncouplers. In the presence of excess substrates, H^+ gradient also collapses when the limited amount of oxygen is exhausted (H^+-leak). The reuptake rate of H^+ (collapse of the H^+ gradient) is usually greatly accelerated by the presence of uncouplers, as shown in fig.1. The concomitant respiration rate is also enhanced by the presence of uncouplers (data not shown).

The H^+ extrusion rate of fluorescamine treated mitochondria is decreased as the extent of modification increased. However, the concomitant respiration rate and the apparent net amount of H^+ extruded (ΔH^+) are not significantly affected. This differential inhibition is illustrated in fig.2. It is possible that the observed inhibition of extrusion rate is the result of increased H^+-leak rate. However, as shown in fig.3, the H^+-leak rate as determined by the oxygen-pulse method [12] or as determined by the anaerobic H^+-leak described in fig.1 (data not shown) remains unchanged in modified mitochondria (⩽30 nmol fluorescamine/mg protein).

3.2. *Effects of the modification on respiratory stimulation by uncouplers*

The respiration rate of modified mitochondria, like the normal ones, can be stimulated by the

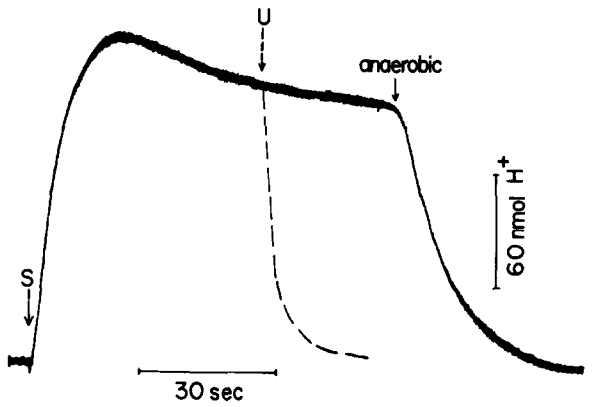

Fig.1. Proton movement associated with the energy state of mitochondria. Mitochondria containing 8 mg protein were suspended and assayed in a medium described in section 2. For the anaerobic H^+-leak assay, the above medium was bubbled with N_2 to reduce oxygen tension to 20% of that in the normal medium. At the arrows indicated with S and U, succinate and uncouplers were added, respectively. At the arrow indicating anaerobic, the concomitant oxygen had effectively come to halt (zero oxygen tension).

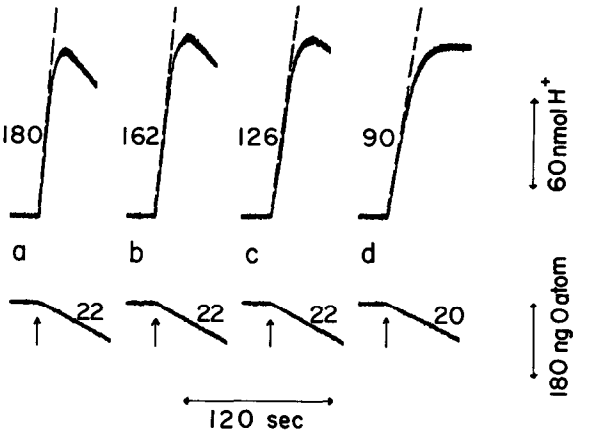

Fig.2. Effects of fluorescamine modification on respiration and concomitant H^+ movement. Mitochondria containing 4.3 mg protein, were modified and assayed as in section 2. Traces a, b, c and d represent H^+ extrusion (upper) and oxygen consumption (lower) kinetics of mitochondria modified with 0, 5, 15 and 25 nmol fluorescamine/mg protein, respectively. The numbers beside the traces represent the corresponding initial H^+ extrusion rate and oxygen consumption rate in nmol H^+/(min.mg) and ng atom O/(min.mg), respectively. Arrows (↑) indicate the addition of succinate.

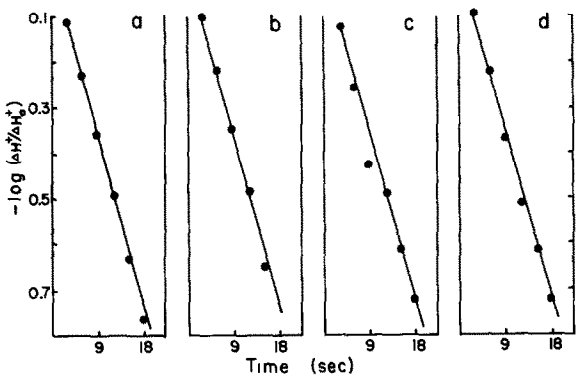

Fig.3. Effects of fluorescamine modification on the H^+-leak process. Mitochondria containing 7 mg protein were modified and assayed as in fig.1,2. After the suspension became anaerobic and ΔH^+ decayed to a stable level, an aliquot (10 μl) of O_2 saturated assay medium was rapidly added to the suspension. The sudden aerobic condition caused a rapid H^+ extrusion. The kinetics of H^+ uptake by mitochondria when suspension become anaerobic again was analyzed by the first-order rate law. $\Delta H^+/\Delta H^+_0$ represents the ratio of extruded protons remaining after a given time interval to that at the time when proton decay was first noted. The apparent first-order rate constants for mitochondria modified with 0 (a), 10 (b), 20 (c) and 30 (d) nmol fluorescamine per mg protein are 0.043, 0.045, 0.041 and 0.046 s^{-1}, respectively.

presence of CCCP or S-13. However, as shown in fig.4, the extent of stimulation is decreased as the extent of modification increased. The possibility that the modification may have decreased the efficiency of the uncouplers is rendered unlikely since the concentration(s) of uncoupler(s) needed for optimal respiratory stimulation is not increased (in fact, slightly decreased) as the modification increased, as shown in fig.4. Similar results were also obtained with DNP, FCCP, and DTFB (data not shown).

3.3. *Effects of the modification on uncoupler-induced H^+ transport*

Since the ability of uncouplers to stimulate respiration is inhibited, one would expect that the transport of H^+ induced by uncouplers (discharge of H^+ gradient) would be hindered in modified mitochondria. However, as shown in fig.5, the apparent kinetics of H^+ transport catalyzed by various concentrations of CCCP, as determined by the procedure mentioned in fig.1, is not affected by the modification. Similar results were also obtained by using S-13, FCCP, DNP, and DTFB.

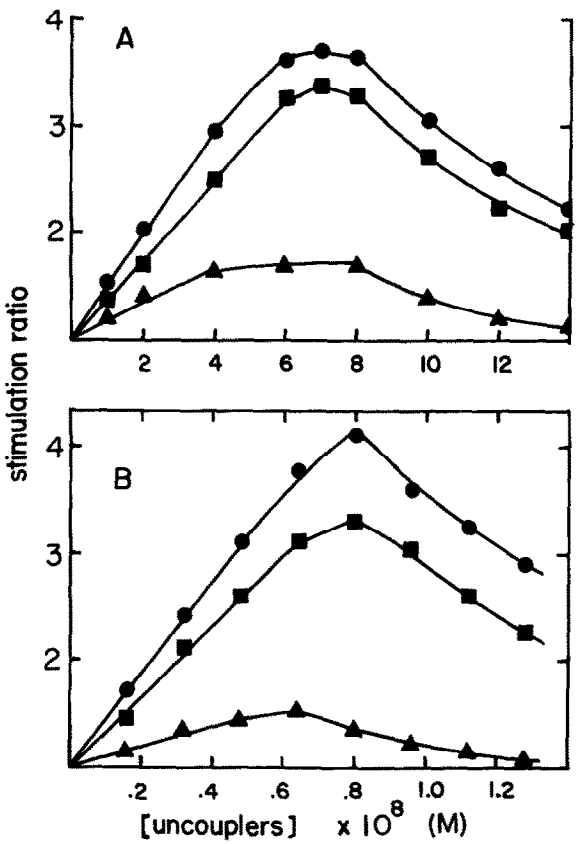

Fig.4. Effect of fluorescamine modification on the stimulation of respiration. Mitochondria containing 4 mg protein, were modified and assayed as described in fig.1 except valinomycin was omitted from the assay medium. In A, the effect of 0 (—●—●—), 5 (—■—■—) and 20 (—▲—▲—) nmol fluorescamine/mg protein on the stimulation of respiration by CCCP is shown. In B, the effect of 0 (—●—●—), 6 (—■—■—) and 25 (—▲—▲—) nmol fluorescamine/mg protein on the stimulation of respiration by S-13 is shown. The stimulation ratio is calculated by dividing the respiration rate after the addition of uncoupler by the rate before the addition. Similar results were obtained in the presence of valinomycin (except smaller extent of stimulation were observed).

4. Discussion

It is generally accepted that uncouplers can discharge the 'high energy state' generated by redox events. The observation that uncouplers enhance proton translocation and respiration in intact mitochondria [7] shows that these two events are tightly

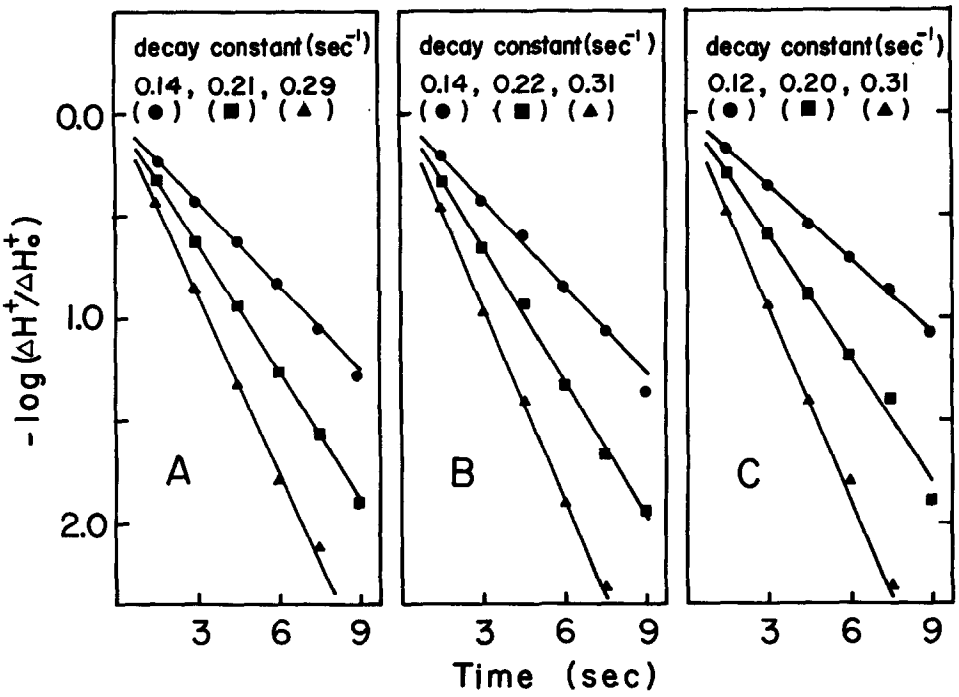

Fig.5. Effect of fluorescamine modification on facilitated H^+ transport by CCCP. Mitochondria were modified with fluorescamine and assayed as in section 2 and fig.1. The H^+ extrusion was started by the addition of succinate. After passing the maximum point of ΔH^+ and attaining steady alkalinisation rate, CCCP was added to discharge the remaining ΔH^+ (at the same level for all the experiment). The rate constants were calculated from the slopes of the lines shown (alkalinisation rate subtracted). $\Delta H^+/\Delta H^+_0$ represents the ratio of extruded protons remaining after a given time interval to that at the time uncoupler was added. A, B and C denote mitochondria modified with 0, 5 and 20 nmol fluorescamine/mg protein. (—●—●—), (—■—■—) and (—▲—▲—) represent final CCCP concentrations of 1.0×10^{-8} M, 4.0×10^{-8} M and 7.0×10^{-8} M, respectively.

coupled. The results presented in this report that the modification of mitochondria by fluorescamine causes an inhibition of respiration stimulation but not the proton translocation induced by uncouplers suggest strongly that the proton movement is indirectly linked to respiration. The data that the modification slows down the proton extrusion but not the concomitant electron transfer associated with the oxidation of succinate support the above suggestion. It should be mentioned that a similar suggestion was made in [13] based on the effects of DCCD on cytochrome oxidase. It is likely that at least an intermediate step, whether chemical or conformational, is required for the coupling of these two events. Thus, an indirect proton extrusion mechanism such as the vectorial Bohr effects [14,15] or the conformational coupling model [16] may be operative in rat liver mitochondria.

Acknowledgement

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