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EFFECTS OF FLUORESCAMINE LABELING ON MITOCHONDRIAL ATPase ACTIVITY

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SUMMARY

Fluorescamine labeling of rat liver mitochondria enhances the ATPase activity. It reached maximum stimulation when mitochondria were treated with 30-34 nmol fluorescamine per mg of mitochondrial protein. This stimulation is inhibited by N,N'-dicyclohexylcarbodiimide. The maximum stimulation caused by labeling is the same as that obtained from uncoupler with optimum concentration. The chemiosmotic potential ($\Delta\mu_{H^+}$) decreases as the labeling increased. However, $\Delta\mu_{H^+}$ is not abolished completely even when ATPase activity reaches a maximum. The results suggest that primary amino groups may be involved in controlling mitochondrial ATPase activity.

Fluorescamine reacts specifically with primary amino groups to form intensely fluorescent products (1). Using fluorescamine labeling, Kraayenhof and Slater (2) showed a close connection between free amino groups and the conformational changes of chloroplast coupling factor 1. Harnischfeger (3) demonstrated that the functions associated with chloroplast coupling factor 1 are also more sensitive to fluorescamine treatment. We have shown that fluorescamine is relatively impermeable to mitochondrial inner membrane and the labeling results are sensitive to the metabolic conditions (4). In the present work, the effects of the labeling on mitochondrial ATPase action are described. The results suggest that primary amino groups may play an important role in mitochondrial ATPase function.

Abbreviations: DCCD, N,N'- dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; S-13, 5-chloro-3-tert-butyl-2'-chloro-4'-nitrosalicylanide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; RLM, rat liver mitochondria.

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MATERIALS AND METHODS

Rat liver mitochondria were isolated as previously described (4). Fluorescamine treated mitochondria were prepared by rapid injection of a small volume of fluorescamine in acetone to mitochondria suspended in the buffer solution which contained 220 mM mannitol, 70 mM sucrose, and 2 mM Hepes at pH 7.4. After centrifugation, treated mitochondria were resuspended to 50 mg/mL in the same buffer. To examine the distribution of fluorescamine labeled groups in mitochondria, the inner membrane, outer membrane, and soluble fraction were separated by the method of Sottocasa, et al. (5). The corrected fluorescence intensity of each fraction was determined by a Perkin-Elmer MPF 44A spectrofluorometer.

The ATPase activity was assayed at room temperature by the method of Pullman, et al. (6). The reaction mixture with a volume of 3 mL contained 6 mM ATP, 3 mM $MgCl_2$, 5 mM phospho-enol-pyruvate, 90 μg pyruvate kinase, and 50 mM Hepes, pH 7.4. The reaction was initiated by the addition of mitochondria and terminated by the addition of 1 mL 40% trichloroacetic acid after 10 minutes. The inorganic phosphate released was determined by measuring the absorbance of the phosphate-molybdate-malachite green complex at 660 nm as mentioned by Hess and Derr (7).

The proton movement associated with ATPase action was monitored by the procedure of Reynafarje and Lehninger (8). The assaying medium with a volume of 2.2 mL contained 120 mM LiCl, 10 mM KCl, 2 μM rotenone, 500 ng valinomycin, mitochondria (5 mg of protein), and 3 mM Hepes, pH 7.3. The reaction was started by the addition of ATP and the H^+ movement was followed by a Markson Model J-445 combined electrode connected to a Corning Model 112 pH meter. The changes of H^+ concentration were recorded by a Varian A-25 recorder.

Pyruvate kinase, phospho-enol-pyruvate, valinomycin, rotenone, Hepes, mannitol, sucrose, and malachite green were obtained from Sigma Co. DCCD was purchased from Aldrich. Fluorescamine, Flaminox solution (1%), and DNP were from Fisher. S-13 was a generous gift from Dr. D. Durst of SUNY at Buffalo.

RESULTS AND DISCUSSION

The enhancement of ATPase activity by fluorescamine treatment: Mitochondria treated with fluorescamine exhibit enhanced ATP hydrolysis activity. As shown in Figure 1, the enhancement reaches a maximum with mitochondria treated with about 34 nmol fluorescamine per mg of protein. When these treated mitochondria were further fractionated, it was found that about 65, 20, and 15% of the total labels were associated with the inner membrane, outer membrane and soluble fraction, respectively. Since the reaction between fluorescamine and primary amino groups is nearly quantitative (85-90% in efficiency) (1,9) and the labels distributed evenly between protein and lipid part of inner membrane (4), we estimated that the ATPase activity reaches maximum when about 10 nmol $-NH_2$ groups of the protein part of inner membrane modified per mg of total mitochondrial protein.

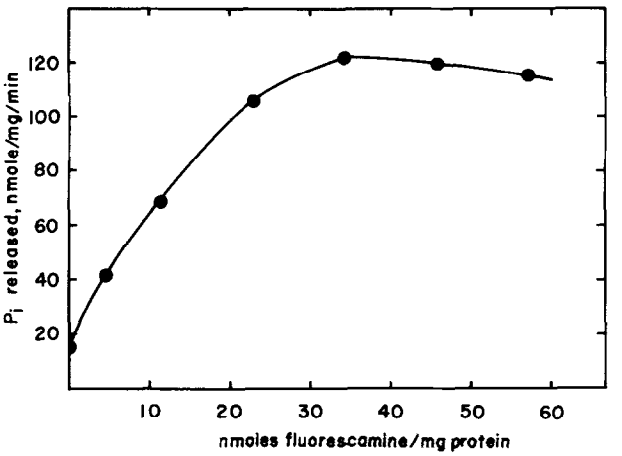

FIGURE 1. The Effects of Fluorescamine Treatment on Mitochondrial ATPase Activity. The mitochondria were treated as described in MATERIALS AND METHODS. The ATP hydrolysis rate measured by inorganic phosphate released is the average rate over a period of 10 minutes. The hydrolysis products of fluorescamine have no effect on the ATP hydrolysis rate (RLM added to assaying media 2 minutes after the addition of fluorescamine).

Effects of DNP and DCCD on ATPase activity of modified Mitochondria:

It is important to establish that the enhanced ATPase activity by the treatment of fluorescamine originates from the ATPase of mitochondrial inner membrane. Thus, the effects of uncoupler and DCCD on the ATPase activity of modified mitochondria were tested. The results are summarized in Table 1. The ATPase activity of normal mitochondria is stimulated by the presence of DNP and is arrested by the presence of DCCD. For modified mitochondria, DCCD also inhibits their ATPase activity. Furthermore, the presence of DNP can not further stimulate the ATPase activity of modified mitochondria beyond the saturation level which is close to the maximum stimulation caused by DNP to normal mitochondria. These results indicate that the enhancement caused by the fluorescamine treatment originates from the modification of membrane bound ATPase system.

ATPase activity associated H^+-movement of modified mitochondria:

Mitchell and Moyle (10) showed that there is a link between ATP hydrolysis and the vectorial transmembranous movement of H^+ in mitochondria. The effects

TABLE 1. Effects of DNP and DCCD on Modified RLM ATPase activity.

	Experiments	Relative ATPase Activity[1]
1.	RLM	1.0
2.	FL-RLM(1)	3.8
3.	FL-RLM(2)	6.2
4.	RLM + DNP	6.3
5.	FL-RLM(1) + DNP	6.2
6.	FL-RLM(2) + DNP	6.5
7.	RLM + DCCD	0.3
8.	FL-RLM(1) + DCCD	0.4
9.	FL-RLM(2) + DCCD	0.4
10.	RLM + DCCD & DNP	0.5
11.	FL-RLM(2) + DCCD & DNP	0.4

[1]Average of three determinations (± 10%), the ATPase activity of normal mitochondria used was 22 nmol P_i released per minute per mg protein. FL-RLM(1) and FL-RLM(2) are mitochondria treated with 16 and 32 nmol fluorescamine per mg of total mitochondrial protein, respectively. The concentrations of DNP and DCCD used were 30 μM and 6 μg/mg protein, respectively.

of fluorescamine treatment on this ATP hydrolysis linked $\Delta\mu_{H^+}$ were examined. As shown in Figure 2, mitochondria treated with 0, 15, 25, and 35 nmol of fluorescamine per mg mitochondrial protein have initial proton ejection rates as 92, 75, 48, and 34 nmol H^+ per minute per mg protein, respectively. Like normal mitochondria, the $\Delta\mu_{H^+}$ of modified mitochondria is decreased by the addition of uncouplers (DNP or S-13). When DNP with optimum concentrations was added to normal mitochondria before the addition of ATP, $\Delta\mu_{H^+}$ was not observed (the slow H^+ release is the result of ATP hydrolysis). It is of interest to note that unlike uncouplers, the maximum stimulation of ATP hydrolysis caused by the fluorescamine is not accompanied by the complete collapse of $\Delta\mu_{H^+}$.

Uncouplers cause the rapid shuttle of H^+ across inner membrane and therefore enhances the ATP hydrolysis (11). Fluorescamine modifies the exposed primary amino groups which include the N-terminal amino acids and ε-amino groups of lysine residues of membrane proteins and the amine groups of phosphatidylethanolamine of membrane lipids. It is very unlikely that the enhancement of ATPase activity caused by labeling has the same mechanism as that for uncoupler

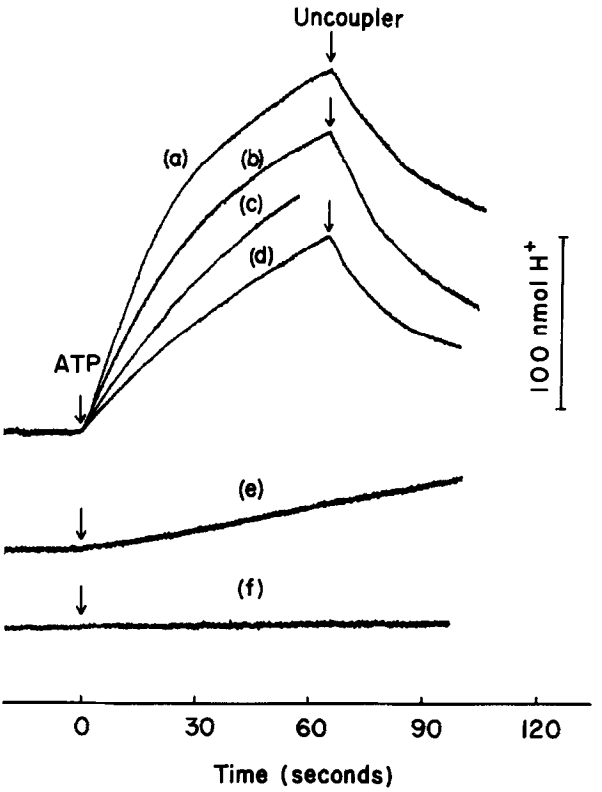

FIGURE 2. The Effects of Fluorescamine Treatment on ATP Hydrolysis Linked H^+ Movement. Mitochondria (5 mg of protein) were incubated in the solution mentioned in MATERIALS AND METHODS for 5 minutes. A 10 µL solution which contained 3 µmol ATP and 1.5 µmol $MgCl_2$ (pH 7.3) was then added to start the H^+ movement. The maximum pH change observed was less than 0.15. The buffer capacity of the system was measured before the addition of ATP and at the end of the experiment by injecting a series of 5 µL of standardized HCl (0.12 N). There was no apparent change of buffer capacity of the mitochondria used. Curves (a), (b), (c), and (d) were obtained with mitochondria treated with 0, 15, 25, and 35 nmol fluorescamine per mg mitochondrial protein, respectively. The addition of uncoupler was made of injecting a small volume of DNP or S-13 such that the final concentration was 30 µM or 5 nM. Curves (e) and (f) were obtained with normal mitochondria incubated with 30 µM DNP and 6 µg DCCD per mg mitochondrial protein before the addition of ATP, respectively. The initial H^+ movement rates are 92, 75, 48, 34, 7, and ∿0 nmol H^+ per minute per mg mitochondrial protein for (a), (b), (c), (d), (e), and (f), respectively.

stimulation, since the flip-flop rates of membrane components are too slow in general (12). Because fluorescamine treatment does not change the apparent maximum ATP hydrolysis rate, it is unlikely that the catalytic site is affected by the modification. Thus, the observed enchanced ATP hydrolysis accompanied

by hindered $\Delta\mu_{H^+}$ generation suggests that either the transferring of ATP to the catalytic site is enhanced or the outward H^+ movement through proton channel is slowed down, or both, by the modification of $-NH_2$ groups.

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