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Energization of Mitochondrial Inner Membranes by L-Malate under Anaerobic Condition is Driven by Energy Derived at Site I of Phosphorylation

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It was shown that addition of L-malate to anaerobic mitochondria, like addition of adenosine triphosphate, caused a red shift of the absorbance maximum, and an increase in the fluorescence of ethidium. These responses of ethidium were sensitive to uncouplers and rotenone. They were not inhibited by antimycin A, KCN or oligomycin. These results show that addition of L-malate to anaerobic, KCN- or antimycin A-inhibited mitochondria caused energization of the mitochondrial inner membranes. Furthermore, this energization of the membranes was reversed by succinate, malonate, and thenoyltrifluoroacetone. Thus, the present results clearly show that L-malate-induced energization of anaerobic mitochondria was driven by energy derived from redox reactions at site I of phosphorylation.

Keywords——energization; ethidium; cytochrome oxidase; oxidative phosphorylation; rat liver mitochondria

Dawson *et al.*[2] showed that both L- and D-malate induced oxidation of cytochrome b-558 in anaerobic mitochondria from *Arum spadix*, pea, mung bean and rat liver. Muraoka and Sugiyama[3] showed that L-malate also caused oxidation of cytochrome a in anaerobic rat liver mitochondria. Then we found that the effect of L-malate on cytochrome a in anaerobic mitochondria was insensitive to oligomycin but that it was reversed by all the uncouplers and detergents tested.[4] From these results, we suggested that addition of L-malate may cause energization of the mitochondrial inner membranes and that oxidation of cytochrome a caused by L-malate in anaerobic mitochondria may result from a L-malate-induced, energy-linked reversal of electron transfer at site II of phosphorylation.[4] However, it was still unknown why L-malate caused energization of mitochondria under anaerobic conditions.

As described in this paper, to clarify the nature of the L-malate-induced energization of mitochondria under anaerobic conditions, we studied the response of ethidium in L-malate-treated anaerobic, KCN-, or antimycin A-inhibited mitochondria to L-malate since ethidium reports an energized state of mitochondria[5-10] and it specifically binds to hydrophobic proteins (chargerin) in an energized state which may have an essential role in the energy transfer in mitochondrial inner membranes.[11-14] The present results clearly show that the L-malate-induced energization of mitochondria under anaerobic conditions was driven by energy derived at site I of phosphorylation.

Experimental

Ethidium bromide was purchased from Tokyo Kasei, Tokyo (Japan). Other reagents were as described previously.[4] Rat liver mitochondria were isolated by the method of Hogeboom,[15] as described by Myers and Slater.[16]

All reactions were carried out in medium consisting of 25 mM Tris–HCl buffer, 50 mM sucrose, 5 mM $MgCl_2$, 2 mM ethylendiaminetetraacetic acid (EDTA) and 15 mM KCl, with other components as indicated in the legends to the figures, in a final volume of 3 ml at pH 7.1. All measurements were carried out at room temperature (approx. 20 °C).

Absorbance changes of cytochrome $a+a_3$ and ethidium were examined in a Hitachi, model 556, dual-wavelength, double-beam spectrophotometer using wavelength pairs of 608 nm minus 630 nm for cytochrome $a+a_3$, and 510 nm minus 485 nm for ethidium. Fluorescence changes of ethidium were determined with a Hitachi, model MPF 3, spectrophotometer, using a wavelength of 485 nm for excitation and measuring fluorescence at 616 nm.

Results and Discussion

As shown in Fig. 1(A), addition of adenosine triphosphate (ATP) to anaerobic mitochondria caused oxidation of cytochrome a and a red shift of ethidium absorption in an energy dependent fashion. The red shift of ethidium was measured as described in Experimental, using a wavelength pair of 510 nm minus 485 nm. Figure 1(B) shows that addition of L-malate to anaerobic mitochondria also induced a red shift of ethidium. The effect of L-malate was like that of ATP, except that the time required to attain the maximal absorbance changes was longer. Addition of 0.15 μM carbonylcyanide *m*-chlorophenylhydrazon (CCCP) reversed this effect (Fig. 1(B)). It was also observed that addition of L-malate to anaerobic mitochondria increased the fluorescence of ethidium and that this effect was sensitive to uncouplers. The amounts of various uncouplers required to

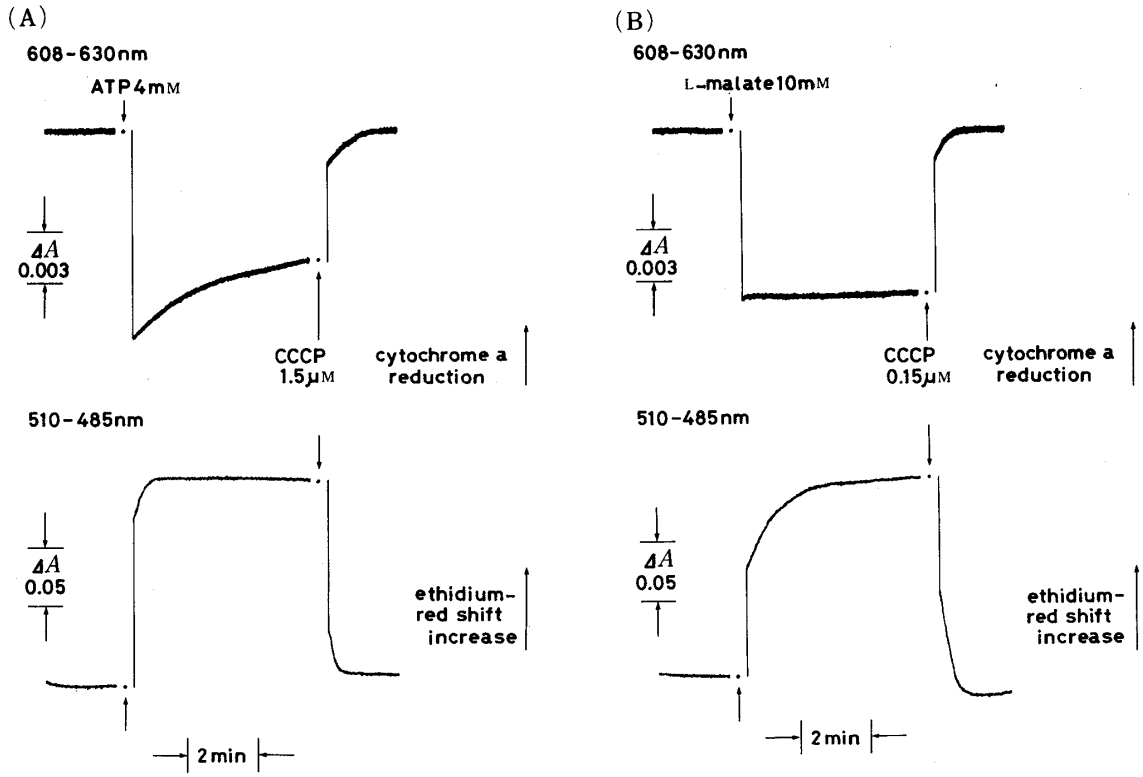

Fig. 1(A). Effects of ATP on the Red Shift of Ethidium and on Cytochrome a in Anaerobic Mitochondria

Anaerobic mitochondria were induced with 10 mM glutamate. Then 3 μg of rotenone, 0.5 mM fumarate and 0.05 mM succinate were added to the mitochondria, followed by 4 mM ATP and 1.5 μM CCCP.

Fig. 1(B). Effects of L-Malate on the Red Shift of Ethidium and on Cytochrome a in Anaerobic Mitochondria

Anaerobic mitochondria were induced with 10 mM glutamate. Then 10 mM L-malate and 0.15 μM CCCP were added as indicated. In the case of the measurements of the red shift of ethidium, 100 μM ethidium was also added before ATP or L-malate. The mitochondrial suspensions for (A) and (B) contained 11.9 mg protein/3 ml.

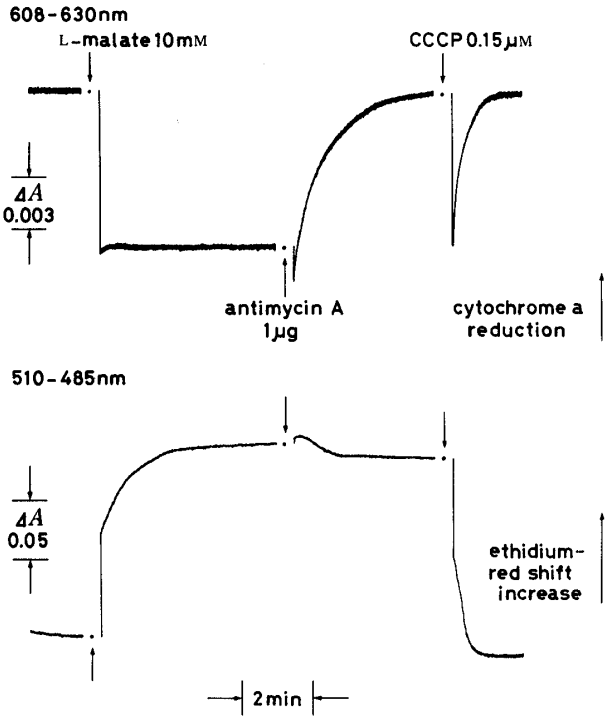

Fig. 2. Effects of Antimycin A on the Red Shift of Ethidium and on Cytochrome a in Anaerobic, L-Malate-Treated Mitochondria

Conditions were as for Fig. 1(B) except that 1 μg of antimycin A was added as indicated.

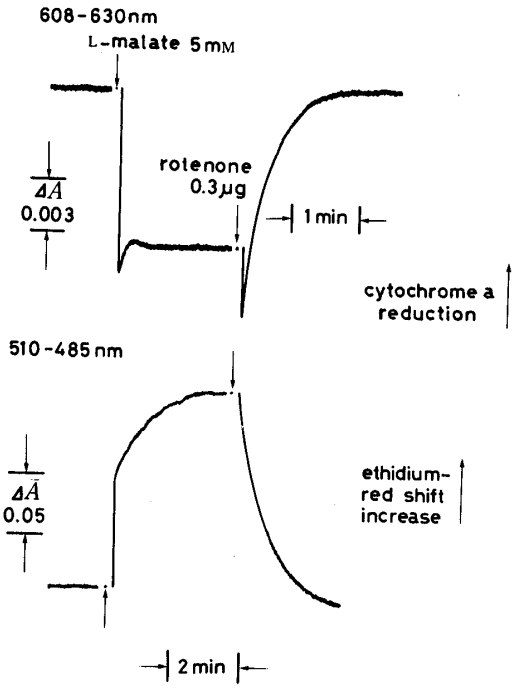

Fig. 3. Effects of Rotenone on the Red Shift of Ethidium and on Cytochrome a in Anaerobic, L-Malate-Treated Mitochondria

Conditions were as for Fig. 1(B) except that 0.3 μg of rotenone was added as indicated.

inhibit the effect of L-malate on the red shift of ethidium were consistently approximately one-tenth of the amounts required to inhibit the effect of ATP on this shift (*cf.* ref.4). Figure 1 also shows that addition of ATP or of L-malate to anaerobic mitochondria resulted in oxidation of cytochrome a. Essentially the same results as in Fig. 1 were obtained with KCN (3 mM)-treated mitochondria under aerobic conditions (data not shown).

Addition of antimycin A reversed the oxidation of cytochrome a in L-malate-treated anaerobic mitochondria, as in ATP-treated anaerobic mitochondria, but it did not reverse the L-malate-induced red shift of ethidium, as shown in Fig. 2. Figure 3 shows that addition of rotenone reversed both the oxidation of cytochrome a and the red shift of ethidium in L-malate-treated anaerobic mitochondria. Malonate (10 mM) and thenoyltrifluoroacetone[17] (0.3 mM), which are inhibitors of succinate dehydrogenase, also completely reversed both reactions (data not shown, but essentially the same results as Fig. 3 were obtained). Addition of 10 mM succinate also reversed both reactions in L-malate-treated mitochondria (data not shown).

These data clearly show that L-malate-induced energization of mitochondria was driven by energy derived from redox reactions at site I of phosphorylation, and that L-malate-induced oxidation of cytochrome a in anaerobic mitochondria occurred by a reversal of electron transfer at sites II and III (redox complexes III and IV) driven by energy derived at site I (redox complex I). The electron acceptor of this reaction could be fumarate since the formation of fumarate from L-malate is catalyzed by fumarate hydratase in mitochondria.

The present results also suggest that chargerin, which binds ethidium in an energized state,[11-13] has an essential role in this energy transfer from redox complex I to redox complexes III and IV (*cf.* ref. 18). This possibility is supported by the fact that anti-chargerin II antibody completely inhibited reverse electron flow from succinate to nicotinamide adenine

nucleotide coupled with succinate oxidation by O_2 in mitoplasts.[14] However, further study is required to confirm this.

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References and Notes

1) Present address: *Taiho Pharmaceutical Co., Ltd., Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771–01, Japan.*
2) A. P. Dawson, G. F. Cox, and M. J. Selwyn, *Biochim. Biophys. Res. Commun.*, **32**, 579 (1968).
3) S. Muraoka and Y. Sugiyama, *FEBS Lett.*, **46**, 263 (1974).
4) T. Higuti, M. Sato, S. Mizuno, M. Yokota, Y. Sugiyama, Y. Nishitani, M. Sekiya, and I. Tani, *Biochim. Biophys. Acta*, **449**, 10 (1976).
5) C. Gitler, B. Rubalcava, and A. Caswell, *Biochim. Biophys. Acta*, **193**, 479 (1969).
6) A. Azzi, P. L. Gherardini, and M. Santato, in "Energy Transduction in Respiration and Photosynthesis," ed. by E. Quagliariello, S. Papa, and C. R. Rossi, Adriatica, Bari, 1971, p. 621.
7) A. Azzi and M. Santato, *Biochem. Biophys. Res. Commun.*, **44**, 211 (1971).
8) G. K. Radda and J. Vanderkooi, *Biochim. Biophys. Acta*, **265**, 509 (1972).
9) B. Chance, *Proc. Natl. Acad. Sci. U.S.A.*, **67**, 560 (1970).
10) T. Higuti, N. Arakaki, and A. Hattori, *Biochim. Biophys. Acta*, **548**, 166 (1979).
11) T. Higuti, T. Ohe, N. Arakaki, and Y. Kotera, *J. Biol. Chem.*, **256**, 9855 (1981).
12) T. Higuti, *Mol. Cell. Biochem.*, **61**, 37 (1984).
13) T. Higuti, M. Takigawa, Y. Kotera, H. Oka, J. Uchida, R. Arakaki, T. Fujita, and T. Ogawa, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1331 (1985).
14) T. Higuti, J. Uchida, M. Takigawa, T. Ueda, and K. Hirano, in "Energy Transduction in ATPases," ed. by S. Fleischer, M. F. Morales, S. Ebashi, M. Nakao, Y. Kagawa, M. Futai, and Y. Mukohata, Tokyo, 1986, p. 496.
15) F. H. Hogeboom, in "Methods in Enzymology," Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1955, p. 16.
16) D. K. Myers and E. C. Slater, *Biochem. J.*, **67**, 558 (1957).
17) B. D. Nelson, B. Norling, B. Persson, and L. Ernster, *Biochem. Biophys. Res. Commun.*, **44**, 1321 (1971).
18) E. C. Slater, J. A. Berden, and M. A. Herweijer, *Biochim. Biophys. Acta*, **811**, 217 (1985).