Biochimica et Biophysica Acta, 449 (1976) 10–22

BBA 47168

ENERGIZATION OF MITOCHONDRIAL INNER MEMBRANES CAUSED BY L-MALATE

TOMIHIKO HIGUTI, MASAMI SATO, SYOZI MIZUNO, MAKOTO YOKOTA, YASUO SUGIYAMA*, YUKARI NISHITANI, MISUZU SEKIYA and ISAMU TANI

Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi, Tokushima, 770 (Japan)

(Received March 22nd, 1976)

SUMMARY

It was found that 0.06 μg antimycin A/mg mitochondrial protein, an amount sufficient to inhibit electron transfer between cytochromes *b* and c_1 completely, fully reversed the oxidation of cytochrome *a* caused by L-malate in anaerobic mitochondria. The effect of L-malate on cytochrome *a* was insensitive to oligomycin, but all the uncouplers and detergents tested reversed the oxidation of cytochrome *a* caused by L-malate in anaerobic mitochondria. It was also found that addition of L-malate to anaerobic mitochondria, like addition of ATP, decreased the fluorescence of 1-anilinonaphthalene-8-sulphonate, and that subsequent addition of uncouplers reversed this effect. The effect of L-malate on the fluorescence of the dye was insensitive to oligomycin. The present findings suggest that addition of L-malate may cause energization of the mitochondrial inner membranes and that the oxidation of cytochrome *a* caused by L-malate in anaerobic mitochondria may result from an L-malate-induced, energy-linked reversal of electron transfer in site II.

INTRODUCTION

Dawson et al. [1] showed that both L- and D-malate induced the oxidation of cytochrome *b*-558 in anaerobic mitochondria from *Arum spadix*, pea, mung bean and rat liver. Muraoka and Sugiyama [2] showed that L-malate also caused the oxidation of cytochrome *a* in anaerobic rat liver mitochondria. However, the reason for the anomalous redox changes of cytochromes *b*-558 and *a* caused by L-malate is still unknown. To examine this question, we compared the effects of ATP and L-malate on the fluorescence of 1-anilinonaphthalene-8-sulphonate (ANS) and on cytochrome *a* in anaerobic mitochondria.

Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SF 6847, 3,5-di-*tert*-butyl-4-hydroxy-benzylidenemalononitrile.

* Present address: Biological Research Lab. Takeda Chemical Industries, Ltd., Higashiyodogawa, Osaka, Japan.

MATERIALS AND METHODS

3,5-Di-*tert*-butyl-4-hydroxy-benzylidenemalononitrile (SF 6847) was a gift from Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan). L-Malate, D-malate, oligomycin and carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP) were purchased from Sigma Chemicals Co., St. Louis, Mo. Antimycin A was a product of Kyowa Fermentation Industry, Tokyo (Japan).

Rat liver mitochondria were isolated by the method of Hogeboom [3], as described by Myers and Slater [4] except that 0.25 M sucrose containing 2 mM Tris (pH 7.4) was used for homogenization and two washings. Protein was determined by the biuret method as described by Cleland and Slater [5], and from the content of cytochrome $a+a_3$ in mitochondria measured as the absorbance of dithionite-reduced minus oxidized mitochondrial suspension at pH 7.4, at the wavelength pair of 605–630 nm in a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer.

All reactions were carried out in medium consisting of 25 mM Tris · HCl buffer/50 mM sucrose/5 mM $MgCl_2$/2 mM EDTA/15 mM KCl, with other components as indicated in the legends to the figures and table, in a final volume of 3 ml at pH 7.1.

Absorbance changes and difference spectra of cytochrome $a+a_3$ were examined in a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer (with a slit width of 1 nm), as described previously [6]. Fluorescence changes of ANS were determined with a Hitachi, Model MPF 3, spectrofluorometer, using the wavelength of 370 nm for excitation and measuring fluorescence at 470 nm. A glass filter (V-Y 43) was placed in front of the photomultiplier to eliminate the actinic light. In all experi-

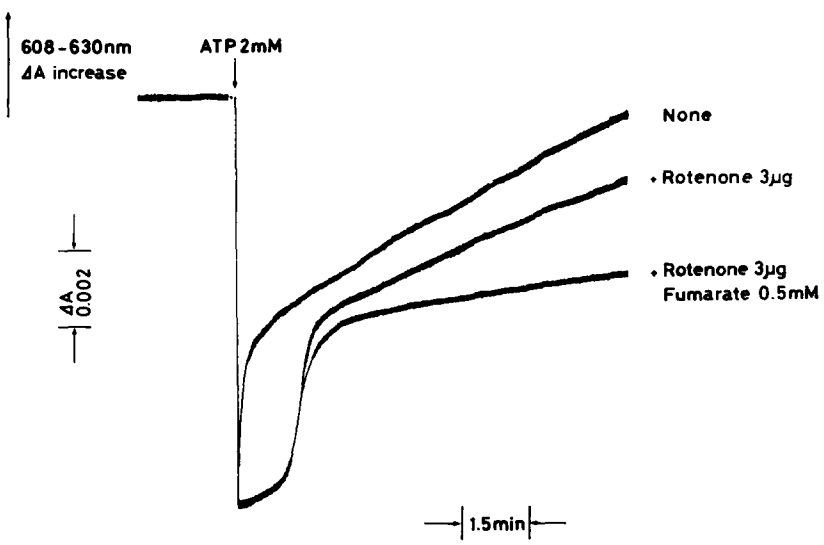

Fig. 1. Effects of rotenone and fumarate on the effect of ATP on cytochrome *a* in anaerobic mitochondria. The rat liver mitochondrial suspension contained 4.5 mg protein/3 ml. 2 mM ATP was added to anaerobic mitochondria induced with 10 mM glutamate. 3 μg of rotenone or 3 μg of rotenone+0.5 mM fumarate were added before the ATP.

(A)

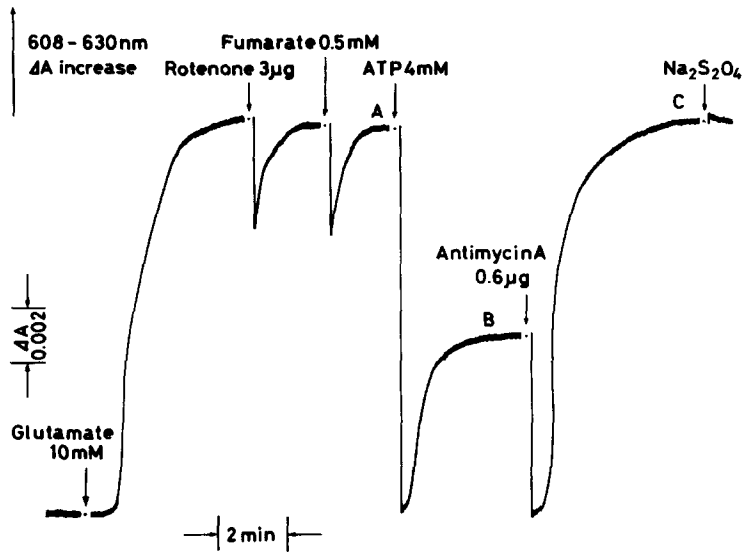

Fig. 2. See opposite page for legend.

ments nitrogen gas was passed through the reaction mixture for 2 min before addition of mitochondrial suspension to shorten the time required to attain anaerobiosis. To minimize contamination with oxygen, the surface of the reaction mixture was covered with liquid paraffin. All measurements were carried out at room temperature (approx. 25 °C).

RESULTS

Reversals of the effects of ATP and L-*malate on cytochrome a by antimycin A*

Addition of ATP to anaerobic mitochondria causes oxidation of cytochrome *a* [7–9]. However, as the oxidation of cytochrome *a* driven by ATP is unstable and is reversed with time, it is very difficult to examine the nature of this effect. So we first tried to stabilize the effect. Fig. 1 shows that the oxidation of cytochrome *a* by ATP was remarkably stabilized by the additions of rotenone and fumarate to anaerobic mitochondria induced by glutamate. Fig. 2 (A and B) shows that the oxidation of cytochrome *a* by ATP is reversed by the addition of antimycin A, in good accordance with the results reported previously [10, 11]. On the other hand, Muraoka and Sugiyama [2] showed that L-malate-induced oxidation of cytochrome *a* is reversed by antimycin A. In agreement with the report of Muraoka and Sugiyama [2], Fig. 3 shows that addition of L-malate to anaerobic mitochondria induced by glutamate resulted in oxidation of cytochrome *a* having an α-band at 608 nm, and this was reversed by subsequent addition of antimycin A. It is well known [12] that antimycin A inhibits electron transfer between cytochromes *b* and c_1 and the curve relating the

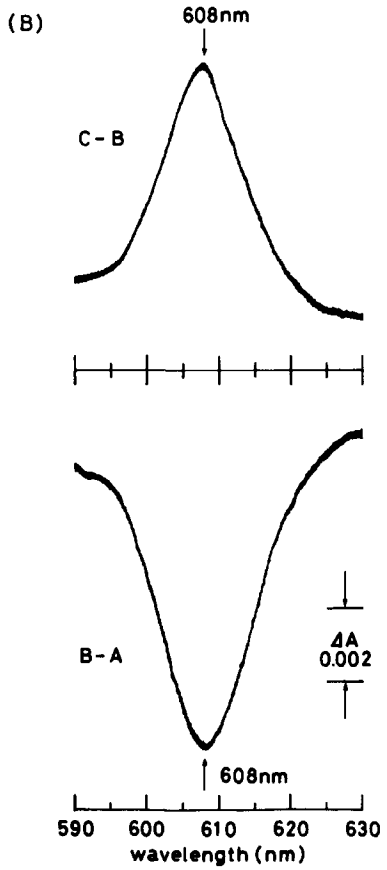

Fig. 2. (A) Reversal by antimycin A of the effect of ATP on cytochrome *a* in anaerobic mitochondria. The rat liver mitochondrial suspension contained 10.0 mg protein/3 ml. Anaerobiosis was induced with 10 mM glutamate, and then 3 μg of rotenone, 0.5 mM fumarate, 4 mM ATP and 0.6 μg of antimycin A were added as indicated. (B) Difference spectra of cytochrome *a*. Conditions were the same as for A except that 10.3 mg protein of rat liver mitochondria were used. Curve B—A: after anaerobiosis had been attained by adding 10 mM glutamate, the rat liver mitochondrial suspensions in the sample and reference cuvettes were both supplemented with 3 μg of rotenone and 0.5 mM fumarate and then 4 mM ATP was added to the sample cuvette only. Curve C—B: after anaerobiosis had been induced with 10 mM glutamate, the rat liver mitochondrial suspensions in the sample and reference cuvettes were both supplemented with 3 μg of rotenone, 0.5 mM fumarate, and 4 mM ATP and then 0.8 μg of antimycin A was added to the sample cuvette only. The difference spectrum was taken as described in the Materials and Methods.

degree of inhibition to the amount of antimycin A is sigmoidal. We examined the effects of various concentrations of antimycin A on the effect of ATP and L-malate on cytochrome *a*. Fig. 4 shows that antimycin A caused similar inhibitions of the effects of ATP and L-malate on cytochrome *a* and electron transfer between cytochrome *b* and c_1. The amount of antimycin A causing complete reversal of the effects of ATP and L-malate on cytochrome *a* was about 0.06 μg/mg mitochondrial protein, as is also shown in Fig. 4. Our data on the amount of antimycin A required for maximal inhibition are in good agreement those of Estabrook [13]. Therefore, it seems likely

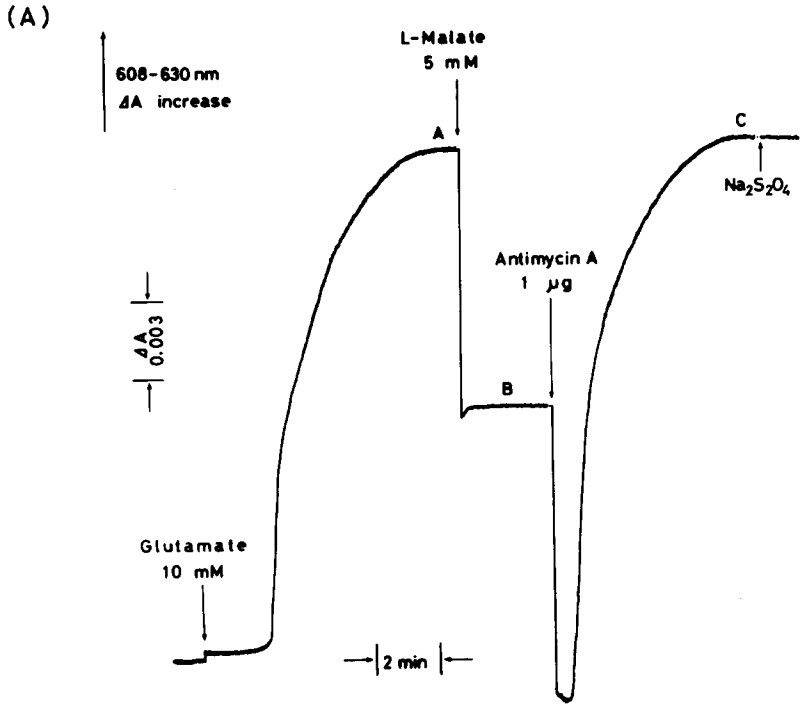

Fig. 3. See opposite page for legend.

that the reversal by antimycin A of the effect of L-malate on cytochrome *a* may be ascribed to the inhibition of reversed electron transfer at site II, like its reversal of the effect of ATP on cytochrome *a* [7–9, 11].

The effect of L-malate on cytochrome *a* was also observed in anaerobic mitochondria induced with 5 mM ascorbate (+100 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) as substrate and in aerobic, 1 mM KCN-inhibited mitochondria (data not shown). Addition of D-malate in place of L-malate did not cause any redox change of cytochrome *a*.

Reversals of the effects of ATP and L-*malate on cytochrome a by uncouplers*

Muraoka and Sugiyama [2] found that 10 μM pentachlorophenol inhibits the oxidation of cytochrome *a* caused by L-malate in anaerobic mitochondria. So, we compared the influences of increasing concentrations of uncouplers on the effects of ATP and L-malate on cytochrome *a*. All the uncouplers tested reversed the effect of L-malate on cytochrome *a*, as shown in Fig. 5 and Table I. However, the amounts of these uncouplers required for 50 % and 100 % inhibitions of the effect of L-malate on cytochrome *a* were approximately one-tenth of the amounts required for 50 % and 100 % inhibitions of the effect of ATP on cytochrome *a*, irrespective of the species of uncoupler used, as shown in Table I. The effect of ATP on cytochrome *a* was sensitive to oligomycin (2 μg/mg protein), but insensitive to aurovertin (20 μM). However, the effect of L-malate on cytochrome *a* was insensitive to both these inhibitors.

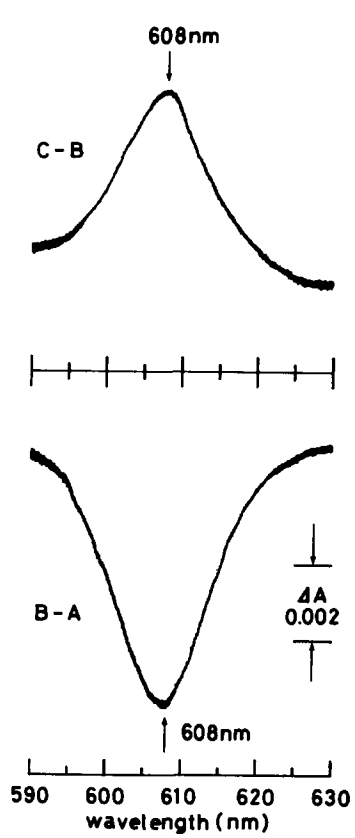

Fig. 3. (A) Reversal by antimycin A of the effect of L-malate on cytochrome *a* in anaerobic mitochondria. The rat liver mitochondrial suspension contained 12.6 mg protein/3 ml. Anaerobiosis was induced with 10 mM glutamate, and then 5 mM L-malate and 1 μg of antimycin A were added as indicated. (B) Conditions were the same as for A except that 11.5 mg protein of rat liver mitochondria were used. Curve B—A: after anaerobiosis had been induced with 10 mM glutamate, 5 mM L-malate was added to the sample cuvette only. Curve C—B: after anaerobiosis had been induced with 10 mM glutamate, the rat liver mitochondrial suspensions in the sample and reference cuvettes were both supplemented with 5 mM L-malate and then 0.8 μg of antimycin A was added to the sample cuvette only. The difference spectrum was taken as described in the Materials and Methods.

It was also found that Triton X-100 (0.0015 % w/v) and sodium dodecyl sulphate (0.01 % w/v) completely reversed the effect of L-malate on cytochrome *a*.

Effect of L-*malate on ANS fluorescence in anaerobic mitochondria*

The above results suggest that addition of L-malate may cause energization of the inner membranes in anaerobic mitochondria. Therefore, we tested the effect of L-malate on ANS fluorescence in anaerobic mitochondria. It is generally accepted [14–19] that ANS shows a change of the energy state of the mitochondrial inner membranes, that ATP decreases the fluorescence of ANS in anaerobic mitochondria and that uncouplers reverse such an effect, as shown in Fig. 6A. Fig. 6B shows that

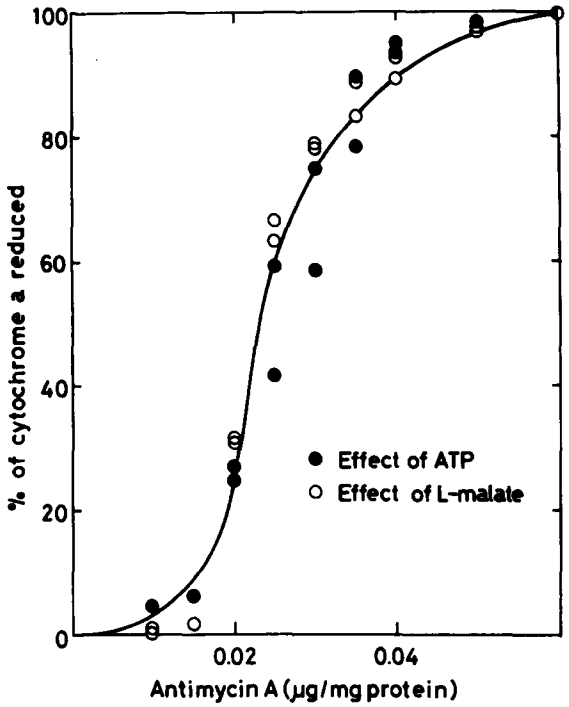

Fig. 4. Influence of varying antimycin A concentrations on the effects of ATP and L-malate on cytochrome *a* in anaerobic mitochondria. Conditions were the same as for Figs. 2A and 3A except that 10.1 mg protein of rat liver mitochondria and antimycin A at the indicated concentrations were used. For convenience, 100 % oxidation of cytochrome *a* was taken as the decrease in the absorption difference of 608 minus 630 nm caused by additions of 4 mM ATP or 5 mM L-malate to anaerobic mitochondria induced with 10 mM glutamate. The mean value of the decrease in absorption difference of 608 minus 630 nm caused by additions of 4 mM ATP and 5 mM L-malate, respectively were 51 % and 54 % of that caused by addition of dithionite to oxidized mitochondria.

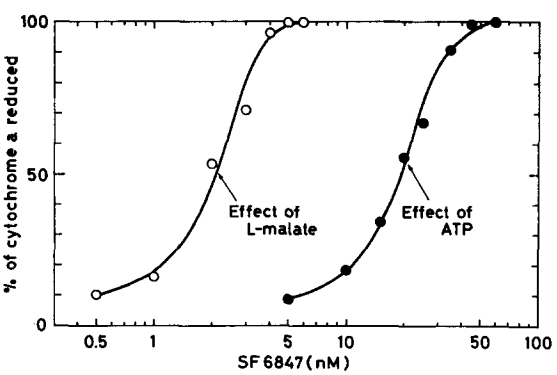

Fig. 5. Reversals of the effects of ATP and L-malate on cytochrome *a* by varying SF 6847 concentrations. Conditions were the same as for Figs. 2A and 3A except that 9.4 mg protein of rat liver mitochondria and SF 6847 in place of antimycin A were used. 100 % oxidation of cytochrome *a* was assumed to be as described in the legend to Fig. 4.

TABLE I

CONCENTRATIONS OF UNCOUPLERS REQUIRED FOR 50 % AND 100 % INHIBITIONS OF THE EFFECTS OF ATP AND L-MALATE ON CYTOCHROME *a* IN ANAEROBIC MITOCHONDRIA

Conditions were the same as for Fig. 5 except that 12.3 mg protein of rat liver mitochondria and the indicated uncouplers were used.

Uncoupler	Effect of ATP on cytochrome *a*		Effect of L-malate on cytochrome *a*			
	Conc. of uncoupler (M) required for		Conc. of uncoupler (M) required for			
	50 % inhibition (a)	100 % inhibition (b)	50 % inhibition (c)	100 % inhibition (d)	(a)/(c)	(b)/(d)
Flufenamic acid	$3.0 \cdot 10^{-5}$	$1.0 \cdot 10^{-4}$	$2.7 \cdot 10^{-6}$	$8.0 \cdot 10^{-6}$	11	13
Pentachlorophenol	$2.0 \cdot 10^{-6}$	$1.8 \cdot 10^{-5}$	$2.0 \cdot 10^{-7}$	$1.8 \cdot 10^{-6}$	10	10
CCCP	$7.0 \cdot 10^{-8}$	$3.0 \cdot 10^{-7}$	$3.4 \cdot 10^{-9}$	$3.0 \cdot 10^{-8}$	20	10
SF 6847	$2.0 \cdot 10^{-8}$	$6.0 \cdot 10^{-8}$	$2.1 \cdot 10^{-9}$	$6.0 \cdot 10^{-9}$	9.5	10

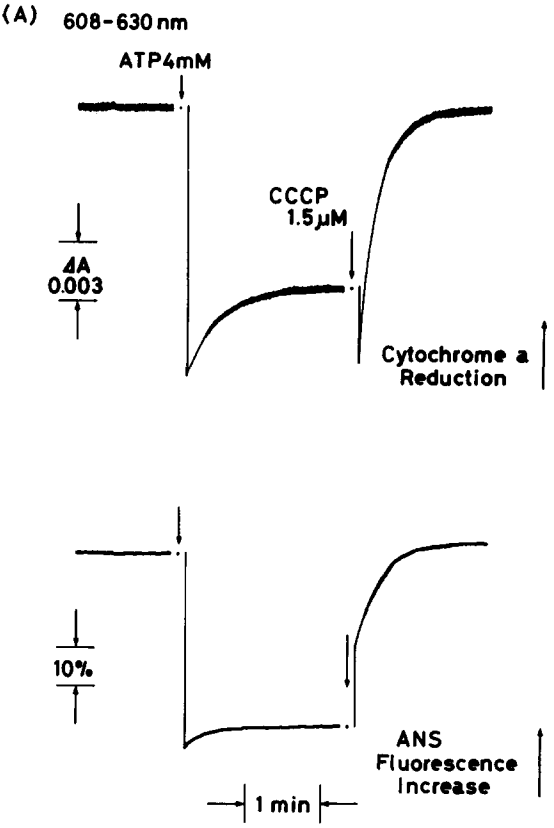

Fig. 6. See opposite page for legend.

L-malate also induced a decrease of ANS fluorescence in anaerobic mitochondria, and that subsequent addition of 0.15 μM CCCP reversed this effect. These effects of L-malate and ATP on ANS fluorescence were similar, except that L-malate caused only about three-quarters as much decrease in ANS fluorescence as ATP. Moreover, only about one-tenth as much uncoupler was required to reverse the effect of L-malate on ANS fluorescence as to reverse that of ATP. When ANS was omitted from the reaction mixture, no change in fluorescence at 470 nm was observed on addition of L-malate to anaerobic mitochondria. Addition of antimycin A (Fig. 7) or KCN to L-malate-treated or ATP-treated anaerobic mitochondria did not cause any change in ANS fluorescence. The effect of ATP on ANS fluorescence in anaerobic mitochondria was sensitive to oligomycin, whereas the effect of L-malate on the fluorescence was not (data not shown).

DISCUSSION

The present work showed that the L-malate-induced oxidation of cytochrome *a* was not inhibited by oligomycin. However, all the uncouplers and detergents tested reversed the effects of both L-malate and ATP on cytochrome *a*. It was also found that

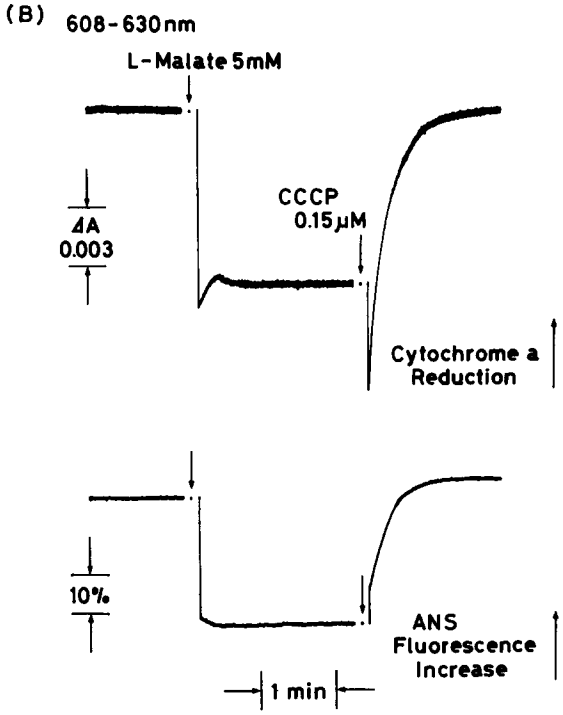

Fig. 6. (A) Effect of ATP on ANS fluorescence in anaerobic mitochondria. Conditions were the same as for Fig. 2A except that 12.1 mg protein of rat liver mitochondria were used and 50 μM succinate and 100 μM ANS were added before ATP and 1.5 μM of CCCP as indicated. Change in fluorescence of ANS was measured as described in the Materials and Methods. (B) Effect of L-malate on ANS fluorescence in anaerobic mitochondria. Conditions were the same as for Fig. 3A except that 12.1 mg protein of rat liver mitochondria were used and 5 mM L-malate and 0.15 μM CCCP were added as indicated. Change in fluorescence of ANS was measured as described in the Materials and Methods.

addition of L-malate to anaerobic mitochondria caused a decrease of ANS fluorescence, and that subsequent addition of uncouplers reversed this effect. Similar results have been obtained on the effect of ATP on ANS fluorescence in anaerobic mitochondria and its reversal by uncouplers [14–19]. However, the effect of ATP on ANS fluorescence in anaerobic mitochondria is sensitive to oligomycin whereas that of L-malate is not.

It is generally accepted [14–19] that reduced ANS fluorescence in mitochondria indicates an energized state of the mitochondrial inner membranes. We also found that addition of L-malate to anaerobic or KCN-inhibited mitochondria, like addition of ATP [20, 21], caused a red shift of the absorbance maximum of ethidium and increase in its fluorescence. Moreover, these responses of the dye were due to an increase in the affinity of the membrane for the dye (to be published). It seems very likely, therefore, that L-malate may cause energization of the mitochondrial inner membranes. We also found that the inhibitory effects of antimycin A on oxidation of cytochrome *a* in anaerobic mitochondria by L-malate and by ATP were very similar. Therefore, the oxidation of cytochrome *a* induced by L-malate may be due to L-

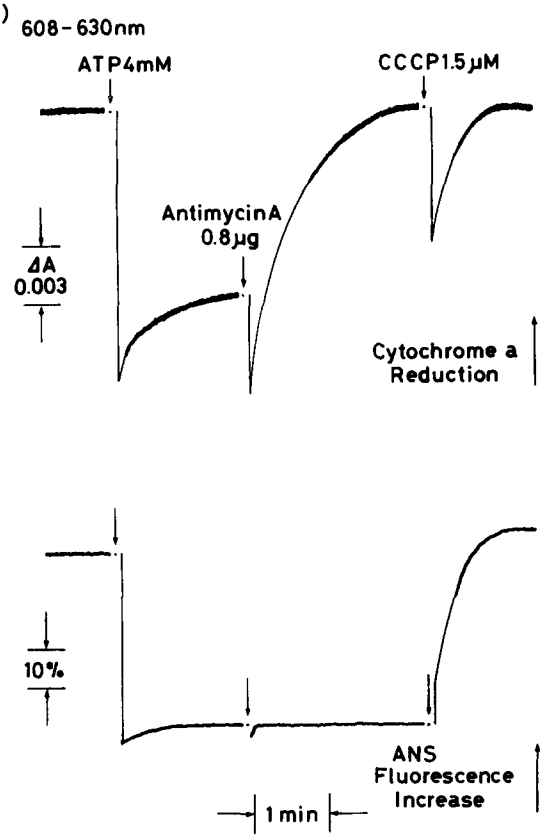

Fig. 7. See opposite page for legend.

malate-induced, energy-linked, reversed electron transfer at site II, like the effect of ATP on cytochrome *a* [7–9, 11].

The amounts of uncouplers required for inhibitions of the effects of L-malate on cytochrome *a* and on ANS fluorescence were about one-tenth of the corresponding amounts required for inhibitions of the effects of ATP on cytochrome *a* and ANS fluorescence, irrespective of the species of uncoupler used. This presumably indicates that the kinetics of energization of mitochondrial inner membranes by L-malate and by ATP are different, probably because different mechanisms are involved. Further comparative studies on the effects of L-malate and ATP on ANS fluorescence, cytochrome *a*, *b*-type cytochromes and other redox components in mitochondria will be reported in subsequent papers. Addition of antimycin A to anaerobic, L-malate-treated mitochondria did not cause any change in ANS fluorescence. It seems likely that antimycin A did not inhibit energization of the mitochondrial inner membranes by L-malate, but prevented the oxidation of cytochrome *a* by inhibiting the reversed electron transfer at site II.

Dawson et al. [1] reported that both L- and D-malate induced oxidation of cytochrome *b*-558 in mitochondria. However, we did not observe the oxidation of cytochrome *b*-558 on addition of D-malate to anaerobic mitochondria, although this

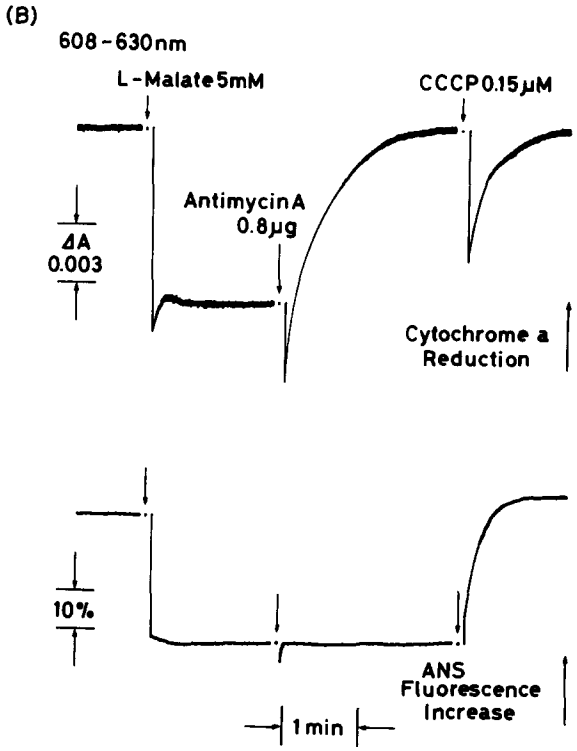

Fig. 7. (A) Effect of antimycin A on ANS fluorescence in anaerobic, ATP-treated mitochondria. Conditions were the same as for Fig. 6A except that 0.8 μg of antimycin A was added as indicated. (B) Effect of antimycin A on ANS fluorescence in anaerobic, L-malate-treated mitochondria. Conditions were the same as for Fig. 6B except that 0.8 μg of antimycin A was added as indicated.

occurred on addition of L-malate. We also found that addition of ATP caused oxidation of cytochrome *b*-558 in anaerobic mitochondria (to be published). Thus, oxidation of cytochrome *b*-558 induced by L-malate may also be caused by energization of the mitochondrial inner membranes.

The present findings suggest that the effect of L-malate on cytochromes *a* and *b*-558 may take place through an L-malate-induced, energy-linked reversal of electron transfer in the respiratory chain.

The present findings do not provide any information on the kind of energization of anaerobic mitochondrial inner membranes caused by L-malate. However, a possible explanation of the present phenomena is as follows. A proton gradient is formed by exchange diffusion of L-malate $^{2-}$ and a trebly charged anion (for example, citrate^{3-}) across the inner mitochondrial membrane, as reported previously [22, 23]. This proton gradient (negative inside) may be the energized state of the membranes induced by L-malate. Trebly charged anions may have to be formed continuously from L-malate (not the D form) transported into the matrix space to maintain the proton gradient. This explanation seems to be supported by the findings that the oxidation of cytochrome *a* induced by L-malate [2] or ATP [9] and the increase in the affinity of the membrane for the ethidium cation induced by L-malate or ATP (un-

published observations) were reversed by increasing the pH of the suspending medium from 7.0 to 8.5, because this could be due to disappearance of the proton gradient. However, further detailed studies are required to confirm this interpretation. This will be discussed in a subsequent paper.

ACKNOWLEDGEMENTS

The authors thank Professor Takekazu Horio (Institute for Protein Research, Osaka University) for helpful suggestions and continuous encouragement. This work was supported by grants 030301 and 078060 from the Ministry of Education of Japan.

REFERENCES

1 Dawson, A. P., Cox, G. F. and Selwyn, M. J. (1968) Biochem. Biophys. Res. Commun. 32, 579–587
2 Muraoka, S. and Sugiyama, Y. (1974) FEBS Lett. 46, 263–267
3 Hogeboom, F. H. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. 1, pp. 16–18, Academic Press, New York
4 Myers, D. K. and Slater, E. C. (1957) Biochem. J. 67, 558–572
5 Cleland, K. W. and Slater, E. C. (1953) Biochem. J. 53, 547–556
6 Higuti, T., Mizuno, S. and Muraoka, S. (1975) Biochim. Biophys. Acta 396, 36–47
7 Wikström, M. K. F. (1972) Biochim. Biophys. Acta 283, 385–390
8 Wikström, M. K. F. (1974) Ann. N.Y. Acad. Sci. 227, 146–158
9 Higuti, T., Sugiyama, Y., Sato, M. and Muraoka, S. (1975) Biochem. Biophys. Res. Commun. 62, 602–607
10 Wikström, M. K. F. and Saris, N. E. L. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds.), pp. 77–88, Elsevier, Amsterdam
11 Caswell, A. H. (1968) J. Biol. Chem. 243, 5827–5836
12 Slater, E. C. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), Vol. X, pp. 48–57, Academic Press, London
13 Estabrook, R. W. (1962) Biochim. Biophys. Acta 60, 236–248
14 Azzi, A. and Vainio, H. (1971) in Probes of Structure and Function of Macromolecules and Membranes (Chance, B., Lee, C. P. and Blasie, J. K., eds.), Vol. I, pp. 209–218, Academic Press, London
15 Radda, G. K. and Vanderkooi, J. (1972) Biochim. Biophys. Acta 265, 509–549
16 Packer, L., Donovan, M. P. and Wrigglesworth, J. M. (1969) Biochem. Biophys. Res. Commun. 35, 832–837
17 Azzi, A. (1969) Biochem. Biophys. Res. Commun. 37, 254–260
18 Azzi, A., Gherardini, P. and Santato, M. (1971) J. Biol. Chem. 246, 2035–2042
19 Jasaitis, A. A., Chu, L. V. and Skulachev, V. P. (1973) FEBS Lett. 31, 241–245
20 Azzi, A. and Santato, M. (1971) Biochem. Biophys. Res. Commun. 44, 211–217
21 Azzi, A., Gherardini, P. L. and Santato, M. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C. S., eds.), pp. 621–637, Adriatica Editrice, Bari
22 McGivan, J. D. and Klingenberg, M. (1971) Eur. J. Biochem. 20, 392–399
23 Papa, S., Lofrumento, N. E., Kanduc, D., Paradies, G. and Quagliariello, E. (1971) Eur. J. Biochem. 22, 134–143