Activation of malate-linked reductions of NAD and flavoproteins in Ascaris muscle mitochondria by phosphate

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Mitochondria isolated from the muscle of Ascaris lumbricoides have quite similar characteristics of mammalian mitochondria with respect to their phosphorylation activity, oxidase activities of NADH and succinate, sensitivity toward uncouplers, and their membrane structure. They have, however, some unique features which are uncommon among mammalian mitochondria. They contain a "shortened" electron transport chain which has no spectroscopically detectable cytochromes c_1, a, and a_3 (Chance & Parsons, 1963). The content of cytochrome c is less than one-tenth of mammalian heart muscle in a mg protein basis. Furthermore, none of oxidase activities appears to be related with cytochrome c oxidase (Bueding & Charms, 1952). Pyruvate (Kmetec & Bueding, 1961) and malate (Seidman & Entner, 1961) are oxidized via flavoproteins and only one phosphorylation is coupled during the electron transport process. Oxidation of substrates is activated by Mn++ and accompanies formation of H_2O_2 (Laser, 1944). From these observations it has been suggested that the terminal oxidase of Ascaris mitochondria is Mn++ catalyzed flavin enzyme (Bueding et al., 1955).

In this paper results are presented showing that flavin enzymes in Ascaris are similar to those found in mammalian mitochondria. It is also shown that malate, succinate, and fumarate are the only Krebs cycle intermediates which reduce both NAD and flavoproteins, and cytochromes b and c type as well. These reductions are found to be activated by ATP, pyrophosphate, phosphate, and arsenate. Mg++ is essential for ATP and pyrophosphate, whereas both Mg++

and Mn++ are stimulatory for arsenate and phosphate. The same effect can be also obtained by lowering the pH of the mitochondrial suspension below 6. FCCP and Ca++ inhibit these reductions. The activation by phosphate and arsenate is abolished upon disruption of mitochondrial membrane. The fragmented mitochondria require only Mn++ for the maximal extent of reduction. It is concluded that phosphate facilitates entry of malate by activating reaction (or enzyme) which has the optimal pH below 6.

Experimental procedures. Adult Ascaris lumbricoides var. suis were obtained from local slaughter houses near Philadelphia. Mitochondria were prepared according to the method of Seidman & Entner (1961) with minor modifications. After removal of low speed sedimenting cell debris, suspension was spun down at 8,000 rpm for 15 min. Mitochondria were then washed twice with a buffered sucrose by repeated centrifugations at the same high speed for 5 min.

The medium used for preparation was 0.25 M sucrose containg 20 mM TRIS-Cl, pH 7.4, 1 mM EDTA, and 0.15 % bovine serum albumin.

Changes in absorbance and fluorescence of flavoprotein were measured with a combined double beam spectrophotometer and fluorometer by the same technique used previously (Chance et al., 1967). The low temperature spectra of cytochromes of Ascaris were obtained with a wavelength scanning split beam spectrophotometer (Estabrook, 1956). Difference spectra of cytochrome a_3^{+2}.CO compound was obtained by the photodissociation technique (Chance et al., 1965).

Results and Discussions. Fig. 1 illustrates fluorometric and spectrophotometric responses of Ascaris muscle mitochondria in a series of reduction-oxidation transitions caused by substrates and respiratory inhibitors. Malate causes partial reductions of both flavoprotein and NAD that are activated by an ATP supplement. Rotenone gives a further reduction of fluorescent flavoprotein and a very small oxidation of weakly fluorescent flavoprotein. At this point NADH shows a maximal fluorescence. It is apparent from kinetics that at least two types of flavoprotein are reduced by malate. The initial electron accep-

tor from malate has a lower fluorescence than the one which is reduced over the course of 2 min after malate addition. Thus, the ratio of % fluorescence change to % absorbance change (f/a ratio) is a minimum (0.7) during the first 20 sec and then gradually increases thereafter. The overall f/a ratio after malate, ATP, and rotenone additions is 3.3. Further detailed studies which will be reported elsewhere indicate that these two flavoproteins are a highly fluorescent lipoate dehydrogenase and a very weakly fluorescent NADH dehydrogenase. Further addition of succinate causes an abrupt decrease in absorbance

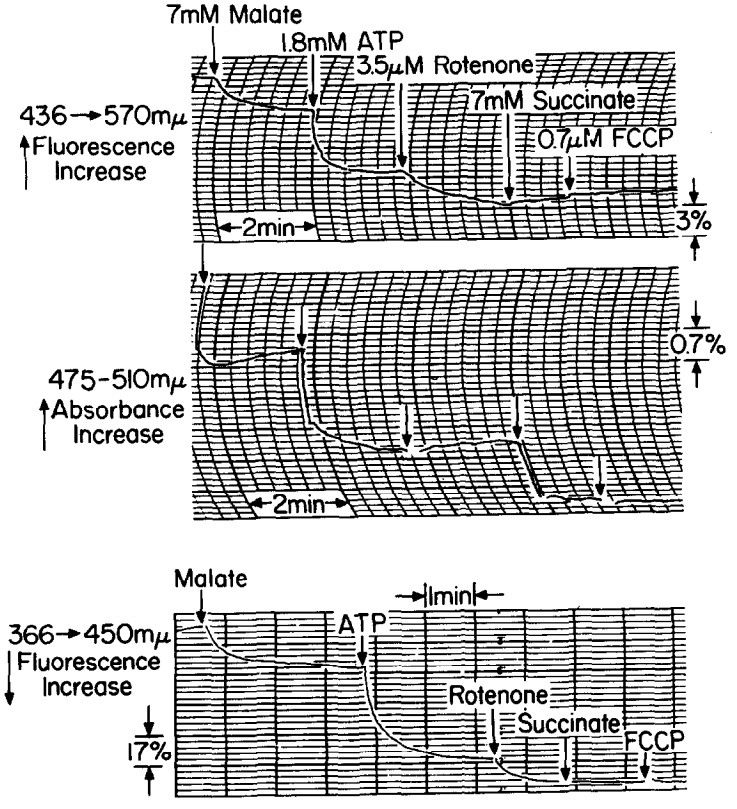

Fig. 1. Changes of fluorescence of flavoprotein (excitation at 436 mμ, measurement at 570 mμ), absorbance of flavoprotein (measurement at 475 mμ with reference at 510 mμ), and fluorescence of NADH (excitation at 366 mμ, measurment at 450 mμ) upon reduction and oxidation by substrates and inhibitors. Mitochondria (2.1 mg protein/ml) were suspended in 20 mM TRIS-Cl, pH 7.4, 1 mM Mg++, 0.075 M sucrose, and 0.225 M mannitol.

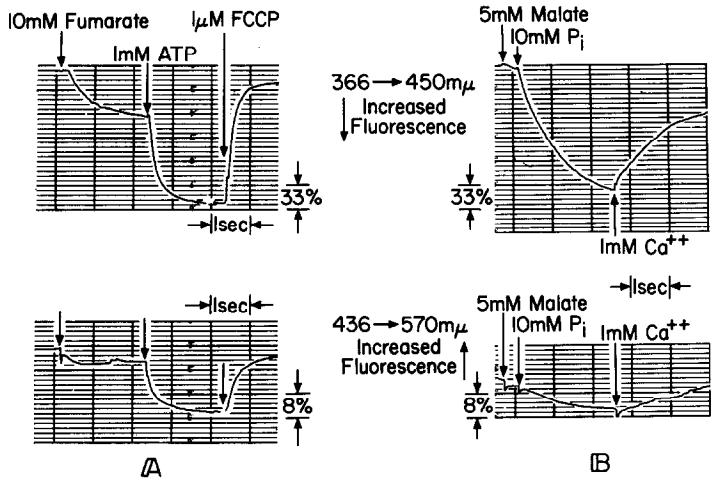

Fig. 2. Reversal of malate-linked NAD and flavoprotein reductions by FCCP [1] and Ca++. Mitochondria, 1.3 mg protein/ml. Other conditions same as in Fig.1.

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but no corresponding fluorescence change, giving a f/a ratio nearly zero. These data divide flavoproteins in Ascaris mitochondria into two categories: one, flavoproteins on the substrate side of the rotenone block, reducible by malate and ATP; and the other, a flavoprotein on the oxygen side of the rotenone block, reducible by succinate. In the presence of rotenone addition of FCCP[1] does not cause oxidation of the reduced flavoprotein. However, in the absence of rotenone FCCP oxidizes the fluorescent flavoprotein and reduces the weakly fluorescent flavoprotein.

When fumarate or succinate is employed instead of malate, ATP exerts the same effect (Fig. 2A). This suggests that at the level of malate, ATP-activated reduction occurs and that malate is formed by succinate dehydrogenase and fumarase. No significant reduction is observed with other Krebs cycle intermediates, or with β-hydroxybutyrate, α-glycerophosphate, and palmitoyl-L-carnitine. The complete reduction of NAD and flavoprotein can be also obtained, without ATP, by NADH and dihydrolipoamide, and by malate in the presence of

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[1] Abbreviation : FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

NADP and Mn++. This is consistant with the presence of diaphorase and malic enzyme (Kmetec & Bueding, 1961; Seidman & Entner, 1961).

As shown in Fig. 2B, addition of phosphate causes reductions of both NAD and flavoprotein essentially to the same extent as when ATP is added. Pyrophosphate and arsenate give the same result, whereas acetate shows no effect. ATP and pyrophosphate require Mg++ for maximal activity. In case of phosphate and arsenate both Mg++ and Mn++ are stimulatory. The reversal of reduction by FCCP or Ca++ suggests that the reduction is an energy-linked reaction, yet oligomycin and rotenone do not inhibit the ATP effect in the presence of succinate. Thus, the reduction cannot be attributed to an energy-linked reversed

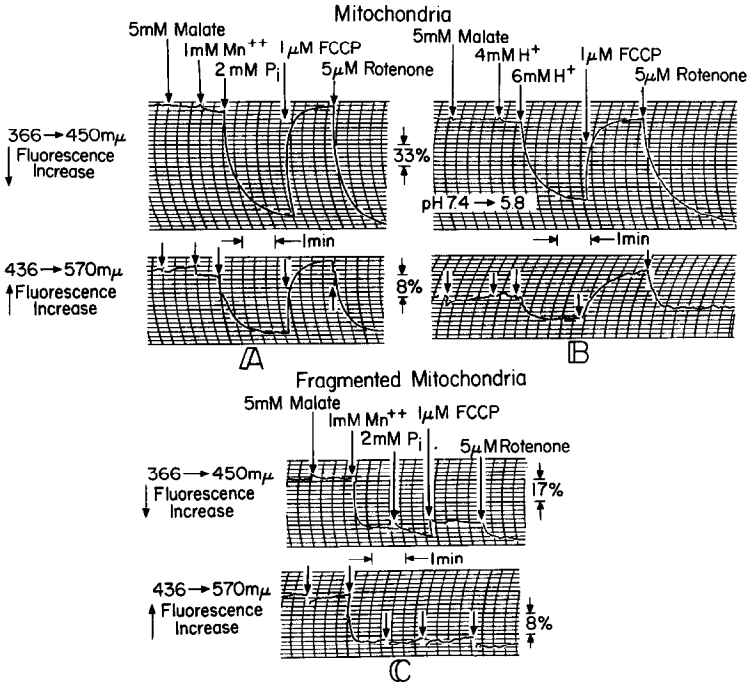

Fig. 3. Comparison of malate-linked reductions of NAD and flavoproteins in intact mitochondria and in fragmented mitochondria. A: activation of reduction by phosphate in intact mitochondria; B: activation of reduction by the lowering of pH of the suspension of intact mitochondria; and C: activation of reduction by Mn++ in fragmented mitochondria. Mitochondria were disrupted by repeated freeze and thawing four times. Mitochondria, 1.5 mg protein/ml. Other conditions same as in Fig. 1.

electron transport (Chance & Hollunger, 1963). The possibility of removal of oxalacetate by ATP is also ruled out by the failure of cystein sulfinic acid to activate the reduction.

When mitochondria are ruptured by repeated freeze and thawing four times, the stimulatory effect of phosphate as well as the reversal by FCCP is completely abolished (cf. Fig. 3A and C), and only Mn++ is required for maximal extent of reduction. The small effects of phosphate and of FCCP observed in Fig. 3C may be due to the residual unbroken mitochondria. Incubation of fragmented mitochondria with FCCP does not inhibit malate-linked reduction, demonstrating that the FCCP effect is indeed an energy-linked reaction rather than inhibition on malate dehydrogenase.

It was also found that the lowering of pH of the mitochondrial suspension below 6 activated reduction (Fig. 3B), just as ATP. It is apparent that ATP and pyrophosphate merely provide phosphate by a Mg++ activated phosphatase, thereby activating entry of malate that is optimal below pH 6. It is not clear, at present, whether phosphate activates permease itself (Chappell & Haarhoff, 1967), or

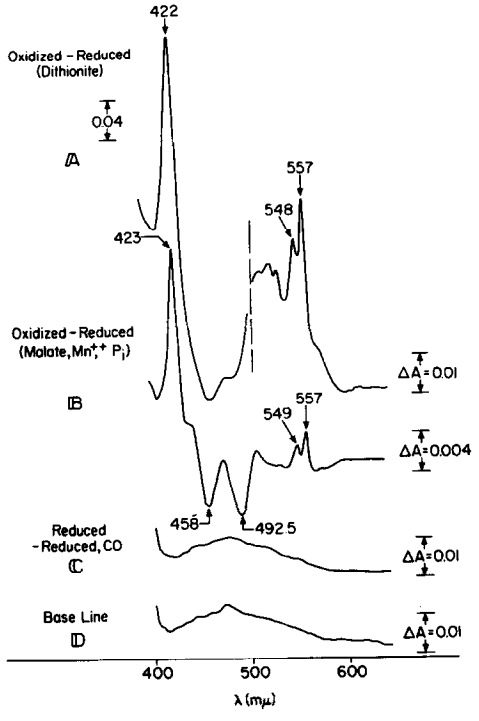

Fig. 4. Low temperature spectra of the reduced respiratory components in Ascaris muscle mitochondria. A: oxidized - dithionite reduced; B: oxidized - malate reduced in the presence of Mn++ and phosphate; C: illuminated, reduced - CO saturated, reduced; and D: base line.

a reaction which facilitates malate-linked reduction, because in the absence of phosphate malate causes reduction if mitochondria are supplemented with

Mn++ and NADP. Thus, it is possible that the permeation involves a product of the malate reaction.

Difference spectra of Ascaris mitochondria treated with malate, Mn++, and phosphate (77°K) shows α-bands of cytochromes b and c type at 557 mμ and 549 mμ. In the Soret region, the trough of flavoprotein appears as two bands at 458 mμ and 492.5 mμ and the peak of cytochrome b type shows at 423 mμ, which is somewhat different from the spectra reported previously (Chance & Parsons, 1963). Cytochromes c_1, a, and a_3 are not detected. The absence of cytochrome oxidase is verified by the lack of its CO compound, the peak and trough of which appear at 589 mμ and 605 mμ. Although the extent of reduction of cytochromes b and c type is less than 5 % of the total dithionite reducible content (cf. Fig. 4A), these data demonstrate that they do communicate with the flavoprotein chain.

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References

Bueding, E., and Charms, B., J. Biol. Chem., 196, 615 (1952).
Bueding, E., Entner, N., and Farber, E., Biochim. Biophys. Acta, 18, 305 (1955).
Chance, B., and Hollunger, G., J. Biol. Chem., 238, 418 (1963).
Chance, B., and Parsons, D. F., Science, 142, 1176 (1963).
Chance, B., Schoener, B., and Yonetani, T., in Oxidases and related redox systems, T. E. King, H. S. Mason, and M. Morison (Eds.), N. Y., Wiley & Sons, Inc., 1965, p. 609.
Chance, B., Ernster, L., Garland, P. B., Lee, C. P., Light, P. A., Ohnishi, T., Ragan, C. I., and Wong, D., Proc. Nat. Acad. Sci., 57, 1498 (1967).
Chappell, J. B., and Haarhoff, K. N., in Biochemistry of mitochondria, E. C. Slater, Z. Kanuiga, and L. Wojtczak (Eds.), Academic Press, 1967, p. 75.
Estabrook, R. W., J. Biol. Chem., 223, 781 (1956).
Kmetec, E., and Bueding, E., J. Biol. Chem., 236, 584 (1961).
Laser, H., Biochem. J., 38, 333 (1944).
Seidman, I., and Entner, N., J. Biol. Chem., 236, 915 (1961).