THE AFFINITY OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION MECHANISMS FOR PHOSPHATE AND ADENOSINE DIPHOSPHATE*

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There is now increasing evidence for the occurrence of specific carrier systems in the mitochondrial membrane that permit certain metabolites to cross it freely, possibly by exchange-diffusion mechanisms.[1-5] One of these systems is specific for adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and it appears to be the site at which atractyloside inhibits a number of mitochondrial reactions dependent on external ADP or ATP.[1-11]

In this communication it is shown that the normally high affinity of oxidative phosphorylation mechanisms in intact mitochondria for both ADP and phosphate, as expressed by their respective Michaelis constants, is greatly decreased when membrane structure is disrupted by exposure to digitonin or to sonic energy. The decrease in affinity for ADP and for phosphate is in proportion to the severity of the disruptive procedure. These changes are evidently not the result of all-or-none inactivation of an increasing fraction of the phosphorylating enzyme molecules as mitochondrial structure is disrupted, since the mitochondria retain considerable phosphorylation activity with high P:O ratios.

To account for these observations, which confirm an earlier suggestion,[12] it is proposed that the very high affinity of oxidative phosphorylation mechanisms for ADP and phosphate in intact mitochondria is primarily a reflection of the high affinity of specific transport systems in the mitochondrial membrane that permit penetration of ADP and of phosphate, rather than of the affinity of the internal phosphorylating enzymes *per se*.

Methods.—Mitochondria were prepared from beef heart by the Nagarse method of Hatefi *et al.*[13] and from rat liver according to Schneider.[14] Digitonin particles were prepared from rat liver and beef heart mitochondria by the method of Wadkins and Lehninger[15] and sonic particles from beef heart mitochondria according to Linnane and Ziegler.[16] Oxygen uptake was measured polarographically with the Clark oxygen electrode, phosphate uptake by the method of Nielson and Lehninger,[17] and ATPase activity by following appearance of inorganic phosphate. The K_M values for ADP and phosphate in the oxidative phosphorylation tests were determined as the concentrations of ADP or phosphate giving one-half maximum rates of phosphate uptake. In the ATPase tests, the K_I for ADP is defined as that concentration giving half-maximal inhibition of the ADP-sensitive portion of the total ATPase activity in the assay system described.

Results.—Effect of ADP concentration on oxidative phosphorylation: Figure 1 shows the effect of ADP concentration on the rate of phosphate uptake observed during oxidative phosphorylation tests on intact beef heart mitochondria, as well as on digitonin and sonic particles derived from them. The apparent K_M for ADP, about 30 μM, agrees favorably with the value of about 20–30 μM previously

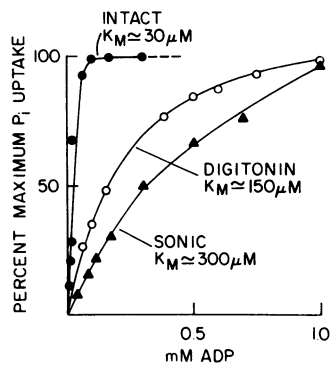

FIG. 1.—Effect of ADP concentration on rate of phosphate uptake by freshly isolated intact mitochondria and submitochondrial particles. The test system contained 49 mM KCl, 22 mM NaCl, 10 mM NaF, 5 mM $MgCl_2$, 11 mM K_2HPO_4, 2.5 mM KH_2PO_4 (containing P_i^{32}), 0.2% bovine serum albumin, 15 mM glucose, 150 μg yeast hexokinase, and 2–3 mg mitochondrial protein. The final pH was 7.2. The substrates used in the assays with the various particles were intact mitochondria, 10 mM pyruvate plus 10 mM malate; digitonin particles, 20 mM β-hydroxybutyrate plus 0.5 mM nicotinamide-adenine dinucleotide (NAD); sonic particles, 0.25 mM NAD, 20 μg yeast alcohol dehydrogenase, and 100 mM ethanol. ADP concentration was varied as indicated. The final volume was 2.0 ml. Incubation was for various periods not exceeding 5 min at 25°. The maximum P:O ratios in these experiments were about 3.0, 2.0, and 1.5, and the maximum acceptor control ratios were about 5.5, 1.5, and 1.0 for intact mitochondria, digitonin particles, and sonic particles, respectively.

observed by Chance and Williams.[18] Submitochondrial particles prepared with digitonin, which in separate experiments not recorded here gave maximum P:O ratios in the range 2.0–2.5, showed a marked increase in the K_M for ADP, to values of about 150–170 μM. Maximum rates of phosphate uptake by digitonin particles required at least 1.0 mM ADP, or some 10–15 times the concentration required by intact mitochondria. Disruption of beef heart mitochondria by sonic oscillation, a more drastic procedure yielding particles with a maximum P:O ratio of 1.0–1.5, decreased the affinity for ADP still more, to K_M values of about 300 μM (Fig. 1).

In similar experiments with frozen-thawed beef heart mitochondria and with rat liver digitonin particles, which show maximum P:O ratios of 1.5–2.2,[15] similar increases in K_M values for ADP were observed, to about 150 and 250 μM, respectively.

Effect of phosphate concentration on the P:O ratios: The effect of increasing concentrations of inorganic phosphate on the rate of phosphate uptake in various beef heart mitochondrial preparations is shown in Figure 2. It is seen that disruption of the mitochondria markedly lowered the affinity of the oxidative phosphorylation mechanisms for phosphate. The apparent K_M for phosphate in intact beef heart mitochondria was found to be about 0.25 mM, in agreement with earlier reports indicating K_M values in the range 0.25–1.0 mM.[19] The K_M values for phosphate in beef heart digitonin and sonic particles were much higher, about 3.0 and 6.0 mM, respectively. In similar experiments with rat liver digitonin particles, the K_M for phosphate was found to be about 1.5–2.0 mM.

A similar large increase in K_M for phosphate to a value of 6.0 mM was noted by Schatz and Racker in ETP_H particles of beef heart mitochondria.[20]

Effect of ADP concentration on the inhibition of ATPase activity: It is widely recognized that ATPase activity of mitochondria reflects in large part the reversibility of the oxidative phosphorylation mechanisms (reviews, refs. 21, 22). It has been found that ADP inhibits ATPase activity of intact mitochondria,[23, 24] of sonic particles,[25–27] and of digitonin particles.[28] ADP also inhibits the soluble ATPase of beef heart mitochondria described by Pullman *et al.*[29] In Figure 3 are shown data on the effect of ADP concentration on the inhibition of ATPase activity in intact beef heart mitochondria, as well as in digitonin and sonic particles. In intact mitochondria the concentration of ADP producing half-maximal inhibition of the ADP-

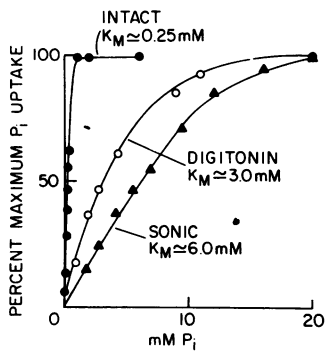

FIG. 2.—Effect of orthophosphate concentration on rate of phosphate uptake by mitochondria and submitochondrial particles. The reactions were carried out as described in Fig. 1 with ADP held constant at 1.0 mM. Varying concentrations of orthophosphate were added as indicated, in a final volume of 2.0 ml. Incubation was for 5 min at 25°.

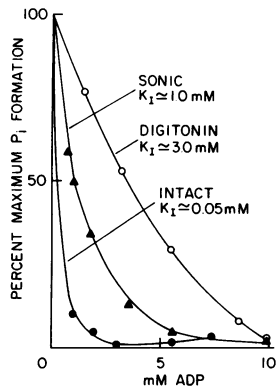

FIG. 3.—Effect of ADP concentration on ADP-inhibited component of ATPase activity of intact mitochondria and submitochondrial particles. The test system contained 16.6 mM Tris chloride, pH 7.4, 3.0 mM $MgCl_2$, 6.0 mM ATP, about 500 μg of mitochondrial protein and varying concentrations of ADP as indicated, in a final volume of 0.60 ml. Incubation was for 10 min at 30°.

sensitive portion of the total ATPase activity was very low, about 50 μM. The concentration of ADP required to produce half-maximal inhibition of digitonin and sonic particles was greatly increased, to give K_M values of about 3.0 and 1.0 mM, respectively. In experiments with frozen-thawed beef heart mitochondria, half-maximal inhibition was observed at about 100 μM ADP, and with rat liver digitonin particles half-maximal inhibition required over 1.0 mM ADP. The Mg^{++} concentration in such experiments may be raised to 20 mM without altering the relatively low affinity for ADP in the particles derived from beef heart mitochondria. Half-maximal inhibition of the purified beef heart mitochondrial ATPase of Pullman *et al.*[29] was reported to be given by 1.5 mM ADP.

The data reported here are in full consonance with the early suggestion of Kielley and Kielley[23, 24] that loss in affinity for ADP as an inhibitor may be responsible for the large increase in ATPase activity occurring when mitochondrial structure is disrupted.

Effect of atractyloside concentration on phosphate uptake: Atractyloside is a specific inhibitor for the entrance of ADP (or ATP) to specific phosphorylation sites in intact mitochondria.[3, 4, 6–9] It has a very high affinity for the ADP binding or transport system and its inhibitory effect may be reversed by ADP.[30] From these considerations it appeared likely that the apparent affinity of the membrane transport system for atractyloside would also decrease on disruption of membrane structure, just as data in Figure 1 show that affinity for ADP decreases. Experiments in Figure 4 are in agreement with this expectation. In intact mitochondria the affinity for atractyloside as inhibitor, with ADP held constant at 1.0 mM, is very high; 50 per cent maximal inhibition was given by less than 1.0 μM atractyloside. Phosphate uptake in sonic particles was not at all inhibited even at 10 μM atractyloside. In agreement with Vignais *et al.*,[7] phosphate uptake in digitonin particles was found

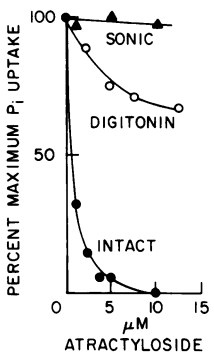

FIG. 4.—Loss of apparent affinity for atractyloside. Experimental details as in Fig. 1; ADP concentration was held constant at 1.0 mM.

to be only partially sensitive to atractyloside. Most important, however, is the finding that the apparent affinity for atractyloside in digitonin particles decreased greatly, to K_I values exceeding 5.0 μM. These findings, together with those in Figure 1, indicate that in sonic particles there is no significant atractyloside-sensitive facilitated diffusion of ADP into the mitochondria; presumably all the ADP entering these particles enters by simple physical diffusion. In digitonin particles, on the other hand, a significant fraction of the ADP may enter the phosphorylation sites by atractyloside-sensitive facilitated diffusion. The data also suggest that the properties of the remaining atractyloside-sensitive ADP transport activity have become altered by digitonin treatment so that its affinity for atractyloside is decreased.

Discussion.—The experiments reported here show that there is an apparently gradual loss of affinity of mitochondrial phosphorylation mechanisms for ADP and for phosphate as mitochondrial structure is disrupted, rather than an all-or-none loss of activity of an increasing fraction of phosphorylating enzyme molecules. One way of accounting for these findings is to assume that the internal ADP-phosphorylating enzyme system *per se* undergoes damage during mitochondrial disruption, in such a way that its affinity for ADP decreases gradually without loss of specific catalytic activity in bringing about ATP formation coupled to electron transport along the respiratory chain. There is some possible support for this view. Pullman *et al.*[29] have isolated from beef heart mitochondria in highly purified form an ATPase (F_1 factor) which can restore oxidative phosphorylation in pretreated beef heart mitochondria and which forms a complex with ADP.[31] Although the apparent Michaelis affinity of the F_1 coupling factor for ADP in reconstructed oxidative phosphorylation systems has not been reported, Pullman *et al.*[29] have shown that the F_1 factor has relatively low affinity for ADP when tested as an inhibitor of its ATPase activity (K_M = 1.5 mM) and that phosphate has no significant capacity to inhibit. The low affinity of F_1 for ADP as an inhibitor of ATPase activity may be due to some damage to the enzyme during the extraction and purification procedure, a change which must evidently take place even on such mild treatment as freezing and thawing of the mitochondria. Such a large decrease in the intrinsic affinity of the enzyme for ADP, without loss of its functional activity in forming ATP, is not incompatible with existing knowledge of enzyme properties; changes in substrate affinity have been observed to occur when certain allosteric enzymes undergo desensitization.[32]

However, an alternative hypothesis may be proposed, which is better able to account for changes not only in affinity, but also in nucleotide specificity, as mitochondrial structure is disrupted. It is possible that the normally high affinity of intact mitochondria for ADP and phosphate during oxidative phosphorylation is intrinsically a reflection of the high affinity of specific membrane translocase or exchange-diffusion systems for adenine nucleotides and for phosphate, rather than of the in-

ternal phosphorylating enzyme(s) *per se*. When membrane structure is disrupted, the specific transport systems for ADP and phosphate are inactivated and the membrane itself loses its intrinsic impermeability to phosphate and ADP which may then enter in a diffusion-controlled process dependent on external nucleotide concentration. Such a change could obviously account for the necessity to add much higher concentrations of ADP to disrupted mitochondria in order to realize maximum rates of oxidative phosphorylation, particularly if the intrinsic affinity of the internal phosphorylating enzymes is lower than that of the membrane carrier system. This hypothesis can also provide an explanation for the fact that ADP is specific as a phosphate acceptor for oxidative phosphorylation in intact mitochondria, whereas UDP, CDP, GDP, etc., may also serve as acceptors in disrupted mitochondria, into which various nucleotides may penetrate nonspecifically.

There is now growing evidence that inhibition of oxidative phosphorylation and ATP-dependent processes in mitochondria by atractyloside[4, 6–8] is due to an atractyloside-sensitive exchange-diffusion carrier for ADP and ATP in themit ochondria.[2–5, 9, 11, 30] This carrier system does not transport nucleoside di- and triphosphates other than ADP and ATP. It has a very high affinity for ADP; recent measurements in this laboratory indicate it to have a half-maximal activity at about 30 μM external ADP in rat liver mitochondria, which agrees very closely with the K_M for ADP as phosphate acceptor in intact mitochondria, i.e., about 20–30 μM.[18] On disruption of mitochondrial structure, the loss in atractyloside sensitivity is partial in digitonin particles (cf. ref. 7) and essentially complete in sonic particles. Parallel with the loss in atractyloside sensitivity, there is a decrease in the affinity for ADP as phosphate acceptor, as is shown in this paper.

The decreased affinity for inorganic phosphate in the submitochondrial systems described here and in the ETP_H preparations of Schatz and Racker[20] requires special consideration, since phosphate is not of course translocated by the specific, atractyloside-sensitive carrier for ADP. However, recent work by Chappell[1, 2] suggests that the phophate anion may enter and leave intact mitochondria by the action of a phosphate-specific membrane-linked transport system, probably in exchange for OH^-. Damage to membrane structure could also facilitate nonspecific concentration-dependent diffusion of phosphate into mitochondria and therefore account for the observed decrease in the affinity for phosphate in submitochondrial systems.

The two hypotheses for the decreased affinity for phosphate and for ADP in disrupted mitochondria are not necessarily mutually exclusive; it is possible that the substrate affinities of *both* the membrane carrier and of the internal phosphorylating enzyme(s) are very high in intact mitochondria, and that both decrease on disruption of membrane structure. However, our findings are in better agreement with a model in which the intrinsic affinity of the internal phosphorylating enzyme system is lower than the affinity of the specific membrane transport systems. This model is also supported by recent reports indicating that ADP and ATP are present in rather high concentrations (5–8 mM) in the internal aqueous phase of intact mitochondria.[3, 33] Presumably the internal nucleotides are retained in such high concentration by Donnan equilibria; the major intramitochondrial cations are Mg^{++} and K^+, which cannot pass through the membrane (review, ref. 5).

The general hypothesis offered here requires no significant modification to be applicable to the chemiosmotic mechanism of oxidative phosphorylation proposed by

Mitchell.[34] It is also consistent with observations on specific transport systems in mitochondria for succinate and isocitrate,[2] which appear to be genetically determined. Such systems are present in rat liver mitochondria but are lacking in intact housefly mitochondria,[2] which are unable to oxidize external succinate or isocitrate but acquire this ability if the membrane is disrupted.[35]

Summary.—Disruption of mitochondrial membrane structure with digitonin or sonic irradiation leads to greatly decreased affinity of oxidative phosphorylation mechanisms for ADP and for phosphate, as indicated by increased Michaelis constants, without a parallel decline in the P:O ratio. Simultaneously, there is a decreased affinity for ADP as an inhibitor of ATPase activity and a decreased sensitivity to and affinity for atractyloside, a specific inhibitor of ADP transport. It is proposed that the normally very high affinity of oxidative phosphorylation mechanisms for ADP and phosphate in intact mitochondria is a reflection of the high affinity of specific membrane-linked transport systems for ADP and for phosphate, rather than of the affinity of the internal phosphorylating enzymes *per se*.

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