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INTERACTION OF HOMOGENEOUS MITOCHONDRIAL ATPASE FROM RAT LIVER WITH ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE

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Mitochondrial ATPase from rat liver mitochondria contains multiple nucleotide binding sites. At low concentrations ADP binds with high affinity (1 mole/mole ATPase, K_D = 1–2 μM). At high concentrations, ADP inhibits ATP hydrolysis presumably by competing with ATP for the active site (K_I = 240–300 μM). As isolated, mitochondrial ATPase contains between 0.6 and 2.5 moles ATP/mole ATPase. This "tightly bound" ATP can be removed by repeated precipitations with ammonium sulfate without altering hydrolytic activity of the enzyme. However, the ATP-depleted enzyme must be redissolved in high concentrations of phosphate to retain activity. AMP-PNP (adenylyl imidodiphosphate) replaces tightly bound ATP removed from the enzyme and inhibits ATP hydrolysis. AMP-PNP has little effect on high affinity binding of ADP. Kinetic studies of ATP hydrolysis reveal hyperbolic velocity vs. ATP plots, provided assays are done in bicarbonate buffer or buffers containing high concentrations of phosphate. Taken together, these studies indicate that sites on the enzyme not directly associated with ATP hydrolysis bind ATP or ADP, and that in the absence of bound nucleotide, P_i can maintain the active form of the enzyme.

INTRODUCTION

Central to our understanding of the terminal enzymatic steps of oxidative phosphorylation is a complete understanding of the mechanism of action of the oligomycin-sensitive ATPase. Through the efforts of a number of laboratories we now recognize that the overall structure of the ATPase is very complex, consisting of 10 or 11 polypeptides, and requiring phospholipids for activity (Fig. 1). Of the four major functional units of this enzyme complex, the headpiece, called F_1, has been studied in greatest detail with respect to both its structural and catalytic properties. Structurally, the F_1 molecule is thought to contain five different types of chains in the stoichiometric ratio A_3B_3CDE (1, 2). The six A and B subunits are thought to be arranged in a hexagonal array (Fig. 2 A). There is still some debate about this structure, and about the origin of the smaller subunits. For example, as indicated in Fig. 2 B, Kozlov and Mikelsaar (3) believe subunits C, D, and E are all derived from a larger precursor at an early stage in the purification.

Functionally, the intact oligomycin-sensitive ATPase participates in both ATP formation and ATP utilization (Fig. 3 A). The possibility that these separate functions involve separate nucleotide binding sites has been proposed (4–6). Presumably, the direction of energy flux is regulated by the ATPase inhibitor protein of Pullman and

Monroy (7–9), but to date there is no unambiguous data in the literature to indicate that this type of regulation occurs in intact mitochondria. Nevertheless, it is of interest that the ATPase inhibitor has now been purified not only from heart mitochondria, but in our laboratory also from rat liver mitochondria (6) and from mitochondria isolated from several strains of yeast (10, 11).

Consistent with the thesis of multiple functional sites on mitochondrial ATPase are the recent findings that F_1 preparations from a variety of sources contain tightly bound ATP (4, 12, 13). The role of this tightly bound ATP is of some concern to all of us and represents one of the central themes of this conference. Is it associated directly with the process of oxidative phosphorylation via conformational changes in F_1, as implied by the work of Boyer and his colleagues (14, 15), Fig. 3 B, or conversely, is it essential for some other purpose?

My talk today will focus on the interaction of mitochondrial F_1 from rat liver with ATP, ADP, and P_i and the possible relevance of these interactions to the functions of the enzyme.

METHODS

Most of the methods employed in this study have been described in detail elsewhere. Purification of the ATPase from rat liver mitochondria was carried out as described by Catterall and Pedersen (15). Binding of ADP and deoxy ADP was carried out by using an ammonium sulfate precipitation technique or an equilibrium dialysis procedure (16). AMP-PNP binding was assayed in the presence of 0.2 mM $MgCl_2$ by the equilibrium dialysis procedure described for ADP (16). ATPase activity was usually assayed by using either a phosphate release assay (5) or a spectrophotometric assay (15). However, when ATP hydrolysis was followed in high phosphate media, the release of ^{32}P from γ -labeled ^{32}P-ATP was followed. In this case, assays were terminated with activated charcoal and centrifuged to remove excess labeled ATP bound to the charcoal. Aliquots of the supernatant fraction were assessed for radioactivity, as described previously (16). The amount of ATP tightly bound to freshly isolated mitochondrial ATPase was determined by the firefly assay (17) after denaturing the enzyme with 0.5 M PCA, centrifuging to remove denatured enzyme, and neutralizing with KOH.

RESULTS AND DISCUSSION

Interaction with ADP

These results have been described in detail in previous publications (5, 16) and will be dealt with only briefly here. As noted in Table I, the purified ATPase from rat liver mitochondria binds about 1 mole of ADP per mole ATPase with high affinity (K_D = 1–2 μM) (16). Binding is specific for ADP and d ADP. In the absence of added Mg^{++}, it is inhibited about 75% by aurovertin, an inhibitor of oxidative phosphorylation. In the presence of added Mg^{++}, aurovertin activates the enzyme.

In addition to binding with high affinity to purified ATPase, ADP also appears to interact with the hydrolytic site of the enzyme. This is indicated by the finding that ADP is a weak competitive inhibitor of the enzyme with a K_I (ADP) of about 240–300 μM (5). The much lower apparent affinity of ADP for the ATP hydrolytic site than for the site

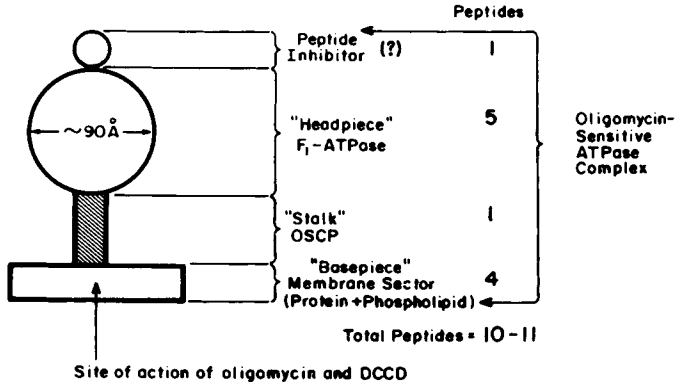

Fig. 1. Component parts of the oligomycin-sensitive ATPase complex. A question mark is placed adjacent to the ATPase inhibitor peptide because it has not been established whether the inhibitor of Pullman and Monroy (7) is separate from F_1 or constitutes one of its subunits.

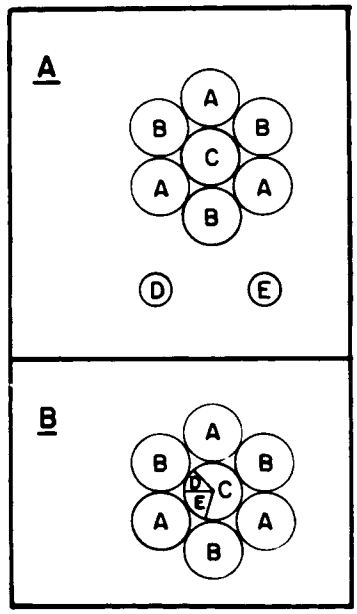

Fig. 2. Models proposed for the arrangement of subunits in the F_1 part of the oligomycin-sensitive ATPase complex. A. Hexagonal model proposed by Catterall and Pedersen (1, 5). The small subunits, D and E, could be associated with any one of the larger subunits. B. Hexagonal model of Kozlov and Mikelsaar (3). The C, D, and E subunits are though to be derived from a larger precursor.

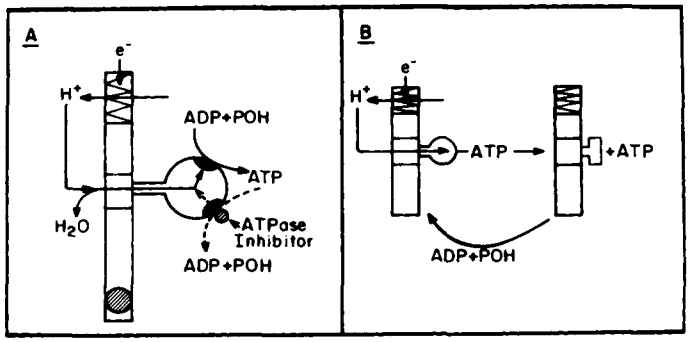

Fig. 3. Models demonstrating how the oligomycin-sensitive ATPase complex might participate in ATP formation and ATP utilization. A. Model depicting two nucleotide binding sites on F_1, one involved in ATP formation and one involved in ATP utilization. B. Model depicting how ATP tightly bound to F_1 might be associated with oxidative phosphorylation.

involved in binding ADP with high affinity would suggest that these two sites are distinct. This suggestion is supported by additional experiments which show that the hydrolytic site can be inhibited by preincubation with Mg^{++}, or by high sucrose concentrations without significantly altering high affinity binding of ADP (5, 16).

Interaction with ATP, AMP-PNP, and P_i

Tightly Bound ATP Associated with Freshly Prepared ATPase. Table II summarizes results obtained with six different ATPase preparations. ATP is referred to as "tightly bound" because it has not been removed during purification of the enzyme, but rather when the purified enzyme is subject to acid treatment (see Methods). Values range from as low as 0.6 mole ATP/mole ATPase to as high as 2.5 mole ATP/mole ATPase.

Removal and Replacement of ATP. As indicated in Table II, acid precipitation is not necessary to remove the bulk of the tightly bound ATP. Instead, removal can be effected by repeated precipitation of the enzyme with ammonium sulfate. In the experiment shown, 1.4 moles of the original 1.6 moles of ATP bound per mole of enzyme were removed by precipitation five times with ammonium sulfate. ATP could not be replaced as such. This may be because the tightly bound ATP is associated with sites on the enzyme distinct from the hydrolytic site, but once removed and added back, the ATP becomes accessible to the hydrolytic site. Relevant here is the finding that even in the absence of added Mg^{++} the rat liver enzyme catalyzes the hydrolysis of ATP at low but significant rates (16).

ATP, once removed, can be replaced with the nonhydrolyzable ATP analog AMP-PNP. Equilibrium dialysis experiments indicate that between 2 and 3 moles of AMP-PNP can be rebound per mole of ATP-depleted enzyme.

Effect of Removal of ATP on Hydrolytic Activity – The Important Role of P_i. It is of considerable interest to know whether removal of tightly bound ATP alters the catalytic activity of mitochondrial ATPase. Experiments summarized in Table IV show that

removal of about 87% of the tightly bound ATP has little effect on enzyme activity provided the depleted enzyme is redissolved in high concentrations of potassium phosphate (250 mM KP_i + 5 mM EDTA). However, after ATP removal, if the enzyme is dissolved in potassium buffers other than P_i, there is a significant loss of enzyme activity. This experiment shows that an ATP-depleted ATPase can be retained in fully active form provided that high P_i concentrations are present. It also suggests that in the absence of P_i, ATP may be playing a stabilizing role. However, additional studies will have to be carried out to test this latter possibility.

TABLE I. Interaction of Purified Mitochondrial ATPase with ADP*

Binding Property	$-Mg^{++}$	$+Mg^{++}$
Moles bound/mole ATPase	0.91	0.65
K_D (μM)	0.96	2.1
Specificity	ADP, d ADP	Not determined
Aurovertin sensitivity	Inhibits (~ 75%)	Activates

Inhibition of ATPase Activity Property	Result
Concentration for 50% inhibition	~ 1.0 mM
Type of inhibition	Competitive
K_I (ADP)	240–310 μM
Specificity	ADP, d ADP

*Details of experimental procedure appear in references 5 and 15.

TABLE II. ATP Associated with Freshly Isolated ATPase*

ATPase Purification	Mitochondria → Submitochondrial particles → 5x wash → Incubate with ATP → Sonic extract → DEAE → Sephadex G–25
Number of preparations	6
Moles ATP/mole ATPase (range)	0.6–2.5
Average value	1.4

*For experimental details see reference 15 and Methods.

TABLE III. Removal and Replacement of ATP*

	Mole/mole ATPase
ATP bound to isolated enzyme	1.6
ATP removed after 5x precipitation	1.4
Amount replaced with ATP	0.2
Amount replaced with AMP-PNP	2–3

*For experimental details see Methods.

TABLE IV. Effect of Removal of Bound ATP on Catalytic Activity of Purified ATPase

Conditions for Removal	Bound ATP*		ATPase activity*	
	Before Removal	After Removal	Before Removal	After Removal
Ammonium sulfate precipitation (5x): redissolve in KP_i	1.6	0.20	29	27
Ammonium sulfate precipitation (5x); redissolve in K_2SO_4	1.9	0.13	32	9

*Bound ATP reported as moles/mole ATPase; ATPase activity reported as μmoles ATP hydrolyzed/min/mg protein. KP_i was present at 250 mM in 5 mM EDTA. K_2SO_4 was present at 200 mM.
In a previous study (5) it was reported that the purified rat liver ATPase contained no more than 1 mole of P_i/mole ATPase. In that study, assays were performed on an enzyme preparation that had been precipitated several times with ammonium sulfate.
For experimental details see Methods.

Effect of AMP-PNP on ATPase Activity and Binding of ADP. Another important question about the sites on purified ATPase which interact with ATP concerns their relation to both the hydrolytic site of the enzyme and to the site involved in binding ADP with high affinity. One approach to obtaining at least a partial answer to this question is to examine the effects of AMP-PNP on ATPase activity and on high affinity binding of ADP.

As noted in Table V, AMP-PNP is a potent inhibitor of hydrolytic activity (K_I = 2.2 μM), and appears to be competitive with ATP for the hydrolytic site (6). Since we know that more than 1 mole of AMP-PNP can be bound per mole of ATP-depleted enzyme, this result would suggest that either more than one hydrolytic site is present on the enzyme, or that AMP-PNP binds not only to the hydrolytic site but to sites not associated with hydrolysis as well. The question of multiple catalytic sites will be dealt with below.

Results also shown in Table V indicate that sites which interact with AMP-PNP are distinct from the high affinity ADP binding site. This site is saturated by as little as 15 μM ADP (16), but requires close to 10 mM AMP-PNP to remove 50% of the ADP. This concentration of AMP-PNP is about 333 times higher than the concentration of AMP-PNP (30 μM) necessary fully to restore AMP-PNP to an ATP-depleted enzyme.

Effect of Inhibitors on Binding of AMP-PNP. Results presented in Table VI show that aurovertin at a farily high concentration (40 μg/ml) is the only inhibitor tested which markedly alters binding of AMP-PNP. Azide, atractyloside, and bongkregic acid have little or no effect at the concentrations tested. Similar results were obtained when the effects of these inhibitors were tested on the high affinity binding of ADP. Thus, nucleotide binding is inhibited at least partially by aurovertin, but not by other inhibitors known to alter nucleotide binding to mitochondria.

Hydrolysis of ATP Catalyzed by Purified ATPase. It is of interest to know whether the purified ATPase has multiple hydrolytic sites. A first approach to answering this question is to examine the kinetics of ATP hydrolysis. Such a study should reveal whether

TABLE V. **Effect of AMP-PNP on ATPase Activity and on Binding of ADP***

ATPase Activity Property	Result
Concentration for 50% inhibition	~ 50 μM
Type of inhibition	Competitive
K_I (AMP-PNP)	~ 2.2 μM

ADP Binding AMP-PNP (mM)	ADP Bound (mole/mole ATPase)	% Inhibition
0	1.1	0
1.8	0.7	26
9.1	0.52	53

*For experimental details see Methods and reference 6.

there are multiple interacting sites. It will not reveal whether there are multiple sites that do not interact with one another.

The hydrolytic properties of the purified enzyme are summarized in Table VII. When assays are carried out in bicarbonate buffer (30 mM) or in buffers containing high concentrations of potassium phosphate (250 mM), initial rates of hydrolysis are linear and velocity vs. ATP curves are hyperbolic. There is no suggestion of interacting ATP hydrolytic sites. The K_m of ATP hydrolysis is about 0.1 mM in phosphate buffer and between 0.2–0.4 mM in bicarbonate buffer. Bicarbonate is an activator of ATPase activity. As reported previously, specific activities are 2- to 3-fold higher when assays are carried out in bicarbonate buffer (6). These observations are entirely consistent with those reported by Mitchell and Moyle (18) for membrane-bound ATPase of rat liver.

In Tris-Cl buffers one observes a different phenomenon. Normally, we store our enzyme in high phosphate medium where it exhibits linear rates and a K_m of about 0.1 mM ATP (see above). (As already mentioned in this report, phosphate will maintain the enzyme in an active form even when bound ATP is removed.) When the enzyme is transferred to the assay system in Tris-Cl, phosphate is diluted to less than 2 mM. At low ATP concentrations, an initial rapid burst of hydrolysis is observed which levels off to a linear rate. At high ATP concentrations the rate remains linear. A plot of initial rates vs. ATP concentration yields a hyperbolic curve. However, if the linear rate observed after the initial burst is plotted vs. ATP concentration, nonhyperbolic velocity vs. ATP curves are observed. The apparent K_m's in the two cases are 0.1 mM ATP and 1.0 mM ATP, respectively.

We believe that the best interpretation of these results at the present time is as follows. When the enzyme is transferred from a high phosphate medium where it is kinetically identical to the membrane-bound enzyme (low K_m) to a P_i-free medium of lower ionic strength, it rapidly changes its conformation to a form which has a higher K_m for ATP. The low K_m form of the enzyme (membrane form) can be only maintained in high concentrations of phosphate and/or high concentrations of ATP.

These results do not rule out the possibility that the purified ATPase contains multiple catalytic sites which do not interact with one another. In fact, the recent report of Ebel and Lardy (19) suggests this might be the case by comparing kinetics of ATP

TABLE VI. Effect of Inhibitors on Binding of AMP-PNP to ATPase*

Inhibitor	Concentration	cpm ^{3}H-AMP-PNP Bound	% Inhibition
None	–	3445	0
Aurovertin	40 μg/ml	1684	51
Azide	200 μM	3407	1.1
Atractyloside	6 μM	3769	0
Bongkregic acid	6 μM	3523	0

*AMP-PNP was present at 1.2 μM and purified ATPase was present at 300 μg/ml. For experimental details see Methods.

TABLE VII. Hydrolysis of ATP Catalyzed by Purified ATPase*

Property	Assay Buffer		
	Tris-Cl	Phosphate	Bicarbonate
Kinetics (Velocity vs time)	Nonlinear at low ATP Linear at high ATP	Linear	Linear
Kinetics (Velocity vs ATP)	Hyperbolic†	Hyperbolic	Hyperbolic
K_m (ATP)	0.1 mM	0.1 mM	0.2–0.4 mM
Specific activity (μmoles P_i/min/mg)	25	25	55–70
Aurovertin sensitivity	62% inhibition	Not tested	Not tested

*Where indicated, final concentrations in the assay medium were 65 mM Tris-Cl, pH 7.5, 250 mM potassium phosphate, pH 7.5, 30 mM sodium bicarbonate, pH 7.5, 0.5 μg/ml aurovertin, and 2μg/ml mitochondrial ATPase.

†Hyperbolic plots are not obtained in Tris-Cl buffer if initial rates are not plotted at both high ATP concentration and low ATP concentration. At low ATP concentration it is easy to overlook the initial rate which is very rapid. In such cases velocity vs ATP plots will not be hyperbolic, but rather tend toward a more sigmoid type of shape.

hydrolysis of rat liver ATPase in activating and nonactivating buffer systems.

Finally, it should be noted that aurovertin, similarly to its inhibitory effects on the binding of ADP and AMP-PNP, also inhibits ATP hydrolysis by about 62%.

CONCLUSIONS

To date we believe we can make the following tentative conclusions about the interaction of ATP, ADP, and P_i with purified mitochondrial ATPase of rat liver. (1) The enzyme contains multiple nucleotide binding sites. Although one or more of these sites appears to be associated with the hydrolytic site, at least one high affinity ADP binding site and at least one ATP binding site do not appear to be directly associated with hydrolysis. (2) Tightly bound ATP associated with the freshly isolated ATPase can be removed (87%) without altering hydrolytic activity provided the enzyme is maintained in a high phosphate medium. (3) Kinetic studies do not reveal multiple interacting sites for

ATP hydrolysis. However, they do not rule out multiple noninteracting hydrolytic sites. (4) The membrane-bound form of mitochondrial ATPase exhibits a low K_m for ATP (0.1 mM) (18). To maintain the membrane-bound form of the enzyme, the soluble enzyme requires P_i and/or ATP. (5) Aurovertin inhibits binding of ADP, binding of AMP-PNP, and ATP hydrolysis. This is consistent with the view (20) that aurovertin induces a conformational alteration in the molecule, making its interaction with ADP and ATP less favorable.

We do not know what the functions of the various nucleotide binding sites are. One hypothesis supported by some data is that the high affinity site for ADP may be directly involved in oxidative phosphorylation because the K_D of this site for ADP is in the same range as the K_D of oxidative phosphorylation (16). The hydrolytic site may be involved in ATP-dependent activities. Hydrolysis can be inhibited by AMP-PNP without inhibiting oxidative phosphorylation (4, 6). Other sites involved in binding ATP very tightly appear to be necessary to maintain the active or stable form of the enzyme. However, it seems reasonable also to suspect that these ATP binding sites may play some hitherto unknown role either in the mechanism of ATP formation or in the mechanism of ATP utilization.

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