ADENOSINE TRIPHOSPHATASE FROM RAT LIVER MITOCHONDRIA: SEPARATE SITES INVOLVED IN ATP HYDROLYSIS AND IN THE REVERSIBLE, HIGH AFFINITY BINDING OF ADP

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Summary

Homogeneous ATPase from rat liver mitochondria binds one mole of ADP per mole of enzyme reversibly, and with high affinity (K_D = 1-2 μM). The high affinity binding site is highly specific for ADP and dADP. AMP does not bind. Agents which inhibit ATP hydrolysis have little inhibitory effect on the high affinity binding of ADP. These agents include adenylyl imidodiphosphate (AMP-PNP), azide, sucrose, and the divalent cation Mg^{++}. AMP-PNP inhibits ATPase activity in phosphorylating membrane preparations of rat liver mitochondria by about 90 percent, but is without effect on ATP synthesis. These results are consistent with the view that the purified soluble, and the membrane-bound ATPase of rat liver mitochondria contain separate sites involved in ATP hydrolysis and in the reversible, high affinity binding of ADP.

Purified mitochondrial ATPase preparations (F_1) from both bovine heart and rat liver have been shown to contain two reversible binding sites for ADP (1, 2). One of these sites binds ADP with very high affinity ($K_D \leq 2$ μM) whereas the other site binds ADP with much less affinity and is thought to be involved in ADP product inhibition of ATP hydrolysis (1, 3). The two sites associated with reversible ADP binding on the heart enzyme appear to bind ADP somewhat more tightly than the liver enzyme which may account for the observation of Slater's laboratory that 2 moles of tightly bound ADP are associated with the enzyme as isolated (4). Recent work has demonstrated also that both the heart and liver enzymes have multiple binding sites for ATP (4-6). The total number of nucleotide binding sites on both enzymes, and their relation to site(s) involved in ATP hydrolysis has not been clearly established. Experiments described in this report focus on the latter question as it relates to the reversible, high affinity binding site for ADP of the rat liver mitochondrial ATPase.

MATERIALS AND METHODS

Purified inner membranes of rat liver mitochondria were prepared exactly as described by Chan, Greenawalt and Pedersen (7), and suspended in a medium containing 200 mM D-mannitol, 70 mM sucrose, 2.0 mM HEPES buffer, pH 7.4, and 0.05% (w/v) crystalline bovine serum albumin.

Purified mitochondrial F_1 of rat liver was prepared exactly as described by Catterall and Pedersen (8) and stored in lyophilized form until used in assays.

ATPase activity was assayed spectrophotometrically in a 1 ml system containing 65 mM Tris-Cl, 4.8 mM $MgCl_2$, 4.0 mM ATP, 0.4 mM NADH, 0.6 mM phosphoenolpyruvate, 5 mM KCN, 1 unit of lactic dehydrogenase, and 1 unit pyruvate kinase, pH 7.5 (8). ATPase activity was assayed also by following the release of P_i in a 1 ml assay medium containing 50 mM Tris-Cl, 5 mM $MgCl_2$, and 5 mM ATP, pH 7.5. In both assays initial rates of ATP hydrolysis were measured.

Binding of ADP and other nucleotides was assayed by an ammonium sulfate precipitation assay exactly as described by Catterall and Pedersen (2). Similar results were obtained with an equilibrium dialysis method (2).

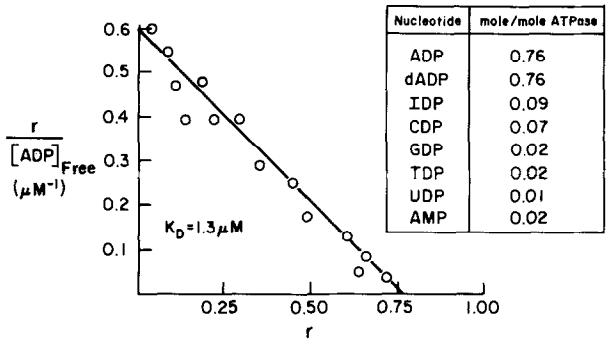
Oxidative phosphorylation was measured as described by Chan, Greenawalt, and Pedersen (7) in a 3 ml system containing 220 mM D-mannitol, 70 mM sucrose, 0.5 mM EDTA, 2.5 mM KP_i, 5.4 mM succinate, 1.8 mM $MgCl_2$, 0.71 mM ADP, 0.18 mg/ml bovine serum albumin, and 10^6 CPM [^{32}P]orthophosphate, pH 7.5.

Unlabelled nucleotides were obtained from P. L. Biochemicals. [^{3}H]ADP, [^{3}H]GDP, and [^{3}H]AMP were purchased from New England Nuclear. All other labelled nucleoside diphosphates were purchased from Amersham/Searles. [^{3}H]-labelled and unlabelled AMP-PNP was purchased from ICN Pharmaceuticals, Inc. Azide, sucrose, and EDTA were from Baker Chemical Co.; ammonium sulfate from Schwarz-Mann; 2,4-dinitrophenol from Eastman Organic Chemicals, and oligomycin from Sigma Chemical Co. Atractyloside was generously donated by Professor R. Santi and Dr. A. Bruni of the University of Padova, Italy.

RESULTS AND DISCUSSION

Purified rat liver F_1 has been shown in previous studies in this laboratory to bind one mole of ADP reversibly, and with high affinity (2, 3). The experiment summarized in Figure 1 is consistent with these previous studies, and shows also that the high affinity site is highly specific for ADP and dADP as ligands. Some binding of other nucleotides does occur but in all cases this is less than 0.1 mole nucleotide/mole ATPase. At high concentrations ADP also inhibits the hydrolysis of ATP (K_i = 0.24 - 0.31 mM) implicating a second, lower affinity site for binding ADP (3).

F_1 preparations depleted of adenine nucleotides by precipitating the enzyme 5 times with ammonium sulfate also contain a single, reversible, high affinity binding site for ADP. Consequently, high affinity binding of



Nucleotide	mole/mole ATPase
ADP	0.76
dADP	0.76
IDP	0.09
CDP	0.07
GDP	0.02
TDP	0.02
UDP	0.01
AMP	0.02

Fig. 1. Binding of ADP by the purified F_1 ATPase of rat liver mitochondria. ADP binding was assayed by an ammonium sulfate precipitation method in the absence of added Mg^{++} (2). The moles ADP bound/mole ATPase is represented by r. Inset: Specificity of ADP binding. Assay conditions were identical to those used for ADP binding.

TABLE I

Effect of inhibitors of ATPase activity on the reversible, high affinity binding of ADP to mitochondrial ATPase[a].

Inhibitor	Concentration	ATPase Activity[b]	% Inhibition	ADP Binding[c]	% Inhibition	K_D[d]
None	-	28.0	0	0.75-0.96	0	0.9-1.3
Azide	0.1 mM	9.1	68	0.87	0	1.0
Sucrose	1.5 M	1.4	95	0.80	6	1.1
Mg^{++}	5.0 mM	14.0	50	0.70	18	2.1
AMP-PNP	0.5 mM	2.0	90	0.61	29	1.2

[a] For assay conditions see MATERIALS AND METHODS. To observe inhibition with Mg^{++}, incubation of F_1 and Mg^{++} was carried out for 5 min at 25° in the absence of other assay components of ATPase activity.

[b] μmoles ATP hydrolyzed x min^{-1} x mg^{-1}

[c] moles ADP/mole ATPase

[d] Dissociation constant for ADP, μmolar

ADP seems not to be simply the result of exchange of ADP with nucleotides already bound to F_1 as isolated.

Results summarized in Table I show that a variety of agents inhibit ATP hydrolysis. These include sucrose, Mg^{++}, and the ATP analog AMP-PNP (9). Of these agents, AMP-PNP was the most potent inhibitor of ATP hydrolysis and was examined in greater detail for both its effect on high affinity binding of ADP to F_1 and for its effect on oxidative phosphorylation in inner membrane vesicles.

As shown in Figure 2A, at low concentrations, AMP-PNP is a potent inhibitor of both the purified rat liver ATPase and of the ATPase activity catalyzed by inner membrane vesicles of rat liver mitochondria. These re-

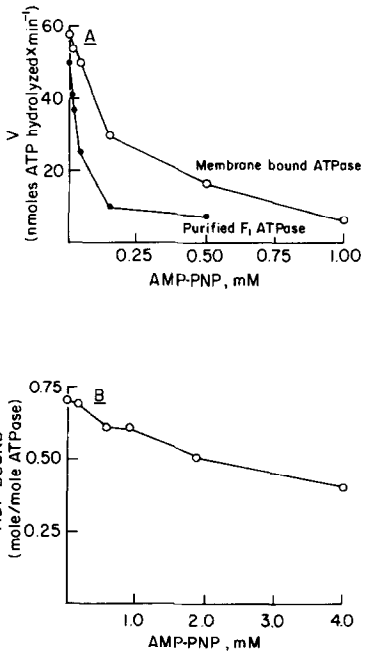


Fig. 2. A. Effect of AMP-PNP on ATPase activity catalyzed by membrane-bound ATPase, and purified F_1 ATPase. Assay conditions were exactly as described under MATERIALS AND METHODS. Where indicated rat liver inner membranes, 20 μg, and rat liver F_1, 2.4 μg, were present in the assay medium. B. Effect of AMP-PNP on the reversible, high affinity binding of ADP. ADP binding was assayed by an ammonium sulfate precipitation assay (2).

sults are entirely consistent with results reported by Penefsky (10) for the heart system. In contrast to its potent inhibitory effect on ATP hydrolysis, however, AMP-PNP had little inhibitory effect on either high affinity ADP binding to F_1 (Fig. 2 B) or on oxidative phosphorylation catalyzed by inner membrane vesicles of rat liver (Table 2). Concentrations of AMP-PNP of 0.5 mM and greater do result in the removal of significant amounts of ADP from purified F_1, but these concentrations are in great excess of that necessary to provide half-maximal inhibition of ATP hydrolysis.

The simplest explanation of the results presented here, which show that the ATPase activity of F_1 can be inhibited without altering binding of ADP, is that the purified F_1-ATPase has separate sites specialized respectively for ATP hydrolysis and for ADP binding. The results showing that AMP-PNP

TABLE 2

Effect of AMP-PNP on oxidative phosphorylation catalyzed by inner membrane vesicles of rat liver mitochondria[a].

Addition	Concentration	P/O[b]
None	-	0.70
AMP-PNP	0.27 mM	0.72
AMP-PNP	1.10 mM	0.84
Oligomycin	0.5 μg/ml	0.03
2,4-dinitrophenol	0.10 mM	0
Atractyloside[c]	10 μM	0.61

[a] For assay conditions see MATERIALS AND METHODS.

[b] Inner membranes prepared by the Digitonin-Lubrol method (7) phosphorylate only at site II. The maximal P/O ratio with succinate is 1.0.

[c] Control carried out to show that nucleotide permeation is not a problem. Membranes are for the most part inverted (7).

is a potent inhibitor of ATP hydrolysis in inner membrane vesicles, but is without effect on either ATP synthesis or reversible, high affinity ADP binding to F_1 are subject to several interpretations. These have been discussed in a recent review (11) and are mentioned only briefly below.

According to the first interpretation the reversible, high affinity ADP binding site may be directly involved in oxidative phosphorylation whereas the Mg^{++}-stimulated ATPase site(s) may be involved in some other function, perhaps ATP-dependent activities. This suggestion is supported also by data which show that the affinity of oxidative phosphorylation for ADP in rat liver membranes (K_m = 3-4 μM) is in the same range as the affinity of rat liver F_1 for ADP (2). Penefsky (10) has already discussed this view and presented evidence to show that in the heart system AMP-PNP fails to inhibit oxidative phosphorylation but does inhibit ATP-dependent activities.

The alternate view is that site(s) on F_1 involved in Mg^{++} stimulated ATPase activity participate in both oxidative phosphorylation and in ATP-dependent activities. High affinity sites involved in binding ADP and ATP (4-6) may simply be involved in regulation similar to the hypothesized role for the ATPase inhibitor peptide (12). If this view is correct, one must assume that AMP-PNP can inhibit ATPase activity in nonphosphorylating membranes but is dissociated when oxidative phosphorylation is initiated.

Experiments are currently under way in this laboratory to determine whether either one of these views is correct, or whether a third interpretation involving cooperation of the ATPase site(s) and the reversible, high affinity ADP binding site is necessary to account for ATP synthesis and ATP-dependent activities of rat liver mitochondria.

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