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SPECIFICITY OF NUCLEOTIDE BINDING AND COUPLED REACTIONS UTILISING THE MITOCHONDRIAL ATPase

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Summary

1. Tightly bound ATP and ADP, found on the isolated mitochondrial ATPase, exchange only slowly at pH 8, but the exchange is increased as the pH is reduced. At pH 5.5, more than 60% of the bound nucleotide exchanges within 2.5 min.

2. Preincubation of the isolated ATPase with ADP leads to about 50% inhibition of ATP hydrolysis when the enzyme is subsequently assayed in the absence of free ADP. This effect, which is reversed by preincubation with ATP, is absent on the membrane-bound ATPase. This inhibition seems to involve the replacement of tightly bound ATP by ADP.

3. Using these two findings, the binding specificity of the tight nucleotide binding sites was determined. *iso*-Guanosine, 2′-deoxyadenosine and formycin nucleotides displaced ATP from the tight binding sites, while all other nucleotides tested did not. The specificities of the tight sites of the isolated and membrane-bound ATPase were similar, and higher than that of the hydrolytic site.

4. The nucleotide specificities of 'coupled processes' nucleoside triphosphate-driven reversal of electron transfer, nucleoside triphosphate-$^{32}P_i$ exchange and phosphorylation were higher than that of the hydrolytic site of the ATPase and similar to that of the tight nucleotide binding sites.

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Abbreviations: ϵ-ATP, N^1,N^6-ethenoadenosine triphosphate; 8-BrATP, 8-bromoadenosine triphosphate; AMP-PNP, adenosine β,γ-imidotriphosphate; GMP-PNP, guanosine β,γ-imidotriphosphate; N^1,*O*-ATP, adenosine-N^1-oxide triphosphate; rro-ATP 2,2′[1-(9-adenyl)-1′-(triphosphoryl-oxymethyl)-dihydroxydiethyl ether; and similarly for the respective diphosphates; NTP, NDP, nucleoside tri-, diphosphate; ANS, 1-anilino-8-naphthalene sulphonate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid; MES, 2-(*N*-morpholino)-ethane sulphonic acid; TES, tris(hydroxymethyl)methylamino ethane sulphonic acid.

5. The different nucleotide specificities of uncoupled ATP hydrolysis and coupled processes can be explained even if both processes involve a single common site on the ATPase molecule. This model requires that energy can be 'coupled' only when it is released/utilised in the nucleotide binding steps of the mechanism.

6. Adenosine β,γ-imidotriphosphate (AMP-PNP) is not a simple reversible inhibitor of the ATPase, since incubation requires preincubation and is not reversed when the compound is diluted out, or by addition of ATP. This compound inhibits the isolated and membrane-bound ATPase equally well. Its guanosine analogue does not act in this way.

7. In submitochondrial particles, ADP inhibited uncoupled hydrolysis of ATP much more effectively than coupled hydrolysis, the latter being measured both directly (from ATP hydrolysis in the absence of uncoupler) or indirectly, by monitoring ATP-driven reduction of NAD^+ by succinate.

8. The effects of ADP and AMP-PNP were interpreted as providing evidence for two of the intermediates in the proposed scheme for coupled triphosphate hydrolysis.

Introduction

The nucleotide binding properties of the coupling ATPase (F_1) of mitochondria, and other coupling membranes, are complex. One ATPase molecule contains probably three 'tight' binding sites for nucleotides, defined by their ability to retain ATP and/or ADP even when ambient nucleotide levels have been reduced to undetectable ($<10^{-11}$ M) (for a review see ref. 1).

The molecule also bears one or more weaker binding sites, with binding constants in the μM range (see, for example ref. 2), but the study of these is hampered by a slow exchange of nucleotide into the tight binding sites [1,3].

The hydrolytic activity of the isolated [3], and the uncoupled membrane-bound [4] ATPase involve only the weaker nucleotide binding site(s). There are, however, indications that the other nucleotide binding sites might be involved in coupled processes. For example, in submitochondrial particles, the nucleotide specificity of phosphorylation * [5], triphosphate-driven reversal of electron transfer [6], and NTP-$^{32}P_i$ exchange [7] appears to be higher than the specificity of uncoupled hydrolysis. In addition, the tightly bound nucleotides become exchangeable with added nucleotide only during coupled turnover of the ATPase [4].

In the work detailed below, the nucleotide specificity of the tight binding sites of the beef heart mitochondrial ATPase was investigated, and compared with the nucleotide specificity of various coupled processes in submitochondrial particles. A rapid, simple procedure for screening nucleotides for their ability to fit the 'tight' sites is described. The specificity of these sites, and of coupled processes, were found to be similar, and much higher than that of uncoupled triphosphate hydrolyses.

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* Strictly, the term 'specificity' applies to the enzyme molecule participating in the reaction. For brevity, we have referred to the 'nucleotide specificity of a reaction', rather than 'the nucleotide specificity of the coupling ATPase when it participates in that reaction'.

Studies on inhibition of ATPase activity by AMP-PNP, GMP-PNP and ADP were also carried out. ADP was found to inhibit uncoupled ATP hydrolysis much better than coupled hydrolysis. AMP-PNP, but not GMP-PNP was shown to trap the enzyme in an inactive enzyme-nucleotide complex. These results are consistent with the view that energy coupling involves a tight 'enzyme-nucleotide' complex, and that energy release or input is associated with nucleotide binding rather than hydrolysis.

Some of these results have been reported in preliminary form elsewhere [8,9].

Methods

Mitochondrial ATPase was prepared by the method of Knowles and Penefsky [10], and freed from ambient nucleotide as described previously [3]. This preparation contained about 2 mol ATP and 1 mol ADP/mol ATPase.

Submitochondrial particles were prepared according to Löw and Vallin [6] and freed from ambient nucleotides by repeated centrifugation [4]. It is essential that the particles used be free of nucleotides in solution, as traces of ADP can lead to observation of phosphorylation in particular, via myokinase, nucleoside diphosphate kinase, or directly if a glucose-hexokinase trap is used. The P/O ratio (P_i esterified/oxygen consumed) in these particles was typically 1.5 using NADH as substrate and 1.1 using succinate, when ADP was used as phosphate acceptor. Using NADH as substrate, ADP stimulated respiration rate 1.8—1.9 times.

ATP and ADP were obtained from Boehringer, ITP *, IDP *, GTP, GDP, ϵ-ATP, ϵ-ADP, 8-BrATP, 8-BrADP, 2′-dATP and 2′-dADP from Sigma. AMP-PNP and GMP-PNP were obtained from P-L Biochemicals. N^1,*O*-ATP, N^1,*O*-ADP, *iso*-GTP and *iso*-GDP were made essentially as described by Bârzu and coworkers [11,12], except that for conversion of the N^1,*O*-adenine nucleotides to the *iso*-guanine nucleotides, illumination by a medium pressure mercury immersion lamp was used. rro-ATP and rro-ADP were prepared as described by Boos et al. [13]. Formycin di- and triphosphates were kind gifts of Dr. D. Trentham, University of Bristol. The purity of all nucleotides was checked by thin-layer chromatography on polyethylenimine cellulose, using 0.85 M LiCl as eluant [14].

[^{3}H]ATP and [^{3}H]GTP were obtained from the Radiochemical Centre, Amersham, Bucks. After addition of carrier triphosphate, all traces of ADP were removed from the [^{3}H]ATP solution as described previously [3], and of GDP and other impurities from [^{3}H]GTP by passage down a Dowex 1 × 2 column (bicarbonate form) and elution with a 0.1—1.1 M gradient of NH_4HCO_3. The NH_4HCO_3 was removed by evaporation under vacuum.

N^1,*O*-[^{14}C]ATP and *iso*-[^{14}C]GTP were prepared as for the unlabelled nucleotides except that [^{14}C]ATP (Radiochemical Centre, Amersham) was included with carrier ATP in the starting material.

Phosphorylation was measured as described by Thayer and Hinkle [15]. 3.8 ml 250 mM sucrose, 20 mM HEPES, 40 mM glucose, 2 mM $MgCl_2$, brought

* Inosine nucleotides from Boehringer contained significant amounts of ADP and ATP.

to pH 7.5 with NaOH containing in addition 10 mM succinate (or 1 mM NADH as indicated), 10 mM potassium [^{32}P]phosphate (pH 7.5) (100 cpm/nmol) and 20 units (μmol/min) hexokinase (ammonia-free) was incubated at 30°C and 500 μM NDP (NTP) added. The reaction was started by adding submitochondrial particles (200 μg) (preincubated with 10 mM succinate if succinate was substrate). Oxygen uptake was monitored using a Yellow Springs oxygen electrode (YSI 5331) and the reaction was stopped by 5% trichloroacetic acid when about 80% of the oxygen had been consumed. NTP-$^{32}P_i$ exchange was measured in the same buffer containing 1 mM KCN, 10 mM potassium [^{32}P]-phosphate (pH 7.5) (200 cpm/nmol), 4 mM MgNTP and 1 mM NDP. The reaction was started by adding submitochondrial particles (200 μg) and stopped after 1 or 2 min with 5% trichloroacetic acid.

The measurement of $^{32}P_i$ incorporation into organic phosphate, and of radioactivity levels in general were as described previously [4]. Protein was determined by the method of Lowry et al. [16] for soluble protein or Cleland and Slater [17] for membrane preparations. Ammonia-free hexokinase, alcohol dehydrogenase and pyruvate kinase were obtained from Sigma biochemicals.

Results

pH dependence of exchange of bound nucleotides

It has been previously shown that, at pH values above 7, the exchange of tightly bound with added nucleotide is slow and incomplete, even under conditions when the enzyme is hydrolysing ATP. The kinetics of this exchange are inhomogeneous, approx. 0.6 mol nucleotide per mol ATPase exchange in an initial rapid phase ($t_{1/2} \leqslant 1$ min) while less than 20% of the remaining 2.4 mol/mol exchange in the next 18 h [3]. This may reflect differences in the three nucleotide binding sites present.

Fig. 1 shows that this exchange is pH dependent, increasing at low pH values to about 1.6 mol/mol ATPase at pH 5.5, measured over a 2.5 min period. Values of 1.5—1.8 mol exchange/mol ATPase are routinely obtained under these conditions if the free nucleotide is subsequently removed by repeated ammonium sulphate precipitation of the enzyme [3] and 2.2—2.5 mol exchanged/mol ATPase if free nucleotide is removed by Dowex treatment [18] (Table I). This may reflect an irreversible effect of high concentrations of SO_4^{2-} on the tight nucleotide binding sites [19]. In contrast, at pH 8.0, only 0.5—0.8 mol nucleotide exchange/mol enzyme in either case. It is not clear whether this increase in exchange observed is due to an increase in extent of the fast phase or in rate of the slow phase of exchange, since at pH 5.5 the enzyme is unstable over long periods of time and a time course cannot be measured.

Fig. 1 also includes, for comparison, the pH profile of the hydrolytic activity of the isolated ATPase [20]. The activity shows the opposite dependence on pH, i.e. it is low at low pH and a maximum around pH 8.0. This is consistent with the view [3] that the tightly bound nucleotides do not participate directly in hydrolysis by the isolated enzyme. Brief exposure (less than 5 min) of the enzyme to pH 5.5 does not lead to irreversible loss of activity (not shown, see ref. 10).

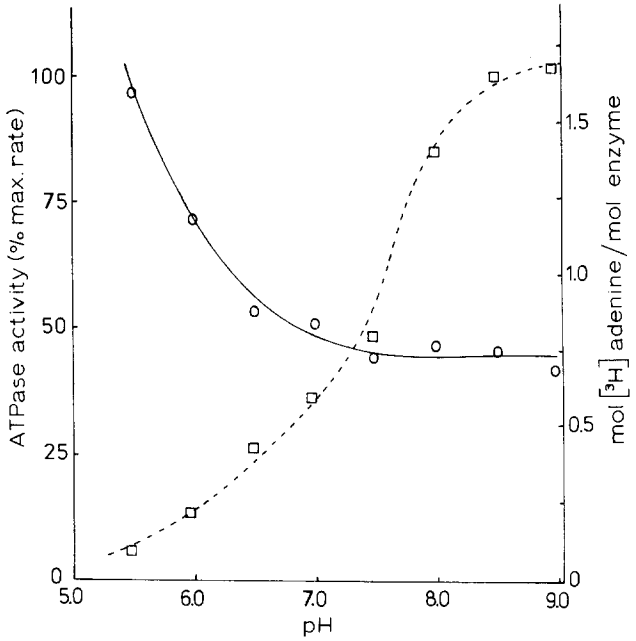

Fig. 1. pH dependence of exchange of tightly bound nucleotides of the isolated ATPase. Washed mitochondrial ATPase (1 mg) was incubated in 0.5 ml of a buffer containing 250 mM sucrose, 2 mM EDTA, 500 μM [^{3}H]ATP (2000 cpm/nmol) and 20 mM buffer brought to the required pH with Tris base (pH 5.5, 6.0, MES; pH 6.5—7.5, TES; pH 8.0, 8.5, acetate; and pH 9, glycine). After 2.5 min, the pH was brought to 8.0 with Tris base (2 M) or acetic acid (1 M) and free nucleotides removed from the solution by repeated precipitation with ammonium sulphate [3]. Protein and radioactivity of the final solution were measured as in Methods. Similar results were obtained if (a) all buffers contained 20 mM Tris and varying amounts of acetate [8] or (b) free nucleotides were removed by Dowex 2 (cf. Table I). The pH profile of hydrolytic activity is taken from the data of Pullman et al. [20]. □- - - - -□, hydrolytic activity; ○———○, nucleotide exchange.

TABLE I

INCORPORATION OF NUCLEOTIDES INTO THE TIGHT BINDING SITES OF THE ISOLATED AND MEMBRANE-BOUND ATPase

Exchange of nucleotides into the tight binding sites of isolated ATPase was measured by incubation at pH 5.5 for 2.5 min, as described in Fig. 1, except that other labelled nucleotides (500 μM) replaced [^{3}H]ATP as indicated. Free nucleotides were removed by passing the enzyme through a Dowex 2 × 8 column as in ref. 18. Exchange of nucleotides into the tight binding sites of the membrane-bound ATPase was measured as previously [4]. Free nucleotides were removed by centrifugation. Where indicated oligomycin or unlabelled ATP was added prior to the labelled nucleotide. Doubling the amount of radioactive nucleotide had little effect on the amount of label bound (in the absence of unlabelled nucleotide). The error indicated represents a range of four readings on three different enzyme preparations. Results are expressed in mol radioactive nucleotide/mol ATPase.

Labelled nucleotide added	Isolated ATPase			Submitochondrial particles		
	Further additions					
	None	ITP (500 μM)	2′-dATP (500 μM)	None	Oligomycin (25 μg/mg)	ATP (50 μM)
[^{3}H]ATP	2.28 ± 0.2	2.50	1.48	0.94 ± 0.06	0.36	—
iso-[^{14}C]GTP	2.04	—	—	1.04	—	—
[^{3}H]GTP	0.44	—	—	0.36	—	—
N^1,*O*-[^{14}C]ATP	0.15	—	—	0.29	—	0.38

Exchange of bound ATP and ADP with other nucleotides

It might seem that the most obvious way to establish the specificity of the tight binding sites of the ATPase would be to remove the tightly bound nucleotide and to measure which nucleotides could then bind tightly to the enzyme. However, because of the very high affinity of these sites for nucleotide, emptying them is not a simple matter. Of the two published procedures for removing tightly bound ATP and ADP from the isolated beef heart ATPase, one, due to Leimgruber and Senior [19] seems to modify irreversibly the enzyme while the other, due to Garrett and Penefsky [21], is, in our hands, not readily reproducible.

We have concentrated rather on approaches to increase the (normally very slow) exchange of the tightly bound ATP and ADP with added nucleotide. It has been previously shown that at low temperatures, the exchange is increased and ADP (and added ATP) can readily displace ATP from the tight binding sites while the GTP can do so only poorly [22].

Table I shows the level of incorporation of added, labelled nucleotides into the tight binding sites of the isolated ATPase during a 2.5 min incubation at pH 5.5. Considerable incorporation of ATP or *iso*-GTP occurs, while GTP (in agreement with ref. 22) and N^1,*O*-ATP are incorporated to a very limited extent only. The ability of unlabelled nucleotides to compete with [^{3}H]ATP for the tight sites is also shown. Addition of 2′-dATP considerably lowers the binding of [^{3}H]ATP, while ITP has little effect. It is concluded, therefore, that 2′-dATP can also bind to the tight binding sites of the ATPase while ITP cannot.

Using a 2.5 min incubation at pH 5.5 to increase exchange, we have also shown that formycin triphosphate, a fluorescent nucleotide, can exchange into the tight binding sites, yielding a fluorescent enzyme while ϵ-ATP, which is also fluorescent, cannot. Since the quantum yield of bound formycin is not known, however, the incorporation cannot be quantitated. The fluorescence of the bound formycin is enhanced by nucleotide binding at the hydrolytic site of the ATPase (unpublished results).

Submitochondrial particles may be labelled simply by incubation with the labelled NTP. Energisation of the membrane (via hydrolysis) leads to increased exchange [4]. A similar specificity is observed to that on the isolated enzyme, (Table I) viz. ATP and *iso*-GTP become significantly incorporated into the tight binding sites while GTP and N^1,*O*-ATP are incorporated to a very limited extent (similar to that seen with ATP if hydrolysis is inhibited by oligomycin). Even if the membrane is energised by ATP, incorporation of N^1,*O*-[^{14}C]ATP remains small, confirming that lack of incorporation does not result from the inability of this compound to energise the membrane, but from its low affinity for the tight binding sites.

Inhibition of ATP hydrolysis by preincubation with nucleoside diphosphates

An unexpected property of the isolated mitochondrial ATPase is shown in Fig. 2. When the isolated enzyme was incubated with ADP, and a small aliquot of the incubation mixture taken and assayed for ATPase activity in an ATP-regenerating system (where all free ADP is rapidly removed), the ATPase was found to be stably inhibited (at least over a 2 min period). This inhibition was maximally 50—60% (varying slightly between different enzyme preparations,

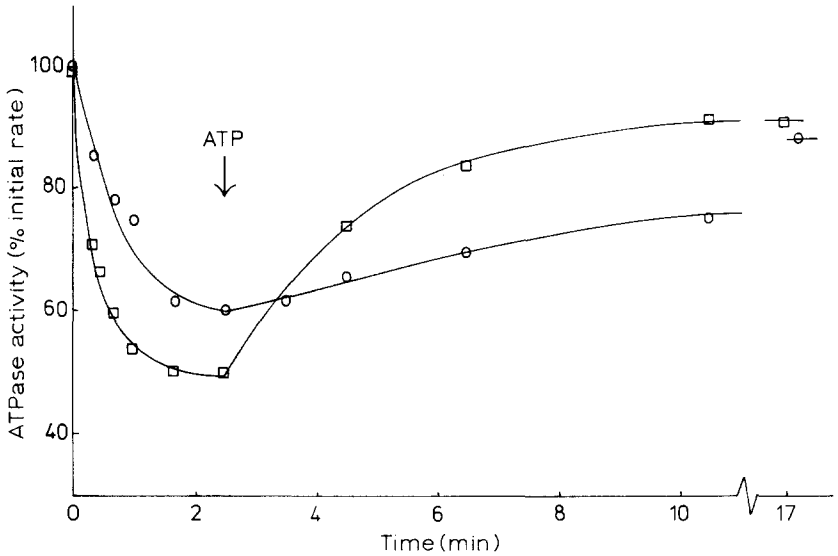

Fig. 2. Effect of preincubation with ADP on soluble ATPase activity. Aliquots containing about 1 unit (μmol/min) soluble mitochondrial ATPase were incubated in 0.2 ml of a buffer containing 250 mM sucrose, 2 mM EDTA and either 20 mM TES brought to pH 6.5 with Tris, or 20 mM Tris brought to pH 8.0 with acetic acid. ADP (200 μM) was added at zero time and ATP (2 mM) at 2.5 min as indicated. 10-μl aliquots were removed at various times and assayed for ATPase activity in an ATP-regenerating system as described in ref. 22. □——□, pH 6.5; ○——○, pH 8.0.

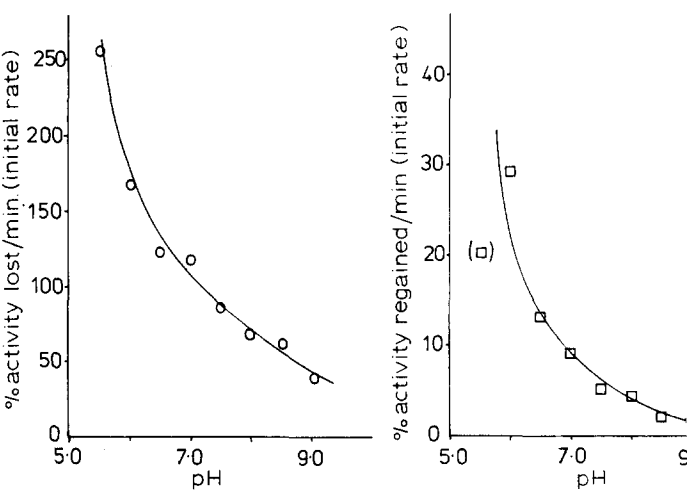

Fig. 3. pH dependence of inactivation and reactivation rates of the ATPase during preincubation with adenine nucleotides. Inactivation and reactivation were followed as in Fig. 2 in the range of buffers described in Fig. 1. Rates were calculated from the tangent at the origin in the curves (see Fig. 2) obtained, the origin being the time of ADP addition (t = 0 min) for inactivation and of ATP addition (t = 2.5 min) for reactivation. ○——○, inactivation; □——□, reactivation.

see below) and was reversed by the subsequent addition of ATP to the preincubation medium (Fig. 2). ITP hydrolysis was similarly inhibited by preincubation with ADP (not shown).

Both the inactivation and reactivation processes were relatively slow, and the rate of each was higher at lower pH (Fig. 2). The rates of inactivation and reactivation as a function of pH are shown in Fig. 3. Both processes show a similar pH dependence, although the reactivation rate was some five times slower than the inactivation rate. (The point at pH 5.5 of the reactivation process is abnormally low because of irreversible denaturation of the enzyme over the long incubation time used in the reactivation experiments.)

ADP-induced inactivation occurred only on the isolated ATPase, and not on the membrane-bound ATPase whether in untreated submitochondrial particles or in particles stripped of their intrinsic ATPase and reconstituted with the same preparation of isolated ATPase (Fig. 4). The inactivation is thus an artifact of isolation of the ATPase and unlikely to be involved in regulation in vivo.

The dependence of the inhibition on ADP concentration is hyperbolic and a $c_{1/2}$ value of 25 μM could be estimated (not shown). This may represent the dissociation constant of ADP from an unknown 'regulatory' site on the enzyme but, as discussed below, probably represents the competition of added ADP with bound ATP. In the latter case, the true dissociation constant will be considerably lower than 25 μM.

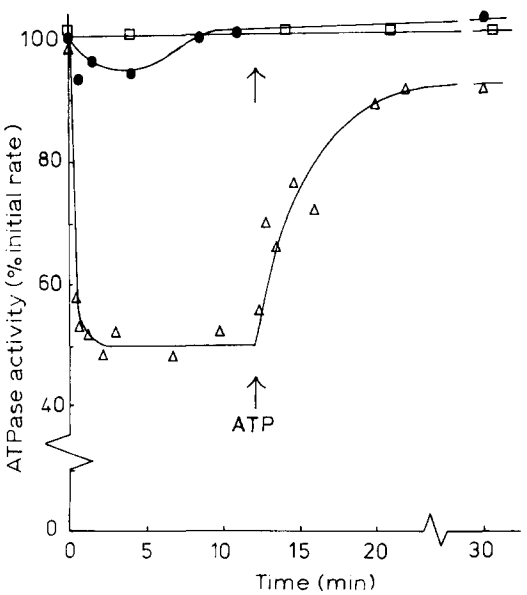

Fig. 4. Effect of preincubation with ADP on the soluble and particulate ATPase activities. Incubation was carried out as in Fig. 2 except that the buffer pH was 6.7 and 800 μM ADP was added at t = 0, and 2 mM ATP at t = 12 min. Where indicated, submitochondrial particles depleted of the ATPase inhibitor by filtration through Sephadex G-50 [47] or submitochondrial particles stripped of endogenous ATPase by treatment with 2 M urea and recombined with the soluble ATPase [48] replaced the soluble ATPase. △———△, soluble ATPase; □———□, submitochondrial particles; ●———●, stripped and reconstituted submitochondrial particles.

TABLE II

INHIBITION OF THE MITOCHONDRIAL ATPase BY PREINCUBATION WITH VARIOUS NUCLEOSIDE DIPHOSPHATES

The isolated ATPase was preincubated with NDP as indicated and the plateau level of inhibition measured, as in Fig. 3, at different nucleotide concentrations. $c_{1/2}$ represents the concentration required to produce half-maximal inhibition. The level of inhibition is expressed relative to the maximal inhibition caused by ADP; thus a relative level of 100% represents 55% inhibition of the ATPase activity. The error indicated represents the range of five duplicate values.

Nucleotide	Maximum inhibition relative to ADP	$c_{1/2}$
ADP	100 ± 3	20 μM
iso-GDP	110	3 μM
2'dADP	93	25 μM
Formycin diphosphate	95	45 μM
IDP	18	— (>1 mM)
GDP	18	—
ε-ADP	28	—
N^1,O-ADP	14	—
rro-ADP	2	—
8BrADP	20	—
No addition	4	—

Table II shows the amount of inhibition observed after preincubation of the isolated mitochondrial ATPase with various nucleoside diphosphates, relative to that observed with ADP. The $c_{1/2}$ values for inhibition are also tabulated. ATP reversed the inhibition in all cases (not shown).

The nucleoside diphosphates fall into two groups. ADP, 2'-dADP, formycin diphosphate and *iso*-GDP cause inhibition while the second group, containing the other nucleotides tested, do not. This division is similar to that between nucleotides which fit into the tight binding sites on the ATPase and those which do not, as defined above.

In the course of this work, it was noted that some ATPase preparations showed a lower sensitivity to preincubation with ADP. This proved to be enzyme of lowered specific activity, which might be induced by pretreatment or ageing of the enzyme preparation. For example, gel filtration in the absence of ATP reduced the specific activity of the ATPase from 85 to 55 μmol/min per mg (cf. refs. 3 and 18) and the inhibition by ADP was reduced from 51 to 33%. This indicates that such treatment modifies the enzyme in some way (as distinct from inactivating a certain proportion of it). The nature of this modification is uncertain.

Inhibition of ATP hydrolysis by incubation with nucleoside β,γ-imidotriphosphates

In agreement with Philo and Selwyn [23], we find that AMP-PNP induces a slow inhibition of the enzyme during preincubation with (but assay in the absence of) this nucleotide. This effect is specific for the adenine nucleotide, GMP-PNP having no significant effect on the enzyme activity (Table III).

Unlike the inactivation by ADP, inhibition induced by AMP-PNP is not

TABLE III

INHIBITION OF THE MITOCHONDRIAL ATPase BY PREINCUBATION WITH NUCLEOSIDE β,γ-IMIDOTRIPHOSPHATES

The inhibition of the isolated or membrane-bound ATPase was followed as in Fig. 4, in the presence of the nucleotide indicated. The plateau levels before, and after the addition of ATP are recorded below. AMP-PNP inhibited the enzyme rather more slowly ($t_{1/2}$ = 1.5 min) than ADP ($t_{1/2}$ ≈ 0.5 min). Errors represent the range of 4 or 5 duplicate readings.

Nucleotide added	Final activity (percent initial activity)	
	Isolated ATPase	Membrane-bound ATPase
None	100 ± 3	100 ± 8
ADP (500 μM)	43	102
ADP (500 μM) + ATP (2.5 mM)	108	97
None	100	100
AMP-PNP (500 μM)	12.5	14
AMP-PNP (500 μM) + ATP (2.5 mM)	13	14
None	100	100
GMP-PNP (500 μM)	89	102
GMP-PNP (500 μM) + ATP (2.5 mM)	84	n.d.

n.d., not determined.

TABLE IV

NUCLEOTIDE SPECIFICITY OF REVERSED ELECTRON FLOW

NTP-driven reduction of NAD^+ by succinate was measured essentially as described by Ernster and Lee [25]. Submitochondrial particles (0.5 mg) were incubated at 30°C in 1 ml of buffer I (200 mM sucrose/100 mM KCl/50 mM Tris/2 mM $MgCl_2$, to pH 8.0 with HCl) containing in addition 2 mM KCN, 5 mM succinate and 1 mM NAD^+ for 5 min, and the reaction started by addition of NTP. Reduction of NAD^+ was followed at 340 nm. NTP hydrolysis was measured in buffer I at 30°C, in the presence of 1 μM FCCP, monitoring P_i release continuously by the method of Hurst [28]. In the absence of FCCP, nucleotide triphosphatase activity was 1.1—1.2 times lower, but the rates relative to ATP were unaffected. The errors given indicate the range of four readings on a given preparation of particles.

Nucleotide	Concentration	Hydrolysis rate (nmol/min per mg protein)	Reversal rate (nmol/min per mg protein)
ATP	100 μM	540	35
	250 μM	940	70 ± 5
	1 mM	1470 ± 75	140 ± 9
2'-dATP	240 μM	590	26
	1.2 mM	1040	75
iso-GTP	500 μM	460	34
	1 mM	610	84
ITP	2.5 mM	590	4
	5 mM	630 ± 20	7
GTP	1.2 mM	430	<2.5
	2.5 mM	500	5
ε-ATP	2 mM	240	<2.5
N^1,*O*-ATP	2 mM	130	<2.5
8BrATP	2 mM	100	<2.5
rro-ATP	2 mM	70	<2.5

reversed by subsequent addition of ATP, although addition of ATP before AMP-PNP slows down inhibition by the latter (not shown). Further, AMP-PNP inhibits both the membrane bound and isolated ATPase (Table IV), and the half time for inhibition of each is about the same (about 1.5 min) (not shown). This suggests that the mechanism of inhibition induced by AMP-PNP is different from that of ADP-induced 'irreversible' inhibition. This is also seen from the extent of the two processes, ADP-induced inhibition reaching a maximum at about 50% (Table II) while inhibition with AMP-PNP may proceed almost to completion (Table III). Inhibition by AMP-PNP can in fact be explained as due to an interaction at the hydrolytic site of the enzyme [22]. while ADP-induced inhibition requires a more complex explanation (see below).

NTP-driven reduction of NAD^+ by succinate

The rates of NTP-driven reversal of electron transfer, at various concentrations of NTP, are compared with the rates of triphosphate hydrolysis in Table IV. Several features are noteworthy.

First, in agreement with other workers [6,24—26] we found the reversal of electron transfer to be 'inefficient' in the sense that far more than 1 mol NTP was hydrolysed per mol NAD^+ reduced. In the case of ATP, for example, about 10 mol were hydrolysed/mol NAD^+ reduced. This is not due to the energy requirement of the reaction, since initially virtually no ADP, fumarate or NADH was present making the energy requirement very small. (Also addition of ADP increases the efficiency, see below.) It seems likely that much hydrolysis was uncoupled under these conditions.

TABLE V

ABILITY OF NUCLEOTIDES TO PARTICIPATE IN COUPLED PROCESSES

Phosphorylation, in the presence of 500 μM NDP using 10 mM succinate as electron donor, and NTP $\rightleftharpoons$ P_i exchange, in the presence of 4 mM NTP and 1 mM NDP, were measured as described in Methods, and reversal of electron transfer and NTP hydrolysis as in Table IV. The results are expressed as percentage of the rate obtained using the adenine nucleotide, the maximum rate (V) being used in the case of reversal and hydrolysis rates. Phosphorylation rates were of the order of 200 nmol · min^{-1} · mg $protein^{-1}$ using ADP as substrate and succinate as electron donor, and were unaltered in all cases if NTP replaced NDP as substrate. NTP-P_i exchange was not linear in time, but the ratios given here, taken over a constant time interval of 1 min, were independent of whether the rate of exchange from $t = 0$ to $t = 1$ min or $t = 1$ to $t = 2$ min was used. Over the latter time period, the ATP $\rightleftharpoons$ P_i exchange rate was 140 nmol · min^{-1} · mg $protein^{-1}$.

Nucleoside moiety	Reversal	ATP-P_i exchange	Phosphorylation	Hydrolysis
Adenosine	100 ± 6%	100 ± 8%	100 ± 4%	100 ± 5%
2'-Deoxyadenosine	44	14	50	73
iso-guanosine	48	48	45	41
Inosine	5	4	16 (5) *	34
Guanosine	4	2	7	34
ϵ-Adenosine	<2.5	<2	11	16
N^1,*O*-Adenosine	<2.5	<2	5	10
rro-Adenosine	<2.5	<2	<2	5
8Br-Adenosine	<2.5	n.d.	<2	6

* 1 mM NADH replaced succinate as substrate.
n.d., not determined.

Secondly, the rate of reversal was not related to the hydrolysis rate. At comparable rates of hydrolysis of ATP, dATP, *iso*-GTP, GTP and ITP, only the first three were able to drive reversal. Since the free energy for the hydrolysis of each is about the same (and in any case higher than the free energy needed to 'drive' reversal under these conditions), the difference must lie in the ability of the coupled membrane to use the energy of hydrolysis in these compounds.

Some reversal of electron transfer (about 30% maximal rate) occurred in the presence of ITP if ADP was included in the medium (not shown). This process presumably involved transphosphorylation of ADP by ITP (not involving the coupling ATPase), forming ATP which then drives the reversal. Similar processes may have occurred during the long preincubation with ITP found to be necessary to induce ITP-driven reversal (in unwashed MgATP particles) by Löw et al. [24].

Large amounts of ITP did not stimulate reversal driven by small amounts of ATP; in fact a slight inhibition was observed. This rules out the possibility that ATP is necessary for a control process which switches on reversed electron flow, while any nucleotide can drive the reversal.

Inhibition of coupled ATP hydrolysis by ADP

In agreement with Huang and Mitchell [26], we find that ADP inhibits ATP hydrolysis by submitochondrial particles very strongly, while it has much less effect on the rate of ATP-driven reversal of electron transfer. For example, at 1.4 mM ATP, addition of 500 μM ADP inhibits ATP hydrolysis by 75% and reversal of electron transfer by only 25%. The effect is even more marked at low ATP concentrations, at 250 μM ATP, 500 μM ADP decreases ATP hydrolysis by over 90% without any decrease in reversal rate. As a result, therefore, the reversal process becomes more 'efficient', approaching an efficiency of 1 ATP hydrolysed per NAD^+ reduced.

These results were interpreted by Huang and Mitchell [26] in terms of a complex kinetic model in which ATP and ADP can bind simultaneously to the enzyme. In this model, they assumed that ATP hydrolysis by coupled and uncoupled ATPase molecules showed identical properties. We have shown previously that the ATPase takes up different conformations during uncoupled and coupled hydrolysis [4], and bearing this in mind, would propose a rather simpler model.

The assumptions involved in our model are: (1) That a population of submitochondrial particles bears coupled and uncoupled ATPase molecules (whether these are carried on different particles, or whether one particle carries both types of ATPase is not considered here). (2) That ADP acts as a simple, competitive inhibitor of the ATPase [27] i.e. it competes with ATP for the active site. (3) That the uncoupled and coupled ATPases have different K_i values for ADP.

From the above data on the reversal of electron transfer, we would suggest that ADP inhibits uncoupled ATP hydrolysis much more than coupled hydrolysis, i.e. the K_i^{ADP} for uncoupled hydrolysis is much lower than for coupled hydrolysis. The experiment of Fig. 5 supports this conclusion.

ATP hydrolysis was measured at varying ADP concentrations in the presence and absence of uncoupler (FCCP). In coupled particles, about 20—25% of the

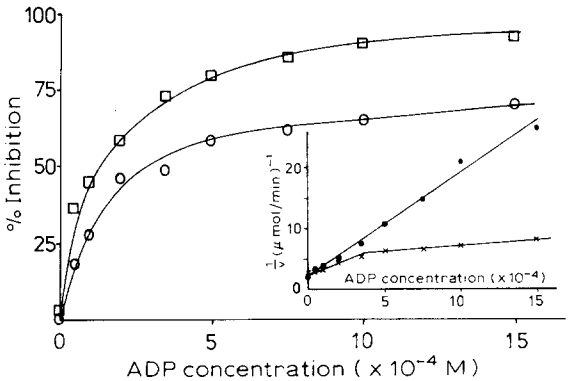

Fig. 5. Effect of ADP on 'coupled' and uncoupled ATP hydrolysis. ATP hydrolysis in the presence of 2 mM ATP and varying amounts of ADP was measured as in Table IV. The large diagram plots percent inhibition (= {1 − [(rate + ADP)/(rate − ADP)]} × 100) against ADP concentration. □———□, hydrolysis in the presence of 1 μM FCCP; ○———○, hydrolysis in the absence of FCCP. The insert (a) plots reciprocal velocity against ADP concentration, for the same data. ●———●, hydrolysis in the presence of 1 μM FCCP; X———X, hydrolysis of FCCP.

hydrolytic activity was resistant to inhibition by ADP, while in uncoupled particles the ATPase was completely inhibited. The existence of two populations of ATPase molecules in coupled particles, one highly susceptible to ADP and one much less so, is clearly seen from a Dixon plot of this data (Fig. 5a). Presumably the two populations correspond to molecules carrying out coupled and uncoupled ATP hydrolysis.

In contrast, hydrolysis of ITP was strongly inhibited by ADP even in the absence of uncoupler. 250 μM ADP inhibited hydrolysis of 5 mM ITP by 76 ± 2% (average of four readings on two separate particle preparations). This can be compared to the data in Fig. 5, where the maximum inhibition attained, at 1.5 mM ADP, was only 70%. This is consistent with the view that ITP hydrolysis is uncoupled (Tables IV and V).

At higher ADP concentrations, a decrease in inhibition of ITP hydrolysis was observed, presumably due to a significant rate of ADP phosphorylation via NDP kinase. This made further study of ITP hydrolysis inconclusive.

Specificity of other coupled processes

NTP-driven reduction of NAD^+ by succinate was chosen for the examination of nucleotide specificity for two reasons: (i) It has a relatively high K_m for NTP and is little affected by small traces of ATP which may be present as a contaminant, and (ii) Reversal can be compared at identical rates of hydrolysis of the different nucleotides. Thus, the problem of a non-linear dependence of the reversal rate on the rate of hydrolysis can be avoided. NTP-$^{32}P_i$ exchange meets the first criterion quite well, although not the second. In the experiments of Table V, NTP and NDP concentrations were chosen arbitrarily (although NTP was maintained above the K_m for hydrolysis in all cases). Phosphorylation, on the other hand, meets neither criterion. In particular, phosphorylation has a very low K_m for ADP, and it is possible that at the concentrations of NDP used

TABLE VI

NUCLEOTIDE SPECIFICITY OF THE ENERGY-LINKED TRANSHYDROGENASE REACTION

Transhydrogenase was measured at 30°C in 250 mM sucrose, 50 mM Tris, 2 mM $MgCl_2$ brought to pH 8.0 with HCl, to which had been added 80 mM ethanol, 0.6 mM KCN, 0.5 mg/ml alcohol dehydrogenase (ammonia free), 6 mM hydrazine sulphate and 2 μM rotenone [30]. The reaction was started by addition of NTP as indicated. NTP hydrolysis was measured, by the method of Hurst [28] in the same buffer, containing in addition 1 μM FCCP. The values are expressed as percentage of maximum rate obtained using ATP, after subtracting the non-energy-linked transhydrogenase (about 15 nmol/min per mg). 100% represents 189 nmol/min per mg for the transhydrogenase and 1.36 μmol/min per mg for the ATPase. The error given represents the range of five values, and the other figures, the average of two duplicates.

Nucleotide	Energy-linked transhydrogenase	Hydrolysis
ATP (2 mM)	100 ± 11	100 ± 5
ATP (270 μM)	66	47
iso-GTP (750 μM)	65	27
2′-dATP (2 mM)	66	73
2′-dATP (400 μM)	52	55
ITP (5 mM)	33	43
GTP (2 mM)	3 (21 *)	37
N^1,*O*-ATP (5 mM)	20	11
8BrATP (2 mM)	4	7

* Value after 4 min incubation. Other values are initial rates.

here (500 μM), contamination by ADP may lead to significant levels of phosphorylation.

Nonetheless, Table V indicates that both NTP-$^{32}P_i$ exchange and phosphorylation show a marked specificity for adenosine, deoxyadenosine and *iso*-guanosine when compared to uncoupled NTP hydrolysis. It is also notable that, if NADH replaced succinate as an electron donor in phosphorylation, the rate of phosphorylation and P/O ratio increased when ADP was the phosphate acceptor but decreased when IDP was the acceptor (cf. also ref. 29). This confirms the suspicion that the low rate of 'IDP phosphorylation' may be due to a secondary reaction, not involving the coupling ATPase directly. It was concluded that NTP-driven reversal of electron transfer, NTP-$^{32}P_i$ exchange and phosphorylation showed a common high specificity for nucleotides.

Both energy-dependent transhydrogenase and ANS fluorescence enhancement showed a somewhat lower specificity for nucleotides. As Table VI shows, the transhydrogenase is certainly driven by ITP, as has been previously demonstrated by Ernster and co-workers [31]. Comparing similar rates of hydrolysis, however, ATP, *iso*-GTP and probably 2′-dATP appeared to be more efficient at driving the transhydrogenase than ITP or GTP.

The enhancement of ANS fluorescence on energisation of coupled membranes is often taken as a measure of the energised state, and its rate has been suggested to reflect the potential across the membrane [32]. Investigation of these phenomena with the range of nucleotides used in the above experiments showed no correlation between the extent of the ANS response, its rate, the rate of nucleotide hydrolysis or ability of the nucleotide to drive reversal of electron transfer. For example, at a rate of hydrolysis about 30% V^{ATP}, ATP gave an enhancement of 1.57 with $t_{1/2}$ = 6.5 s, ITP an enhancement of 1.48 with $t_{1/2}$ = 7 s and GTP an enhancement of 1.27 with $t_{1/2}$ = 11 s. Even N^1,*O*-

ATP, at a hydrolysis rate of only 7% V^{ATP}, gave a significant enhancement of fluorescence (1.20), with $t_{1/2}$ = 8.5 s. The physical meaning of these parameters thus remains uncertain.

Discussion

Specificity of the tight binding sites

The tightly bound nucleotides of the coupling ATPase are operationally defined as those nucleotides remaining bound to the enzyme when all detectable free nucleotides are removed from the solution. At low pH, these bound nucleotides (here about 2 mol ATP and 1 ADP/mol enzyme) exchange rapidly with added ATP (Fig. 1). Using radioactivity labelled, or fluorescent ATP analogues under these conditions, it was shown that only ATP, 2′-dATP, formycin triphosphate and *iso*-GTP were able to bind tightly to the isolated ATPase (Table I).

These results were confirmed and extended using the finding that ADP preincubation inhibits the isolated ATPase even when ATP hydrolysis is assayed in the absence of free ADP (Fig. 2). Since this inhibition persists when all free ADP is removed, the inhibitory ADP binding site accords with our operational definition of a tight binding site on the enzyme. It is likely that this site is one of the three known tight binding sites, since further tight sites seem improbable. This view is supported by the pH dependence of the rates of inhibition and reactivation by ADP (Fig. 3), which, like the exchange of the bound nucleotides with added ATP (Fig. 1), are low at high pH (8—9) and high at low pH (5.5—6). Further, on the membrane-bound ATPase, where the tightly bound nucleotides are more stably bound, [4,19] the effect is altogether absent.

We would propose, therefore, that ADP displaces ATP from one of the tight binding sites during preincubation. Replacement of tightly bound ATP by ADP, as shown previously [22], can lead to a loss of hydrolytic activity of the ATPase. ADP-induced inactivation therefore provides a rapid, simple procedure for screening other nucleotides for their ability to occupy the tight binding sites (at least in this system). Again we find a strong preference of the tight sites for adenosine, 2′d-adenosine, *iso*-guanosine and formycin nucleotides (Table II).

From these results with four very closely related nucleotides (see Fig. 6), the specificity of the tight binding sites appears similar on the membrane-bound and isolated ATPase (Table I). A similar high specificity on the membrane-bound ATPase of chloroplasts has recently been demonstrated [33].

The specificity of the tight binding sites for nucleotides is clearly much greater than that of the hydrolytic site of the ATPase. All these triphosphates are hydrolysed well by the isolated and membrane-bound ATPase and yet most show only a low affinity for the tight binding sites. V for ATP and ITP on the isolated enzyme, for example, are about equal, while the ITP apparently binds to the tight binding sites very poorly. This is a further confirmation that the hydrolytic and tight binding sites are separate on the isolated enzyme.

The structural features which promote nucleotide binding to the tight sites are clearly subtle and not completely delineated by this work. However, even

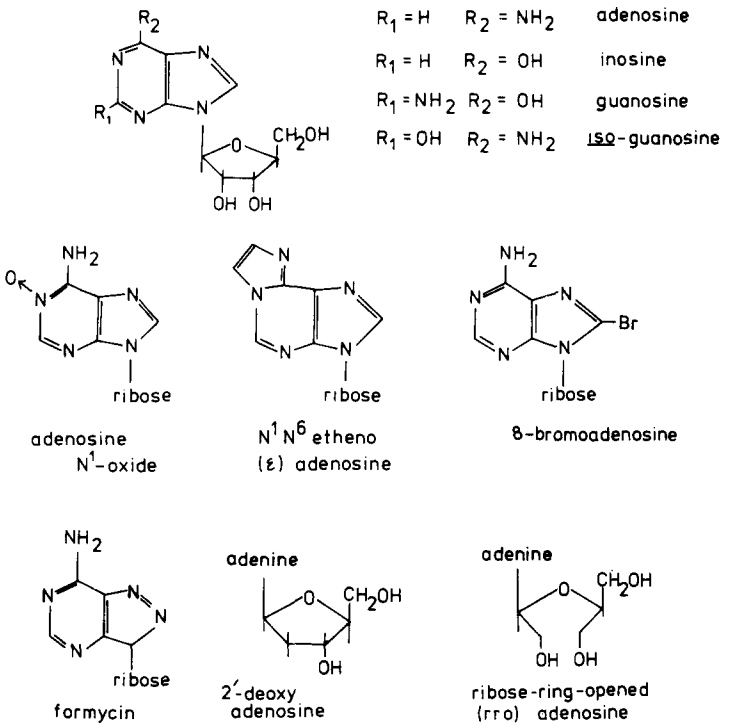

Fig. 6. Structures of nucleotides used in this study.

a small change at the N^1 or C^6 position of the adenine moiety is strongly deleterious to tight binding (see Fig. 6) while small changes at C^2, C^8 and on the ribose can be tolerated. (Larger changes here, such as in 8-BrATP and rro-ATP lead to poor binding to both hydrolytic and tight sites.)

Specificity of coupled processes

An attempt was made to correlate specificity of coupled processes with the specificity of the tight binding sites. That they show a common, high specificity for nucleotides has been well established in the case of NTP-driven reversal of electron transfer (Table IV), and subject to the problems outlined above, in the case of phosphorylation and NTP-$^{32}P_i$ exchange (Table V). This finding is a necessary, but not a sufficient, conditions for these coupled processes to involve the tight binding sites of the ATPase. It is insufficient (and specificity studies in general somewhat difficult to interpret) since specificity may be introduced through kinetic as well as thermodynamic (binding) effects. For example, a given nucleotide substitution might markedly decrease NDP off and on rates, say, while leaving other rate constants unchanged. In this case, NTP-$^{32}P_i$ exchange (which need not involve ADP release from the enzyme) would be unaffected, while both phosphorylation and hydrolysis rates would be greatly reduced.

Thus, in the minimal hypothesis for coupled ATPase activity, outlined below, the enzyme is treated as if only one nucleotide binding site were involved in all processes. An extension to involve more sites is left for the reader.

It is instructive to compare these results with those obtained in chloroplasts, where the tight nucleotide binding sites and NTP-$^{32}P_i$ exchange reactions show a high nucleotide specificity [33—35], while phosphorylation appears less specific [33,36].

The wider specificity of the other two reactions tested, the transhydrogenase reaction and the enhancement of ANS fluorescence, represent a rather different problem. Both these, and NTP-driven reversal, can be compared at a given rate of triphosphate hydrolysis, and from Tables IV and VI we see that, unlike reversal of electron transfer, transhydrogenation and the ANS response can be driven quite effectively by ITP and N^1,*O*-ATP. If all three reactions are driven by the same energy 'pool', this finding is difficult to explain. It has been suggested [31] that the transhydrogenase may indeed utilise a different energy 'pool'. A fuller explanation of these results thus requires a more adequate knowledge of the physical processes involved in these two 'reactions'.

A model for coupled ATPase activity

In developing the simplest possible model for coupled ATP hydrolysis, we hope to account for the following facts on the basis of an ATPase with only one type of ATP binding site per molecule (althought there may, of course, be more than one of these).

(1) AMP-PNP binds to the ATPase in a virtually 'irreversible' manner, trapping the enzyme in a form incapable of ATP hydrolysis. GMP-PNP does not form this tight complex (Table III).

(2) The hydrolysis of only some hydrolysable nucleotides can drive coupled processes (Table IV).

(3) ADP inhibits uncoupled ATP hydrolysis much better than coupled hydrolysis (Fig. 5).

(4) Neither AMP-PNP nor ATP inhibit phosphorylation of ADP [23,37].

In the past some of these findings have been explained on the basis of regulatory nucleotide binding sites on the ATPase [7,26], but this is, in fact, not a necessary postulate. It is also shown above (Table V) that specificity studies do not necessitate postulating separate hydrolytic and phosphorylation sites on the enzyme.

The simplest ATPase mechanism possible can be written as follows:

$$E + ATP \rightleftharpoons E \cdot ATP \rightarrow E \cdot ADP \cdot P_i \rightleftharpoons E + ADP + P_i \qquad (i)$$

(the single arrow representing a step with a large drop in free energy)

Since at a constant rate of free energy production by hydrolysis, only some nucleotides can drive coupled processes, it seems likely that the energy release step is one at which nucleotide recognition is important, i.e. a binding step rather than a hydrolytic step. We can then expand this mechanism to give

$$E + ATP \overset{1}{\rightleftharpoons} E \cdot ATP \overset{2}{\rightarrow} E^* \cdot ATP \overset{3}{\rightleftharpoons} E \cdot ADP \cdot P_i \overset{4}{\rightleftharpoons} E + ADP + P_i \qquad (ii)$$

This mechanism involves a tight enzyme-nucleotide complex $E^* \cdot ATP$. When AMP-PNP binds to the enzyme active site, it will form $E^* \cdot$ AMP-PNP, for which further hydrolysis is blocked and, being in an energy trough, this complex is stable. Nucleotides (e.g. GTP) which cannot form tight complexes will hydrolyse by the route

$$E + NTP \overset{1}{\rightleftharpoons} E \cdot NTP \overset{2}{\rightleftharpoons} E^{*} \cdot NTP \overset{3}{\rightarrow} E \cdot NDP \cdot P_i \overset{4}{\rightleftharpoons} E + NDP + P_i \qquad \text{(iia)}$$

using the same site and the same enzyme intermediates, but with energy release at step 3 where it cannot be utilised by the membrane. The $E^{*} \cdot$ GMP-PNP complex is thus less stable, and GMP-PNP does not act as an irreversible inhibitor.

This work thus provides further evidence for the model of energy transduction where ATP binding (release) is the energy yielding (requiring) step, originally proposed by Slater [38] and Boyer et al. [39], and supported experimentally by the energy independence of the $P_i \rightharpoonup H_2{}^{18}O$ exchange [40]. The lack of inhibition of phosphorylation by ATP or AMP-PNP, and of coupled hydrolysis by ADP cannot be explained on this model (ii). By applying the kinetic analysis of Cleland [41], however, these results can still be explained on a single site enzyme if ADP and ATP (AMP-PNP) do not combine with identical, or rapidly equilibrating forms of the enzyme. This gives the slightly altered equation:

$$E + ATP \overset{1}{\rightleftharpoons} E \cdot ATP \overset{2}{\rightarrow} E^{*} \cdot ATP \overset{3}{\rightleftharpoons} E^{*} \cdot ADP \cdot P_i \overset{4}{\rightarrow} E + ADP + P_i \qquad \text{(iii)}$$

In this equation, there are two energy-releasing steps, 2 and 4, both concerned with nucleotide binding. Conversely, in phosphorylation, both ADP (or P_i) binding and ATP release are energy-requiring steps. This is again suggested by the extended analysis of $P_i \rightharpoonup H_2{}^{18}O$ exchange by Boyer and coworkers [42,43], and is similar to the mechanism proposed for the myosin ATPase [44].

During uncoupled ATP hydrolysis, K_i^{ADP} becomes much lower, implying that $E^{*} \cdot ADP \cdot P_i$ and E in equilibrium, and thus more of the enzyme can be 'trapped' as the $E \cdot ADP$ complex. This means that the energy of hydrolysis is allowed to dissipate earlier, in step 3 for example, in an unusable form (see above) as depicted in Eqn. iiia. This implies that, during coupled ATPase turnover, the enzyme is constrained in some way to maintain $E^{*} \cdot ADP \cdot P_i$ as a 'high energy' form.

$$E + ATP \overset{1}{\rightleftharpoons} E \cdot ATP \overset{2}{\rightarrow} E^{*} \cdot ATP \overset{3}{\rightarrow} \underset{\underset{E \cdot ADP \cdot P_i}{\|}}{E^{*} \cdot ADP \cdot P_i} \overset{4}{\rightleftharpoons} E + ADP + P_i \qquad \text{(iiia)}$$

(indistinguishable)

Reaction iii contains two irreversible steps, i.e. steps which require energy input during phosphorylation. Kayalar et al. [43] proposed that, to allow only one energy input during the phosphorylation cycle, the two steps could be linked by a conformational change of the enzyme in a 2-site, flip-flop, mechanism. In our scheme, this implies that reactions 2 and 4 are linked together. It can then be seen why such a linkage is prevented in the uncoupled enzyme, since reactions 2 and 4 are no longer energetically balanced. This is in accordance with our previous demonstration of conformational changes during coupled ATP hydrolysis only [4].

Ligand binding and ATPase structure

The effects of low pH (above) and low temperature [22] on the isolated ATPase are similar, i.e. both cause a reversible denaturation of the enzyme (above) followed, over a longer time, by an irreversible denaturation (not

shown here). In both cases, the intermediate 'reversibly denatured' form (the '9 S' form in the case of the cold-treated enzyme [22]) exchanges its tightly bound nucleotides very readily with added nucleotides, unlike the 'native' or '12 S' form. It is difficult to establish whether this intermediate represents a physiological form, but on the model for phosphorylation proposed by Slater et al. [45], in which energy leads to dissociation of the bound nucleotides, this form may correspond to the energised form of the enzyme (cf. ref. 22). If so, the involvement of H^+ in promoting this state may provide a clue to the mechanism of energy transduction (cf. ref. 46).

This work also confirms that the tight nucleotide binding sites and the 'hydrolytic site(s)' of the ATPase are separate but interact with each other. This will be considered in detail elsewhere, but it should be noted here that ADP cannot be considered a simple competitive inhibitor of the isolated ATPase in kinetic analyses. The role of this 'allosteric' inhibition remains unclear, especially since, on the, presumably more physiological, membrane-bound ATPase the ADP-induced inactivation is absent (Fig. 4). However, the intersite interaction is presumably maintained in the membrane-bound ATPase, and its nature may well provide a clue to the complex behaviour of this enzyme in vivo.

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