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STUDIES OF THE KINETICS OF THE ISOLATED MITOCHONDRIAL ATPase USING DINITROPHENOL AS A PROBE *

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Summary

1. Dinitrophenol and maleate anions increase V^{ATP} on the 'washed', isolated, mitochondrial ATPase. Hydrolyses of *iso*-GTP and 2'-deoxy ATP are also stimulated, while hydrolyses of other nucleoside triphosphates (ITP, GTP etc.) are not.

2. Preincubation with ATP, *iso*-GTP or 2'-deoxy ATP results in a metastable enzyme form with a raised V and a reduced K_m. Dinitrophenol stimulates both ATP and ITP hydrolyses by this form.

3. The Arrhenius plot of ATP (but not ITP) hydrolysis by the isolated ATPase shows a break at about 18°C, apparently because the rate limiting step of hydrolysis changes as the temperature rises.

4. Adenylyl β,γ-imidodiphosphate (Ado*PP*[NH]*P*) inhibits ITP hydrolysis in a pseudofirst order reaction. Its binding is competitive with ITP. If the enzyme is preincubated with ATP, the rate of Ado*PP*[NH]*P* binding increases. It is concluded that Ado*PP*[NH]*P* inhibits by binding to the hydrolytic site of the enzyme.

5. We conclude that ATP hydrolysis is limited by diphosphate release and ITP hydrolysis by bond splitting. Energy release during ATP hydrolysis is maximal at the ATP binding step, and during ITP hydrolysis at bond splitting.

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* Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/172/48038/635 (1981) 412—428.
The supplementary information includes the effect of dinitrophenol on ITP hydrolysis by the isolated ATPase and inhibition of ATP hydrolysis by ADP. The results are plotted according to Dixon (Biochem. J. 55, 170—171 (1952)).

Introduction

Dinitrophenol interacts with several enzymes that handle nucleotides. Various kinases and dehydrogenases are inhibited by dinitrophenol [1], the myosin ATPase is stimulated [2], and in ATP phosphoribosyltransferase (EC 2.4.2.17) dinitrophenol can replace ATP as a 'parasite' substrate [3]. The mitochondrial ATPase, F_1, is also stimulated by dinitrophenol [1,4—6] in an interaction unrelated to its uncoupling activity [5—7]. Covalent dinitrophenyl derivatives of ATP have been used to probe the nucleotide binding sites of F_1 [8].

We show here that dinitrophenol stimulation of F_1 hydrolytic activity is limited to certain nucleotides (ATP, dATP *iso*-GTP) above 20°C. Hydrolysis of other ATP analogues, such as ITP, GTP, N^1-oxido ATP and N^1,N^6-etheno ATP, is inhibited by dinitrophenol, as is the hydrolysis of ATP below 20° C. Maleate [9] and methanol [10] have also been shown to stimulate ATP hydrolysis at 30°C, but not ITP hydrolysis.

The simplest explanation for these results is that the rate limiting step of ATP hydrolysis at 30°C is different from that of ITP (cf. Ref. 9). From the temperature dependence of hydrolysis, and other kinetic data, we conclude that during ATP hydrolysis at 30°C, product release limits ATPase turnover, and that this is the step stimulated by dinitrophenol. Using ITP as substrate, however, the rate limiting step of the untreated enzyme occurs earlier, probably at the bond-splitting step.

The first step in hydrolysis, the ATP-binding step, was investigated using Ado*PP*[NH]*P* as an ATP analogue. Ado*PP*[NH]*P* inhibits F_1 in a time-dependent manner, forming a very tight F_1-Ado*PP*[NH]*P* complex [11,12]. We show here that this complex is due to Ado*PP*[NH]*P* binding at the active site of the ATPase, and not due to interaction at one of the other nucleotide binding sites [13].

Using these data, and a simple kinetic model for triphosphate hydrolysis, it is possible to derive all the individual rate constants of the ATPase reaction from the data given herein.

It is interesting that those nucleotides whose hydrolysis is stimulated by dinitrophenol are also those that fit into the 'tight nucleotide' binding sites of the ATPase [12]. The explanation for this is unknown.

Materials and Methods

Nucleotide triphosphate hydrolysis was measured at pH 8 either by the continuous assay of Rosing et al. [14], in which an ATP-regenerating system was present, or by monitoring H^+ release essentially as described by Van de Stadt et al. [15]. The ATPase was prepared by the method of Knowles and Penefsky [16], and was 'washed' by repeated precipitation [14] before use. Assay of the unwashed enzyme yielded confusing results since a mixture of two different forms of the ATPase (containing different levels of 'tight' ATP) were present (see below).

The nucleotide analogues were prepared as described [12]. Inhibitor-depleted submitochondrial particles were prepared as described by Racker and Horstmann [17]. Dinitrophenol and dicoumarol were obtained from Merck Biochemicals.

Results

Effect of dinitrophenol on ATP hydrolysis

Fig. 1 shows the rate of ATP hydrolysis at saturating ATP concentrations (>7 K_m) in the presence of varying amounts of dinitrophenol. An ATP regenerating system was used in the assay so no product inhibition (by ADP) occurred. Dinitrophenol, at low concentrations, increased the maximum rate of the ATPase by up to 60—70% at 30° C. Half maximal stimulation was at about 0.15 mM dinitrophenol. This value did not change when ATP concentration was reduced from 2 mM to 200 μM, suggesting that ATP and dinitrophenol do not compete for a common site. Higher concentrations of dinitrophenol inhibit the enzyme, but may not eliminate its activity completely (see Fig. 2).

The effect of stimulatory amounts of dinitrophenol on K_m^{ATP}, V^{ATP} and

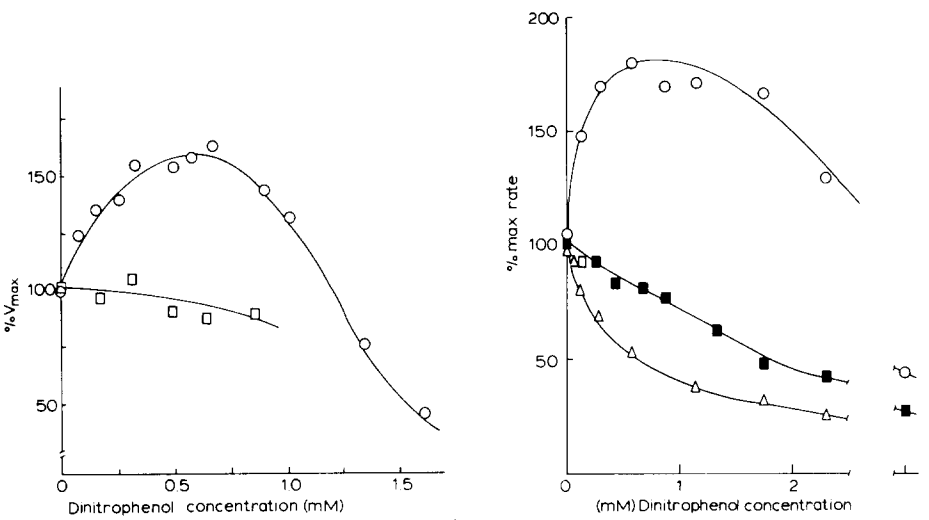

Fig. 1. The effect of dinitrophenol on ATP hydrolysis by the isolated ATPase. 2 μg 'washed' isolated ATPase [25] or 20 μg 'AS' particles [17] was added to 1 ml buffer I (250 mM sucrose, 50 mM KCl, 50 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA brought to pH 8.0 with HCl) containing 2 mM Mg-ATP, 0.17 mM NADH, 2 mM phospho*enol*pyruvate, 3 U/ml pyruvate kinase and 3 U/ml lactate dehydrogenase, and dinitrophenol as indicated. ATP hydrolysis at 30° C was followed by monitoring absorbtion at 340 nm. The path length was 0.2 cm and a double beam spectrophotometer (Pye-Unicam SP 1800) was used with a solution containing dinitrophenol in the reference beam to eliminate the absorption due to dinitrophenol alone. 100% activity for the isolated enzyme = 89 μmol · min^{-1} · mg^{-1} protein, and for the membrane-bound enzyme, 100% activity = 11.3 μmol · min^{-1} · mg^{-1} protein. ○———○, isolated ATPase; □———□, membrane-bound ATPase.

Fig. 2. The effect of dinitrophenol on hydrolysis of various nucleoside triphosphates. 'Washed' ATPase was dissolved in 100 mM Tris, 1 mM EDTA brought to pH 8.0 by HCl. A portion of this solution containing 6 μg ATPase was added to 4.5 ml of assay buffer (1 mM Tris, 50 mM NaCl, 100 μM EDTA and 1 mM $MgCl_2$) containing Mg-NTP and dinitrophenol as indicated and adjusted to pH 8.0 with HCl or NaOH in the reaction chamber. NTP hydrolysis at 30° C was monitored by measuring H^+ release in a continuously recording pH meter [15]. The system was calibrated by addition of known amounts of succinic acid. Initial rates (2—15 s) for those substrates where the rate decreased with time. ○———○, 2 mM Mg-ATP; ■———■, 2.5 mM Mg-dATP, △———△, 6 mM Mg-ITP. *iso*-GTP gave a curve identical to ATP, and GTP, N^1,N^6-etheno ATP gave curves identical to ITP when plotted in this way. The V values for the last two nucleotides, however, were around 45 μmol · min^{-1} · mg^{-1}, i.e. about 50% of that observed with the other nucleotides.

TABLE I

KINETIC PARAMETERS OF THE MITOCHONDRIAL ATPase

The rate of hydrolysis of ATP by F_1 was measured as in Fig. 2 except that ATP-Mg levels were varied between 0.1 and 2 mM, and ADP levels between 0.0125 and 1.25 mM. Dinitrophenol, where present, was added to a final concentration of 0.8 mM. K_m etc. were estimated graphically.

	K_m^{ATP} (mM)	V^{ATP} (μmol · min^{-1} · mg^{-1})	K_i^{ADP} (μM)
No dinitrophenol	0.29	86	28
+ dinitrophenol	0.26	146	65

K_i^{ADP} is shown in Table I. In contrast to Cantley and Hammes [5], we find that the major effect of dinitrophenol is to increase V (by about 70% at 30°C) and that its effect on K_m is negligible. The reason for the discrepancy is uncertain, but it may well reflect an effect of ADP on enzyme activity in their case.

ADP binds strongly to the ATPase, inhibiting it both by competition at the active site ($K_i \approx 100$ μM) (instantaneous) and by (slower) binding to an allosteric inhibitory site ($K_i < 1$ μM) [12,18]. Thus, ADP inhibition observed varies with the length of time taken for measurement. We obtain an apparent K_i value of 30 μM (cf. Ref. 20) when initial rate measurements are taken over 2—15 s as here (data deposited with BBA), but a value of 10 μM using the (slower) procedure of Van de Stadt et al. [15]. The method employed by Cantley and Hammes [5,19] allows the initial rate to be measured only after a 15-s period has elapsed, a period during which some ADP inhibition would have developed. Despite its two sites of action, ADP appears in kinetic plots to act as a simple competitive inhibitor ([20], data deposited with BBA), probably because it competes with ATP at both sites of action.

Dinitrophenol, at stimulatory concentrations, decreases inhibition of the ATPase by ADP, K_i rising from 27 to 65 μM (Table I). Since we can demonstrate no effect of dinitrophenol on ADP binding to the tight ADP binding site ([5]; our unpublished results), this appears to represent an effect on ADP binding to the catalytic site(s) of the enzyme. In agreement with Cantley and Hammes [5], we find no effect of dinitrophenol on the uncoupled, membrane bound ATPase at saturating ATP levels (Fig. 1). This supports the view that the stimulation of hydrolysis on the isolated ATPase is not the cause of the uncoupling action of dinitrophenol [5—7].

Effect of dinitrophenol on hydrolysis of other triphosphates

At 31°C, ITP is hydrolysed with a V about equal to that of ATP (90 μmol · min^{-1} · mg^{-1}) (see Fig. 3). In contrast, however, ITP hydrolysis is inhibited by dinitrophenol. The K_i value for dinitrophenol (0.7 mM) is independent of ITP concentration over the range $3\,K_m$ to $0.1\,K_m$, indicating non-competitive inhibition, and the inhibition appears to involve only one class of dinitrophenol binding site, Dixon plots for the inhibition being linear (data deposited). *Iso*-GTP and 2′-dATP show a pattern of stimulation and inhibition like ATP (Fig. 2). In contrast N^1,N^6-etheno ATP, N^1-oxido ATP and GTP behave like ITP and show simple inhibition. At low concentrations (approx. 0—0.3 mM) therefore,

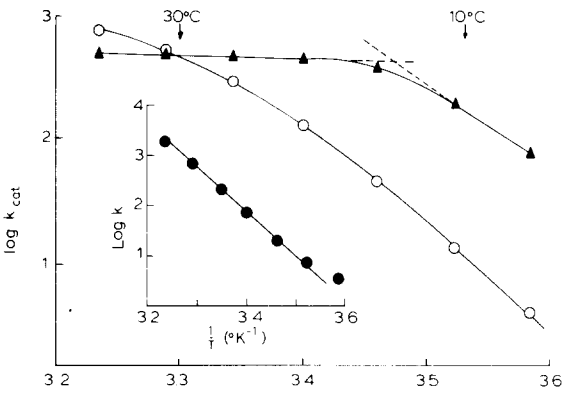

Fig. 3. Temperature dependence of NTP hydrolysis. Hydrolysis was measured as in Fig. 2 using 2 mM Mg-ATP or 6 mM Mg—ITP as substrate. To ensure that the enzyme was saturated with substrate at all temperatures, measurements were made in which the substrate concentration was halved. At no temperature did this change cause a drop in rate of more than 15% ▲———▲, ATPase, ○———○, ITPase. The insert shows a plot of log $c_{1/2}$ vs. $1/T$ for dinitrophenol inhibition of ITP hydrolysis. The $c_{1/2}$ values were obtained from Fig. 4a.

dinitrophenol increases V for hydrolysis of ATP and *iso*-GTP, but not of ITP and GTP, etc. For an agent to raise V for a reaction, it must of necessity increase the rate of the limiting step. A simple model for these effects, therefore, suggests that dinitrophenol stimulates one of the steps of the enzyme catalysed reaction which is rate limiting to ATP hydrolysis but not to ITP hydrolysis (for example), i.e. that ATP and ITP hydrolyses are limited by different chemical steps.

This view is supported by the finding that other agents which are chemically distinct from dinitrophenol can stimulate ATP hydrolysis without stimulating ITP hydrolysis. These agents include maleate (Ref. 9, Table II) and methanol [10]. These agents presumably bind to different sites on the enzyme from dini-

TABLE II

EFFECT OF MALEATE AND DINITROPHENOLATE ANIONS ON NUCLEOTIDE HYDROLYSIS BY F_1

The rates of hydrolysis of ATP (2 mM) and ITP (6 mM) were measured as in Fig. 2, except that meleate (as the potassium salt) or dinitrophenol were added as indicated. The results are expressed relative to the rate of ATP hydrolysis in the normal assay buffer. 100% = 86 μmol · min^{-1} · mg^{-1} ± 1.8 (S.E. of ten readings). Other readings are the average of two or three duplicates. n.d., not determined.

Temperature (°C)	Maleate added	ATPase rate		ITPase rate	
		— dinitrophenol	+ 0.5 mM dinitrophenol	— dinitrophenol	0.5 mM dinitrophenol
31	none	100	180	100	52
	25 mM	133	190	n.d.	n.d.
	75 mM or 100 mM	159	194	91	n.d.
6	none	17.4	<0.3	0.86	<0.03
	40 mM	16.4	n.d.	n.d.	n.d.

trophenol, maleate certainly does since an enzyme fully stimulated with maleate can be further stimulated by dinitrophenol (Table II).

Temperature dependence of hydrolysis

The Arrhenius plot (log V vs. $1/T$) for ATP hydrolysis by the isolated ATPase shows a break at about 18°C (Fig. 3). Above this temperature, V increases only very slightly with temperature (E_a low, approx. 3 kcal · mol · K^{-1}), while below this temperature V increases very sharply (E_a high, approx. 30 kcal · $mol^{-1} \cdot K^{-1}$). This is consistent with previous results [36]. Control experiments showed that substrate concentrations used in the experiments of Fig. 3 were sufficient to saturate the enzyme at all temperatures used here.

Lenaz and coworkers [22] also find a break in the Arrhenius plot of the mitochondrial ATPase, but in its membrane-bound form. They interpret this phenomenon as indicating a phase change in this enzyme at about 18°C. This interpretation is not consistent with the other results given in Fig. 3. First, an Arrhenius plot of ITP hydrolysis, while curved, does not show an obvious break (if present at all, a break would be upwards of 25°C). Second, when the binding of dinitrophenol to its inhibitory site (measured as in Fig. 4) is used as a probe of enzyme conformation, a Van't Hoff plot (Fig. 3, insert) (log K_d vs. $1/T$) is linear. This shows that ΔH for binding is constant over the range 6—36°C, indicating that no phase change is likely to occur in the enzyme between these temperatures.

A more likely interpretation of the sharp break in the Arrhenius plot for ATP hydrolysis is a change in rate limiting step of the reaction. At 30°C, for example, ATP hydrolysis would be limited by one step and at 10°C by another.

Effects of dinitrophenol on hydrolysis also vary with temperature (Fig. 4). Over the entire range, ITP hydrolysis is simply inhibited by dinitrophenol. The $c_{1/2}$ for dinitrophenol, however, varies strongly with temperature, binding becoming much stronger as the temperature is lowered. Assuming the $c_{1/2}$ value corresponds to the K_d for dinitrophenol, as it should for a simple, noncompetitive inhibitor, ΔH for the binding of dinitrophenol to its inhibitory site on F_1 can be calculated (Fig. 3) to be −40 kcal/mol. This is a surprisingly high value which may indicate (a) specific, binding site(s) for this probe.

Below about 18°C, both ATP and ITP hydrolysis are simply inhibited by dinitrophenol (Fig. 4). Above 18°C (the 'transition' temperature for ATP hydrolysis) the stimulatory effect of dinitrophenol becomes noticeable, increasing as the temperature increases. This is consistent with the model proposed (below 18°C, both ATP and ITP hydrolysis are limited by the same step) that one inhibited by dinitrophenol. Above 18°C, the rate limiting step for ITP hydrolysis remains the same (high temperature sensitivity, inhibited by dinitrophenol) while that of ATP hydrolysis changes (low temperature sensitivity, stimulated by dinitrophenol).

Maleate behaves in a manner similar to dinitrophenol, stimulating ATP hydrolysis above the transition temperature, but not below it (Table II), as it must on this model.

It is interesting, though apparently fortuitous, that hydrolysis of ATP and ITP by the isolated enzyme occurs at approximately equal rates at 30°C (90 μmol · $min^{-1} \cdot mg^{-1}$) in spite of having different rate limiting steps. This is a

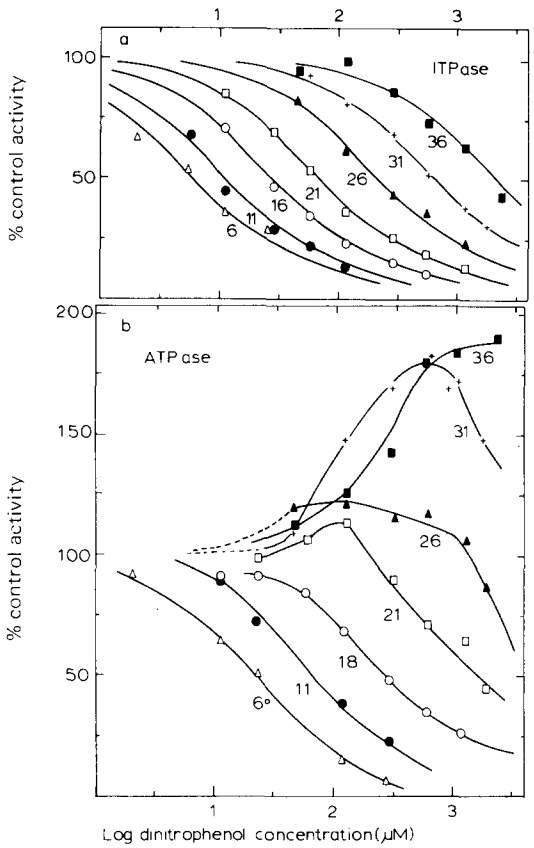

Fig. 4. Temperature dependence of dinitrophenol effects on F_1. Measurement of hydrolysis rates in the presence of varying amounts of dinitrophenol was as in Fig. 2, except that the temperature of the reaction medium was varied as indicated. The results are plotted as a series of % response vs. log dose curves. The 100% values are those recorded in Fig. 3. In the case of ITP hydrolysis, all curves are identical in shape but related by translation along the abscissa. (a) ITP hydrolysis. (b) ATP hydrolysis.

result of the much higher affinity of the ATPase for ADP than for IDP. Above 30°C ITP is hydrolysed faster than ATP, while below 30°C the opposite is true (Fig. 3).

Preincubation of the enzyme with nucleoside triphosphates

We have shown previously that preincubation of F_1 with ADP leads to an enzyme in which V is reduced to about 50%. This form of F_1 is stable for several minutes in an ATP regenerating system [12,21].

Preincubation with certain triphosphates (ATP, dATP and *iso*-GTP) results in an enzyme with an increased V (Table III) (cf. Ref. 14). The effect is most marked with dATP in the preincubation. The triphosphate-treated enzyme is somewhat less stable than the diphosphate treated enzyme, and declines in activity, towards the control level, during a 2—3 min period (Fig. 5).

The triphosphate-treated enzyme is characterised by another remarkable property. That is, hydrolysis of both ATP (not shown) and ITP (Fig. 6) by this

TABLE III

F_1 ACTIVITY AFTER PREINCUBATION WITH NUCLEOSIDE TRIPHOSPHATES

F_1 was freed of ambient nucleotides by reprecipitation, and preincubated with 10 mM NTP as indicated, in 250 mM sucrose, 10 mM Tris acetate, 2 mM EDTA, pH 7.5, at a protein concentration of 1—2 mg/ml. The results are expressed relative to the hydrolysis by the untreated enzyme (100%) viz. 90 ± 2 μmol · min^{-1} · mg^{-1} for ATP, 91 ± 1.5 μmol · min^{-1} · mg^{-1} for ITP and 51 ± 1.5 μmol · min^{-1} · mg^{-1} for N^1-oxido ATP. n.d., not determined.

Hydrolysis of	Preincubated with (% untreated rate)							
	ATP	dATP	*iso*-GTP	ITP	GTP	N^1-oxido ATP	ATP	No addition
ATP	110	164	n.d.	96	106	94	94	100
ITP	129	168	129	94	103	94	94	100
N^1-oxido ATP	121	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	100

form of F_1 is stimulated by low concentrations of dinitrophenol. On the untreated enzyme, hydrolysis of ATP but not ITP is dinitrophenol stimulated. Similar effects are seen when N^1-oxido ATP is being hydrolysed; the untreated enzyme is inhibited by dinitrophenol, while the ATP-preincubated enzyme is stimulated.

Table III also shows that the only triphosphates capable of eliciting these effects are ATP, dATP and iso-GTP. ITP, GTP, N^1-oxido ATP and N^1,N^6-etheno ATP show no such effects. The specificity of this site is thus identical to that of

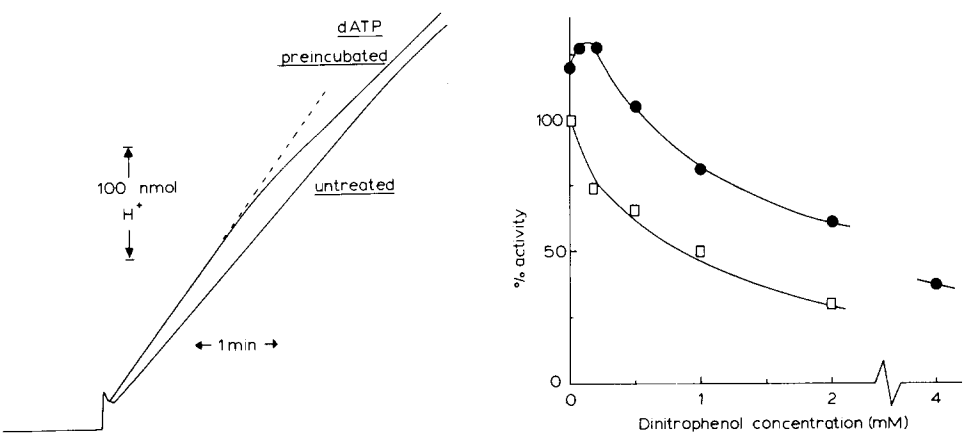

Fig. 5. Effect of dATP incubation on time course of triphosphate hydrolysis. F_1 (100 μg) was freed from ambient nucleotides [25], and incubated in 0.2 ml 250 mM sucrose, 20 mM Tris sulphate, 0.2 mM EDTA pH 7.5, with (left hand curve) or without (right hand curve) 5 mM dATP present. After 3 min, 5 μl of this solution was taken for assay of Mg-ITPase activity at 30°C, using a recording pH meter [15]. A small amount (5 μM) dATP was added to the control assay mixture so that the final dATP concentrations in the assay were the same in both cases.

Fig. 6. Effect of dinitrophenol on ITP hydrolysis in the preincubated enzyme. F_1 was treated and assayed as in Fig. 5 except that ATP replaced dATP in the preincubation, and the indicated amounts of dinitrophenol were added to the assay mixture. ●———●, F_1 preincubated with 5 mM ATP; □———□, untreated F_1.

TABLE IV

KINETICS OF HYDROLYSIS BY F_1 AFTER PREINCUBATION WITH NUCLEOTIDES

Preincubations with ADP [12], or ATP (Table III) were performed as described. K_m values were taken between 100 μM and 4 mM Mg-ATP (10 mM Mg-ITP) over which range the plot was linear. Control values were as in Table III. +, stimulation at low concentrations; —, inhibition.

Treatment	ATP hydrolysis			ITP hydrolysis		Proposed structure
	K_m (μM)	V (% control)	Dinitrophenol effect	V (% control)	Dinitrophenol effect	
None	300	100	+	100	—	$E(ATP)_2$ (ADP)
ATP preincubation	120	110 (165) *	+	130 (168) *	+	$E(ATP)_3$
ADP preincubation	320	45	+	40	—	$E(ADP)_2$ (ATP)

* Preincubated with dATP.

the 'tight binding' sites of the ATPase [12], which suggests that the interaction involved occurs at these 'tight binding' sites.

It appears, therefore, that 'loading' the tight binding sites of F_1 changes the rate limiting step of hydrolysis to the dinitrophenol stimulated step even when ITP and N^1-oxido ATP are being hydrolysed. This feature explains why equivocal results may be obtained if the 'unwashed' enzyme (presumably a mixture of forms) is used in the assays.

Table IV summarises the kinetic properties of the ATP-pretreated, ADP-pretreated and 'washed' enzyme. The nucleotide specificity, persistance and pH-dependence of these effects are characteristic of the tight binding sites of F_1. Since the nucleotide composition of washed F_1 is $E(ATP)_2(ADP)_1$ we propose $E(ATP)_3$ and $E(ADP)_2(ATP)$ as possible results of preincubation with ATP and ADP, respectively, although it must be emphasised that these assignments are only tentative. Notably, however, the ATP-pretreated enzyme has a 2.5-fold lower K_m^{ATP}, a raised V and a different dinitrophenol stimulation pattern from the 'washed' enzyme.

Inhibition of F_1 by AdoPP[NH]P

Ado*PP*[NH]*P* is a powerful ($K_i < 1$ μM) inhibitor of F_1, which can bind to both the catalytic and the 'tight binding' sites of the ATPase [20,23]. The kinetics of Ado*PP*[NH]*P* inhibition of F_1 can be measured as in Fig. 7. In the presence of Ado*PP*[NH]*P*, the rate of ITP hydrolysis by F_1 falls off in time, while in its absence, the rate is constant. By drawing tangents to the product-time curve obtained in the presence of Ado*PP*[NH]*P*, the hydrolysis rates at various times can be measured (cf. Ref. 15).

Fig. 8 shows the results of such an experiment. A plot of log rate vs. time for ITP hydrolysis is linear. The linearity of this plot suggests a single type of site on F_1 for inhibition by Ado*PP*[NH]*P*. The inhibition rate (the slope of such a line) is first order in Ado*PP*[NH]*P* concentration between 0.25 and 5 μM. Above 5 μM the rates become too fast to measure by this technique.

The rate of inhibition is decreased by increasing substrate (ITP) (Fig. 7), suggesting a competition between Ado*PP*[NH]*P* and ITP at the site of action.

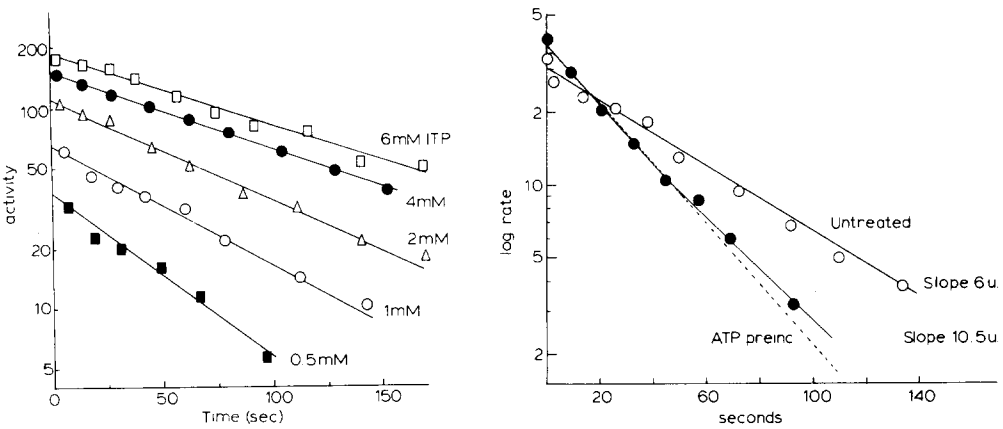

Fig. 7. Time course of ITP hydrolysis in the presence of Ado*PP*[NH]*P*. F_1 was freed from ambient nucleotides [25] and ITPase activity measured using a recording pH meter [15]. The lines are derived from a series of (curved) product-time graphs measured at 1 μM Ado*PP*[NH]*P* but various Mg-ITP concentrations as indicated. The activity at t_1, t_2, etc. (arbitrary units) is the tangent to a curve at t_1, t_2, etc. The half-times calculated for the inhibition are, at the given ITP concentrations: 0.5 mM, 37 s; 1 mM, 50 s; 2 mM, 60 s; 4 mM, 78 s; 6 mM, 84 s.

Fig. 8. Effect of ATP preincubation on Ado*PP*[NH]*P* inhibition. ITPase activity in the presence of 2 μM Ado*PP*[NH]*P* and 4 mM Mg-ITP was measured as in Fig. 7, except that, in the case of the filled symbols, the enzyme was incubated with 5 mM ATP for 3 min prior to assay. A small amount of ATP was added to the control enzyme assay (open symbols) so that the final concentration of ATP in both assay mixtures was 1 μM. The half-times for inhibition are 40 s for the untreated enzyme, and 25 s for the pretreated enzyme. $E(ATP)_3$ slowly dissociates in the assay mixture (Fig. 5) and so the line corresponding to the pre-incubated enzyme curves slightly.

Since ITP is known to be unable to bind to the 'tight' nucleotide binding sites [12,18], these results suggest that Ado*PP*[NH]*P* is an active site inhibitor of the ATPase.

Further evidence for this view is obtained from studies on the ATP-loaded enzyme. If Ado*PP*[NH]*P* acted by binding to the 'tight' sites of the ATPase, preincubation of the enzyme with ATP would block its action, at least for several minutes, since the tight nucleotide sites are now stably filled. However, Fig. 8 shows that the preincubated enzyme is inhibited by Ado*PP*[NH]*P* faster than the untreated enzyme. This suggests that the preincubated enzyme has developed a higher affinity for Ado*PP*[NH]*P*. Taken in conjunction with the lowered K_m for ATP, this finding again suggests that Ado*PP*[NH]*P* binds to the active site of the enzyme.

Although Ado*PP*[NH]*P* is a powerful ATPase inhibitor, it nonetheless binds non-covalently. If F_1 is preincubated with Ado*PP*[NH]*P*, and the F_1-Ado*PP*-[NH]*P* complex thus formed is diluted into an ITPase assay mixture, an increase in activity is observed as Ado*PP*[NH]*P* dissociates from the enzyme. By following the increase in rate in time, the rate of dissociation of F_1-Ado*PP*-[NH]*P* can be determined (Fig. 9).

Effect of Mg^{2+} on AdoPP[NH]P interaction

From Fig. 7 we see that, when added to the assay mixture Ado*PP*[NH]*P* inhibits with a half-time of a few seconds, while previously we have shown that,

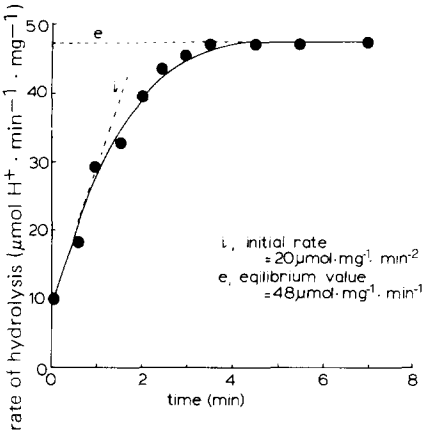

Fig. 9. Release of Ado*PP*[NH]*P* from F_1. ITPase activity was measured as in Fig. 8, except that F_1 was preincubated with 5 μM Ado*PP*[NH]*P*, and Ado*PP*[NH]*P* was omitted from the assay medium.

in a preincubation, far higher concentrations of Ado*PP*[NH]*P* require several minutes to act [12]. This is due to an effect of Mg^{2+} on the F_1-Ado*PP*[NH]*P* interaction, leading to an increase in affinity (or on-rate) for nucleotide at the active site. This is shown in Fig. 10. The rate of inhibition due to preincubation with Ado*PP*[NH]*P* increases very markedly if Mg^{2+} is also present in the incubation. Similar results were found with the membrane-bound ATPase. It is not clear whether this effect is due to Mg^{2+} binding to F_1 or to Ado*PP*[NH]*P*, although we have previously demonstrated an effect of Mg^{2+} on the conformation of F_1 in the absence of nucleotide [31].

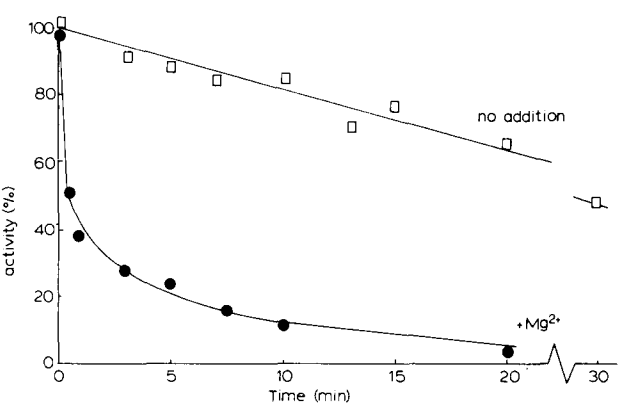

Fig. 10. Effect of Mg^{2+} on Ado*PP*[NH]*P* inhibition of F_1. F_1 was preincubated with 10 μM Ado*PP*[NH]*P* in the presence (●——●) or absence (□——□) of 1 mM free $MgCl_2$. Samples were taken for assay at various times, as previously [32] except that 0.8 μM Ado*PP*[NH]*P* was added to the assay mixture to slow down the dissociation of bound Ado*PP*[NH]*P*. (Similar results were obtained if Ado*PP*[NH]*P* were omitted from the assay medium). The rates shown are the initial rates of hydrolysis. 100% activity = 88 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for the enzyme pretreated in the absence of Mg^{2+}, and 53 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for that preincubated with Mg^{2+}. Note that, in the absence of Mg^{2+}, inhibition is zero order, suggesting that a conformational change in the enzyme precedes Ado*PP*[NH]*P* binding in this case.

Discussion

Dinitrophenol binding to the ATPase

Figs. 1 and 2 suggest that dinitrophenol can bind to 2 types of 'effector' site on the coupling ATPase. At 30°C, binding to the high affinity site ($K_{1/2} \approx 0.15$ mM) leads to a stimulation of hydrolysis of ATP and some other triphosphates. Binding to the low affinity site ($K_i = 0.7$ mM) leads to inhibition. The effects are noncompetitive (data deposited), and therefore both sites are likely to be separate from the active site of the enzyme. Studies of the binding of dinitrophenol to the ATPase are consistent with there being one site per enzyme of each type, although the data on the lower affinity site are not sufficiently precise to rule out a larger number [5,6].

The rate limiting step of triphosphate hydrolysis

At 30°C, ATP hydrolysis is stimulated by low concentrations of dinitrophenol, and by maleate, while ITP hydrolysis is not (Fig. 2, Table II). If we assume the dinitrophenol and maleate binding site(s) to be always present, then the rate limiting steps for ATP and ITP hydrolysis must be different, i.e. in the case of ITP hydrolysis, these effectors stimulate a step which is not a rate limiting one, and no effect is observed. The proposal that the rate limiting steps for ATP and ITP hydrolysis differ at 30°C is supported by a consideration of the temperature dependence of the hydrolysis.

What, then, is the rate limiting step for ATP hydrolysis? Several lines of evidence suggest that this is the release of ADP from the enzyme. First, dinitrophenol increases the K_i^{ADP} about 2-fold (Table I). This can be simply explained if it increases the ADP-off rate by a factor of 2. If this step were rate limiting, V would also increase by a factor of about 2, and K_m would be unaltered, as is indeed the case (Table I).

Secondly, the low activation energy for the ATPase reaction (Fig. 3) suggests that the rate-limiting step is not a chemical transformation, i.e. that it probably involves an enzyme-binding step.

Thirdly, at very low ATP concentrations, it is deduced that product release is rate limiting from the $H_2^{18}O$-P_i exchange experiments of Choate et al. [24]. Although rate of product release increases with ATP concentration, it is quite likely that this rate still limits substrate turnover at saturating substrate concentrations.

Finally, the myosin ATPase, which seems to be closely analogous to the F_1-ATPase in mechanism (i.e. hydrolysis does not involve a phosphoryl enzyme) also has the ADP-off rate as the rate-limiting step [29]. Incidentally, this hydrolysis is also stimulated by dinitrophenol [2].

We would thus propose that ADP release limits ATP hydrolysis by F_1 at 30°C. ITP hydrolysis must be limited by some other step and, since we are dealing with changes in V (i.e. at saturating substrate), the bond splitting step is a likely candidate, and is consistent with the high values for E_a (Fig. 3). Dinitrophenol, at high concentrations, inhibits this step completely (or nearly so) and thus can also inhibit ATP hydrolysis at high concentrations.

Over a range of nucleotides, we find that the hydrolysis of some, like ATP

and *iso*-GTP, are stimulated by low concentrations of dinitrophenol, while others are not. This reflects a difference in the rate limiting steps of the reaction, ATP and *iso*-GTP being limited by product release and ITP etc. by the bond splitting step. dATP is an intermediate case (Fig. 2), in this case we expect bond splitting and product release steps to be similar in rate. Dinitrophenol thus provides a useful probe to show either the rate limiting step in hydrolysis of an unknown nucleotide analogue (which may be useful in assessing its ability to energise the membrane-bound ATPase [12]), or in assessing whether in modifying the ATPase (chemically or by changing its nucleotide content), its rate limiting step has altered.

Mode of action of AdoPP[NH]P

Ado*PP*[NH]*P* is a very powerful inhibitor of the F_1-ATPase in both its isolated and membrane bound forms. At 1 μM the half-time for inhibition is about 1 min (Fig. 7). Philo and Selwyn [11] in 1974 suggested that the tight binding of Ado*PP*[NH]*P* to F_1 was indicative of a mechanism of ATP hydrolysis where energy was released when ATP bound to the enzyme (cf. Refs. 25, 26).

This suggestion relies on the assumption that inhibition is due to Ado*PP*-[NH]*P* binding to the hydrolytic site(s) of the ATPase. This assumption was previously unproven and, since Ado*PP*[NH]*P* can bind to both the hydrolytic and 'tight binding' sites of F_1 [23], there remained the possibility that Ado*PP*[NH]*P* might inhibit by binding to a tight binding 'regulatory' site on the enzyme [13,27,28].

We show above that Ado*PP*[NH]*P* inhibition of ATP hydrolysis does indeed involve Ado*PP*[NH]*P* binding to the hydrolytic site of F_1. Loading the tight binding sites with ATP increases the rate of Ado*PP*[NH]*P* inhibition of F_1 (Fig. 8), which clearly could not occur if Ado*PP*[NH]*P* had to occupy the tight binding sites itself. The increase in rate (about 2-fold) is comparable with the decrease in K_m^{ATP} seen in the ATP-loaded enzyme (Table IV), confirming that this interaction is at the active site of the ATPase. Competition of Ado*PP*-[NH]*P* with ITP which cannot occupy the tight binding sites [12] supports this conclusion (above). In agreement with Ref. 11, therefore, we conclude that tight Ado*PP*[NH]*P* binding indicates a large release of energy when ATP binds to the active site of F_1.

Given that Ado*PP*[NH]*P* acts as an ATP analogue at the active site(s) of F_1, its on and off rates can be taken as estimates of the on and off rate for ATP binding to the ATPase. The off rate, for example, can be calculated from Fig. 9

($k_{-1} = 4 \cdot 10^{-3} s^{-1}$) and k_1, from $k_{-1}/K_d \geqslant 4 \cdot 10^6\ M^{-1} \cdot s^{-1}$.

The rates of the individual steps of the ATPase reaction

Our results are compatible with the simplest possible kinetic model for NTP hydrolysis by F_1.

$$E + NTP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot NTP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E \cdot NDP \cdot P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + NDP + P_i \qquad (1)$$

At high NTP concentrations, k_{-2} seems to be slow relative to k_3 since $H_2^{18}O$-P_i

exchange is abolished [24]. Under these conditions,

$$K_m = (k_2 + k_{-1})/k_1 \text{ and } 1/k_{cat} = 1/k_2 + 1/k_3 \quad [30] \qquad (2)$$

Using this model and the above data, it is possible to calculate all the rate constants of the above reaction for ATP hydrolysis at 30°C. The results are shown in Table V. It should be noted that the only a priori assumption made in deriving these constants is the kinetic model (the simplest model), all other conclusions are drawn from experimental data. The methods used to derive these constants are given in the appendix.

Considering ATP hydrolysis, Table V shows that the slowest step (k_3) is ADP release from the enzyme. The bond splitting step is some 10-times faster, and indeed approaches the maximum possible (diffusion limited) value, since $k_2/K_m = 10^8$ l · mol^{-1} · s^{-1} [30]. Binding of ATP and ADP to the enzyme also appear to be fairly close to the diffusion limited rate.

The reverse of the reaction, however, is much slower, limited by ATP release at $k_{-1} = 4 \cdot 10^{-3}$ s^{-1}. k_{-2} is also relatively slow (15 s^{-1}), but is probably faster in the membrane bound form of the ATPase, since P_i-$H_2^{18}O$ exchange occurs even at relatively high ATP levels on the membrane bound ATPase [24]. Thus for ATP synthesis (turnover > 100 s^{-1}) k_{-1} must be increased, in keeping with suggestions that energy is used to release ATP from the enzyme [25,26].

From k_2 and k_{-2}, we can estimate an equilibrium constant of ATP hydrolysis on the ATPase of about 10^3. This indicates a shift of the ATP-ADP equilibrium towards ATP from the value in free solution, but not so far as occurs in myosin, where $K_{eq} = 9$ (see Ref. 30). This explains the failure to observe ATP synthesis by isolated F_1 at levels down to 1 ATP : 100 ADP (Harris, D.A., unpublished results). On the membrane, however, k_{-2} is larger (above) and the equilibrium is further displaced towards ATP, i.e. the free energy drop on bond splitting will be greatly reduced below that in free solution. Thus, as in myosin, the major free energy release during ATP hydrolysis by F_1 probably occurs when ATP binds to the enzyme.

F_1 is apparently less efficient at splitting ITP, k_2 being lower than for ATP. Since ITP cannot replace ATP in exchange reactions on the mitochondrial

TABLE V

The rate constants are for the reaction mechanism shown in the Discussion (Eqn. 1). The calculations are detailed in the Appendix.

	k_1 (l · mol^{-1} · s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	k_3 (s^{-1})	k_{-3} (l · mol^{-1} · s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m
ATPase (31°C)	$5 \cdot 10^7$	$4 \cdot 10^{-3}$	$1.7 \cdot 10^4$	15	$1.5 \cdot 10^3$	$5 \cdot 10^7$	$1 \cdot 10^3$	$3 \cdot 10^6$
ITPase (31°C)	$5 \cdot 10^7$?	$>10^4$	$1.7 \cdot 10^3$	$<<1$	$>5 \cdot 10^3$	$5 \cdot 10^7$	$1 \cdot 10^3$	$5 \cdot 10^5$
ATPase (6°C)	$5 \cdot 10^7$?	?	$0.14 \cdot 10^3$	—	$1.5 \cdot 10^3$	$5 \cdot 10^7$?	$1.7 \cdot 10^2$	$>5 \cdot 10^5$
ITPase (6°C)	$5 \cdot 10^7$?	?	8.9	—	$5 \cdot 10^3$?	$5 \cdot 10^7$?	8.6	$>4 \cdot 10^3$

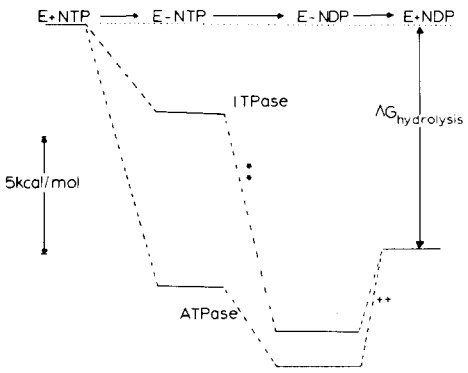

Fig. 11. Energy profile for hydrolysis of ATP and ITP. The values on the ordinate are calculated from Table III, and refer to 31°C. They are only approximate since the expression used ($\Delta G^{0\prime} = -RT\ln k_n / k_{-n}$) gives a value for $\Delta G^{0\prime}$, not ΔG, and the concentrations of the reaction intermediates are not known. The rate-limiting steps at 31°C for ITP (**) and ATP (++) hydrolysis are shown.

membrane [13], we conclude that k_{-2} for ITP hydrolysis is similarly reduced, to a very low value, while k_{-1} is relatively fast as judged by experiments with inosine β,γ-imidotriphosphate [12]. This is compatible with the view that, during ITP hydrolysis, energy is liberated mainly during the bond splitting step (and as such, cannot be used to drive energy linked processes) [12]. A comparable change in the mechanism of triphosphate hydrolysis by myosin is seen when GTP replaces ATP [34].

The scheme of Eqn. 1 and Table V does not rule out the possibility of conformational changes in the enzyme mechanism. ADP release, for example, may be limited by a prior conformational change with $k = 1.5 \cdot 10^3\ s^{-1}$. In fact, any of the three steps shown may be limited by conformational steps accompanying them. This seems unlikely since the reverse of these conformational changes must be fast (k_1, k_{-3} were calculated using simple equilibrium constants) but cannot be ruled out [12]. However, this does not affect the conclusions drawn here.

These results can be expressed as a free energy profile for the reaction as shown in Fig. 11.

Boyer and coworkers have shown that k_3 is ATP dependent [24]. The value of k_3 that we calculate is the maximal value, at saturating ATP concentrations. It was necessary to assume the form of the dependence to calculate k_{-2} and we assume, from Boyer and coworkers, a flip-flop mechanism for the enzyme which leads directly to a prediction for this dependence. The evidence presented by Choate et al. [24], however, is not conclusive of a flip-flop mechanism in that ATP dependence of exchange could be due to ATP acting through a 'regulatory' site, either to decrease k_{-2} or to increase k_3. Stronger evidence is perhaps provided by Ref. 27, which shows that ITP (which cannot fit into the noncatalytic sites of F_1) [12] stimulates ATP hydrolysis at suboptimal ATP concentrations *, and the more recent paper from Boyer's group [35].

* The reader should note that a bent $1/v$ in $1/s$ plot is not indicative of a flip-flop mechanism, i.e. a reaction in which two (or more) identical sites alternate as the active site. Such a mechanism would predict a linear plot [9,33].

Appendix

Calculation of rate constants for the ATPase reaction

1. At the break in Arrhenius plot for ATP hydrolysis (Fig. 3) at 18°C the rate limiting step of the enzyme changes. At this point, $k_2 = k_3$, using the symbols of Eqn. 1. By Eqn. 2, $1/k_{cat} = 1/k_2 + 1/k_3 = 2/k_3$ [30] i.e. at 18°C, $k_3 = 1.5 \cdot 10^3\ s^{-1}$. From Fig. 3, k_3 is virtually independent of temperature (E_a = 3 kcal/mol) and so at 30°C, $k_3 = 1.5 \cdot 10^3\ s^{-1}$.

2. From Fig. 3, k_2 is strongly dependent on temperature (rate limiting below 18°C, E_a = 29 kcal/mol). Assuming E_a remains constant between 6 and 36°C, at 18°C, $k_2 = 1.5 \cdot 10^3\ s^{-1}$. Therefore at 30°C, $k_2 = 1.7 \cdot 10^4\ s^{-1}$.

3. Subject to the provisions above, ADP is a competitive inhibitor of the enzyme. If $K_i^{ADP} = 30\ \mu M = k_3/k_{-3}$, we have: $k_{-3} = 5 \cdot 10^7\ l \cdot mol^{-1} \cdot s^{-1}$, which is not far from the diffusion limited rate.

4. To find k_{-2} we use the observation that k_3 is dependent on ATP. If we assume a flip-flop mechanism [24] then this dependence on ATP is the same as that of the overall reaction velocity, i.e.

$$k_3 = k_3^{max} \frac{([ATP])}{(K_m + ATP)} \tag{4}$$

P_i-H_2O exchange is a competition between product release (k_3) and reversal of bond splitting (k_{-2}). When these are about equal, we would expect about one excess ^{18}O to be incorporated into P_i (in addition to the single directly incorporated ^{18}O) since product release occurs on average about once per reversal. From Fig. 2 [24], this occurs at about 3 μM ATP (about 0.01 K_m).

Under these conditions $k_3 = 1.5 \cdot 10^3/100 = 15\ s^{-1}$. Thus k_{-2} under these conditions also equals 15 s^{-1}. There being no reason to assume otherwise, under saturating conditions, the value is taken of $k_{-2} = 15\ s^{-1}$.

5. k_1 and k_{-1} are estimated from experiments using Ado*PP*[NH]*P* as an ATP analogue. We show above that Ado*PP*[NH]*P* binds to the active site of the ATPase (Fig. 8), and that it is a powerful inhibitor of the enzyme. Fig. 9 shows that k_{-1} for Ado*PP*[NH]*P* from F_1 is very low and thus presumably the same holds for ATP. (Note that Ino*PP*[NH]*P* is a poor inhibitor of ITP hydrolysis [12,27] with K_i around 10 mM. Thus k_{-1} for ITP hydrolysis is around 10^7-times faster than for ATP hydrolysis).

6. If k_{-1} is negligible, $K_m = k_2/k_1$ (Eqn. 2). We have:

$K_m = 3 \cdot 10^{-4}$ M, and $k_2 = 1.7 \cdot 10^4\ s^{-1}$

Therefore $k_1 = 5 \cdot 10^7\ l \cdot mol^{-1} \cdot s^{-1}$, which again is close to the diffusion limited rate.

7. From Fig. 9, we can estimate k_{-1} for AMP-PNP as around $4 \cdot 10^{-3}\ s^{-1}$, and $K_d^{AdoPP[NH]P}$ as around 10^{-9} M. These values will approximate the corresponding values for ATP; if anything, K_d^{ATP} is likely to be lower than 10^{-9} M.

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