Inhibition of the Soluble Adenosine Triphosphatase from Mitochondria by Adenylyl Imidodiphosphate

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1. Adenylyl imidodiphosphate is an inhibitor with high affinity for the soluble ATPase (adenosine triphosphatase) from mitochondria. 2. The reaction of the inhibitor with the ATPase is slow and estimates for the association and dissociation reaction rate constants are given. 3. The number of binding sites for the inhibitor appears to be doubled in the presence of 2,4-dinitrophenol. 4. Adenylyl imidodiphosphate is less effective as an inhibitor of the ATPase activity of this enzyme than of the inosine triphosphatase activity. It is also less effective on the ATPase of frozen-thawed or intact mitochondria and did not inhibit ADP-stimulated respiration by intact mitochondria.

The bridge oxygen atom between the β and γ phosphorus atoms in ATP can be replaced by a CH_2 group, giving adenylyl methylenediphosphate, or by an NH group, giving adenylyl imidodiphosphate. Yount *et al.* (1971) have reported that these compounds are stable and that they are not substrates for a number of enzymes which hydrolyse ATP, including the mitochondrial ATPase.†

The apparent failure of the mitochondrial ATPase to hydrolyse these compounds means they may be useful in elucidating the mode of action of this enzyme. The data reported here show that not only is ATP(β,γ-NH) not significantly hydrolysed by the soluble ATPase from mitochondria, but also that it is an inhibitor with a high affinity for this ATPase. However, the high affinity of the inhibitor causes some uncertainty as to whether ATP(β,γ-NH) is in fact hydrolysed by the ATPase, since although we were not able to observe hydrolysis of ATP(β,γ-NH) by the ATPase we cannot exclude the possibility that undetectably small amounts were hydrolysed, giving rise to a product which was an inhibitor of high affinity, and that this inhibitory product then prevented hydrolysis of any more ATP(β,γ-NH). A useful feature of ATP(β,γ-NH) is that its reaction with the ATPase is sufficiently slow to be studied by normal assay techniques.

Materials and Methods

The preparation of the soluble ATPase from ox heart mitochondria was as described previously

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† Abbreviations: ATPase, adenosine triphosphatase; ITPase, inosine triphosphatase; ATP(β,γ-NH), adenylyl imidodiphosphate; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

(Philo & Selwyn, 1973), except that Tris–HCl buffers were replaced by Tris–H_2SO_4 buffers of the same Tris molarity in all cases. The enzyme assay system was as described previously (Philo & Selwyn, 1973), as was the method of preparation of rat liver mitochondria and the method of estimation of mitochondrial protein (Selwyn *et al.*, 1970).

The assay media used were: ITPase medium, 50mM-KCl, 0.25mM-Hepes buffer, 0.5mM-MgCl₂, 0.5mM-ITP, pH7.6; ATPase medium, 50mM-KCl, 0.25mM-Hepes buffer, 1mM-MgCl₂, 1mM-ATP, pH7.6. Preincubation of the enzyme with ATP(β,γ-NH) was done for 5min at 30°C with 10μl of stock enzyme solution being added to 90μl of 110μM-MgCl₂, 55mM-KCl, 275μM-Hepes buffer, pH7.6, and ATP(β,γ-NH) at various concentrations. MgCl₂ was included in this mixture because no inhibition was found to occur in the absence of bivalent metal ions. A period of 5min was chosen because incubation for times up to 30min did not show any significant increase in the amount of inhibition over that found at 5min.

Incubation of frozen-thawed rat liver mitochondria (4–6mg of protein) with ATP(β,γ-NH) and oligomycin was done in 5ml of 1mM-MgCl₂, 50mM-KCl, 0.25mM-Hepes buffer, pH7.6, and the reaction was started after 5min by addition of 0.1ml of 50mM-ITP.

Assays to determine the rate of reaction of ATP(β,γ-NH) with the ATPase were carried out by adding various amounts of ATP(β,γ-NH) to the ITPase assay medium before addition of the enzyme. Concentrations of ATP(β,γ-NH) stock solutions were determined from the E_{260} by using a value for the extinction coefficient of 15litre·mmol⁻¹·cm⁻¹, based on the assumption that the value is the same as for ATP.

ATP, ITP and ATP(β,γ-NH) were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ,
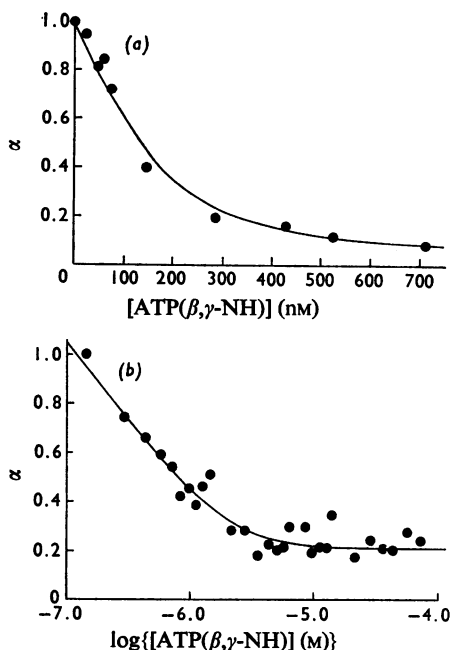
Fig. 1. *Inhibition of the soluble mitochondrial ATPase by adenylyl imidodiphosphate*

ATPase was incubated for 5min with adenylyl imidodiphosphate and then assayed for either (*a*) ITPase activity or (*b*) ATPase activity. Conditions for incubation and assay were as stated in the Materials and Methods section.

U.K. ATP(β,γ-NH) was supplied as the tetralithium salt and was stated by Boehringer to be: ATP(β,γ-NH) (from N) 80%, Li 4.5%, water 12%. Hepes buffer was obtained from Stuart Kinney Co., London W1A 4ES, U.K., or from Sigma (London) Chemical Co., Kingston-upon-Thames KY2 7B11, U.K. Oligomycin was also obtained from Sigma (London) Chemical Co.

Results and Discussion

When a sample of the ATPase which had been preincubated with ATP(β,γ-NH) was added to the ITPase medium the rate of ITP hydrolysis increased over the course of several minutes, presumably owing to dissociation of the enzyme-inhibitor complex. This made it necessary to measure initial rates. Hence the ITPase assay was preferable on account of the difficulty of measuring initial rates with ATP as substrate (Philo & Selwyn, 1973). However, the inhibitions of the ATPase and ITPase activities of this enzyme by ATP(β,γ-NH) are not identical processes. Fig. 1 shows the inhibition of these activities by different concentrations of ATP(β,γ-NH). The inhibition of the ITPase activity (Fig. 1*a*) tended to completion at 700nM-ATP(β,γ-
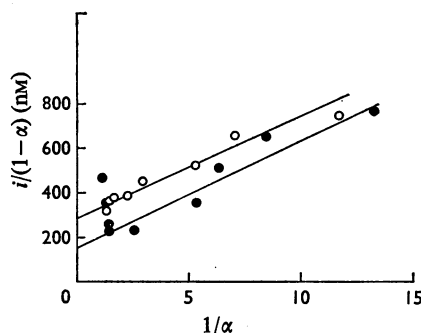

Fig. 2. *Plot to determine K_i for adenylyl imidodiphosphate and the concentration of sites to which it binds*

α = inhibited rate/uninhibited rate; *i* is the concentration of ATP(β,γ-NH) in the incubation mixture. Incubaiton was as described in the Materials and Methods section and assays were done in the ITPase assay medium; $\bullet$, no 2,4-dinitrophenol; $\circ$, 500μM-2,4-dinitrophenol.

NH), whereas the ATPase activity (Fig. 1*b*) was only 80% inhibited by 70μM-ATP(β,γ-NH). This suggests two alternative possibilities: first, that the enzyme-ATP(β,γ-NH) complex will hydrolyse ATP at a decreased rate, but will not hydrolyse ITP; secondly, that the enzyme sample used contained at least two forms of the enzyme, one capable of hydrolysing ITP, and probably ATP, which bound ATP(β,γ-NH) and was then inactive and another form, which would hydrolyse ATP but not ITP and which is not inhibited by ATP(β,γ-NH). Further kinetic and equilibrium-binding studies would be necessary to resolve this point. The data for ATP(β,γ-NH) inhibition of the ITPase activity can be analysed to determine the K_i for ATP(β,γ-NH) and the concentration of the site to which it binds by use of eqn. (1) (Dixon & Webb, 1964, p. 332):

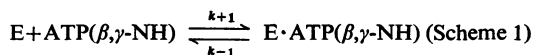$$\frac{i}{1-\alpha} = \frac{K_i}{\alpha} + e \qquad (1)$$

where *i* is the concentration of inhibitor, α is the inhibited rate/uninhibited rate, and *e* is the concentration of the binding site for the inhibitor. This equation applies strictly only in the case of non-competitive inhibitors, but it can be used with competitive inhibitors provided the inhibitor is only slowly displaced by substrate (Myers, 1952; Dixon & Webb, 1964, p. 334). This procedure gives the results (Fig. 2): $K_i = 48 \pm 6.2$nM, ATP(β,γ-NH)-binding site $= 26.9 \pm 10.1$ pmol/unit, where a unit is defined as that amount of enzyme which will produce an initial rate of 1μmol of ATP hydrolysed/min under the standard assay conditions (Philo & Selwyn, 1973).

When 500 μM-2,4-dinitrophenol was added to the preincubation mixture the results obtained were: $K_i = 39 \pm 3.5$ nM, ATP(β,γ-NH)-binding site = 55.8 ± 4.4 pmol/unit (Fig. 2). Although there is considerable scatter on Fig. 2 these results indicate a significant increase and perhaps a doubling of the number of ATP(β,γ-NH)-binding sites in the presence of 2,4-dinitrophenol. This doubling may be due to the phenol causing the release of one of the tightly bound adenine nucleotides (Harris *et al.*, 1973) and permitting ATP(β,γ-NH) to bind in its place.

The ITPase activity of frozen-thawed rat liver mitochondria was only inhibited by about 40% by 2.8 μM-ATP(β,γ-NH), but it was also only inhibited by 40% by 5 μg of oligomycin or by both 2.8 μM-ATP(β,γ-NH) and 5 μg of oligomycin. An attempt was made to determine the concentration of ATP(β,γ-NH)-binding sites in the frozen-thawed mitochondria by plotting the inhibition of the oligomycin-sensitive ITPase by ATP (β,γ-NH) according to eqn. (1), but the value obtained was not significantly different from zero. The K_i was estimated to be about 250 nM.

The ATPase of intact rat liver mitochondria was also incompletely inhibited by ATP(β,γ-NH), but in this case a greater inhibition was produced by oligomycin than by ATP(β,γ-NH). Atractyloside and ATP(β,γ-NH) together produced approximately the same amount of inhibition as oligomycin, which suggests that ATP(β,γ-NH) either was not translocated into intact mitochondria or did not bind to ATPase in intact mitochondria. ATP(β,γ-NH) also produced no effect on coupled respiration in rat liver mitochondria at a concentration of 17.5 μM, which also suggests these two alternative possibilities. Since it has been reported (M. Klingenberg, personal communication, cited in Yount *et al.*, 1971) that ATP(β,γ-NH) is translocated into mitochondria the second alternative seems more likely.

If the enzyme is added to the ITPase assay medium containing ATP(β,γ-NH) the inhibition takes a few minutes to reach a steady value, and when ATP(β,γ-NH) is present at concentrations much greater than the enzyme the time-course of the inhibition appears to be a first-order decay of enzyme activity. With ATP(β,γ-NH) in great excess a reaction such as that in Scheme 1 would be pseudo-first-order, and varying the concentration of ATP(β-γ-NH) produces the expected change in the apparent first-order rate constant.

$$E + \text{ATP}(\beta,\gamma\text{-NH}) \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot \text{ATP}(\beta,\gamma\text{-NH}) \quad \text{(Scheme 1)}$$

The reaction progress curves were fitted to eqn. 2:

$$x = p_1 t + p_2 (1 - e^{-k't}) \quad (2)$$

where x is the product concentration, t is time, $k' = k_{+1}q[I] + k_{-1}$, k_{+1}, k_{-1} are the rate constants in Scheme 1, [I] is the inhibitor concentration, q is a fac-

tor which allows for protection by the substrate and is calculated from the Michaelis equation and the substrate concentration, p_1 and p_2 were used as empirical constants, but $p_1 = v_0 k_{-1}/k'$ and $p_2 = v_0 k_{+1} q[I]/(k')^2$, where v_0 is the rate of ITP hydrolysis that would occur in the absence of inhibitor. A plot of k' against [I] allows determination of k_{-1} and $k_{+1}q$, and Fig. 3 shows a plot of k' values for the progressive inhibition of ITPase activity by different concentrations of ATP(β,γ-NH). Evaluation of k_{+1} is rendered uncertain by lack of knowledge about the competition between ITP and ATP(β,γ-NH) and hence uncertainty in the value of q. However, even if ITP and ATP(β,γ-NH) are strictly competitive the fraction of the enzyme protected by ITP is unlikely to exceed 0.33, since the K_m for ITP is 1 mM and the assay medium contained 0.5 mM-ITP. The corrected value of k_{+1} could be anything between 10.3×10^3 and 15.4×10^3 M$^{-1} \cdot$ s^{-1}, depending on the extent of protection by ITP. The intercept on this graph also gives an estimate, albeit not an accurate one, of the value of the rate constant for dissociation of the inhibitor, k_{-1}.

An estimate for k_{-1} can also be obtained by incubating the enzyme with ATP(β,γ-NH) in a small volume before assay. The dilution produced on adding the enzyme to the assay was arranged to change the ATP(β,γ-NH) concentration from 10 K_i to 0.1 K_i. The reaction progress curves which this produced, owing to the time-dependent dissociation of the ATP(β,γ-NH), were analysed by making two simplifying approximations; (1) that the change in ATP(β,γ-
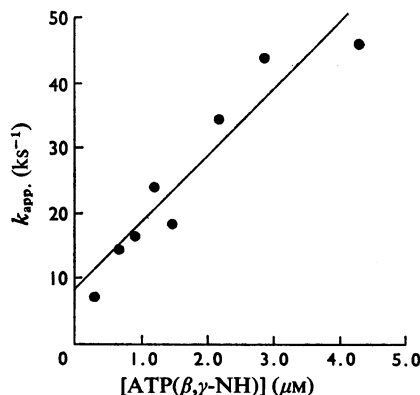

Fig. 3. *Relationship between the apparent first-order rate constant for the reaction of ATPase and ATP(β,γ-NH) and the ATP(β,γ-NH) concentration*

Values of apparent first-order rate constants ($k_{app.}$) were obtained by analysis of ITPase reaction progress curves as described in the text. The best-fit straight line was obtained by the method of least squares.
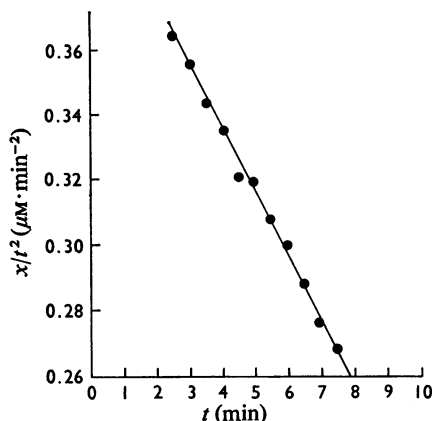
Fig. 4. *Plot to determine the rate constant for the dissociation of the postulated ATPase–ATP(β,γ-NH) complex*

The enzyme was incubated with ATP(β,γ-NH) and assayed as described in the text. x is product concentration (P_1 in this case) and t is time.

NH) concentration was such that the process could be considered as the enzyme going from all complexed with ATP(β,γ-NH) to all free enzyme and that no significant back reaction occurred; (2) that the change in ITP concentration during the assay was sufficiently small to be neglected.

On these assumptions the equation for the progress curve should be:

$$x = v_t \left(t + \frac{e^{-k_{-1}t} - 1}{k_{-1}} \right) \qquad (3)$$

where x is product concentration, t is time, k_{-1} is as indicated above and v_t is the final uninhibited rate. By expanding the exponential and rearranging this can be approximated to:

$$\frac{x}{t^2} = \frac{v_t k_{-1}}{2!} - \frac{v k_{-1}^2 t}{3!} \qquad (4)$$

Thus by plotting x/t^2 against t (Fig. 4) a straight line of intercept on the t axis of $3/k_{-1}$ should be obtained. This gives a value of $2.3 \times 10^{-3}\,\text{s}^{-1}$ for k_{-1}, compared with $8.9(\pm 4.1) \times 10^{-3}\,\text{s}^{-1}$ from Fig. 3.

As can be seen from Figs. 3 and 4 the estimate of $k_{-1} = 2.3 \times 10^{-3}\,\text{s}^{-1}$ from Fig. 4 is likely to be the more accurate. Even so, by using this value and one of $15.4 \times 10^3\,\text{M}^{-1}\cdot\text{s}^{-1}$ for k_{+1} Scheme 1 predicts that K_i, which should be k_{-1}/k_{+1}, will have a value of 150 nM. This is clearly not in agreement with the measured value of K_i (48 nM), but there is the question of systematic error to be considered before rejecting Scheme 1 on this basis. The calculated estimates of errors on the parameters account for random errors only and there may be biases in the methods used which are sufficiently large to produce the observed difference

in the two K_i estimates. One obvious source of bias is the difference in conditions under which K_i is measured compared with k_{+1} and k_{-1} (see the Materials and Methods section). In particular the absence of ITP from the incubation mixture for measuring K_i and its presence in the assays for measuring k_{+1} and k_{-1} may be the critical difference. Also, owing to the method of storage of the enzyme (Philo & Selwyn, 1973) the incubation mixture used for measuring K_i contained a higher concentration of glycerol than did the assays for measuring k_{+1} and k_{-1}.

Boyer *et al.* (1974) have proposed a mechanism for mitochondrial oxidative phosphorylation in which the energy for the phosphorylation of ADP is required not at the phosphorylation step but at a step in the dissociation of ATP from the enzyme. Their mechanism entails a sequential change from tightly bound to loosely bound ATP. In view of the close structural similarity of ATP and ATP(β,γ-NH) the present finding that the mitochondrial ATPase had a very high affinity for ATP(β,γ-NH) may be significant. The difference in apparent free energies of binding for ATP and ATP(β,γ-NH) can be calculated by assuming the dissociation constant for ATP is roughly the same as the K_m (400 μM; Philo & Selwyn, 1973) and the value obtained is $27\,\text{kJ}\cdot\text{mol}^{-1}$ ($6.5\,\text{kcal}\cdot\text{mol}^{-1}$). This could well be in accord with the hypothesis of Boyer *et al.* (1974), the non-hydrolysable ATP(β,γ-NH) coming into equilibrium in the tightly bound state, while the energy from hydrolysis of ATP maintains the enzyme–ATP complex mainly in the loosely bound state.

Fig. 5 shows the energy-profile diagrams for some postulated reactions of the ATPase. Fig. 5(*a*) shows a
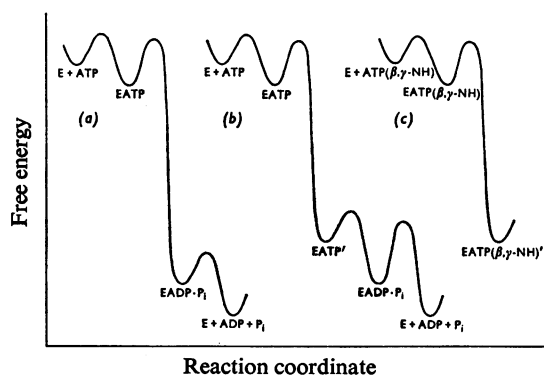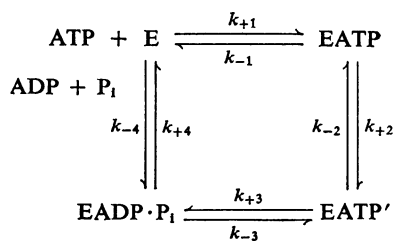

Fig. 5. *Energy profile diagrams of some postulated reactions of the mitochondrial ATPase*

(*a*) Conventional view of enzymic hydrolysis of ATP; (*b*) reaction mechanism for ATP hydrolysis incorporating a change from loosely to tightly bound ATP; (*c*) reaction mechanism for the non-hydrolysable substrate analogue ATP(β,γ-NH). EATP′ and EATP(β,γ-NH)′ represent tightly bound forms of the enzyme–adduct complex.

$$\text{ATP} + \text{E} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{EATP}$$

Scheme 2.

expressions for K_m are complicated. A more tractable expression is obtained if we consider the effect of hydrolysis on the ratio of the forms EATP′ to EATP, i.e. tightly bound to loosely bound ATP. In the absence of hydrolysis, which is the case for the ratio EATP(β,γ-NH)′ to EATP(β,γ-NH) this ratio is k_{+2}/k_{-2}, but when hydrolysis occurs the ratio EATP′/EATP becomes:

$$\frac{k_{+2}}{k_{-2}+k_{+3}k_{+4}/(k_{-3}+k_{+4})}$$

The basis of the hypothesis of Boyer *et al.* (1974) is that $k_{-2}/k_{+2} \ll 1$, in which case k_{-2} is also likely to be small in relation to $k_{+3}k_{+4}/(k_{-3}+k_{+4})$. Thus the faster the rate of hydrolysis the larger will be the values of k_{+3} and k_{+4} and the smaller will be the proportion of enzyme–adduct complex in the tightly bound form.

conventional view of the enzymic hydrolysis of ATP with small, but favourable, free-energy changes occurring at the binding of ATP and with the release of the products; the major free-energy change occurs at the hydrolytic step. Fig. 5(*b*) shows the ATPase reaction incorporating a change from loose to tight binding of ATP, with this step involving the greatest free-energy change. The binding of ATP(β,γ-NH) to the ATPase is shown in Fig. 5(*c*), including a change from loose to tight binding similar to that shown in Fig. 5(*b*) for ATP. As there is no hydrolysis of the ATP(β,γ-NH) it remains tightly bound to the ATPase.

Scheme 2 shows the ATPase reaction incorporating the additional step for the transformation from loosely bound ATP, EATP, to tightly bound ATP, EATP′. The steady-state kinetics of enzyme reactions with such additional steps are given in standard texts, such as Dixon & Webb (1964, p. 98), but the

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