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A kinetic study of the interaction between mitochondrial F_1 adenosine triphosphatase and adenylyl imidodiphosphate and guanylyl imidodiphosphate

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1. The presence of 5′-adenylyl imidodiphosphate, a non-hydrolysable analogue of ATP, in the solution used to assay the soluble bovine heart mitochondrial F_1-ATPase produced slow competitive inhibition. If the enzyme was preincubated with the inhibitor before the substrate, MgATP, was added, a partial re-activation was obtained. 2. The slow inhibitory process showed first-order rate kinetics, and therefore it seems likely that a conformational change of the enzyme occurs following a faster binding process. A reaction scheme is suggested. At pH 7.8 the rate constant for the inhibition reaction was calculated to be $6.7 \times 10^{-2}\,s^{-1}$ and that for the re-activation $3.8 \times 10^{-3}\,s^{-1}$, with $K_{eq.}$ 17.6, indicating that the inhibited enzyme–inhibitor complex will be favoured over the non-inhibited enzyme–inhibitor complex. 3. The presence of 5′-guanylyl imidodiphosphate in the solution used to assay F_1-ATPase produced rapid competitive inhibition, which was then slowly reversed until a steady state was reached. This might be explained by a rapid but reversible shift of the inhibition pathway induced by this non-hydrolysable analogue of ATP. A complex rate constant for the displacement of the inhibitor by the substrate of $7.6 \times 10^{-3}\,s^{-1}$ was calculated. 4. The results are discussed in the light of other recent observations about binding of 5′-adenylyl imidodiphosphate to F_1-ATPase and with reference to the binding-site-change mechanism of hydrolysis of ATP by F_1-ATPase.

The F_1-ATPase of mitochondria is the key enzyme in oxidative phosphorylation. This enzyme can be separated from the mitochondrial inner membrane in a soluble form, but it can then catalyse only ATP hydrolysis. Several recent reviews on F_1-ATPase have been published (Penefsky, 1979; Senior, 1979; Cross, 1981). Up to date many aspects of its structure and molecular mechanism of catalysis remain unclear. Some of the more important of them are as follows. (i) Stoicheiometry of subunits: both $\alpha_2\beta_2$ and $\alpha_3\beta_3$ possibilities have been proposed; but recent work seems to support the latter possibility (see Cross & Nalin, 1982, and references cited therein). (ii) Number and the type of nucleotide-binding sites (Harris, 1978; Slater *et al.*, 1979): in general, exchangeable or loosely bound and non-exchangeable or tightly bound nucleotides are distinguished; the first type can be related to catalytic sites, but we know little about the role of the latter, although their direct implication in catalysis seems unlikely (Gresser *et al.*, 1979; Harris & Baltscheffsky, 1979). (iii) Mechanism of catalysis: much recent work supports the energy-dependent binding-site-change mechanism originally proposed by Boyer and co-workers (Boyer *et al.*, 1973; Kayalar *et al.*, 1977).

[$\beta\gamma$-Imido]ATP and [$\beta\gamma$-imido]GTP are structural analogues of ATP and GTP respectively, introduced by Yount *et al.* (1971), that are not hydrolysed by mitochondrial F_1-ATPase. The interaction of [$\beta\gamma$-imido]ATP with this enzyme was first studied by following fluorescence changes in aurovertin (Penefsky, 1974). [$\beta\gamma$-Imido]ATP has also been shown to be a potent competitive inhibitor of F_1-ATPase activity (Schuster *et al.*, 1975*a*), of the ATP-dependent reduction of NAD^+ by succinate and of ATP–[^{32}P]P_i exchange; however, it does not affect the ATP synthesis from ADP and P_i (Penefsky, 1974). [$\beta\gamma$-Imido]ATP inhibits F_1-

Abbreviations used: F_1-ATPase, ATPase (EC 3.6.1.3) of mitochondrial coupling factor 1; [$\beta\gamma$-imido]ATP, 5′-adenylyl imidodiphosphate; [$\beta\gamma$-imido]GTP, 5′-guanylyl imidodiphosphate.

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ATPase in a time-dependent manner, forming a relatively stable complex (Philo & Selwyn, 1974; Harris *et al.*, 1978), and Harris *et al.* (1981) have shown that it inhibits by binding to the hydrolytic site of the enzyme. The stoicheiometry of the binding has been studied, and the presence of three exchangeable binding sites that are distinct from non-catalytic sites, i.e. tight sites, has been suggested (Cross & Nalin, 1982). However, they reported a K_i of 14nM, which is significantly lower than other previously reported values (Penefsky, 1974; Melnick *et al.*, 1975; Schuster *et al.*, 1975*a*).

The effect of [$\beta\gamma$-imido]GTP on F_1-ATPase activity has been studied less. It induces complex kinetic behaviour when it is present in the assay medium (Schuster *et al.*, 1975*a*); however, no inhibition is observed when the enzyme is preincubated with [$\beta\gamma$-imido]GTP but assayed in its absence (Harris *et al.*, 1978).

F_1-ATPase shows hysteretic behaviour on inhibition by preincubation with ADP (Di Pietro *et al.*, 1980). Furthermore, it has been shown that ADP and [$\beta\gamma$-imido]ATP inhibit F_1-ATPase by acting on the same site (Baubichon *et al.*, 1981; Krull & Schuster, 1981; Ortiz-Flores *et al.*, 1982). We study in the present paper the kinetic characteristics of the interaction between [$\beta\gamma$-imido]ATP and F_1-ATPase giving rise to a time-dependent inhibition. This is in turn compared with the effect produced by [$\beta\gamma$-imido]GTP. We suggest that these inhibitions also follow hysteretic patterns. Possible explanations of the hysteretic behaviour of F_1-ATPase by the binding-site-change mechanism are discussed.

Materials and methods

ATP, phosphoenolpyruvate, NADH, pyruvate kinase and lactate dehydrogenase were from Sigma Chemical Co. (Poole, Dorset, U.K.). [$\beta\gamma$-Imido]ATP and [$\beta\gamma$-imido]GTP were from P–L Biochemicals (Milwaukee, WI, U.S.A.). All the other chemicals used were of analytical grade.

Bovine heart mitochondria were prepared as described by Ferguson *et al.* (1977), and mitochondrial F_1-ATPase was purified by the method of Knowles & Penefsky (1972). The enzyme has typical specific activities of 90–100 units (μmol/min)/mg at 30°C when assayed as described previously (Fernández-Belda *et al.*, 1982). The enzyme was stored as a suspension in $(NH_4)_2SO_4$ solution at 4°C. Portions were removed when required and centrifuged at 1500*g* for 10min in the cold, to eliminate most of the $(NH_4)_2SO_4$; then the pelleted enzyme was resuspended in 250mM-sucrose/2mM-EDTA/10mM-Tris/acetate buffer, pH7.5, at room temperature. This F_1-ATPase preparation contained loosely bound nucleotides.

Inhibition of F_1-ATPase activity by [$\beta\gamma$-imido]ATP was studied at pH7.8, with MgATP concentrations of 0.2, 0.7 and 2mM. The [$\beta\gamma$-imido]ATP concentration was varied between 6 and 40μM. In other experiments F_1-ATPase was incubated with [$\beta\gamma$-imido]ATP in assay medium from which the MgATP had been excluded; after a time period between 5 and 30min the reaction was started by adding MgATP. The inhibition induced by [$\beta\gamma$-imido]GTP was studied at pH7.8 with substrate concentrations of 0.05, 0.125 and 0.25mM and analogue concentrations between 10 and 80μM. Reactions were started by adding the enzyme to the mixture. At the concentrations used here, [$\beta\gamma$-imido]ATP and [$\beta\gamma$-imido]GTP do not inhibit the enzymes of the assay coupled system (Schuster *et al.*, 1975*b*). Protein was assayed by a procedure involving the use of Folin's reagent (Besandoun & Weinstein, 1976).

Results

Inhibition of F_1-ATPase activity by [$\beta\gamma$-imido]ATP

Fig. 1 shows that the presence of [$\beta\gamma$-imido]ATP in the assay medium produced a slow time-dependent inhibition of F_1-ATPase. This observation agrees with those by previous authors (Penefsky, 1974; Melnick *et al.*, 1975). From the analysis of the

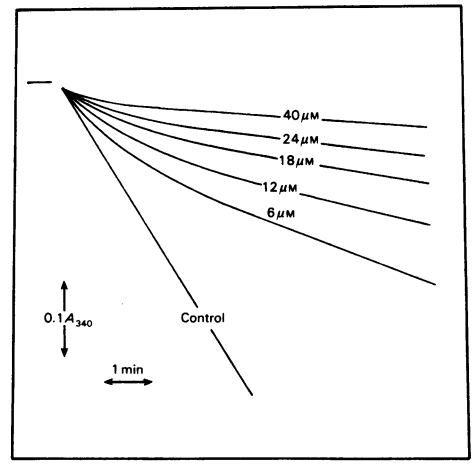


Fig. 1. *Inhibition of F_1-ATPase by [$\beta\gamma$-imido]ATP*
Reactions were initiated by adding 0.017 unit of F_1-ATPase to 1ml of a solution containing 0.7mM-ATP, 0.7mM-$MgCl_2$, 2mM-phosphoenolpyruvate, 0.24mM-NADH, 7.68 units of pyruvate kinase, 12.7 units of lactate dehydrogenase, 50mM-triethanolamine/HCl buffer, pH7.8, and the indicated concentration of [$\beta\gamma$-imido]ATP. The spectrophotometric traces obtained with increasing [$\beta\gamma$-imido]ATP concentrations are shown.

steady-state kinetics, by means of a Dixon plot (Fig. 2), the inhibition was deduced to be of competitive type with a K_i of 0.5 μM. Fig. 2 shows that the kinetics did not differ from the Michaelis–Menten type. This observation is in agreement with the results obtained by Penefsky (1974) but is at variance with those reported by Schuster *et al.* (1975*a*). These last authors claimed that co-operativity effects and non-competitive inhibition were implicated in this interaction. The reason for this discrepancy is unclear.

Fig. 3 shows that during the first few minutes the time-dependent inhibition of the enzyme was reversible to a certain extent. When the reaction was initiated by adding 0.012 unit of F_1-ATPase to the assay mixture containing 0.7 mM-MgATP and 6 μM-[$\beta\gamma$-imido]ATP, the activity decreased with time until a steady-state rate was reached. On the other hand, if the reaction was initiated by adding MgATP to the enzyme preincubated for 5 min with [$\beta\gamma$-imido]ATP, the enzymic activity was almost completely inhibited initially but recovered slowly with time, until it reached the same steady-state rate as that seen when the reaction was initiated by adding F_1-ATPase (i.e. without preincubation). However, if the enzyme was preincubated with [$\beta\gamma$-imido]ATP for longer periods of time (20–30 min), the lag periods were progressively increased (results not shown).

Harris *et al.* (1978) proposed a simple scheme for the hydrolysis of ATP by the mitochondrial ATPase where a modified enzyme complex symbolized by E*·ATP was involved; on binding of [$\beta\gamma$-imido]ATP, E*·[$\beta\gamma$-imido]ATP is formed. Further steps, i.e. hydrolysis, would be blocked for the last complex.

On the basis of this suggestion, the mechanism of inhibition of F_1-ATPase by [$\beta\gamma$-imido]ATP would be as shown in Scheme 1.

It was assumed that k_{+3} and k_{-3} were of the same order as k_{+1} and k_{-1} respectively but were signifi-

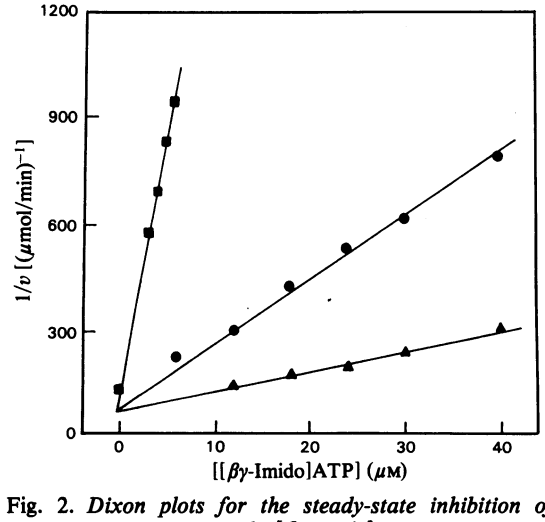


Fig. 2. *Dixon plots for the steady-state inhibition of F_1-ATPase by [$\beta\gamma$-imido]ATP*

Different MgATP concentrations were used, in an assay system otherwise similar to that described in Fig. 1 legend. ■, 0.2 mM-MgATP; ●, 0.7 mM-MgATP; ▲, 2 mM-MgATP. $K_i = 0.5\ \mu$M.

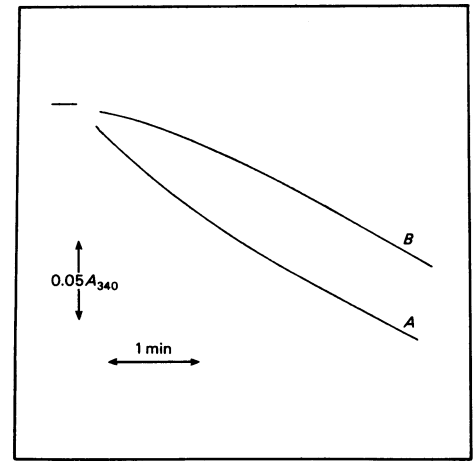
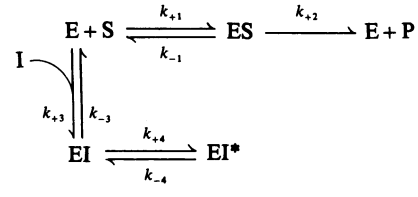


Fig. 3. *Activity of F_1-ATPase after preincubation with [$\beta\gamma$-imido]ATP*

F_1-ATPase activity was followed spectrophotometrically. For curve *A* the reaction was initiated by addition of 0.012 unit of F_1-ATPase to an assay system similar to that described in Fig. 1 legend, containing 6 μM-[$\beta\gamma$-imido]ATP. For curve *B* 0.012 unit of F_1-ATPase was added to an assay system similar to that described in Fig. 1 legend, containing 6 μM-[$\beta\gamma$-imido]ATP, but without MgATP, and, after 10 min of preincubation, the reaction was initiated by adding the MgATP.



Scheme 1. *Mechanism of inhibition of F_1-ATPase by [$\beta\gamma$-imido]ATP*

E is F_1-ATPase; S is MgATP; P is MgADP and P_i; I is [$\beta\gamma$-imido]ATP; EI is the complex F_1-ATPase–[$\beta\gamma$-imido]ATP; EI* is a modified and inactive form of the enzyme–inhibitor complex.

catively higher than k_{+4} and k_{-4} respectively. This indicated that the initial alterations of F_1-ATPase with [$\beta\gamma$-imido]ATP were rapid, and that the transformation of either EI into EI* or EI* into EI was the slow step that produced the time-dependent inhibition (or re-activation). Therefore when [$\beta\gamma$-imido]ATP is added to the assay mixture it will rapidly bind to F_1-ATPase molecules, and this complex will slowly give the inhibited form of the enzyme, (E·[$\beta\gamma$-imido]ATP)*. As a consequence a time-dependent inhibition, eventually reaching a steady state, will be observed.

The partial re-activation found when the reaction was started by adding MgATP will be due to the displacement by the substrate of [$\beta\gamma$-imido]ATP previously bound to the enzyme during the pre-incubation period. This displacement will favour the mechanism leading to formation of product.

Given these characteristics of the hydrolysis of ATP by F_1-ATPase in the presence of [$\beta\gamma$-imido]ATP, the rate constants of the slow inhibition can be evaluated by studying the transition period of the process. A detailed account of the kinetic approach used is included in the Appendix.

It can be deduced [see eqn. (13) in the Appendix] that $\ln(P_s - P) = \ln P_s^0 - k_{app.}t$, where P_s is the value of the asymptotic straight line of the curve of accumulation of product at each particular time, P is the amount of product actually accumulated at each particular time, P_s^0 is the value of the asymptotic line at time zero, $k_{app.}$ is the rate constant and t is the time.

It was deduced from a Guggenheim analysis (Guggenheim, 1926) of the traces shown in Fig. 1 that the inhibition reaction followed pseudo-first-order kinetics, in the transition state; therefore a plot of $\ln(P_s - P)$ versus t will give a straight line, and this was indeed obtained (Fig. 4). $k_{app.}$ (in s^{-1}) can be evaluated from these plots, corresponding to the slopes of the straight lines. $k_{app.}$ values increased with increasing inhibitor concentrations (Table 1), and they were higher when substrate concentrations decreased (Fig. 5), indicating a competitive mechanism of inhibition.

Fig. 5 shows a plot of $1/k_{app.}$ versus $1/$[[$\beta\gamma$-imido]ATP] at different ATP concentrations; hyperbolic curves that crossed on the ordinate were obtained. A k_∞ value of 7.18×10^{-2} s^{-1} for ATPase inhibition by [$\beta\gamma$-imido]ATP was calculated. This value corresponds to the value of $k_{app.}$ when the inhibitor concentration tends to infinity [see eqn. (14) in the Appendix].

From the extrapolation of the asymptiotic lines of the inhibition curves (Fig. 1), for $t = 0$, P_s^0 can be calculated. By using these P_s^0 values, v_0, i.e. the initial rate, will be evaluated from:

$$P_s^0 = \frac{v_0 - v_s}{k_{app.}}$$

[see eqn. (15) in the Appendix], where v_s represents

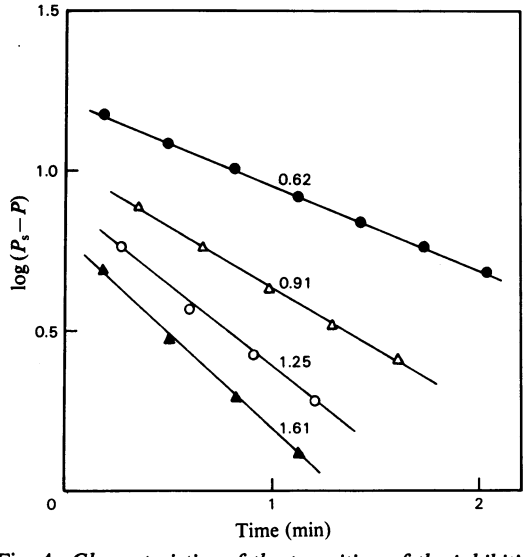


Fig. 4. *Characteristics of the transition of the inhibition of F_1-ATPase by [$\beta\gamma$-imido]ATP*
Activities were measured as indicated in Fig. 1 legend, with an MgATP concentration of 0.7 mM and different concentrations of [$\beta\gamma$-imido]ATP: ●, 6 μM; △, 12 μM; ○, 18 μM; ▲, 24 μM. $k_{app.}$ values, in min^{-1}, are given in each case, calculated after eqn. (13) of the Appendix.

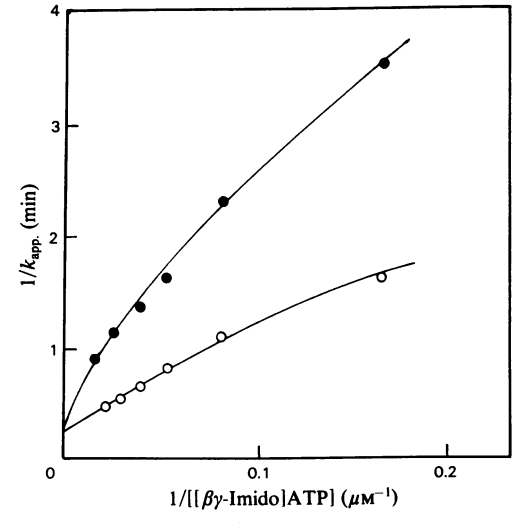


Fig. 5. *Graphical calculation of $k_{app.}$ for the inhibition of F_1-ATPase by [$\beta\gamma$-imido]ATP*
Conditions were as indicated in Fig. 1 legend. MgATP concentrations were: ○, 0.7 mM; ●, 2 mM.

Table 1. *Kinetic parameters for the inhibition of F_1-ATPase by [βγ-imido]ATP*

Measurements were done in the assay medium described in the Materials and methods section except that the pH was 7.8 and the MgATP concentration was 0.7mM. $k_{app.}$ represents the rate constant of the inhibition process, v_0 is the initial rate and v_s is the rate in the steady state.

Concn. of [βγ-imido]ATP ...	Kinetic parameter 6μM	12μM	18μM	24μM
$10^2 \times k_{app.}$ (s^{-1})	1.03	1.51	2.08	2.68
v_0 (nmol/min)	14.53	13.33	12.58	11.58
v_s (nmol/min)	4.29	3.43	2.36	1.88

the rate in the steady state. From knowledge of v_0 and v_s (Table 1) k_{+4} and k_{-4} (rate constants of the first-order slow process) can be calculated from:

$$k_{-4} = \frac{v_s k_{app.}}{v_0}$$

and

$$k_\infty = k_{+4} + k_{-4}$$

At pH 7.8, k_{+4} is $6.7 \times 10^{-2}\,s^{-1}$, k_{-4} is $3.8 \times 10^{-3}\,s^{-1}$ and $K_{eq.}$ is 17.6. Therefore k_{+4} is higher than k_{-4}, indicating that EI* will be favoured over EI and thus explaining the stability of the inhibition of the ATPase (Philo & Selwyn, 1974; Harris *et al.*, 1978).

Inhibition of F_1-ATPase activity by [βγ-imido]GTP

Incubation of F_1-ATPase with [βγ-imido]GTP in the solution in which ATPase is assayed resulted in an immediate inhibition with a delayed (slow) re-activation of the enzyme until a steady state was reached. This is shown in Fig. 6, where the ATPase reaction was started by adding 0.013 unit of F_1-ATPase to assay mixture containing MgATP and different concentrations of [βγ-imido]GTP.

From analysis of the spectrophotometric traces corresponding to the steady state by means of a Dixon plot (Dixon, 1953) (Fig. 7), it was found that [βγ-imido]GTP gave a competitive inhibition of the same type as that produced by [βγ-imido]ATP: a K_I value of 12.3μM was calculated.

These results can also be explained in terms of Scheme 1. [βγ-Imido]GTP will evoke a rapid establishment of the inhibition pathway, but if this pathway is reversible then with time the F_1-ATPase will be re-activated as a result of a slower displacement of the inhibitor of the substrate from the enzyme binding site.

Frieden (1970) has studied this type of reaction, in which slow displacement of an inhibitor by substrates or by activators occurs. A complex first-order reaction for the transition is obtained, which depends on the several rate constants involved and on the concentrations of substrate and inhibitors. By plotting $\log(P_s - P)$ versus time (Guggenheim, 1926), an estimate of the constant can be made from the lag period of the curve of accumulation of product with time (Fig. 6). It was found to be $7.6 \times 10^{-3}\,s^{-1}$ in these conditions.

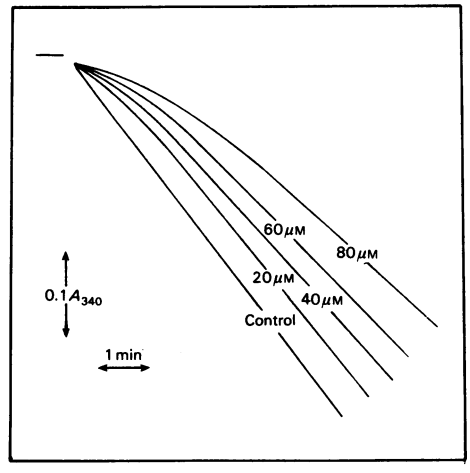


Fig. 6. *Inhibition of F_1-ATPase by [βγ-imido]GTP*

Spectrophotometric traces of the F_1-ATPase assays are shown. Reactions were initiated by addition of 0.013 unit of F_1-ATPase to an assay system similar to that described in Fig. 1 legend, except that the MgATP concentration was 0.125mM, containing the stated concentrations of [βγ-imido]GTP.

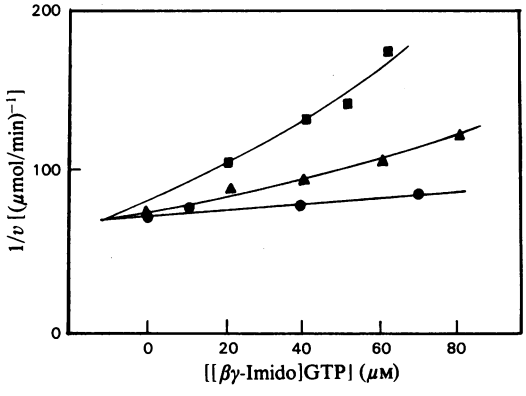


Fig. 7. *Dixon plots for the steady-state inhibition of F_1-ATPase by [βγ-imido]GTP*

Different MgATP concentrations were used, in an assay system similar to that indicated in Fig. 6 legend. ■, 0.2mM-MgATP; ▲, 0.7mM-MgATP; ●, 2mM-MgATP. $K_I = 12.3\,\mu M$.

Discussion

Enzyme inhibitors that are structural analogues of a substrate may interact with the active centre and can therefore be used to study the kinetic characteristics of enzyme-catalysed reactions.

Most of the studies published in this field have been conducted under steady-state conditions, assuming that an equilibrium between the different species participating in the reactions is rapidly established; however, this is not always true. For example, in time-dependent inhibitions the addition of the enzyme to the assay medium containing the inhibitor yields a relatively rapid initial rate that then decreases until the steady-state rate is reached. They have the kinetic characteristics of the hysteretic type of inhibition (Frieden, 1970).

It should be noted that the time-dependent inhibition gives transition-state kinetics in the time scale of the steady-state kinetics, and hence initial rate and steady-state rates, which are normally synonymous, are different in this case.

$$E + ATP \overset{1}{\rightleftharpoons} E \cdot ATP \overset{2}{\rightleftharpoons} E^* \cdot ATP \overset{3}{\rightleftharpoons} E^* \cdot ADP \cdot P_i \overset{4}{\rightleftharpoons} E + ADP + P_i$$

(Scheme 2)

It has been shown (Cross & Nalin, 1982) that F_1-ATPase can bind 3 mol of [$\beta\gamma$-imido]ATP/mol, at three exchangeable sites. One of these sites binds [$\beta\gamma$-imido]ATP slowly with high affinity, and the other two bind this nucleotide more rapidly but with lower affinity. In the conditions of [$\beta\gamma$-imido]ATP concentration and the time scale of our experiments we are probably observing the binding of 1 mol of [$\beta\gamma$-imido]ATP/mol to one of the 'rapid sites' (see Fig. 2 of Cross & Nalin, 1982). Nevertheless we observed maximum inhibition. This is in agreement with a previous suggestion that [$\beta\gamma$-imido]ATP is an inhibitor of the type 'one-third of the sites' (Cross & Nalin, 1982), i.e. occupancy of one-third of the sites is sufficient for maximum inhibition.

We calculated that the K_i for [$\beta\gamma$-imido]ATP inhibition is 0.5 μM, which is close to values reported by some authors (Penefsky, 1974; Melnick *et al.*, 1975) but significantly differs from the 14 nM value reported by Cross & Nalin (1982). However, this discrepancy must be due to the fact that in the last-mentioned work the concentrations of [$\beta\gamma$-imido]ATP used for the assay were in the nanomolar range and therefore the K_i value refers to the higher-affinity site only. As stated above, we have been concerned with the lower-affinity sites, which indeed have a K_d value for [$\beta\gamma$-imido]ATP of about 1.0 μM (Cross & Nalin, 1982), i.e. of the same order of magnitude as the K_i value reported by us.

The slow inhibition that follows first-order kinetics strongly suggests the existence of a conformational change of the enzyme–inhibitor complex leading to a inhibited form of the enzyme. A similar explanation was given by Tondre & Hammes (1973) for the binding of N^1N^6-etheno-ADP to F_1-ATPase. Therefore this provides support for our suggested Scheme 1.

The inhibition by [$\beta\gamma$-imido]GTP is more rapid than that of [$\beta\gamma$-imido]ATP, but at the same time is more easily reversed by MgATP. It was shown previously (Harris *et al.*, 1978) that analogues of ATP such as GTP will not form a tight complex with the enzyme and therefore could not be hydrolysed in a coupled way. Therefore the simplified scheme for a single-site enzyme (Harris *et al.*, 1978) will be transformed into:

$$E + GTP \overset{1}{\rightleftharpoons} E \cdot GTP \overset{2}{\rightleftharpoons} E^* \cdot GTP \overset{3}{\rightarrow} E^* \cdot GDP \cdot P_i \overset{4}{\rightleftharpoons} E + GDP + P_i$$

(Scheme 3)

[Note that step 4 of Schemes 2 and 3 might be irreversible (Harris *et al.*, 1978)]. Scheme 3 explains why the E*·[$\beta\gamma$-imido]GTP complex is less stable than E*·[$\beta\gamma$-imido]ATP and is easily displaced by ATP, diverting the enzyme back towards the productive pathway (Scheme 1).

It is interesting to relate the behaviour of F_1-ATPase, showing hysteretic inhibition by [$\beta\gamma$-imido]ATP and [$\beta\gamma$-imido]GTP, with the binding-site-change mechanism of catalysis proposed by Boyer and co-workers (Boyer *et al.*, 1973; Kayalar *et al.*, 1977). Fig. 8(A) shows a current version for the hydrolysis of ATP (Cross, 1981). Sites T (tight), O (open) and L (ligand) are present at any one moment. T is a tight-binding site for ligands and catalytically active, L is a loose-binding site for ligands and catalytically inactive, and O is an open site with very low affinity for ligand and catalytically inactive. Apart from these interconvertible sites, other binding sites might exist on F_1-ATPase (see Harris, 1978; Slater *et al.*, 1979).

Cross & Nalin (1982) found three exchangeable sites for the binding of [$\beta\gamma$-imido]ATP to F_1-ATPase, of which two have low affinity and are rapidly filled and one has high affinity but binds [$\beta\gamma$-imido]ATP more slowly.

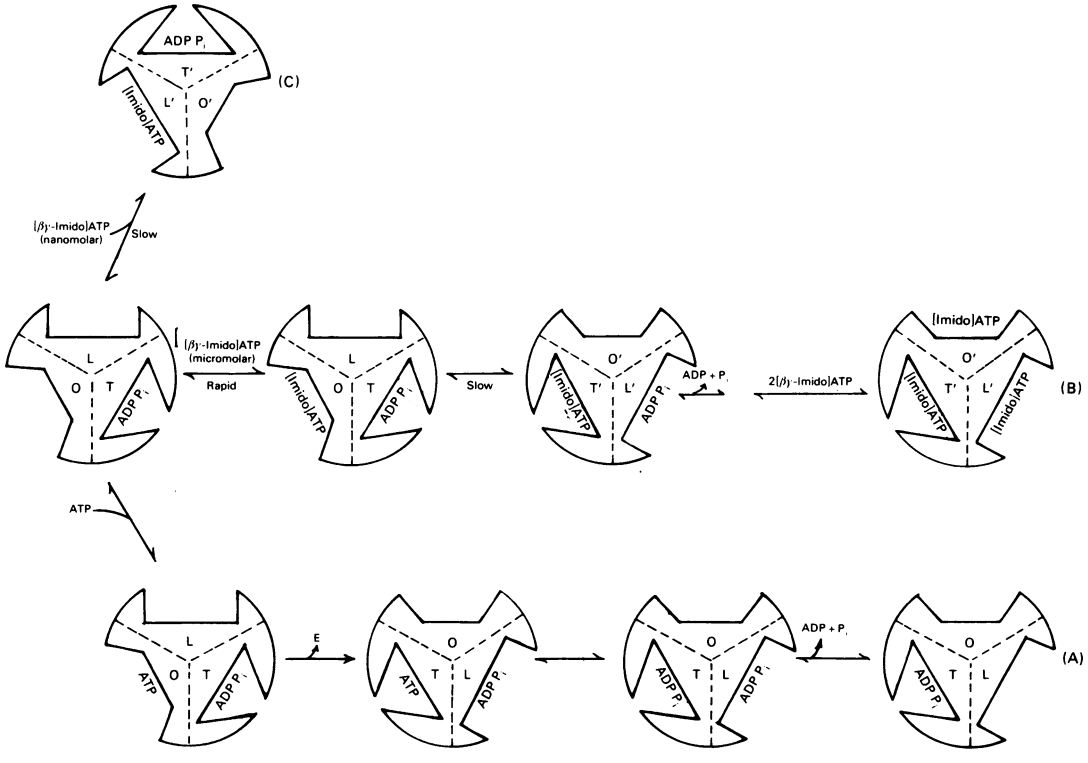


Fig. 8. *Suggested schemes for the hydrolysis of ATP by F_1-ATPase and for the interaction of F_1-ATPase with [$\beta\gamma$-imido]ATP*

These schemes follow the energy-dependent binding-site-change mechanism initially proposed by Boyer *et al.* (1973) as developed by Cross (1981). Three hydrolytic sites per molecule of F_1-ATPase are assumed. L is a loose-binding site for ligands and catalytically inactive; T is a tight-binding site for ligands and catalytically active; O is an open site with very low affinity for ligands and catalytically inactive. T', O' and L' represent modified sites resulting from the binding of [$\beta\gamma$-imido]ATP to the enzyme. The normal pathway of hydrolysis of ATP is shown in (A), where E represents energy. The interaction of [$\beta\gamma$-imido]ATP in micromolar concentration with F_1-ATPase is shown in (B). Finally, the interaction of [$\beta\gamma$-imido]ATP in the nanomolar range with F_1-ATPase is shown in (C).

On the basis of our observations we suggest a tentative scheme to explain the interaction of [$\beta\gamma$-imido]ATP with F_1-ATPase that follows the mechanism proposed by Boyer and co-workers (Boyer *et al.*, 1973; Kayalar *et al.*, 1977) (Fig. 8B).

Incubation of F_1-ATPase with [$\beta\gamma$-imido]ATP in the micromolar range would induce binding of this analogue of ATP to site O, with a $K_i \simeq 1\,\mu$M. As a result of the strain introduced by this nucleotide in the binding site, a slow transition would produce an altered form of the enzyme with sites L', O' and T', somehow different from those of the normal enzyme that has bound ATP. [$\beta\gamma$-Imido]ATP would now be bound to site T'. As [$\beta\gamma$-imido]ATP cannot be hydrolysed, this step would not be totally irreversible. The kinetics studied in the present investigation would correspond precisely to this transition. Further binding of two molecules of [$\beta\gamma$-imido]ATP might occur, and one of these two molecules would bind to site L' with high affinity ($K_i \simeq 1\,\mu$M) but very slowly (~30 min, as shown in Fig. 2 of Cross & Nalin, 1982), i.e. out of the time range of the normal enzymic assay (a few minutes). Incubation of F_1-ATPase with [$\beta\gamma$-imido]ATP in the nanomolar range would produce binding in site L only (Fig. 8C) with a $K_i \simeq 14$ nM and very slowly.

This mechanism would explain the inhibition of the type 'one-third of the sites' at both low and high concentrations of [$\beta\gamma$-imido]ATP.

On the interpretation suggested above, if [$\beta\gamma$-imido]GTP has a rate constant of binding to site O higher than that of ATP binding, it would produce competitive inhibition. In this case the enzyme molecules with bound [$\beta\gamma$-imido]GTP would be diverted into a 'dead-end', but would slowly revert to the active form, since the nucleotide will not be a

good fit to the site, and consequently a lag will be observed in the enzymic activity.

Although this mechanism is speculative at the present moment, we consider that models similar to it may explain many of the observations of 'co-operative' interactions between the catalytic sub-units of F_1-ATPase (see Cross, 1981, and references cited therein).

Also explicable by our scheme is the release and binding of [$\beta\gamma$-imido]ATP from a very-high-affinity site that is strongly influenced by the binding of adenine nucleotides at additional sites (Penefsky, 1979; Chernyak & Kozlov, 1979; Nalin & Cross, 1980) and the observation by Harris *et al.* (1981) of [$\beta\gamma$-imido]ATP inhibition of F_1-ATPase by binding to the hydrolytic site.

Also related to this might be the observed hysteretic inhibition of F_1-ATPase induced by preincubation with ADP (Di Pietro *et al.*, 1980; Baubichon *et al.*, 1981). We consider that a scheme similar to that of Fig. 8 might explain this inhibition. Ortiz-Flores *et al.* (1982) have in fact pointed out that ADP and [$\beta\gamma$-imido]ATP inhibit hydrolysis by acting on the same site. Further experiments are needed to test this hypothesis, however.

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APPENDIX

In Scheme 1 of the main paper the steps involved in the catalysis of formation of product and those involves in the initial interaction of E with I are assumed to be rapid and therefore they would reach a steady state immediately:

$$\frac{d[ES]}{dt} = k_{+1}[S][E] - (k_{-1} + k_{+2})[ES] = 0 \quad (1)$$

$$\frac{d[E]}{dt} = -(k_{+1}[S] + k_{+3}[I])[E] + (k_{-1} + k_{+2})[ES] + k_{-3}[EI] = 0 \quad (2)$$

$$\frac{d[EI]}{dt} = k_{+3}[I][E] - k_{-3}[EI] = 0 \quad (3)$$

If $[E_s]$ represents the concentration of the enzyme involved in the steady state:

$$[E_s] = [E] + [ES] + [EI] \quad (4)$$

whereas in the slow step:

$$-\frac{d[E_s]}{dt} = \frac{d[EI^*]}{dt} = k_{+4}[EI] - k_{-4}[EI^*] \quad (5)$$

therefore:

$$[E_T] = [E_s] + [EI^*] \quad (6)$$

The concentration of enzyme in the steady state, from eqns. (1), (2) and (3), will be:

$$[E_s] = [EI]\left(\frac{K_i}{[I]} + 1 + \frac{K_i[S]}{[I]K_m}\right) \quad (7)$$

being

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad \text{and} \quad K_i = \frac{k_{-3}}{k_{+3}}$$

Substituting in eqn. (5) and keeping in mind eqn. (6):

$$-\frac{d[E_s]}{dt} = k_{+4} \cdot \frac{[E_s]}{\frac{K_i}{[I]} + 1 + \frac{K_i[S]}{[I]K_m}} - k_{-4}[E_T] + k_{-4}[E_s] \quad (8)$$

By integration of eqn. (8), knowing that for $t = 0$ the concentration of E_s is equal to that of E_T, and substituting

$$v = k'[E_s],$$

$$v = k'[E_T]$$

and

$$k_{app.} = \frac{k_{+4}}{\frac{K_i}{[I]} + 1 + \frac{K_i[S]}{[I]K_m}} + k_{-4}$$

the following rate equation can be obtained:

$$v = \frac{k_{-4}}{k_{app.}} \cdot v_0 \cdot \left(1 + \frac{k_{app.} - k_{-4}}{k_{-4}} \cdot e^{-k_{app.}t}\right) \quad (9)$$

The steady-state rate for this system would be obtained when $t \to \infty$ and therefore

$$v_s = \frac{k_{-4}}{k_{app.}} \cdot v_0 \quad (10)$$

As $v = dP/dt$, the integrated equation for the accumulation of product with time will be obtained:

$$P = v_s t + (v_0 - v_s) \cdot \frac{1 - e^{-k_{app.}t}}{k_{app.}} \quad (11)$$

The equation for the asymptote to the curve of accumulation of product will be obtained when $t \to \infty$:

$$P = v_s t + \frac{v_0 - v_s}{k_{app.}} \quad (12)$$

Subtracting P from P_s and taking the logarithm:

$$\ln(P_s - P) = \ln P_s^0 - k_{app.}t \quad (13)$$

$k_{app.}$ can be determined from the slope of the straight line, by means of a Guggenheim analysis for first-order processes.

It is interesting to define the limit of $k_{app.}$ when the concentration of inhibitor tends to infinity:

$$\lim_{[I]\to\infty} k_{app.} = k_{+4} + k_{-4} = k_\infty \quad (14)$$

Hence, keeping constant the concentrations of substrate and of enzyme, and changing the concentration of inhibitor, the value of $k_{app.}$ can be determined for each curve of accumulation of product. Furthermore, by plotting $1/k_{app.}$ versus $1/[I]$, k_∞ can be obtained, corresponding to the intersection with the ordinate:

$$P_s^0 = \frac{v_0 - v_s}{k_{app.}} \quad (15)$$

and, as v_s is the slope of the asymptote and $k_{app.}$ is a known value, v_0 can be determined.

Finally, by using

$$k_{-4} = \frac{v_s k_{app.}}{v_0}$$

k_{-4} can be obtained and from eqn. (14) k_{+4} can be deduced.