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MgATP-INDUCED INHIBITION OF THE ENZYMIC ACTIVITY OF CHLOROFORM-RELEASED OX-HEART MITOCHONDRIAL ATPase

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Summary. Preincubation of the chloroform-released ox-heart mitochondrial ATPase with its substrate, MgATP, results in a time-dependent inhibition of its ATPase activity. The inhibition is irreversible on the time-scale of an ATPase assay. It is not due to the accumulation of ADP and P_i. The extent of the inhibition is proportional to the number of turnovers of the enzyme during the preincubation period. It is suggested that the MgATP-induced inhibition described here is due to an intermediate enzyme-substrate complex of the ATP-hydrolytic pathway becoming converted into an inhibited enzyme species.

Mitochondrial ATPase is known to possess multiple nucleotide-binding sites (1-3): one or more of these are catalytic sites at which MgATP is bound and then hydrolysed. Other sites may serve a regulatory role (2,3). Harris and co-workers have shown that preincubation of the F_1-ATPase with MgATP could result in a time-dependent 'irreversible' inhibition of ATPase activity (4,5). Since preincubation of F_1-ATPase with equal concentrations of MgATP or MgADP resulted in similar degrees of inhibition, they concluded that inhibition induced by preincubation with MgATP was in fact due to MgADP, produced by ATP hydrolysis(4).

In this report we show that the chloroform-released ox-heart mitochondrial ATPase is inhibited by preincubation with MgATP but not by preincubation with MgADP. Hence, the nature of the inhibition appears to be different from that described by Harris et al. with F_1-ATPase (4,6). The inhibition of chloroform-released ATPase activity by preincubation of the ATPase with MgATP is due to ATP hydrolysis. It is not caused by the products of ATP hydrolysis. We propose

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that the process of MgATP-induced inhibition involves an intermediate enzyme complex of the ATP-hydrolytic pathway which can become transformed into an enzyme species which has no ATPase activity.

MATERIALS AND METHODS

Preparation of chloroform-released ATPase. Chloroform-released ATPase was prepared as in Beechey et al. (7,8) with some minor modifications to ensure that the free nucleotide level was low. Ox-heart submitochondrial particles, prepared as described (9), were washed with 0.25M-sucrose, 10mM-Hepes/KOH, pH 7.6 and then resuspended in the same medium at a protein concentration of about 5 mg/ml. After chloroform-treatment as described (7), the dilute solution of ATPase was concentrated to about 0.5-1 mg of protein/ml by ultra-filtration using a Diaflo XM-100A membrane in a stirred Amicon ultra-filtration cell. The buffer was then changed from 10mM-Hepes/KOH, pH 7.6 and the ultra-filtration was continued until about 15 times the volume of ATPase-containing solution had been passed through the membrane. Finally, the ATPase solution was concentrated to between 2 and 4 mg of protein/ml and 0.1 ml aliquots were freeze-dried and then stored at -20°C over silica gel. The specific activity of different preparations lay between 30 and 40 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. No loss of ATPase activity was found on reconstitution of the freeze-dried ATPase, which had been stored for up to six months. As judged by polyacrylamide gel electrophoresis (in the presence and absence of sodium dodecyl sulphate), followed by staining with Coomassie blue the ATPase preparation was over 90% pure (8). The molecular weight of the ATPase was taken to be 360,000.

Measurement of ATPase activity. ATPase activity was measured using a coupled enzyme assay basically as described (9) but the reaction mixture consisted of 50 mM-Hepes/KOH, 5mM-$MgSO_4$, 0.35 mM-NADH, 5mM-sodium phosphoenolpyruvate, 50µg of pyruvate kinase (Sigma, type II)/ml, 50µg of lactate dehydrogenase (Sigma, type II)/ml and ATPase, pH 8.0 in a final volume of 1.0 ml at 30°C. The reaction was started by addition of sodium ATP to a final concentration of 2.5mM.

Measurement of MgATP-induced inhibition. ATPase was preincubated at 30°C with ATP in a medium containing 50mM-$Na_2B_4O_7$, 5mM-$MgSO_4$ at the pH and concentration stated. At intervals, aliquots (20µl) were removed from the preincubation and diluted into the ATPase assay medium, described above. The extent of ATP hydrolysis which had occurred in the preincubation was estimated from the initial decrease in A_{340}, which occurred on addition of the aliquot into the assay medium. ATP (2.5mM) was then added to the ATPase assay medium and the ATPase activity was measured as described above. This latter ATPase activity was used to calculate the degree of inhibition of ATPase exerted by preincubation of the ATPase with MgATP.

Estimation of protein and nucleotides. Protein was estimated as described (10) using the trichloroacetic acid precipitation method. ATP and ADP concentrations were estimated by measurements of A_{260} at pH 7, assuming an extinction coefficient of 15,000 $litre \cdot mol^{-1} \cdot cm^{-1}$.

RESULTS AND DISCUSSION

Preincubation of ox-heart chloroform-released ATPase with its substrate, MgATP, can result in a time-dependent inhibition of ATPase activity (termed MgATP-induced inhibition)(see Fig.1). It should be emphasised that the loss of ATPase activity was measured by dilution of samples from the preincubation me-

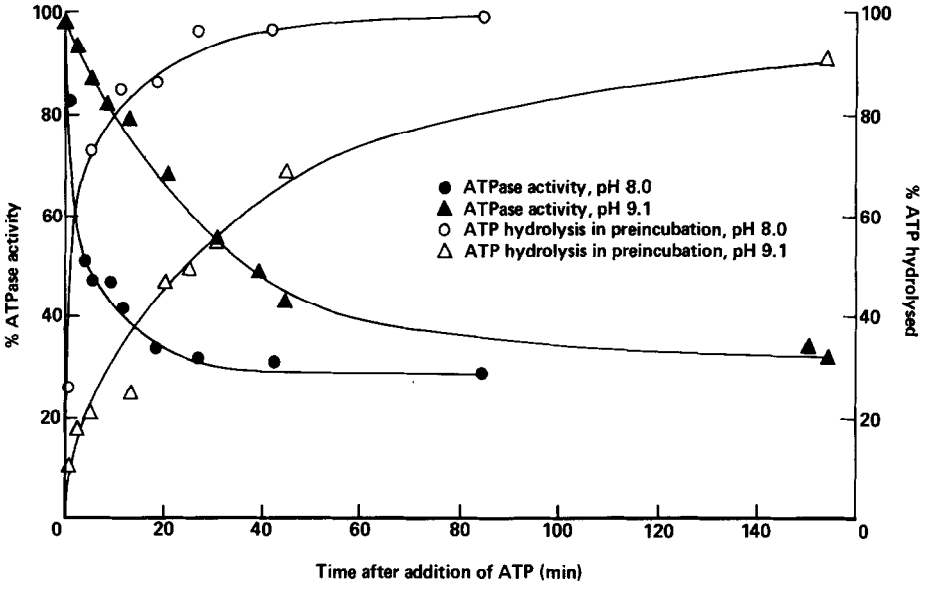

Fig. 1. MgATP-induced inhibition of ATPase activity. ATPase (100 µg protein/ml) was incubated in 50 mM-$Na_2B_4O_7$, 5 mM-$MgSO_4$ at either pH 8.0 or pH 9.1 for 30 min at 30°C. Then the ATPase was further preincubated at 30°C with no additions or with 9.8 mM-ATP. Aliquots (20 µl) were removed at intervals and diluted fifty times into the standard ATPase assay medium and ATPase activity was measured. ATPase activities of the samples preincubated with ATP are expressed as percentages of the activity of controls preincubated without ATP. The extent of the ATP hydrolysis during the preincubation period was measured by estimation of the amount of ADP produced and is expressed as a percentage of the initial amount of ATP in the preincubation.

dium, containing the ATPase and MgATP, into the ATPase assay medium. The same rate of ATP hydrolysis was obtained whether ATP was added immediately or after 10 min incubation in the ATPase assay medium. Thus the inhibition is stable to dilution. The extent and rate of inhibition induced by MgATP is dependent upon the nature of the buffer, the pH of the incubation and the concentrations of ATP and ATPase in the preincubation system.

It can be seen from Fig.1 that the time-course for the MgATP-induced inhibition of ATPase activity is similar to that for the hydrolysis of ATP in the preincubation system. Indeed, the amount of ATP hydrolysis in the preincubation was found to be directly proportional to the loss of ATPase activity (Fig.2). In the system described in Figs.1 and 2, about 25,000 turnovers of the ATPase enzyme occurred in order to induced a 50% inhibition of ATPase activity. In other preincubation media, the number of turnovers needed for this effect differed (data not shown).

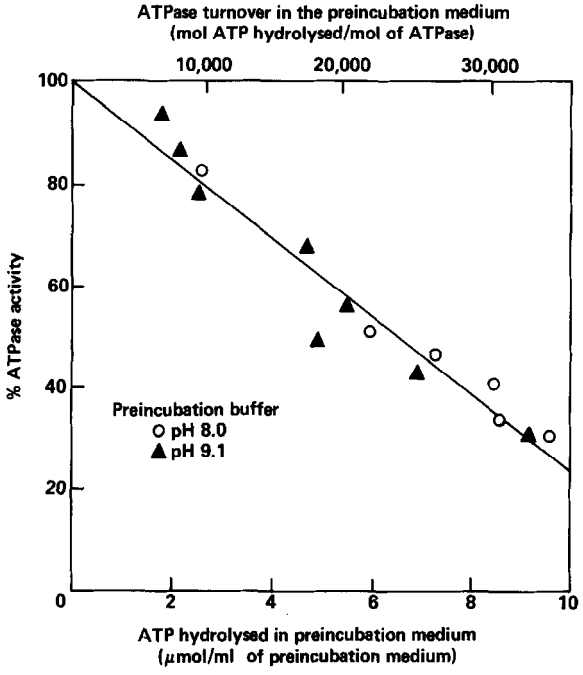

Fig. 2. Relationship between the extent of ATP hydrolysis during the preincubation of ATPase with MgATP and the induced inhibition of ATPase activity. For details see the legend to Fig. 1.

At pH 9.1, the ATPase preparation hydrolysed the ATP in the preincubation medium and also was inhibited by MgATP at a significantly lower rate than at pH 8.0 (Fig.1). In fact the reduction in the rate of ATP hydrolysis in the preincubation medium caused by this change in the pH of the preincubation paralleled the reduction in the rate of MgATP-induced inhibition of ATPase activity. Thus at both pH 8.0 and 9.1 the degrees of induced inhibition were proportional to the amount of hydrolysis of ATP in the preincubation and also possessed the same constant of proportionality (Fig.2).

The maximum inhibition of ATPase activity induced by preincubation with MgATP occurred at the time when all the ATP in the preincubation medium had been hydrolysed (Fig.1). In the experiment described in Fig.1, the maximum inhibition obtained was about 70%. The same value was obtained at both pH values, showing that the rate of ATP hydrolysis in the preincubation medium can change substantially without any change in the maximum degree of inhibition. If more ATP

hydrolysis was allowed to occur, either by increasing the concentration of ATP or by the inclusion of an ATP-regenerating system (pyruvate kinase and phosphoenolpyruvate) in the preincubation system, complete inhibition of ATPase activity could be induced.

The results of these experiments suggest that ATP hydrolysis is involved in the process of MgATP-induced inhibition of ATPase activity. ADP and MgADP are known inhibitors of the F_1-ATPase (2,5) and thus it seemed possible that the inhibition of chloroform-released ATPase activity induced by MgATP could be due to the product(s) of ATP hydrolysis. Preincubation of the chloroform-released ATPase with ADP (0.8μM to 10mM) in the presence of 5mM-$MgSO_4$ followed by assay of ATPase activity by dilution of samples into the ATPase assay medium did not result in any significant degree of inhibition. Similarly preincubation with 10mM-P_i or the combination of 10mM-P_i and 10mM-ADP in the presence of Mg^{2+} did not exert an inhibitory effect on the ATPase activity that was not reversible by dilution. These results suggest that the products of ATP hydrolysis are not the cause of the inhibition induced by MgATP, that has been described above.

Inhibition of ATPase activity, induced by preincubation with MgATP,could be due to a combination of the ATPase with ATP or MgATP, other than at the catalytic site, to form an inhibited enzyme complex, E.(Mg)ATP, i.e. E + (Mg)ATP $\rightleftharpoons$ E.(Mg)ATP, where E is the native enzyme. On the basis of this scheme, the equilibrium amount of inhibited enzyme complex should increase with an increase in either the concentration of ATPase or that of ATP. The data in Table 1 show that this is not the case. Although the extent of inhibition does increase with an increase in the concentration of ATP, an increase in the ATPase concentration, at any specified ATP concentration, results in a decrease in the extent of inhibition.

The data presented in Table 1 are explicable, however, if the process of ATP hydrolysis is the cause of the observed inhibition. The extent of inhibition is determined by the amount of ATP hydrolysed by each molecule of ATPase enzyme. In this experiment a fixed amount of ATP was present in the preincubation so that if the ATPase concentration is low, more ATP can be hydrolysed by each molecule

Table 1. Effects of the ATPase and ATP concentrations on the MgATP-induced inhibition of ATPase activity

Concentration of ATPase in the preincubation medium (μg protein/ml)	Maximum inhibition of ATPase activity	
	Concentration of ATP in the preincubation medium	
	1 mM	10 mM
100	5-10%	70%
1000	0%	5%

ATPase at either 100 or 1000 μg protein/ml was incubated with or without ATP (1 or 10 mM) in a buffer containing 50 mM-$Na_2B_4O_7$, 5 mM-$MgSO_4$, pH 8.0 at 30°C. Aliquots (20 μl or 5 μl) were removed at intervals and diluted into the standard ATPase assay medium for measurement of ATPase activity. The maximum inhibition produced by preincubation with ATP was achieved within 3 h by which time greater than 98% of the ATP in the preincubation had been hydrolysed. The inhibition is expressed as a percentage of a control incubated without ATP.

of ATPase than if the ATPase concentration is high. Thus at the lower ATPase concentration a greater degree of inhibition is obtained after hydrolysing a fixed quantity of ATP.

In summary, ATP hydrolysis appears to be involved in the process of MgATP-induced inhibition of the chloroform-released ATPase activity. The products of ATP hydrolysis do not cause the observed inhibition and so we postulate that an intermediate enzyme complex of the ATP-hydrolytic pathway can become transformed into an inhibited enzyme species. In its simplest form the scheme would be:

$$E + MgATP \rightleftharpoons E{\cdot}MgATP \xrightarrow{A} E + MgADP + P_i$$
$$\qquad\qquad\qquad\qquad\quad \downarrow B$$
$$\qquad\qquad\qquad\qquad\quad E^*$$

where E* is an inhibited form of the ATPase. E* must be derived from an intermediate of the ATP-hydrolytic pathway which is formed prior to an irreversible step, since the combination of ADP and P_i did not induce the formation of E*. Since it takes a large number of turnovers of the ATPase to reach an appreciable inhibition of ATPase activity, the flux through path A must be greater than that through path B. E* does not contain freshly bound adenine nucleotide

(data not shown). It is possible that E* is a conformationally-changed form of the ATPase (11-13) or one in which endogeneous inhibitor-protein (14) is exerting its inhibitory action (6). An unanswered question is whether the process of MgATP-induced inhibition serves any role in vivo in the regulation of the ATP-synthetase complex.

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