MgATP-induced inhibition of the adenosine triphosphatase activity of submitochondrial particles

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1. The ATP-hydrolytic activity of ox heart submitochondrial particles can be increased from 2–3 $\mu\text{mol/min}$ per mg of protein to 10–12 $\mu\text{mol/min}$ per mg of protein by incubation in media containing 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$. This process appears to be due to the partial release of inhibitor protein from the particles. 2. The ATPase activity of submitochondrial particles can be inhibited by incubation with the substrate, MgATP. This inhibition is not due to the accumulation of the hydrolysis products, MgADP and P_i , but could involve the process of ATP hydrolysis. 3. The mechanism of MgATP-induced inhibition of ATPase activity is proposed to involve a conformational change in one of the intermediate enzyme species of the ATP-hydrolytic sequence. 4. MgATP inhibits the ATPase activity of control submitochondrial particles at a higher rate and to a greater extent than it does that of inhibitor-protein-depleted submitochondrial particles, suggesting that the conformational change involves the endogenous inhibitor protein.

It has been demonstrated that the ATP-hydrolytic activity of the soluble ox heart mitochondrial ATPase can be inhibited by incubation with its substrate, MgATP (Lowe *et al.*, 1979a,b; Chernyak *et al.*, 1979; Lowe & Beechey, 1981). This inhibition is not caused by the accumulation of the hydrolysis products, P_i and MgADP, nor is it caused by binding of adenine nucleotide (Lowe *et al.*, 1979b; Lowe & Beechey, 1981). The inhibition induced by MgATP is thought to be due to an intermediate enzyme form of the ATP-hydrolytic pathway becoming conformationally transformed into an inhibited species. It has been further suggested that this conformational change may involve modifications in the interaction between endogenous inhibitor protein and the ATPase molecule (Lowe *et al.*, 1979b; Lowe & Beechey, 1981).

Kurup & Sanadi (1976) have shown that preincubation of ox heart submitochondrial particles

with MgATP led to a decrease of ATPase activity, assayed after sedimentation of the particles and resuspension into an ATPase assay medium. They found that the extent of inhibition depended on the amount of ATP hydrolysed during the preincubation period. During incubation of the particles with MgATP, some proteins were released into solution, and it was suggested that this phenomenon was related to the MgATP-induced inhibition of ATPase activity. Dabbeni-Sala *et al.* (1974) have also found that submitochondrial-particle ATPase can be inhibited by incubation with MgATP, but this effect was ascribed to a change in the association between the ATPase and endogenous inhibitor protein.

In the present paper we show that the ATPase activity of ox heart submitochondrial particles can be reversibly inhibited by incubation with MgATP. The characteristics of the inhibition appear to be similar to those described as occurring with the chloroform-released soluble ATPase preparation (Lowe *et al.*, 1979a,b; Lowe & Beechey, 1981). The ATPase activity of MgATP-particles can be increased to that of AS-particles by incubation in borate buffer of high ionic strength. We suggest that this increase in ATPase activity is associated with a change in the interactions between the ATPase complex and the inhibitor protein, so that a partial dissociation of the inhibitor protein occurs. Submitochondrial particles depleted of inhibitor protein

Abbreviations used: AS-particles, submitochondrial particles partially depleted of inhibitor protein by filtration through Sephadex; MgATP-particles, submitochondrial particles prepared in the presence of MgATP; borate-particles, MgATP-particles incubated in borate buffer to give a high ATPase activity.

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in this way were inhibited less by incubation with MgATP than were untreated particles, suggesting that the mechanism of inhibition does indeed involve interactions between endogenous inhibitor protein and the ATPase.

Methods and materials

Preparation of submitochondrial particles

MgATP-particles. These were prepared from ox heart mitochondria as described by Linnett *et al.* (1978).

AS-particles. These were prepared from ox heart mitochondria (Linnett *et al.*, 1978) as described by Racker & Horstman (1967). As prepared by us, the ATPase activity of these particles was about 8 $\mu\text{mol}/\text{min}$ per mg of protein. A loss of activity occurred after freezing and thawing.

Borate-particles. These were prepared by incubating MgATP-particles at a protein concentration of about 1 mg/ml at 30°C in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7/5$ mM- $\text{MgSO}_4/10\%$ (v/v) methanol buffer, pH 8.0, for 1–6 h. Inhibitor-protein-depleted borate-particles were prepared by centrifuging borate-particles and resuspending them in fresh 50 mM- $\text{Na}_2\text{B}_4\text{O}_7/5$ mM- $\text{MgSO}_4/10\%$ (v/v) methanol buffer, pH 8.0.

Measurement of ATPase activity

Standard ATPase assay. This was as described by Lowe *et al.* (1979b), except that 2 μg of antimycin A/ml of assay medium was also present. In this assay ADP production is coupled to the production of pyruvate, which is determined concurrently by using NADH and lactate dehydrogenase.

Preincubation ATPase assay. Submitochondrial particles were preincubated with MgATP in the presence of an ATP-regenerating system, which consisted of 0.7 mg/ml of pyruvate kinase (Sigma type III) and 71 mM-sodium phosphoenolpyruvate. During the preincubation, ATP hydrolysis results in the production of a stoichiometric amount of pyruvate. The amount of pyruvate produced was determined from the decrease in A_{340} that occurred on addition of 20 μl of the preincubation medium to the standard ATPase assay medium. From a graph of the amount of pyruvate produced against incubation time the initial rate of ATP hydrolysis in the preincubation medium could be evaluated. The amount of ATP hydrolysed could be used to calculate the number of enzyme turnovers that occurred in the preincubation, assuming that 1 molecule of ATP is hydrolysed per turnover and an ATPase content of 0.25 nmol/mg of submitochondrial-particle protein (Ferguson *et al.*, 1976).

Determination of protein and nucleotides

Insoluble protein was measured by the biuret method in the presence of 0.27% (w/v) sodium

deoxycholate (Dawson *et al.*, 1969). Adenine nucleotides were determined by measurements of A_{260} at pH 7, by assuming an absorption coefficient of $15\,000\text{M}^{-1}\cdot\text{cm}^{-1}$.

Assay for the inhibitor protein

A modification of the procedure of Horstman & Racker (1970) was used. AS-particles were resuspended at 1 mg of protein/ml in 20 mM-2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonic acid (Tes), adjusted to pH 6.7 with Tris base. A 30 μl sample of this suspension was incubated with 20 μl of the inhibitor-protein solution and 1 μl of 25 mM-MgATP at 30°C for 30 min. The standard ATPase assay medium (0.95 ml) was then added, and ATP hydrolysis was initiated by addition of 2.5 mM-ATP as described by Linnett *et al.* (1978).

ATP was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) (product no. A6144). Identical results were obtained with ATP from Pabst Laboratories (Milwaukee, WI, U.S.A.) or Boehringer (Mannheim, W. Germany).

Results

Preparation and properties of borate-particles

Characteristics of the activation induced by borate. Incubation of MgATP-particles in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7/5$ mM- $\text{MgSO}_4/10\%$ (v/v) methanol buffer, pH 8.0, leads to a time-dependent increase in the ATPase activity, as measured by dilution of samples into the ATPase assay medium (see Fig. 1). The maximum ATPase activity was obtained after about 4 h incubation. The increase in activity is moderate when the particles are incubated at a high protein concentration (e.g. at 20 mg of protein/ml the activity increased only 1.2-fold), but is 4–5-fold at about 1 mg of particle protein/ml. The increase in activity is not due to solubilization of the ATPase molecule, since the oligomycin-sensitivity of the particles is always greater than 94%, and over 90% of the ATPase can be recovered in the pellet produced by centrifugation at 100 000 g for 30 min.

Of the components of the incubation medium ($\text{Na}_2\text{B}_4\text{O}_7$, MgSO_4 and methanol), borate is the cause of the increase in activity, since (a) replacement of MgSO_4 in the incubation with 0.2 mM-EDTA has no effect on the final specific activity of the ATPase, although the rate of increase was greater, and (b) omission of methanol from the incubation had no effect on the final ATPase specific activity, although the omission did cause a lower rate of increase of activity.

Some preparations of MgATP-particles, which had been stored frozen for 12 months, had a low ATPase specific activity (0.5–1 $\mu\text{mol}/\text{min}$ per mg of protein) and were not activated by incubation with

Fig. 1. Increase in the ATPase activity of MgATP-particles caused by incubation in borate buffer

MgATP-particles were resuspended in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, at a protein concentration of 1 mg/ml and incubated at 30°C. At intervals 20 μ l samples were removed and assayed for ATPase activity in the standard ATPase assay medium. The inhibition of ATPase activity induced by 5 μ g of oligomycin/mg of particle protein was 94–97% at all stages of this experiment.

50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0. It is noteworthy that the same particles were not activated by gel filtration by the method of Racker & Horstman (1967).

Relationship between the activation of submitochondrial-particle ATPase in borate buffer and the loss of the inhibitor protein. The increase in the ATPase specific activity of MgATP-particles during incubation in borate buffer might be attributed to a change in the interaction between the endogenous inhibitor protein and the ATPase complex. If so, inhibitor protein may be released from the ATPase into solution. We tested this hypothesis by incubating MgATP-particles in borate buffer, thus increasing the ATPase activity. The particle suspension was centrifuged and the protein in the supernatant was concentrated by ultrafiltration with a Diaflo UM2 membrane with a molecular-weight cut-off of 1000. Since in addition to the inhibitory activity about 5% of the ATPase activity had been solubilized, ATPase molecules were inactivated by a heat treatment (Warsaw *et al.*, 1968). The resultant solution contained an ATPase-inhibitory activity that was heat-stable. MgATP was necessary for its inhibitory action to be expressed. These properties are consistent with the presence of inhibitor protein in the supernatant (Pullman & Monroy, 1963; Horstman & Racker, 1970). A minimum estimate of the quantity of inhibitor

protein in the supernatant may be found by assuming that heat treatment does not inactivate any inhibitory factor and that maximal inhibition is obtained in the assay. These assumptions are likely to lead to an under-estimate of the inhibitor-protein content. It was found that after treatment of MgATP-particles (1 mg of protein) with borate the supernatant contained sufficient inhibitory material to inhibit completely the ATPase activity of 0.32 mg of AS-particles. It therefore appears that a significant quantity of inhibitor protein is released by incubation of MgATP-particles in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, and thus borate-particles are depleted of inhibitor protein.

MgATP-induced inhibition of ATPase activity

Preincubation of borate-particles with MgATP can lead to a time-dependent decrease in the ATPase activity, as measured by removal of samples into the ATPase assay medium (Fig. 2). During the preincubation ATP is hydrolysed. Once all the ATP in the preincubation mixture has been hydrolysed, the inhibition of ATPase activity is slowly reversed (Fig. 2). Similar results were obtained with MgATP-particles in a preincubation medium based on 50 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes)/KOH/5 mM- MgSO_4 buffer, pH 8.0.

Preincubation of submitochondrial particles with either MgADP or P_i causes little inhibition of ATPase activity, suggesting that the cause of the MgATP-induced inhibition is either the binding of ATP at a non-catalytic site or the process of ATP hydrolysis itself. Evidence that ATP hydrolysis is involved is that incubation of particles with 9.6 mM-ATP (no ATP-regenerating system) resulted in an inhibition by 40%, whereas incubation of the same concentration of ATP in the presence of 0.2 mM-EDTA did not result in any inhibition. In the presence of EDTA the ATP was not hydrolysed.

Direct single-step ATP-binding at a non-catalytic site can be eliminated as the cause of the inhibition, since the initial rate of inhibition would be directly proportional to the concentration of MgATP. In the experiments shown in Fig. 2, the concentration of MgATP was maintained constant by using an ATP-regenerating system. Fig. 2 shows that an increase in the concentration of MgATP in the preincubation medium leads to an increase in the initial rate of the induced inhibition. The initial rates of onset of inhibition are not, however, proportional to the concentration of MgATP in the preincubation medium and tend towards a maximum value. Thus it appears that single-step ATP-binding is not involved in the mechanism of inhibition.

The data in Fig. 3(b) show that, during the initial stages of ATP hydrolysis by submitochondrial

Fig. 2. Preincubation of borate-particles with MgATP: effects on the ATPase activity

Borate-particles were prepared as indicated in Fig. 1, the mixture being incubated for 2 h at 30°C. ATP at various concentrations (●, 6.3 μ M; ▲, 63 μ M; ○, 630 μ M; △, 6600 μ M) was added to a series of mixtures containing 0.79 mg of borate-particle protein/ml, 71 mM-phosphoenolpyruvate, 0.71 mg of pyruvate kinase/ml, 5 mM-MgSO₄, 50 mM-Na₂B₄O₇ and 10% (v/v) methanol, pH 8.0. Incubation was continued at 30°C, and 20 μ l samples were removed at intervals for measurement of ATPase activity and the amount of pyruvate produced. The ATPase activity, measured after dilution into the ATPase assay medium, is plotted as a percentage of the activity of a control incubated without addition of ATP. The ATPase activity of this control was constant at 7 μ mol/min per mg of protein. The arrow indicates the time at which the ATP-producing potential of the ATP-regenerating system was exhausted.

particles, the inhibition of ATPase activity increases directly with the amount of ATP hydrolysed in the preincubation medium. Under these conditions about 10000 turnovers of the enzyme lead to a 20% inhibition of the enzymic activity. After longer periods of preincubation, a maximum inhibition is attained, despite the continuing hydrolysis of ATP.

We have observed that the number of turnovers needed to obtain a specified inhibition increases with an increase in the concentration of ATP in the preincubation medium, so that more ATP is hydrolysed to achieve the same degree of inhibition.

Relationship between MgATP-induced inhibition of ATPase activity and the content of inhibitor protein in submitochondrial particles

We attempted to determine if the mechanism of MgATP-induced inhibition of submitochondrial-

Fig. 3. MgATP-induced inhibition of borate-particles: comparison with particles depleted of inhibitor protein

MgATP-particles were incubated in 50 mM-Na₂B₄O₇/5 mM-MgSO₄/10% (v/v) methanol buffer, pH 8.0, at a protein concentration of 1 mg/ml at 30°C for 6 h. The resultant borate-particles (specific activity 11.5 μ mol/min per mg of protein) were divided into three portions. One was left untreated (tube A). The other two suspensions were centrifuged at 50000 rev./min for 30 min in a Beckman type 65 rotor. The pellet from one tube was immediately resuspended in its own supernatant (tube B). The pellet from the other tube was suspended in fresh 50 mM-Na₂B₄O₇/5 mM-MgSO₄/10% (v/v) methanol buffer, pH 8.0, at a protein concentration of 1 mg/ml (tube C). Incubations were set up at 30°C each containing 0.7 mg of particle protein from tubes A, B and C per ml, 71 mM-phosphoenolpyruvate, 0.7 mg of pyruvate kinase/ml, 50 mM-Na₂B₄O₇, 5 mM-MgSO₄ and 10% (v/v) methanol, pH 8.0. ATP was added to give a final concentration of 0.7 mM. At intervals 20 μ l samples were removed and assayed for ATP hydrolysis during the preincubation period (by assaying the pyruvate formed) and for ATPase activity after dilution into the standard ATPase assay medium. In (a) the ATPase activity is plotted, as a percentage of the activity of a control incubated without ATP, against the incubation time after the addition of ATP. In (b) the percentage inhibition of ATPase activities is plotted against the number of enzyme turnovers during the preincubation period. ●, Tube A, untreated borate-particles; ▲, tube B, borate-

particle ATPase activity involved the ATPase-inhibitor protein by comparing the characteristics of MgATP-induced inhibition of the ATPase activity of control and inhibitor-protein-depleted borate-particles. In the experiment illustrated in Fig. 3 borate-particles were prepared and divided into three portions. One sample was kept as a control. The other two were centrifuged at 110 000 g . One pellet was resuspended in its own supernatant, whereas the other pellet was resuspended in a fresh solution of 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0. The ability of MgATP to inhibit the ATPase activity of these samples was investigated. The ATPase activity of the washed borate-particles was inhibited by MgATP to a much smaller extent than was the ATPase activity of control particles (Fig. 3a). The sensitivity to MgATP of the ATPase activity of the borate-particles that had been centrifuged and resuspended in their own supernatant was identical with that of the control borate-particles. The results presented in Fig. 3(a) suggest that a factor that confers sensitivity of the ATPase to MgATP is present in the supernatant.

The difference between the response of the ATPase of washed and control borate-particles is not due to a difference in their ability to hydrolyse ATP, since these particles had similar specific activities after dilution into the assay medium (10.3 and 11 $\mu\text{mol}/\text{min}$ per mg of protein respectively) and both hydrolysed ATP in the preincubation medium at similar initial rates (0.23 $\mu\text{mol}/\text{min}$ per mg of protein).

The difference in the MgATP-induced inhibition is also reflected in Fig. 3(b), where the relationship between the inhibition of ATPase activity and the number of turnovers of the ATPase molecule is shown. It is shown that depletion of the inhibitor protein from borate-particles results in a large increase in the total number of enzyme turnovers required to achieve inhibition, although the actual number of turnovers required to inhibit an individual enzyme molecule would remain the same. The depletion of the inhibitor protein is also reflected in the low maximum inhibition obtained.

Discussion

In the present paper we have shown that the ATPase activity of MgATP-particles or borate-particles can be inhibited by incubation with MgATP. This inhibition is not caused by the accumulation of hydrolysis products, and we have presented evidence suggesting that ATP hydrolysis rather than ATP-binding is causing the inhibition.

Once all the ATP in the preincubation medium has been hydrolysed, the inhibition is slowly reversed. The characteristics of the inhibition are thus similar to those observed with the soluble mitochondrial ATPase, where the inhibition is caused by ATP hydrolysis and not by ATP-binding as such (Lowe *et al.*, 1979b; Lowe & Beechey, 1981). We suggest that during ATP hydrolysis an intermediate enzyme species of the ATP-hydrolytic pathway can be transformed into an inactive enzyme species. Since 10 000 turnovers of the enzyme are needed to achieve 20% inhibition (see Fig. 3b), this transformation into an inhibited form, at least initially, only occurs once in 50 000 turnovers of the ATPase molecule. Complete inhibition does not occur, since a steady-state situation is set up in which the rate of inhibition caused by MgATP hydrolysis is balanced by a spontaneous regeneration of active enzyme. During this state, ATP hydrolysis takes place, but no further inhibition occurs (Fig. 3b). This continues until all the ATP in the preincubation medium has been hydrolysed. Once this has occurred the inhibition is relieved (Fig. 2).

As with the chloroform-released ATPase (Lowe & Beechey, 1981), the number of ATPase turnovers needed to achieve any specified inhibition increases with increasing ATP concentration in the preincubation. This suggests that a substrate-activation step is also part of the mechanism of ATP hydrolysis catalysed by submitochondrial-particle ATPase.

A difference between the MgATP-induced inhibition of soluble and submitochondrial-particle ATPases is concerned with the relationship between the degree of inhibition and the amount of ATP hydrolysed in order to achieve it. With the chloroform-released ATPase dissolved in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol buffer, pH 8.0, the degree of inhibition of ATPase activity is directly proportional to the amount of ATP hydrolysed by the ATPase in the preincubation medium (Lowe *et al.*, 1979b). With submitochondrial-particle ATPase, however, the degree of inhibition is not directly proportional to the amount of ATP hydrolysed in the preincubation medium; initial ATP hydrolysis causes a greater degree of inhibition than that caused by later ATP hydrolysis (Fig. 3b). A difference can also be seen during examination of the rates of reversal of the inhibition once all the ATP in the preincubation medium has been hydrolysed. With the soluble ATPase the rate of reversal is low, whereas that of MgATP-particle ATPase activity is restored relatively quickly (Fig. 2). It is thought that the inhibited form of the soluble ATPase is formed irreversibly in the presence of MgATP in this medium, whereas that of the MgATP-particle ATPase is formed reversibly even in the presence of MgATP so that a steady-state situation can be achieved (see above).

particles resuspended in their own supernatant; ■, tube C, borate-particles spun down and suspended in fresh buffer.

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