Involvement of the endogenous inhibitor protein in the MgATP-induced inhibition of soluble mitochondrial adenosine triphosphatase activity

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Chloroform-released ATPase from ox heart mitochondria contains significant amounts of inhibitor protein. There is a correlation between processes that affect the interactions between the inhibitor protein and the ATPase molecule and the ability of MgATP to induce an inhibition of ATPase activity. Evidence is presented suggesting that the endogenous inhibitor protein is involved in the process of MgATP-induced inhibition of soluble ATPase activity.

Preincubation of both membrane-bound and solubilized preparations of ox heart mitochondrial ATPase with MgATP can lead to a time-dependent inhibition of ATPase activity (Akimenko *et al.*, 1972; Dabbeni-Sala *et al.*, 1974; Kurup & Sanadi, 1976; Chernyak *et al.*, 1979, 1981; Lowe *et al.*, 1979; Lowe & Beechey, 1981*a,b*; Galante *et al.*, 1981). ATP-hydrolysing activity of the ATPase molecule induces an inhibition of further ATP hydrolysis by the enzyme. A mechanism for the observed inhibition has been proposed in which an intermediate enzyme species of the ATP-hydrolytic pathway can transform into an inhibited form (Lowe & Beechey, 1981*a,b*). This transformation occurs with a probability of about 1 in 5000–200 000 turnovers, depending on the preincubation conditions. It could involve some re-arrangement of the constituent subunits and/or a change in the interaction of the ATPase with endogenous inhibitor protein. Both these explanations have been previously suggested, although little experimental evidence has been obtained (Akimenko *et al.*, 1972; Dabbeni-Sala *et al.*, 1974; Chernyak *et al.*, 1979, 1981; Lowe *et al.*, 1979; Lowe & Beechey, 1981*a,b*). In the case of MgATP-induced inhibition of submitochondrial-particle and factor-V ATPase activity, the inhibitory process is dependent on the presence of the inhibitor protein (Lowe & Beechey, 1981*b*; Galante *et al.*, 1981).

The aim of the present study was to investigate if the inhibitor protein is also involved in the MgATP-

induced inhibition of the soluble chloroform-released ATPase activity.

Experimental

Biological preparations

AS-particles were prepared from ox heart mitochondria (Linnett *et al.*, 1978) as described by Racker & Horstman (1967). The ATPase activity was 7–8 $\mu\text{mol/min}$ per mg of protein.

Chloroform-released ATPase was prepared as described in Lowe *et al.* (1979). F_1 -ATPase, prepared by the method of Knowles & Penefsky (1972), was kindly given by Dr. D. A. Harris and Dr. C. Goldsmith. For use in an experiment, a sample of the ammonium sulphate suspension of the F_1 -ATPase, containing ATP and EDTA, was centrifuged for 10 min at 4°C in a Hawksley Micro-haematocrit centrifuge. The supernatant was discarded, and the pellet was dissolved directly in the desired buffer. Both preparations of F_1 -ATPase gave similar results. The preparation provided by Goldsmith was used for the measurement of inhibitor-protein content.

Sephadex-treated ATPase was prepared by chromatography of chloroform-released ATPase on a column of Sephadex G-25, eluted with a buffer of high ionic strength. Chloroform-released ATPase (0.39 ml; 3 mg of protein/ml) was applied to a column (0.9 cm $\times$ 27 cm) of Sephadex G-25 (fine grade), equilibrated and eluted with 50 mM-Tris/ H_2SO_4 /60 mM- K_2SO_4 /1 mM-EDTA buffer, pH 8.0 (Leimgruber & Senior, 1976). ATPase activity was eluted at an elution volume between 5.4 and 9.0 ml. These fractions were pooled and had a specific acti-

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activity of 65–80 $\mu\text{mol}/\text{min}$ per mg of protein. The protein recovery was about 75%, and that of enzyme activity about 145%. The ATPase was then concentrated by pressure-ultrafiltration with a Diaflo XM-100A membrane in a stirred Amicon cell. The buffer was changed to 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer, pH 8.0, by continuous ultrafiltration, and the solution was freeze-dried in small portions. The final specific activity after reconstitution of the freeze-dried samples was $62 \pm 16 \mu\text{mol}/\text{min}$ per mg of protein. The freeze-dried ATPase could be stored at -20°C indefinitely.

Inhibitory factor was extracted from ATPase in two ways. (a) A solution containing ATPase was heated at 82°C for 5 min, and the denatured proteins were removed by centrifugation, leaving the inhibitory factor in the supernatant (cf. Warshaw *et al.*, 1968). (b) A modification of the procedure of Horstman & Racker (1970) was followed. A solution of the chloroform-released ATPase (0.2 ml; 1 mg of protein/ml) was left in ice for 10 min. Then 1 M-KOH (0.02 ml) was added, followed 40 s later by 0.02 ml of 50% (w/v) trichloroacetic acid. The mixture was centrifuged at 100 000 g for 5 min at 4°C . The supernatant was discarded, and the pellet was washed carefully with 0.04 ml of ice-cold water. The mixture was re-centrifuged for 5 min, and the supernatant was discarded. The pellet was homogenized in 0.1 ml of 0.25 M-sucrose/1 mM-Tris/ H_2SO_4 buffer, pH 7.5, and stored frozen at -20°C , if necessary. A control was run in which 0.2 ml of 1 mg of bovine serum albumin/ml was put through the same procedure. This control did not inhibit in the assay for inhibitor protein.

Assay methods

ATPase activity and MgATP-induced inhibition were measured and performed as described by Lowe & Beechey (1981a). MgATP-induced inhibition was often studied in a medium containing borate and methanol. This medium was chosen because the inhibition is displayed easily in the presence of borate (Lowe & Beechey, 1981a), and methanol stabilizes the ATPase. Methanol did not affect the process of MgATP-induced inhibition. Protein and nucleotides were determined as described in the same paper. Inhibitor protein was assayed as described in Lowe & Beechey (1981b).

Fluorescence of aurovertin B was measured on a Baird Atomic model SFR 100 recording spectrofluorimeter operating in the ratio mode. The excitation wavelength was 368 nm and the emission wavelength was 470 nm.

Materials

Trypsin treated with 1-chloro-4-phenyl-3-tosyl-amidobutan-2-one (1560 units/mg) was obtained

from Worthington Biochemical Corp. Trypsin inhibitor from soya beans (type I-S) and pyruvate kinase (type III) were obtained from Sigma Chemical Co.

Results

We have presented evidence that the inhibitor protein is directly involved in the MgATP-induced inhibition of submitochondrial-particle ATPase activity (Lowe & Beechey, 1981b). The following experiments were designed to test the involvement of the inhibitor protein in the MgATP-induced inhibition of soluble ATPase preparations.

Inhibitor-protein content of chloroform-released ATPase

The ox heart mitochondrial ATPase-inhibitor protein has a molecular weight of 10 000–12 000 (Pullman & Monroy, 1963; Brooks & Senior, 1971; Gomez-Fernandez & Harris, 1978). It can withstand temperatures of 90°C for 5 min. Most of the ATPase proteins are denatured and precipitated under these conditions, and thus this type of treatment was used to attempt a preparation of the inhibitor protein from the chloroform-released ATPase (see the Experimental section). As judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the main components of this preparation were proteins of molecular weight about 10 000 (A. D. Mitchell & P. E. Linnett, unpublished work).

This crude extract was assayed for its inhibitory action on AS-particle ATPase activity. As shown in Fig. 1, the extract was capable of inhibiting the ATPase activity. It was also found that precipitation of the ATPase with trichloroacetic acid (Horstman & Racker, 1970; and the Experimental section) could produce an extract capable of inhibiting the ATPase activity of AS-particles with about the same yield.

Identification of the inhibitory factor as the inhibitor protein. The inhibitory action of the inhibitor protein is destroyed by a brief exposure to low concentrations of trypsin (Pullman & Monroy, 1963). The results presented in Table 1 show that the inhibitory action of the supernatant from heat-treated ATPase was also destroyed by trypsin treatment, indicating its protein nature.

A distinctive property of the inhibitor protein interacting with F_1 -ATPase is that under most conditions its inhibitory action is greatly stimulated by the presence of MgATP. The same effect is seen with the inhibitory factor obtained from the chloroform-released ATPase.

In the presence of 0.5 mM-MgATP the ATPase activity of AS-particles (30 μg of protein) was 94% inhibited by the inhibitory protein obtained from 20 μg of soluble ATPase. In the presence of 0.5 mM-MgSO₄ the inhibition was 13%. The results

Fig. 1. Inhibition of AS-particle ATPase activity by extracts from chloroform-released ATPase and F_1 -ATPase

Inhibitory factor was released from chloroform-released ATPase and F_1 -ATPase by heating at 85°C for 5 min at a protein concentration of 2.8 mg/ml in 10 mM-Hepes/KOH buffer, pH 7.6. The inhibitory factor from both ATPase preparations was assayed as described in the Experimental section. The assay mixture contained $21\mu\text{g}$ of AS-particle protein. The ATPase activity of the AS-particles, as a percentage of a control without added inhibitory factor, is plotted against the quantity of ATPase from which the inhibitory factor in each experiment was derived. The specific activity of the AS-particle ATPase was $7.5\mu\text{mol/min per mg}$ of protein. ●, Inhibitory factor extracted from chloroform-released ATPase; ▲, inhibitory factor extracted from F_1 -ATPase.

were very similar to those obtained by Horstman & Racker (1970).

This evidence suggests very strongly that the inhibitory agent present in denatured chloroform-released ATPase is identical with the inhibitor protein described by Pullman & Monroy (1963).

Quantitative determination of the inhibitor protein. It was found that 0.2 and 0.14 mol of the inhibitor were isolated from 1 mol of chloroform-released ATPase and F_1 -ATPase respectively (see Fig. 1). These are thought to be serious underestimates, since the inhibitor protein is likely to be significantly denatured during extraction. Also, AS-particles have not been shown to be devoid of inhibitor protein and hence may not rebind inhibitor protein maximally. Furthermore, it is unlikely that the maximal inhibition occurs on incubation of inhibitor protein with the AS-particles, since the induced inhibition is extremely sensitive to pH and ionic strength (Horstman & Racker, 1970), neither of which was stringently controlled in our experiment. It was assumed that the ATPase content of the AS-particles was 0.25 nmol/mg of particle protein (Ferguson *et al.*, 1976) and that 1 mol of inhibitor protein inhibits 1 mol of ATPase (Gomez-Fernandez & Harris, 1978).

Modification of the ATPase-inhibitor-protein interaction: its effect on the MgATP-induced inhibition of ATPase activity

If the interaction of the inhibitor protein with the ATPase molecule is involved in the mechanism of

Table 1. *Sensitivity to trypsin of the inhibitory factor derived from heat-denatured chloroform-released ATPase*
Inhibitory factor was derived from chloroform-released ATPase by treatment at 82°C at a protein concentration of 1 mg/ml. The inhibitory action was assayed on AS-particles by using a modification of the assay method described in the Experimental section. Inhibitory factor ($15\mu\text{l}$) was incubated with $20\mu\text{l}$ of 20 mM-Tes (2-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino)ethanesulphonic acid/Tris buffer, pH 6.7, at 30°C , and $2\mu\text{l}$ of a solution containing $4\mu\text{g}$ of trypsin and $2\mu\text{l}$ of a solution containing $20\mu\text{g}$ of trypsin inhibitor. The chemicals were added in the order shown. Trypsin inhibitor was incubated with trypsin for 3 min before any further additions (incubations 2 and 4). Trypsin was incubated with inhibitory factor for 10 min (incubations 2 and 3) before AS-particles were added (see below). In incubation 6 inhibitory factor was incubated with trypsin for 10 min before trypsin inhibitor was added, followed 3 min later by the addition of AS-particles. After the relevant incubations, as described above, $10\mu\text{l}$ of AS-particles (2.1 mg of protein/ml) was added, followed immediately by $1\mu\text{l}$ of 25 mM-MgATP. After 30 min the ATPase activity was assayed.

Treatment	Inhibition* (%)	
	Pullman & Monroy (1963)	Present work
1. Inhibitory factor alone	93	61
2. Trypsin inhibitor + trypsin + inhibitory factor	80	47
3. Trypsin + inhibitory factor	-8	-10
4. Trypsin inhibitor + trypsin	-18	0
5. Trypsin	18	-6
6. Trypsin + inhibitory factor + trypsin inhibitor	-27	—

* Negative values refer to stimulation.

the MgATP-induced inhibition of ATPase activity, then factors that affect the interaction between the inhibitor and ATPase proteins should affect the MgATP-induced inhibition of ATPase activity.

Gel filtration of submitochondrial particles under high-ionic-strength conditions can lead to an increase in ATPase activity (Racker & Horstman, 1967), ascribed to the removal of the inhibitor protein. The passage of chloroform-released ATPase through a similar column also results in a 2-fold increase in its specific activity (see Table 2).

The abilities of MgATP to inhibit control and Sephadex-treated chloroform-released ATPase were therefore compared. Sephadex-treated ATPase was dissolved in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol, pH 9.1, at a protein concentration of 100 $\mu\text{g}/\text{ml}$, and was then incubated with 10 mM-ATP. ATPase activity was measured by dilution into the ATPase-assay medium, and a maximum inhibition of 40–45% was achieved when all the ATP had been hydrolysed. Incubation with 10 mM-ADP caused no inhibition. Under similar conditions, the control ATPase showed 70% inhibition when all the ATP was hydrolysed, and ADP caused no inhibition. Thus the hydrolysis of a fixed amount of ATP with the same ATPase-protein concentration leads to a greater inhibition of ATPase activity with control ATPase than with Sephadex-treated ATPase.

Possible explanations for these results are: (1) it is necessary for the Sephadex-treated ATPase to hydrolyse more ATP than the control ATPase to achieve the same degree of inhibition; (2) because the

Sephadex-treated ATPase has a high ATPase activity it is exposed to MgATP for a shorter time than the control enzyme and hence is not inhibited to the same extent.

To distinguish between these two mechanisms, Sephadex-treated and control ATPase preparations were diluted to the same ATPase activity per unit volume. These were preincubated in 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /10% (v/v) methanol, pH 8.0, in the presence of an ATP-regenerating system with either 71 μM - or 7.1 mM-ATP. Samples were removed for ATPase assay by dilution into a standard ATPase-assay medium. The time courses of ATP hydrolysis in the preincubation mixture and of inhibition of ATPase activity on dilution were followed. The results in Table 2 show that more ATP must be hydrolysed during the preincubation to achieve 50% inhibition of ATPase activity in Sephadex-treated ATPase than in untreated ATPase. This holds over a wide range of ATPase activity in the preincubation mixture (0.037–1.02 $\mu\text{mol}/\text{min}$ per ml). The amount of ATP hydrolysed to give 50% inhibition is different at the two ATP concentrations used (see Table 2). This is accounted for by the model presented in Lowe & Beechey (1981a), in which a substrate-activation step is involved in the mechanism of MgATP-induced inhibition of ATPase activity.

If one assumes that the amount of active enzyme present is proportional to the specific activity of the ATPase, then the number of molecules of ATP hydrolysed (in the preincubation) required to achieve inhibition is similar for the control and Sepha-

Table 2. Comparison of the MgATP-induced inhibition of ATPase activity of control ATPase and Sephadex-treated ATPase

Sephadex-treated ATPase (see the Experimental section) and control ATPase were diluted into 50 mM- $\text{Na}_2\text{B}_4\text{O}_7$ /5 mM- MgSO_4 /70 mM-phosphoenolpyruvate/pyruvate kinase (0.7 mg/ml; Sigma type III)/10% (v/v) methanol pH 8.0, to the protein concentrations stated below. The amounts of the two ATPases used contained the same total amount of ATPase activity. ATP was added, and at intervals 20 μl portions were removed for assay of the ATPase activity and pyruvate production. From the rates of pyruvate production, the ATPase activity in the preincubation mixture was evaluated. From the ATPase activity measured after dilution, the time taken to achieve 50% inhibition was determined. The amount of ATP hydrolysed in the preincubation for the ATPase activity to be 50% inhibited (measured after dilution) was evaluated from a graph of ATPase activity, after dilution, against the amount of ATP hydrolysed (pyruvate produced) in the preincubation. The specific activities of the control and Sephadex-treated ATPases, incubated without ATP, were 64 and 96 $\mu\text{mol}/\text{min}$ per mg of protein respectively.

ATPase	Preincubation mixture				Time taken to achieve 50% inhibition (min)	ATP hydrolysed in preincubation to achieve 50% inhibition (mol of ATP/mol of ATPase)
	[ATPase] (μg of protein/ml)	ATPase activity (initial rate) ($\mu\text{mol}/\text{min}$ per mg)	Total ATPase activity (initial rate) ($\mu\text{mol}/\text{min}$ per ml)	[ATP] (mM)		
Control	81	0.46	0.037	0.071	62	12 000
Sephadex-treated	42	0.88	0.037	0.071	61	17 000
Control	81	12.6	1.03	7.1	6.0	21 500
Sephadex-treated	42	24.3	1.01	7.1	7.4	49 500

dex-treated ATPase preparations. This is consistent with a model in which the ATPase preparation consists of wholly active or inactive molecules; Sephadex-treated ATPase would have a greater proportion of active enzyme than control ATPase. It should be noted that for both preparations with sufficiently long times of incubation the inhibition reached 100%, suggesting that, if the inhibitor protein is involved, each molecule of active Sephadex-treated ATPase still contains at least one molecule of inhibitor protein.

This conclusion would appear to be in contradiction with the previous statement that gel filtration at high ionic strength of submitochondrial particles removes inhibitor protein. However, it is possible that the conditions of high ionic strength may have displaced the inhibitor protein from its inhibitory site on the soluble ATPase, without causing release from the ATPase molecule. Evidence for this proposition is that inhibitor protein can be bound in a non-inhibitory mode (Van de Stadt *et al.*, 1973; Bruni *et al.*, 1977, 1979; Galante *et al.*, 1981), that the inhibition exerted by the inhibitor protein can be relieved by incubation at high ionic strength (Horstman & Racker, 1970; Van de Stadt & Van Dam, 1974a; Gomez-Fernandez & Harris, 1978) and that interactions between the soluble ATPase and the inhibitor protein are different and possibly stronger than the interactions between inhibitor protein and factor-V ATPase, a preparation that resembles submitochondrial particles in a number of features (Galante *et al.*, 1981).

Correlation between MgATP-induced inhibition of ATPase activity and MgATP-induced quenching of the fluorescence of aurovertin B

Aurovertin is a non-competitive inhibitor of mitochondrial ATPase activity. Binding to the F_1 -ATPase causes a 50–100-fold increase in the fluorescence of aurovertin (Chang & Penefsky, 1973; Yeates, 1974; Muller *et al.*, 1977). Chloroform-released ATPase also enhances the fluorescence of aurovertin B. Chloroform-released ATPase that had been completely inhibited by pre-incubation with MgATP in borate buffer causes, however, only a small enhancement of aurovertin B fluorescence. The inhibitor protein can cause a quenching of aurovertin-ATPase fluorescence (Layton *et al.*, 1973; Chang & Penefsky, 1974; Van de Stadt & Van Dam, 1974a,b). Hence we investigated the possibility of a relationship between MgATP-induced inhibition of ATPase activity and MgATP-induced quenching of aurovertin fluorescence.

ATPase was incubated with MgATP and aurovertin B in either Hepes-based or borate-based buffers. [The variations of response of the ATPase to incubation with MgATP in different buffers has been discussed in Lowe & Beechey (1981a)]. The

results in Fig. 2 show that in borate buffer MgATP inhibits ATPase activity in a pseudo-first-order process with respect to remaining enzyme activity. Inhibition reaches 100% before all the ATP has been

Fig. 2. *MgATP-induced quenching of aurovertin fluorescence*

Chloroform-released ATPase (75 μ g of protein/ml) was incubated for 3 h at 30°C in either 50 mM-Hepes/KOH/5 mM-MgSO₄/15 mM-phosphoenolpyruvate/pyruvate kinase (0.75 mg/ml), pH 8.0, or in the same medium but with Hepes/KOH buffer replaced by 50 mM-sodium borate buffer, pH 8.0. Each incubation mixture was then divided into two 0.5 ml portions. To each, 5.5 μ l of 38 μ M-aurovertin B was added. After 5 min, 4 μ l of 100 mM-ATP was added. One sample of incubation mixture in each buffer was placed in the fluorimeter at 30°C, and the changes in fluorescence yield were followed continuously. From the other sample of incubation mixture (in each buffer) 20 μ l portions were assayed for ATPase activity and pyruvate production. A control without added ATP did not change in activity. The fluorescence of the incubation mixture in Hepes buffer is plotted directly on a semi-logarithmic scale against the incubation time after addition of ATP. The fluorescence of the incubation in borate buffer was corrected for the final fluorescence (14 units), after 3 h incubation, when the ATPase was completely inhibited. On addition of ATP to the ATPase-aurovertin complex, in Hepes buffer an initial rapid stimulation of fluorescence was observed, whereas in borate buffer an initial rapid quenching was seen. The ATP-regenerating potential of the incubation in Hepes buffer was exhausted at the point where an arrow ($\downarrow$) is shown. In borate buffer the ATP-regenerating potential was not exhausted during the course of the experiment. ATPase activity: ●, Hepes buffer; ▲, borate buffer. Fluorescence: ○, Hepes buffer; △, borate buffer.

hydrolysed. Simultaneously, the fluorescence of bound aurovertin is quenched in a first-order process. In Hepes buffer MgATP caused a time-dependent inhibition, but after all the ATP had been hydrolysed the inhibition was reversed. In parallel with this inhibition and re-activation of ATPase activity, the fluorescence yield of the aurovertin B first decreased and then increased. Thus, in either buffer, the quenching of aurovertin fluorescence during ATP hydrolysis appears to be related to the MgATP-induced inhibition of ATPase activity. These fluorescence experiments give further indirect evidence that MgATP-induced inhibition and inhibition by the endogenous inhibitor protein are the same process.

Discussion

In the present paper we have provided two indirect pieces of evidence that the endogenous inhibitor protein is involved in the process of MgATP-induced inhibition of soluble ATPase activity. Firstly, there is a correlation between processes that affect the interactions between the inhibitor protein and the ATPase molecule and the ease with which MgATP can induce an inhibition of ATPase activity (see Table 2). Secondly, the enhanced fluorescence of aurovertin B bound to the ATPase is quenched during MgATP-induced inhibition, and, under conditions where the inhibition is reversed, the quenching is also reversed (see Fig. 2). Inhibitor protein similarly causes a quenching of fluorescence when added to the ATPase-aurovertin complex (Chang & Penefsky, 1974).

The depletion of the inhibitor-protein content of submitochondrial particles (Lowe & Beechey, 1981b) and of factor-V ATPase (Galante *et al.*, 1981) prevented the MgATP-induced inhibition of ATPase activity. Similar experiments could not be performed on the soluble ATPase, since there is as yet no method for the preparation of ATPase that is unequivocally devoid of inhibitor protein. This is due to its high affinity for the ATPase and the absence of a quantitative assay for the inhibitor protein. The inhibitor protein can bind to the ATPase molecule in a non-inhibitory mode (Van de Stadt *et al.*, 1973; Bruni *et al.*, 1977, 1979; Galante *et al.*, 1981). Hence estimates of the inhibitor protein based on the enzymic activity of ATPase preparations are likely to be misleading. Furthermore, isolated inhibitor protein has different properties from those of endogenous inhibitor protein (Galante *et al.*, 1981). It might be possible to obtain ATPase by chloroform treatment of submitochondrial particles, depleted of inhibitor protein. The problem, however, would remain of determining how much inhibitor protein is present. An attempt to obtain ATPase by chloroform treatment of inhibitor-protein-

depleted submitochondrial particles (borate-particles prepared as described in Lowe & Beechey, 1981b) did not produce active ATPase, possibly because of the instability of inhibitor-protein-depleted ATPase under these conditions. The present inability to prepare inhibitor-protein-depleted ATPase prevents any direct demonstration of the role of the inhibitor protein in the MgATP-induced inhibition of soluble ATPase activity.

The high ATPase activity of F_1 -ATPase preparations has been ascribed to the absence of inhibitor protein. Hence we examined the ability of MgATP to inhibit F_1 -ATPase activity in the expectation that no inhibition would occur. However, F_1 -ATPase activity is also inhibited by preincubation of the enzyme with MgATP (results not presented). [This inhibition can be clearly distinguished from that caused by MgADP (Gomez-Fernandez & Harris, 1978; Harris *et al.*, 1978; Minkov *et al.*, 1979).] It should be noted that, although no inhibitor protein could be detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of this F_1 -ATPase preparation, it was possible to isolate a protein with inhibitory activity (see Fig. 1) with yields similar to those obtained from the chloroform-released ATPase preparation. These results emphasize the care that has to be taken before assuming that the inhibitor protein is absent from any preparation, as has also been concluded by Galante *et al.* (1981).

In addition to the evidence provided in the present paper, the suggestion that the inhibitor protein is involved is strengthened by the fact that MgATP is necessary for the inhibitor protein to exert its inhibitory effects (Horstman & Racker, 1970; Van de Stadt *et al.*, 1973; Galante *et al.*, 1981). Furthermore, it has been proposed that ATP hydrolysis is essential for the onset of inhibition by inhibitor protein, as is the case for the MgATP-induced inhibition of ATPase activity. In both cases it is envisaged that intermediates of the ATP-hydrolytic pathway are diverted to form inactive enzyme (Van de Stadt *et al.*, 1973; Lowe & Beechey, 1981a; Galante *et al.*, 1981). We propose that the two processes are identical.

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