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PURIFICATION AND PROPERTIES OF ATPase INHIBITOR FROM RAT LIVER MITOCHONDRIA

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SUMMARY

(1) The ATPase inhibitor protein has been isolated from rat liver mitochondria in purified form. The molecular weight determined by sodium dodecyl sulfate gel electrophoresis is approximately 9500, and the isoelectric point is 8.9.

(2) The protein inhibits both the soluble ATPase and the particle-bound ATPase from rat liver mitochondria. It also inhibits ATPase activities of soluble F_1, and inhibitor-depleted submitochondrial particles derived from bovine heart mitochondria.

(3) On particle-bound ATPase the inhibitor has its maximal effect if incubated in the presence of $Mg^{2+} \cdot ATP$ at slightly acidic pH.

(4) The inhibitor has a minimal effect on P_i-ATP exchange activity in sonicated submitochondrial particles. However, unexpectedly the inhibitor greatly stimulates P_i-ATP exchange activity in whole mitochondria while the low ATPase activity of the mitochondria is not affected. The possible mechanism of action of the inhibitor on intact mitochondria is offered.

INTRODUCTION

A natural ATPase inhibitor has been characterized in bovine heart mitochondria [1–6]. This protein inhibits not only ATP hydrolysis, but also all the mitochondrial energy-linked reactions driven by ATP. From the results of equilibrium studies between the mitochondrial ATPase and its natural inhibitor, it has been proposed that the inhibitor exerts a regulatory function during energy conservation [3, 6]. A similar ATPase inhibitor has also been isolated from chloroplasts, which inhibits specifically chloroplast ATPase [7]. Recently, the isolation of an ATPase inhibitor protein from yeast mitochondria has been reported [8]; its physical properties are quite different from those of the inhibitor protein from bovine heart mitochondria. In this paper, we describe the isolation and some characterization of an ATPase inhibitor from rat

* Abbreviations: AS particle, inhibitor-depleted submitochondrial particle, obtained by sonication in the presence of 20 mM EDTA at pH 9.2 and subsequently passed through a Sephadex column.

liver mitochondria and compare its properties to those of inhibitors from other sources. Understanding the properties of the inhibitor protein from liver mitochondria will be of great importance in comparative studies of mitochondria from normal tissues and tumors such as hepatomas and carcinomas.

MATERIALS AND METHODS

Liver mitochondria were isolated from male Sprague-Dawley rats according to the method of Johnson and Lardy [9]. The mitochondrial preparation was either used immediately or stored at −90 °C in 0.25 M sucrose solution (50 mg protein/ml). ATPase inhibitor activity on particle-bound ATPase was measured by the method of Horstman and Racker [4]. In a final volume of 0.5 ml, 50 μg of AS particles (inhibitor-depleted submitochondrial particles, obtained by sonication in the presence of 20 mM EDTA at pH 9.2 and subsequently passed through a Sephadex column) with a specific activity of 2–3 μmol P_i/mg per min were incubated for 20 min at room temperature, with various amounts of inhibitor fractions in the presence of 0.5 mM $MgSO_4$, 0.5 mM ATP, 0.25 M sucrose and 15 mM Tris/sulfate (pH 6.0). The final pH of the incubation mixture was about 6.1. The order of addition was sucrose buffer, particles, inhibitor and $Mg^{2+} \cdot$ ATP. For comparison a control without inhibitor and a zero time control were assayed with each set of determinations. After 20 min, 0.2 ml of 0.1 M Tris/sulfate (pH 7.4) and 0.18 ml of H_2O were added to each tube. The assay was performed in the presence of an ATP-regenerating system containing 15 μg of pyruvate kinase and 5 mM phosphoenolpyruvate [10]. ATPase inhibitor activity on a soluble F_1-ATPase was measured by the method of Pullman and Monroy [1]. Bovine heart mitochondrial F_1 and rat liver mitochondria ATPase were prepared by the methods of Penefsky [11] and Lambeth and Lardy [12], respectively. AS particles were prepared according to the method of Horstman and Racker [4]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for molecular weight determinations were carried out on 10 % gels according to Weber and Osborn [13], using bovine serum albumin, ovalbumin, pyruvate kinase and cytochrome *c* as standards. Isoelectric focusing was carried out according to the procedure of Vesterberg [14], using 5.5 % acrylamide gels in the presence of pH 3–10 ampholine. The p*I* was determined both by a direct microelectrode measurement and by elution of thin sectioned gels. ATP-P_i exchange activities were measured according to the method of Pullman [15] both without and with an ATP-regenerating system. In a volume of 0.45 ml, 0.6 mg of mitochondria or submitochondrial particles was incubated with various amounts of inhibitor in the presence of 1.0 mg bovine serum albumin, 0.5 mM $MgCl_2$, 0.5 mM ATP, 10 mM Tris/sulfate (pH 6.0) for 10 min at room temperature. After the incubation, 50 μl of 0.2 M Tricine (pH 8.0) was added and the mixture was incubated for an additional 5 min at 30 °C before 0.5 ml of substrate containing 20 mM ATP, 20 mM $MgCl_2$ and 40 mM potassium phosphate (pH 7.4) and approximately 250 000 cpm of $^{32}P_i$ was added. Reactions in duplicate tubes were stopped at 4, 7 and 10 min by adding 0.1 ml of 50 % trichloroacetic acid. Phosphate was extracted with water saturated *iso*-butanol and benzene (1 : 1) mixture according to the method of Pullman [15] and radioactive ATP was measured with a Nuclear Chicago gas flow counter. Results were corrected for a zero time control tube in which trichloroacetic acid was added prior to the substrate addition.

RESULTS AND DISCUSSION

Purification of ATPase inhibitor

All procedures were carried out at 0 °C unless otherwise stated. The freshly isolated rat liver mitochondria in 0.25 M sucrose were adjusted to 0.15 M sucrose, 2 mM EDTA and 5 mM Tris/sulfate (pH 7.4) (1.53 g protein). With rapid stirring, 1.0 M KOH was added dropwise to pH 11.6–11.7 within 75 s. After 1 min at this pH, 10.0 M acetic acid was added to lower the pH to 8.0 within 75 s. The mitochondrial suspension was allowed to remain at this pH for 2.5 min and within 1 min the pH was lowered to 5.4 using 1.0 M acetic acid. After 1 min at pH 5.4, the pH was finally raised to 7.4 using 1.0 M KOH and the solution was centrifuged for 20 min at 100 000 $\times g$. To the clear yellow supernatant solid ammonium sulfate was added (39.0 g/100 ml solution) with rapid stirring. After incubating for 30 min, the resulting precipitate was collected by centrifuging at 100 000 $\times g$ for 10 min. The pellet was dissolved in 10 ml of 0.25 M sucrose and the pH adjusted to 7.4 using 1.0 M unneutralized Tris. The protein concentration was 14 mg/ml. To the protein solution, 50 % trichloroacetic acid was added dropwise with rapid stirring to a final concentration of 10 % trichloroacetic acid and the solution was immediately centrifuged at 20 000 $\times g$ for 5 min. The pellet was homogenized in 12 ml of glass distilled water and the pH quickly adjusted to 5.0 with 1.0 M KOH. After centrifuging for 5 min at 100 000 $\times g$, the supernatant was saved and neutralized to pH 7.4 with 0.1 M KOH and 2 M sucrose was added to make a final solution 0.25 M in sucrose. A second ammonium sulfate fractionation was carried out as follows. With rapid stirring 2.43 g of solid ammonium sulfate per 10 ml of the sucrose protein solution was added. The solution was allowed to incubate for 30 min at 0 °C and then centrifuged at 100 000 $\times g$ for 10 min. To the supernatant, 1.32 g of solid ammonium sulfate per 10 ml solution was added to make a final concentration of 60 % ammonium sulfate saturation. The solution was allowed to stir for 30 min and centrifuged as before. The pellet was dissolved in 2 ml of 0.25 M sucrose. The protein solution was placed in a long narrow

TABLE I

PURIFICATION OF RAT LIVER MITOCHONDRIAL ATPase INHIBITOR*

Fraction	Volume (ml)	Protein (mg**)	Total units***	Specific activity (units/mg)	Yield (%)
Mitochondria	42.5	1530			
Alkaline extract	73.0	347	3139	9.0	100
1st ammonium sulfate precipitate	10.04	149	2169	14.6	68.1
Trichloroacetic acid precipitate	16.17	10.5	1120	106.7	35.7
2nd ammonium sulfate precipitate	2.17	2.69	1042	387.4	33.2
Heat-treated solution	2.17	1.79	1267	707.8	40.4

* Inhibitor-depleted AS particles from mitochondria were used as the source of ATPase activity.

** Protein was determined by the method of Lowry et al. [17] scaled down to 1.0 ml.

*** 1 unit of ATPase inhibitor activity is defined [4] as that amount of inhibitor required to produce a 50 % inhibition of 0.2 unit of ATPase activity under the assay conditions (see text). 1 unit of ATPase activity is that amount of enzyme which hydrolyzes 1 μmol of ATP per min under the specified conditions.

test tube and incubated in a water bath at 80 °C for exactly 3 min, and then chilled at 0 °C. The resultant precipitate was removed by centrifugation at 100 000 × *g* for 10 min. A clear solution of the inhibitor in 0.25 M sucrose was obtained. A protocol for a typical purification is shown in Table I.

It is clear from the table that approximately 80-fold purification with 40 % yield of the inhibitor was achieved. The specific activity of the crude extract varies from preparation to preparation; however, the specific activity of the final product uniformly reaches more than 700 as defined [4]. The purification steps are similar to those used for isolating the inhibitor from bovine heart mitochondria [4] with several modifications. Firstly, the alkaline extraction step was carried out in a hypotonic sucrose solution. Results from pilot studies showed approximately a 50 % increase in yield when the extraction was carried out in a hypotonic vs. an isotonic sucrose medium. Secondly, unlike the bovine heart mitochondrial ATPase inhibitor, during the first ammonium sulfate precipitation the majority of the inhibitor activity was precipitated with low ammonium sulfate concentration, but after the trichloroacetic acid step, the inhibitor was precipitated between 40 % and 60 % ammonium sulfate saturation; probably the inhibitor protein was coprecipitated with other proteins before the trichloroacetic acid step. Finally, it was repeatedly observed that heat treatment at 80 °C for 3 min resulted in the highest specific activity and yield. It was also observed that centrifugation at the high *g* force was more efficient to remove contaminating protein during the purification steps.

Molecular weight and isoelectric point of rat liver mitochondrial ATPase inhibitor

The purified inhibitor appeared as a single band in sodium dodecyl sulfate gel electrophoresis. A densitometric tracing of the gel is shown in Fig. 1. The molecular weight is calculated to be 9500 (range from 8900 to 10 500 in different gel runs) based on the known molecular weights of the four standard proteins used. Since the apparent molecular weights of the three inhibitors from bovine heart, yeast and liver are

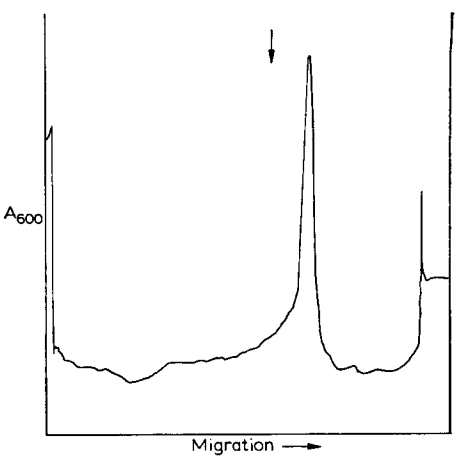

Fig. 1. Densitometric tracing of sodium dodecyl sulfate-polyacrylamide gel of purified ATPase inhibitor isolated from rat liver mitochondria. The gel was stained with Coomassie Blue. The arrow represents the position of cytochrome *c* from the same electrophoresis run.

different (all obtained from sodium dodecyl sulfate gel electrophoresis runs) [8], and since it is known that molecular weights of less than 10 000 cannot be accurately determined by sodium dodecyl sulfate gel electrophoresis, we are presently using gel filtration and equilibrium sedimentation methods to check for these differences in molecular weights of these similar inhibitors. The isoelectric point determination of the inhibitor by isoelectric focusing revealed a value of 8.9. It is interesting to note that the isoelectric points of the three inhibitors vary from fairly acidic to very basic. The amino acid content and partial sequence of the rat liver inhibitor are under current investigation.

Effect of pH on interaction between ATPase and inhibitor

To test the effect of the pH on the interaction between ATPase and the inhibitor, purified inhibitor was preincubated with inhibitor-depleted particle ATPase in the presence of 0.5 mM $Mg^{2+} \cdot$ ATP over a pH range of 4.5–9. After 20 min at room temperature, the pH was brought to 7.4 and the ATPase assay was started by addition of the reaction mixture. As shown in Fig. 2, in general agreement with the bovine heart ATPase for which maximal inhibition occurred at pH 6.7 [4], rat liver inhibitor yielded a maximal inhibition between pH 5.0 and pH 6.0 with a rapid decline in inhibitory activity at higher pH values, though the control ATPase activity without inhibitor was steadily increased by 20 % from pH 5.0 to pH 8.5 during the preincubation.

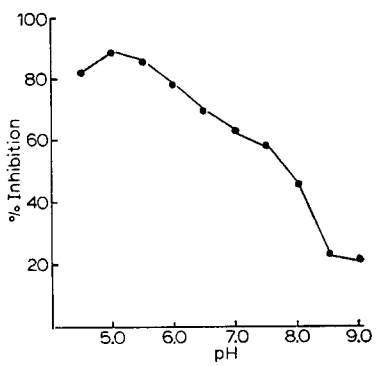

Fig. 2. Effect of pH on inhibition of particle-bound ATPase by inhibitor. The assay of inhibition was performed as described in the text. The pH values indicated were those of the preincubation medium; the enzyme assays were uniformly done at pH 7.4. 0.1 unit of AS particle ATPase and 4 μg of the inhibitor were used. The percent inhibition was calculated from each set of experiments at the same pH in the absence and presence of inhibitor. The actual ATPase activity steadily decreased by about 15 % from pH 8.5 to pH 5.0.

Requirement of $Mg^{2+} \cdot$ ATP for inhibition of ATPase

Horstman and Racker [4] demonstrated that $Mg^{2+} \cdot$ ATP was required to obtain a maximal inhibitory effect of the inhibitor on ATPase. Satre et al. [8] showed that the activity of the inhibitor from yeast could be conferred to particle ATPase by addition of $Mg^{2+} \cdot$ ATP or ATP alone. For the rat liver inhibitor incubated with the particle ATPase at pH 6.2 (Fig. 3) maximal inhibition, was strictly dependent on $Mg^{2+} \cdot$ ATP. Though ATP alone did confer partial inhibitory activity, $MgSO_4$ alone at the concentrations assayed was ineffective in conferring activity.

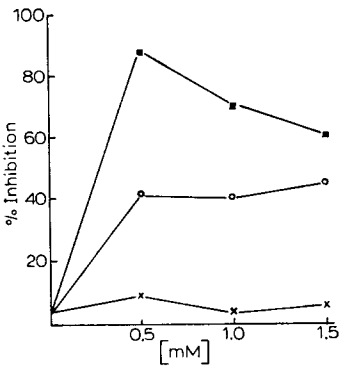

Fig. 3. Effect of $MgSO_4$ and ATP on inhibition of ATPase. 0.1 unit of particle-bound ATPase was preincubated with 4 μg of ATPase inhibitor at pH 6.2 in the presence of $MgSO_4$ (×), ATP (○) and Mg^{2+} · ATP (■). With no $MgSO_4$ and ATP, the inhibition of the ATPase activity by the inhibitor was less than 5 %. See text for the specific assay procdure and conditions.

Cross-reaction of bovine heart and rat liver inhibitors with soluble and particulate ATPases

The specificity and the potency of bovine heart and rat liver inhibitors were measured toward both soluble and particle ATPases from the two tissues. As represented in Fig. 4, it is clear that (a) inhibitor from bovine heart was more potent in inhibiting ATPases from both bovine heart and rat liver mitochondria, (b) inhibitor

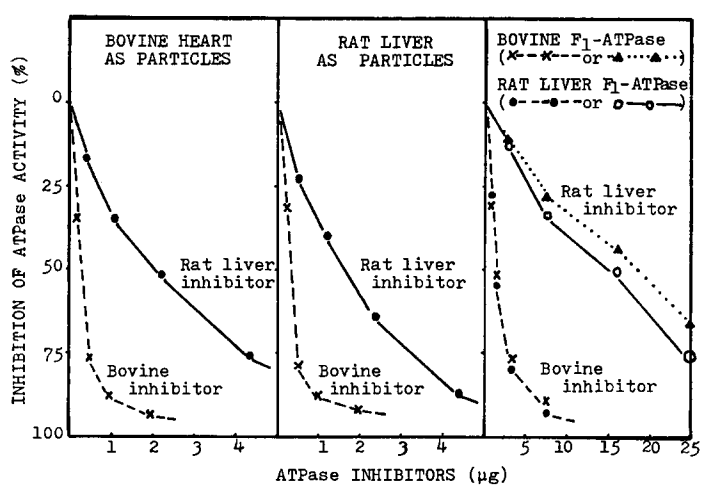

Fig. 4. Cross-reactions of bovine heart and rat liver mitochondrial ATPase inhibitors with inhibitor-depleted submitochondrial particles and purified soluble F_1-ATPases. Approximately 0.1 unit of ATPase regardless of source was used for each set of experiments. The ATPase activities with various amounts of the inhibitors were determined after a 20 min preincubation at room temperature in the presence of 0.5 mM Mg^{2+} · ATP (pH 6.2). Bovine heart mitochondrial ATPase inhibitor was isolated by the procedure of Horstman and Racker [4]. Specific activities of the ATPases were: AS particles from bovine heart, 3.3 units/mg; AS particles from rat liver, 0.82 units/mg; F_1 from bovine heart, 70 units/mg; soluble ATPase from rat liver, 22 units/mg.

from rat liver was slightly more active toward homologous ATPase than toward ATPase from bovine heart and (c) inhibitors from both rat liver and bovine heart were more potent toward particulate ATPases (AS particles) than toward soluble ATPases under the assay conditions. Nevertheless, the two inhibitors cross-react with ATPase from the other tissue very well; indeed, assays on rat liver inhibitor were usually carried out with bovine heart AS particles (see data obtained in Table I) because they are more readily available. Preliminary results (not shown) in our laboratory indicate that the rat liver inhibitor also cross-reacts immunologically with antibodies prepared against the bovine heart inhibitor.

Effect of rat liver ATPase inhibitor on ATP-P_i exchange activities of mitochondria and submitochondrial particles

As shown in Fig. 5A, the rat liver ATPase inhibitor has no or minimal effect on ATP-P_i exchange activity of the sonicated submitochondrial particles. This is in contrast to the ATPase activity of the submitochondrial particles (ATPase activity of the particles was 0.3 units/mg protein), which was inhibited to more than 90 % when 13 μg of the inhibitor was used for ATPase assays. Therefore, it is clear that the inhibitor interacts with the submitochondrial particles but has no effect on its ATP-P_i exchange activity. However, results such as those shown in Fig. 5B indicated that when the rat liver inhibitor was preincubated with intact mitochondria (from both rat liver and heart), the relatively low ATP-P_i exchange activity of the mitochondria was stimulated several-fold, up to the level exhibited by submitochondrial particles. This stimulation of the ATP-P_i exchange activity is unexpected, particularly when separate experiments showed that preincubating the same amount of the mitochondria (0.6 mg) with 26 μg of the inhibitor did not greatly inhibit the ATPase activity of the mitochondria (ATPase activity was 0.07 units/mg protein), presumably the inhibitor did not reach the sites of ATPase enzyme, which are located on the inner side of the inner mitochondrial membrane facing the matrix. These experiments were repeated in the presence of an ATP-regenerating system [1] and similar results were obtained.

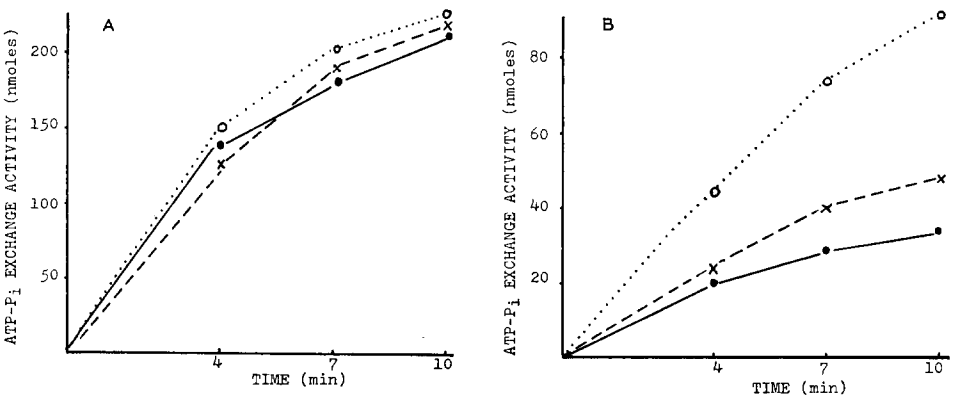

Fig. 5. (A) Effect of rat liver mitochondrial ATPase inhibitor on ATP-P_i exchange activity of sonicated submitochondrial particles from rat liver mitochondria. Zero (●), 13 μg (×) and 26 μg (○) of rat liver inhibitor were used. (B) Effect of rat liver mitochondrial ATPase inhibitor on ATP-P_i exchange activity of intact mitochondria from rat liver. Zero (●), 13 μg (×) and 26 μg (○) of rat liver inhibitor were used.

Furthermore, it was separately determined that, for example, using 0.6 mg of mitochondrial protein with an ATPase activity of 0.07 units/mg, not more than 0.5 μmol of ATP was hydrolyzed by the mitochondrial ATPase under the assay conditions in the absence of the inhibitor. Therefore the initial amount of ATP used (10 μmol) for the ATP-P_i exchange assay was essentially unchanged even in the absence of an ATP regenerating system. Thus the differential effects of the inhibitor on the ATP-P_i exchange activities of intact mitochondria and submitochondrial particles are not due to merely maintaining the rate-limiting level of ATP concentration. This unexpected effect of the inhibitor on whole mitochondria suggests that, though the inner mitochondrial membrane may not be permeable to a protein of molecular weight larger than 9000, the inhibitor protein can interact with the mitochondrial membrane from outside and affect the mitochondrial activity. This is not unreasonable, particularly when one learns that the biosynthesis of the inhibitor in yeast occurs in the cytoplasm (Tzagoloff, A., personal communication).

We are examining whether this observation of stimulating ATP-P_i exchange activity in whole mitochondria by the inhibitor is due to the facilitated adenosine nucleotide transport in intact mitochondria. More interestingly, studies have been initiated in our laboratory to examine the correlation between Mg^{2+}-ATPase and uncoupler-stimulated ATPase [16] and the presence (or absence) of the ATPase inhibitor and possible alteration in the control of oxidative phosphorylation in some tumor mitochondria.

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