# MULTIPLE SITES OF INTERACTION BETWEEN THE ATPase INHIBITOR AND MITOCHONDRIAL MEMBRANE FROM RAT LIVER MITOCHONDRIA

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Received July 12,1976

Summary: The ATPase inhibitor interacts with $F_1$-ATPase and sonicated submitochondrial particles in which the matrix side of the inner membrane is exposed to the suspending medium. On the other hand, preincubating the inhibitor with intact mitochondria in the presence of $Mg^{++}$-ATP greatly stimulates ATP-Pi exchange activity of the intact mitochondria. In contrast, preincubating the inhibitor with submitochondrial particles has no effect on the ATP-Pi exchange activity of the latter. Furthermore, when the inhibitor is incorporated inside the submitochondrial particles by sonicating intact mitochondria in the presence of added inhibitor, the ATP-Pi exchange activity of the so prepared submitochondrial particles is higher than that of control submitochondrial particles. When atractyloside is included in the assay medium, the ATP-Pi exchange activities of intact mitochondria (regardless of preincubation with added inhibitor or not) are completely inhibited while that of submitochondrial particles (regardless of preincorporating the inhibitor inside the particles or not) are not affected. These results indicate that the inhibitor interacts with $F_1$-ATPase on the matrix side and with the adenine nucleotide translocator on the outer side of the inner mitochondrial membrane.

## Introduction

The ATPase inhibitor from bovine heart mitochondria has been characterized (1, 2). This protein inhibits not only ATP hydroylsis, but also all the mitochondrial energy-linked reactions driven by ATP (3). Its regulatory function during energy conservation has been proposed (3, 4). Recently, we reported the isolation of the ATPase inhibitor protein from rat liver mitochondria (5) and offered evidence that many of its physical properties are similar to that of the inhibitor from bovine heart mitochondria and that the two inhibitors cross-react with ATPase from the other tissue quite well. In this communication we wish to describe results of experiments which indicate that the rat liver inhibitor not only interacts with the ATPase on the matrix side of inner mitochondrial membrane

by inhibiting ATPase activities of both soluble $F_1$ and the membrane-bound enzyme, but also interacts with the adenine nucleotide translocator which has been proposed to be on the outer side of the inner mitochondrial membrane (6). Results obtained may indicate that the inhibitor protein actually exerts a regulatory function on the adenine nucleotide translocator.

## Materials and Methods

Liver mitochondria were isolated from male Sprague-Dawley rats according to the method of Johnson and Lardy (7). Liver mitochondrial ATPase inhibitor was obtained as described previously (5). ATPase inhibitor activity on particle-bound ATPase and ATP-Pi exchange activity were measured by the methods of Horstman and Racker (2) and Pullman (8) respectively as modified in our laboratory (5). When atractyloside was used, 50 µM final concentraction was added after the preincubation period, just before the addition of ATP-Pi substrate mix which was added to start the reaction.

For the experiment on the interaction between mitochondria and the inhibitor, 625 µg of freshly isolated rat liver mitochondria were incubated with 70, 140 and 210 µg of the inhibitor for 10 min at room temperature in the presence of 1 mM Mg-ATP, 1 mg bovine serum albumin, 10 mM Tris-$SO_4$ pH 6.0 final volume 0.45 ml. After centrifugation for 10 min at 20,000 x g, the indicated amount of supernatant was assayed for the ATPase inhibitor activity (5). A tube with 140 µg of inhibitor preincubated with the same reagents but without mitochondria was used as a control as indicated. For the preparation of SMP* containing added internal ATPase inhibitor, 21 mg of freshly isolated rat liver mitochondria were suspended in 0.15 M sucrose, 10 mM Tris-Cl, pH 7.4 in the presence of 2.8 mg of ATPase inhibitor in a final volume of 10 ml. The suspension was sonicated for 4 min with a Raytheon Sonicator at 0°C. After removing the unbroken mitochondria by centrifuging at 16,000 x g for 5 min, the SMP were spun down at 100,000 x g for 20 min, washed and finally suspended in 0.25 M sucrose. The control submitochondrial particles were similarly prepared but without added ATPase inhibitor during sonication.

## Results and Discussion

It has been well documented that the ATPase inhibitor protein inhibits $F_1$-ATPase activity, thereby interacting with soluble $F_1$-ATPase (1) as well as sonicated submitochondrial particles in which the matrix side of the inner membrane is exposed to the suspending medium (2). We reported that the inhibitor protein from rat liver mitochondria inhibits the ATPase activity of the inhibitor depleted submitochondrial particles (5). Figure 1 shows the inhibitor also

*Abbreviation: SMP, Sonicated submitochondrial particles

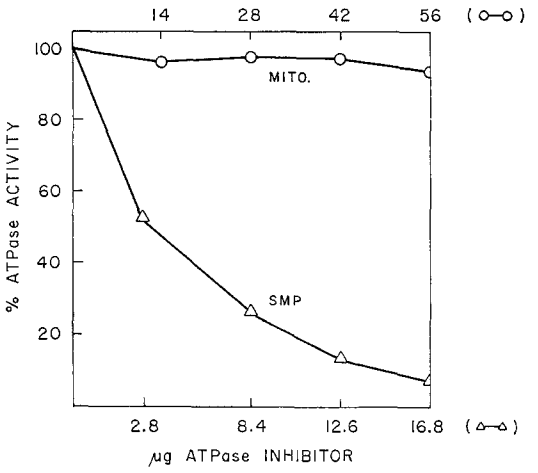


Figure 1. The effect of ATPase inhibitor on ATPase activities of freshly isolated mitochondria (Mito) and sonicated submitochondrial particles (SMP). 600 μg of mitochondrial protein or 250 μg of SMP were incubated with indicated amounts of the ATPase inhibitor at room temperature in the presence of 0.25 M sucrose, 0.5 mM Mg-ATP, 15 mM Tris-$SO_4$, pH 6.0. ATPase activities were measured as described (5). The specific activities of mitochondria and SMP without added inhibitor were 0.066 and 0.52 units/mg. respectively. 1 unit of ATPase activity is that amount of enzyme which hydrolyzes 1 μmole of ATP per min under the specified conditions.

inhibits the ATPase activity of SMP which had not been treated to deplete all the inhibitor protein that the SMP contains. It can be seen that using 250 μg of SMP with specific activity of 0.5 2 μmole/mg/min, approximately 3 μg of the ATPase inhibitor causes 50% inhibition. It is interesting to notice that when inhibitor-depleted particles were used, only approximately 1.5 μg of the inhibitor is necessary to give 50% inhibition. In contrast, as can be seen when the whole mitochondria were used, even large quantities of the inhibitor did not inhibit the ATPase activity at all. It is reasonable to assume that only when the ATPase enzyme (either soluble or membrane-bound) is exposed to the suspending medium where the active site or sites are accessible to added inhibitor protein can the ATPase activity be inhibited. In whole mitochondria, where the ATPase enzyme faces the matrix (10), the active sites are not accessible to the medium, so that added inhibitor can not penetrate into the mitochondrial matrix to affect the ATPase activity.

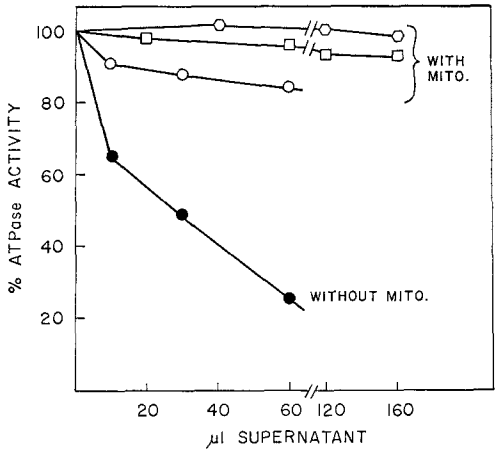


Figure 2. The adsorption of ATPase inhibitor by rat liver mitochondria. 70 µg (◇——◇), 140 µg (□——□) and 210 µg (o——o) of ATPase inhibitor were incubated with 625 µg of freshly isolated mitochondria as described in the text. Indicated amounts of supernatant were assayed for the ATPase inhibitor activity using 0.1 unit of inhibitor-depleted submitochondrial particles as the source of ATPase activity (5). 140 µg of the ATPase inhibitor without added mitochondria (●——●) was preincubated under identical conditions and assayed for the ATPase inhibitor activity as the control.

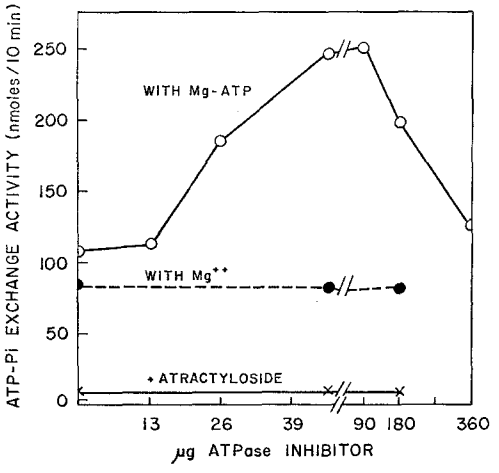


Figure 3. The effect of ATPase inhibitor on ATP-Pi exchange activity of rat liver mitochondria. Indicated amounts of ATPase inhibitor were preincubated with 608 µg of freshly isolated mitochondria in the presence of Mg-ATP (o——o), or $Mg^{++}$ (●——●) and ATP-Pi exchange activities were measured as described previously (5). 50 µM atractyloside in the assay medium (x——x) completely inhibited the exchange activity regardless of preincubation conditions.

However, as shown in Fig. 2, when the inhibitor protein was preincubated with freshly isolated rat liver mitochondria in the presence of Mg-ATP at pH 6.0, considerable amounts of the inhibitor protein are adsorbed by the mitochondria and spun down with the mitochondrial pellet. It is clear from the figure that as much as 70 to 100 μg of the inhibitor protein are adsorbed by 625 μg of mitochondria. In order to convince ourselves that the adsorption is not non-specific, one mg of bovine serum albumin was added in addition to the mitochondria during the preincubation, and similar results were obtained. In order to test whether the inhibitor has any effect on mitochondria, ATP-Pi exchange activity of the freshly isolated mitochondria with and without added inhibitor protein were measured. Results are shown in Figure 3. As indicated, the titration curves clearly show that when preincubated in the presence of $Mg^{++}$-ATP, the inhibitor greatly stimulated the ATP-Pi exchange activity of rat liver mitochondria. In addition, when preincubated in the presence of only $Mg^{++}$ ion, the effect of the inhibitor on mitochondrial ATP-Pi exchange activity was not observed. Furthermore, as indicated, when 50 μM atractyloside was added either to mitochondria alone or to the ATPase inhibitor-preincubated mitochondria, the ATP-Pi exchange activities of both were completely inhibited. This last observation supports the suggestion by Groot (9) that the entry of ATP into mitochondria may be the rate-limiting step in the exchange reaction. Since atractyloside is a specific inhibitor of mitochondrial adenine nucleotide translocator, the complete inhibition of ATP-Pi exchange activity by atractyloside dictates that the adenine nucleotide translocator is the rate-limiting step in intact mitochondria; and the stimulation of the exchange activity by the inhibitor protein may be the result of increased entry of ATP into mitochondria due to the interaction of the inhibitor protein with the adenine nucleotide translocator.

To further tests this hypothesis, the ATP-Pi exchange activities of SMP were tested. Figure 4 shows that the exchange activity of SMP is completely insensitive to atractyloside and the specific activity is three to five times

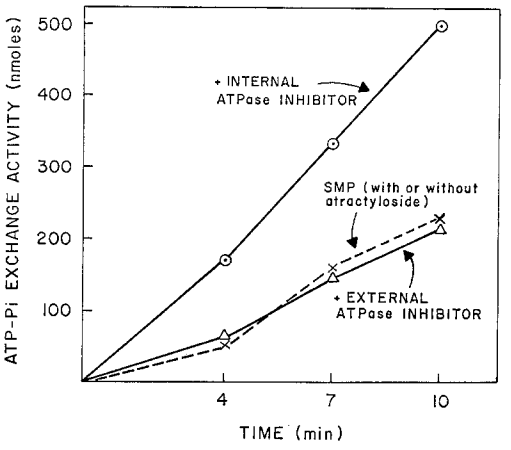


Figure 4. The effect of ATPase inhibitor in ATP-Pi exchange activity of sonicated submitochondrial particles (SMP). 208 µg of SMP were preincubated without (x——x) and with 94 µg of ATPase inhibitor (Δ——Δ) and assayed for ATP-Pi exchange activities as described (5). Adding 50 µM atractyloside in the assay medium in either tube did not affect the exchange activity as indicated. Internally added ATPase inhibitor (0——0) during sonication as described in the text increased the ATP-Pi exchange activity.

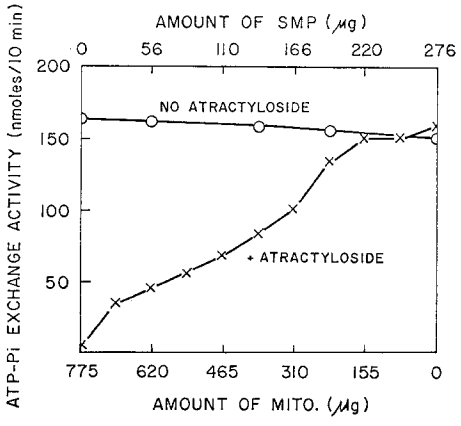


Figure 5. Sensitivity of ATP-Pi exchange activities to atractyloside. 775 µg of freshly isolated rat liver mitochondria and the indicated proportions were mixed with indicated proportions of 276 µg of sonicated submitochondria particles. ATP-Pi exchange activities of the resultant mixtures were measured in the absence of (o——o) and 50 µM atractyloside (x——x) as described (5). The specific exchange activity of mitochondria and SMP were 20.9 and 57.9nmoles/mg/min respectively.

higher than that of intact mitochondria. Thus it is clear that in sonicated "inside out" submitochondrial particles, the adenine nucleotide translocator

is no longer the rate-limiting step in the ATP-Pi exchange activity. When ATPase inhibitor was added in the assay medium, the ATP-Pi exchange activity of SMP was no longer affected.

However, interestingly, when ATPase inhibitor protein was added to SMP internally during sonication, the ATP-Pi exchange activity of the so prepared SMP was stimulated considerably when compared to control SMP prepared without internally added ATPase inhibitor. These results together with those shown in Figure 3 clearly indicate that in addition to the interaction on the matrix side of the inner mitochondrial membrane, where it inhibits the ATPase activity, the inhibitor protein also interacts with the adenine nucleotide translocator on the outer side of the mitochondrial membrane. We further tested the atractyloside sensitivity of ATP-Pi exchange activities of whole mitochondria and SMP. As shown in Figure 5, when mitochondria and SMP were mixed in different proportions, only the ATP-Pi exchange activity contributed by mitochondria was inhibited when atractyloside was added while that portion contributed by SMP was not inhibited at all.

In conclusion, data presented in this communication argue for multiple sites of interaction between ATPase inhibitor and rat liver mitochondria; it interacts with ATPase on the matrix side as well as the adenine nucleotide translocator on the outer side of the inner mitochondrial membrane. Recent reports on the proton-translocating ATPase complex (11, 12) had some bearing on the earlier proposal that the adenine nucleotide transport system and the mitochondrial ATPase may function as a single unit (13). In view of the present communication, the effect of ATPase inhibitor on the reconstituted adenine nucleotide translocator is of great interest. Results reported here also raise the question of the localization of the ATPase inhibitor in intact mitochondria (14, 15). The possibility also exists that the ATPase inhibitor is a mobile component, which is distributed on the inner or outer face of the inner mitochondrial membrane depending on the environmental conditions.

Acknowledgement: This investigation was supported by a grant from American Heart Association, Finger Lakes Chapter, New York, and a Syracuse University Senate Research and Facilities grant. We thank Dr. H. R. Levy for proof-reading the manuscript.

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