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ACTION OF THE MITOCHONDRIAL ATPase INHIBITOR PROTEIN ON THE Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM

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Summary: The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum catalyzes a rapid ATP⇄Pi exchange in absence of a transmembrane Ca^{2+} gradient. The rate of ATP⇄Pi exchange is activated by the natural ATPase inhibitor protein from heart mitochondria. Activation of ATP⇄Pi exchange depends on both the Ca^{2+} and ADP concentration in the medium and is not accompanied by a significant modification of the rate of ATP hydrolysis.

Vesicles derived from the sarcoplasmic reticulum of skeletal muscle retain a highly efficient Ca^{2+} transport system mediated by a membrane bound ATPase. When a Ca^{2+} gradient is formed across the vesicles membrane, the entire process of Ca^{2+} transport can be reversed and, depending on the experimental conditions used, the ATPase can catalyze either the net synthesis of ATP or a rapid ATP⇄Pi exchange. During ATP⇄Pi exchange the ATPase operates simultaneously forward (ATP hydrolysis) and backwards (ATP synthesis from ADP and Pi). When the reversal of the Ca^{2+} transport was described it was proposed that energy for the synthesis of ATP is derived from the transmembrane Ca^{2+} gradient (1-5). Subsequent studies revealed that in the absence of a Ca^{2+} gradient the enzyme catalyzes both the net synthesis of a small amount of ATP and a rapid and continuous ATP⇄Pi exchange (6-9).

Pullman and Monroy (10) isolated a low molecular weight protein from mitochondria that inhibits the ATPase activity of particulate and soluble mitochondrial ATPase. For some years the protein was considered an unidirectional inhibitor of mitochondrial ATPase, since no effect on ATP synthesis was observed. However, in recent years, it was found that the protein also affected ATP synthesis (11, 12), and that it may exert a regulatory action on the catalytic processes of ATP synthesis (13).

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Since both the ATPase from mitochondria and the sarcoplasmic reticulum can catalyze the synthesis of ATP, it was decided to explore whether the inhibitor protein from mitochondria could affect the catalytic properties of the sarcoplasmic reticulum ATPase. In this respect it is of interest that an inhibiting effect of the protein on the ATPase activity of actomyosin has been reported (14, 15). The data of this report show that under suitable conditions, the mitochondrial inhibitor activates the rate of ATP$\rightleftharpoons$Pi exchange catalyzed by the sarcoplasmic reticulum ATPase in the absence of Ca^{2+} gradient without having a significant effect on ATP hydrolysis.

METHODS

The inhibitor protein was prepared from bovine heart mitochondria (16) according to Kanner et al (17). The purification of the inhibitor protein involved precipitation of the cholate solubilized protein between 38 and 45% $(NH_4)_2SO_4$, heating at 75°C for 4 min, and precipitation with ethanol. The inhibitor protein was kept frozen until used. The various preparations of inhibitor protein contained about 2M $(NH_4)_2SO_4$, as inferred from the assay of NH_4^+ (18). The protein was desalted by passage through a column of sephadex G25 as described by Penefsky (19). The content of $(NH_4)_2SO_4$ in the eluted protein varied between 20 and 30 mM. Soluble F_1-ATPase from heart mitochondria was prepared as described elsewhere (20). The inhibitory effect of the protein on this enzyme was measured by incubating 5 μg F_1-ATPase with the inhibitor protein in 20 mM Tris-MES buffer pH 6.6, 1 mM ATP and 1 mM $MgCl_2$. After 15 min of incubation at 25°C, aliquots were withdrawn to assay ATPase activity as previously described (21).

Leaky vesicles reconstituted from purified Ca^{2+}-dependent ATPase were prepared from sarcoplasmic reticulum vesicles of rabbit skeletal muscle as described by MacLennan (22, 23). ATPase activity was assayed by measuring the release of (^{32}P)Pi from (γ -^{32}P)ATP. After precipitation with trichloroacetic acid, the (^{32}P)Pi was extracted as the phosphomolybdate complex with a mixture of benzene and isobutyl alcohol (6). Synthesis of ATP was determined by measuring (γ -^{32}P)ATP formed from ^{32}Pi. After precipitation of the protein with trichloroacetic acid, the (^{32}P)Pi present in the aqueous phase was extracted as a phosphomolybdate complex as described above. The organic phase was discarded, 0.01 ml of 20 mM Pi carrier plus 0.3 ml of acetone were added to the water phase, and this was re-extracted with benzene-isobutyl alcohol. This procedure was repeated 4 times. The water phase was counted in the scintillation counter (6). The assay medium composition is described under Results. The concentrations of ATP, ADP and Pi chosen were optimal for ATP$\rightleftharpoons$Pi exchange as shown in a previous report (24).

RESULTS AND DISCUSSION

Hydrolysis and synthesis of ATP are regulated by the binding of Ca^{2+} to a high (K_2 0.4 to 0.5 μM at pH 7.5) and low affinity (K_s 0.4 to 0.6 mM at pH 7.5) sites of the Ca^{2+}-dependent ATPase located respectively on the outer and inner surface of the sarcoplasmic reticulum membrane (6, 8, 9). With a low Ca^{2+} concentration (0.1 mM in Fig. 1A), the ATPase catalyzes only the hydrolysis of ATP. Raising the Ca^{2+} concentration in the medium leads to both an inhibition of ATP hydrolysis and activation of formation of radioactive

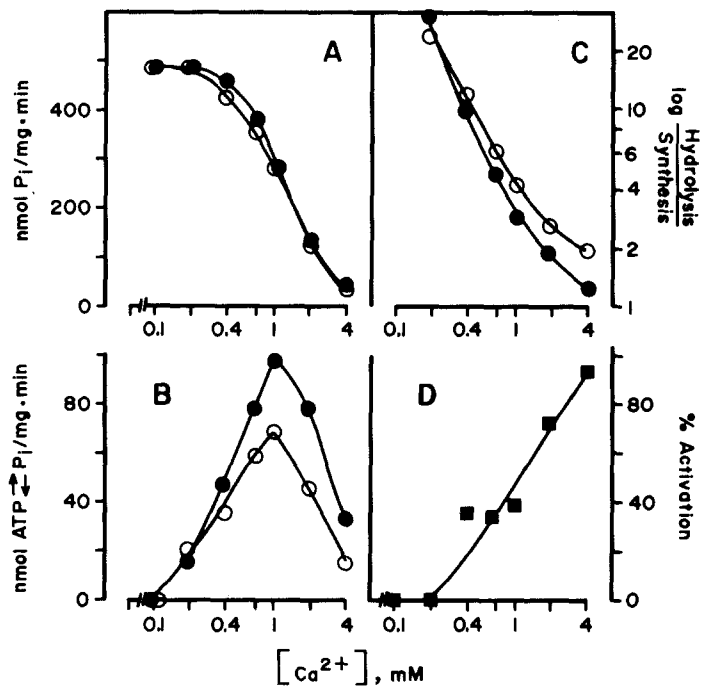

Figure 1.- Effect of mitochondrial inhibitory protein on sarcoplasmic reticulum ATPase . A and B: The assay medium consisted of 40 mM Tris-maleate buffer (pH 7.5), 10 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM ADP, 4 mM Pi and the $CaCl_2$ concentrations shown in the figure. The reaction was performed at 35°C, it was started by the addition of sarcoplasmic reticulum ATPase to a total of 5 ug protein per ml and stopped after 10 min by the addition of 4 volumes 10% (w/v) of trichloroacetic acid. For ATPase activity (A), (γ-^{32}P)ATP and non-radioactive Pi were used. For ATP⇄Pi exchange, (B), non-radioactive ATP and (^{32}P)Pi were used. In A and B, (○) is control without addition or with 15 mM $(NH_4)_2SO_4$, the same values of ATP hydrolysis and of ATP⇄Pi exchange were obtained in these two conditions; (●) addition of mitochondrial inhibitory protein, total of 20 µg protein per ml. The final concentration of $(NH_4)_2SO_4$ in the medium introduced with the inhibitory protein was 13 mM. C: Ratio of the rates of ATP hydrolysis and of ATP formation calculated from the values of A and B. D: Activation of rate of ATP⇄Pi exchange promoted by the inhibitor protein of mitochondria calculated from the values shown in B.

ATP from ADP and (^{32}P)Pi (Figs. 1, A and B). Maximal activation of ATP formation is detected in presence of 1 mM Ca^{2+}, higher Ca^{2+} concentrations are inhibitory. Addition of the inhibitory protein of mitochondria increases the rate of radioactive ATP formation (Fig. 1B). This effect depends on both the Ca^{2+} (Fig. 1B) and ADP concentration (Fig. 2), becoming higher as the concentration of these reactants in the medium is increased. Activation of the rate of ATP⇄Pi exchange by the inhibitor protein was observed in 10 different experiments using 5 different inhibitor protein preparations and 6 different enzyme preparations.

The effect of the inhibitor protein on the rate of ATP hydrolysis was variable. Inhibotory protein concentrations which promote maximal activation of ATP⇄Pi exchange, produced a small activation (20 to 30%) of the rate of

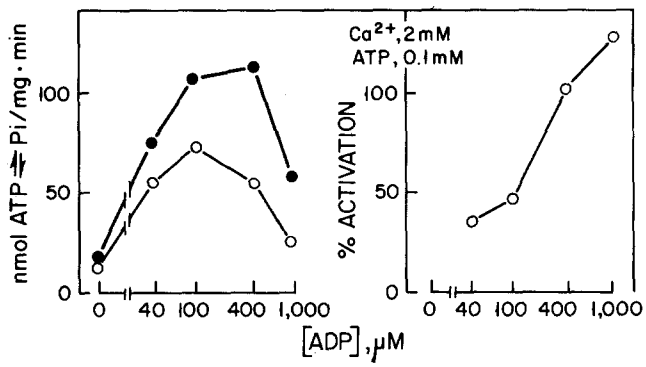

Figure 2.- ADP dependence of the action of the inhibitor protein. Left: Assay medium and conditions were as in fig. 1 except that the $CaCl_2$ concentration was 2 mM and the ADP concentration was as shown in the figure. (○) control without additions; (●) plus mitochondrial inhibitor, total of 20 μg protein per ml. Right: Activation of the rate of ATP⇌Pi exchange promoted by the inhibitor protein of mitochondria.

ATP hydrolysis in 4 experiments, but it had no effect in 6 other experiments. At present we do not know the reason for the discrepancy. Fig. 1 shows a typical experiment in which the inhibitor protein activates the ATP⇌Pi exchange, without a significant effect on the rate of ATP hydrolysis. It has been shown (24) that the ratio of the rates of ATP hydrolysis and ATP synthesis decreases as the Ca^{2+} concentration in the medium is raised. At high Ca^{2+} concentrations this ratio becomes smaller in the presence of the inhibitor protein (Fig. 1C).

The concentration of inhibitor protein required to inhibit F_1-ATPase activity is smaller than that required to activate the velocity of ATP⇌Pi

TABLE I.

Effect of the Inhibitor Protein on the ATP⇌Pi Exchange of the sarcoplasmic reticulum ATPase and on the ATPase activity of soluble F_1-ATPase.

Inhibitor protein μg per ml	F_1ATPase activity μmol Pi/mg. min	Sarcoplasmic Reticulum ATPase nmol ATP⇌Pi/mg. min
0	42	43
0.5	28	-
2.5	16	-
5.0	11	-
8.0	9	-
13	-	56
26	-	68
64	-	70

The effect of the inhibitory protein on the rate of ATP⇌Pi exchange was measured as shown in Fig. 1B using 2 mM $CaCl_2$. The interaction of the inhibitor protein with F_1-ATPase was determined as described under Methods.

exchange catalyzed by the sarcoplasmic reticulum ATPase (Table 1). To attain maximal inhibition of F_1-ATPase (25, 26), the inhibitor protein must be preincubated with the enzyme (Table I). Preincubation does not seem to be required in the case of the sarcoplasmic reticulum ATPase. The same activation of ATP⇄Pi exchange is observed regardless of the preincubation conditions (data not shown). In experiments in which the ATP⇄Pi exchange was measured as a function of incubation time, it was found that the formation of radioactive ATP proceeds linearly for the initial 20 minutes of incubation tested with or without added inhibitor protein; throughout this time, the percent of activation remained constant (data not shown). In these experiments, the conditions were the same as in Fig. 1 using 2 mM $CaCl_2$.

CONCLUSIONS

The mechanism by which the sarcoplasmic reticulum ATPase catalyzes the ATP⇄Pi exchange in the absence of a transmembrane Ca^{2+} gradient was discussed in detail in previous reports (6, 8, 9, 24). The ATP⇄Pi exchange is an index of the reversal of the Ca^{2+} pump (4, 8,9). As far as we know, the inhibitory protein of mitochondria is the first factor found which activates the reversal of the catalytic cycle of the sarcoplasmic reticulum ATPase without having a major effect on the velocity of ATP hydrolysis. The data of Figs. 1 and 2 show that activation of ATP⇄Pi exchange is more pronounced at concentrations of Ca^{2+} and ADP which, in absence of the mitochondrial factor, are inhibitory, as if the mitochondrial factor protected the sarcoplasmic reticulum ATPase from the inhibition promoted by excess of substrate. The finding that this protein may exert a regulatory action of both the ATPase of mitochondria and the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum raises the possibility that these two enzymes originated from a common molecular ancestral, and that there might be some common intermediary steps in the catalytic process of energy transduction of the two systems.

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