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ELECTROCHEMICAL GRADIENT INDUCED DISPLACEMENT OF THE NATURAL ATPase INHIBITOR PROTEIN FROM MITOCHONDRIAL ATPase AS DETECTED BY ANTIBODIES AGAINST THE INHIBITOR PROTEIN

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Summary

Antibodies against the natural ATPase inhibitor protein of bovine heart mitochondria (1) block the inhibitory action of the protein on ATP hydrolysis by soluble or particulate F_1-ATPase. By immunodiffusion tests, inhibitor protein may be detected in Mg-ATP and State 3 submitochondrial particles. The binding of ^{121}I-labeled antibodies to submitochondrial particles that possess their ATPase in the inhibited state is several times lower than in particles that had been exposed to electrochemical gradients and which show higher rates of ATPase activity. The results indicate that electrochemical gradients induce a change in position of the inhibitor protein in relation to F_1-ATPase which results in the appearance of the catalytic properties of the enzyme.

Introduction

Pullman and Monroy (1) isolated from heart mitochondria a protein that inhibited ATP hydrolysis by the mitochondrial ATPase. Later it was proposed (2, 3) that the inhibitor protein regulated the hydrolysis of ATP and all the ATP-dependent reactions of mitochondria. Proteins with similar properties have now been isolated from several sources (4-9). It is also known that the inhibitor proteins interact with the F_1 component of the ATPase complex (10), or more precisely, in the heart system, with its β-subunit (11).

Although the inhibitory effect of the protein on ATP hydrolysis is established, it is not yet clear whether the inhibitor protein also controls the synthesis of ATP. In this respect, some recent experiments (12-14)

Abbreviations: F_1, the component of mitochondrial ATPase that possesses the catalytic site(s); MES, (2 N-morpholino ethane sulfonic acid); State 3 particles; submitochondrial particles as prepared in ref. 15.

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suggest that synthesis of ATP does not occur until electrochemical gradients induce the transfer of the inhibitory protein to a non-inhibitory site.

Although the experimental results (12-14) suggested that the displacement of the inhibitor protein from its inhibitory site is a rate limiting step in ATP synthesis at the level of the ATPase, in the reported studies no direct evidence was provided that firmly indicated that electrochemical gradients induce a change in the position of the inhibitor protein. Moreover, in the earlier studies of Van de Stadt et al (15), it was also concluded that activation of the ATPase by electron transport was due to release of inhibitor protein, but again their conclusions were not supported by direct evidence. As the inhibitor protein may be involved in the regulation of ATP synthesis and other processes (16), it is necessary to determine whether, under the influence of electrochemical gradients, the inhibitor protein undergoes changes in position that result in the appearance of catalytic activity.

In this work it is shown that in phosphorylating submitochondrial particles, the binding of antibodies directed against inhibitor protein increases several fold upon activation of the ATPase by electrochemical gradients. The results indicate that the inhibitor protein controls the activity state of mitochondrial ATPase through changes in its position in F_1-ATPase.

Materials and Methods

Bovine heart mitochondrial (17), Mg-ATP (18), State 3 (15) particles, F_1-ATPase (19), and inhibitor protein (20) were prepared by reported methodologies. ATP hydrolysis was assayed as described by Pullman et al (21).

Antibodies to purified inhibitor protein (one band in 10% sodium dodecyl gels; 22) were raised in rabbits by the injection of 1 ml of a mixture of 1 mg of the inhibitor protein in 1 ml of Freunds complete adjuvant, followed by 3 intravenous injections of inhibitor protein (100 µg/100 µl) at 15 day intervals. One week after the fourth injection, blood of the rabbits was collected by intracardiac puncture. The antisera were assayed by the double immunodiffusion test of Ouchterlony (23). The γ-globulin fraction was obtained by precipitation with 33% $(NH_4)_2\ SO_4$. This procedure was repeated three times; finally the γ-globulins were dialyzed against 9 1 of phosphate buffer saline.

Anti-inhibitor protein γ-globulins were radiolabeled with 1 µCi ^{125}I to a specific activity of 4.1×10^5 cpm per µg of protein employing chloramine T (24). Protein was determined by the biuret method or according to Lowry et al (25).

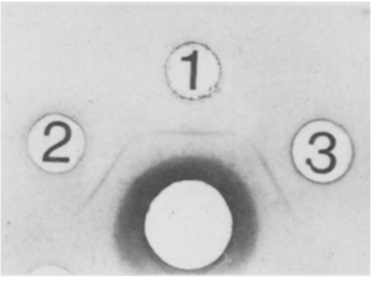

Fig. 1. Immunodiffusion of γ-globulins Against Inhibitor Protein as Tested against Inhibitor Protein (Well 1),Mg-ATP (Well 2), and State 3 (Well 3) Particles. The center well contained anti-inhibitor protein γ-globulins. The plate was prepared with 0.7% agar/50mM tris-HCl pH 7.4/ 1% triton X-100/ 0.2 M sucrose/ 0.05% sodium azide. The particles were lysed with 1% triton X-100 and added to the indicated wells. The gel was incubated for 5 days at 4°C. After band formation, it was washed with buffer for 2 days. Thereafter it was dried and stained with 0.25% Coomasie blue/ 30% methanol/ 7% acetic acid, and subsequently destained with the same solution without the dye.

Results

Figure 1 shows that the γ-globulin fraction of rabbits injected with inhibitor protein cross-reacts with the purified inhibitor protein. The antibodies also detect the inhibitor protein in Mg-ATP and State 3 submitochondrial particles after solubilization with triton X-100. It should be pointed out that ATPase activity of our State 3 particles (1.5 - 2.4 μmol min^{-1} mg^{-1}) is 5-8 times higher than that of Mg-ATP particles (0.3 - 0.6 μmol $min^{-1}mg^{-1}$).

The incubation of antibodies with inhibitor protein abolishes the inhibiting action of the protein on the ATPase activity of soluble or particulate F_1 (Fig. 2). The neutralization curve at various concentrations of antibodies is sigmoidal, compatible with the classical antibody-antigen precipitin reaction with polyvalent antibodies.

After activation of the ATPase system by electrochemical gradient, we have looked very extensively for the presence of inhibitor protein in the external water phase of the particles. No inhibitor protein was found in the external water phase even though in our experimental conditions the hydrolytic activity of the particles increased 5-8 times. If indeed the protein

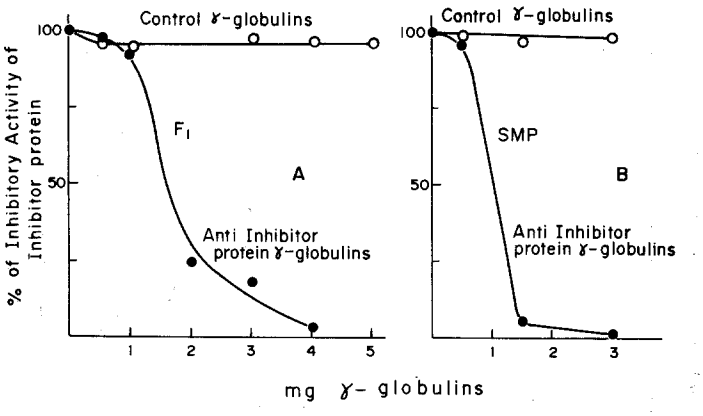

Fig. 2. Effect of γ-globulins against Inhibitor Protein on the Action of Inhibitor Protein on the ATPase Activity of Soluble F_1-ATPase and Sephadex Particles. Inhibitor protein (1 μg) was preincubated for 10 min in 4 mM Mg-ATP and 16 mM tris-MES pH 6.7 and the indicated amounts of control γ-globulins directed against inhibitor protein. At this time soluble F_1 (2.7 μg) (A), or Sephadex submitochondrial particles (SMP) (100 μg) (B) were added. After a further 10 min of incubation, the ATPase activity of the mixture was assayed.

is involved in the activation of ATPase, these results indicated that the inhibitor protein was not released to the external water space, but rather that the protein was transferred to a non-inhibitory site in the particles. Indeed the presence of inhibitor protein in State 3 particles suggests that this is the case (Fig. 1).

If the latter proposal is correct, it could be expected that the amount of antibodies against the inhibitor protein that bind to Mg-ATP particles would differ from that that binds to State 3 particles. Figure 3 shows that the binding of ^{125}I-labeled antibodies against inhibitor protein is higher in State 3 than in Mg-ATP particles. Scatchard plots (26) of the data indicate (inset Fig. 3) that State 3 particles possess a high affinity binding that is absent in the Mg-ATP particles.

Discussion

Previous studies (12, 13) suggested that oxidative phosphorylation did not take place until the inhibitor protein was released from its inhibitory site through a process that was induced by electrochemical gradients. If

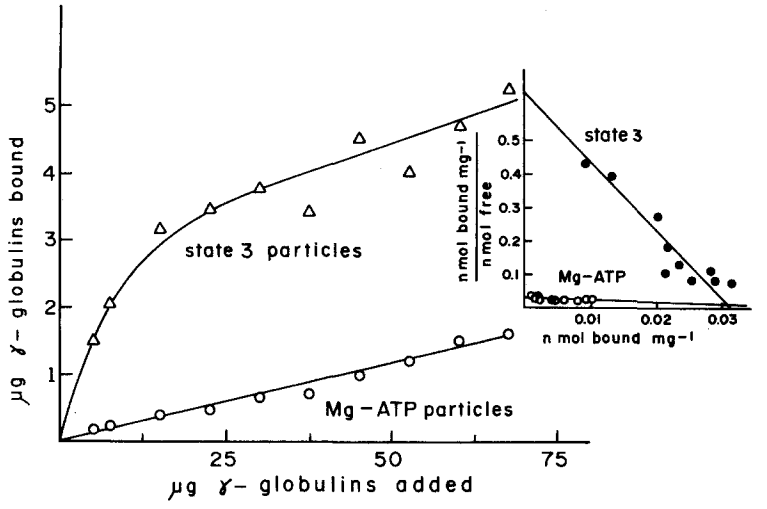

Fig. 3. Binding of ^{125}I-γ globulins against Inhibitor Protein to Mg-ATP and State 3 Particles. Mg-ATP and State 3 submitochondrial particles (1 mg) were incubated overnight in the cold with the indicated amount of ^{125}I-γ globulins in 0.25 M sucrose in a final volume of 200 μl in Eppendorf Microfuge Tubes. Total radioactivity was counted, and this was followed by centrifugation at 25,000 rpm in a 50 Ti rotor (Beckman) for 20 min. The supernatant was discarded, and after washing the pellet 3 times, the tubes were counted again. Binding of ^{125}I-γ globulins was calculated from these values. The specific activity (μmol ATP hydrolyzed $min^{-1}mg^{-1}$) of the Mg-ATP and State 3 particles was 0.38 and 2.2 respectively. The inset shows a Scatchard plot of the data, the line was calculated by linear regression analysis.

this mechanism is correct, it follows that under the influence of electrochemical gradients, the inhibitor protein must undergo a change in position relative to its inhibitory site in F_1-ATPase. This latter step has been inferred only from measurements of enzyme activity which may not be a reliable index of the displacement of the inhibitor protein, since a large number of factors influence the catalytic properties of mitochondrial ATPase.

The results of this work show that more antibodies against the inhibitor protein bind to particles that had been exposed to electrochemical gradients (and which resulted in activation of the ATPase), than in particles in which the ATPase is in the inhibited state. This implies that in its inhibiting site, the antigenic groups of the inhibitor protein are in cryptic state, and that electrochemical gradients induce the exposure of these antigenic

groups. Therefore electrochemical gradients induce a modification of the F_1 component of the ATPase that results in changes of position of the inhibitor protein in relation to the enzyme. Whether these changes are localized to the domain of subunit interactions in F_1-ATPase, or involve a displacement of the inhibitor protein to a second locus in the membrane remains to be established. However the overall data indicate that in mitochondria the displacement of inhibitory protein from its inhibitory site is an essential step in oxidative phosphorylation.

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