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IMMUNOPRECIPITATION OF ATP SYNTHETASE FROM BEEF HEART MITOCHONDRIA

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SUMMARY

Antibody against chloroform-treated beef heart mitochondrial F_1ATPase has been raised in rabbits. The purified antibody inhibits ATPase activity in soluble and membrane bound F_1ATPase. It immunoprecipitates the entire ATP synthetase complex (F_1ATPase plus membrane sector) from mitochondria. The polypeptide profile of the immunoprecipitate is similar to that of oligomycin-sensitive ATPase purified according to Berden and Voorn-Brouwer (Biochim. Biophys. Acta 501, 424-439, 1978).

F_1ATPase, the water soluble globular portion of the mitochondrial ATP synthetase complex containing the active site(s) for ATP hydrolysis and ATP synthetase, is apparently localized on the inner (matrix facing) surface of the mitochondrial inner membrane (1,2). It is associated in the membrane with several other polypeptides, together called F_0 (3) or the membrane sector (1) and including F_6, OSCP and the DCCD binding proteolipid (1-6).

Very little is known about the arrangement of components in the membrane sector. For example, it remains to be established whether this portion of the ATP synthetase spans the mitochondrial inner membrane as expected of a unit acting as a proton channel (7,8).

One way of examining this question is to use radioactive, membrane-impermeable, protein-modifying reagents to tag any subunits of the membrane sector which are exposed in intact mitochondria (where only the cytoplasmic surface of the inner membrane is available for labelling) and in submitochondrial particles (where the matrix-facing surface is outermost). Such experiments are greatly facilitated by using an antibody to precipitate the ATP synthetase from small amounts of highly radioactive membranes (see 9,10 for a description of the usual protocols for labelling experiments).

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To date, F_1ATPase has proved a poor antigen and antibodies against the mammalian enzyme have only been raised in chickens and mice (11). However, we have recently found that chloroform treatment of F_1ATPase greatly enhances its antigenicity. Here we describe the preparation of F_1ATPase-specific antibodies in rabbits. Some properties of these antibodies are given and results are presented which show an interesting effect of sonication on the subunit composition of F_1ATPase.

MATERIALS AND METHODS

Enzyme and Membrane Preparations. Mitochondria were isolated from beef hearts according to Smith (12). Submitochondrial particles were obtained by sonicating the mitochondrial suspension (20 mg/ml) in SPMS buffer (0.25 M sucrose, 10 mM phosphate, 1 mM $MgCl_2$, 1 mM succinate, pH 7.8) three times for 15 seconds at full setting in an MSE sonicator. The suspension was centrifuged at 10,000 rpm in a Sorval SS34 for 10 minutes and the pellet discarded. The supernatant was centrifuged at 78,000 x g for 30 minutes and the pellet resuspended in SPMS buffer.

F_1ATPase was prepared according to Beechey et al. (13) or as described by Senior and Brooks (14). One sample of F_1ATPase and an inhibitor protein preparation (15) were the kind gifts of Dr. A. E. Senior, University of Rochester. Oligomycin-sensitive ATPase preparations were isolated according to Berden and Voorn-Brouwer (4) and Serrano and Racker (5). OSCP was prepared according to MacLennan and Tzagoloff (16). Submitochondrial particles were reacted with 3.5 M sodium bromide as described by Tzagoloff et al. (17).

Antibody Preparation. Antibodies against F_1ATPase have been generated in three rabbits. A sample of F_1ATPase isolated as described by Beechey et al. (12) was used to immunize one rabbit. Aliquots were eluted through a Sepharose 6B column in a buffer containing 10 mM Tris HCl, 1 mM EDTA,

pH 7.4 at 4°C and fractions containing the α and β subunit were injected into the rabbit. The entire F_1ATPase, separated from impurities on the column, was then used in booster injections.

Two other rabbits were immunized with enzyme isolated by the method of Senior and Brooks (14). Samples of this preparation were dissolved in 0.25 M sucrose, 10 mM Tris HCl, 1 mM EDTA, pH 7.4 and then shaken together with 1/2 vol. of chloroform. The aqueous phase was separated by centrifugation and protein precipitated in 50% ammonium sulfate. The antigen (1 mg) was suspended in Freund's complete adjuvant and the immunization of rabbits was performed as described before (18). The IgG fraction was purified as described by Harboe and Inglid (19) and stored in 0.25 M sucrose, 10 mM Tris HCl, 1 mM EDTA, pH 7.4 at -70°.

Double diffusion analysis (20) was carried out on plates prepared with 0.7% agarose containing 1% Triton X100, 0.2 M sucrose, 0.1 M KCl, 0.05% sodium azide, 2% methanol, 0.2 mM EDTA, 1.0 mM ATP, 0.05 M Tris HCl, pH 7.4. Plates were developed for 40 hours at room temperature, washed with the Triton X100 buffer and stained with Coomassie brilliant blue.

For immunoprecipitation experiments, membranes were dissolved at 2.5 mg/ml by incubating for 30 minutes on ice in the same Triton X100 containing buffer used in double diffusion experiments. The solution was centrifuged at 100,000 x g for 40 minutes and aliquots of the supernatant were incubated overnight with antiserum dissolved in the same buffer. Immunoprecipitates, centrifuged down at 5000 rpm (Sorvall SS34) for 15 minutes, were washed once in 0.1% cholate, 50 mM KCl, 50 mM Tris HCl, pH 7.5, once in distilled water and then examined by SDS polyacrylamide gel electrophoresis.

Other Analytical Methods. SDS polyacrylamide gel electrophoresis was conducted with 10% gels (acrylamide to bisacrylamide ratio of 33:1) in Weber-Osborn buffer conditions (21) or in 15% gels (30:1) in the Swank-Munkres buffer system which contains 8 M urea in addition to SDS (22).

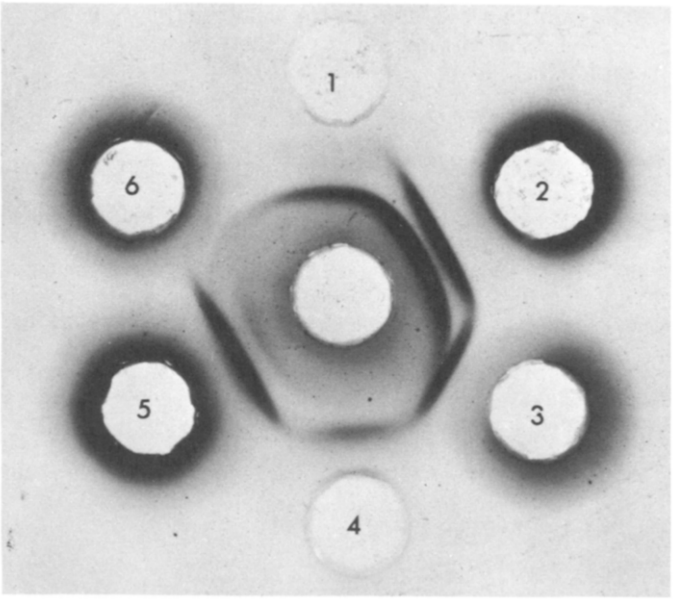

Fig. 1. Double diffusion analysis of F_1ATPase antibody. Antiserum generated against chloroform-treated F_1ATPase was reacted against; in well 1, supernatant from the sodium bromide treatment of submitochondrial particles (0.5 mg/ml). 2. F_1ATPase (1 mg/ml). 3. Oligomycin-sensitive ATPase (1 mg/ml) prepared according to Berden and Voorn-Brouwer (4). 4. Submitochondrial particles (2.5 mg/ml) dissolved in 1% Triton X100, 0.5 M sodium sulfate, 2% methanol, 0.2 M sucrose, 0.2 mM EDTA, 1 mM ATP, 50 mM Tris HCl, pH 7.5. 5. Submitochondrial particles (2.5 mg/ml) dissolved in Triton X100, 0.1 M KCl, 0.05% azide, 2% methanol, 0.2 M sucrose, 0.2 mM EDTA, 1 mM ATP, 50 mM Tris HCl, pH 7.4. 6. Pellet from sodium bromide treatment of submitochondrial particles in well 5 (2 mg/ml).

F_1ATPase activity was assayed as described by Serrano and Racker (5). Protein concentrations were determined by the method of Lowry et al. (23).

RESULTS AND DISCUSSION

Antibodies raised against beef heart F_1ATPase cross-reacted in double diffusion experiments with purified F_1ATPase, with oligomycin-sensitive ATPase, with submitochondrial particles dissolved in Triton X100, and with the F_1ATPase-containing supernatant obtained by treating submitochondrial particles with 3.5 M sodium bromide (Figure 1). However, there was no cross reaction with the pellet obtained by sodium bromide treatment of submitochondrial particles (Figure 1, well 6). This pellet contained

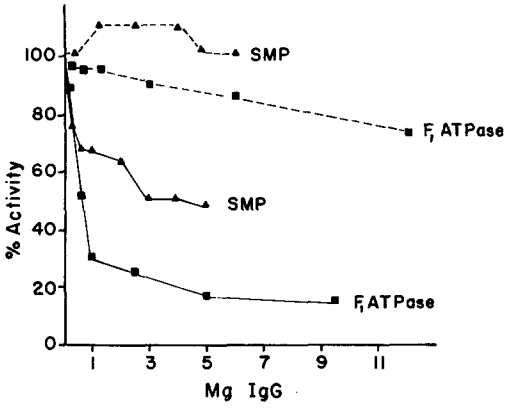

Fig. 2. Antibody inhibition of isolated and membrane-bound F_1ATPase. The dotted lines are for control experiments done with blank antisera; □–□–□– isolated F_1ATPase; △–△–△– submitochondrial particles, F_1ATPase activity of submitochondrial particles. 100% activity of isolated F_1ATPase was 70 µmoles of ATP hydrolyzed/min/mg protein. The optimal value for ATPase activity of submitochondrial particles was 1.0 µmoles of ATP hydrolyzed/min/mg protein--no uncouplers were added. The membrane-bound ATPase was 85% oligomycin sensitive.

the membrane sector of the ATP synthetase, the ADP-ATP translocase, electron transfer complexes as well as many other mitochondrial inner membrane components. Moreover, the F_1ATPase antibody did not cross react with complexes II, III or cytochrome c oxidase when tested separately in double diffusion experiments. Two precipitation lines were seen against F_1ATPase (Figure 1, well 2). The inner line probably came from reaction of antibodies with faster migrating individual subunits generated by cold inactivation of the enzyme (see Ref. 1). This precipitation band predominated in the sodium bromide-treated F_1ATPase.

The F_1ATPase antibodies were a potent inhibitor of the ATPase activity of isolated enzyme but were less effective in inhibiting the activity of membrane-bound F_1ATPase (Figure 2), possibly because of steric effects.

The polypeptide profile of protein immunoprecipitated by the F_1ATPase antibodies from detergent solubilized mitochondria is shown in Figure 3. Oligomycin-sensitive ATPase prepared according to Berden and Voorn-Brouwer (4) was run in the same slab gel for comparison. Both preparations contained

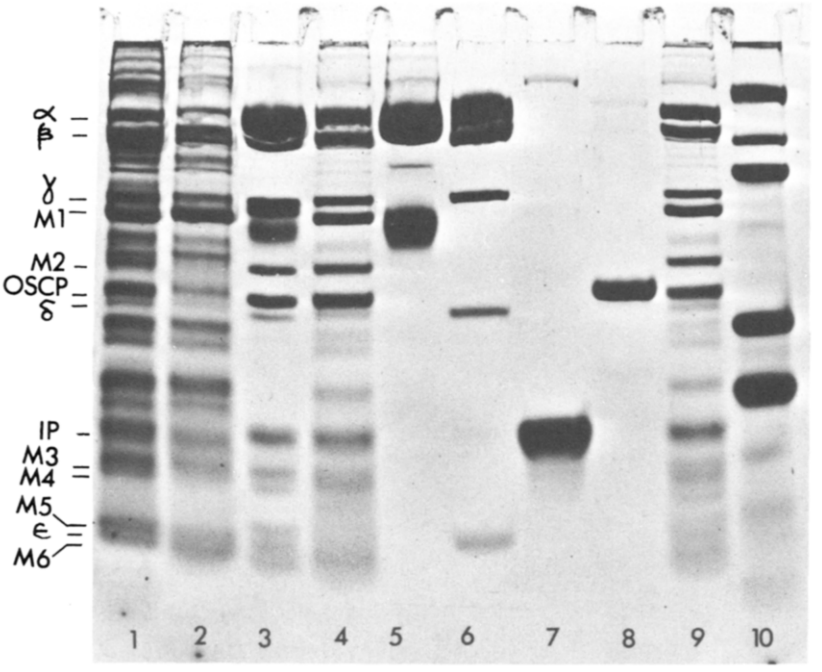

Fig. 3. SDS polyacrylamide gel electrophoresis of different F_1ATPase preparations and fractions. The gel used contained 15% acrylamide (1:30 cross linker) and electrophoresis was carried out in the Swank-Munkres buffer system. Well 1. Submitochondrial particles (200 µg). 2. The pellet obtained in the first centrifugation step in the isolation procedure of Berden and Voorn-Brouwer (4) which is depleted in oligomycin-sensitive ATPase. 3. Protein immunoprecipitated from submitochondrial particles (1.5 mg) by F_1ATPase antibody. 4. Oligomycin-sensitive ATPase prepared according to Berden and Voorn-Brouwer (4), 150 µg. 5. IgG 150 µg. 6. F_1ATPase 120 µg. 7. F_1 inhibitor 50 µg. 8. OSCP 50 µg. 9. Same as 4. 10. Molecular weight standards, bovine serum albumin, ovalbumin, glyceraldehyde-3-PO_4 dehydrogenase, whale myoglobin and cytochrome c.

the five subunits of F_1ATPase (well 6), F_1ATPase inhibitor (well 7) OSCP (well 8) and several polypeptides assumed to be intrinsic to the membrane sector (see Ref. 4).

The migration of the α and β subunits of F_1ATPase and of the γ subunit of F_1ATPase and 29,000 dalton components were altered slightly in the immunoprecipitate so that the components in each doublet ran closer together than in purified oligomycin-sensitive ATPase. This is probably due to the presence of large amounts of IgG subunits in these two regions of the gel. The effect

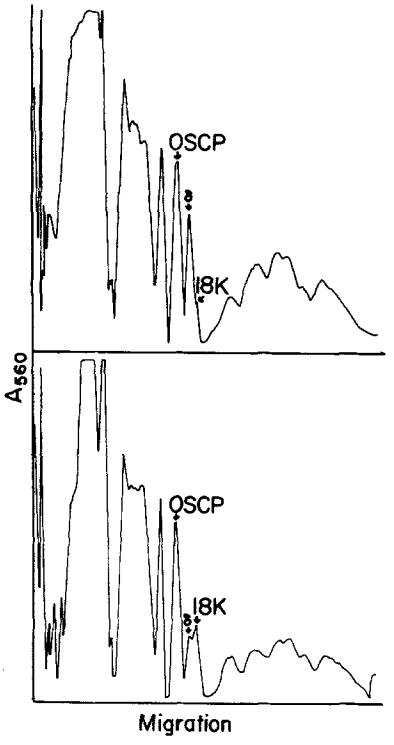

Fig. 4. Densitometric traces of the ATP synthetase complex (plus IgG bands) immunoprecipitated from mitochondria (upper trace) and submitochondrial particles (lower trace) as run on 15% polyacrylamide gels (1 part bisacrylamide to 30 parts acrylamide) in the Swank-Munkres buffer system.

is seen in Fig. 3 but is most evident in the labelling experiments being conducted at present, when these components are radioactively labelled and can be clearly distinguished from antibodies.

Figure 4 compares the gel profile of ATP synthetase immunoprecipitated from mitochondria (upper trace) and submitochondrial particles (lower trace). The two traces are almost identical except that the δ subunit is present in much higher amount in the enzyme obtained from mitochondria. This suggests that the binding of the δ subunit to F_1ATPase is weakened by sonication (to generate submitochondrial particles) and it could be that the variability in number and stoichiometry of subunits (see Refs. 1 and 2 for review) results in part from the use of differently treated membrane preparations as starting material for isolation of F_1ATPase.

In summary, procedures are described by which antibodies against beef heart mitochondrial F_1ATPase can be raised in rabbits. Such antibodies immunoprecipitate the entire ATP synthetase complex (F_1ATPase plus membrane sector) from the mitochondrial inner membrane. We are using the F_1ATPase antibody in conjunction with membrane-impermeant, protein modifying reagents to determine the arrangement of the membrane sector of ATP synthesis in the mitochondrial inner membrane. F_1ATPase antibodies may also prove a useful probe with which to study the functioning of the ATP synthetase in the mitochondrial inner membrane.

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