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IMMUNOLOGICAL AND RECONSTITUTION STUDIES ON THE ADENOSINE TRIPHOSPHATASE COMPLEX FROM *RHODOSPIRILLUM RUBRUM*

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Summary

Studies on restoration of membrane-bound adenosinetriphosphatase (ATP phosphohydrolase, EC 3.6.1.3) from *Rhodospirillum rubrum* show that the δ-subunit is capable of binding to the F_1 factor or to the F_0 moiety of the F_0-F_1 ATPase complex. This subunit is thus likely involved in linking the F_0 and F_1 factor.

During solubilization of the oligomycin-sensitive F_0-F_1 ATPase complex with Triton X-100 the detergent becomes specifically associated with the lipophilic F_0 part of the enzyme complex.

Crossed immunoelectrophoresis, agglutination tests, and kinetic studies with anti-F_1 ATPase antibodies reveal a reaction of immunological identity of membrane-bound ATPase, F_0-F_1 ATPase, and F_1 ATPase.

Introduction

Membrane-bound proton-translocating ATPases consist of two functional components, the water-soluble entity (F_1 ATPase), which catalyzes ATP hydrolysis, and the detergent-soluble hydrophobic F_0 component, an integral membrane protein, which probably contains the proton-translocating moiety of the enzyme [1].

The hydrophilic F_1 component may be detached from photosynthetic membranes of *Rhodospirillum rubrum* by ultrasonication [2—4] and can be purified to homogeneity [5].

When solubilized by the nonionic detergent Triton X-100 under suitable con-

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ditions, the enzyme complex (F_0-F_1 ATPase) shows ATPase activity that is inhibited by various antibiotics [6,7].

Recently, crossed immunoelectrophoresis was applied to reveal the antigenic relationship of the F_1- and F_0-F_1 ATPase of *Micrococcus luteus* [8]. Berzborn et al. [9] followed the course of preparation and the degradation of urea-treated F_1 ATPase from *R. rubrum* by using various F_1 ATPase antisera. Moreover, they demonstrated by immunodiffusion studies that the F_1 ATPase of *R. rubrum* is located on the outer surface of the membrane [9].

In our present investigation, antibodies raised against the F_1 ATPase have been used to study the distribution of antigenic specificities in various ATPase preparations. Although progress has been made in studying the composition and in separating the membrane-bound ATPase complex from *R. rubrum* [6,7] little is known about the spatial arrangement of the ATPase components in the membrane. The present studies are designed to reveal more information on the binding of the highly purified F_1 part to the membrane-bound F_0 moiety in *R. rubrum* chromatophores.

Experimental

Materials. Sepharose 4B-iminobispropylaminyl-*N*-acetyl-homocysteyl-6-thio-9-β-D-ribofuranosylpurine 5′-triphosphate (Sepharose 4B/complex I) was a gift from Dr. F.W. Hulla, Frankfurt. Sepharose CL-6B, Sepharose 4B, Sepharose CL-4B were obtained from Deutsche Pharmacia, Freiburg, and phosphatidylcholine was from Sigma Chemical Co., St. Louis. All other chemicals were of analytical grade, purchased from Merck, Darmstadt.

Preparation of membrane-bound and soluble ATPase activities. Photosynthetic membranes (chromatophores) from *R. rubrum* were prepared according to a published procedure [10] and solubilization of the oligomycin-sensitive F_0-F_1 ATPase complex by Triton X-100 was carried out as described recently [7].

The water-soluble F_1 ATPase was released from chromatophores by ultrasonication in the presence of 0.0015 or 0.015 M EDTA [2,3]. The sonicated membranes were centrifuged at 100 000 × *g* for 2 h to separate the F_1 ATPase-depleted chromatophore fragments (pellet) from the soluble F_1 ATPase (supernatant). The pellet was washed with 0.02 M glycylglycine/NaOH (pH 8.0) containing 0.0015 M EDTA and stored in 0.2 M glycylglycine/NaOH (pH 8.0) at 0°C.

Purification of the soluble F_1 ATPase to homogeneity involved fractionated $(NH_4)_2SO_4$ precipitation, followed by gel filtration on Sepharose CL-6B equilibrated with 0.05 M Tris-HCl (pH 7.5) and affinity chromatography on Sepharose 4B/complex I equilibrated with the same buffer [11,12]. The F_1 ATPase was eluted by application of an ATP gradient ranging from 0—0.005 M. All purification steps were carried out at 0—4°C.

Release of the F_1 ATPase from reconstituted chromatophore fragments was carried out by use of the chloroform method described by Beechey et al. [13] and further purification was achieved by gel filtration on Sepharose CL-6B equilibrated with 0.05 M Tris-HCl (pH 7.5). Chloroform extraction of the F_1 ATPase irreversibly destroys the depleted membranes.

Immunological techniques. Immunization of rabbits and purification of antibodies against partially purified F_1 ATPase (gel filtration step) from *R. rubrum* was carried out as described elsewhere [8,14]. Monospecific anti-F_1 ATPase antibodies were prepared by immunoaffinity chromatography [8] with highly purified F_1 ATPase as affinity ligand. Monospecificity of the so purified anti-F_1 ATPase antibodies was verified by crossed immunoelectrophoresis against partially purified F_1 ATPase (gel filtration step). The monospecific anti-F_1 ATPase antibodies were concentrated to 3 mg protein per ml.

Crossed immunoelectrophoresis was performed in 1.5% agarose gels containing barbital buffer (I = 0.02, pH 8.6). The Triton X-100 concentration in the gels was 0.01—1%. In certain experiments detergent-free gels were used. Test for agglutination of chromatophores or F_1 ATPase-depleted chromatophore fragments were carried out on microscope slides. The extent of agglutination was observed at room temperature in a Leitz phase-contrast microscope at a magnification of X400. In some tests purified F_1 ATPase was added to the antibodies before mixing with the chromatophore suspension.

Analytical methods. ATPase activity was determined continuously by measurement of the liberated inorganic phosphate as described by Arnold et al. [15]. Specific activity is given in units per mg protein. 1 unit is defined as the number of μmol inorganic phosphate liberated during 1 min at 37°C. The determination of protein was carried out either according to Lowry et al. [16] with bovine serum albumin as standard or in the presence of Triton X-100, according to Soper and Pedersen [17]. Bacteriochlorophyll was estimated by measuring the absorbance of the chromatophores at 880 nm. The extinction coefficient given by Clayton [18] was used.

Analytical disc gel electrophoresis was carried out in 5% polyacrylamide gels according to system 1a described by Maurer [19]. Dissociation into subunits was performed at 100°C for 1 min in the presence of 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol. Sodium dodecyl sulfate disc gel electrophoresis was carried out in 12% polyacrylamide gels containing 0.1% SDS according to Laemmli [20].

Results

Isolation of F_1 ATPase and restoration of membrane-bound Mg^{2+}-ATPase activity

Ultrasonication of *R. rubrum* chromatophores in a buffer of low ionic strength in the presence of the chelating agent EDTA led to release of the F_1 ATPase from the membrane-bound enzyme complex. As shown in Table I about 23% of the Mg^{2+}-ATPase activity remained in the membrane. After purification by affinity chromatography the F_1 ATPase was homogeneous as revealed by polyacrylamide disc gel electrophoresis (see Fig. 1) and crossed immunoelectrophoresis (see Fig. 3a). In Fig. 2a SDS disc gel electrophoresis of the F_1 ATPase preparation confers the presence of four different polypeptide chains usually referred to as α-, β-, γ- and δ-subunits [21,22]. The amount of the δ-subunit was very low compared to F_1 ATPases prepared by other solubilization methods from *R. rubrum* chromatophores [21,22].

Optimal activity of the present F_1 ATPase was measured in 0.05 M Tris-HCl

TABLE I

REBINDING OF F_1 ATPase FROM *R. RUBRUM* TO F_1ATPase DEPLETED CHROMATOPHORE FRAGMENTS

F_1ATPase-depleted chromatophore fragments were suspended in 1.3 ml of a mixture containing 0.02 M glycylglycine/NaOH (pH 8.0), 560 μg highly purified F_1 ATPase, and 0.01 M $MgCl_2$ as indicated. The final bacteriochlorophyll concentration was 0.12 mg/ml. After incubation at 25°C in the dark for 1.5 h samples of 0.6 ml of the mixture were taken for ATPase assay. Mg^{2+}-ATPase activity was determined in 0.05 M Tris-HCl (pH 8.0), containing 0.001 M $MgCl_2$ and 0.001 M ATP as described in Experimental.

Fraction	Specific Mg^{2+}-ATPase activity (U/mg bacteriochlorophyll)	Activity (%)
(a) untreated chromatophores	4.59	100
(b) depleted chromatophores	1.06	23
(c) * depleted chromatophores + purified F_1ATPase	3.76	82
(d) depleted chromatophores + $MgCl_2$ + purified F_1ATPase	4.82	105

* In fraction (c) 2.5 mg purified F_1ATPase were suspended in 5.8 ml incubation mixture before chloroform treatment.

(pH 8.0) containing 0.001 M Ca-ATP^{2-}. Mg^{2+} could not replace Ca^{2+} in the ATPase reaction.

Rebinding of highly purified F_1 ATPase to F_1 ATPase-depleted chromatophore fragments was carried out for 1.5 h in the absence or presence of Mg^{2+}. As shown in Table I membrane-bound Mg^{2+}-ATPase activity could largely be

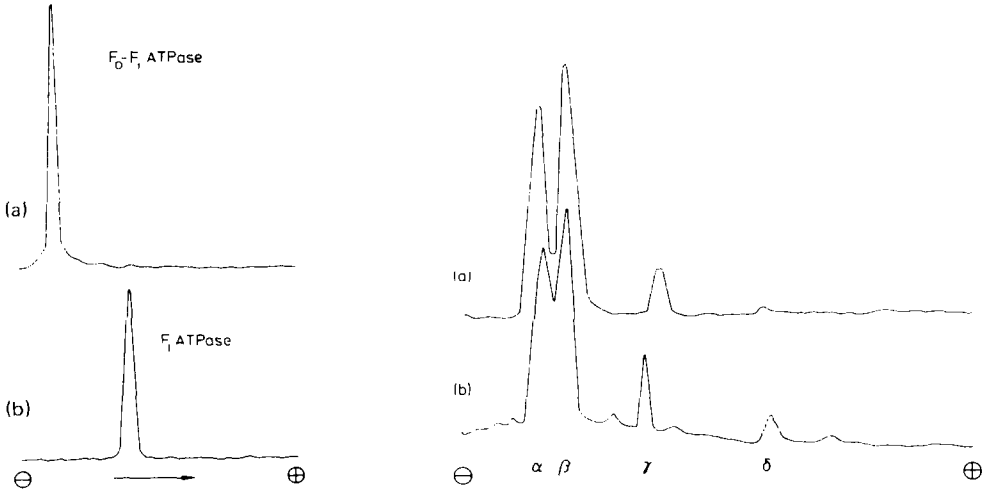


Fig. 1. (a) Analytical polyacrylamide disc gel electrophoresis (5% polyacrylamide) of 50 μg F_0-F_1 ATPase (upper densitometric trace) and (b) 40 μg F_1 ATPase in presence of 0.1% Triton X-100. The gels were stained with Coomassie brilliant blue G 250.

Fig. 2. SDS disc gel electrophoresis of (a) 75 μg purified F_1 factor solubilized by EDTA-ultrasonic treatment from *R. rubrum* chromatophores (upper densitometric trace) and (b) 75 μg purified F_1 factor solubilized by chloroform treatment from reconstituted chromatophore fragments (lower trace). Electrophoresis was run as described in Experimental. The gels were stained with 0.25% (w/v) Coomassie brilliant blue G 250.

restored without adding bivalent cations [4]. Complete restoration was achieved in the presence of Mg^{2+}. Restored membrane-bound ATPase activity was sensitive to oligomycin (90% inhibition at 10 μg per ml). No Mg^{2+}-ATPase activity could be detected when F_1 ATPase, Mg^{2+}, and ATP were added to depleted membranes immediately before ATPase assay.

The purified F_1 factor detached from reconstituted chromatophore fragments by chloroform treatment is composed of at least the α-, β-, γ- and δ-subunits as revealed by SDS disc gel electrophoresis (see Fig. 2b). Compared with the densitometric trace of the purified F_1 ATPase, used for rebinding, the amount of the δ-subunit is markedly increased.

Stability of the detergent-solubilized ATPase complex

Solubilization by Triton X-100 and gel filtration on Sepharose CL-4B yielded a homogeneous and stable F_0-F_1 ATPase complex not contaminated by F_1 ATPase (see Fig. 1). Optimal ATPase activity of the F_0-F_1 complex was measured in 0.05 M Tris-HCl (pH 8.0), containing 0.001 M Ca-ATP^{2-} and 0.003—0.025% Triton X-100 (v/v). At detergent concentrations $\geqslant$0.03% ATPase activity was considerably reduced ($I_{50\%}$ = 0.1% Triton X-100).

The buffer optimal for stabilizing the F_0-F_1 ATPase activity with respect to oligomycin sensitivity contains 0.05 M Tris-HCl (pH 8.0), 0.005 M $MgCl_2$, 0.001 M $CaCl_2$, 0.01% dithiothreitol (w/v), 5% glycerol (w/v), and 5 mg phosphatidylcholine per ml. When stored in this buffer at 4°C no loss in enzymic activity (0.39 U/mg) and oligomycin sensitivity could be detected within 2 weeks. 50% inhibition of F_0-F_1 ATPase activity was achieved at an oligomycin concentration of about 20 μg/ml [7]. Storage of the F_0-F_1 ATPase without adding phospholipids resulted in a loss of oligomycin sensitivity ($t_{1/2}$ = 2 days) linked with an increase in oligomycin-insensitive F_1 ATPase activity (0.53 U/mg).

Analysis of F_0-F_1 ATPase and F_1 ATPase from R. rubrum in crossed immunoelectrophoresis

When stored in the absence of phospholipids, immunoelectrophoresis of soluble F_0-F_1 ATPase with an antiserum to partially purified F_1 ATPase yielded two fusing precipitin peaks (see Fig. 3b) which exhibited ATPase activity. The peak with lower electrophoretic mobility represented the F_0-F_1 ATPase complex, whereas the precipitin peak with higher electrophoretic mobility was identified as the F_1 ATPase (see Fig. 3a).

To obtain some information about the hydrophilic and/or amphiphilic character of the two soluble ATPase fractions we have made use of the different behaviour of F_1 ATPase and F_0-F_1 ATPase in crossed immunoelectrophoresis. It was possible to run F_1 ATPase in detergent-free agarose gels. The incorporation of Triton X-100 (0—0.1%, v/v) into the gel had no effect on the electrophoretic mobility and did not interfere with the immunoprecipitation reaction. In detergent-free crossed immunoelectrophoresis the F_0-F_1 ATPase complex denatured in the gel near its origin, but we were able to run the F_0-F_1 complex in the presence of 0.01% (v/v) Triton X-100. Triton X-100 concentrations $\geqslant$0.1% interfered with the immunoprecipitation reaction.

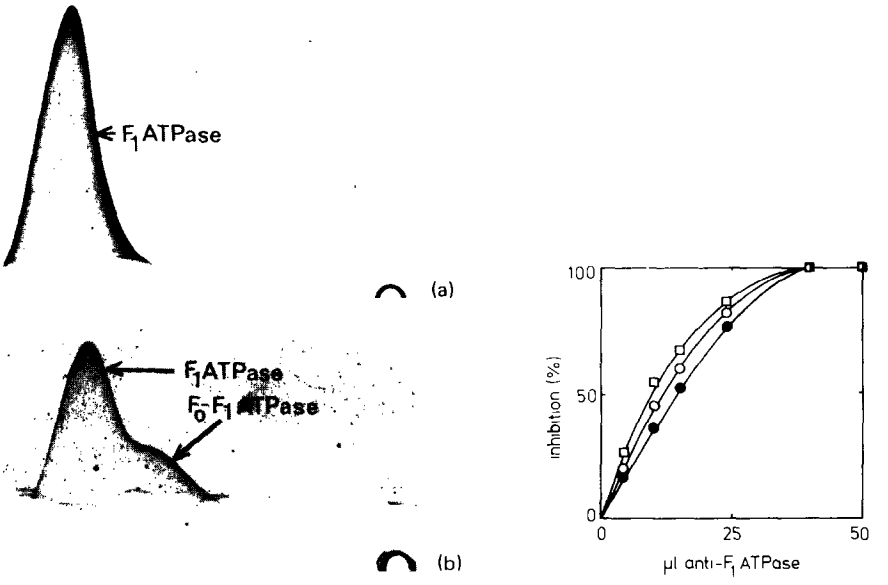


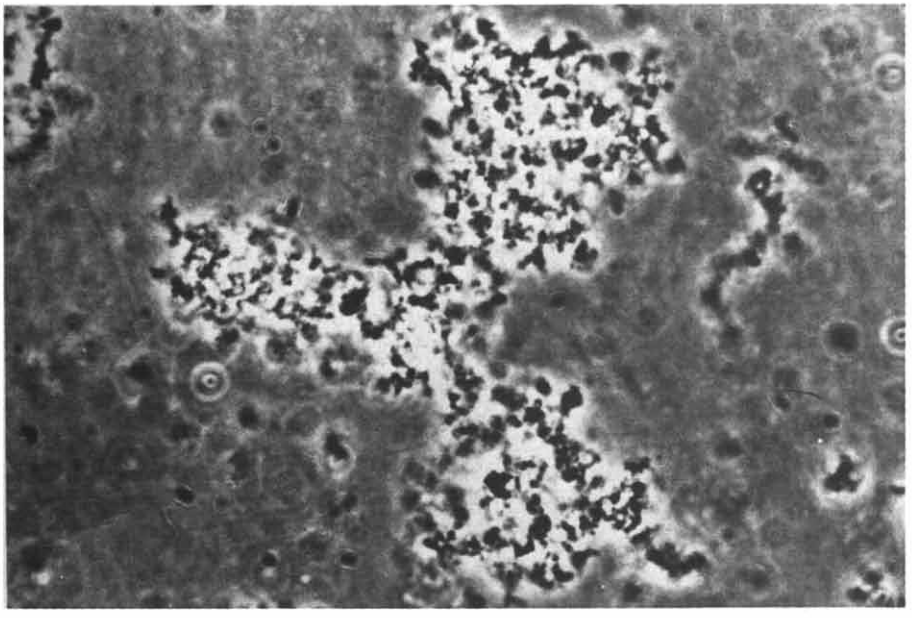
Fig. 3. (a) Crossed immunoelectrophoresis of 5 µg purified F_1 ATPase from *R. rubrum*. Concentration of antibodies: 3% (v/v). First dimension electrophoresis (anode to the left) was carried out for about 100 min at 300 V. Second dimension electrophoresis (anode at the top) was run for 18 h at 80 V in agarose containing antibodies against partially purified F_1. (b) Crossed immunoelectrophoresis of 3 µg of F_0-F_1 ATPase stored for 8 days in absence of stabilizing phospholipids (for details see Results). Electrophoresis conditions as indicated in (a), but 0.01% Triton X-100 (v/v) was added to agarose.

Fig. 4. Effect of anti-F_1 ATPase antibodies on ATPase activities from *R. rubrum*. Concentration of antibodies was 0.1 mg/ml. 50 mU of each ATPase preparation were incubated in 10 ml 0.05 M Tris-HCl (pH 8.0) at 37°C for 10 min with anti-F_1 ATPase antibodies as indicated and then assayed for enzymic activity as described for F_0-F_1 ATPase in Results (substrate = Ca-ATP^{2-}). Control experiments, in which immunoglobulin G from rabbit replaced the anti-F_1 ATPase antibodies, did not show any inhibition. □———□, soluble F_1 ATPase (5 µg protein); ○———○, soluble F_0-F_1 ATPase (128 µg protein); ●———●, chromatophores (454 µg protein). Specific activities (U/mg) are 9.6, 0.39, and 0.11, respectively.

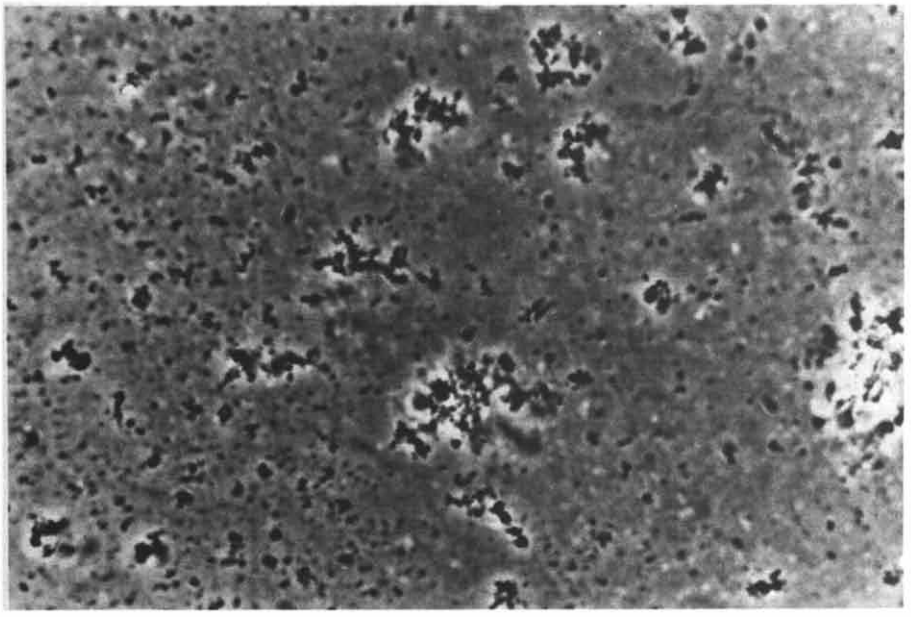
Specificity of anti-F_1 ATPase antibodies from R. rubrum

Anti-F_1 ATPase antibodies from *R. rubrum* were reacted with membrane-bound ATPase, water-soluble F_1 ATPase and detergent-solubilized F_0-F_1 ATPase. Interaction between the various ATPase preparations and sufficient amounts of antibodies led to a complete reduction in enzymic activity. For half maximal inhibition, however, different concentrations of anti-F_1 ATPase antibodies were required (Fig. 4). The inhibition was noncompetitive. Preincubation of the various ATPase fractions with Ca-ATP^{2-} had no protective effect on the subsequent formation of the enzyme-antibody complex.

Agglutination of untreated chromatophores by anti-F_1 ATPase antibodies was completed within 10 min (Fig. 5a). Agglutination was prevented by adding purified F_1 ATPase to the antibodies before mixing with the chromatophores. Only partial agglutination of F_1 ATPase-depleted but F_0-containing chromatophore fragments was observed after addition of anti-F_1 ATPase antibodies (Fig. 5b). Agglutination of reconstituted chromatophore fragments was again complete.



(a)



(b)

Fig. 5. (a) Agglutination of untreated chromatophores by anti-F_1 ATPase antibodies. 15 μl membrane suspension (0.4 mg bacteriochlorophyll/ml) and 85 μl anti-F_1 ATPase antibodies (3 mg protein/ml) were mixed and observed as described in Experimental. (b) Partial agglutination of depleted chromatophore fragments by anti-F_1 ATPase antibodies. For details see (a) and Experimental.

Discussion

Very little is known about the spatial arrangement of ATPase components in the membranes of photosynthetic bacteria. For other bacteria Futai et al. [23], Abrams et al. [24] and Smith and Sternweis [25] have demonstrated that the δ-subunit is required for attaching the F_1 ATPase to depleted membranes. Our results obtained with *R. rubrum* indicate that the δ-subunits remain largely bound to the membrane-integrated F_0 moiety after ultrasonic removal of the F_1 factor which is mainly composed of three different subunits (α, β, γ). After rebinding of this F_1 factor to the depleted chromatophore fragments, subsequent extraction of the membranes by chloroform leads to removal of the F_1 ATPase which in this case is composed of at least four subunits (α, β, γ, and δ). These findings support the suggestion that the δ-subunit is likely involved in linking the F_1 factor and the F_0 component in the F_0-F_1 ATPase complex of *R. rubrum*.

Restoration of membrane-bound ATPase activity in *R. rubrum* chromatophore fragments without addition of bivalent cations is coincident with findings of Smith and Sternweis [25]. They have demonstrated that a complex between δ-subunits and δ-deficient F_1 ATPase from *Escherichia coli* can be formed in absence of bivalent cations.

As revealed by immunochemical analysis the F_0-F_1 ATPase complex from *R. rubrum* does not undergo drastical conformational changes when solubilized with a nonionic detergent. Furthermore, in accordance with the suggestion of Helenius and Simons [26], Triton X-100 does not produce any dissociation of the F_0-F_1 ATPase during solubilization. Contrary to the F_0-F_1 complex, the behaviour of F_1 ATPase is not affected by Triton X-100 in crossed immunoelectrophoresis. Therefore, the nonionic detergent may exclusively cover the hydrophobic F_0 part of the enzyme. The fusing immunoprecipitin lines of F_1 ATPase and the F_0-F_1 complex indicate that parts of the antigenic determinants of the F_0-F_1 ATPase are structurally identical with those of the F_1 ATPase. Recently these observations have also been demonstrated for the F_0-F_1 ATPase complex from *Micrococcus luteus* [8].

Inasmuch as anti-F_1 ATPase antibodies reacted with the F_1 ATPase and inhibited ATPase activity in isolated chromatophores but failed to bind to F_1 ATPase-depleted membranes, the location of the F_1 factor on the outer face of the chromatophore membranes is corroborated [9]. The noncompetitive type of inhibition of all three states of the enzyme by anti-F_1 ATPase antibodies indicates that the side of antibody action on the enzyme is different from the catalytic site.

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