The Isolation and Subunit Structure of Streptococcal Membrane Adenosine Triphosphatase*

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ABSTRACT: The adenosine triphosphatase (ATPase) released in a soluble form from streptococcal membrane ghosts [Abrams, A. (1965), *J. Biol. Chem 240*, 3675] was isolated in pure form using a combination of $(NH_4)_2SO_4$ precipitation and zone sedimentation. Analysis of the purified enzyme by acrylamide gel electrophoresis showed a single protein band. This protein band exhibited ATPase, guanosine triphosphatase (GTPase), and inosine triphosphatase (ITPase) activity when tested by direct application of ATP, GTP, and ITP solutions, respectively, to the gel. In gels containing 8 or 10 M urea five protein bands, designated x, y, α, β, and γ in the order of increasing mobilities, were observed. Their mobilities were all greater than that for the intact enzyme. The x and y proteins were completely converted to the α protein by mercaptoethanol or dithiothreitol and the amounts of α, β, and γ were then found by densitometry to be in a ratio close to 2:2:1. This pattern was not changed by increasing the concentration of thiol reagent. It is concluded that the streptococcal membrane ATPase is made up of three different types of subunits, α, β, and γ, in noncovalent association and that the α subunit is a sulfhydryl protein which oxidizes readily to disulfide polymers.

Some years ago we reported that *Streptococcus fecalis* contains an ATPase[1] which is firmly bound to the membrane ghosts produced by lysis of protoplasts (Abrams *et al.*, 1960). In the electron microscope such membrane ghosts are seen to consist principally of plasma membranes (Abrams *et al.*, 1964); their phospholipid and associated RNA components have been investigated extensively (Ibbott and Abrams, 1964; Abrams *et al.*, 1964; Nielsen and Abrams, 1964; Vorbeck and Marinetti, 1965). In further studies of the membrane ATPase a means was devised for releasing the enzyme in a soluble form (Abrams, 1965). It was found that exhaustive washing of the ghosts in the absence of multivalent cations led eventually to an abrupt and largely selective release of the enzyme into the wash fluid. Since the presence of low concentrations of multivalent cations, *e.g.*, Mg^{2+} or spermidine^{3+} completely prevented the release it was concluded that such cations are required for attachment of the enzyme to membranes.

Examination of the solublized enzyme preparation by means of zone sedimentation and gel electrophoresis showed that the enzyme had a sedimentation constant of about 13 S and that it migrated as a single electrochemical species with a large net negative charge at pH 8.5. However, the preparation contained a number of other proteins besides the ATPase (Abrams, 1965).

In the present report we will describe the isolation of the membrane ATPase of *S. fecalis* in a practically pure form. In addition we have examined the substrate specificity of the purified enzyme by means of a direct staining technique for enzyme activity on gels after zone electrophoresis. Finally, in order to gain some information concerning its submolecular structure, the enzyme was examined electrophoretically in gels containing high concentration of urea. As will be seen, the findings indicate that the ATPase consists of three different types of subunits.

An ATPase, particularly one that is membrane associated, has not previously been isolated from any bacterial species. Membrane ATPases are widely distributed among higher organisms but only a few of these, such as the ATPase in *Tetrahymena* cilia and animal mitochondria, have been obtained in a soluble purified form (Gibbons and Rowe, 1965; Pullman *et al.*, 1960; Penefsky and Warner, 1965). The various membrane ATPases in higher organisms have attracted much attention in recent years since they are implicated in the mechanisms of active transport of cations, oxidative phosphorylation, and contractile processes. While various proposals have been made, the true physiological role of the streptococcal membrane ATPase described in this report still remains to be elucidated (Abrams, 1965).

Materials and Methods

Preparation of Solubilized Membrane ATPase. The preparation of protoplasts and membrane ghosts from

* From the Department of Biochemistry, University of Colorado School of Medicine, Denver, Colorado 80220. *Received September 23, 1966.* This study was supported in part by a grant from the National Institutes of Health, U. S. Public Health Service (GM 05810). This work was presented in part at the conference on Microbial Protoplasts, Spheroplasts, and L-Forms held at Brook Lodge, Mich., Nov 10–11, 1966.

[1] The following abbreviations are used: ATPase, adenosine triphosphatase; GTPase, guanosine triphosphatase; ITPase, inosine triphosphatase; 2-ME, 2 mercaptoethanol; DTT, dithiothreitol.

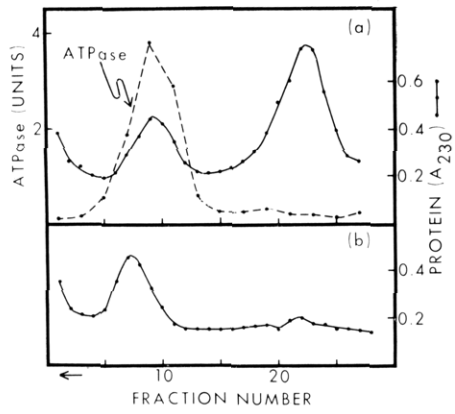

FIGURE 1: Purification of membrane ATPase by zone sedimentation. (a) Several portions of the $(NH_4)_2SO_4$ precipitated ATPase each containing about 3 mg of protein were layered onto 5 ml of 4–16% sucrose gradients in 0.033 M Tris–Cl buffer, pH 7.5. Centrifugation was carried out for 16 hr at 5° at 34,000 rpm in the Spinco Model L2 centrifuge. Fractions (0.2 ml) were collected from the bottom of the tube and diluted with 0.8 ml of Tris–Cl buffer. The absorbancy of each fraction was measured at 230 mμ and 25 μl from every odd fraction was assayed for ATPase activity. The ATPase was recovered from pooled fractions 5–12 by $(NH_4)_2SO_4$ precipitation. Electrophoretic analysis is shown in Figure 2. (b) Repeated zone sedimentation of ATPase isolated as in a. Electrophoretic analysis is shown in Figure 3.

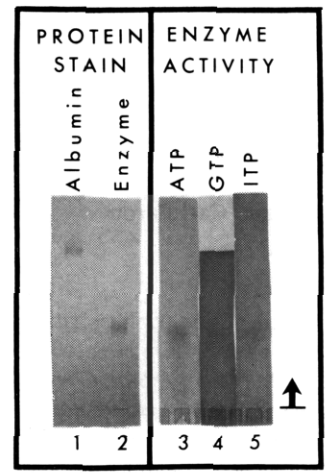

FIGURE 2: Electrophoretic analysis of purified ATPase and the specificity of the enzyme. Sample 1, bovine serum albumin (10 μg); samples 2–5, ATPase (4.4 μg of protein, 0.25 unit). Samples 1 and 2 were stained with Amido Black. The gel was run for 100 min at 300 v. Samples 3–5 were stained for nucleoside triphosphatase activity (see Methods) using different substrates as indicated.

S. fecalis (ATCC no. 9790) and the procedure for releasing the membrane ATPase in a soluble form have been described previously (Abrams, 1965). For a typical preparation of solubilized ATPase in the amounts needed for the subsequent purification procedure described here we started with cells harvested in the stationary phase of growth from 4 l. of growth medium. After the exhaustive washing of membrane ghosts as described previously, 72% of the membrane ATPase was released into the 8–10th wash of 100 ml each. These wash fluids were pooled and constitute the starting material for the isolation of the pure enzyme described in this paper.

Gel electrophoresis. Electrophoresis was carried out in the vertical direction in 3-mm thick 5% polyacrylamide gel slabs with running tap water as the coolant using the apparatus described by Raymond (1962) (manufactured by E. C. Corp., Philadelphia, Pa.). The gels were prepared with Tris–glycine buffer, pH 8.5 (0.015 M Tris–0.07 M glycine) with or without urea as indicated, and they were prerun for 30 min. The electrode compartments contained the same buffer as the gel except that in the case of 8 and 10 M urea gels, the compartment buffer contained 3 M urea. All samples were diluted with the solvent used in the gels and 20 μl containing about 5–20 μg of protein were introduced into the gel. Bovine serum albumin was always run as a reference protein for the purpose of comparing mobilities in one gel with another. A potential of 17 v/cm was applied to the gel and the direction of migration of the proteins was toward the positive electrode.

Detection of Protein and Enzyme Activity on the Gels. Proteins were detected by staining with 1% amido black in methanol–water–acetic acid (5:5:1) for about 30 min followed by electrolytic destaining of the background. To locate directly the position of ATPase activity the gel was incubated with a solution of 0.005 M ATP and 0.005 M $MgCl_2$ in 0.1 M Tris–Cl, pH 7.5, at 38° usually for about 0.5 hr. The excess ATP solution was then withdrawn and the P_i liberated by the enzyme reaction was detected by immersing the gel in a solution, at room temperature, containing 9.4% perchloric acid, 1% ammonium molybdate, 0.25% reducing mixture (sodium sulfite–sodium bisulfite–1 amino-2-napthol-4 sulfonic acid, 6:6:1). In a few minutes a blue band appears against an almost colorless background, denoting the position of the enzyme. Eventually the entire background becomes blue. In order to detect GTPase and ITPase activity on the gel, GTP or ITP was used as the substrate.

Other Methods. Quantitative assays of ATPase activity were performed as previously described (Abrams, 1965). A unit of activity is defined as that amount of enzyme which splits 1 μmole of ATP/min at 38°. Protein was determined by the method of Lowry *et al.* (1951), or by absorbancy at 230 mμ when indicated, using bovine serum albumin as a standard. The absorbancy method was employed particularly for measuring the small amounts of purified ATPase protein in the fractions collected after zone sedimentation.

Experimental Procedures and Results

Isolation of the ATPase. The starting material for the isolation procedure to be described is the highly dilute semipurified soluble ATPase extracted from the membrane ghosts as previously described (see Methods). The procedure for isolating the enzyme from the extract is carried out in essentially three steps which will be outlined briefly before giving the details. The first step is a treatment with RNase and precipitation of the enzyme with 80% saturated $(NH_4)_2SO_4$; the second step takes advantage of the high molecular weight of the enzyme and consists of sedimentation of the concentrated solution of the enzyme in a sucrose density gradient so as to obtain a discrete zone of ATPase separated from other proteins; the third step is a reprecipitation with $(NH_4)_2SO_4$ of the enzyme separated by zone sedimentation. The details of the isolation procedure are as follows.

A. TREATMENT WITH RNASE AND PRECIPITATION WITH $(NH_4)_2SO_4$. RNase (1 μg/ml) was added to the membrane extract and the mixture was placed in a dialysis bag and then immersed in four times its volume of saturated $(NH_4)_2SO_4$ (4°) adjusted previously to pH 7.5–8.0. After equilibration overnight at 4°, the precipitate which formed was removed by centrifugation at 4° and then taken up in about 1 ml of water. The concentrated enzyme solution was dialyzed a few hours against water to remove most of the $(NH_4)_2SO_4$. This was necessary in order to layer the solution onto sucrose gradients.

B. PURIFICATION OF THE ENZYME BY ZONE SEDIMENTATION. Portions of the concentrated enzyme solution, usually 0.25 ml, were layered onto 5-ml sucrose gradients and then centrifuged in a swinging bucket rotor. Figure 1a gives the conditions of centrifugation and illustrates a typical sedimentation profile of protein and ATPase activity. It is clear that after centrifugation there is a symmetrical peak of protein in the lower portion of the tube which coincides closely with a single peak of ATPase activity. The specific enzyme activity (units per milligram determined at three different points in the peak for three different enzyme preparations was very nearly the same (59 ± 6 units/mg) (Table I). This constancy of specific activity indicates that the enzyme separated by zone sedimentation is homogeneous or very nearly so. It should also be noted in Figure 1a that the ATPase zone is well resolved from the bulk of nonactive protein which sediments much more slowly.

For the determination of protein in the fractions collected after zone sedimentation we used A_{230} rather than A_{280} readings since the latter were very low. The A_{260} values were even lower than the A_{280} and, therefore, it may be concluded that the ATPase separated on the gradient was free of contaminating nucleotide material. It is worth pointing out that the prior treatment of the crude membrane extract with RNase is essential to achieve this result for without it there is a severe overlap of the enzyme peak with slower sedimenting RNA containing impurities.

For the third and final step in the isolation, the fractions which contained the ATPase (fractions 5–12, Figure 1a) were pooled and the enzyme was precipitated once again with 80% saturated $(NH_4)_2SO_4$ by dialysis as described above. After dissolving the precipitate in about 1 ml of water, the solution was dialyzed against water. A slight cloudiness in some preparations was removed by centrifugation. The yield and the specific activity of a typical purified enzyme preparation is shown in Table I.

Analysis of the purified enzyme by acrylamide gel electrophoresis shows one protein band (Figure 2, sample 2). Three additional samples of the purified enzyme were also run on the same gel (Figure 2, samples 3–5), and each was tested for enzyme activity by direct reaction on the gel with ATP, GTP, and ITP, respectively. The results show a single zone of liberated P_i for each substrate and these zones coincide with the position of the enzyme when it is stained for protein with amido black. Thus, there can be little doubt that the same enzyme splits all three substrates. This result confirms earlier work which indicated that ATPase and GTPase activities reside in a protein of the same molecular weight as judged by zone sedimentation analysis (Abrams, 1965).

We have observed that the purified enzyme may contain very small amounts of enzymatically active aggregates which migrate into the gel only a short distance. It should be emphasized that this slow-migrating enzyme activity was observed only after prolonged incubation of the gel with ATP and that no protein was detected in that region.

The Subunit Structure of the Membrane ATPase. The fairly high sedimentation constant of the streptococcal membrane ATPase, about 13 S (Abrams, 1965), suggested that it was probably composed of subunits. In order to demonstrate subunits associated by means of noncovalent forces the purified enzyme was electrophoresed in gels containing 8 M urea. As a result five protein bands appeared instead of the single band observed in gels without urea. To satisfy ourselves that this finding was not due to contaminations, however unlikely this seemed, the purified enzyme was recycled once more through the purification procedure by means of another zone sedimentation and $(NH_4)_2SO_4$ precipitation as described in the previous section. As expected

TABLE I: Recovery and Specific Activity of Purified ATPase Isolated from Streptococcal Membranes.

	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)
Membrane ghosts, washed IX	786	570	0.7
Purified enzyme	1.5	86	59 ± 6[a]

[a] Average of nine determinations from three different points in a zone of sedimentation of three different enzyme preparations (see text and Figure 1a).

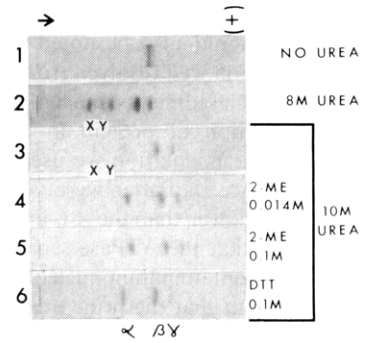

FIGURE 3: Electrophoresis of membrane ATPase in acrylamide gels containing urea, and the effect of thiol reagents. The ATPase preparation was purified twice through zone sedimentation (Figure 1b, see text). Sample 1, no urea, run for 100 min at 300 v; samples 2–6; with urea as indicated, run for 240 min at 300 v. Some samples were treated with thiol reagents as indicated, prior to application to the gel. Samples 2–6 contained about twice as much protein as sample 1.

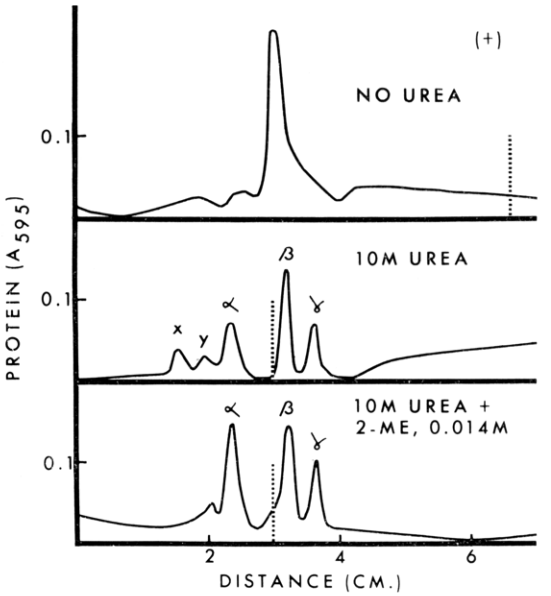

FIGURE 4: Densitometry of gel electrophoretic patterns of membrane ATPase. The patterns are, from top to bottom, samples 1, 3, and 4 of Figure 3. The gel was scanned with a Photovolt densitometer using a 595-mμ filter and the optical density was recorded continuously. Samples 3 and 4 contained about twice as much protein as sample 1. The vertical dotted line in each pattern represents the position of bovine serum albumin run in the gel at the same time as the ATPase sample.

the result of the repeated zone sedimentation, illustrated in Figure 1b, shows only one protein band; gel electrophoresis of the protein precipitated from the zone sedimentation band also shows only one protein band (Figure 3, sample 1). When this recycled purified enzyme was electrophoresed in 8 M urea, five protein bands were observed as shown in Figure 3, sample 2, thus corroborating the results obtained with the enzyme that was carried through the purification procedure only once. It is evident then that the five protein bands appearing after electrophoresis in gels with 8 M urea are derived from the ATPase molecule and no other protein. They are designated x, y, α, β, and γ in the order of increasing mobility. Further confirmation comes from quantitative densitometry of the gel which shows that the sum of the protein in the five bands was reasonably accounted for by the protein in the intact enzyme (Figure 4).

The recycled purified ATPase was examined next in gels containing 10 rather than 8 M urea, and as can be seen in Figure 3, sample 3, the same five bands appeared, with no apparent change in their relative quantities. Since increasing the urea concentration caused no further change it seems that the maximum degree of dissociation of the protein into subunits attainable with urea was achieved. There was the possibility however that one or more of the bands was composed of polypeptide chains linked covalently through disulfide bonds. In order to test this possibility, samples of the recycled purified enzyme were treated with two different concentrations of mercaptoethanol, and also with dithiothreitol, and then examined again by gel electrophoresis in 10 M urea. The results are shown in Figure 3, samples 4–6. It can be easily seen first of all that after treatment with the thiol reagents, the x and y bands disappeared and the intensity of the α band increased markedly, while no apparent quantitative change was evident in the β and γ bands. Secondly, quantitative densitometry of the gel (Figure 4 and Table II) shows that the increase in the α band is accounted for by the loss of the x and y bands. Finally it should be noted that

TABLE II: Analysis of Electrophoretic Pattern of Purified ATPase.[a]

Electrophoretic Component	Rel Mobility (albumin = 1)	Total Protein (%) −ME	Total Protein (%) +ME
Intact enzyme	0.45	100	
x	0.52	12	0
y	0.65	5	0
α	0.75	22	43
β	1.07	41	37
γ	1.20	20	19

[a] The relative amounts of protein were calculated from the areas under the peaks, measured with a planimeter, in the densitometric tracings shown in Figure 4. The total area in one pattern is taken at 100. x, y, α, β, and γ are the protein bands observed in gels containing urea (Figure 3).

after treatment with thiol reagents the relative amounts of α, β, and γ proteins are very nearly in the ratio of 2:2:1 (Figure 4, Table II) and the same result was obtained with both mercaptoethanol and dithiothreitol over a ten fold concentration range of these reducing agents. These results suggest that the x and y proteins are disulfide polymers of the α subunit and therefore it may be concluded that the ATPase is actually composed of three different types of subunits, the ones designated α, β, and γ, in a noncovalent type of association.

Discussion

The ATPase which we have isolated from streptococcal membranes appears to be homogeneous as judged by zone sedimentation analysis and by zone electrophoresis in acrylamide gels. After electrophoresis of the enzyme in gels containing urea five protein bands appear, designated x, y, α, β, and γ in the order of increasing electrophoretic mobility. However, the two slower moving proteins, x and y are completely eliminated by thiol reagents and they are apparently converted to the α protein. Thus, when thiol-reducing agents are present, urea splits the ATPase into only three proteins, α, β, and γ. Furthermore, the three proteins are always found in an apparent ratio of 2:2:1 despite attempts to change this pattern by increasing the urea concentration in the gel or by increasing the concentration of thiol reagents (Figures 3 and 4, Table II). On the basis of the evidence obtained thus far, we conclude first of all that the native streptococcal membrane ATPase is made up of three different types of subunits, α, β, and γ, in a noncovalent type of association and secondly that the α subunit is a sulfhydryl protein which oxidizes easily but only partially to disulfide polymers (proteins x and y) under the conditions used. Consistent with this view is the fact that the α, β, and γ proteins migrate faster than the intact enzyme and that the x and y proteins migrate slower than the α protein, as expected in gels with molecular seiving properties such as polyacrylamide (Table II). It also seems likely from the results obtained with thiol reagents that α, β, and γ are single polypeptide chains.

An evaluation of the number of each type of subunit in a molecule of ATPase must await a determination of their molecular weights. However, if we assume that the three types of subunits have about the same molecular weight and in view of the observed proportions of 2:2:1, then a tentative subunit composition may be formulated as $\alpha_{2n}\beta_{2n}\gamma_n$. A minimal molecular weight of the intact ATPase of approximately 350,000 has been reported on the basis of zone sedimentation (Abrams, 1965). Since the molecular weight of single polypeptide chains are usually somewhere between 10,000 and 100,000, it is reasonable to expect that the value of n lies between 1 and 5.

It is of interest to compare the streptococcal membrane enzyme with other ATPases whose subunit structures have been studied. The ATPase of protozoan cilia (Gibbons and Rowe, 1965), animal muscle myosin (Small *et al.*, 1961), and animal mitochondria (Penefsky and Warner, 1965) like the one from *S. fecalis* have high molecular weights and are composed of many subunits. However, in contrast to the bacterial enzyme, they all appear to contain only one type of subunit.

Although there is no evidence we may surmise that one of the subunits in the ATPase contains the active site while another has some other function such as a regulatory function as in the case of aspartate transcarbamylase (Gerhart and Schachman, 1965) which has two nonidentical subunits. Since the streptococcal membrane ATPase contains a third type of subunit, an interesting possibility to consider for its function is that it contains the site for attachment to membranes.

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