ROLE OF MG^{2+} IONS IN THE SUBUNIT STRUCTURE AND MEMBRANE BINDING PROPERTIES OF BACTERIAL ENERGY TRANSDUCING ATPASE

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SUMMARY - ATPase extracted from Streptococcus faecalis membranes was purified by preparative slab gel electrophoresis in the presence of Mg^{++} (plus Mg^{2+} ATPase) and without Mg^{2+} (minus Mg^{2+} ATPase). The subunit composition and membrane binding capacity of both preparations was then examined. The plus Mg^{2+} ATPase had 5 types of subunits ($\alpha\beta\gamma\delta\varepsilon$) and reattached normally to depleted membranes. The minus Mg^{2+} ATPase had the $\alpha\beta\gamma$ and ε chains, but no δ chain, and failed to reattach to membranes. These data indicate that Mg^{2+} or a similar cationic ligand anchors the δ chain to the core enzyme complex and that the δ chain in turn is needed for membrane attachment. For the plus Mg^{2+} ATPase the data are consistent with the subunit stoichiometry and arrangement, $(\alpha_3\beta_3\ \gamma\ \varepsilon)\text{-}Mg^{2+})_n\text{-}(\delta)$.

INTRODUCTION - The ATPase associated with the plasma membrane in the fermentative organism, Streptococcus faecalis, is a 385,000 dalton multisubunit complex believed to be responsible for coupling ATP hydrolysis to solute accumulation (1-7). Over the last decade, considerable progress has been made in elucidating the molecular properties of the S. faecalis enzyme and related energy transducing ATPases found in aerobic bacteria, mitochondria and chloroplasts (8-13) According to the widely accepted chemiosmotic theory of Mitchell (14) all these ATPases are proton translocators involved in the generation or the utilization of a proton motive force. However, the relation between substructure and the energy transduction function is not well understood (15,16).

Some years ago some studies were initiated to help define the factors involved in the association of the S. faecalis ATPase with the plasma membrane (17). It was found that the membrane-bound enzyme could be readily solubilized by repeatedly washing the isolated membranes with dilute buffered salt solutions without Mg^{2+} ions (1). In the presence of Mg^{2+} ions the solubilized enzyme reattached normally to the depleted membranes as evidenced by a variety of criteria including restoration of sensitivity to inhibition by dicyclohexylcarbodiimide

(5,17). Subsequently, Baron and Abrams (18) presented evidence that a protein factor was associated with the ATPase via Mg^{2+} ions and that this protein, called nectin, was required for reattachment to depleted membranes. Nectin separated from the enzyme during fractionation by gel filtration or DEAE-cellulose chromatography (19) in the absence of Mg^{2+} ions. The nectin-free ATPase failed to reattach to depleted membranes unless nectin and Mg^{2+} ions were added to the rebinding test system. The mol. wt. of nectin, estimated by gel filtration under nondissociating conditions, was about 37,000 daltons (18), but the factor was not characterized by SDS-gel electrophoresis.

Recently reported subunit analyses of the S. faecalis ATPase indicated a subunit structure $\alpha_3\beta_3\gamma$ in which α = 60,000, β = 55,000 and γ = 37,000 daltons (4,12). In the present communication we wish to offer evidence that the S. faecalis ATPase contains two additional minor subunits, δ = 20,000 and ε = 12,000, and that the δ chain is held in the enzyme complex by Mg^{2+} ions. In addition we have found that the δ subunit is needed for reattachment to the membranes as has already been reported for the E. coli ATPase system by a number of investigators (20-22).

To demonstrate the Mg^{2+}-dependent association of the δ subunit with the S. faecalis ATPase complex we isolated the enzyme by preparative electrophoresis at pH 8 in gel slabs in the presence and absence of Mg^{2+} ions. To our knowledge there has been no previous report other than the study of nectin (18), indicating that Mg^{2+} ions or other multivalent cations are ligands that link subunits together in the bacterial or the related eucaryotic ATPases.

METHODS - Preparation of cells, plasma membranes and solubilized ATPase. S. faecalis cells (ATCC #9790), harvested from 30 liters of growth medium, were converted to protoplasts by treatment with lysozyme in 0.2m sucrose, 0.2m glycylglycine, pH 7.2, and then lysed by osmotic shock to produce membrane ghosts (19). The enzyme was solubilized by an aqueous wash procedure (1,19). In brief, the membranes are first washed successively in 2 mM $MgCl_2$ and in 2 M LiCl which removes some membrane components and contaminants, but not the ATPase. The washing is continued with low ionic strength Tris Cl pH 7.5 buffer which results in release of the ATPase provided that Mg^{2+} ions are not present. The yield is about 1500 units. The depleted membranes were lyophilized and saved for subsequent assays of reattachment of the solubilized enzyme (19). (See below).

Purification of the ATPase by column chromatography. The crude solubilized ATPase preparations, in 20 mM Tris Cl pH 7.5 - 10 mM $MgCl_2$ was successively fractionated by chromatography on DEAE-Bio-Gel A, gel filtration on Agarose (1.5 M) rechromatography on DEAE-Bio-Gel A. The procedures used were similar

to those already described except that the preliminary short heating step at 80° was omitted (19); $MgCl_2$ and 10% glycerol were added to all solutions to help stabilize the enzyme. Mg^{2+} ions also prevent loss of the attachment factor, nectin (18).

Preparative slab gel electrophoresis in the presence and absence of Mg^{2+} ions. We employed a water cooled vertical slab gel apparatus manufactured by E.C. Apparatus Co., St. Petersburg, Fla. We used 6 mm thick 5% polyacrylamide slabs prepared in 2 mM Tris and 70 mM glycine, pH 8. To prepare the Mg^{2+} containing system, 2 mM $MgSO_4$ was added to the Tris glycine buffer which was used both to prepare the gel and for the electrode compartment buffer. About 70 units of enzyme in 2 ml of 20 mM Tris Cl pH 7.5, 10 mM $MgCl_2$ and 10% sucrose was layered into a single slot on top of both slabs. From 400-450 volts were applied across the slabs for 3-4 hours. The enzyme migrated about 6 cm, sufficient for separating it from virtually all contaminating proteins. The enzyme zone in each slab was located by cutting out two thin guide strips along the direction of migration and staining one of them for protein with Coomassie Blue and the other for ATPase activity by the method of Abrams and Baron (2).

To extract the enzyme from the slab gels the enzyme zone was excised and macerated using a syringe and needle and then incubated in 15 ml 20 mM Tris Cl, pH 7.5, 10 mM $MgCl_2$ and 10% glycerol at 38° for 30 min. Several extractions yielded about 40% of the starting amount of ATPase. The extracted enzyme from each slab was concentrated on a small DEAE Bio-Gel A column, eluted with a small volume of 0.5 M KCl, and then dialyzed against Tris-Mg buffer plus 10% glycerol to remove the KCl.

SDS-gel analysis. Gel analysis under dissociating conditions was carried out in 12% polyacrylamide cylindrical gels containing 1 mM EDTA, 0.1% SDS and 0.1 M phosphate, pH 7, (23). The enzyme sample was dissociated by heating at 100° for 3 minutes in 5% SDS, 10% mercaptoethanol and 0.01 M phosphate, pH 7.0. The gel was stained for protein with 0.05% Coomassie Blue in CH_3OH, H_2O, acetic acid (5:5:1) and destained in the same solvent. To obtain subunit mol. wts. we used as protein standards BSA, γ globulin, ovalbumin, myoglobin and cytochrome C.

Assay of reattachment of depleted membranes. The reattachment assay was carried out by the pelleting method developed earlier for assaying the attachment factor, nectin (18,19). In essence, from a concentrated ATPase stock solution, 0.3 units is mixed with a suspension of depleted membranes (0.25 mg membrane protein), in 10 mM $MgCl_2$ and 100 mM Tris Cl pH 7.5 in a final volume of 0.3 ml. The mixture is incubated for 0.5 hours at 38° and then centrifuged. The pellet is washed in Tris-Mg^{2+} buffer, resuspended, and then assayed for ATPase activity.

EXPERIMENTAL AND RESULTS - Effect of gel electrophoresis with and without Mg^{2+} ions on ATPase subunit composition. In order to determine the role of Mg^{2+} in the ATPase substructure it was desirable, if not essential, to have for study electrophoretically pure enzyme preparations. To this end the ATPase extracted from S. faecalis membranes was first purified to near homogeneity by chromatographic procedures in the presence of Mg^{2+} ions. Such enzyme preparations reattach normally to depleted membranes. We then isolated electrophoretically fairly large amounts of the enzyme, at pH 8 in two different polyacrylamide gel slab systems. In one gel system we introduced 2 mM Mg^{2+} while in the other Mg^{2+} ions were omitted. The electrophoretically homogeneous enzyme

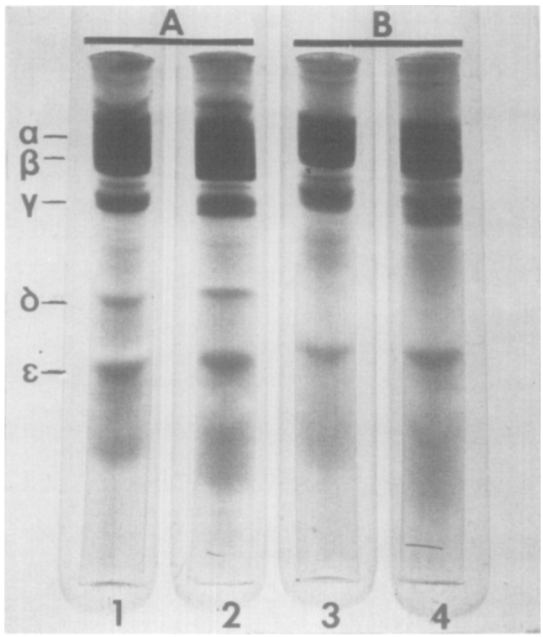

Fig. 1. SDS-gel electrophoresis of ATPase purified electrophoretically with and without Mg^{2+}. A. Plus Mg^{2+} ATPase. B. Minus Mg ATPase. Gels 1 and 3 were loaded with 2 ATPase units; gels 2 and 4 were loaded with 3 ATPase units.

preparations, which we will refer to as plus Mg^{2+} ATPase and minus Mg^{2+} ATPase, were then extracted from the gel slabs. The subunit compositions of the plus Mg^{2+} ATPase and the minus Mg^{2+} ATPase, were then determined using conventional SDS-gel electrophoretic analysis in tubes. The results are pictured in Fig. 1.

The plus Mg^{2+} ATPase yielded 5 types of subunits, $\alpha,\beta,\gamma,\delta$ and ε, while the minus Mg^{2+} ATPase had α,β,γ and ε but no δ. It should be noted that in these subunit analyses we overloaded the gels with respect to the larger subunits, α and β, in order to ensure visualization of the smaller polypeptide chains, δ and ε, which together account for only about 10% or less of the total enzyme protein. Due to the overloading, α and β merge into one large band instead of resolving as has been observed when smaller amounts of the enzyme are analyzed in SDS gels or urea gels (2,3,4,12).

Since the δ subunit was retained during electrophoresis in the presence of Mg^{2+} ions while it was selectively lost in the absence of Mg^{2+} ions we may conclude that the Mg^{2+} ions act to connect the δ subunit to the remainder of

the enzyme complex. Notably the Mg^{2+} dependent association of the δ chain with the enzyme is reminiscent of the behaviour of nectin, the membrane attachment factor associated with the enzyme described in earlier work (18). (See Discussion).

Rebinding to membranes of the plus Mg^{2+} ATPase and minus Mg^{2+} ATPase. As described in the previous section, ATPase was extracted from gels after electrophoretic purification in the presence and absence of Mg^{2+} ions. The plus Mg^{2+} ATPase and the minus Mg^{2+} ATPase, whose subunit compositions are presented in Fig. 1, were tested for the ability to rebind to ATPase-depleted membranes. The results of the rebinding test are given in Table I, Expt. #1. The data show that the plus Mg^{2+} ATPase, which contains the δ subunit, reattached normally to depleted membranes. By contrast, the minus Mg^{2+} ATPase, in which the δ subunit is absent, failed almost completely to reattach to the depleted membranes. Table I also shows a confirmatory set of data obtained in a completely independent experiment (Expt. #2) using enzyme preparations derived from another batch of cells.

We wish to emphasize that in the rebinding test system, Mg^{2+} ions are added and are therefore not a limiting factor in the assay. Thus we may conclude that the δ subunit in S. faecalis is needed for the attachment of the enzyme to the membrane. This function of the δ chain in the S. faecalis ATPase corresponds to the function ascribed to the nectin factor in S. faecalis (18), and the δ subunit in the E. coli membrane ATPase system (20-22).

DISCUSSION - It seems likely that the S. faecalis ATPase is a metalloenzyme in which the metal ligand anchors the δ subunit to the catalytically active core complex consisting of the other types of subunits, α,β,γ and ε. This conclusion stems from the observation that 2 mM Mg^{2+} prevents selective loss of the δ subunit which occurs during gel electrophoresis without Mg^{2+} at pH 8 (Fig. 1). We surmise that the free Mg^{2+} ions in the gel replace the natural metal ions which presumably tend to dissociate from the enzyme under the conditions that prevail during gel electrophoresis without Mg^{2+}. Whether the natural metal ion in the enzyme is actually Mg^{2+} or perhaps some other multi-

TABLE I

Effect of Gel Electrophoretic Purification With and Without Mg^{2+} on the Membrane Binding Capacity and Subunit Composition of S. faecalis ATPase

Expt. #	Electrophoresis Conditions	Subunit Types	Reattachment (ATPase units bound/0.25 mg membrane protein)
1	plus Mg^{2+}	$\alpha\beta\gamma\delta\varepsilon$	0.22 ± 0.04
	minus Mg^{2+}	$\alpha\beta\gamma$-ε	0.07 ± 0.01
2	plus Mg^{2+}	$\alpha\beta\gamma\delta\varepsilon$	0.22 ± 0.01
	minus Mg^{2+}	$\alpha\beta\gamma$-ε	0.03 ± 0.003

The ATPase used for the electrophoretic purification experiments was isolated in the presence of Mg^{2+}. (See Methods). Tests of reattachment prior to electrophoresis gave values of 0.23 units bound/0.25 mg membrane protein. The average deviation of 6 replicate determinations are shown for the reattachment assays. A unit is defined as the amount of enzyme which liberates 1 μmol Pi per min. at 38°. See Fig. 1 for subunit analysis obtained in Expt. #1.

valent cation remains to be seen.

A tentative subunit formulation, which incorporates Mg^{2+} as an integral part of the enzyme in a manner suggested by the data, is the following: $(\alpha_3\beta_3\ \gamma\ \varepsilon)$ - $Mg^{2+})_n$-δ. This subunit stoichiometry is consistent with a mol. wt. of 385,000 for the complete enzyme reported by Schnebli et al. (3) and the approximate mol. wts. of the subunits derived from SDS-gel electrophoretic analysis, α = 55-60,000, β = 50-55,000, γ = 35,000, δ = 20,000, ε = 12,000 (Fig. 1 and Ref. 4,12). Not shown in this formula are the tightly bound ligands, non-exchangeable ATP and ADP, that have been found in the S. faecalis enzyme (4,24,25). According to the subunit arrangement shown above the removal of Mg^{2+} would be expected to result in the loss of only the δ subunit and yield the enzyme, $\alpha_3\beta_3\ \gamma\ \varepsilon$, with only 4 types of chains as is seen in Fig. 1. Except for showing a metal ion ligand as an integral component of the protein the

structural formulation for the complete S. faecalis ATPase given above is the same as the 5 subunit formula suggested for the E. coli ATPase (26) and the mitochondrial ATPase (27,28).

We have also shown in this report that the loss of the δ subunit, accomplished by gel electrophoresis without Mg^{2+} ions, yields a catalytically active enzyme that fails almost completely to reattach to depleted membranes (Table I). From this finding we may infer that the δ subunit is required for attachment. However, we do not wish to exclude the possibility that the Mg^{2+} ligand itself (or both Mg^{2+} and the δ chain) in the enzyme makes contact with the receptor site for the enzyme in the membrane.

In earlier work it was reported that the S. faecalis ATPase contained a protein factor, named nectin, which was needed for attachment to depleted membranes and which separated from the enzyme during column chromatographic purification of the enzyme in the absence of Mg^{2+} ions. It would appear that the δ subunit corresponds to nectin except for the apparent 2-fold difference in their molecular wts, which are approximately 20,000 and 37,000 daltons respectively (Fig. 1 and Ref. 18). It should be pointed out however, that the mol. wt. of nectin was estimated by gel filtration under non-denaturing conditions whereas the δ subunit mol. wt. was determined electrophoretically under dissociating conditions, i.e. in SDS after reduction with mercaptoethanol (Fig. 1). Therefore, it is possible that nectin may have been a functionally active dimer of the isolated δ chain. In this regard it is significant that nectin has a tendency to aggregate to high mol. wt. functionally active forms (19).

We have found here that the S. faecalis ATPase needs Mg^{2+} ions to hold the δ chain, which in turn is needed to attach the ATPase complex to the plasma membrane. It remains to be seen whether Mg^{2+}, or possibly some other multivalent cation functions similarly in the related energy transducing membrane ATPase found in mitochondria, chloroplasts and in other bacteria.

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