TIGHTLY BOUND ADENINE NUCLEOTIDE IN BACTERIAL MEMBRANE ATPASE

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Received September 11,1973

SUMMARY - Earlier studies have shown that Streptococcus faecalis organisms incorporate [^{32}P]-orthophosphate into membrane ATPase (Abrams and Nolan, BBRC (1972) 48, 982). We have now found that [^{14}C]adenine as well as ^{32}Pi is incorporated into the enzyme in vivo. The in vivo labelled ATPase, solubilized and then purified by zonal sedimentation, contained labelled ATP, ADP and Pi. Binding experiments in vitro showed that labelled ATP, ADP and Pi bind to the isolated solubilized ATPase at concentrations of 5×10^{-5}M. The ATPase-ligand complexes formed in vitro like those formed in vivo were sufficiently stable to be isolated by zonal sedimentation and polyacrylamide gel electrophoresis. The stoichiometry of in vitro binding of the labelled compounds was about 1 mol ATP, 1 mol ADP and 0.1 mol Pi per mol of enzyme.

INTRODUCTION - There is considerable evidence indicating that the energized transport of certain solutes by Streptococcus faecalis depends on ATP and the action of an ATPase localized in the plasma membrane (1-4). We have studied this enzyme extensively as knowledge of its properties could help to elucidate the energy transduction mechanism which at present is largely unknown. In previous work methods were developed for solubilizing and purifying the S. faecalis ATPase to homogeneity and several molecular features of the protein were investigated such as its size (mol wt = 385,000), shape, subunit structure and amino acid composition (5-9). Through studies of the dissociation and reassembly of the ATPase-membrane complex in vitro, various interactions of the enzyme with other membrane components and with multivalent cations were also delineated (2,3,10,11). Recently, a new feature of the S. faecalis ATPase came to light when it was found that ^{32}P-orthophosphate is incorporated into the enzyme by intact cells (12). The incorporated ^{32}P remained firmly associated with the catalytically active enzyme through several stages of purification including zonal sedimentation and polyacrylamide gel electrophorsis Moreover the bound ^{32}P was not displaced from the isolated enzyme by

addition of non-radioactive Pi or ATP. We concluded that the native ATPase contains tightly bound phosphate groups and that these groups do not represent the transient catalytic intermediates involved in the enzymatic hydrolysis of ATP. However, we did not determine in what chemical form the stably bound phosphate groups were actually present.

In this communication we report that both ^{32}Pi and [^{14}C]adenine are incorporated into the active ATPase *in vivo*. ATP, ADP and Pi were identified as components of the isolated labelled enzyme. We also tested the binding of labelled ATP, ADP and Pi to the catalytically active ATPase *in vitro* and found that they bind tightly at quite low concentrations (50 μM). Preliminary determination of the stoichiometry of this tight *in vitro* binding indicates that the ATPase binds about 20 times more nucleotide than Pi.

METHODS - *S. faecalis* cells were harvested in the stationary phase of growth from 2400 ml of a medium consisting of 1% tryptone, 0.5% yeast extract, 1% glucose, 0.76% KCl and 3 mM K_2HPO_4. To label the ATPase with ^{32}P *in vivo*, the cells were washed and incubated for 30 min at 38°C in 100 ml of a solution containing 20 mM Tris Cl pH 7.5, 10 mM KCl, 1% glucose and 5 mC carrier-free ^{32}Pi. (New England Nuclear) (12). For *in vivo* labelling of the enzyme with adenine the washed cells were incubated, as described above, with 0.1 mC of 8-[^{14}C]-adenine (41.3 mC/mmol.).

Membranes were isolated by converting the cells to protoplasts which were then lysed by osmotic shock as described previously (12). To solubilize the membrane-bound ATPase the membranes were subjected to an aqueous wash procedure which releases the enzyme selectively (6,12). The solubilized enzyme preparation was concentrated to a small volume, about 1.0 ml, by pressure ultrafiltration prior to further purification by zonal sedimentation.

The method used to assay ATPase activity was described previously (5). One unit of enzyme is that amount which yields 1 μmol Pi per minute.

Zonal sedimentation was carried out for about 20 hours at 30,000 x g at 4° in a 4-16% sucrose gradient containing 20 mM Tris Cl pH 7.5 and 10 mM $MgCl_2$ (6).

The gel electrophoretic analysis of the labelled ATPase was done in a 5% polyacrylamide gel slab using a Tris-glycine buffer at pH 7.5 (7). To locate the band of ATPase activity in the gel we employed the staining procedure of Abrams and Baron (7). The location of ^{3}H in the gel was determined by slicing the gel into 2.8 mm segments which were incubated overnight in 1 ml NCS-H_2O (9:1) and then counted by means of liquid scintillation.

To identify labelled ATP, ADP, and Pi we used paper electrophoresis at pH 3.8 (13) and thin layer chromatography on polyethylenimine-cellulose sheets (Brinkman) employing 1.2 M LiCl as the solvent (14). For these analyses we first treated the labelled ATPase with 0.01 N HCl for 30 min at room temperature. The acid treated enzyme samples were co-electrophoresed or co-chromatographed with authentic ATP, ADP and AMP whose positions were located by ultraviolet light. ^{32}Pi in the sample was identified by comparison with known ^{32}Pi run alongside. Radioactivity was located by liquid scintillation

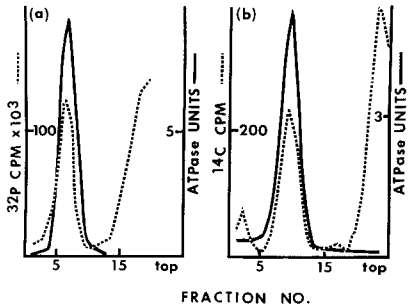
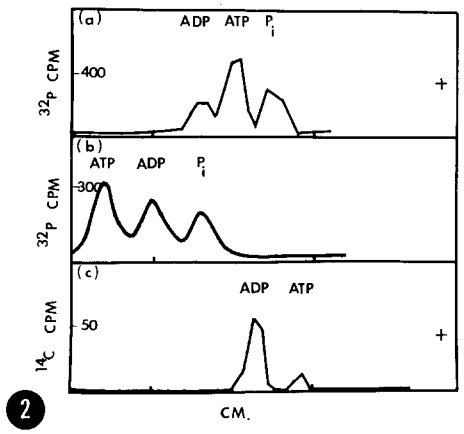


Fig. 1 Zonal sedimentation of ATPase labelled in vivo with ^{32}Pi or with ^{14}C-adenine. (a)ATPase isolated from cells incubated in ^{32}Pi. (b)ATPase isolated from cells incubated in [^{14}C]adenine.

counting of strips or by direct scanning.

EXPERIMENTAL AND RESULTS - The in vivo incorporation of ^{32}Pi and ^{14}C-adenine into the membrane ATPase. Solubilized ATPase preparations obtained from cells that had been incubated with ^{32}Pi or ^{14}C-adenine were purified by zonal sedimentation in a sucrose gradient (Fig. 1a and 1b). As can be seen the sedimentation profiles show that both ^{32}Pi (Fig. 1a) and ^{14}C-adenine (Fig. 1b) are incorporated in a tightly bound form. As in our earlier work (12) polyacrylamide gel electrophoresis analysis of the gradient isolated ^{32}P labelled ATPase confirmed the tight association of ^{32}P with the enzyme.

Identification of radioactive components in the in vivo labelled ATPase. The ATPase that had been labelled in vivo with ^{32}Pi or ^{14}C-adenine and purified by zonal sedimentation (Fig. 1) was treated with 0.01 M HCl in order to dissociate the labelled components from the protein. (This acid treatment may not completely strip all labelled moieties from the protein). The acid treated preparations were then analyzed by paper electrophoresis and by thin layer anion exchange chromatography (Fig. 2). The results obtained by both methods indicate that [^{32}P]ATP, [^{32}P]ADP and ^{32}Pi are present in the acid treated ^{32}P labelled ATPase preparation (Fig. 2a and b). This result was substantiated by paper electrophoretic analysis of the [^{14}C]adenine-labelled ATPase which showed the presence of [^{14}C]ATP and [^{14}C]ADP (Fig. 2c). We did not detect any free labelled AMP in the acid-treated ATPase. We wish to point



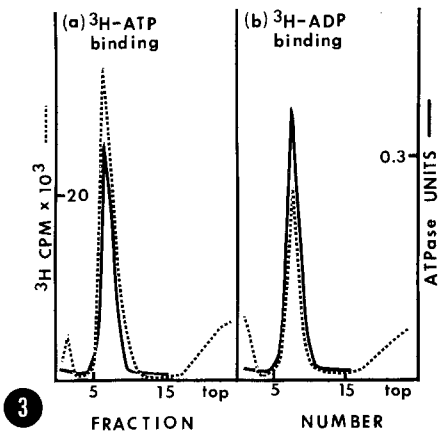


Fig. 2 Analysis of the radioactive components in the ATPase labelled in vivo with ^{32}P or [^{14}C]adenine. The labelled enzyme was purified by zonal sedimentation as shown in Fig. 1 and then analyzed after treatment with 0.01 M HCl. (a)Paper electrophoresis of the ^{32}P labelled components. (b)Thin layer anion exchange chromatography of the ^{32}P labelled components. (c)Paper electrophoresis of the ^{14}C-adenine labelled components.

Fig. 3 Binding of Nucleotides to ATPase in vitro.
(a)ATPase, 2.25 units, was incubated with 5×10^{-5}M [^{3}H]ATP (7.70×10^8 cpm per µmol) for 70 min. at 4° in 0.22 ml of a solution containing 40 mM EDTA, 10 mM Mg^{2+} and 20 mM Tris Cl, pH 7.5. Using the same buffer-EDTA solution the mixture was diluted 2-fold and dialyzed for 6 hours at 4° to remove the bulk of unbound nucleotide. Binding to the ATPase was then determined by zonal sedimentation.
(b)Same as (a) except that the ATPase, 2.25 units, was mixed with 5×10^{-5} M [^{3}H]ADP (7.74×10^8 cpm per µmol).

out that the relative amounts of radioactivity found in the labelled ATP, ADP, and Pi (Fig. 2) do not necessarily reflect the actual relative molar quantities of these components since their specific radioactivities may be quite different. It should be mentioned also that the relative amounts of radioactive ATP, ADP, and Pi that we found in the enzyme varied in different experiments.

Binding of ATP, ADP, and Pi to the ATPase in vitro. Since the foregoing experiments indicated that ATP, ADP and Pi bind to the ATPase in vivo it was of interest to determine if these compounds would bind to the enzyme in vitro. A soluble ATPase preparation to be used for testing in vitro binding was

first precipitated with 80% saturated $(NH_4)SO_4$, then redissolved and dialyzed.[1] Samples of the enzyme were then incubated with [^{3}H]ATP, [^{3}H]ADP or ^{32}Pi of known specific activity at concentrations of 5×10^{-5}M at pH 7.5 in the presence of EDTA (Fig. 3). After the incubation period the mixtures were dialyzed against Tris buffer at pH 7.5 containing EDTA in order to remove the bulk of unbound labelled compounds. To measure binding each of the mixtures was analyzed by rate zonal sedimentation. Fig. 3 depicts the results obtained with [^{3}H]ADP and [^{3}H]ATP and clearly indicates that these nucleotides bind tightly to the enzyme.[2] We then determined the nucleotide composition of the labelled enzyme isolated from the sucrose gradient. (See Methods). We found only [^{3}H]ADP in the ATPase that had been incubated with [^{3}H]ADP. However in the enzyme that had been incubated with [^{3}H]ATP, we found both labelled ATP and ADP in about equal amounts. It seems, therefore, that partial enzymatic hydrolysis of the ATP yields some free ADP which then binds to the enzyme. It is possible also that some bound ATP may have been converted directly to bound ADP on the surface of the enzyme.

We calculated the stoichiometry of binding of the labelled nucleotides from the data in Fig. 3 using a value of 30 units per mg for the specific activity of the pure ATPase and 385,000 daltons for the molecular weight (8,9). The stoichiometry of [^{3}H]ATP binding appeared to be about 2 mols per mol of enzyme. However, as mentioned above, the presumed bound ATP actually consisted of roughly equal amounts of ATP and ADP. The stoichiometry of ADP binding (Fig. 3b) was about 1 mol per mol of enzyme.

^{32}Pi was also bound to the enzyme in vitro. However, the stoichiometry

[1] Precipitation with $(NH_4)_2SO_4$ removes a considerable portion of the bound phosphate compounds as judged from experiments with in vivo labelled ^{32}P-enzyme. We also observed that the stoichiometry for [^{3}H]ATP binding increased from 0.6 to 2 moles nucleotide per mol of enzyme after the $(NH_4)_2SO_4$ precipitation. It appears that some of the bound nucleotide initially present in the native enzyme is removed by salt precipitation thus opening up additional sites for nucleotide binding in vitro.

[2] The same experiment carried out without the use of EDTA gave the same amount of bound nucleotide indicating that free multivalent metals are not involved in binding.

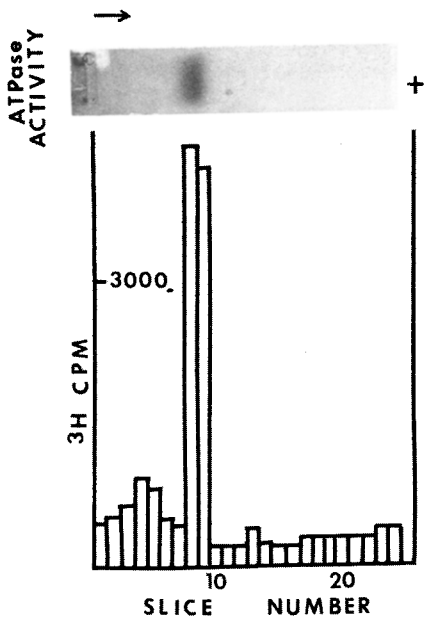


Fig. 4 Polyacrylamide gel electrophoresis of the ATPase-[^{3}H]ADP complex formed in vitro. ATPase that had been incubated with [^{3}H]ADP in vitro was purified by zonal sedimentation as shown in Fig. 3b. Two samples of the purified ATPase were then electrophoresed in two tracks next to each other in a gel slab. After electrophoresis one track was sliced and counted; ATPase activity was located in the other track. The profile of radioactivity is shown in the lower part of the figure; the ATPase activity shown at the top appears as a blue colored band of reduced phosphomolybdate (7).

of ^{32}Pi binding was only about 0.1 mol per mol of enzyme.

The observed values for in vitro stoichiometry are probably minimal as the native enzyme presumably contains some tightly bound nucleotide and Pi to begin with as has been indicated by the in vivo labelling experiments described earlier (see Footnote 1). It should be noted also that we have not as yet examined the stoichiometry as a function of time and concentration of the ligands.

To confirm the in vitro binding of labelled ADP and ATP indicated by the zonal sedimentation analysis shown in Fig. 3, we took the enzyme that was collected from the gradient and analyzed it further by gel electrophoresis. Fig. 4 illustrates the result obtained with the ATPase that had been incubated with [^{3}H]ADP. It shows that the zone of ATPase activity and radioactivity in the gel coincide. Similarly, gel electrophoretic analysis of the ATPase that

had been incubated with [^{3}H]ATP also showed a co-electromigration of ATPase activity and radioactivity.

DISCUSSION - Tight binding of ATP, ADP and Pi to the soluble active ATPase occurs *in vitro* at concentrations of only 50 μM. (Figs. 3 and 4). Since the intracellular concentrations of ATP, ADP and Pi are much higher it is to be expected that a similar direct binding to the membrane-bound enzyme would also occur *in vivo*. This would account for the observed presence of labelled ATP, ADP and Pi in the ATPase isolated from cells after incubation in ^{32}Pi or ^{14}C-adenine (Figs. 1 and 2).

The presence of ATP, ADP and Pi firmly fixed in the native active enzyme raises the question of its physiological significance. The long term stability exhibited by the isolated enzyme-ligand complex produced *in vitro* and *in vivo* indicates that the firmly bound adenine nucleotides and Pi are to be distinguished from the ATP, ADP and Pi transiently associated with the enzyme during the course of ATP hydrolysis. It has been reported that the ATPase of chloroplasts contains tightly bound ADP which might be an intermediate in coupled ATP synthesis from AMP and Pi (15). The possibility that the tightly bound nucleotides and Pi in the *S. faecalis* ATPase are involved in oxidative phosphorylation seem remote. The *S. faecalis* organism is a homolactic fermentor lacking cytochromes, and does not carry out oxidative phosphorylation except possibly under extraordinary circumstances (16). The evidence presently available makes it more likely that the *S. faecalis* ATPase mediates energized transport of solutes such as K^+ and amino acids by utilizing ATP generated via substrate level phosphorylation (1-4). Conceivably the tightly bound ATP, ADP and Pi could play a role in the energy coupling phase of active transport by influencing the catalytic activity, cation binding, or the conformational state of the membrane-bound ATPase.

ACKNOWLEDGEMENTS. This work was supported by Grant 05810 from the National Institute of General Medical Sciences.

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