CELLULAR INCORPORATION OF ^{32}P-ORTHOPHOSPHATE INTO THE MEMBRANE ATPASE OF *STREPTOCOCCUS FAECALIS*

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SUMMARY. We have found that *Streptococcus faecalis* cells incorporate $^{32}P_i$ into their plasma membrane ATPase. The labeled ATPase was isolated from cells grown in a $^{32}P_i$ containing medium and also from non-growing cells suspended in $^{32}P_i$ and glucose. To demonstrate that the enzyme contained ^{32}P it was solubilized and then purified by chromatography on DEAE-cellulose, rate-zonal sedimentation and polyacrylamide elctrophoresis. Since the ^{32}P remained firmly fixed to the catalytically active enzyme we conclude that it contains phosphate as a structural constituent. We estimate that one molecule of the ATPase contains 5 to 10 atoms of P.

INTRODUCTION. The adenosinetriphosphatase (ATPase) associated with the plasma membrane of *Streptococcus faecalis* (1) can be solubilized and isolated in essentially pure form (2,3,4,5). Studies of the molecular properties of this enzyme have shown that it is a multimeric protein of fairly high molecular weight containing non-identical subunits. The amino acid analysis of the enzyme revealed nothing unusual about its composition except perhaps for the finding that the terminal amino group is blocked (5). As for the physiological role of the membrane ATPase in *S. faecalis* there is considerable evidence, both biochemical and genetic, that it mediates the utilization of ATP generated by anaerobic glycolysis to drive the transport of monovalent cations and amino acids (6,7,8,9,10).

In this communication we wish to report some experiments indicating that the fully active *S. faecalis* ATPase contains phosphate as a firmly bound constitutent. As far as we are aware, there have been no previous reports that any membrane ATPase, either from procaryotic or eucaryotic organisms, contains phosphate as a stably bound component. However, there is evidence that the terminal phosphate of ATP is introduced transiently

into the Na + K stimulated membrane ATPase of animals during the hydrolysis of the substrate (11,12,13). To demonstrate the presence of phosphorous in the Streptococcal ATPase we fed ^{32}P-inorganic phosphate to intact cells, then isolated the enzyme and determined its content of radioactivity. The $^{32}P_i$ was supplied to either growing cells or to a suspension of non-growing cells in glucose. Under both of these conditions we found that the ATPase became radioactively labeled.

METHODS. The ATPase was assayed as previously described (2) using the method of Fiske and SubbaRow for measuring P_i (14). One unit of ATPase is that amount which catalyzes the release of 1 µmole of P_i per minute from ATP under the assay conditions. Column chromatography of the enzyme was carried out on DEAE-cellulose (Whatman DE32) according to the procedure of Schnebli et. al.(4). The procedures used for zonal sedimentation and gel electrophoresis are described in the figure legends. Radioactivity was determined by liquid scintillation.

EXPERIMENTAL AND RESULTS. Isolation of radioactively labeled ATPase from cells grown in $^{32}P_i$. S. faecalis cells (ATCC 9790) were grown in 2400 ml of medium containing 1% tryptone, 0.5% yeast extract, 1% glucose, 0.76% KCl, 3 mM K_2HPO_4 and 50 mC $^{32}P_i$ (New England Nuclear, > 99% pure, carrier free). The specific radioactivity was 4.3×10^{12} cpm/mol P. The medium was inoculated with 5 ml of a fully grown culture and incubated at 38°C overnight. To obtain the membranes and release the bound ATPase in a soluble form, we followed a procedure slightly modified from that developed previously in this laboratory (15,2). After harvesting and washing the cells by centrifugation they were converted to protoplasts using lysozyme together with glycylglycine as an osmotic stablizer. The protoplasts were pelleted and lysed by osmotic shock in the presence of DNAase. The resulting membrane ghosts were then isolated by centrifugation. The membranes, now freed of the bulk of the extracellular and intracellular $^{32}P_i$, were subjected to a series of 10 washings as follows: one wash with 2 mM $MgCl_2$; two washes with 2 M LiCl-0.25 M Tris Cl, pH 7.5; four washes with 33 mM Tris Cl, pH 7.5; three washes with 1 mM Tris Cl. This washing procedure further reduced contaminating $^{32}P_i$ in the membranes and also achieved release of the enzyme into the 5th to the 9th wash fluids. The

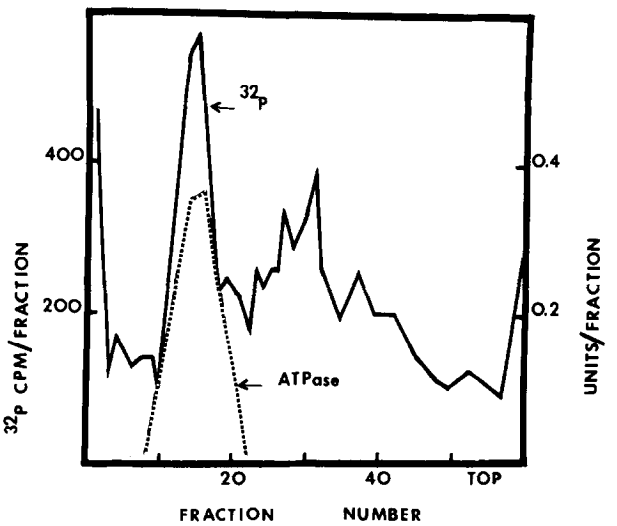

Fig. 1. Zonal sedimentation of ATPase from cells grown in ^{32}P-orthophosphate. - A 0.8 ml sample of ATPase previously fractionated by DEAE-cellulose column chromatography, was sedimented on a 4-16% linear sucrose gradient containing 20 mM Tris Cl, pH 7.5 and 10 mM $MgCl_2$. Sedimentation was carried out in a Model L-2 Beckman ultracentrifuge at 34000 g for 18 hours at 4°C using a No. 41 head; 0.2 ml fractions were collected and alternate fractions were assayed for ^{32}P and for ATPase activity. An aliquot from the ATPase peak fraction was also taken for analysis on polyacrylamide gel as described in Fig. 2.

pooled enzyme extracts were dialyzed, incubated with RNAase and heat treated at 55° as previously described (4).

To show that the solubilized enzyme was radioactively labeled we subjected it to 3 successive purification steps - first chromatography on DEAE-cellulose, then zonal sedimentation and finally electrophoresis in a polyacrylamide gel. Because there was considerable ^{32}P in all the DEAE column fractions it was not possible to tell at this stage of purification if ^{32}P was associated specifically with the ATPase peak. However, when the ATPase peak from the DEAE column was sedimented in a sucrose gradient, the zone of enzyme activity was found to be coincident with a peak of ^{32}P as shown in Fig. 1. To confirm that the ^{32}P was associated with the enzyme, the ATPase in the peak tube of the sucrose

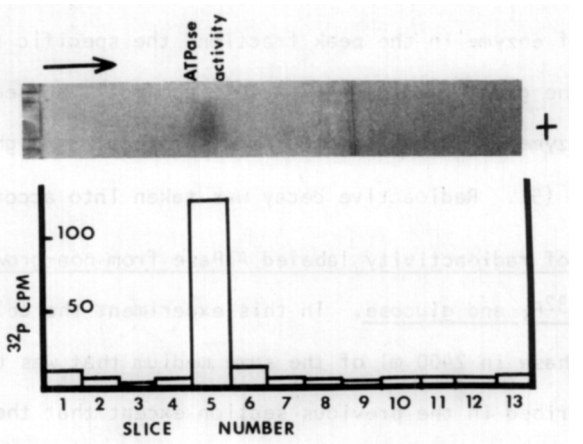

Fig. 2. Polyacrylamide gel electrophoresis of ATPase from cells grown in ^{32}P-orthophosphate. - The sample of ATPase was previously fractionated by DEAE-cellulose chromatogrpahy and zonal sedimentation (see Fig. 1). Electrophoresis was carried out on 3 mm thick 5% polyacrylamide gel slabs (E-C Apparatus, Philadelphia) using a discontinuous buffer system. The polyacrylamide gel contained 0.38 g bisacrylamide, 7.12 g acrylamide, 15 ml Tris Cl pH 9.5, 15 g sucrose and H_2O to a final volume of 150 ml. The buffer compartments contained Tris glycine pH 8.5 (1.78 g Tris and 5.24 g glycine per liter). A 0.1 ml sample of ATPase, taken from the peak fraction after zonal sedimentation (Fig. 1), was mixed with 0.1 ml saturated sucrose. From this solution 0.1 ml was layered on the gel. Electrophoresis was carried out for 1 hour at 400 volts. The enzyme activity was located directly on the gel using the Fiske-SubbaRow reagents as described previously (3,16). The position of the enzyme in the gel, visualized by a blue colored band, is shown by the photograph in the upper part of this figure. To locate the ^{32}P in the gel it was cut into 1 cm slices which were minced and then counted by liquid scintiallation in Aquasol (New England Nuclear). The ^{32}P profile of the gel is shown in the lower part of the figure.

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gradient was analyzed further by polyacrylamide gel electrophoresis. After running the enzyme on the gel, the gel was stained to locate the enzyme activity (3,16), and then sliced and counted to locate the ^{32}P. The result, illustrated in Fig. 2, clearly demonstrates that the radioactivity and enzyme activity in the gel coincide.

From the zonal sedimentation analysis shown in Fig. 1, we estimate that the stoichiometry of ^{32}P incorporation into the ATPase is between

5 to 10 moles of P per mole of the enzyme. This estimate is based on the cpm/unit of enzyme in the peak fraction, the specific radioactivity of the P_i in the growth medium (4.3×10^{12} cpm/mol), a specific activity of the pure enzyme of 30 units/mg (4) and a molecular weight of 385,000 for the enzyme (5). Radioactive decay was taken into account.

Isolation of radioactivity labeled ATPase from non-growing cells incubated with $^{32}P_i$ and glucose. In this experiment the cells were grown to stationary phase in 2400 ml of the same medium that was used in the experiment described in the previous section except that the P_i was not radioactive. After harvesting the cells they were washed twice and suspended in 100 ml of 20 mM Tris Cl pH 7.5 buffer containing 10 mM KCl, 1% glucose and 5 mC of carrier-free $^{32}P_i$. (Without glucose there was little or no incorporation of ^{32}P). Non-radioactive P_i was not added to the suspension. After 45 minutes of incubation at 38°C membrane ghosts were prepared and the ATPase was solubilized using the same procedure as that described in the previous section. To help remove any adsorbed $^{32}P_i$ from the membranes we added 10 mM non-radioactive K phosphate to the first wash fluid. The solubilized ATPase was concentrated to a small volume by pressure ultrafiltration and then isolated by zonal sedimentation in a sucrose gradient. The zone of ATPase activity was collected and dialyzed free of sucrose and then divided into two equal samples. To one of these samples 1 mM non-radioactive K phosphate was added and then both samples of ATPase were isolated again by zonal sedimentation. The results are shown in Fig. 3a and 3b. From the outcome it is clear first of all that the peaks of ATPase activity and ^{32}P are coincident. Secondly the specific radioactivity of the enzyme, expressed as cpm/unit of enzyme, were the same whether or not cold P_i was added. This result demonstrates that the ^{32}P associated with the enzyme is not displaceable by non-radioactive P_i. Finally and most importantly, it should be noted in Fig. 3a and 3b that the specific radioactivity of the enzyme was essentially

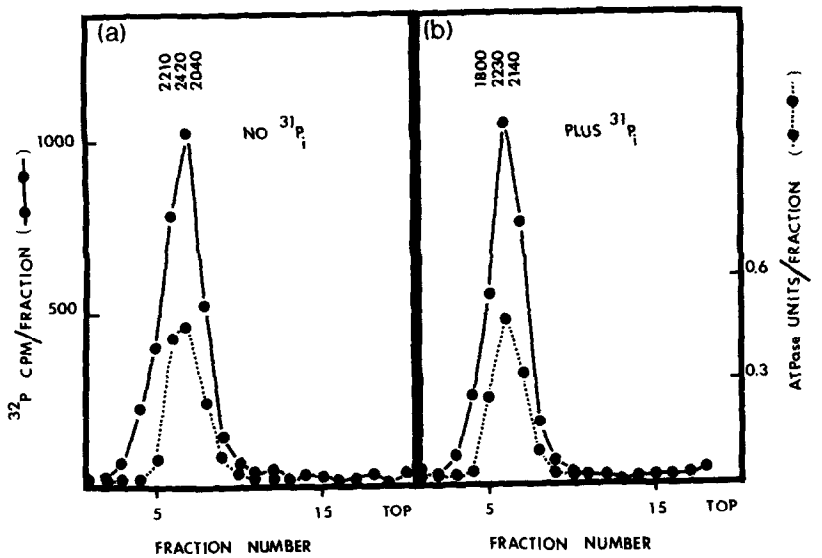

Fig. 3. Zonal sedimentation of ATPase from non-growing cells fed $^{32}P_i$ and glucose. - ^{32}P labeled ATPase which had been isolated previously by zonal sedimentation was divided into two equal samples and reisolated by zonal sedimentation. To one of the samples 1 mM non-radioactive P_i was added prior to centrifugation (panel b). No P_i was added to the other sample (panel a). The sucrose gradient and the centrifugation conditions were as described in Fig. 1; 0.5 ml fractions were collected and assayed for ATPase activity and radioactivity. The numbers above the 3 peak fractions give the specific radioactivity of the enzyme in those fractions as cpm/unit of enzyme.

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constant throughout the peak of enzyme activity (about 2000 cpm/unit of enzyme). This constancy of specific radioactivity leaves little doubt that the ^{32}P label is specifically associated with the enzyme.

As a further check on the assocation of ^{32}P with the enzyme, we took the ATPase from the sucrose gradient shown in Fig. 3a and examined it by gel electrophoresis in the same way as was described in the previous section (see legend of Fig. 2). Two samples of the enzyme were applied to the gel, one with non-radioactive P_i added and the other without added P_i. Again the ATPase activity and ^{32}P were coincident and there was no displacement of radioactivity from the enzyme by non-radioactive P_i. In a similar experiment we treated the ^{32}P-labeled ATPase with

trypsin and found that both the enzyme and the radioactivity failed to appear on the gel after electrophoresis, indicating that the ^{32}P was associated with protein.

DISCUSSION. In the experiments we are reporting here we found that the membrane ATPase of Streptococcus faecalis became radioactively labeled after feeding the cells ^{32}P-orthophosphate. The incorporated ^{32}P was not displaced from the enzyme by non-radioactive inorganic phosphate and it remained firmly fixed to the catalytically active enzyme through successive purification procedures which included anion exchange chromatography on DEAE-cellulose, zonal sedimentation and gel electrophoresis We may conclude therefore that the enzyme contains phosphate groups as a bona fide structural constituent. However, we cannot specify at this time in what chemical form the phosphate is associated with the enzyme or by what cellular metabolic process it becomes incorporated. Although phosphate, as a fixed component of a membrane ATPase, has not been reported previously it is perhaps not a surprising finding since a number of other non-oxidative enzymes are known to contain phosphate groups in a variety of chemical forms (17). It remains to be seen whether or not the phosphate in the S. faecalis membrane ATPase is present in a chemical form which corresponds to any of those found in other proteins. The solubilized S. faecalis ATPase is known to be associated with nectin, a small protein involved in attaching the enzyme to the membrane (18,6). It is therefore possible that the ^{32}P is in the nectin part of ATPase-nectin complex.

It has been reported that the particulate ATPase preparations from animal plasma membranes become labeled transiently with ^{32}P during the hydrolysis of [$\gamma^{32}P$] ATP (11,12). However, in a previous study of the S. faecalis ATPase catalytic mechanism (4) we could find no evidence for the formation of a phospho-enzyme catalytic intermediate like the one formed by the animal plasma membrane ATPase. We would like to stress that the $^{32}P_i$ incorporated into the bacterial ATPase by intact cells

remains fixed in the active enzyme over a long time period. Therefore the ^{32}P labeled enzyme can hardly represent a transient catalytic intermediate.

It is interesting that non-multiplying cells, as well as growing cells, incorporated $^{32}P_i$ into the membrane ATPase. This could mean that a small amount of net synthesis of the ATPase is still occurring in the stationary cells. Alternatively, there might be a metabolic turnover of the phosphate group, or a phosphate containing compound, in the enzyme.

Studies are presently under way to elucidate the chemistry of the phosphate associated with the Streptococcal ATPase and its physiological significance.

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