NON-COVALENTLY BOUND ADENINE NUCLEOTIDES IN ADENOSINE TRIPHOSPHATASE OF Escherichia coli

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SUMMARY --- Homogeneous membrane-bound ATPase (EC 3.6.1.3)of Escherichia coli purified by the method of Kobayashi and Anraku (J. Biochem. (1974) 76, 1175) contained non-covalently bound adenine nucleotides. It was estimated that there were 2.2 mol ATP, 0.4 mol ADP and 0.1 mol inorganic phosphate per mol of the enzyme. No AMP was detected. A rapid, quantitative analytical method for measuring bound nucleotides was developed. The method involved treatment of the crude enzyme with specific antiserum for ATPase. Evidence showing that this method was specific and reliable was presented. Using this method, the stability and release of bound nucleotides under various conditions were examined. It was found that strain AN120 (unc A) and strain AN382 (unc B) have five identical subunits as ATPase with bound nucleotides.

INTRODUCTION --- There is now much evidence that molecules of coupling factors contain firmly, but non-covalently bound adenine nucleotides. Bound nucleotides have been found in purified beef heart mitochondrial F_1 (1,2), chloroplast CF_1 (3), and Streptococcus faecalis ATPase and also in various membranes, such as submitochondrial particles (5), chloroplast thylakoid membranes (3), and the chromatophores of Rhodospirillum rubrum (6). The molar contents of bound nucleotide reported were not the same, although different procedures were employed. In several current hypotheses of the mechanism of energy transduction and ATP synthesis (7-9) it has been proposed that bound nucleotides may function in the process of the oxidative and photophosphorylation (7,8,10). However, such functions have not yet been established.

In the studies on the function of ATPase (11-13) we have demonstrated that E. coli cells incorporated organic phosphate into the enzyme molecules when the culture medium was supplemented with inorganic ^{32}P-orthophosphate (^{32}Pi) and

that these labelled compounds remained associated with the ATPase throughout its purification (14). This paper reports studies showing that highly purified ATPase contained ATP, ADP, and Pi, but not AMP. A rapid analytical method was established for measuring bound nucleotides using specific antiserum against ATPase (15,16). Results using this method showed that an unc A mutant contained cross-reacting material with identical subunits to ATPase and bound nucleotides.

MATERIALS AND METHODS

Media and Growth of Cells --- E. coli W3092 (17) was grown aerobically at 37° in semidefined, low phosphate medium containing (per liter) 3 g of NaCl, 1 g of KCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.011 g of $CaCl_2$, 0.1 g of KH_2PO_4, 5 g of casamino acids (Difco), 5 g of succinic acid and 11 g of Tris, pH 7.2. The medium was supplemented with carrier free ^{32}Pi and ^{3}H-amino acid mixture or ^{3}H-leucine as indicated. Cells were grown for more than 10 generations and harvested in the late exponential phase of growth, as described previously (14) Strains AN180, AN120 and AN382 were kindly provided by Dr. F. Gibson (18), and were grown in the same medium, but with 2 g of glucose in place of succinate.

Analysis of Nucleotides Bound to ATPase --- Crude ATPase was obtained from the labelled cells by washing cell membranes with 2 mM Tris-HCl, pH 7.2, as described previously (11). It was purified and assayed by the method of Kobayashi and Anraku (14). Purified ATPase (140 μg) was lyophilized and dissolved in 50 μl of 10 M urea and the ^{32}P-nucleotides released were analyzed by thin layer chromatography on a polyethyleneimine-cellulose plate (Merck). Authentic ATP, ADP, and AMP were added to this sample solution and the whole mixture was applied on the plate as band (6 cm in width). The nucleotides were located under ultraviolet light and Pi was detected by a published method (19). Each band separated was then scraped and the radioactivities were measured in a Packard Liquid Scintillation Spectrometer, Model 3330. Scintillater used was Packard Insta-Gel. The solvents used were 1.2 or 0.8 M LiCl (20) and 0.38 or 0.50 M KH_2PO_4 (21). Plates were washed with methanol for 10 min (20) before use.

Analytical Method Using Anti-ATPase Serum --- Antiserum agaist purified ATPase was prepared by the method of Futai et al (15,16). Rapid analysis of bound nucleotides using this antiserum was carried out as follows: The crude ATPase fraction (0.2-0.5 unit per 200 μl) was treated with 40 μl of antiserum for 2 hr at 20°. Then the mixture was centrifuged at 1,000 xg for 10 min and the precipitate was washed three times with 0.2 ml volume of solution containing 0.2 M NaCl-2 mM Tris-HCl (pH 7.2)-1% Triton X-100 (22). Then the material was dissolved in 0.1 ml of 0.1 M glycine-HCl (pH 3.0)-7M urea and the nucleotides released were analyzed as described above.

The washed precipitate was analyzed by gel electrophoresis in 7.5% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) (23). Samples were dissolved in 9 M urea-0.1% SDS-0.1 M β-mercaptoethanol and incubated for 16 hr at 37°. The resulting solutions were heated for 10 min at 100° and then mixed with one fifth volume of 50% glycerol. After electrophoresis the gels were stained (24) and then cut into 1.5 mm wide sections. Radioactivities were measured after incubating the sections with 0.4 ml of H_2O_2 for 6 hr at 60°. Protein (25) and Pi (26) were determined by published methods. ^{3}H-L-Amino acid mixture (NET-250) and ^{3}H-L-leucine were obtained from New England Nuclear Co. and ^{32}Pi from CEA, France. ATP and ADP were obtained from Boelinger. The other chemicals used were of analytical grade.

The specific radioactivity of ^{32}P was determined by measuring the total radioactivity and the total phosphorus content (26) in 100,000 xg supernatant

Table I. Molar contents of bound nucleotides and total phosphorus atoms per mole of ATPase of various preparations

Analytical method and enzyme preparation[a]	Specific activity[b]	Molar content[c]				Total phosphorus atoms
		ATP	ADP	AMP	Pi	
A) Antiserum untreated						
Glycerol-enzyme	71.7	1.9	0.7	n.d.[d]	0.5	7.3
B) Antiserum treated						
Crude enzyme	8.5	2.4	0.6	n.d.	0.5	8.5
DEAE-cellulose-enzyme	21.7	2.5	0.4	n.d.	n.d.	7.9
Glycerol-enzyme	71.7	2.2	0.4	n.d.	0.1	7.5

a) E.coli W3092 was grown in medium containing 2 mCi of ^{32}Pi and 0.15 mCi of ^{3}H-amino acid mixture per liter and crude enzyme was prepared as described in the METHODS. DEAE-Cellulose- and glycerol-enzymes were obtained from this crude enzyme according to the method of Kobayashi and Anraku (14). The solvents used was 0.5 M KH_2PO_4 and the Rf values were: ATP, 0.23; ADP, 0.49; AMP, 0.61; Pi, 0.76.
b) Units/mg protein; for definition, see ref. 14.
c) The specific radioactivities of ^{32}P and ^{3}H-protein were determined as 1.85×10^3 cpm per ng atom of phosphorus and 3.57×10^4 cpm per mg protein, respectively. A factor of 0.85 was used in order to correct the amount of ^{3}H-ATPase in the precipitate (see text). These values were used in the calculation of molar contents of nucleotides and total phosphorus atoms per mole of ATPase (molecular weight, 300,000 (16)).
d) n.d., not detected (less than 3 pmol AMP or Pi per band).

of the extract from sonicated, labelled cells. The specific radioactivity of ^{3}H-protein was determined by measuring the radioactivity of ^{3}H and the protein content (25) in the fraction of the 100,000 xg supernatant precipitated by hot 5% trichloroacetic acid.

RESULTS AND DISCUSSION

Identification of Bound Nucleotides in Purified ATPase --- The adenine nucleotides bound to purified ATPase (glycerol-enzyme in Table I) were identified as ATP and ADP using several solvent systems. The enzyme also contained small amount of Pi, but not AMP. Table I shows the molar contents of nucleotides and Pi per molecule of ATPase (molecular weight, 300,000 (16)). There were 7.3 mol of total phosphorus per purified enzyme. When alkali-treated ATPase has been prepared (27) and incubated with hexokinase and glucose, as described previously (12) before chromatography, no radioactivity was found in the position of ATP but that in the position of ADP increased with stoichiometric for-

nation of ^{32}P-labelled glucose-6-phosphate, indicating that ATP was bound non-covalently to the enzyme.

Our conventional purification procedure (14) involves chromatography on DEAE-cellulose and centrifugation in a glycerol density gradient, and this procedure takes about 6 days. Thus during this procedure some bound nucleotides may well be degraded or liberated. To examine this possibility and to obtain accurate values for the molar contents of nucleotides, we developed a rapid analytical method, as described in the METHODS.

Determination of Bound Nucleotides by a Rapid Method Using Anti-ATPase Serum --- This method only takes about 2 hr from the time of preparation of the crude ATPase fraction and seems to be specific and reliable judged by the following criteria: 1) No precipitate formed on treatment of crude ATPase with normal serum and the crude membrane fraction after dissolving in 0.1% SDS contained no cross-reacting material other than ATPase. 2) Anti-ATPase serum precipitated all the ATPase activity under the standard conditions used and precipitation of the ^{3}H-labelled protein in the crude ATPase fraction was inhibited quantitatively by addition of purified, unlabelled ATPase. 3) Five subunits of *E. coli* ATPase were shown by polyacrylamide gel electrophoresis in the presence of 0.1% SDS (16). Using this system, it was found that the precipitated materials contained five subunits identical to those of ATPase and that about 85% of the total ^{3}H-protein in the precipitate was present in these subunits (Fig. 1). 4) When the purified enzyme (glycerol-enzyme in Table I) was treated with antiserum, the radioactivities of ^{32}P and ^{3}H recovered in the precipitate were found to be 100 and 85%, respectively. This indicated that the glycerol-enzyme contained minor impurities which had no bound phosphorus compounds.

This method was used to measure the contents of bound nucleotides in ATPase at different stages of purification (14). Table I shows that the three preparations with specific activities as indicated contained about the same amount of bound ATP and ADP, although the content of Pi was slightly high in the crude

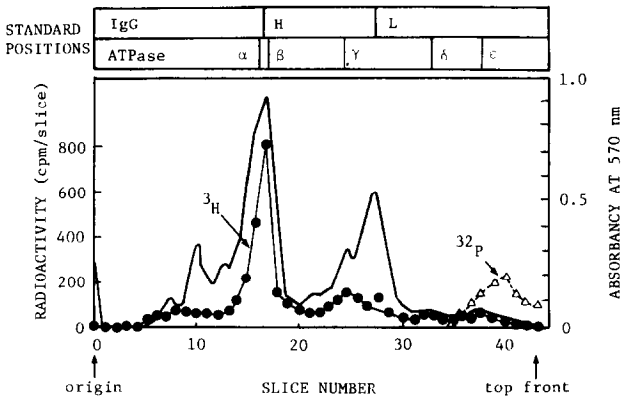

Fig.1. Polyacrylamide gel electrophoresis of the immunoprecipitate of ATPase. Crude ATPase was obtained from strain W3092 labelled with ^{3}H-amino acid mixture and ^{32}Pi as described in legend to Table I. Electrophoresis in the presence of 0.1% SDS was carried out as described in the METHODS. After electrophoresis, the gel was stained and protein bands were located by densitometry (solid line). Then the gel was cut into sections and the radioactivities of the sections were measured. The standard positions of ATPase (α,β,γ,δ,ε) (16) and immunoglobulin IgG (heavy chain(H), light chain(L)) are shown in the top of the Figure.

enzyme. No AMP was also detected. The molar contents of bound nucleotides and the total phosphorus content in the glycerol-enzyme were found to be essentially the same irrespective of the analytical methods used and it seemed that small portion of the bound ATP was degraded to ADP and Pi upon lyophilization of enzyme followed by treatment with 10 M urea (Table I). These results strongly suggest that the non-covalently bound nucleotides (2 mol ATP and 0.5 mol ADP per mol ATPase) remained associated with enzyme throughout purification.

Stabilities of Bound Nucleotides --- The stabilities of bound nucleotides in solubilized ATPase in the crude fraction were studied. The bound ATP was found to be stable and not to be released from enzyme under the following conditions: 1) Incubation with hexokinase and glucose for 10 min at 24° (for details, see ref.12). 2) Incubation with 4 mM ATP or ADP in 2 mM Tris-HCl, pH 7.2, containing 2 mM $MgCl_2$ for 15 min at 37°. Addition of 10 mM NaN_3 or 20 μM m-chlorocarbonylcyanide phenylhydrazone had no effect. However, about 50% of the bound ADP was released under all the conditions mentioned above. Bound nucleotides were found to be released when ATPase was inactivated completely:

Table II. Bound nucleotides in cross-reacting materials and ATPase activities of various strains

Strain[a]	Specific activity	Molar content				Total phosphorus atoms	Specific radioactivity[b]	
		ATP	ADP	AMP	Pi		^{32}P	^{3}H
AN180 (parent)	2.0	1.7	0.8	0.4	0.3	7.4	2.17×10^3	3.71×10^5
AN120 (unc A)	0.03	1.5	0.5	0.2	0.1	5.8	1.46×10^3	2.64×10^5
AN382 (unc B)	2.7	1.1	0.5	0.1	0.2	4.6	1.45×10^3	1.93×10^5

a) Cells were grown in medium containing 2 mCi of ^{32}Pi and 0.4 mCi of ^{3}H-leucine (55 Ci per mmol) per liter and crude enzyme fractions were prepared as described in the METHODS. For other details, see legend to Table I.
b) The specific radioactivities of ^{32}P and ^{3}H-protein were determined (see METHODS) and expressed as cpm per ng atom of phosphorus and cpm per mg protein, respectively.

About 50% of the bound nucleotides in the glycerol-enzyme was released upon dialysis against 2 mM Tris-HCl, pH 7.2, for 6 days at 4°.

Studies on ATPase Mutants --- A crude extract of strain AN120 (unc A) was found to contain a cross-reacting material which had the five identical subunits as ATPase (data not shown). The precipitate thus obtained contained bound ATP and ADP in amounts comparable to those in crude fraction from strain AN180 (parent) (Table II). Recently, Günther and Mariß reported that strain AN120 showed ATPase activity when grown under appropriate conditions (28). However, as shown in Table II, no ATPase activity was found in the crude extract of strain AN120 used, confirming a previous report (18).

The ATPase activity of strain AN382 (unc B) was as high as that of the parent strain, although the mutant did not grow on respiratory substrates, such as succinate. It was also noted that the molar content of ATP and the total phosphorus atom per mole of ATPase in strain AN382 were lower than those in strain AN180 and that there was small amount of bound AMP in the ATPase of the parent strain. Reasons for this finding were not studied further. We have already found that a few other unc A mutants as well as AN120 contain cross-reacting materials with bound nucleotides (unpublished data).

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