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PURIFICATION AND PROPERTIES OF MEMBRANE-BOUND COUPLING FACTOR-LATENT ATPASE FROM MYCOBACTERIUM PHLEI

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The membrane bound coupling factor-latent ATPase was solubilized from the membrane vesicles of Mycobacterium phlei by using 0.25 M sucrose or low ionic strength buffer. Purification of the solubilized enzyme by use of Sepharose-ADP conjugate gel yielded a homogenous preparation of latent ATPase which was purified about 216-fold in a single step with an 84% yield. The enzyme exhibits a specific activity of 39 μmoles of ATP hydrolyzed per min per mg protein. The purified enzyme exhibits coupling factor activity. Electrophoresis in two dissociating solvent systems indicates that the enzyme contains at least three major polypeptides of molecular weights 56,000, 51,000, and 46,000 daltons, and two minor polypeptides of 30,000 and 17,000 daltons. Equilibrium binding studies of ADP with purified coupling factor-latent ATPase reveal the presence of two nucleotide binding sites per molecule with an apparent Ka of 8.1×10^{-5} M.

By use of affinity chromatography, another latent ATPase has been isolated from the solubilized enzyme, which does not exhibit coupling factor activity.

A number of bacterial systems have been used to study membrane-related phenomena; however, in most instances little is known about the nature of the bioenergetic processes and the vectorial orientation of membrane components. The membrane vesicles from Mycobacterium phlei have been characterized with regard to the nature of the respiratory chains, the sequence of the electron transport carriers, the sites of phosphorylation (1, 2), the conformational states of the membrane during substrate oxidation (3), and the active transport of metabolites (4). Membrane vesicles of M. phlei suspended and centrifuged in low ionic strength buffer or sucrose yielded a membrane preparation which was capable of carrying out oxidation with succinate and NAD^+-linked substrate but was unable to couple phosphorylation to this oxidation (5). These membranes are depleted of membrane bound coupling factor and are referred to as DETP. The coupled phosphorylation was restored when the sucrose-solubilized fraction was added to the DETP in the presence of Mg^{++} ions. This sucrose supernatant fraction also exhibited latent ATPase activity which was unmasked by trypsin treatment. Studies were undertaken to determine whether the coupling factor and latent ATPase of M. phlei were associated with the same protein or different proteins, and also to elucidate the molecular properties of the ATPase which might provide insight into the mechanism of energy transduction. Similar enzymes have also been purified from other bacterial membranes (6), mammalian mitochondria (7–9), and yeast mitochondria (10).

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This paper describes the use of a Sepharose-ADP conjugate for the purification to homogeneity of membrane-bound coupling factor-latent ATPase from M. phlei. By using affinity chromatographic procedures it has also been possible to separate two proteins which exhibit latent ATPase activity: one exhibits coupling factor activity and the other fails to restore phosphorylation. Some of the physicochemical characteristics of the purified membrane-bound coupling factor-latent ATPase have been described elsewhere (11).

EXPERIMENTAL PROCEDURE

Preparation of Membrane Vesicles and Solubilization of Membrane-Bound Coupling Factor-Latent ATPase

Mycobacterium phlei ATCC 354 was grown and harvested as described earlier (12). Membrane vesicles (ETP) were prepared by sonication of cells as described by Brodie (13). Membrane-bound coupling factor was solubilized from ETP by suspending ETP in 0.25 M sucrose in the absence of inorganic ions followed by centrifugation at 105,000 $\times$ g for 90 min. The resulting pellet is referred to as the depleted membrane vesicles (DETP electron transport particles depleted of membrane-bound coupling factor-latent ATPase) and a sucrose supernatant fraction. Sucrose density gradient centrifugation of membrane vesicles was carried out by the procedure of Higashi et al. (5) for preparation of the depleted membrane vesicles and a fraction (BCF_4) which contains coupling factor-latent ATPase activity.

Measurement of Oxidation and Coupled Phosphorylation

Oxygen consumption was measured manometrically with a Gilson differential respirometer at 30°C. The main compartment of the vessel contained 100 μmoles of HEPES-KOH buffer, pH 7.5, 15 μmoles of $MgCl_2$, 15 μmoles of inorganic phosphate (potassium salt, pH 7.5), 3 mg of yeast hexokinase (P-L Biochemicals), 50 μmoles of glucose, membrane vesicles and coupling factor at the protein concentration indicated in the legends, and water to a final volume of 2.0 ml. The side arm of the vessel contained 25 μmoles of KF, 5 moles of ADP or AMP and substrate; 100 μmoles of succinate or 100 μmoles of ethanol, 1 μmole of NAD^+, and 50 μmoles of hydrazine. The reaction was carried out for 10–40 min and terminated by the addition of 1.0 ml of 10% trichloroacetic acid. Inorganic phosphate in the trichloroacetic acid supernatant was measured by the method of Fiske and SubbaRow (14).

Succinate-Oxidation Stimulating Activity

The reaction system contained depleted membrane vesicles (2 mg protein), 50 mM HEPES-KOH buffer, pH 7.5, 10 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.5, and 2.5 mM succinate as a substrate. Oxygen consumption was measured with a Clark oxygen electrode at 30°C. Stimulation of the rate of succinate oxidation by the addition of the partially purified coupling factor is expressed either in percentage stimulation or μ atoms of oxygen consumption above the level observed with depleted membrane vesicles.

Assay of Latent ATPase

Unmasking of ATPase by trypsin treatment was carried out in a reaction mixture

containing 50 mM HEPES-KOH buffer, pH 7.4, 0.5 mg bovine pancreas trypsin, and 0.1–0.5 ml of a sample solution (1.0–3.0 mg protein) at 30°C for 10 min followed by the addition of 1.0 mg of soybean trypsin inhibitor. ATPase activity of the sample was determined in the presence of 4 mM $MgCl_2$ and 10 mM ATP at 30°C (11).

Binding Measurements

Binding of nucleotides to the enzyme was studied by the gel filtration method of Hummel and Dreyer (15) as described by Hilborn and Hammes (16). An ultrafiltration method with Diaflo UM-10 membranes was used to measure the binding of [^{14}C]-ADP to the purified coupling factor-latent ATPase, as described by Hilborn and Hammes (16).

Preparation of Sepharose-ADP

CH-Sepharose-4B (2 gm) was extensively washed with 0.5 NaCl and water. To this, ADP (160 mg, dissolved in 0.5 ml water) was added and the pH adjusted to 5.1 with 1.0 N HCl. Then 1.0 ml of 10 mM 1-ethyl-3(3-dimethyl amino propyl)-carbodiimide was added dropwise with stirring at 25°C. The pH was repeatedly adjusted to 5.1 over a period of 1 hr with sodium hydroxide and the suspension was stirred on a shaker for 24 hr at room temperature. The gel was washed with several volumes of 5.0 mM Tris-acetate buffer, pH 8.3, containing 0.5 M NaCl, water, 50 mM Tris-acetate buffer, pH 5.0, containing 0.5 M NaCl, and finally with 50 mM Tris-acetate buffer, pH 8.0, until the eluate was free of unreacted ligand, as determined spectrophotometrically. The extent of coupling was determined on the exhaustively washed gel by phosphate analysis and UV absorption data. It was observed that the extent of coupling of ADP was 0.4–0.8 μmoles of ADP per gm of packed wet gel. The terminal phosphate of Sepharose-ADP conjugate was not involved in coupling since the quantitative enzymatic conversion to Sepharose-ATP was observed using membrane vesicles of M. phlei and reaction mixture for oxidative phosphorylation.

Gel Electrophoresis

Analytical disc gel electrophoresis at alkaline pH under nondissociating conditions was performed according to the general procedure described by Davis (17). Dissociating disc gel electrophoresis at alkaline pH under reducing conditions was carried out with the same gels and buffers as above, except for incorporation of 0.1% sodium dodecyl sulfate (SDS) and 0.002 M EDTA into the separation gel, and 0.14 M phosphate, 0.1% SDS, and 0.002 M EDTA into the stacking gel. ATPase samples were prepared for dissociating gel electrophoresis by dissolving aliquots containing 1.0 mg protein in 1.0 ml of 10 mM Tris-phosphate, pH 8.0, containing 1 mM EDTA, 10% sucrose, 2 mM phenylmethyl sulfonyl fluoride, 2% SDS, 0.002% bromphenol blue, and 1% β-mercaptoethanol. Samples were immediately heated at 100°C for 5 min and then run on 7.5% and 10% acrylamide gels containing 0.1% SDS. Gels were removed when the marker dye front reached the end of the tube and stained overnight in a fresh solution of 0.1% Coomassie brilliant blue in 50% methanol-10% acetic acid and then destained electrophotometically for 15 min in 7% acetic methanol-10% acetic acid and destained electrophotometically for 15 min in 7% acetic acid with a Canalco quick gel destainer. The stained gels were scanned at 555 nm using a Beckman DU spectrophotometer with a Gilford linear transport assembly and recorder.

Protein Determination

Protein concentration was determined either by the method of Lowry et al. (18), with bovine serum albumin as standard, or spectrophotometrically at 280 nm (19).

Materials

CH-Sepharose-4B was purchased from Pharmacia Fine Chemicals and 1-ethyl-3 (3-dimethyl amino propyl)-carbodiimide from Sigma Chemical Company. All other chemicals were of reagent grade purity.

RESULTS

Physicochemical Properties of the Coupling Factor-Latent ATPase and the Component which Stimulates Succinate Oxidation

The membrane bound latent ATPase was solubilized from membrane vesicles by washing with 0.25 M sucrose in the absence of Mg^{++} or K^{+} ions (5). The solubilized sucrose supernatant fraction exhibited latent ATPase activity, and coupling factor activity (11). In addition, the sucrose supernatant fraction also stimulated the rate of succinate oxidation when it was added to the depleted membrane vesicles (Table I). It was therefore necessary to establish whether a single enzyme was exhibiting these catalytic activities or whether there was more than one protein involved. Thus attempts were made to purify the sucrose supernatant fraction.

The sucrose supernatant fraction was first adsorbed on a DEAE-cellulose column which had been pre-equilibrated with 5 mM Tris-HCl buffer, pH 8.0 containing 0.25 M sucrose. The column was eluted with the same buffer containing a linear gradient of KCl from 0.1 to 0.4 M. Coupling factor activity, succinate-oxidation-stimulating activity, and latent ATPase activity were found to be eluted from the column in the same fraction (Fig. 1). Furthermore, the sedimentation pattern of the sucrose supernatant fraction in the sucrose density gradient centrifugation (0.6-1.2 M) (Fig. 2), as well as its molecular gel filtration pattern on a G-200 Sephadex column (data not shown), indicated that these three activities were associated with the same fraction and could not be separated from one another. These results also indicated that the molecular sizes of these factor(s) were almost identical and that it was even possible that the same molecular species catalyzes phosphorylation, ATP hydrolysis, and succinate-oxidation-stimulating activity.

Chromatography of Sucrose Supernatant Fraction on a CH-Sepharose-ADP Conjugate

The surcrose supernatant fraction (10.0 mg protein) was applied to a Sepharose-ADP conjugate column (12 × 0.8 cm), equilibrated with 50 mM Tris-acetate buffer, pH 8.0. The column was washed with 25 ml of 50 mM Tris-acetate buffer, pH 8.0, then with 30 ml of the same buffer containing 20 mM sodium pyrophosphate and 0.25 M sucrose. Further elution was carried out with 0.5 M KCl in 50 mM Tris-acetate buffer, pH 8.0 (Fig. 3). The pyrophosphate-eluted fraction possessed high latent ATPase activity (after removal of inhibiting ions by dialysis) and coupling factor activity (11). However, succinate-oxidation-stimulating activity was not associated with this fraction. This pyrophosphate-eluted latent ATPase-coupling factor fraction was found by gel electrophoresis to be homogeneous. The enzyme was purified about 216-fold and recovered with an 84%

TABLE I. Coupling Factor-Latent ATPase and Succinate Oxidation-Stimulating Activity of Sucrose Supernatant

	Oxidation	Phosphorylation	P/O	Latent ATPase nmoles Pi/min/ mg protein	Succinate oxidation stimulation (%)
ETP	6.0	7.0	1.17	76	–
DETP	3.2	0	0.0	0.0	100
DETP + sucrose supernatant	6.0	6.8	1.13	80	214
Sucrose supernatant	0.0	0.0	0.0	58	0

The measurement of oxygen consumption, phosphorylation, and stimulation of succinate oxidation were carried out as described in Experimental Procedure. The amount of sucrose supernatant used was 3 mg protein. The assay of latent ATPase was carried out as described in Experimental Procedure.

yield in a single step (Table II). The conventional purification procedure for the enzyme involves five steps, and only 7% of the original amount of enzyme is recovered with 10-fold purification (11).

The KCl eluate (Table II) exhibited latent ATPase activity but extremely little or no coupling factor activity (Fig. 3). This suggested that there may be two kinds of latent ATPase enzymes which may have a different molecular structure (subunit composition) and function. Further studies are in progress to elucidate the subunit composition of the latent ATPase eluted with KCl.

Physicochemical Characteristics of Coupling Factor-Latent ATPase

In contrast to ATPase obtained from mitochondria, the purified enzyme from M. phlei was found to be cold stable (0° –4°C). The enzyme has a molecular weight of 250,000 ± 10,000, as estimated by gel filtration (11). Trypsin treatment of the purified enzyme resulted in a marked stimulation of ATPase activity and an inactivation of the coupling factor activity. This differential effect of trypsin on the coupling factor and ATPase activities would appear to indicate that the active site for coupling factor activity differs from the active site for ATPase activity.

The subunit composition was determined by electrophoresis of a sample of purified ATPase under dissociating conditions in the presence of β-mercaptoethanol. Electrophoretic analysis on 7.5% and 10% acrylamide gels revealed the presence of at least three major and two minor subunits (Fig. 4). Molecular weights were determined by comparing the mobilities of the subunit bands to the mobilities of several standard proteins (bovine serum albumin, yeast alcohol dehydrogenase, heavy and light chains of human gamma globulin, and egg white lysozyme) according to the method of Weber and Osborn (20). The results indicated that the three major subunits of M. phlei have approximate molecular weights of 54,000, 51,000, and 46,000 daltons, and that the two minor polypeptides have molecular weights of about 30,000 and 17,000 daltons. In six different gels of either 7.5% or 10% acrylamide, the molecular weights estimated did not differ by more than 9% from the values reported above.

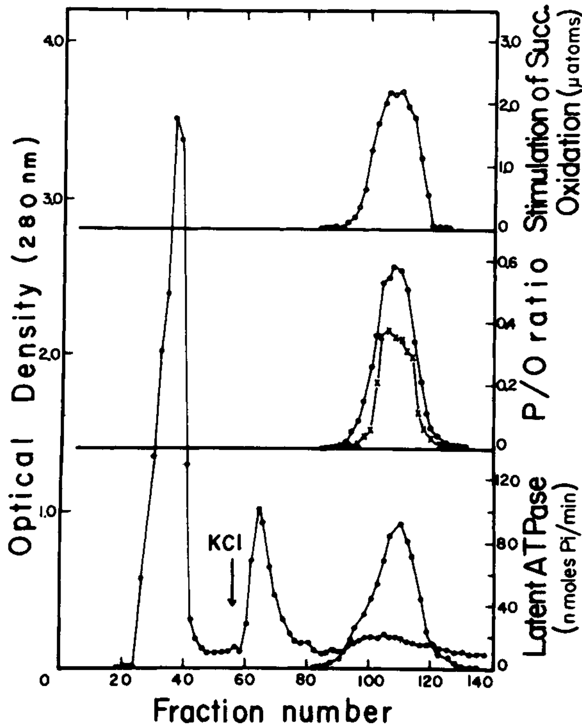

Fig. 1. DEAE-cellulose chromatography of sucrose supernatant fraction. The surcose supernatant fraction (370 ml containing 560 mg protein) was loaded on a DEAE-cellulose column (2.5 x 20 cm) which had been equilibrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.25 M sucrose. After washing with 200 ml of the same buffer, the column was eluted with KCl linear gradient (0.1–0.4 M, total volume 1,000 ml). Flow rate was 90 ml/hr. Fractions (11 ml) were collected and the following activities were examined in each fraction. (a) Stimulation of succinate oxidation catalyzed by depleted membrane vesicles (DETP). (b) Oxidative phosphorylation was assayed using generated NADH (o—o) or succinate (x—x) as substrate. (c) Latent ATPase (0.5 ml of each fraction) was treated with trypsin and analyzed for latent ATPase activity. Succinate oxidation was assayed by use of nonphosphorylating particles (DETP, containing 5.0 mg protein) while generated NADH oxidation was assayed by use of 3.8 mg DETP protein in the presence of 1.0 ml of eluted fractions. Oxidative phosphorylation was measured in each fraction with succinate or generated NADH as substrate with DETP preparation.

It should be noted that the major subunit peak of 46,000 daltons may be a proteolytic degradation product of the 54,000 and 51,000 dalton subunits, since recent studies of Abrams (21) on the subunits of ATPase from Streptococcus faecalis demonstrated that under proteolytic conditions the two major subunits yielded an additional subunit of molecular weight approximately 49,000 daltons.

Binding of [^{14}C]-ADP to Purified Coupling Factor-Latent ATPase

Incubation of the purified enzyme with the radioactive nucleotide resulted in bind-

TABLE II. Purification of Membrane-Bound Coupling Factor-Latent ATPase by Affinity Chromatography Procedure

Fraction	Total protein (mg)	Latent ATPase: Total Activity μmoles Pi/min	Latent ATPase: Specific Activity μmoles Pi/min/ mg protein	Yield	Purification-fold
Sucrose supernatant	10.0	1.80	0.18	100.0	–
CH-Sepharose ADP					
1. pyrophosphate eluate	0.4	1.56	39.00	86.7	216
2. 0.5 M KCl eluate	0.078	0.06	3.3	3.3	18

The affinity chromatography procedure for the purification of coupling factor-latent ATPase is described in the text. Latent ATPase activity was measured as described in the Experimental Procedure.

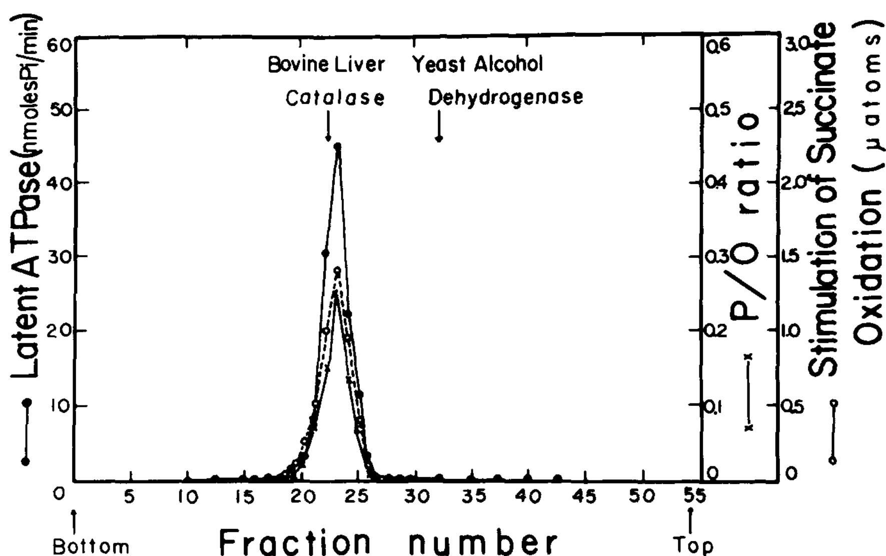

Fig. 2. Sedimentation pattern of sucrose supernatant in sucrose density gradient. Linear density gradient was made in a Spinco tube by mixing 0.6 M and 1.2 M sucrose (total volume, 4.6 ml). The samples of sucrose supernatant (3–5 mg protein), crystalline yeast alcohol dehydrogenase (1 mg protein), and crystalline bovine liver catalase (0.4 mg protein) suspended in 0.1 ml of 0.25 M sucrose were layered on the top of the sucrose gradient and centrifuged at 40,000 rpm for 18 hr in an SW 50.1 rotor with a Spinco L-2 centrifuge. Fractions of three drops (approximately 0.1 ml) were collected by puncturing the tube. Succinate oxidation-stimulating activity and oxidative phosphorylation were measured in the presence of sucrose-washed particles (nonphosphorylating particles), and latent ATPase was measured in each fraction as described in legends to Fig. 1.

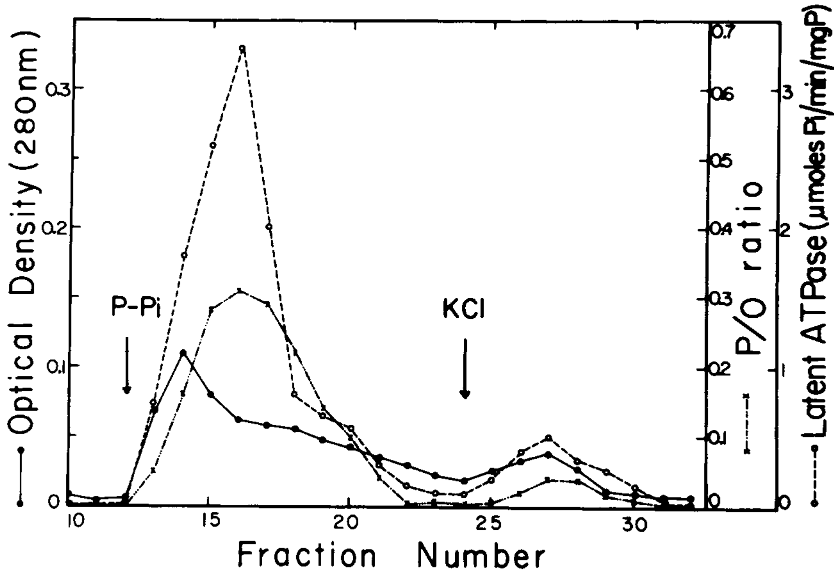

Fig. 3. Affinity chromatography of the membrane-bound coupling factor-latent ATPase on CH-Sepharose-ADP conjugate. The purification procedure is described in the text. Protein was estimated by spectrophotometric absorbance at 280 nm and the latent ATPase activity was assayed in each fraction (0.5 ml) as described under Experimental Procedure. Oxidative phosphorylation was assayed with 1.0 ml of eluted fraction and depleted membrane vesicles (2.5 mg protein) as described in the Experimental Procedure.

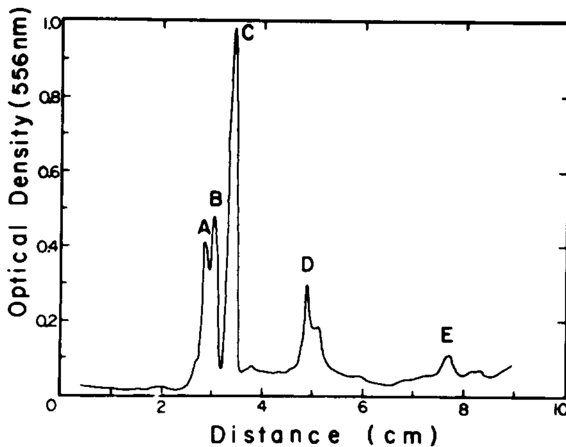

Fig. 4. Electrophoretic analysis of purified ATPase on polyacrylamide gel containing SDS. Gel electrophoresis of purified ATPase (100 μg protein) was run under dissociating conditions on 10% acrylamide gels containing 0.1% SDS at pH 8.8, as described in the text. The gel was scanned at 556 nm using a Gilford linear transport assembly. The subunit designations are given above the respective peaks in the tracing.

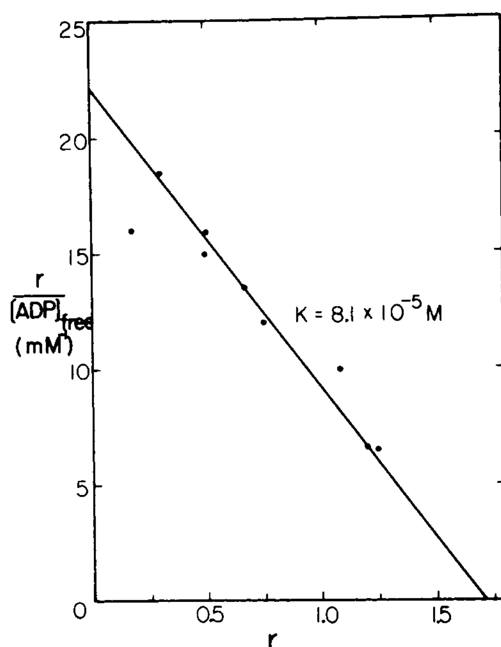

Fig. 5. Binding of ADP to purified coupling factor-latent ATPase in the presence of Mg^{++} ions. The incubation mixture contained 50 mM HEPES-KOH, pH 7.4, 5 mM $MgCl_2$, 90 μg of purified coupling factor-latent ATPase, [^{14}C]-ADP at 1×10^{-6} M, and graded amounts of unlabeled ADP from 10^{-6} M to 10^{-4} M in a total volume of 1.0 ml. The incubation was carried out at 30°C for 60 min and filtered on a UM-10 Amicon filter. r, Amount of ADP bound (moles per mole of the enzyme); K is affinity constant. Data are plotted as recommended by Scatchard (22).

ing as measured by gel filtration, ultrafiltration, and equilibrium dialysis. The number of moles of ADP bound per mg protein was found to be 4.1 nmoles by the ultrafiltration method in the presence of Mg^{++} ions. In order to determine the number of binding sites and the binding constant for ADP, the amount of ADP bound to the purified coupling factor-latent ATPase was determined as a function of free ADP added to the system and the data were analyzed by a Scatchard plot (22) (Fig. 5). The calculations show that coupling factor-latent ATPase has an association constant, K, for the interaction with [^{14}C]-ADP of 8.1×10^{-5} M at 30°C. The number of binding sites of ADP per molecule of enzyme was found to be 1.7, by using molecular weight based on gel filtration method as 250,000.

DISCUSSION

ATPase has been solubilized and purified from a number of micro-organisms, Streptococcus faecalis, E. coli, Bacillus megatarium, and M. lysodeikticus (6), as well as from mammalian, chloroplast and yeast mitochondria (23) by conventional chromatographic procedures. The purification of the membrane-bound coupling factor-latent

ATPase by affinity chromatography procedure using CH-Sepharose-ADP conjugate permitted the enzyme purification to be carried out on a more rational basis, shortened the purification procedure, and provided a means of obtaining homogeneous coupling factor-latent ATPase. Moreover, affinity chromatographic procedures permitted the separation of another latent ATPase (from KCl eluate) which differs from the coupling factor in that it fails to restore phosphorylation. The lack of ability to restore phosphorylation may be due to the absence of a component or to a difference in the subunit structure.

The purified coupling factor-latent ATPase has a molecular weight of 250,000 ± 10,000, as estimated by the gel filtration method (11). Latent ATPase activity has an absolute requirement for Mg^{++} ion. Unmasking of latent ATPase activity by trypsin treatment stimulates ATPase activity, while coupling factor activity is completely inactivated, which suggests that the catalytic sites of action for ATP hydrolysis and coupling to ATP synthesis may be different. Like other bacterial and mammalian mitochondrial ATPases, the purified enzyme is inhibited by dicyclohexylcarbodiimide (DCCD) only when it is membrane bound (24). The M. phlei latent ATPase exhibits greater affinity for ATP as compared to other nucleoside triphosphates such as ITP, GTP, UTP, and ϵ-ATP (1,N^6-ethenoadenosine triphosphate), while CTP and deoxy ATP are not hydrolyzed (11). ADP acts as a competitive inhibitor of the purified latent ATPase with a K_i of 5×10^{-3} M. Binding studies show that about 1.7 binding sites of ADP are present per molecule of the enzyme.

The subunit composition of M. phlei ATPase, as determined by SDS gel electrophoresis, consists of at least three major polypeptides 54,000, 51,000, 46,000 daltons (A, B, and C) and two other polypeptides of 30,000 and 17,000 daltons. The subunit composition of purified ATPase from mitochondria, chloroplasts (23) and several species of bacteria has been determined. The subunit pattern of M. phlei ATPase is similar to that observed for mammalian and bacterial ATPases since they also exhibit major subunits in the range of 50,000–60,000 daltons and a third major subunit of about 30,000 daltons. The significance of the 46,000 dalton polypeptide as a subunit of M. phlei ATPase is questionable since a 49,000 dalton polypeptide has been found to be a proteolytic degradation product derived from the 60,000 and 55,000 dalton subunits of S. faecalis ATPase. Further studies are in progress to determine the effect of trypsin treatment on the subunit composition, and the nature of binding of nucleotides to latent ATPase from M. phlei.

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REFERENCES

1. Asano, A., and Brodie, A. F., J. Biol. Chem. 239:4280 (1964).
2. Asano, A., and Brodie, A. F., J. Biol. Chem. 240:4002 (1965).
3. Kalra, V. K., Aithal, H. N., and Brodie, A. F., Biochem. Biophys. Res. Commun. 46:979 (1972).
4. Brodie, A. F., Hirata, H., Asano, A., Cohen, N. S., Hinds, T. R., Aithal, H. N., and Kalra, V. K., in "Membrane Research," C. Fred Fox, (Ed.). Academic Press, New York, p. 445 (1972).
5. Higashi, T., Bogin, E., and Brodie, A. F., J. Biol. Chem. 244:500 (1969).
6. Abrams, A., and Smith, J. B., in "The Enzymes," Vol. X, P. D. Boyer, (Ed.). Academic Press, p. 396 (1974).
7. Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E., J. Biol. Chem. 235:3322 (1960).
8. Catterall, W. A., and Pederson, P. L., J. Biol. Chem. 247:7969 (1972).
9. Lambeth, D. O., and Lardy, H. A., Eur. J. Biochem. 22:355 (1971).
10. Schatz, G., Penefsky, H. S., and Racker, E., J. Biol. Chem. 242:2552 (1967).
11. Higashi, T., Kalra, V. K., Lee, S. H., Bogin, E., and Brodie, A. F., J. Biol. Chem. (In press).
12. Brodie, A. F., and Gray, C. T., J. Biol. Chem. 219:853 (1956).
13. Brodie, A. F., J. Biol. Chem. 234:398 (1959).
14. Fiske, C. H., and SubbaRow, Y., J. Biol. Chem. 66:375 (1925).
15. Hummel, J. P., and Dreyer, W. J., Biochim. Biophys. Acta 63:530 (1962).
16. Hilborn, D. A., and Hammes, G. G., Biochemistry 12:983 (1973).
17. Davis, B. J., Ann. N.Y. Acad. Sci. 121:404 (1964).
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193:265 (1951).
19. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M., in "Data for Biochemical Research," 2nd ed., Oxford Univ. Press, New York, p. 625 (1969).
20. Weber, K., and Osborn, M., J. Biol. Chem. 244:4406 (1969).
21. Abrams, A., Jensen, C., and Morris, D., ICN-UCLA Winter Conferences on Molecular and Cellular Biology (Abstr.), Squaw Valley (1975).
22. Scatchard, G., Ann. N.Y. Acad. Sci. 15:660 (1949).
23. Penefsky, H. S., in "The Enzymes," Vol. X, P. D. Boyer, (Ed.). Academic Press, New York, p. 375, (1974).
24. Kalra, V. K., and Brodie, A. F., Arch. Biochem. Biophys. 147:653 (1971).