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Isolation, Characterization, and Reconstitution of a Solubilized Fraction Containing the Hydrophobic Sector of the Mitochondrial Proton Pump[1]

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Abstract

The hydrophobic sector of the mitochondrial ATPase complex was purified by sequential extraction with cholate and octylglucoside, by further differential solubilization with guanidine and cholate in the presence of phosphatidylcholine, and by fractionation with ammonium sulfate. A polypeptide with a mass of 28,000 dalton was present in the purified hydrophobic section which was cleaved by trypsin, resulting in loss of reconstitution activity. In contrast, dicyclohexylcarbodiimide-binding proteolipid remained unimpaired after exposure to trypsin. The $^{32}P_i$-ATP exchange activity of the reconstituted ATPase complex was inhibited by *p*-hydroxymercuribenzoate, which reacted primarily with the 28,000-dalton protein, as monitored by acrylamide gel electrophoresis with ^{14}C-labeled inhibitor. The function of a 22,000-dalton polypeptide and of some minor components in the region of the proteolipid remains unknown. An examination of the phospholipid requirements for reconstitution of an active complex revealed an unexpected discrepancy. With an excess of phosphatidylethanolamine, optimal reconstitution of $^{32}P_i$-ATP exchange and ATP synthesis in the presence of bacteriorhodopsin and light was achieved; at a high phosphatidylcholine:phosphatidylethanolamine ratio, the rate of ATP synthesis remained high, but the rate of $^{32}P_i$-ATP exchange dropped precipitously. A new procedure is described for the reconstitution of

[1]Abbreviations: DCCD—*N,N'*-dicyclohexylcarbodiimide; STE-DTT buffer—sucrose (250 mM), Tricine-KOH (50 mM), EDTA (5 mM), DTT (5 mM), pH 8.0; F_o—a membranous preparation from mitochondria conferring oligomycin (or rutamycin) sensitivity to F_1; F_1, F_6—coupling factors 1 (ATPase) and 6; OSCP—oligomycin-sensitivity-conferring protein; BSA—bovine serum albumin; SDS—sodium dodecyl sulfate; DTT—dithiothreitol; STE buffer—sucrose (250 mM), Tricine-KOH (50 mM), EDTA (5 mM); TUA particles—submitochondrial particles prepared by stepwise exposure of light-layer submitochondrial particles to trypsin and urea, then sonic oscillation in the presence of dilute ammonia (pH 10.4); OG-cholate buffer—glycerol (20%), Tricine (50 mM), $MgSO_4$ (5 mM), DTT (5mM), cholate (0.5%), octylglucoside (0.5%), pH 8.0; *p*-HMB—*p*-hydroxymercuribenzoate.

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the ATPase complex with purified phospholipids which is stable for at least 15 days.

Key Words: Mitochondrial ATPase; proteolipid; dicyclohexylcarboidiimide; *p*-hydroxymercuribenzoate; phospholipid requirements for reconstitution of ATPase; bacteriorhodopsin; ATP synthesis; reconstitution of the ATPase complex; 28,000-dalton polypeptide; octylglucoside.

INTRODUCTION

The ATP-driven proton pump of mammalian mitochondria, chloroplasts, and microorganisms is a complex structure consisting of eight to ten subunits (Alfonzo and Racker, 1979; Nelson, 1976; McCarty, 1979; Kagawa, 1978; Futai and Kanazāwa, 1980). The functions of these subunits are slowly being elucidated. The five nonidentical subunits of F_1 from bacteria have been successfully separated and reconstituted (Kagawa, 1978; Dunn and Futai, 1980), but the subunits of the hydrophobic sector (F_o) have been more difficult to dissociate and their functions are still ambiguous. Reports of the successful reconstitution of a DCCD-sensitive proton channel with a pure proteolipid isolated by extraction with solvents have appeared (Nelson *et al.*, 1977; Criddle *et al.*, 1977), but these preparations have not as yet been successfully incorporated into a functional proton pump. The failure of these attempts points to the participation of other subunits in the formation of a native proton channel. Several hydrophobic polypeptides associated with active preparations have been implicated as potential subunits (Alfonzo and Racker, 1979; Kagawa, 1978).

We have reported in a symposium paper (Alfonzo and Racker, 1979) on the reconstitution of a highly purified preparation of the hydrophobic sector of the mitochondrial proton pump and on the role of the 28,000-dalton subunit. It is the purpose of this paper to describe this preparation in detail, to present some recent experiments on the properties of the 28,000-dalton subunit, and to report some new and curious observations on the properties of the reconstituted ATPase complex which catalyzes ATP synthesis from ADP and P_i in the presence of light and bacteriorhodopsin.

Materials and Methods

Tris (hydroxymethyl) aminomethane, DL-dithiothreitol, defatted bovine serum albumin, NaATP, soybean trypsin inhibitor and yeast hexokinase (type F-300), egg phosphatidylcholine, and egg lysophosphatidylcholine were obtained from Sigma Chemical Company; DCCD, guanidine-HCl, and urea from Schwarz-Mann; TPCK-trypsin (224 U/mg) from Worthington

Biochemical Corporation; cholic and deoxycholic acid from Sigma were recrystallized as previously described (Schneider *et al.*, 1972); octylglucoside was either synthesized (Banerjee *et al.*, 1979) or purchased from Calbiochem. Soybean phospholipids (asolectin) were obtained from Associated Concentrates and extracted with acetone (Kagawa and Racker, 1971). Cardiolipin from bovine heart was obtained from Avanti Biochemical, Inc.; ^{3}H-cholate from New England Nuclear; $^{32}P_i$ (164 mCi/mmol) from ICN; [^{14}C]DCCD from Research Products International Corporation; and [^{14}C]*p*-hydroxymercuribenzoate from ICN.

Mitochondrial phospholipids were extracted from 200-ml packed heavy layer mitochondria with 4 liters of chloroform–methanol (2:1) for 2 hr with stirring under nitrogen at room temperature. The suspension was filtered and 800 ml of 0.04% $MgCl_2$ was added. After vigorous stirring, the two phases were separated by low-speed centrifugation in 250-ml glass bottles and the lower layer was taken to dryness. The dried material was suspended in a minimal volume of ether, 500 ml of acetone was added and the mixture was stirred for 30 min under nitrogen. The suspension was centrifuged and the pellet was suspended in 250 ml of ether. The mixture was centrifuged and the supernatant was taken to dryness and resuspended in about 30 ml chloroform. Further purification was carried out as described (Ragan and Racker, 1973) and the preparations of phosphatidylcholine and phosphatidylethanolamine were stored in chloroform-methanol (4:1) at −70°C.

Mixed mitochondria from bovine heart (Green *et al.*, 1957), submitochondrial particles (Racker, 1962), TU and TUA particles (Bulos and Racker, 1968), F_1 (Horstman and Racker, 1970), F_6 (Kanner *et al.*, 1976), partially purified OSCP (MacLennan and Tzagoloff, 1968), and pure OSCP (Senior, 1971) were prepared as described in the references.

Assays

1. Rutamycin-Sensitive ATPase Assay. The components were mixed together in a volume of 0.2 ml in the following order: 100 μl of STE-DTT buffer, 2 μg F_1, 0.5μmol $MgSO_4$, 10 μg partially purified OSCP, 5–100 μg F_o protein, 2.5—5 μg F_6, and 200–500 μg of soybean phospholipids. Rutamycin (2 μg) was added where indicated. After incubation for 15–30 min at 30°C, the ATPase reaction was started by addition of 0.5 ml of an assay mixture containing 5 mM NaATP, 5 mM $MgSO_4$, and 50 mM Tricine-KOH (pH 8.0). After 5 or 10 min of incubation at 30°C, the reaction was stopped with either 0.1 ml of 50% trichloroacetic acid or by adding the molybdate–H_2SO_4 reagent used for the determination of P_i (Lohmann and Jandrassik, 1926).

2. $^{32}P_i$-ATP Exchange Assay. This assay involves two steps: (a) incorporation of F_o into phospholipid vesicles, and (b) reconstitution of coupling factors.

(a) Phosphatidylethanolamine:phosphatidylcholine (1:1 ratio) at a concentration of 12.5 mM plus 250 μg cardiolipin/ml were dried under N_2 and sonicated to clarify in 36 mM Tricine-KOH (pH 8.0), 100 mM KCl, 0.5% cholate, and 5 mM DTT. Between 3–15 μg protein in 5 μl was added to 50 μl of the vesicles, frozen in liquid N_2 then thawed at room temperature. The vesicles, which are stable for several hours, were kept on ice.

(b) To reconstitute the coupling factors, 40 μl vesicles were added to 40 μl STE buffer, plus 20 μg partially purified OSCP, 20 μg F_1, and 5 μl 0.1 M Mg-ATP. The mixture was incubated for 30 min at room temperature. The exchange reaction was started by the addition of 1 ml of an exchange mixture containing 20 mM K $^{32}P_i$ (4000 cpm/nmol), 0.25 M sucrose, 10 mM $MgSO_4$, 10 mM ATP, 2 mg of defatted BSA, and 30 mM Tricine-KOH (pH 7.6). The reaction was carried out for 10–15 min at 37°C and stopped by the addition of 0.1 ml of 50% trichloroacetic acid. Denatured protein was removed by centrifugation at 5,000 *g* for 10 min at 4°C. An aliquot (0.5 ml) of the supernatant was used for isobutanol–benzene/ammonium molybdate extraction (Lindberg and Ernster, 1956) and 1 ml of the water phase was mixed with 7 ml of liquid scintillation fluid (Liquiscent or ACS) and counted in a Beckman liquid scintillation counter.

3. ATP Synthesis with Bacteriorhodopsin. This assay involves three steps: (a) incorporation of bacteriorhodopsin into phospholipid vesicles, (b) incorporation of F_o into the bacteriorhodopsin phospholipid vesicles, and (c) reconstitution of coupling factors.

(a) Phospholipids (25 mM phosphatidylethanolamine/phosphatidylcholine, 1:1 ratio, and cardiolipin, 500 μg/ml all from bovine heart) were sonicated to clarity in 50 mM Tricine-KOH (pH 8.0) and 5 mM DTT, and an aliquot (50 μl) was mixed with 30 μl (100–150 μg) of bacteriorhodopsin in a medium containing, in a final concentration, 0.1 M KCl, 36 mM Tricine-KOH (pH 8.0), 5 mM DTT, and 0.5% cholate in a final volume of 100 μl. The mixture was sonicated for 1 min at room temperature. The samples were then chilled at 4°C and kept on ice.

(b) F_o fractions (5–20 μg) were added to the above-described bacteriorhodopsin vesicles (containing 0.5% cholate). After freezing in liquid N_2 and thawing at room temperature for 15 min, the vesicles were sonicated for 45 to 60 sec at 5°C (Kasahara and Hinkle, 1976).

(c) An aliquot (40 μl) was reconstituted with coupling factors as described for the $^{32}P_i$-ATP exchange assay. They were then transferred to a 32°C water bath and 1.0 ml of "ATP synthesis mixture," containing 20 mM K $^{32}P_i$ (0.5 mCi/mmol), 4 mM $MgSO_4$, 2 mM ATP, 32 mM glucose, 0.3 mM EDTA, 0.275 M sucrose, and 1 mg/ml defatted BSA, 20 mM Tricine-KOH, and 50 units of hexokinase was added (final pH was 7.6).

The reaction was carried out for 20–30 min under saturating light

intensity (Racker and Stoeckenius, 1974). The reaction was stopped by turning off the light and adding 0.1 ml of 50% trichloracetic acid. Insoluble material was removed by centrifugation at 5,000 *g* for 10 min. An aliquot (0.5 ml) of the supernatant was taken for extraction with isobutanol/benzene/molybdate (Lindberg and Ernster, 1956). An aliquot (1 ml) of the aqueous phase was mixed with 7 ml of liquid scintillation fluid and counted as described for the $^{32}P_i$-ATP exchange reaction.

4. [^{14}C]DCCD Binding. TUA particles (60 ml containing 5 mg/ml) were incubated for 6 hr with 462 nmol of [^{14}C]DCCD (6.5 mCi/mmol). [^{14}C]DCCD-labeled TUA particles were layered on top of 12.5 ml of 0.45 M sucrose and 20 mM Tricine-KOH (pH 8.0), and centrifuged for 60 min at 125,000 *g*. The sediment was dispersed in 65 ml of 0.25 M sucrose, 0.5 mM EDTA, and 20 mM Tricine-KOH (pH 8.0). Aliquots were taken and precipitated by addition of absolute ethanol to 90% (v/v). The suspension was centrifuged at 10,000 *g* for 10 min.

All pellets were dispersed in 1% SDS and counted for ^{14}C in a Beckman liquid scintillation counter.

5. Phospholipid Determination. Total phosphorus was determined by ashing the phospholipid with magnesium nitrate and digesting in 1.0 N HCl for 20 min at 100°C (Ames and Dubin, 1960).

6. Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed in 8 M urea–SDS (Swank and Munkres, 1971) with 10% acrylamide:0.65% bisacrylamide (9 × 0.5 cm). Samples were incubated with the detergents at 37°C for 2 hr before application to the gels and run at 1 mA/tube for 17 hr. Gels were fixed for 8 hr in 20% isopropyl alcohol/10% trichloroacetic acid and stained for 6 hr in 0.2% Coomassie Brilliant Blue–30% methanol–10% acetic acid at 37°C and destained in 10% acetic acid, and 7% methanol. The last destaining solution contained 10% glycerol. Gels were scanned at 550 nm with a Gilford spectrophotometer equipped with a linear scanner. Apparent molecular weights were calculated using ovalbumin, chymotrypsin, myoglobin, and cyanogen bromide-cytochrome *c* fragments as standards.

The [^{14}C]DCCD labeling was detected by slicing gels in 1-mm fractions and digesting with 1.0 ml of NCS (Amersham/Searle—tissue solubilizer) for 12 hr. Acetic acid (1.5 ml, 10%) and 8 ml liquid scintillator (ACS) were added and samples counted without corrections for quenching.

Miscellaneous. Halobacterium halobium (S_9) strain was grown (Lanyi and MacDonald, 1979), and bacteriorhodopsin separated (Oesterhelt and Stoeckenius, 1974), purified (Becker and Cassim, 1975), and measured for proton pumping in the presence of 0.2 μg valinomycin (Racker and Stoeckenius, 1974) as described in the references. Protein was determined according to Bensadoun and Weinstein (1976). Sonicated phospholipids were

prepared as follows. Asolectin or purified lipids (25 μmol) were taken to dryness in a small Pyrex tube under a stream of nitrogen. A small volume of ether was added and again evaporated, rotating the tube so that the walls of the tube were evenly coated. When absolutely dry, 0.5 to 1 ml of buffer was added, and nitrogen was blown into the tube, which was then sealed with parafilm and sonicated to clarity (this takes 5–20 min depending on the phospholipid mixture and on the presence or absence of detergents) in a cylindrical ultrasonic tank made by Laboratory Supplies Co., Inc. (29 Jefry Lane, Hicksville, New York 11801).

Purification of the Hydrophobic Sector (F_o) Step 1. TUA particles (25–30 mg/ml) were diluted to 18–20 mg protein/ml to yield a solution containing 0.25 M sucrose, 1 mM DTT, 1 mM $MgSO_4$, 0.5 mM EDTA, 10 mM Tris-SO_4 (pH 7.4), and 10% saturated ammonium sulfate. The suspension was mixed by gentle stirring and 20% sodium cholate (pH 8.0) was added slowly to a final concentration of 1.5%. After 15 min at 0°C with continuous stirring, the suspension was centrifuged at 176,000 *g* for 30 min. The insoluble material was discarded, and, to the yellowish supernatant, 0.2 ml of saturated ammonium sulfate (pH 8.0) per milliliter of supernatant was added. The mixture was stirred for 10 min on ice, and the precipitated material was removed by centrifugation at 105,000 *g* for 15 min. The ammonium sulfate concentration was raised to 35% by addition of 0.15 ml of saturated ammonium sulfate per milliliter of supernatant. After 10 min of stirring on ice and centrifugation as above, the precipitate was removed. To the supernatant, 0.25 ml of ammonium sulfate per milliliter of supernatant was added with stirring at 0°C. After centrifugation as above, the pellet (35–48 P) was taken up in a small volume (3 ml/g of original TUA particles) of STE-DTT buffer.

Step 2. The 35–48 P fraction (4 ml containing 15 mg/ml) was diluted with 15 ml STE-DTT buffer, and 1 ml of 10% octylglucoside was slowly added. After 15 min at 0°C, the suspension was centrifuged for 30 min at 200,000 *g* and the supernatant was carefully removed.

Step 3. The octylglucoside extract (20 ml, 2 mg/ml) was diluted 10 times in ice-cold STE-DTT buffer, and this suspension was kept on ice for 15 min with occasional gentle mixing by inversion of the container. The suspension was then centrifuged at 105,000 *g* for 15 min, and the sediment was dispersed into 15 ml of STE-DTT buffer and then added to 75 ml of a solution containing 1.5 M guanidine-HCl in STE-DTT buffer, 6 mM $MgSO_4$, 2.4 mM DTT, and 60 mM Tricine-KOH (pH 8.0). The final protein concentration was 0.3–0.4 mg/ml. After 15 min at 0°C, the suspension was centrifuged for 15 min at 180,000 *g*. The sediment was rinsed with 2 volumes of 20% glycerol, 5 mM $MgSO_4$, 2 mM DTT, and 50 mM Tricine-KOH (pH 8.0), and dispersed by homogenization in 10 ml of the same buffer.

Table I. Purification of F_o ($^{32}P_i$-ATP Exchange Assay)[a]

Fraction	Volume	Protein, mg/ml	Units, total	S.A., nmol/min/mg F_o protein	Fold purification
TUA particle	36.0 ml	27.0	87,480	90	—
35–45 ammonium sulfate fraction from cholate extract	4.0 ml	12.0	14,859	317	3.5
Octylglucoside extract from 35–45 P	20.0 ml	1.5	14,100	470	5.2
0–40 precipitate	2.0 ml	2.6	2,267	436	4.8
40–58 precipitate	2.0 ml	2.0	2,568	642	7.1

[a]Between 5–15 μg F_o in no more than 5 μl volume was added to 50 μl liposomes from purified mitochondrial lipids as described under Materials and Methods.

Step 4. The dispersed fraction (10 ml) was mixed with 2 ml of 5% cholate containing 25 mM egg phosphatidylcholine in 0.25 M sucrose, 0.5 mM EDTA, 50 mM Tricine-KOH (pH 8.0), and 2 mM DTT. The suspension cleared up and was kept on ice for 30 min with occasional tube inversion. After centrifugation for 30 min at 250,000 *g*, 0.66 ml of saturated ammonium sulfate (pH 8.0) was added to each milliliter of the clear supernatant and the mixture was centrifuged at 105,000 *g* for 15 min.

Step 5. The pellet (0–40 P) was collected and the supernatant was further fractionated by raising the ammonium sulfate concentration to 58% saturation. The sediment (40–58 P) was saved and the supernatant was discarded. Both 0–40 P and 40–58 P were taken up in half volume of the original F_o fraction in octylglucoside-cholate buffer. The 0-40 P contained

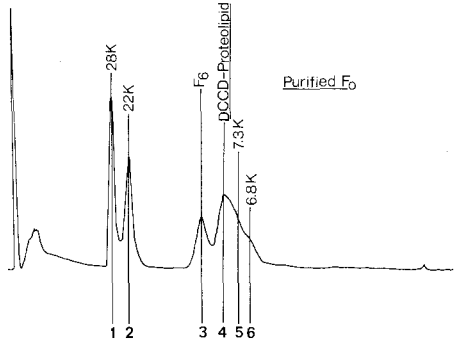

Fig. 1. Polyacrylamide gel eletrophoresis pattern of purified F_o. Purified F_o was prepared as described under Materials and Methods. Polyacrylamide gel electrophoresis was performed according to the procedure of Swank and Munkres (1971).

about 4 mg protein/ml, and the 40–58 P about 2 mg protein/ml. In most experiments, the 40–58 P fraction was used. The specific activity of various fractions is shown in Table I.

Preparation of Stable Proteoliposomes Catalyzing $^{32}P_i$-ATP Exchange. Phosphatidylethanolamine (6.25 μmol), phosphatidylcholine (6.25 μmol), and cardiolipin (250 μg) were dried as previously described under Materials and Methods and sonicated to clarity in a final volume of 1.0 ml containing 36 mM Tricine-KOH, pH 8.0, 100 mM KCl, 0.5% cholate, and 5 mM DTT. To 250 μl of sonicated liposomes, 25 μl of F_o (1.5–2.5 mg/ml) was added. The mixture was frozen in liquid nitrogen and thawed at room temperature. Sonication was not necessary with this mixture of lipids. The proteoliposomes were then passed through a phenyl sepharose column (1.0 ml packed in a Pasteur pipette) that was previously equilibrated with STE-DTT buffer. The active proteoliposomes were eluted with the same buffer and appeared in the void volume in a final volume of 400 μl.

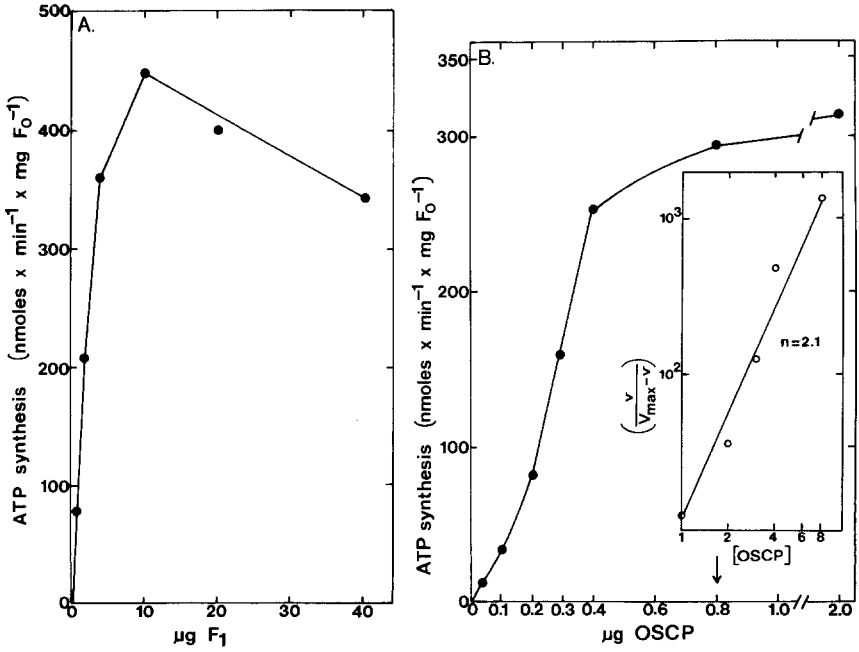

Fig. 2. Titration of F_1 and OSCP in the preparation of vesicles catalyzing light-driven ATP synthesis. (A) ATP synthetase with bacteriorhodopsin was reconstituted as described under Materials and Methods. The reconstitution volume was increased form 100 to 600 μl and varying amounts of F_1 were added to 40-μl samples in the presence of 5 μg of pure OSCP. (B) The experimental conditions were as described for A except that 10 μg F_1 were added and pure OSCP was titrated as described.

Results

Properties of the Purified F_o Complex

As can be seen from Fig. 1 polyacrylamide gel electrophoresis of F_o revealed as a major component a 28,000-dalton protein, a 22,000-dalton protein, a broad peak of probably three components in the region of the proteolipid, and preceding it a band corresponding to F_6. The latter explains the lack of dependence on F_6 in the reconstitution experiments.

Purified F_o was fully competent in the synthesis of ATP when reconstituted together with bacteriorhodopsin and exposed to light. A titration of the requirement for F_1 and OSCP is shown in Fig. 2. About 2 mol of OSCP were needed for each mole of F_1-ATPase. A calculation of the Hill coefficient (the inset in Fig. 2B) yielded a value of 2, suggestive of a cooperative interaction.

An analysis of the phospholipid requirements for the reconstitution of an active complex revealed an unexpected discrepancy, as shown in Fig. 3. The synthesis of ATP in the presence of light and bacteriorhodopsin was actively catalyzed by proteoliposomes reconstituted at a phosphatidylcholine:phosphatidylethanolamine ratio varying over a wide range. In contrast, the

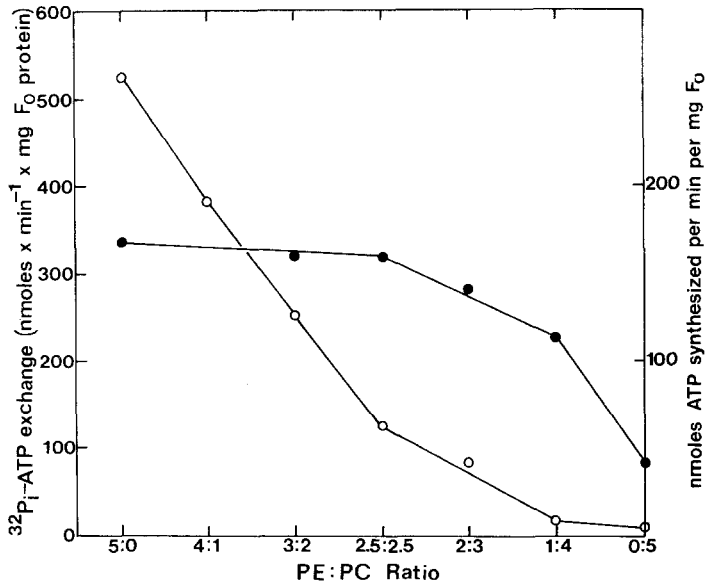

Fig. 3. Effect of phospholipid composition of ATP synthesis and $^{32}P_i$-ATP exchange in F_o-bacteriorhodopsin vesicles. Reconstitution and assay were performed as described under Materials and Methods. ●, ATP synthesis; ○, $^{32}P_i$-ATP exchange.

$^{32}P_i$-ATP exchange rate was dependent on the presence of an excess amount of phosphatidyl-ethanolamine, as observed previously (Kagawa *et al.*, 1973). At a ratio of 1:4 or 2:3 the $^{32}P_i$-ATP exchange rate was barely detectable while the ATP synthesis proceeded at a high rate. Measurements of rutamycin-sensitive ATPase revealed a sensitivity to excess phosphatidylcholine similar to that shown by the $^{32}P_i$-ATP exchange as shown in Fig. 4.

Our explanation of this phenomenon centers on a control of the back reaction by the mitochondrial inhibitor since it preferentially inhibits reactions involving ATP (hydrolysis, $^{32}P_i$-ATP exchange) without interfering with the forward reaction of ATP formation from ADP and P_i (Pullman and Monroy, 1963; Gómez-Puyou *et al.*, 1979). Although a preparation of F_1, free of inhibitor, yielded virtually identical data, residual amounts of inhibitor

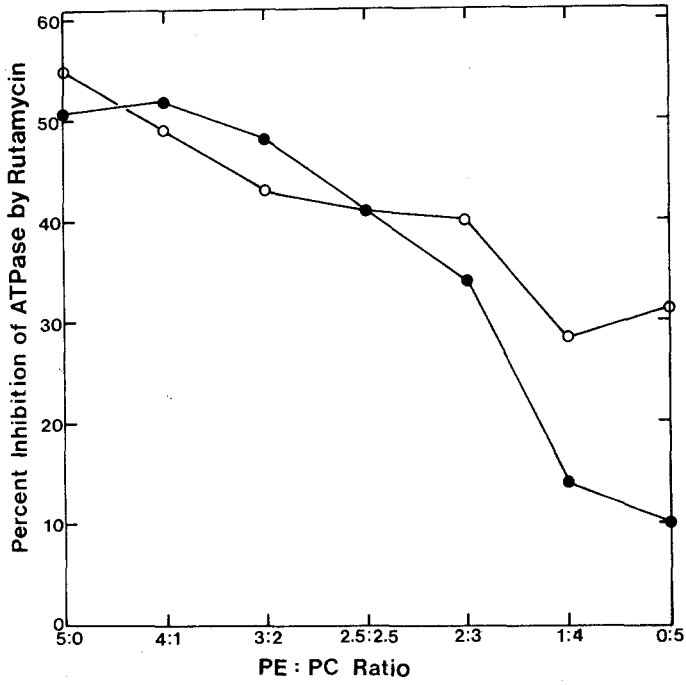

Fig. 4. Effect of phospholipid composition on rutamycin-sensitive ATPase. in reconstituted F_o vesicles. Stable proteoliposomes were prepared as described under Materials and Methods except that phosphatidylethanolamine and phosphatidylcholine were used in different ratios and that 30 μl of concentrated F_o (11.6 mg/ml) was added to 250 μl of liposomes. The samples were frozen and thawed and sonicated for 5 min each. After passage through phenyl sepharose, 65 μl of the vesicles was incubated with 15 μl STE-DTT, 5 μl F_1 (2 mg/ml), 5 μl 0.1 M Mg-ATP, and 10 μl crude OSCP. After 30 min at room temperature, 20 μl was assayed as described under Materials and Methods. The same vesicles were reassayed after 30 hr (open circles). The ATPase activity (100%) was 43 μmol/min/mg.

may still have been present in the F_o preparation. It is therefore possible that the presence of an excess phosphatidylcholine facilitates the regulation of the back reaction by the inhibitor without interfering with ATP synthesis.

The Role of an SH Group in the 28,000-Dalton Component of F_o

It has been shown that a DCCD-sensitive polypeptide from chloroplasts functions as a proton channel in reconstituted vesicles (Nelson *et al.*, 1977). Although these experiments have been repeated in our laboratory (by Dr. N. Nelson and others), there are three observations that suggest that the native channel is a more complex structure than a simple polymer of the proteolipid: (1) The closing of the proton channel was very slow, requiring at 20 μM DCCD up to 50 min to reach maximum. (2) All our attempts to obtain a DCCD-sensitive proteolipid from bovine mitochondria by extraction with butanol or other solvents have thus far failed. (3) No active proton pump has been reconstituted with the pure proteolipid without other hydrophobic

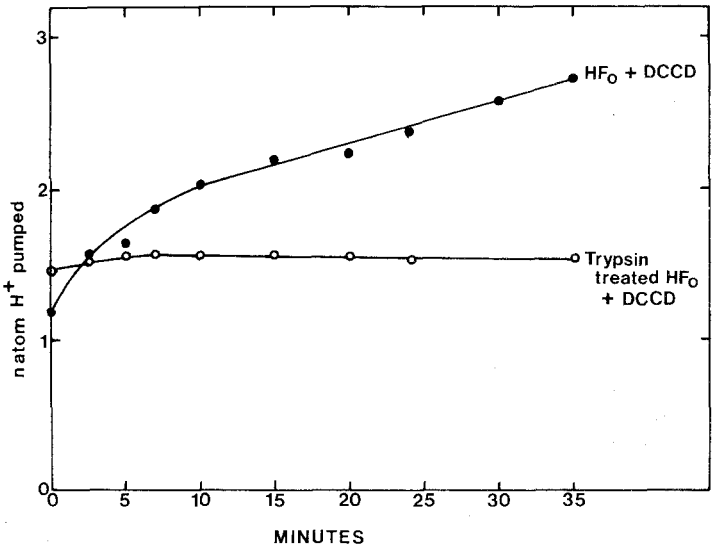

Fig. 5. H^+ pumping in vesicles reconstituted with bacteriorhodopsin in the presence of F_o or trypsin-treated F_o. The F_o-bacteriorhodopsin liposomes were prepared as described under Materials and Methods. After sonication, 300-μl samples were diluted to 1 ml with a buffer containing 5 mM $MgSO_4$, 150 mM KCl, 1 mM DTT, 2 mM Tricine-KOH (pH 7.0), and layered on top of a 6% Ficoll solution containing 150 mM KCl, 1 mM DTT, and 2 mM Tricine-KOH, pH 7.0. Samples were centrifuged at 200,00 *g* for 15 min and the vesicles at the interphase were collected using a Pasteur pipette. An aliquot of 50 μl of this interphase material was used in the determination of H^+ pumping as described under Materials and Methods. DCCD was dissolved in ethanol, and 1 μl of 500 μM solution was added at zero time. A control experiment using 1 μl of ethanol had no effect on the pumping activity.

components. On the other hand, incorporation of purified F_o, prepared as described under Materials and Methods, into liposomes containing bacteriorhodopsin gives rise to a proton leak that can be closed with low concentrations of DCCD (Fig. 5). Moreover, after exposure of the preparation to trypsin, which does not impair DCCD reactivity of the proteolipid (Alfonzo and Racker, 1979) but results in loss of the $^{32}P_i$-ATP exchange activity (Fig. 6), the proton leak is no longer repaired by DCCD.

We have shown previously (Alfonzo and Racker, 1979) that trypsin hydrolyzes F_6 and a 28,000-dalton component of F_o which is required for the $^{32}P_i$-ATP exchange activity. Several properties of the 28,000-dalton component, particularly its instability, suggested that it may require an SH group for activity. As shown in Table II, both $^{32}P_i$-ATP exchange and ATPase synthetase activity were inhibited by *p*-hydroxymercuribenzoate, and dithio-

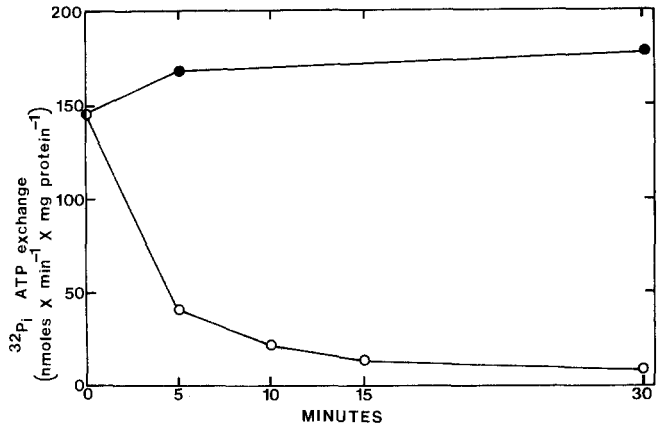

Fig. 6. Effect of trypsin on $^{32}P_i$-ATP exchange. F_o (1 mg) was treated with 100 μg of TPCK-trypsin at room temperature for different periods of time as shown. The reaction was terminated by adding 200 μg trypsin inhibitor. The samples were diluted 10-fold with STE-DTT buffer and kept on ice for 15 min. The samples were centrifuged at 55,000 rpm for 30 min. Pellets were dispersed in OG-cholate buffer. $^{32}P_i$-ATP exchange activity was reconstituted and measured using the octylglucoside dilution method with asolectin (Racker *et al.*, 1979). (A) In a final volume of 88 μl, the following components were added: 1.5 μmol of sonicated soybean phospholipids, 1.5 mM $MgSO_4$, 7% glycerol, 1 mM DTT, 50 mM Tricine-KOH (pH 8.0), 1% cholate, and 0.4% octylglucoside. F_o fractions (25–150 μg protein) were added to the mixture and incubated on ice for 1–4 hr. (B) In a finale volume of 100 μl, the following components were added: 25 μl of 50 mM STE–5 mM DTT, 5 μl of 0.1 M ATP Mg^{+2}, 10 μl of F_1 (2 mg/ml), 10 μl of partially purified OSCP (3 mg/ml), 25 μl of a solution containing reconstituted F_o vesicles from (A), 10 μl of F_6 (0.3–0.5 mg/ml), 10 μl of 60% glycerol, 15 mM $MgSO_4$, 15 mM DTT, and 150 mM Tricine-KOH (pH 8.0). Samples were incubated for 30–60 min at room temperature and exchange was measured as described under Materials and Methods. ●, control; ○, + trypsin.

Table II. Effect of *p*-HMB on ATP Synthesis and $^{32}P_i$-ATP Exchange Activities of F_o[a]

Treatment of vesicles	ATP-synthesis, nmol/min/mg	$^{32}P_i$-ATP exchange, nmol/min/mg
180 μl vesicles + 20 μl *p*-HMB diluted without DTT	3.8	0
180 μl vesicles + 20 μl *p*-HMB diluted with DTT	80.6	68.3
180 μl vesicles diluted without DTT	97.1	68.6
180 μl vesicles + 20 μl K glycylglycine pH 10.7, diluted without DTT	98.2	93.8

[a]Liposomes were prepared with 15 μmol phosphatidylethanolamine:phosphatidylcholine at a 1:1 ratio plus 100 μg cardiolipin and sonicated to clarity in 0.6 ml 50 mM Tricine-KOH (pH 8.0). These liposomes were diluted with 0.6 ml of a solution containing 1.8 mg bacteriorhodopsin, 200 μmol KCl, 6 mg cholate, and 30 mM Tricine. This mixture was sonicated for 10 min. F_o (324 μg) was added and the mixture was frozen in liquid nitrogen, thawed at room temperature, and again sonicated for 10 min. The stock solution of 0.1 M *p*-HMB was prepared in 50 mM K glycylglycine, pH 10.7. After 1 hr of incubation on ice, the mixture was diluted to 1 ml using a buffer containing 0.15 M KCl, 50 mM Tricine-KOH, pH 8.0, with or without 5 mM DTT, and layered on top of 6% Ficoll solution. The sample was centrifuged at 200,000 *g* for 15 min. The material that remained in the interphase was collected and 80 μl of these vesicles was used for the reconstitution of $F_o \cdot F_1$-ATPase complex by addition of F_1, OSCP, and ATP-Mg^{+2}, and assayed for ATP synthesis or $^{32}P_i$-ATP exchange as described in Table I.

threitol protected against this inactivation. As shown in Fig. 7, interaction of F_o with [^{14}C]*p*-hydroxymercuribenzoate yielded two radioactive peaks on SDS gel chromatograms. The major peak was seen in the region of the 28,000-dalton polypeptide; the second was in the region of the 6800-dalton polypeptide. This polypeptide has been a variable component of the F_o preparation and may be a proteolytic degradation product of the 28,000-dalton polypeptide. The observation (Alfonzo and Racker, 1979) that the 28,000-dalton polypeptide is cleaved to a 20,000-dalton polypeptide is consistent with this possibility.

Preparations of Stable Proteoliposomes Catalyzing $^{32}P_i$-ATP Exchange

Passage of reconstituted proteoliposomes through a phenyl sepharose column as described under Materials and Methods removed variable amounts of unincorporated protein (usually 60% of total protein), free liposomes (about 50% of total lipid), and 95% of cholate and potassium (measured with tracer ^{3}H-cholate and ^{86}Rb). As can be seen from Fig. 8, the proteoliposomes, which were quite unstable at 4°C before passage through phenyl sepharose, became remarkably stable afterwards. It should be noted that only proteoliposomes reconstituted with purified phospholipids were stable for two weeks or longer, while proteoliposomes prepared with asolectin lost activity within 24 to 48 hr. Phosphatidylethanolamine vesicles (though highly active) lost activity when passed through the phenyl sepharose column.

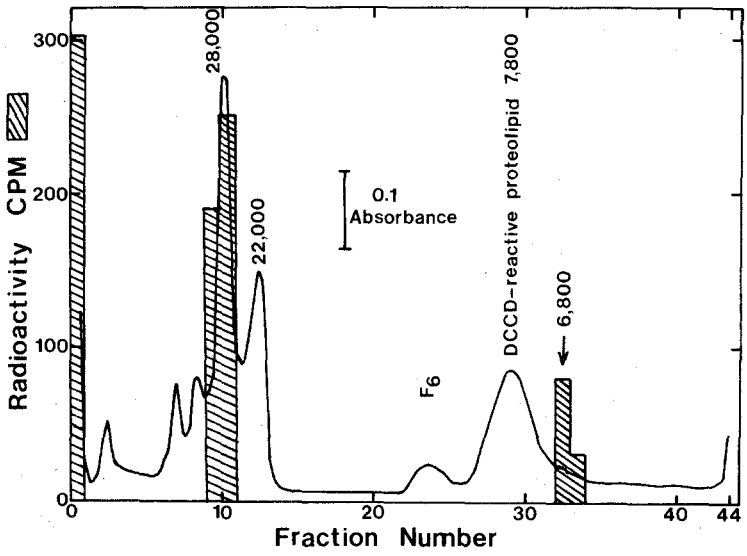

Fig. 7. Radioactivity in polypeptide chains after exposure of F_o to [^{14}C]*p*-hydroxymercuribenzoate. F_o was incorporated into phospholipid vesicles by freeze–thaw–sonication as described under Materials and Methods. F_o (300 μg protein) was incubated in 0.5 ml with 0.2 μmol of [^{14}C]*p*-hydroxymercuribenzoate (5 μCi/μmol) at 0°C for 1 hr. The sample was then applied to a Sepharose 4B column equilibrated in STE buffer to remove unbound *p*-hydroxymercuribenzoate. The vesicles in the void volume (0.6 μl) were mixed with 30 μl of 20% cholate and incubated for 15 min at 0°C. After addition of 0.6 ml saturated ammonium sulfate and centrifugation at 200,00 *g* for 30 min, 20 μg of the pellets were electrophoresed (Swank and Munkres, 1971) without β-mercaptoethanol. For radioactivity measurements, 100 μg of F_o protein was used and the gel was cut into 1 mm slices and digested with NCS for 12 hr at 50°C. Samples were counted for ^{14}C in 8 ml of ACS (aqueous counting scintillant) from Amersham-Searle in a Beckman liquid scintillation counter.

Discussion

What is the specific function of the 28,000-dalton polypeptide which is a required component for the reconstitution of a functional H^+ pump from bovine heart mitochondria? Where is it located? Why is it not present in preparations of the ATPase complex from chloroplasts (Pick and Racker, 1979) or from some bacteria (Kagawa, 1978)?

We assume that the location of the 28,000-dalton polypeptide is on the M side of the inner mitochondrial membrane at the orifice of the proton channel because of its accessibility to trypsin added to submitochondrial particles that were deprived of F_1. It appears to serve as a connecting structure between the attachment factors of F_1 (F_6 and OSCP) and the DCCD-reactive proteolipid. We propose that the 28,000-dalton polypeptide

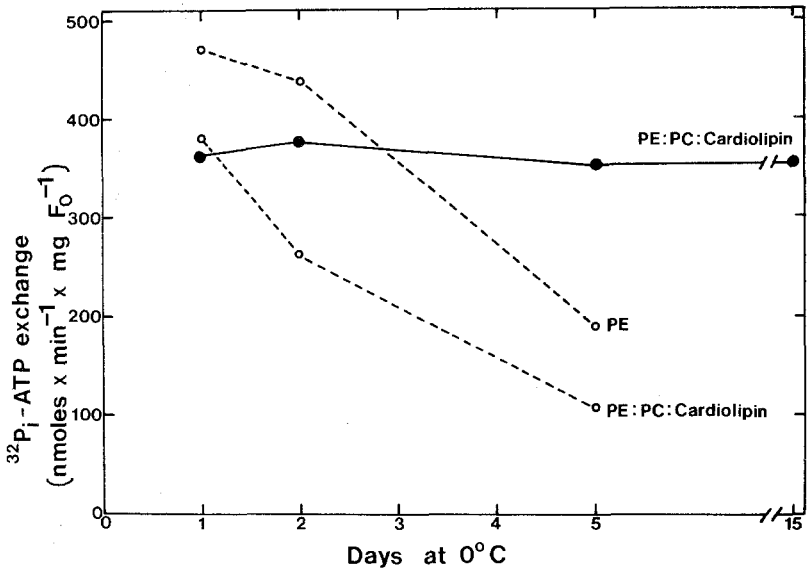

Fig. 8. Stability of reconstituted liposomes. Stable vesicles were prepared as described under Materials and Methods. Phosphatidylethanolamine vesicles were prepared at 12.5 mM in the same buffer. Open circles indicated vesicles not passed through phenyl sepharose and kept at 0°C. Closed circles indicate vesicles passed through phenyl speharose.

participates in the opening and closing of the channel required for the operation of the Mg cycle mechanism as previously suggested (Racker, 1979). The major justification for the assignment of this function to the 28,000-dalton polypeptide rests on early observations (Kagawa and Racker, 1966) that a component of F_o inhibits the ATPase activity, provided most of the phospholipids have been removed. We have shown recently (Alfonzo and Racker, 1979) that this inhibition of ATPase activity is lost on exposure of the 28,000-dalton polypeptide to trypsin.

The question of why the 28,000-dalton component cannot be seen in purified preparations of the $F_o \cdot F_1$ complex from chloroplasts or bacteria may be of special interest from the evolutionary viewpoint. Other membrane components also vary in complexity depending upon the organism. For example, cytochrome oxidase from bovine heart mitochondria (Carroll and Racker, 1977) or yeast (Schatz and Mason, 1974) show six to seven subunits, whereas bacterial cytochrome oxidase (Ludwig and Schatz, 1980; Sone *et al.*, 1979) has one or two subunits. It appears (Ludwig and Schatz, 1980) that the bacterial enzyme acts primarily as an electron pump (half loop) whereas the mitochondrial enzyme pumps protons as well, thereby increasing the efficiency of ATP synthesis. In a similar manner, we propose that the aquisition

of the 28,000-dalton subunit has served to increase the efficiency of closing and opening of the channel and thereby raised the yield of ATP. On the other hand, it is possible that the function of the 28,000-dalton component is taken over by a smaller polypeptide in bacteria and chloroplasts. In purified F_o preparations, a 15,000-dalton polypeptide has been recorded in bacteria (Kagawa, 1978) and chloroplasts (Pick and Racker, 1979).

The unexpected difference in the effect of the phospholipid composition on the activity of the $^{32}P_i$-ATP exchange reaction and ATP synthesis remains unexplained. The lack of a $^{32}P_i$-ATP exchange in chloroplasts (Nelson *et al.*, 1972) and in submitochondrial particles to which ATPase inhibitor has been added (Gómez-Puyou *et al.*, 1979) appears to be a related phenomenon since the forward reaction of ATP generation remains unimpaired.

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