Proton-Pumping *N,N'*-Dicyclohexylcarbodiimide-Sensitive Inorganic Pyrophosphate Synthase from *Rhodospirillum rubrum*: Purification, Characterization, and Reconstitution†

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ABSTRACT: A new method has been developed for the isolation of the proton-pumping *N,N'*-dicyclohexylcarbodiimide-sensitive PP_i synthase (H^+-PP_i synthase) from chromatophores of *Rhodospirillum rubrum*. The H^+-PP_i synthase was purified by extraction of chromatophores with a mixture of nonanoyl-*N*-methylglucamide and cholate, by fractionation with poly(ethylene glycol) 4000, hydroxyapatite chromatography, and affinity chromatography. The purified enzyme is homogeneous and has a specific activity of 20.4 μmol of PP_i hydrolyzed min^{-1} mg^{-1} at pH 7.5 and 20 °C. The hydrolytic activity of the enzyme was stimulated by addition of phospholipids and Triton X-100. Of the lipids tested, cardiolipin proved to have the maximal activating effect. Reconstitution of the H^+-PP_i synthase by the freeze–thaw technique yielded an uncoupler-stimulated and *N,N'*-dicyclohexylcarbodiimide-sensitive PP_i hydrolytic activity. The subunit composition of the purified H^+-PP_i synthase was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. One band was obtained after silver staining with an apparent molecular weight of 56 000. The oligomeric structure of the H^+-PP_i synthase is discussed.

The membrane-bound inorganic pyrophosphatase (H^+-PP_i synthase),[1] which has been found in some phototrophic bacteria (Nore et al., 1990) but not in chloroplasts (Baltscheffsky et al., 1988), functions as an alternative coupling factor. The protonmotive force created by the light-driven cyclic electron transport in phototrophic bacteria can drive the phosphorylation of P_i to PP_i by the H^+-PP_i synthase. The enzyme also catalyzes the hydrolysis of PP_i and the concomitant translocation of protons across the plasma membrane (Shakhov et al., 1982; Nyrén & Baltscheffsky, 1983). In other words, the H^+-PP_i synthase functions as a proton pump, and its activity is regulated by the polarity and strength of the prevailing protonmotive force over the plasma membrane (Strid et al., 1986, 1987a).

The H^+-PP_i synthase from the phototrophic bacteria *Rhodospirillum rubrum* has been solubilized and partially purified previously (Rao & Keister, 1978; Nyrén et al., 1984). The catalytic portion of the enzyme is located at the cytoplasmic surface of the plasma membrane of the cells. Chromatophores (Ribi press vesicles) pump protons inward via the H^+-PP_i synthase during PP_i hydrolysis, creating a protonmotive force (positive and acidic inside).

Although the H^+-PP_i synthase has been purified and partially characterized, the methods so far available do not give a preparation pure enough for raising antibodies or for amino acid sequencing. For instance, six to seven discrete bands could be found on Coomassie brilliant blue R-250 stained SDS–PAGE gels (Baltscheffsky & Nyrén, 1986). Furthermore, the PPase activity after purification was not sensitive toward DCCD in contrast to the activity in chromatophores (Nyrén et al., 1984; Baltscheffsky et al., 1982). The present paper describes a new method for purification of a DCCD-sensitive H^+-PP_i synthase from *R. rubrum*. The purified enzyme shows a single band of M_r 56 000 on silver-stained SDS–PAGE gels.

Materials and Methods

Chemicals. All biochemicals were of analytical grade and obtained from commercial sources.

Growth of Bacteria and Preparation of Chromatophores. *R. rubrum* (strain S1) was grown, and chromatophores were prepared and stored and BChl was determined according to methods described earlier (Strid et al., 1987b; Bose et al., 1961; Clayton, 1963).

Detergent Extraction. H^+-PP_i synthase was purified as follows. Ten milliliters of chromatophores (about 60 mg/mL protein or 2.0 mM BChl) was incubated (on ice for 20 min with gentle stirring) in 70 mL (20 °C) of 50 mM Tris-HCl buffer (pH 8.4 at 4 °C) containing 1.5% (w/v) MEGA-9, 0.5% (w/v) cholate, 25% (v/v) ethylene glycol, 0.75 M $MgCl_2$, 0.5 mM EDTA, and 0.2 mM DTT and then centrifuged at 200000*g* for 60 min. The supernatant containing solubilized proteins was collected.

Poly(ethylene glycol) Fractionation. Solubilized proteins were fractionated with PEG 4000. A 50% (w/v) solution was prepared in 0.1 M Tris–HCl (pH 8.4 at 4 °C). The PEG solution at 20 °C was added to the supernatant (solubilized proteins) up to 11.75% (w/v) saturation, and the mixture was stirred for 20 min on ice before being centrifuged 20 min at 30000*g*. The pellet was discarded and the supernatant brought to 14% (w/v) saturation, stirred, and centrifuged as before.

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[1] Abbreviations: BChl, bacteriochlorophyll; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; H^+-PP_i synthase, membrane-bound proton-translocating PP_i synthase; MEGA-9, nonanoyl-*N*-methylglucamide; PEG, poly(ethylene glycol) 4000; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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The precipitate, enriched in H^+-PP_i synthase, was collected and resuspended at a protein concentration of 25 mg/mL (4 mL) in 50 mM Tris-HCl (pH 8.4 at 4 °C), 30% (v/v) glycerol, 0.2 mM EDTA, and 0.4 mM DTT (buffer A) containing 2% (v/v) Triton X-100 and 0.1 M $MgCl_2$. The resuspended pellet (P2) could be stored frozen at −80 °C.

Hydroxyapatite Chromatography. A 3.5 × 10 cm column was packed at 4 °C with 10 g of hydroxyapatite (Calbiochem; high resolution) at 1 mL/min in a solution containing 10% (v/v) ethylene glycol and 10 mM $MgCl_2$. The packing was controlled with a small amount of methyl red dissolved in the above-mentioned solution. Three milliliters of P2 was diluted with 3 mL of buffer A containing 0.1 M $MgCl_2$ and then applied to the hydroxyapatite column, which had been pre-equilibrated with buffer A containing 0.3% (v/v) Triton X-100 and 0.1 M $MgCl_2$. The column was developed with the same buffer at a flow rate of 1 mL/min. Fractions (10 mL) were collected and assayed for PPase activity. Those fractions showing highest activity (fractions 5–6) were pooled and concentrated to a final volume of 0.5–1 mL by ultrafiltration through an Amicon YM 30 membrane and kept frozen at −80 °C.

Affinity Chromatography. Inorganic pyrophosphate (PP_i) was bound to AH Sepharose 4B by a simple one-step procedure: 110 mL of swollen and washed AH Sepharose 4B (22 g) was mixed with 220 mL of sodium pyrophosphate, 0.1 M, at pH 6.0. An aqueous solution of EDAC (2.5 g dissolved in a minimal volume) was added dropwise over 5 min. The reaction was allowed to proceed for 24 h at room temperature with gentle stirring. The gel was washed thoroughly with 0.5 M NaCl, followed by distilled water, and finally with 50 mM Tris-HCl (pH 8.4 at 4 °C) and 10% ethylene glycol. The gel was packed in a column (3.0 × 20 cm) at 4 °C and equilibrated with buffer A. Concentrated fractions from the hydroxyapatite column were desalted by passing 0.2-mL aliquots through 1-mL Sephadex G-50 (medium) columns according to Penefsky's procedure (Penefsky, 1977). The column buffer was buffer A containing 10 mM $MgCl_2$, and the centrifugation was performed in a clinical centrifuge for 2 min at top speed. The desalted sample was loaded onto the affinity column at a rate of 0.5 mL min^{-1}, followed by a wash with 20 mL of buffer A containing 1 mM $MgCl_2$ and 0.3% (v/v) Triton X-100 at a rate of 2.5 mL min^{-1}. The protein possessing PPase activity was eluted with buffer A containing 25 mM PP_i, 10 mM $MgCl_2$, and 0.3% (v/v) Triton X-100 at the same rate and collected in 10-mL fractions. Fractions containing the highest PPase activity (12–13) were pooled and concentrated to a final volume of 0.5 mL. The preparation could be stored frozen at −80 °C.

The following solutions were used for regeneration of the affinity column: (a) 50 mL of 10% (v/v) ethylene glycol; (b) 200 mL of 0.4 M $MgCl_2$, 0.5% (v/v) Triton X-100, and 10% (v/v) ethylene glycol; (c) 100 mL of 1 M NaCl, 0.5% (v/v) Triton X-100, and 10% (v/v) ethylene glycol; (d) 200 mL of 10% (v/v) ethylene glycol; (e) starting buffer.

Glycerol Gradient Centrifugation. To decrease the Triton X-100 concentration, the purified H^+-PP_i synthase was subjected to glycerol gradient centrifugation. The H^+-PP_i synthase preparation (concentrated by ultrafiltration through an Amicon YM 30 membrane after the affinity chromatography step) was desalted as above except that the column buffer was 50 mM Tris-HCl (pH 8.4 at 4 °C), 33% (v/v) ethylene glycol, 10 mM $MgCl_2$, 0.2 mM EDTA, and 0.4 mM DTT (buffer B). The desalted sample was layered onto 13 mL of a 4–26% (v/v) linear glycerol gradient in buffer B plus 0.1% (v/v) Triton X-100 and centrifuged at 36000 rpm for 22 h in a Beckman SW-40 Ti rotor at 4 °C. The gradient was collected in 0.4-mL fractions from the bottom of the tube. Proteins were analyzed by SDS–PAGE, and fractions containing pure H^+-PP_i synthase were combined, frozen, and stored at −80 °C without significant loss of activity over at least 6 months.

Reconstitution in Liposomes. The freeze–thaw technique of Kasahara and Hinkle (1977) was used to reconstitute the purified H^+-PP_i synthase into liposomes: 50 μL of purified H^+-PP_i synthase after glycerol gradient centrifugation was added to 0.1 mL of liposomes [40 mg/mL soybean phospholipids sonicated for 10 min in 10 mM Tris-HCl (pH 7.5 at 4 °C), 0.5 mM DTT, 0.5 mM EDTA, 50 mM KCl, and 0.05% (w/v) sodium cholate] and rapidly frozen in liquid nitrogen. After being thawed at 4 °C, the preparation was stored on ice. The PPase activity was measured in 0.1 M glycylglycine (pH 7.5 at 20 °C).

Assay of PPase Activity. The method used was a modification of Rathbun's method (Rathbun & Betlach, 1969) as described earlier (Nyrén et al., 1986). Twenty micrograms of cardiolipin was added when required. The PPase activity of chromatophores was tested in 0.1 M glycylglycine buffer (pH 7.5 at 20 °C) in the presence of 1 μM FCCP. The specific activity was expressed in units defined as micromoles of PP_i hydrolyzed during 1 min by 1 mg of protein at 20 °C.

SDS–PAGE. One-dimensional 12.5% acrylamide Midget SDS–PAGE (Pharmacia LKB Biotechnology) was performed as described by Laemmli (1970) with the exception that 20% (v/v) glycerol was included in the gels. Protein was detected by Coomassie brilliant blue R-250 (Zehr et al., 1989) or by silver staining (Morrissey, 1981). Samples were treated with 1% SDS and 1% 2-mercaptoethanol overnight at 37 or at 80 °C for 5 min prior to electrophoresis.

Protein Determination. Protein was determined by the Lowry method (Lowry et al., 1951) and with the Bio-Rad protein assay reagent (Bradford, 1976). Bovine serum albumin or ovalbumin was used as the standard.

Results

Solubilization and PEG Fractionation of H^+-PP_i Synthase. The H^+-PP_i synthase from *R. rubrum* can be solubilized by many different detergents and combinations of detergents. Sodium cholate (Rao & Keister, 1978), Triton X-100 (Nyrén et al., 1984), octyl glucoside, MEGA-9, deoxycholate, and combinations of those detergents (Nyrén, unpublished experiments) were efficient for solublization of H^+-PP_i synthase. In all cases, a high concentration of $MgCl_2$ was necessary for an efficient solubilization to take place, which implies that ammonium sulfate fractionation could not be used as the next purification step if the protein sample was not desalted in advance. On the other hand, PEG 400–6000 could be used as precipitant in the presence of high concentrations of $MgCl_2$. We found that PEG 4000 was the most efficient of the polymers for H^+-PP_i synthase precipitation. However, increased purity of the preparation after PEG fractionation was obtained only when the H^+-PP_i synthase was solubilized with a combination of 1.5% (w/v) MEGA-9 and 0.5% (w/v) cholate. Most of the solubilized H^+-PP_i synthase (over 60%) was precipitated at 11.75–14% saturation of PEG 4000. The specific activity of the preparation was increased from 0.35 to 0.84 unit in this step. In order to stabilize the membrane protein, 30% (v/v) glycerol was included in all subsequent chromatographic steps. Neither 20% (v/v) glycerol nor 25% (v/v) ethylene glycol was sufficient for optimal stability.

Hydroxyapatite Chromatography. The H^+-PP_i synthase has previously been purified by hydroxyapatite chromatog-

Table I: Purification of the H^+-PP_i Synthase from *R. rubrum*[a]

fraction	protein		PPase activity[b]			
	mg	%	units × mg	%	units	PF[c]
chromatophores	610	100	136	100	0.22	1.0
MEGA-9/cholate extract	384	63	136	100	0.35	1.6
PEG fraction, 11.75–14% (w/v)	105	17.2	88.7	65	0.84	3.8
hydroxyapatite	3.5	0.6	27.3	20	7.8	35.5
affinity chromatography	0.8	0.13	16.3	12	20.4	92

[a] See text for details. [b] Activity was measured in the presence of cardiolipin. [c] PF stands for purification factor.

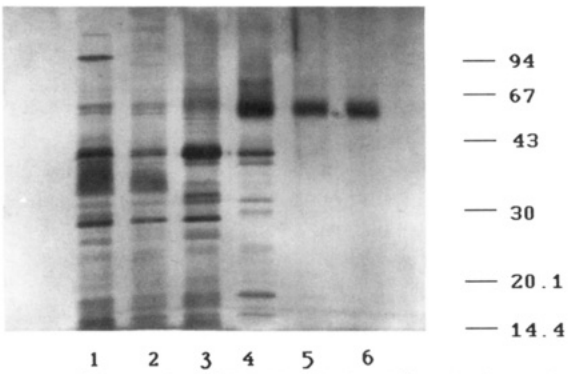

FIGURE 1: Silver-stained SDS–PAGE (12.5%) gel of *R. rubrum* H^+-PP_i synthase at different stages of purification. Lane 1, *R. rubrum* chromatophore membranes (2.5 μg); lane 2, crude extract (MEGA-9/cholate treatment) (1 μg); lane 3, 11.75–14% (w/v) PEG fraction (4 μg); lane 4, H^+-PP_i synthase after hydroxyapatite chromatography (2 μg); lane 5, H^+-PP_i synthase after affinity chromatography (0.6 μg); lane 6, purified H^+-PP_i synthase after glycerol gradient centrifugation (0.6 μg). Markers indicate the positions of various standard proteins (×10^{-3}).

raphy (Nyrén et al., 1984) with good results, although the preparation was contaminated with several proteins. However, we found that the purification was very much dependent on the batch of hydroxyapatite used. We have examined the effect of the $MgCl_2$ concentration in the elution buffer on the degree of purification obtained by several different batches of hydroxyapatite obtained from Calbiochem. We found that for some batches of hydroxyapatite the H^+-PP_i synthase activity could be eluted at very low concentrations of $MgCl_2$ (10 mM) and thereby a nearly pure preparation of the enzyme could be obtained. For other batches, a much higher concentration was necessary, and a highly contaminated preparation was obtained. To overcome this serious problem, we decided to use hydroxyapatite only as a rough purification step working for all batches of hydroxyapatite, and to include an affinity chromatography step. The concentration of Triton X-100 in the elution buffer was critical. At 0.3% (v/v), the bulk of the contaminating proteins was bound to the column whereas the H^+-PP_i synthase passed through. At higher detergent concentrations, more of the contaminating proteins were eluted together with the H^+-PP_i synthase, and at lower concentrations, the enzyme was bound to the column and could not be eluted with 0.1 M $MgCl_2$. In this step, a 9-fold increase in the specific activity of the preparation was obtained.

Affinity Chromatography. After chromatography on hydroxyapatite, the concentrated and desalted protein solution was applied to a column of AH Sepharose 4B with bound inorganic pyrophosphate. A typical purification scheme is summarized in Table I. A purification of 92-fold was obtained with 12% recovery. The specific activity increased from 0.22 to 20.4 units.

SDS–PAGE. The purity of the protein fractions was analyzed by SDS–PAGE with 12.5% gels (Figure 1). The purified enzyme showed a single protein band (this was also observed at lower protein loadings) on a silver-stained gel with a molecular weight of 56 000, and this band could be recognized as a minor component of the chromatophore preparation. The H^+-PP_i synthase only diffusely stained with Coomassie brilliant blue (not shown).

Table II: Effects of Lipids and Triton X-100 on the Activity of the Purified H^+-PP_i Synthase[a]

lipid/detergent	μg	% activity
control	0	100
phosphatidylglycerol	20	400
phosphatidylethanolamine	20	450
soybean asolectin (22% phosphatidylcholine)	40	780
cardiolipin	20	960
Triton X-100	100	480

[a] Phospholipids were added to purified H^+-PP_i synthase (after glycerol gradient centrifugation) in the assay mixture and preincubated 3 min before addition of substrate. The amounts of lipids indicated gave optimal activity.

Effect of Lipids. The activity of the purified H^+-PP_i synthase was stimulated by addition of phospholipids. Of the phospholipids tested, cardiolipin proved to have the highest activating effect whereas the effect of phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine was lower (Table II). Triton X-100 alone reconstituted the enzyme activity to about 50% or to the same extent as a phosphatidylglycerol and phosphatidylethanolamine. It is worth noting that the major lipids found in *R. rubrum* whole cells (Wood et al., 1965) and in *R. rubrum* chromatophores (Costes et al., 1978) are phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, and *O*-ornithylphosphatidylglycerol. No phosphatidylcholine has been detected. Our interpretation of the effect of phospholipids and Triton X-100 is that the hydrophobic parts of the H^+-PP_i synthase have to be covered by either detergents or lipids to retain activity and that cardiolipin has a more profound effect which is necessary for full activity.

Cardiolipin had different effects on the PPase activity of the H^+-PP_i synthase preparation after different stages of the isolation procedure. After the first step in the purification scheme, the PPase activity was only slightly stimulated by cardiolipin (5%), indicating that lipids are copurified with the enzyme. After PEG fractionation, the 11.75–14% fraction was clearly dependent on lipids for full activity (stimulation by 110%). Most of the lipids were probably obtained in the 14–18% fraction since combination of this fraction with the 11.75–14% fraction restored full activity. In the subsequent step, no further lipids seem to be lost: 69% activation by cardiolipin after the hydroxyapatite chromatography. After the affinity step and the glycerol gradient step, the level of phospholipids was substantially reduced (more than 6- and 9-fold activation with cardiolipin, respectively).

Reconstitution. For H^+ transport measurements, the H^+-PP_i synthase was reconstituted into liposomes by the freeze–thaw procedure (Nyrén & Baltscheffsky, 1983). The activity of the reconstituted H^+-PP_i synthase was more than 4-fold higher after the addition of FCCP. In contrast, no effect of FCCP on isolated H^+-PP_i synthase was found. The maximal turnover was the same for isolated (in the presence of

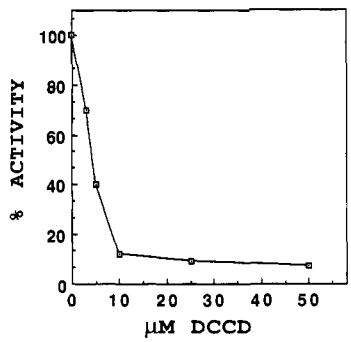

FIGURE 2: Relative rate of PP_i hydrolysis by purified H^+-PP_i synthase as a function of DCCD concentration. Purified H^+-PP_i synthase (after glycerol gradient centrifugation) was preincubated for 10 min with the DCCD concentration indicated. The 2-mL reaction medium contained 50 mM Tris-HCl, pH 7.5, 0.75 mM $MgCl_2$, and 20 μg of cardiolipin. The reaction was started by addition of 0.5 mM PP_i and was terminated 10 min later with 3% (w/v) TCA. Other experimental conditions are found under Materials and Methods.

cardiolipin) and uncoupled reconstituted H^+-PP_i synthase, which indicates that the incorporation occurred unidirectionally with the PP_i-binding site located at the outside of the proteoliposome, especially since PP_i cannot permeate the liposome membrane. The reconstitution experiment clearly shows that the purification procedure results in an intact enzyme with both the PPase activity and the proton-pumping activity retained and that the two activities are coupled to each other. It is interesting to note that the proteoliposomes were better coupled than the chromatophores for which the PPase activity was only doubled in the presence of FCCP.

DCCD Sensitivity. DCCD inhibits both ATP and PP_i hydrolysis in *R. rubrum* chromatophores (Baltscheffsky et al., 1982). However, after purification according to Nyrén et al. (1984) and Kondrashin et al. (1980), the isolated H^+-PP_i synthase was not inhibited by DCCD. In contrast, the method presented in this paper gave a DCCD-sensitive preparation of the H^+-PP_i synthase (Figure 2). I_{50} for the PP_i hydrolysis was nearly 5 μM, which is comparable to the value obtained for chromatophores (Baltscheffsky et al., 1982).

Substrate Specificity and Effectors. The hydrolytic activity of the purified H^+-PP_i synthase was very specific for PP_i. The activity with a number of other compounds containing the pyrophosphate moiety, e.g., ATP, ADP, imidodiphosphate, methylenediphosphonate, tripolyphosphate, and tetrapolyphosphate, was zero. Imidodiphosphate and methylenediphosphonate acted as competitive inhibitors of PP_i hydrolysis (not shown). The enzyme specifically required Mg^{2+} for activity. There was no detectable activity in the presence of Ca^{2+}, Fe^{2+}, Ni^{2+}, Ba^{2+}, or Sr^{2+}. The activity with Mn^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} was 11, 2, 2, and 1% of that with Mg^{2+}, respectively. Added nucleotides, with the exception of ATP, slightly stimulated the PP_i hydrolysis activity. The nucleotides tested were ATP, ADP, AMP, NAD^+, NADH, $NADP^+$, and NADPH. NADPH gave the highest stimulating effect (about 26%).

Discussion

The purification procedure described in this paper gives a purer preparation than earlier protocols (Rao & Keister, 1978; Nyrén et al., 1984). The main improvements compared to earlier methods are the extraction of chromatophores with a combination of MEGA-9 and cholate, PEG fractionation, an affinity chromatography step, and the fact that the enzyme is not bound to the hydroxyapatite column, which minimizes the activity loss. The method is fast and simple, and the purified enzyme obtained is stable for several months at −80 °C.

After purification of the H^+-PP_i synthase, a lipid-depleted or partly lipid-depleted preparation is obtained, which possesses minimal PPase activity. The enzyme could be reactivated by interaction with detergents or phospholipids, in particular cardiolipin. The results indicate that there is no absolute requirement for any phospholipid for this H^+-PP_i synthase preparation. However, the preparation of H^+-PP_i synthase might still contain residual phospholipids that have not been completely removed from the enzyme. The effect of phospholipids on the PPase activity points to a dual role for them in the H^+-PP_i synthase. Phospholipids seem to have a nonspecific function: to cover the hydrophobic parts of the enzyme and to hold the enzyme in a solubilized and active form. The latter is an effect similar in action to that of detergents (Triton X-100 stimulates the activity to the same extent as phosphatidylglycerol and phosphatidylethanolamine). In contrast to a nonspecific effect by phospholipid, there also seems to be a more specific requirement of H^+-PP_i synthase for cardiolipin. A similar dependence on cardiolipin has been observed for several integral membrane proteins of mitochondria, such as the NADH–dehydrogenase complex (Fry & Green, 1981), the cytochrome *c* oxidase (Robinson et al., 1980; Fry & Green, 1980), the cytochrome bc_1 complex (Fry & Green, 1981), and the phosphate carrier (Kadenbach et al., 1982). In addition to the activating effect, cardiolipin also has a stabilizing effect on the enzyme. If cardiolipin, to a final concentration of 0.1 mg/mL, is added to the pure H^+-PP_i synthase, the activity is stable for several days at 4 °C. However, the activity is totally lost if the enzyme is frozen in the presence of lipids.

The *R. rubrum* H^+-PP_i synthase seems to consist of a single polypeptide of about M_r 56 000, which is a minor constituent of the chromatophore membrane fraction (Figure 1). The H^+-PP_i synthase probably spans the plasma membrane since it operates as a proton pump. At the moment, however, we do not know the number of subunits in the functional enzyme. SDS–PAGE of the purified sample gave a single band if the protein was incubated with SDS at 37 °C overnight or at 80 °C for 5 min. In contrast, if the sample was heated to 100 °C in the presence of SDS for 5, 10, or 15 min, two, three, and four bands, respectively, appeared after SDS–PAGE (not shown). The apparent molecular weights of the different bands were 56 000, 148 000, (2 × 74 000), 230 000 (3 × 77 000), and 300 000 (4 × 75 000). The appearance of the bands of higher molecular weight was paralleled by the disappearance of the lower molecular weight bands. These results might indicate a dimeric or a tetrameric structure of the functional enzyme. It might also suggest an aggregation of H^+-PP_i synthase polypeptides under denaturating conditions at high temperatures. As the H^+-PP_i synthase probably is a highly hydrophobic protein, and since such proteins bind an anomalously high amount of SDS (Fish & Bogorad, 1986), the apparent molecular mass of 56 000 by SDS–PAGE might be an underestimation. The true molecular mass might be closer to 75 000 as indicated by the above results. If the purified preparation was concentrated by ultrafiltration through an XM 300 membrane, about 75% of the solubilized enzyme was rejected by the membrane and 25% passed through. The latter result indicates a molecular weight of around 230 000 for the native enzyme plus bound Triton X-100. If we assume that one micelle of Triton X-100 (M_r 90 000) binds to each protein (Helenius & Simons, 1975; Weiss & Kolbe, 1979), a molecular weight for the native enzyme of 140 000 is obtained, indicating that the enzyme might be a dimer of identical subunits. In addition, data from Rao and Keister (1978) (using gel filtration) indicate a molecular weight of less than 200 000 for

the H^+-PP_i synthase plus bound cholate. This is also in accordance with a dimeric structure for the enzyme [by assuming 0.6 mg of cholate/mg of protein (Helenius & Simons, 1972)].

The amount of H^+-PP_i synthase is about 1% of the total protein content of the chromatophore membrane. This value is low as compared to the relative abundance of the F_1F_0-ATP synthase (20%) in *R. rubrum* chromatophores (Norling et al., 1989).

Although the oligomeric structure of the H^+-PP_i synthase obtained after the new purification procedure is still unclear, it is evident that the enzyme has retained its proton-pumping activity. After reconstitution of the purified enzyme into liposomes, the hydrolytic activity was stimulated 4-fold by the addition of FCCP, a factor of 2 higher than the value obtained for the chromatophore activity. The incorporation probably occurred undirectionally with the active site situated on the outside of the proteoliposomes. The results show that the proteoliposomes are highly coupled and well adapted for closer studies of the H^+-PP_i synthase.

Previous studies have shown that both PP_i hydrolysis (Baltscheffsky et al., 1982) and PP_i synthesis (Nyrén et al., 1986) in chromatophores are inhibited by DCCD. The present study shows that the isolated and highly purified H^+-PP_i synthase also is inhibited by DCCD, a property which is shared by all F_1F_0-ATP synthases (Senior & Wise, 1983), cytochrome bc_1 complexes (Esposti et al., 1983; Clejan & Beattie, 1983), and cytochrome oxidase (Casey et al., 1980). In these systems, DCCD inhibits primarily proton translocation, and it seems likely that the effect of DCCD on the H^+-PP_i synthase also is due to inhibition of the vectorial proton translocating activity.

The H^+-PP_i synthase presents a simple model system, and perhaps the best one, for studies of the mechanism of phosphorylation coupled to proton translocation. The purification method presented in this paper enables us to examine the protein more closely.

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