Reconstitution of highly purified proton-translocating pyrophosphatase from *Rhodospirillum rubrum*

Yuri A. Shakhov*, Pål Nyrén and Margareta Baltscheffsky+

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Received 20 July 1982

Pyrophosphatase *ATPase* *Coupling factor* (Rhodospirillum rubrum) *Proton pump* *Liposome*

1. INTRODUCTION

Membrane-bound PPase is of interest as it functions in various organisms as a coupling factor between electron transport and PP_i synthesis and is involved in the energy transduction pathway as an independent alternative to the ATPase system [1–4]. PP_i hydrolysis in chromatophores of *Rhodospirillum rubrum* caused a change in the fluorescence of added 8-anilino-naphtalene-1-sulfonic acid [5], an uptake of phenyl dicarbaundecaborane anion [6] and a pH change of the outer medium [7]. Therefore, it was concluded that chromatophore PPase translocates protons across the membrane coupled to PP_i hydrolysis, apparently in a manner corresponding to that of the chromatophore ATPase.

Further investigation of membrane-bound PPase has been impeded by the lack of an efficient method of isolation. Although a method of purifying the PPase has been reported [8], only recently has a method of obtaining a highly purified membrane-bound PPase from *R. rubrum* chromatophores been developed [10].

A partly purified membrane-bound PPase from chromatophores of *R. rubrum* has been reconstituted into liposomes and generates an electric current [9]. However, an unanswered question of great significance is whether the pure membrane-bound PPase can function as a proton pump.

Here, we describe the reconstitution of this purified PPase into liposomes and the function of the enzyme as a proton pump.

2. MATERIALS AND METHODS

Rhodospirillum rubrum (strain S1) was grown anaerobically in light at 30°C in the medium of [11]. After 40 h of growth (the end of the logarithmic phase) cells were harvested, washed and chromatophores were prepared by mechanical disruption in a Ribi cell fractionator in 0.2 M glycylglycine (pH 7.4) at 20 000 lb · in^{-2}. Cell debris was removed by centrifugation at 10 000 × *g* for 60 min and the supernatant was further centrifuged at 100 000 × *g* for 90 min. The pellet was washed twice, first with 0.3 M NaCl in 0.2 M glycylglycine (pH 7.4) and finally with 0.2 M glycylglycine (pH 7.4) with centrifugation at 100 000 × *g* for 60 min following.

The PPase assay was done in a medium containing 50 mM Tris–HCl (pH 8.0), 0.5 mM $Na_4P_2O_7$ and 0.75 mM $MgCl_2$ at 30°C. In the case of purified PPase 0.2 mg · ml^{-1} of acetone-washed soybean phospholipids sonicated to clarity were added. The ATPase activity was measured in

* Present address: A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 177234, USSR

+ To whom correspondence should be addressed

Abbreviations: PPase, inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTE, dithiothreitol; IDP, imidodiphosphate

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

50 mM Tris–HCl (pH 8.0) containing 4 mM ATP and 2 mM $MgCl_2$ at 30°C. When the enzymatic activities were measured in liposomes, $5 \cdot 10^{-2}$ M KCl was added. Total volume was 2 ml. Reactions were stopped by addition of 1 ml 10% cold trichloroacetic acid. Inorganic orthophosphate was determined as in [12]. Protein was determined according to [13].

The membrane-bound PPase was solubilized from chromatophores by extraction with Triton X-100 in presence of ethylene glycol and $MgCl_2$ [10]. A high level of purification was obtained with hydroxylapatite chromatography of the Triton extract. The enzyme preparation had spec. act. 15.2 μmol $PP_i \cdot mg^{-1} \cdot min^{-1}$. After hydroxylapatite chromatography the protein was concentrated from ~15–1 ml on a Diaflo YM-5 ultrafiltration membrane with some loss of activity and then desalted on a column of Sephadex G-25 course (0.5 cm diam.; 25 cm length). The column was equilibrated with 50 mM Tris–HCl buffer (pH 8.0), 25% (v/v) ethylene glycol, 5 mM $MgCl_2$, 0.2 mM DTE and 0.04% Triton X-100. At this stage, the enzyme preparation spec. act. was 7.5 μmol $PP_i \cdot min^{-1} \cdot mg^{-1}$.

The freeze–thaw technique of [14] was used to reconstitute the PPase into liposomes: 40 mg soybean phospholipids were supplemented with 1 ml medium, containing 10 mM Tris–HCl (pH 7.5), 0.5 mM DTE, 0.5 mM EDTA, 50 mM KCl and 0.05% recrystallized Na-cholate. The suspension was flushed with argon and sonicated in a covered test tube for 20 min at 20–25°C in a bath-type sonicator model G112 SP1T, Laboratory Supplies Co. (Hicksville NY). The PPase preparation (0.2 ml, containing 50–100 μg protein) was then added to 0.3–0.4 ml liposomes, sonicated for 10 s and rapidly frozen in a dry-ice–ethanol bath. After thawing at room temperature, the preparation was stored in ice.

The F_0F_1 ATPase complex was solubilized with Na-cholate and octyl-α-D-glucopyranoside and purified from crude chromatophore extract by ammonium sulfate precipitation and sucrose-gradient centrifugation [15]. The preparation spec. act. was 0.2 μmol $ATP \cdot min^{-1} \cdot mg^{-1}$. The ATPase was reconstituted into liposomes as follows: 0.1 ml ATPase fraction (500 μg protein) was supplemented with 0.2 ml PPase (50–100 μg protein) and 0.5 ml sonicated liposomes. The mixture was frozen and thawed as above.

All chemicals were purchased from Sigma (St Louis MO) except DCCD which was from Fluka AG (Bucks).

3. RESULTS AND DISCUSSION

Reconstitution experiments were partly purified PPase from *R. rubrum* chromatophores included a dialysis procedure to remove 2% cholate. Cholate was essential for solubilization and for subsequent stabilization of activity during the purification [9]. However, the dialysis procedure requires the presence of detergent and has been used widely with cholate or deoxycholate but is not suitable for membrane proteins extracted with non-ionic detergents, such as Triton X-100. Triton is not removed by dialysis. Triton also interferes with the formation of vesicles by sonication methods.

As mentioned in section 2, the new method of PPase solubilization and purification was done in the presence of Triton X-100 and the stability of the enzyme was dependent on the presence of both $MgCl_2$ and ethylene glycol during Triton extraction. Attempts to remove Triton or replace it with 2% Na-cholate (with or without added phospholipids) led to the loss of activity of aggregation of protein.

It was found, in agreement with [8], that the stability of solubilized PPase was strongly dependent on the presence of $MgCl_2$ in the storage medium. For example, removing $MgCl_2$ by dialysis led to the complete loss of enzyme activity. However, Mg^{2+} at > 10 mM led to the appearance of an insoluble precipitate of phospholipids when the enzyme preparation was supplemented with 50 mg/ml soybean asolectin solution. Consequently, the concentrations of Triton and $MgCl_2$ were reduced to levels suitable for incorporation of the PPase into vesicles, by chromatographing the enzyme preparations on columns of Sephadex G-25 coarse equilibrated with 5 mM $MgCl_2$ and 0.04% Triton X-100. Substantial losses of activity did not occur and the desalted enzyme was stable.

Because the freeze–thaw method was not affected by up to 0.05% Triton [16] we used this method for reconstitution of the PPase into phospholipid vesicles. PPase subjected to the freeze–thaw procedure retained 90% of its original activity (table 1). The addition of the uncoupler FCCP,

Table 1

Effect of various compounds of the PPase activity before and after its incorporation into liposomes

Addition / PPase act.	Control	FCCP (1.5 μM)	Valinomycin (10 μg/ml)	Nigericin (10 μg/ml)	Valinomycin + nigericin	Oligomycin (10 μg/mg protein)	DCCD (100 μM)	NaF (10 mM)	IDP (1 mM)
Incorporated									
(nmol P_i/min)	37	50	46	54	55	36	36	6	21
(%)	100	135	124	146	149	97	97	16	57
Solubilized									
(nmol P_i/min)	40	39	40	40	39	38	39	4	18
(%)	100	98	100	100	98	95	98	10	45

PPase activity was measured in the same mixture with phospholipids added before (solubilized PPase) and after (incorporated PPase) freezing–thawing. The assay medium contained $5 \cdot 10^{-2}$ M KCl and 50 μl PPase preparation was added. For details see section 2

which collapsed the electrochemical potential difference of H^+ across the membrane, led to stimulation of PPase activity in liposomes. Likewise, the activity increased when the membrane was made permeable to K^+ by adding valinomycin (to collapse the membrane potential). In this case, an inward translocation of H^+ was accompanied by an outward translocation of K^+. The stimulatory effect was also achieved when nigericin, a H^+/K^+ exchanger, was used. The effects of the 2 antibiotics were not additive. The compounds used did not affect PPase activity before incorporation of the enzyme into liposomes (table 1).

From the results obtained we infer that PP_i hydrolysis by membrane-bound PPase is coupled to proton translocation into the liposomes.

In [17] the PPase activity of native *R. rubrum* chromatophores was stimulated by the addition of valinomycin + KCl and valinomycin + nigericin + KCl. Moreover, the stimulatory effects were about the same as those described here for the reconstituted system.

Neither the membrane-bound PPases [18,19] nor the reconstituted enzyme in our experiments (table 1) were inhibited by oligomycin. Both PPases were strongly inhibited by 1×10^{-2} M fluoride. Imidiodiphosphate, a competitive inhibitor of PPases, decreased enzymatic activity 2-fold.

The membrane-bound PPase of chromatophores is inhibited by DCCD [20]. The titration curves with added DCCD were similar for both PPase and ATPase activities of chromatophores. For example, 65–75% of inhibition occurred for both enzymes after 10 min or 5 h of preincubation with 100 μM DCCD. It was suggested that PPase contains an identical or similar proton channel to that of the ATPase [20]. Also, the activity of PPase preparations from both beef heart and rat liver mitochondria was markedly stimulated by the addition of ATPase [21], which itself did not cleave PP_i under these conditions.

However, our experiments show that after solubilization the PPase lost the DCCD-sensitivity and treatment with 100 μM DCCD for 10 min did not change the activity of reconstituted PPase (table 1). Similar results were obtained in [9]. It was suggested that this phenomenon was due to the effect of detergent. For example, the addition of deoxycholate or Triton X-100 to both ATPases purified from bovine heart mitochondria and spinach chloroplast results in loss of the sensitivity to DCCD [16,22]. But in our work the ATPase, purified in the presence of Triton X-100, was DCCD-sensitive even after incorporation (see table 2) into vesicles. This difference remains difficult to explain.

To examine the presumed interaction between membrane-bound PPase and ATPase we preincubated the mixture of PPase and ATPase preparations for 10 min before incorporation into liposomes as in section 2.

Table 2 shows that in this case the stimulation of PPase by added uncoupler and valinomycin was the same as in table 1. The ATPase activity in lipo-

Table 2

Effect of various compounds on the PPase and ATPase incorporated into liposomes

Activity \ Additions	Control	FCCP (1.5 μM)	Valinomycin (10 μg/ml)	DCCD (100 μM)	Oligomycin (10 μg/mg protein)
PPase					
(nmol P_i/min)	45	58	54	44	45
(%)	100	129	120	98	100
ATPase					
(nmol P_i/min)	23	51	46	12	7
(%)	100	222	200	52	30

Liposomes (25 μl and 200 μl) were added to PPase and ATPase assay media, respectively

somes was activated 2-fold by FCCP, as was also found for chromatophore-bound ATPase.

The results obtained show that pure membrane-bound PPase, isolated from *R. rubrum* chromatophores, and incorporated into phospholipid vesicles, can function alone as a H^+-pump.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Swedish Natural Research Council to M.B; Yu.S. thanks the Swedish Institute for 10 months' scholarship. We wish to thank Professor Herrick Baltscheffsky for active support and Professor Harvey Penefsky for helpful discussions.

REFERENCES

[1] Baltscheffsky, H., Von Stedingk, L.-V., Heldt, H.-W. and Klingenberg, M. (1966) Science 153, 1120.
[2] Baltscheffsky, M. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R. eds) pp. 595–613, Plenum, New York.
[3] Mansurova, S.E., Shakhov, Yu.A. and Kulaev, I.S. (1977) FEBS Lett. 74, 31–34.
[4] Shakhov, Yu.A., Mansurova, S.E. and Kulaev, I.S. (1981) Biochem. Internat. 3, 139–145.
[5] Azzi, A., Baltscheffsky, M., Baltscheffsky, H. and Vainio, H. (1971) FEBS Lett. 17, 49–52.
[6] Isaev, P.I., Liberman, E.A., Samuilov, V.D., Skulachev, V.P. and Tsofina, L.M. (1970) Biochim. Biophys. Acta 216, 22–29.
[7] Moyle, J., Mitchell, R. and Mitchell, P. (1972) FEBS Lett. 23, 233–236.
[8] Rao, P.V. and Keister, D.L. (1978) Biochem. Biophys. Res. Commun. 84, 465–473.
[9] Kondrashin, A.A., Remennikov, V.G., Samuilov, V.D. and Skulachev, V.P. (1980) Eur. J. Biochem. 113, 219–222.
[10] Nyrén, P. and Baltscheffsky, M. (1982) in preparation.
[11] Bose, S.K., Gest, H. and Ormerod, J.G. (1961) J. Biol. Chem. 236, 13–14.
[12] Rathbun, W.B. and Betlach, V.M. (1969) Analyt. Biochem. 28, 436–445.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, K.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Kasahara, M. and Hinkle, R.C. (1977) J. Biol. Chem. 252, 7384–7390.
[15] Rott, R. and Nelson, N. (1981) J. Biol. Chem. 256, 9224–9228.
[16] Pick, U. and Racker, E. (1979) J. Biol. Chem. 254, 2793–2799.
[17] Baltscheffsky, H., Baltscheffsky, M. and Von Stedingk, L.-V. (1969) Prog. Photosynth. Res. 3, 1313–1318.
[18] Baltscheffsky, M., Baltscheffsky, H. and Von Stedingk, L.-V. (1966) Brookhaven Symp. Biol. 19, 246–257.
[19] Mansurova, S.E., Shakhov, Yu.A., Beljakova, T.N. and Kulaev, I.S. (1975) FEBS Lett. 55, 94–98.
[20] Baltscheffsky, M., Baltscheffsky, H. and Boork, J. (1982) in: Electron Transport and Photophosphorylation (Barber, J. ed) Top. Photosynth. vol. 4, pp. 249–272. Elsevier Biomedical, Amsterdam, New York.
[21] Racker, E. (1962) Proc. Natl. Acad. Sci. USA 48, 1659–1663.
[22] Serrano, R., Kanner, B.I. and Racker, E. (1976) J. Biol. Chem. 251, 2453–2461.