Biochimica et Biophysica Acta, 766 (1984) 630–635
Elsevier

BBA 41586

PURIFICATION OF THE MEMBRANE-BOUND PROTON-TRANSLOCATING INORGANIC PYROPHOSPHATASE FROM *RHODOSPIRILLUM RUBRUM*

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(Received February 8th, 1984)

Key words: Proton pump; Inorganic pyrophosphatase; Photophosphorylation; Chromatophore

The proton translocating membrane-bound inorganic pyrophosphatase of *Rhodospirillum rubrum* S1, has been solubilized with good yield from chromatophores using Triton X-100 (9–10 oxyethylene groups) in the presence of high concentrations of $MgCl_2$ and ethyleneglycol. The enzyme has been purified 80-fold by hydroxylapatite column chromatography, to a state of near homogeneity, according to polyacrylamide-gel-electrophoresis. The enzyme appears to be a very hydrophobic integrally bound membrane protein. Phospholipids or Triton X-100 reconstitutes the enzyme activity after solubilization and purification. The purified enzyme preparation has a specific activity of 24 units. Both the purified and the chromatophore-bound enzyme are inhibited by *N*-ethylmaleimide, 4-chloro-7-nitrobenzo-2-oxo-1,3-diazol (NBF-Cl), sodium fluoride, imidodiphosphate, methylenediphosphonate and the antibiotic Dio-9 (energy-transfer inhibitor). In the solubilized state the purified enzyme is not stimulated by uncouplers or inhibited by dicyclohexylcarbodiimide in contrast to the chromatophore-bound pyrophosphatase. When reconstituted into liposomes the purified enzyme regains the stimulation by uncouplers.

Introduction

Inorganic pyrophosphate (PP_i) is produced as a byproduct in a variety of biosynthetic reactions. The hydrolysis of PP_i to P_i is catalyzed by inorganic pyrophosphatase (E.C. 3.6.1.1.). The non-sulfur purple bacterium *Rhodospirillum rubrum*, contains both soluble and membrane-bound inorganic pyrophosphatase activities.

Baltscheffsky et al. have shown that not only ATP, but also PP_i can be formed as a phosphorylated end product in electron-transport-coupled photophosphorylation [1,2]. This alternative reaction is catalyzed by a membrane-bound inorganic pyrophosphatase activity. PP_i was shown to act as an energy donor to the electron-transport chain at the cytochrome level [3]. The capability of PP_i as energy donor, in *R. rubrum* was also suggested from the observations that both PP_i and ATP can serve as energy donors for energy-linked transhydrogenation [4], succinate-linked NAD^+ reduction [5], cytochrome redox changes [6] carotenoid band shift [3] and proton uptake [7]. Further, it has been demonstrated that PP_i can be used to drive the synthesis of ATP [8]. Membrane-bound inorganic pyrophosphatase activity has also been found in other species of photosynthetic bacteria [9–12] as well as in other organisms. Kulaev and coworkers have demonstrated that PP_i is formed as a phosphorylated energy-rich compound coupled to electron transport in mitochondria of lower [13] and higher heterotrophic organisms [14,15] as well

Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NBF-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol; BChl, bacteriochlorophyll; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate.

as in chloroplasts from algae and higher plants [16]. It has been claimed that the membrane-bound inorganic pyrophosphatase serves as a coupling factor for electron-transport driven PP_i formation in bovine heart mitochondria [17].

Klemme and Gest [18,19] made a very comprehensive study of the soluble enzyme, and have shown that the enzyme probably is under allosteric control.

Recently, it has been shown that a partly purified inorganic pyrophosphatase isolated from *R. rubrum* chromatophore membranes can be incorporated in a phospholipid membrane and act as a PP_i-dependent electric generator [20]. As one may assume the structural organization of the inorganic pyrophosphatase to be considerably simpler than that of the ATP-synthetase complex from energy-transducing membranes, purification and physicochemical characterization of this enzyme would seem to be of great interest. An earlier published method for the solubilization and purification of the enzyme [21], yields an only partially purified product of limited stability and comparatively low specific activity.

In this paper, we describe a simple method for extensive purification of the membrane-bound inorganic pyrophosphatase from *R. rubrum* with good activity. We also report on a preliminary characterization of the enzyme. We have shown that this purified membrane-bound pyrophosphatase incorporated into phospholipid vesicles can function as an H^+-pump [22]. This preparation has also been used for reconstitution of PP_i-driven ATP-synthesis in a liposomal system containing both the inorganic pyrophosphatase and the F_0F_1 ATPase complex [23].

Materials and Methods

Materials

Triton X-100, dithioerythritol, FCCP, imidodiphosphate, methylenediphosphonate, *N*-ethylmaleimide, Trizma base and asolectin (crude soybean phospholipids used after an acetone wash [24]) were purchased from Sigma, $Na_4P_2O_7$, $MgCl_2$ and ethylene glycol from Merck, and NBF-Cl from Serva. Hydroxylapatite was obtained from Bio-Rad and DCCD from Fluka AG (Bucks., U.K.).

Preparation of chromatophores

R. rubrum strain S1 was grown anaerobically in light at 30 °C in the medium described by Bose et al. [25]. After 40 h of growth (at the end of the log phase), cells were harvested and washed, and chromatophores were prepared as described previously [22].

Assays

The inorganic pyrophosphatase activity was assayed in a reaction mixture containing 0.75 mM $MgCl_2$, 0.5 mM $Na_4P_2O_7$, 1 ml 0.1 M Tris-HCl (pH 7.5), asolectin, when required, and H_2O, in a total volume of 2 ml. Asolectin was prepared by sonication in 0.1 M Tris HCl (pH 7.5) until fairly clear. The assay mixture was incubated at 30 °C and the reaction was terminated after 10 min by addition of 1 ml 10% trichloroacetic acid. In the blanks, trichloroacetic acid was added before the sample. With crude fractions in which a visible protein precipitate was formed, the mixture was chilled and centrifuged, and the supernatant fluid was collected. A volume of 0.4 ml of the supernatant fluid was used for P_i determination. P_i was assayed colorimetrically, in a total volume of 3 ml, as described by Rathbun et al. [26]. The specific activity was expressed in units defined in terms of micromoles of PP_i hydrolyzed in 1 min by 1 mg protein. Protein was determined according to the modified Lowry method described in Ref. 27. Bacteriochlorophyll was determined using the in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm, as reported by Clayton [28].

Polyacrylamide gel electrophoresis (5% gels) was performed by the procedure described by Davis [29] except that 20% (v/v) ethyleneglycol, 0.1 mM dithioerythritol and 0.1% (v/v) Triton X-100 were included in the gel. The electrolyte buffer contained 0.1% (v/v) Triton X-100. To localize the inorganic pyrophosphatase activity the gel was incubated for 20 min at 30 °C in a solution identical with that used for the assay of the enzyme, rinsed with water, and immediately immersed in the triethylaminemolybdate reagent of Sugino and Miyoshi [30], which specifically precipitates P_i. A sharp, discrete zone, corresponding to the enzyme activity in the gel appeared within a few min. Sephadex centrifuge columns were used to desalt the sample before Triton X-100 electrophoresis [31].

Solubilization of the enzyme. All manipulations were performed at 0–5 °C, unless otherwise stated. 3 ml chromatophores (60–70 mg protein per ml, about 1.5 mM BChl) were mixed with 21 ml of a 50 mM Tris-HCl (pH 8.4) buffer containing 2.5% (v/v), Triton X-100, 0.75 M $MgCl_2$, 25% (v/v) ethyleneglycol and 0.2 mM dithioerythritol. After gentle stirring on ice for 20 min the suspension was centrifuged at 215000 × *g* for 60 min. The supernatant was decanted into a beaker and stored on ice in the dark or was kept frozen at −70 °C.

Hydroxylapatite chromatography. 8 g Bio-Gel HTP were rehydrated in a 50 mM Tris-HCl (pH 8.4) buffer containing 0.1% (v/v) Triton X-100/25% (v/v) ethylene glycol/66 mM $MgCl_2$/0.2 mM dithioerythritol/0.5 mM EDTA. The rehydrated gel was packed into a column (3.2 × 10 cm) and equilibrated with the same buffer. 6 ml of the solubilized enzyme was diluted 10-fold with the same buffer as mentioned above, except that $MgCl_2$ and Triton X-100 were omitted. The sample was applied to the column, with a flow rate of 1–2 ml per min. The column was washed with 30 ml of the equilibration buffer plus 30 ml of the same buffer with 130 mM $MgCl_2$. The enzyme was eluted with 0.2 M $MgCl_2$ in the above-mentioned buffer and fractions of 6 ml were collected. Those fractions showing highest inorganic pyrophosphatase activity (fraction 5–8) were pooled and concentrated to a final volume of 1 ml by ultrafiltration through an Amicon YM 5 (340000 Pa) or XM 100 A (170000 Pa) membrane and kept frozen at −70 °C.

Results and Discussion

Phosphate determination

Rathbun et al. [26] have described a method to estimate the enzymatically produced P_i, in the presence of CTP and ATP. This method can be used also for P_i determination in the presence of PP_i. The sensitivity of the method is, however, dependent of the PP_i concentration. At PP_i concentration lower than 0.35 mM, the method is as sensitive as without PP_i. But at higher concentrations the method gradually loses its sensitivity, and misleading results may be obtained, if special attention is not given to the remaining PP_i concentration.

Solubilization and stabilization of the enzyme

The inorganic pyrophosphatase has previously been solubilized from *R. rubrum*, G9, with 1.76% sodium cholate and 0.88 M $MgCl_2$ [21]. However, the combination of Triton X-100, $MgCl_2$ and ethylene glycol was more effective, giving a 30–40% higher yield of enzyme. Since ethylene glycol suppresses hydrophobic interactions, and salt supresses electrostatic interactions, a medium containing both these agents together with detergent could be expected to give efficient solubilization of membranes. The solubilization effect was dependent on the concentrations of the different reagents. At higher detergent concentration than 2.2% (v/v) Triton X-100, the activity in the extract decreased, Fig. 1. Not only Triton X-100, but also $MgCl_2$ was necessary for the solubilization. Without $MgCl_2$ nearly no activity was solubilized, as was also found by Rao and Keister [21]. We obtained the best solubilization with a final concentration of 0.66 M $MgCl_2$. The solubilized enzyme was fairly stable for at least 5–10 days at

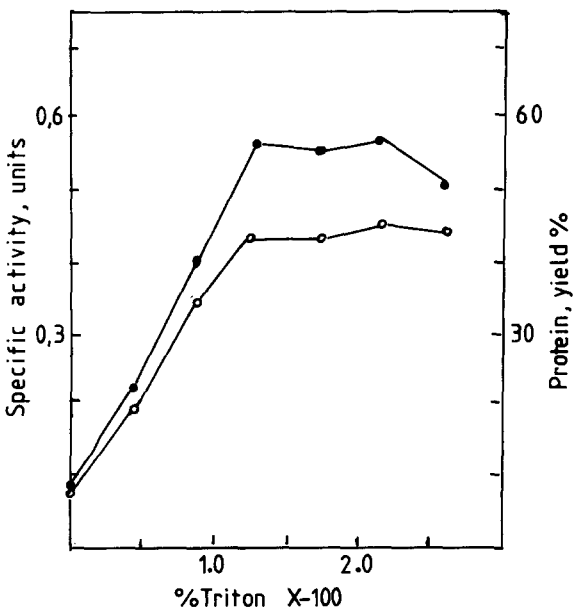

Fig. 1. Solubilization of the inorganic pyrophosphatase complex with Triton X-100. Chromatophores were solubilized with 22% (v/v) ethylene glycol/0.66 M $MgCl_2$/0-2.66% (v/v) Triton X-100. After incubation and centrifugation for 1 h at 100000 × *g*, the supernatant was assayed for inorganic pyrophosphatase activity and protein. O——O, protein; ●——●, activity.

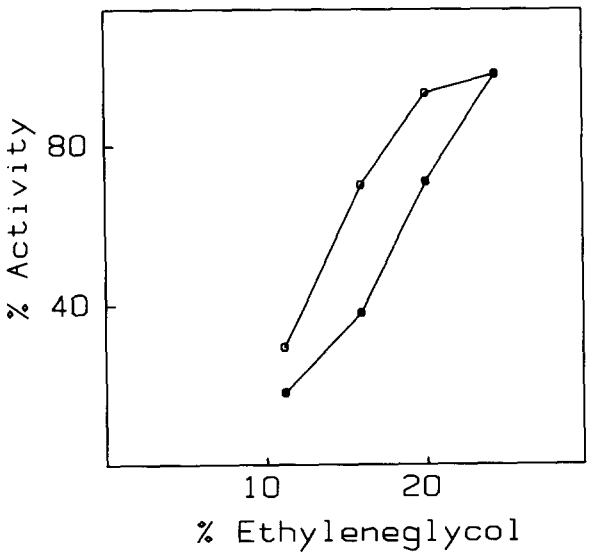

Fig. 2. Stabilization of the solubilized inorganic pyrophosphatase by ethyleneglycol. The solubilized inorganic pyrophosphatase was diluted 8-times to give the final specified concentration of ethylene glycol and $MgCl_2$. The diluted enzyme solution was stored on ice for 5 days and after that assayed for inorganic pyrophosphatase activity. O——O, 0.12 M $MgCl_2$; ●——●, 0.74 M $MgCl_2$.

0 °C in the dark. The stability was due to the high concentration of ethylene glycol present (Fig. 2). The solubilized enzyme could be stored frozen at −70 °C for at least 6 months without loosing activity. After solubilization, the addition of 3 mg sonicated L-phosphatidylcholine (from soybeans) per mg protein increased the activity up to 40%. Triton X-100 alone reconstitutes the enzyme activity to at least 70%. This is in contrast to the cholate-solubilized enzyme which is totally dependent on phospholipids for activity [21] and where detergent cannot reconstitute the activity.

Purification of the enzyme on hydroxylapatite

The solubilized enzyme is purified on an hydroxylapatite column, as described in the Methods section. The concentration of Triton X-100, 0.1% (v/v) in the equilibration buffer, was found to be critical. If the Triton X-100 concentration was higher, for instance 0.2% (v/v), the enzyme was not retained by the hydroxylapatite. In contrast, if the detergent concentration was lower, for instance

TABLE I

PURIFICATION OF THE MEMBRANE-BOUND INORGANIC PYROPHOSPHATASE FROM CHROMATOPHORES

Purification step	Total protein (mg)	Specific activity (μmol PP_i/min per mg protein)	Total activity (μmol PP_i/min)
Chromatophores (uncoupled 1.5 μM FCCP)	79	0.30	23.7
Triton X-100-extract	40	0.52	20.8
After hydroxylapatite	0.17	24	4.2

0.05% (v/v), a very high $MgCl_2$ concentration, at least 0.7 M, was necessary for the elution of the enzyme. After elution of the enzyme, the fractions showing highest inorganic pyrophosphatase activity were concentrated by ultrafiltration. The choice of filter depended on the intended use of the purified enzyme. The YM5 membrane was unfavourable when the enzyme was used for reconstitution into liposomes, because Triton X-100 micelles were also concentrated (the micellar molecular weight is higher than 90000 at high ionic-strength according to Robson and Dennis [32]).

Table I gives a summary of the specific activity and yield of fractions. A 80-fold purification was obtained, and the recovery was about 20% of the original activity. This purification method results in a more than 10 times purer preparation of the membrane-bound inorganic pyrophosphatase than the previously reported method [21].

Native gel electrophoresis

When 20 μg of the purified inorganic pyrophosphatase was subjected to electrophoresis on a polyacrylamide gel with 0.1% (v/v) Triton X-100, a heavy protein band was observed (Fig. 3) at the same position as that obtained after staining the gel for inorganic pyrophosphatase activity. Sometimes two minor bands with faster and slower mobilities were observed. Their origin is uncertain.

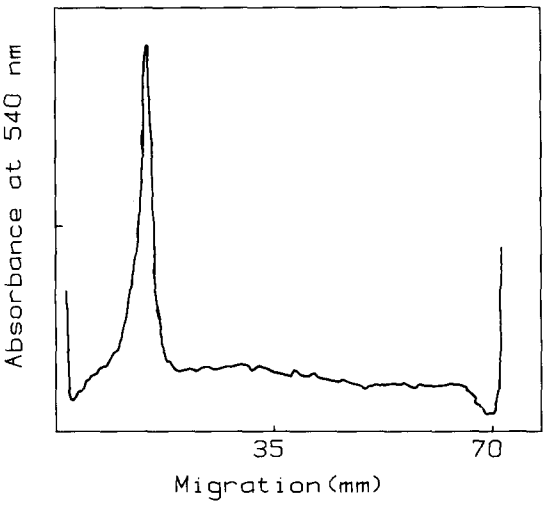

Fig. 3. Densitometric trace of Triton X-100 polyacrylamide gel after electrophoresis of purified inorganic pyrophosphatase from *R. rubrum*. 20 μg pyrophosphatase was run and the gel was stained for protein with Coomassie Blue.

Effects of different compounds on the inorganic pyrophosphatase activity

A 50% stimulation of the chromatophore bound inorganic pyrophosphatase activity was observed with 1.5 μM FCCP. The purified inorganic pyrophosphatase was not stimulated by uncouplers unless it was reconstituted into liposomes [22]. DCCD (100 μM), which inhibited the inorganic pyrophosphatase activity of chromatophores to 65–75% did not inhibit the purified enzyme. The presence of Triton X-100 may be responsible for the lack of sensitivity of the inorganic pyrophosphatase to DCCD [33,34]. Fluoride, methylene-diphosphonate and imidodiphosphate, known inhibitors of inorganic pyrophosphatase activity, inhibited the purified inorganic pyrophosphatase to a high degree. *N*-Ethylmaleimide has been shown to inhibit the chromatophore inorganic pyrophosphatase after preincubation at 0 °C but not at 30 °C [35]. *N*-Ethylmaleimide has the same effect

TABLE II

EFFECT OF SOME SUBSTANCES ON MEMBRANE-BOUND AND PURIFIED INORGANIC PYROPHOSPHATASE

Particles corresponding to 120 μg protein and purified enzyme corresponding to 15 μg protein were assayed. For the methylene-diphosphonate and imidodiphosphate treatment 12.5 mM $MgCl_2$ was used instead of 0.75 mM. The DCCD, *N*-methylmaleimide and NBF-Cl treatment was performed by incubation of particles and enzyme for 10 min at 0 or 30 °C. Dio-9 was incubated with particles and enzyme for 20 min at 0 °C. The inorganic pyrophosphatase reaction was initiated by adding PP_i.

Addition	Concn.	Activity of inorganic pyrophosphatase (% of control)	
		membrane-bound	purified
FCCP	1.5 μM	150	100
DCCP (0 °C)	100 μM	30	97
NaF	5 mM	83	32
	10 mM	64	11
	20 mM	45	4
Methylenedi-	0.1 mM	84	64
phosphonate	0.2 mM	74	36
Imidophosphate	0.1 mM	48	24
	0.2 mM	34	12
NBF-Cl (°C)	0.25 mM	33	23
	0.50 mM	18	7
NBF-Cl (30 °C)	0.25 mM	82	12
	0.50 mM	61	1
N-methylmaleimide	1 mM	30	20
(0 °C)	2 mM	13	13
N-methylmaleimide	1 mM	99	84
(30 °C)	2 mM	85	76
Dio-9 (0 °C)	15 μg/ml	80	40
	30 μg/ml	60	30

on the purified enzyme. An absolute requirement of preincubation at 0°C for obtaining inhibition by *N*-ethylmaleimide of inorganic pyrophosphatase activity can be interpreted by assuming that a temperature-dependent conformation change is necessary in order to expose one (or more) sulphydryl groups, essential for the inorganic pyrophosphatase activity, to the action of the inhibitor. The inhibition of the chromatophore inorganic pyrophosphatase activity by NBF-Cl is also dependent on the preincubation temperature, in the same manner as the inhibition by *N*-ethylmaleimide. However, the purified enzyme is inhibited by NBF-Cl independent on the preincubation temperature. This difference might indicate that a temperature-dependent change in the membrane structure is necessary for the action of NBF-Cl on the chromatophore inorganic pyrophosphatase activity. The inactivation of the membrane-bound inorganic pyrophosphatase by NBF-Cl might be due to a chemical modification of a tyrosine residue as has been shown to be the case for the mitochondrial ATPase [36]. The energy-transfer inhibitor Dio-9 was an effective inhibitor of the purified inorganic pyrophosphatase, as it is for the membrane energization due to PP_i in chromatophores [37]. In the solubilized form the inorganic pyrophosphatase activity loses the stimulation by uncoupler as well as the inhibition by DCCD, inferring that the proton translocating function of the enzyme is also lost. On the other hand, the inhibitors more likely to inhibit at groups involved in catalysis, NBF-Cl, *N*-ethylmaleimide, Dio-9, methylenediphosphonate and imidodiphosphate, are still effective with the solubilized enzyme.

Acknowledgements

The authors wish to thank Dr. Y.A. Shakov and M.Sc. O. Breig for their stimulating discussions and great help. This work was supported by a grant from the Swedish Natural Science Research Council to M. Baltscheffsky.

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