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ENERGY-LINKED REACTIONS IN PHOTOSYNTHETIC BACTERIA. X. SOLUBILIZATION OF THE MEMBRANE-BOUND ENERGY-LINKED INORGANIC PYROPHOSPHATASE OF RHODOSPIRILLUM RUBRUM*

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Summary: The energy-linked membrane-bound inorganic pyrophosphatase of Rhodospirillum rubrum, G-9, has been solubilized with good yield from chromatophores using cholate in the presence of $MgCl_2$. The enzyme has been partially purified using ammonium sulfate fractionation and gel chromatography. After fractionation the enzyme requires phospholipid for activity. The solubilized enzyme is specific for PP_i and requires Mg^{2+} for activity as has been found for other PP_iases.

Rhodospirillum rubrum is a unique organism that can couple the synthesis of inorganic pyrophosphate to light-induced electron transport (1). The importance of PP_i[1] in energy conservation is suggested from the observations that both PP_i and ATP can serve as an energy-donor for energy-linked transhydrogenation (2), succinate-linked NAD^+ reduction (3), cytochrome reduction (4) and proton uptake (5). Further, we demonstrated that PP_i can be used to drive the synthesis of ATP (6).

Our studies on the mechanism of the membrane-bound PP_iase

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1. Abbreviations used are: PP_i, inorganic pyrophosphate; PP_iase, inorganic pyrophosphatase (EC 3.6.1.1); PE, L-α-phosphatidyl ethanolamine; PC, L-α-phosphatidyl choline; Bchl, bacteriochlorophyll.

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began with a study of the energy-linked $P_i \leftrightarrows PP_i$ exchange reaction catalyzed by the enzyme (7), and are continuing with this paper which reports the solubilization of the enzyme. After considerable effort, we have developed a method for solubilizing the enzyme in good yield. The enzyme is hydrophobic after solubilization and after fractionation requires phospholipid(s) for activity. Detergents do not reconstitute the enzyme activity as have been found with some membrane enzymes. The solubilized enzyme is specific for PP_i and requires Mg^{++} for activity as is typical of several soluble PP_iases (8,9). We have partially purified the enzyme and carried out initial studies on the phospholipid and Mg^{++} requirements.

Materials and Methods. The carotenoid-less mutant of R. rubrum, strain G-9, was grown in the synthetic medium of Cohen-Bazire et al. (10) supplemented with 0.1% yeast extract and 0.1% casamino acids. Cultures were grown in filled glass-stoppered bottles using a 5% inoculum. After inoculation, the cultures were placed in the dark for 8-16 hours to allow the cultures to become anaerobic. The cells were then grown in the light ($\sim$ 200 ft. candles of incandescent light) for 72 hours at 28°. Chromatophores were prepared as previously described (11) and the pellets were frozen and stored at -70° until used. PP_iase activity was determined at 40°C in the following reaction mixture: Tris-Cl, pH8, 50mM; PP_i 1mM; $MgCl_2$, 0.75 mM and 1mg/ml L-α-phosphatidyl ethanolamine (PE Sigma Cat. #P4264) when required. PE was prepared by sonicating 3 mg/ml in 20 mM Tris-1mM EGTA, pH8, until fairly clear. The reaction was terminated by the addition of trichloroacetic acid to 5%. Inorganic phosphate was determined by the standard Fiske-SubbaRow procedure.

Chemicals were obtained from the Sigma Chemical Co. Natural lipid was extracted from chromatophores with chloroform : methanol (2:1), evaporated to dryness, and suspended in Tris-EGTA as above. Lipid phosphorous was determined using the method of Chen (12) as modified by Ames and Dubin (13). Protein was determined using the Lowry procedure (14) or the method of Ross and Schatz (15) when sulphydryl compounds were present.

RESULTS

Solubilization of the enzyme. Many techniques which have been successfully used for solubilizing other membrane-bound enzymes

including organic solvents, chaotrophic agents and several detergents were tried with limited or no success with the membrane-bound PP_iase. Occasionally a small amount of soluble enzyme was observed when detergent and a salt such as KCl or $(NH_4)_2SO_4$ was used in combination. A detailed examination of the effect of several salts in the presence of 1.5-2% cholate led to the selection of cholate and $MgCl_2$ as the most effective combination. The concentration dependence of these reagents is shown in Table I. Experiment 1 illustrates that a relatively high concentration of $MgCl_2$ is required while experiment 2 illustrates the cholate requirement. Higher concentrations of cholate solubilized more protein and gave less total recovery of activity. The concentrations which were finally determined to give good solubilization are shown in experiment 3. Note that 73 percent of the initial activity was recovered and that most of the activity was solubilized as judged by lack of sedimentation at 226,400 X g (maximum rcf) for 90 minutes. Although this is a very arbitrary criteria of solubilization, a better criteria is presented in Figure 1 and will be discussed below.

In an attempt to obtain a more purified solubilized enzyme we thought that a sequential treatment of the chromatophores with cholate followed by $MgCl_2$ or the reverse may be helpful. This treatment gave very little solubilization as is shown in Table II. Only when cholate and $MgCl_2$ were present together was any significant solubilization obtained.

Partial Purification of the Enzyme (Table III)

Solubilization and Ammonium Sulfate Fractionation (P_{50-60}). Chromatophores of R. rubrum G-9 were suspended in pH8 Tris (10mM)-glycerol (20%)-DTT (5mM) to an A_{880} of about 0.4. $MgCl_2$ (2.5M) and sodium cholate (5%, pH8) were added dropwise to give a final

Table I. Effect of $MgCl_2$ and Cholate on Solubilization of the Membrane-bound PP_iase

	Treatment		% of Activity	Total Activity
	$MgCl_2$ (mM)	Cholate (%)	in Supernatant	Recovered %
Expt. 1	0	0	11	98
	5	1	11	84
	50	1	19	68
	200	1	32	64
	500	1	50	70
Expt. 2	200	0.1	13	100
	200	0.5	23	70
	200	1.0	32	64
	200	2.0	44	61
Expt. 3	880	1.76	66	73
	880	0	4	72
	0	1.76	0	25

Chromatophores were suspended in Tris (10 mM)-glycerol (20%)-DTT (5 mM) and $MgCl_2$ and cholate were added dropwise. After stirring for 1 hour at 4°C the suspensions were centrifuged for 90 minutes at 226,400 x g (maximum rcf).

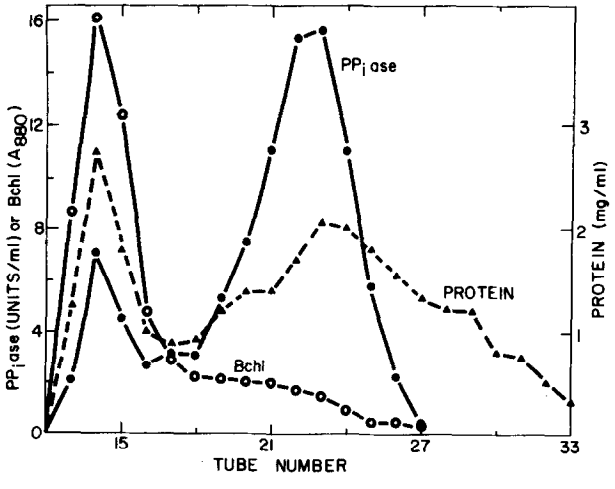

Fig. 1. Chromatography of P_{50-60} on Biogel A1.5M. Fifty-two mg protein was loaded on the column and eluted at 6 ml per hour and collected in 1.5 ml fractions. See text for additional details.

concentration of 0.88 M and 1.76%. The mixture was stirred for one hour at 4°C. A saturated solution of ammonium sulfate (pH8)

Table II. $MgCl_2$ and Cholate Together are Required for Solubilization

	$MgCl_2$ (mM)	Cholate (%)	% of Activity in Supernatant	Total Activity Recovered %
	Chromatophores			
1.	880	0	5	72
2.	0	1.76	8	41
	Insoluble pellet of 1.			
3.	0	1.76	0	25
4.	880	1.76	53	55
	Insoluble pellet of 2.			
5.	880	0	0	60
6.	880	1.76	97	56

Chromatophores were suspended and treated as in Table I. The pellets from the 90 minute centrifugation of 1 and 2 were treated with cholate or $MgCl_2$ or both in order to demonstrate that both reagents together were required for solubilization.

Table III. Purification of PP_iase

	Fraction	Total ml	Total Protein (mg)	Total Units	Specific Activity	% Recovery
a.	Chromatophores	36	585	585	1.0	100
b.	P_{50-60}	1.4	52	258	5.0	44
c.	Biogel A1.5M Tubes 21-23	4.5	7.7	81	10.5	14

See text for details.

was then added dropwise to give a 30% final concentration. After 30 minutes, the precipitated solids were removed by centrifugation. More ammonium sulfate was added to give a 50% final concentration and the solids again removed as above. Ammonium sulfate solution was again added to 60% concentration, the solids were collected

by centrifugation and suspended in as small a volume as possible of Tris (10 mM)-glycerol (20%)-DTT (5 mM)-cholate (2%). Solubilization of the pellets into concentrated solution requires about one hour with stirring although this occurs more rapidly if only low protein concentrations are desired. The preparation is quite stable for several months at this stage if frozen and stored at -70°C. It has very low activity and requires phospholipid for activity. This is shown in Table IV and will be discussed below.

P_{60-75}. The supernatant from the 60% step was brought to 75% by adding solid $(NH_4)_2SO_4$. The solids are removed by centrifugation to yield a loose pellet which appears to be enriched in phospholipids.

Gel Chromatography on Bio-Gel A1.5M. Bio-Gel A1.5M, 200-400 mesh (Bio·Rad Laboratories) was equilibrated with Tris (10 mM)-glycerol (20%)-$MgCl_2$ (0.5M)-cholate (2%)-DTT (5 mM) at pH8 and poured into a 1.5 X 50 cm column. The enzyme was eluted with the same buffer mixture and collected in 1.5 ml fractions. The elution profile is shown in Figure 1. Some activity and the bulk of the remaining bacteriochlorophyll elutes in the void volume. The bulk of the enzyme applied to the column elutes at about 1.6 times the void volume. We have not carefully calibrated this column but this indicates a molecular weight of less than 200,000 including the bound detergent which has not yet been determined. This fractionation by gel chromatography is good evidence that the enzyme has been solubilized.

Collection of the three most active fractions (21-23) gives a yield of 14% and a 10-fold purification from the chromatophores. The enzyme is quite stable frozen at -20°C. Attempts to remove the buffer and detergent have led to loss of activity and aggre-

Table IV. Reconstitution of PP_iase

Fraction	Additions	μmole P_i/min
P_{50-60} (0.61 mg protein)	---	0.03
P_{60-75}(a) (0.3 ml)	---	---
P_{69-75}(b) (0.1 ml)	---	0.12
P_{50-60}	P_{60-75}	0.43
P_{50-60}	P_{60-75}	0.38
P_{50-60}	PE	0.44
PE	---	---

The P_{60-75} contains a small amount of activity when freshly prepared but loses activity on storage. P_{60-75}(a) was an older fraction which had no activity alone whereas P_{60-75}(b) was freshly prepared and had some activity. They were titrated to give maximum activity with the P_{50-60} fraction at the levels listed. PE containing 50 μg phosphorous was added.

gation of the protein. Storage of the enzyme with phospholipid added also yields an inactive preparation perhaps indicating cold lability when phospholipid is present.

Reconstitution of PP_iase Activity. After ammonium sulfate fractionation, we found that the separated fractions had little activity. Combining the P_{50-60} and the P_{60-75} fractions restored activity. Since the P_{60-75} fraction appeared to be a lipid-rich fraction, we substituted PE and found that it would restore full activity. These results are shown in Table IV.

The concentration dependence of several phospholipids is shown in Figure 2. There appears to be little specificity for any of those tested for obtaining maximum activity. However, at low concentrations the natural lipid and cardiolipin were most effective. It should be noted that all of the lipids tested

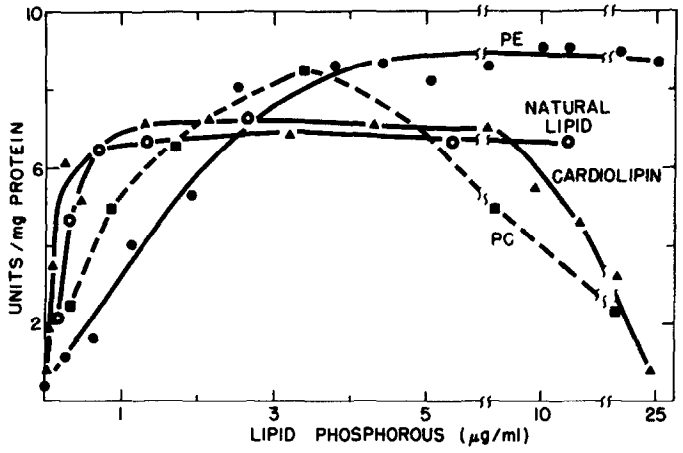

Fig. 2. Reconstitution of PP_iase activity with phospholipids. Partially purified enzyme following gel chromatography was used. Phospholipids, suspended as described in Materials and Methods, was added to the enzyme in the assay mixture and preincubated 6 minutes before adding substrate.

here are found as natural constituents of the R. rubrum membranes (16) and thus the reactivation by these lipids may not be a good criteria of specificity.

Specificity. AMP, ADP, ATP and β-glycerophosphate were hydrolyzed at 0, 2.7, 1.3 and 1.4%, the rate of PP_i with the P_{50-60} preparation. Thus even at this relatively impure stage the enzyme is quite specific for PP_i. Mg^{++} is required for activity and the optimal Mg^{++}/PP_i ratio is about 0.75. Preliminary work suggests that this less than stoichiometric requirement for Mg^{++} is due to strong inhibition by the Mg_2PP_i complex. The Mg_2PP_i complex is also a substrate for the enzyme but the activity with this substrate is much lower than with the $MgPP_i^{-2}$ complex.

Discussion. For several years we have been interested in the energy-transduction coupled to PP_i synthesis or hydrolysis by R. rubrum and have published several studies on energy-linked reactions catalyzed by the membrane-bound PP_iase in

chromatophores. Now with the solubilization of the enzyme it is possible to study it's properties apart from the membranes. Soluble PP_iases are small molecules compared with energy-transducing ATPases and the R. rubrum soluble PP_iase has a reported molecular weight of about 100,000 (17). The solubilized membrane-bound enzyme appears to be considerably smaller than the ATPase complex and thus may provide a less complex molecule with which to pursue studies of energy-coupling. The enzyme appears to have a number of interesting catalytic properties including the lipid requirement and a complex Mg^{++} requirement in which both $MgPP^{-2}$ and Mg_2PP_i are substrates. Studies on these requirements and on reconstitution experiments will be the subject of future publications.

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