A membrane-bound pyrophosphatase from respiratory membranes of *Rhodospirillum rubrum*

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A pyrophosphatase activity was found in respiratory membranes of *Rhodospirillum rubrum*. This activity was specific for pyrophosphate and was inhibited by dicyclohexylcarbodiimide, NaF and pyrophosphate analogues, but not by oligomycin or LiCl. The divalent cation selectivity was $\text{Zn}^{2+} > \text{Mg}^{2+} > \text{Co}^{2+} > \text{Ca}^{2+}$, and the pH dependence was the same as that of the membrane-bound pyrophosphatase of chromatophores (optimum pH 6.5). The pyrophosphate hydrolysis activity of respiratory membranes was inhibited by 1-butanol. The enzyme was solubilized by Triton X-100, and the activity of the solubilized enzyme was stimulated by phospholipids. The respiratory-membrane enzyme ran in native electrophoresis with the same R_F as the membrane-bound pyrophosphatase of chromatophores. Antibodies raised against both enzymes cross-reacted with each other. These findings show that a membrane-bound pyrophosphatase is present in respiratory membranes of *R. rubrum* and is similar to the enzyme of photosynthetic membranes.

Introduction

The photosynthetic bacterium *Rhodospirillum rubrum* is a member of the family *Rhodospirillaceae*. These organisms are remarkably versatile in obtaining growth energy through alternative mechanisms. They can grow in the dark chemotrophically in the presence of O_2 by the oxidation of substrates, or phototrophically in the absence of O_2 .

In phototrophically grown bacteria, extensions and modifications of the cytoplasmic membrane are formed and can be easily isolated. These pigmented membranes, called chromatophores, contain the photosynthetic electron transport and ATP synthesis enzymes. H. Baltscheffsky *et al.* (1966) reported that chromatophores contain a membrane-bound pyrophosphatase which is coupled reversibly to the H^+ gradient (Guillory & Fisher, 1972; Moyle *et al.*, 1972). In chemotrophically dark-grown cells, the fragments derived from the cytoplasmic membrane are enriched in respiratory chain activity (Taniguchi & Kamen, 1965; Thorne *et al.*, 1969). These membranes contain very low concentrations of bacteriochlorophyll and carotenoids. However there have been no reports of a bound pyrophosphatase in respiratory membranes.

In this work, we report the existence and characteristics of a membrane-bound pyrophosphatase activity in

respiratory membranes of *R. rubrum*, and compare this enzyme with the membrane-bound pyrophosphatase of chromatophores.

Methods

Cell cultures and membrane preparations. *Rhodospirillum rubrum* (ATCC 11170) was grown chemotrophically in the dark at 30 °C. We used 1 litre Erlenmeyer flasks, which contained 200 ml of the growth medium described by Cohen-Bazire *et al.* (1957); intense aeration was provided by gyratory shaking. Cells were harvested during the exponential phase and contained 0.3 µg bacteriochlorophyll (mg cell protein)⁻¹. The respiratory membranes were prepared as described by Fenoll & Ramírez (1984). For phototrophic growth, the cells were grown anaerobically in light from tungsten lamps (30 W) 30 cm from the flasks and at 30 °C in the same medium as above. The bacteria were harvested in the exponential growth phase and contained 25 µg bacteriochlorophyll (mg protein)⁻¹. The chromatophores were prepared by ultrasonic rupture of the cells and centrifugation as previously described (Celis *et al.*, 1985). For experiments with divalent cations, the residual Mg^{2+} was eliminated from the respiratory membranes and chromatophores by washing them with 5 mM-EDTA, pH 7.5, 5 mM-EGTA, pH 7.5, and 10 mM-Tris/HCl, pH 7.5, followed by a second washing with 10 mM-Tris/HCl, pH 7.5. Cytoplasmic pyrophosphatase of *R. rubrum* was partially purified as described by Klemme & Gest (1971).

Pyrophosphatase and ATPase activities. Hydrolysis of PP_i and ATP was determined in the dark with a Green safety light. The solutions contained: for PP_i hydrolysis, 50 mM-Tris/maleate, pH 6.5, 2.0 mM-sodium pyrophosphate and 5.0 mM- MgCl_2 ; for ATP hydrolysis, 50 mM-

Tris/acetate, pH 7.5, 3.0 mM-ATP, 5.0 mM-MgCl₂, 1.45 mM-phosphoenol pyruvate and 7 µg pyruvate kinase. The final concentration of chromatophores or respiratory membranes was 1 mg protein in 0.5 ml mixture. The temperature was 30 °C. The reaction was stopped by adding trichloroacetic acid to a final concentration of 6.0%. Phosphate was determined in the supernatant as described by Sumner (1944).

Protein and bacteriochlorophyll determinations. Protein was measured by the method of Lowry. Bacteriochlorophyll content was determined according to Clayton (1963).

Solubilization of membrane-bound pyrophosphatase. The solubilization of the pyrophosphatase of respiratory and photosynthetic membranes was done according to the procedure of Nyrén *et al.* (1984) as follows: 1 ml of respiratory or photosynthetic membranes (70 mg protein ml⁻¹) was suspended in 7 ml buffer containing 50 mM-Tris/HCl, pH 8.2, 25% (v/v) ethylene glycol, 600 mM-MgCl₂, 0.2 mM-dithioerythritol and various concentrations of Triton X-100. After gentle stirring on ice for 20 min, the suspension was centrifuged at 110000 g for 2 h, and the supernatants were analysed for pyrophosphatase activity. The phospholipids were prepared as follows: L- α -lecithin from soybean, commercial grade (Sigma) was washed with acetone and ether (Kagawa & Racker, 1966) and 60 mg ml⁻¹ of washed phospholipids were suspended in 10 mM-Tris/HCl, pH 7.5, and sonicated to give a uniform suspension which was used for experiments.

Gel electrophoresis. The solubilized respiratory and photosynthetic pyrophosphatases were analysed by non-denaturing electrophoresis using the method of Davis (1964) on slab gels (1.5 mm thickness). Electrophoresis, with 10% acrylamide running gels and 4.5% stacking gel, was carried out at 4 °C in 24.8 mM-Tris, 191.8 mM-glycine buffer, pH 8.3, for 5 h at 30 mA per gel. To localize the pyrophosphatase activity, the gels were incubated for 30 min at 37 °C in 50 mM-Tris/HCl, pH 8.5, 3 mM-sodium pyrophosphate and 5 mM-MgCl₂. The gels were then washed once with water and immersed in a solution at 45 °C of 10% ascorbate and 0.42% ammonium molybdate in 0.5 M-H₂SO₄ (1:6, v/v) until blue bands showing the position of enzyme were clearly visible. Gels were rinsed quickly with distilled water, and immediately scanned at 660 nm with a Beckman DU-50 spectrophotometer gel scanner.

Antibodies. The bands with pyrophosphatase activity were excised and homogenized. Mouse antibodies were raised against these mixtures by the intramuscular injection of 0.1 ml of the preparation (7 µg protein) in 0.1 ml of Freund's complete adjuvant and a intraperitoneal injection of 0.9 ml (60 µg protein) of the mixture without adjuvant. This procedure was repeated on days 7 and 21 after the first inoculation. Bleeding was performed 7 d after the last injection and antisera were used for ELISA experiments.

Two-dimensional denaturing (sodium lauryl sulphate) polyacrylamide gel electrophoresis of the bands with pyrophosphatase activity showed a band around 56 kDa, and no contaminants were detected.

Enzyme-linked immunosorbent assay (ELISA). Indirect ELISA was done as described by Nakamura *et al.* (1986). ELISA immunoplates (Dynatech Immulon) were adsorbed with solubilized respiratory and photosynthetic pyrophosphatase, which reacted with the antisera. Anti-mouse antibodies conjugated with horse-radish peroxidase were then added and the colour reaction was developed with orthophenylenediamine and H₂O₂ as substrate and stopped with 2.5 M-H₂SO₄. Absorbance was read at 492 nm in a Bio-Rad EIA reader.

Reproducibility. The experiments shown in each Figure or Table were all repeated two or more times to confirm that the results were reproducible. Representative data are presented.

Table 1. Comparison of specific activities of pyrophosphatase (PP_iase) and ATPase in chromatophores and respiratory membranes of *Rhodospirillum rubrum*

	$\mu\text{mol P}_i \text{ h}^{-1}$ (mg protein) ⁻¹		$\mu\text{mol P}_i \text{ h}^{-1}$ (mg Bchl) ⁻¹	
	PP _i ase	ATPase	PP _i ase	ATPase
Chromatophores	6.8	2.4	119.0	42.1
Respiratory membranes	3.7	4.1	50320.0	55983.0

Results and Discussion

The pyrophosphatase and H⁺-ATPase activities of respiratory membranes and chromatophores are shown in Table 1. When the PP_i hydrolysis activity was normalized with respect to protein, the specific activity found in respiratory membranes was 30–50% of that found in chromatophores; however, when the ATPase activity was compared, the ATPase activity was higher in respiratory membranes than in chromatophores. When the specific activities for hydrolysis of PP_i and ATP were normalized with respect to bacteriochlorophyll, very high activities of both enzymes were found. This was due to the decrease in bacteriochlorophyll content in respiratory membranes. Since it is known that ATP hydrolysis is catalysed by the same enzyme in chromatophores and respiratory membranes (Melandri *et al.*, 1971) these findings strongly imply that the PP_i hydrolysis found in respiratory membranes was not due to chromatophore contamination.

Effects of inhibitors and substrate specificity of the pyrophosphatase of respiratory membranes

The pyrophosphatase and H⁺-ATPase activities in chromatophores and respiratory membranes were compared in their sensitivity to classical inhibitors of these activities. These data are shown in Table 2. M. Baltscheffsky *et al.* (1966) found that oligomycin strongly inhibits the H⁺-ATPase of chromatophores, but not the membrane-bound pyrophosphatase, which we confirmed. Dicyclohexylcarbodiimide (DCCD) is an inhibitor of the H⁺ channel of both mitochondrial and chromatophore H⁺-ATPases (Schmid *et al.*, 1981; Sebald & Hoppe, 1981) and also inhibits the pyrophosphatase activity (Baltscheffsky *et al.*, 1982). Table 2 shows that the H⁺-ATPase and pyrophosphatase of chromatophores and respiratory membranes were inhibited by 480 µM-DCCD. NaF is a specific inhibitor of cytoplasmic and membrane-bound pyrophosphatases (Keister & Minton, 1971a) but not H⁺-ATPase. The same was found in this work for the pyrophosphatase

Table 2. *Effect of various inhibitors on the ATPase and pyrophosphatase activities of chromatophores and respiratory membranes*

Conditions of measurement as in Methods.

Compound	Concentration	Inhibition (%)		
		ATPase	Pyrophosphatase	
		Chromatophores*	Chromatophores†	Respiratory membranes‡
Oligomycin	20 µg ml ⁻¹	85.3	1.5	4.7
DCCD	480 µM	85.0	65.1	87.4
NaF	10 mM	8.6	100.0	100.0
Imidodiphosphate	20 mM	—	80.0	74.0
Methylenediphosphate	20 mM	0.0	50.0	60.0
LiCl§	2 M	96.6	0.0	0.0¶

* 100% activity corresponds to 2.5 µmol P_i h⁻¹ (mg protein)⁻¹.† 100% activity corresponds to 6.65 µmol P_i h⁻¹ (mg protein)⁻¹.‡ 100% activity corresponds to 3.55 µmol P_i h⁻¹ (mg protein)⁻¹.

§ LiCl inhibition measurements were made as follows: 10 mg protein from respiratory membranes or chromatophores was suspended in 3 ml 100 mM-glycylglycine, pH 8.0, and 10% (w/v) sucrose, and then diluted to 50 ml at final concentration of 250 mM-glycylglycine, pH 8, and 2 M-LiCl, incubated for 10 min at 4 °C, with agitation, then centrifuged at 110000 g for 60 min. The pellets were resuspended in the initial buffer and their activities measured as in Methods.

|| 37% stimulation.

¶ 76% stimulation.

activity of respiratory membranes. Moreover, the competitive inhibitors methylenediphosphate and imidodiphosphate inhibit the membrane-bound pyrophosphatase of chromatophores (Keister & Minton, 1971*b*; Nyren & Baltscheffsky, 1983) and we found that they also inhibited the pyrophosphatase of respiratory membranes. LiCl, at high concentrations (2 M) inhibits the H⁺-ATPase of chromatophores, because it releases the β -subunit from F₁ (Pilosoph *et al.*, 1977). However, although the membrane-bound pyrophosphatase of chromatophores is said to be insensitive to this compound (Guillory & Fisher, 1972), we found this enzyme was activated. The respiratory membranes pyrophosphatase activity was also activated by LiCl (Table 1). In summary, the pattern of sensitivity to inhibitors of the pyrophosphatase of respiratory membranes was the same as that found in chromatophores.

To assay the specificity of the pyrophosphatase present in respiratory membranes, α -glycerophosphate, β -glycerophosphate, D-glucose 1-phosphate, D-glucose 6-phosphate, AMP and *p*-nitrophenylphosphate were tested at 2 mM concentration (data not shown). Except for ADP, which is hydrolysed at 20% the rate of PP_i, none of these compounds were hydrolysed by the enzyme at a rate of more than 2% of the rate of PP_i. Thus, the pyrophosphatase of respiratory membranes is highly specific for PP_i as substrate.

Effect of pH and divalent cations

To compare the pyrophosphatase of respiratory membranes with the one present in chromatophores, two kinetic characteristics were studied: the pH dependence and the divalent cation requirement. The pH profile obtained for the PP_i hydrolysis in chromatophores and respiratory membranes is shown in Fig. 1. The pH optimum for chromatophores was 6.5, as reported previously (Celis & Romero, 1987). Although the pyrophosphatase activity in respiratory membranes was lower than in chromatophores, the pH optimum was the same. Moreover, the optimum pH for cytoplasmic pyrophosphatase of *R. rubrum* is 8.6–9.5 (Klemme & Gest, 1971). This comparison indicates that the pyrophosphatase activity found in respiratory membranes was not due to cytoplasmic pyrophosphatase contamination.

The membrane-bound pyrophosphatase of chromatophores, like most microbial pyrophosphatases, requires Mg²⁺ for hydrolysis (Lathi, 1983). Mg²⁺ forms a Mg-PP_i complex which is the true substrate for the enzyme (Randahl, 1979; Celis *et al.*, 1985). Recently, we reported that the Zn-PP_i complex at low concentrations is a better substrate than the Mg-PP_i complex (Celis & Romero, 1987). Fig. 2 shows the cation dependence of the pyrophosphatase of respiratory membranes. Both

Fig. 1. pH profiles of pyrophosphatase of chromatophores and respiratory membranes. The incubation media contained 50 mM-Tris/maleate at the pH values indicated in the abscissa, 2 mM-sodium pyrophosphate, 5 mM-MgCl₂ and 1 mg of protein from chromatophores (○) or respiratory membranes (●).

Fig. 2. Effect of divalent cations on the pyrophosphatase activity of respiratory membranes from *Rhodospirillum rubrum*. The assay medium contained: 50 mM-Tris/maleate, pH 6.5, 0.67 mM-sodium pyrophosphate and the indicated concentration of Co²⁺ (○), Ca²⁺ (□), Mg²⁺ (■) or Zn²⁺ (●).

Mg-PP_i and Zn-PP_i were good substrates for the pyrophosphatase activity, and Co-PP_i was less efficient. Very low activity was found with Ca-PP_i. At high concentrations (>1.0 mM), Zn²⁺ inhibited the activity completely and Mg²⁺ inhibited it partially. This was probably due to the presence of free Zn²⁺ and free Mg²⁺ at these high concentrations (Celis & Romero, 1987). The profile of divalent cation requirement obtained for respiratory-membrane pyrophosphatase was the same as that obtained by Celis & Romero (1987) for the membrane-bound pyrophosphatase of chromatophores.

These observations strongly suggest two points. On the one hand, the pyrophosphatase found in respiratory membranes is very similar to that of chromatophores, and may be the same enzyme. On the other hand, the pyrophosphatase is a different molecular entity from the H⁺-ATPase.

Fig. 3. Effect of 1-butanol on the activity of pyrophosphatase of chromatophores, respiratory membranes and cytoplasmic pyrophosphatase. The reaction mixture was as in Methods, and contained 50 μg protein of cytoplasmic pyrophosphatase (PP_iase) where indicated. The maximal activity values (100%) were: for chromatophores (○), 6.5 μmol P_i h⁻¹ (mg protein)⁻¹; for respiratory membranes (●), 3.4 μmol P_i h⁻¹ (mg protein)⁻¹; and for cytoplasmic PP_iase (□), 720 μmol P_i h⁻¹ (mg protein)⁻¹.

Evidence that the pyrophosphatase of respiratory membranes is a membrane-bound enzyme

M. Baltscheffsky *et al.* (1966) showed that 1-butanol is a specific inhibitor of membrane-bound pyrophosphatase of chromatophores of *R. rubrum* but inhibits neither the cytoplasmic pyrophosphatase nor H⁺-ATPase. Fig. 3 shows inhibition as a function of 1-butanol concentration. The pyrophosphatase of respiratory membranes was inhibited in the same fashion as that of chromatophores. This result further indicates that the pyrophosphatase activity of respiratory membranes was due to a membrane-bound enzyme and is not the result of cytoplasmic pyrophosphatase contamination.

To support the above suggestion, the respiratory-membrane pyrophosphatase was solubilized with different concentrations of Triton X-100 (Fig. 4). In (a), the specific activity in the supernatant is shown. When the concentration of Triton X-100 was increased, enzyme was liberated from the membrane. Moreover, the specific activity was enhanced when soybean phospholipids were added to the measurement reaction mixture. More than 80% of the initial activity of the membrane was released with Triton X-100 (Fig. 4b). Other treatments, such as exposure to high ionic strength or washings with chelators, did not release the enzyme (data not shown).

In summary, the respiratory-membrane pyrophosphatase is an integral membrane protein according to the criteria of Singer & Nicolson (1972). This conclusion is

Fig. 4. Solubilization of the membrane-bound pyrophosphatase from respiratory membranes with Triton X-100. The solubilization was done as indicated in Methods. (a) The supernatant fractions were analysed for pyrophosphatase activity as in Fig. 1 at pH 6.5. The final concentration of supernatant protein in the assay tubes was 3 mg. The activity was measured with (○) and without (●) phospholipids. (b) Percentage extraction, expressed on the basis of activity (with phospholipids, ○) and protein (●).

Fig. 5. Densitometric trace of non-denaturing polyacrylamide gel after electrophoresis of solubilized membrane-bound pyrophosphatases. Triton X-100 solubilized photosynthetic (a) and respiratory-membrane-bound (b) pyrophosphatases were run and the gels were stained for hydrolytic activity as stated in Methods.

supported by the following facts: the respiratory-membrane pyrophosphatase was inhibited by 1-butanol, which inhibits only the membrane-bound pyrophosphatase (M. Baltscheffsky *et al.*, 1966) (Fig. 3), and the enzyme activity was released from the membrane by treatment with Triton X-100 and requires phospholipids for maximal activity.

Structural similarities between membrane-bound pyrophosphatases of respiratory and photosynthetic membranes

To determine the structural similarities between the two membrane-bound pyrophosphatases, an electrophoretic analysis of the activities was performed (Fig. 5). A

Fig. 6. Cross-reactivity of antibodies raised against photosynthetic and respiratory membrane-bound pyrophosphatases. In panel (a), solubilized photosynthetic membrane-bound pyrophosphatase (640 μg protein per well) was used as the basis for the indirect ELISA assay. In panel (b), solubilized membrane-bound pyrophosphatase from respiratory membranes (640 μg protein per well) was used as the basis for the assay. Antisera vs. photosynthetic membrane-bound pyrophosphatase (●); antisera vs. respiratory membrane-bound pyrophosphatase (○).

single band for both enzymes migrated to the same position, suggesting the same overall charge. Furthermore, the cross reactivity of mouse antibodies to the two proteins was tested by ELISA, using membrane-bound pyrophosphatase of photosynthetic membranes as base for antibody union assay (Fig. 6a) or membrane-bound pyrophosphatase from respiratory membranes (Fig. 6b). The binding was higher for the homologous antigen and antibody in each case, but the heterologous antibodies bound efficiently to the heterologous antigen, indicating structural similarities between the two enzymes.

The collected results suggest that membrane-bound pyrophosphatase is the same molecular entity in both photosynthetic and respiratory growing cells. The findings constitute the first evidence of the existence of a membrane-bound pyrophosphatase in respiratory membranes of the photosynthetic bacterium *R. rubrum*.

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