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Evidence of an Essential Carboxyl Residue in Membrane-Bound Pyrophosphatase of *Rhodospirillum rubrum*

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Chemical modifications with water-soluble carbodiimides (EDC and CMC) were performed to elucidate whether some carboxyl residues are involved in the catalytic activity of membrane-bound pyrophosphatase of *Rhodospirillum rubrum*. EDC and CMC cause a loss of hydrolytic activity following pseudo-first-order kinetics up to 10 min of reaction. The enzyme was completely protected against EDC inhibition by PPi or Mg^{2+}, whereas PPi or Mg^{2+} gave partial protection against CMC inactivation. Mg-PPi protected completely against the inhibition caused by both carbodiimides. These data suggest that the carboxyl moiety modified by EDC is at the active site. At longer times of inactivation with both carbodiimides, we could not observe a linear relationship in semilogarithmic plots of residual activity versus time, indicating that at least two carboxyls are involved in the inactivation, which correlates with the partial protection against CMC inactivation by PPi. We found that the activator site for Mg^{2+} is apparently at or near the active site of the enzyme. This is supported by the fact that PPi protects completely the activator effect of this divalent cation.

KEY WORDS: *Rhodospirillum rubrum*; membrane-bound pyrophosphatase; chemical modification; water-soluble carbodiimides; inactivation kinetics; active site residues.

INTRODUCTION

The chromatophore membrane of the photosynthetic bacteria *Rhodospirillum rubrum* contains a membrane-bound pyrophosphatase (EC 3.6.1.1) (Baltscheffsky *et al.*, 1966), which catalyzes the synthesis or hydrolysis of PPi linked to the electrogenic translocation of protons in a fully reversible process (Baccarini-Melandri and Melandri, 1978; Baltscheffsky, 1978). As for most inorganic pyrophosphatases, the real substrate for hydrolysis is the Mg–PPi^{2-} complex (Lahti, 1983) and the Mg–Pi complex for the synthesis (Guillory and Fisher, 1972; Celis *et al.*, 1985). Recent data suggest that Mg^{2+} and Zn^{2+} ions exert a regulatory action on the catalytic properties of the enzyme (Randahl, 1979; Celis and Romero, 1987). Hydrophobic carbodiimide, *N,N'*-Dicyclohexylcarbodiimide (DCCD), inhibits the membrane-bound pyrophosphatase (Baltscheffksy *et al.*, 1982), both in the chromatophore membrane and in the isolated state (Nyrén *et al.*, 1991). This hydrophobic compound is known to abolish proton pumping activity in other proton-translocating enzymes (Sebald and Hoppe, 1981). We are interested in understanding the molecular mechanism by which the negatively charged substrate interacts with the active site and how Mg^{2+} interacts in its regulatory site(s). As an important approach to this purpose we are attempting to obtain information about specific amino acids involved in substrate and activator binding to the enzyme, in particular the carboxyl moieties of glutamic and aspartic acids that could be involved in Mg^{2+} binding. In the present study, we investigated the presence of carboxyl residues in the membrane-bound pyrophosphatase by chemical modification with two water-soluble carbodiimides. The results suggest the existence of at least one essential carboxyl residue involved

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in the active site of the enzyme, which probably has also a role in the binding of free Mg^{2+}.

MATERIAL AND METHODS

1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) were obtained from Sigma, Chemical Co. (St. Louis, Missouri). All salts and buffers were of the highest grade available.

Bacterial Growth and Preparation of Chromatophores

Wild-type *Rhodospirillum rubrum* (ATCC 11170) were grown anaerobically in the light at 30°C in the medium described by Cohen-Bazire *et al.* (1957). Bacteria were harvested in the late exponential phase. The cells were washed with 50 mM MOPS and 50 mM KCl, pH 7.5, and chromatophores were prepared by sonication as in Celis and Romero (1987). The chromatophore preparation was kept at 4°C and used for the modification experiments within the next 3 days. Protein was determined by the method of Lowry *et al.*, 1951.

Pyrophosphatase Assay

The hydrolytic and modification reactions were determined in the dark with a green safety light. The medium contained 50 mM Tris-maleate, pH 6.5, 2 mM sodium PPi, pH 6.5, and 2 mM $MgCl_2$. After 5 min incubation at 30°C in a final volume of 0.5 ml, the reaction was arrested by adding trichloroacetic acid to a final concentration of 6.0%. Phosphate was determined in the supernatant as described (Sumner, 1944).

Reaction of Membrane-Bound Pyrophosphatase with Water-Soluble Carbodiimides

Carbodiimide stock solutions ($\sim$ 50 mM) were freshly prepared immediately before each experiment to avoid decomposition (Gilles *et al.*, 1990). The modification reaction was performed by preincubating 1 mg of protein of chromatophores with EDC or CMC (at concentrations indicated in the figure legends) in 50 mM Mes and 100 mM taurine, pH 6.0, at room temperature. At this pH the water-soluble carbodiimides are almost totally specific for carboxyl groups (Hoare and Koshland, 1967). Aliquots were taken at various times and quenched by rapid addition of a 10-fold dilution with the pyrophosphatase assay mixture. The extent of modification was determined by measuring the residual pyrophosphatase activity. In the experiments for studying the effect of free Mg^{2+} or PPi on the rate of inactivation of membrane-bound pyrophosphatase by EDC or CMC, these compounds were added to the preincubation medium and assayed as described before. The reaction was performed without preincubation only in the studies on the effect of Mg–PPi on the modification.

The measured EDC and CMC $t_{1/2}$ decomposition, under our conditions of pH 6.0 pyrophosphatase treatments, were 6.85 h for both carbodiimides (data not shown).

RESULTS

Incubation of EDC with membrane-bound pyrophosphatase results in a time-dependent loss of the hydrolytic activity. The semilog plots of residual activity vs. time at various concentrations of EDC yield straight lines up to 10 min of reaction (Fig. 1A). The hydrolytic activity was measured for 5 min for all assays. A plot of pseudo-first-order constants of inactivation (K_{obs}) vs. EDC concentration gives a linear relation, indicating, as described previously (Gomi and Fujioka, 1983; Church *et al.*, 1985; Morjana and Scarborough, 1989), that an irreversible complex is formed between the carboxyls of the enzyme and EDC (Fig. 1B); the second-order rate constant for the inactivation, calculated from this plot, was $16.9\ M^{-1}\,min^{-1}$. The reaction order for inactivation with respect to EDC concentration was calculated from a plot of log K_{obs} vs. log EDC concentration (Fig. 1C), and the stoichiometry obtained from the slope is 0.87, indicating that approximately 1 mol of EDC inactivated 1 mol of membrane-bound pyrophosphatase at this time interval (10 min).

Figure 2A shows the inactivation effect of CMC (which molecularly is a bulkier reagent than EDC) on the membrane-bound pyrophosphatase. A linear relationship was obtained for the inactivation in the semilog plots until 10 min. The plot of K_{obs} vs. CMC concentration gives a straight line, suggesting again the formation of an irreversible complex (Fig. 2B), and a second-rate constant of $8.07\ M^{-1}\,min^{-1}$. The double logarithmic plot of K_{obs} for inactivation vs. CMC concentration (Fig. 2C) has a slope of 0.89, suggesting again a 1 : 1 stoichiometry. Comparing Fig.

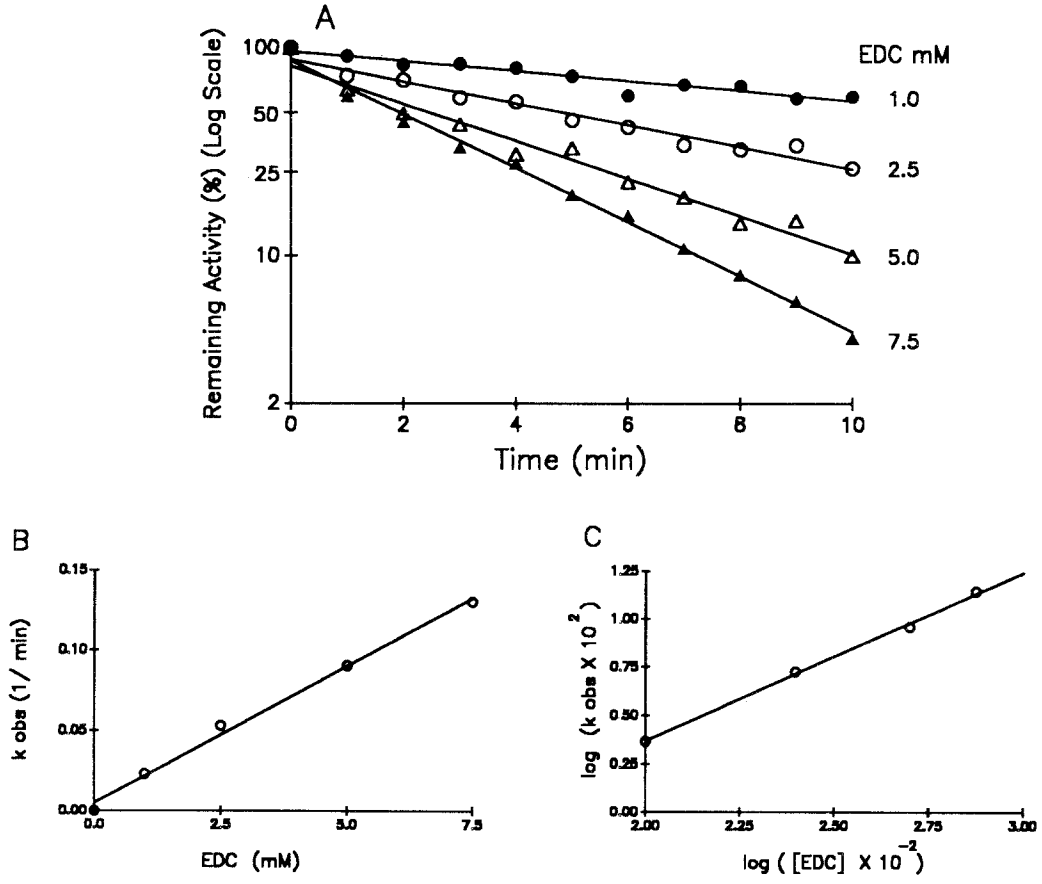


Fig. 1. Inactivation of membrane-bound pyrophosphatase by EDC. (A) Semilog plot of percent remaining activity vs. incubation time. Chromatophores were incubated in Mes 50 mM, pH 6.0, with 100 mM Taurine, pH 6.0, and incubated at room temperature in the presence of 0–7.5 mM EDC. At intervals, samples were withdrawn for the assay of hydrolytic activity as described under Materials and Methods. (B) Pseudo first-order rate constants as a function of EDC concentration. (C) Log–log plot of the same data.

1A with Fig. 2A, it is apparent that EDC has a faster K_{obs} than CMC (at 5 mM, K_{obs} EDC = 0.090 min^{-1} and K_{obs} CMC = 0.046 min^{-1}), probably reflecting the smaller molecular size of EDC.

Protection of Mg^{2+}, PPi, and Mg–PPi against the Inactivation of the Membrane-Bound Pyrophosphatase by EDC and CMC

To investigate whether the inactivation of the membrane-bound pyrophosphatase by water-soluble carbodiimides (CMC and EDC) is due to a modification in the active site or to an indirect effect on other carboxyls, out of the active site, which in turn modify the active site, the protection of PPi, Mg^{2+}, and the substrate (Mg–PPi complex) against inactivation was studied.

The protection of free PPi or Mg on the preincubation with EDC is shown in Fig. 3A and 3C. The plot of $1/K_{obs}$ against PPi concentration (Fig. 3B) gives a linear relation, suggesting a complete protection with an abscissa intercept equal to K_{PPi} = 3 mM. Mg also protects completely from the inactivation of EDC (Fig. 3D). From the $1/K_{obs}$ vs. Mg concentration plot, a straight line is obtained with a K_{Mg} = 4.6 mM.

PPi and Mg also protected against the loss of activity produced by CMC (Fig. 4A and 4C). Replots of $1/K_{obs}$ vs. PPi or Mg concentration are curved (Fig. 4B and 4D), suggesting that PPi and Mg clearly acted as partial protectors against the inactivation by CMC. Similar behaviors have been observed in other enzymes (adenosine 5′-phosphosulfate kinase from *Penicillium chrysogenum:* Renosto *et al.*, 1985). This suggests that CMC could be interacting with other carboxyl residues that are not directly involved in the

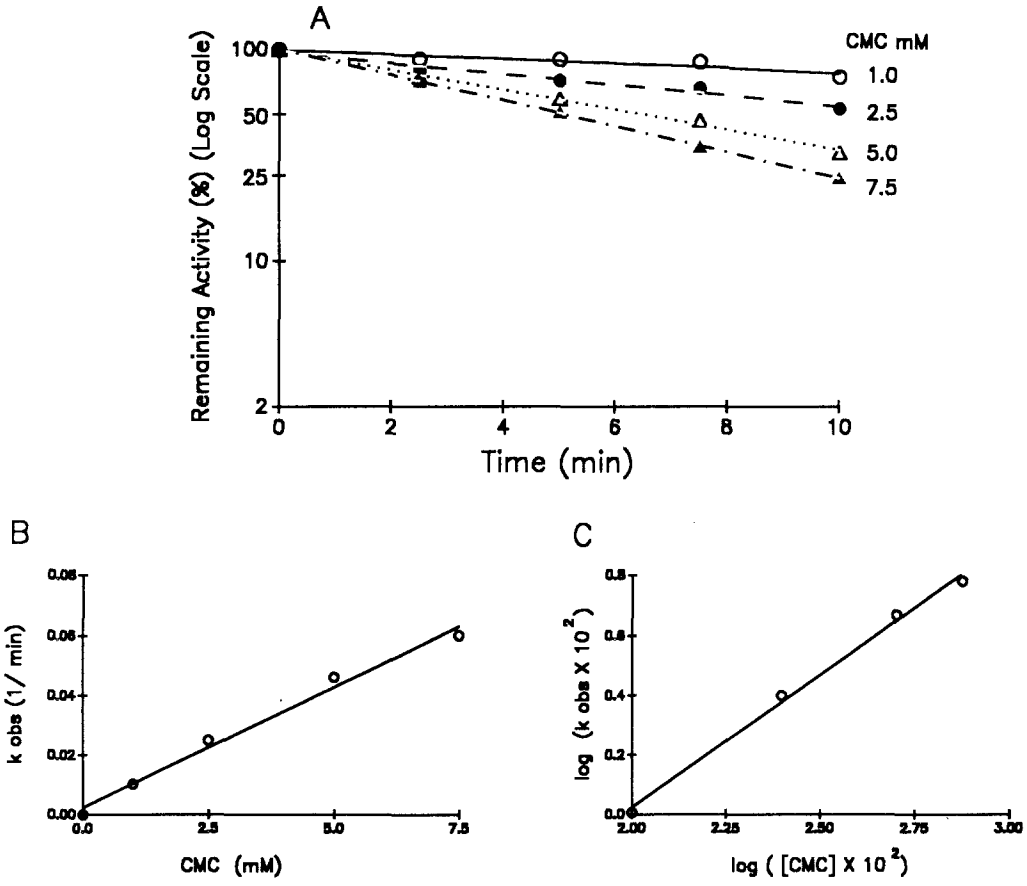


Fig. 2. Inactivation of membrane-bound pyrophosphatase by CMC. (A) Semilog plot of percent remaining activity vs. incubating time. Chromatophores were treated as in Fig. 1, but incubated in the presence of different concentrations of CMC. (B) Pseudo first-order rate constants against EDC concentrations. (C) Log–log plot of the same data.

active site and their modification affects the activity only partially. This possibility will be analyzed below.

The substrate Mg–PPi added simultaneously with the water-soluble carbodiimides protected completely against inactivation (data not shown). This effect was also observed in the presence of free Mg^{2+}. These data, together with those of Figs. 3 and 4, strongly suggest that the carboxyl moiety modified by water-soluble carbodiimides is at the active site.

Effect of EDC on the Activation of Membrane-Bound Pyrophosphatase by Mg^{2+}

It has been demonstrated that Mg^{2+} exerts a regulatory action on the catalytic properties of membrane-bound pyrophosphatase (Randahl, 1979; Celis and Romero, 1987). Recent data demonstrate that free Mg^{2+} is an essential activator of the hydrolytic reaction, and increased concentrations of Mg^{2+} produce a higher affinity for the substrate (Sosa *et al.*, 1992).

Mg^{2+} could interact with a negative carboxyl moiety in order to produce the activation of the enzyme. This regulatory site could be in or out of the active site. To obtain an insight into these possibilities, we performed the experiment depicted in Fig. 5. Modification with EDC was performed in the preincubation step, in the absence (△, ▲) and in the presence (□, ■) of PPi to protect the carboxyls involved in the active site (as demonstrated in Fig. 3A). As control, the preincubation step was performed without EDC and PPi. After preincubation, hydrolytic activity was measured with (filled symbols) or without (empty symbols) free Mg^{2+}.

The results show that the enzyme is activated in the same way by free Mg^{2+}, in the presence of EDC plus PPi (□, ■), and in their absence (○, ●); hence, we

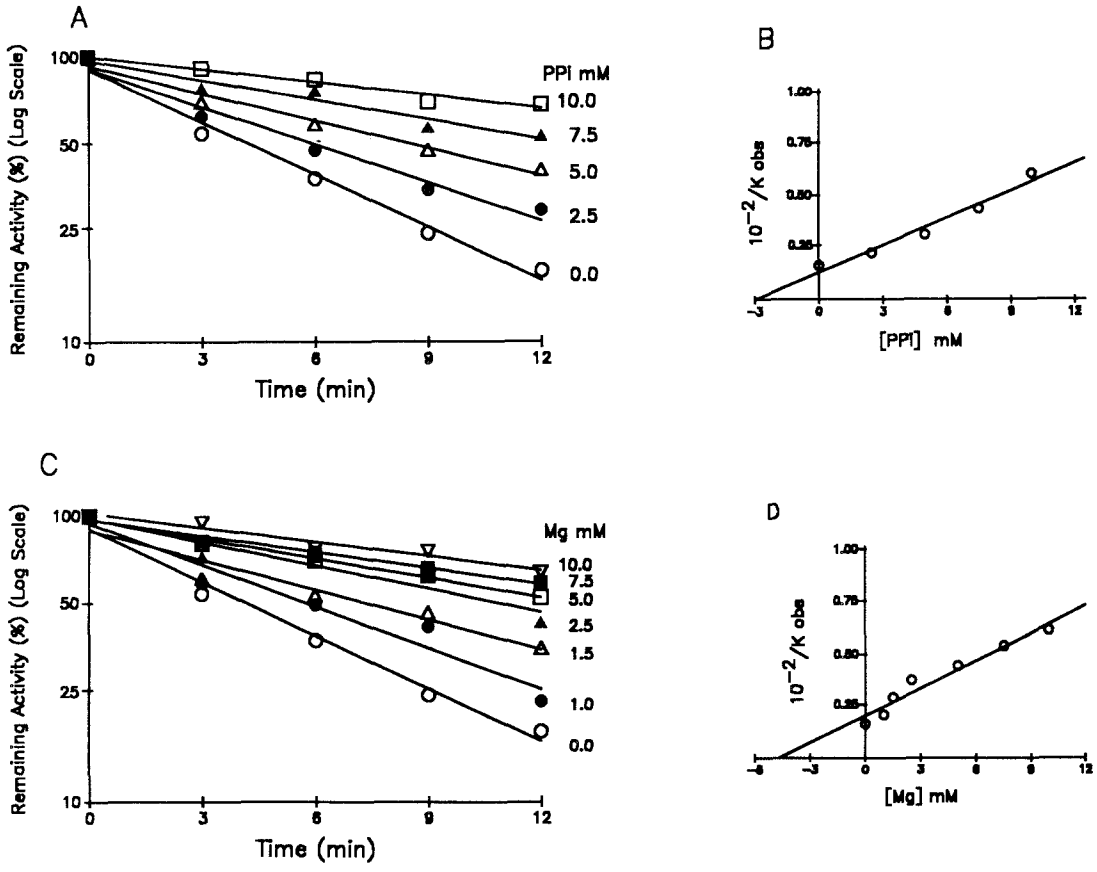


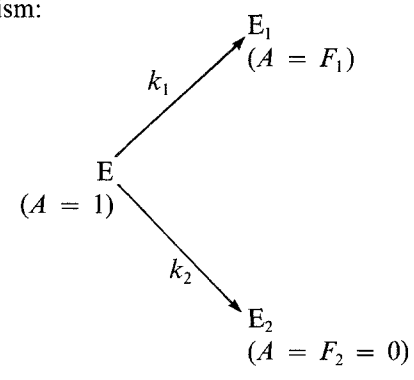
Fig. 3. Effect of PPi and Mg^{2+} on the inactivation of membrane-bound pyrophosphatase by EDC. Chromatophores were incubated with 3 mM EDC as in Fig. 1, but in the presence of the indicated concentrations of PPi (A) or Mg^{2+} (C). (B, D) plots of $10^{-2}/K_{obs}$ vs. PPi or Mg^{2+} concentrations, respectively.

can conclude that the activator site for Mg^{2+} is part or near the active site since it was protected by PPi.

Inactivation of Membrane-Bound Pyrophosphatase by CMC and EDC at Longer Times

At longer incubation times, more than 10 min, we could not observe a linear relationship in the semilogarithmic plots of residual activity against time (data not shown), suggesting that inactivation follows more complex kinetics. This behavior is not due to decomposition of the reagents, since at 75 min only 9% of them have been decomposed (see Materials and Methods). The curves obtained fit with Ray's and Koshland's (1961) model (Fig. 6A and 6B) in which two residues are involved in the modification, one giving complete inactivation (the first slope) and the other giving a residual activity [Eq. (1)].

This is shown schematically by the following mechanism:



Scheme 1

$$\frac{A}{A_0} = (1 - F_1)\, e^{-(k_1+k_2)t} + (F_1)\, e^{-k_2 t} \qquad (1)$$

in which E denotes the enzyme in which amino acid x_1 has been modified, and E_2 in which amino acid x_2 has

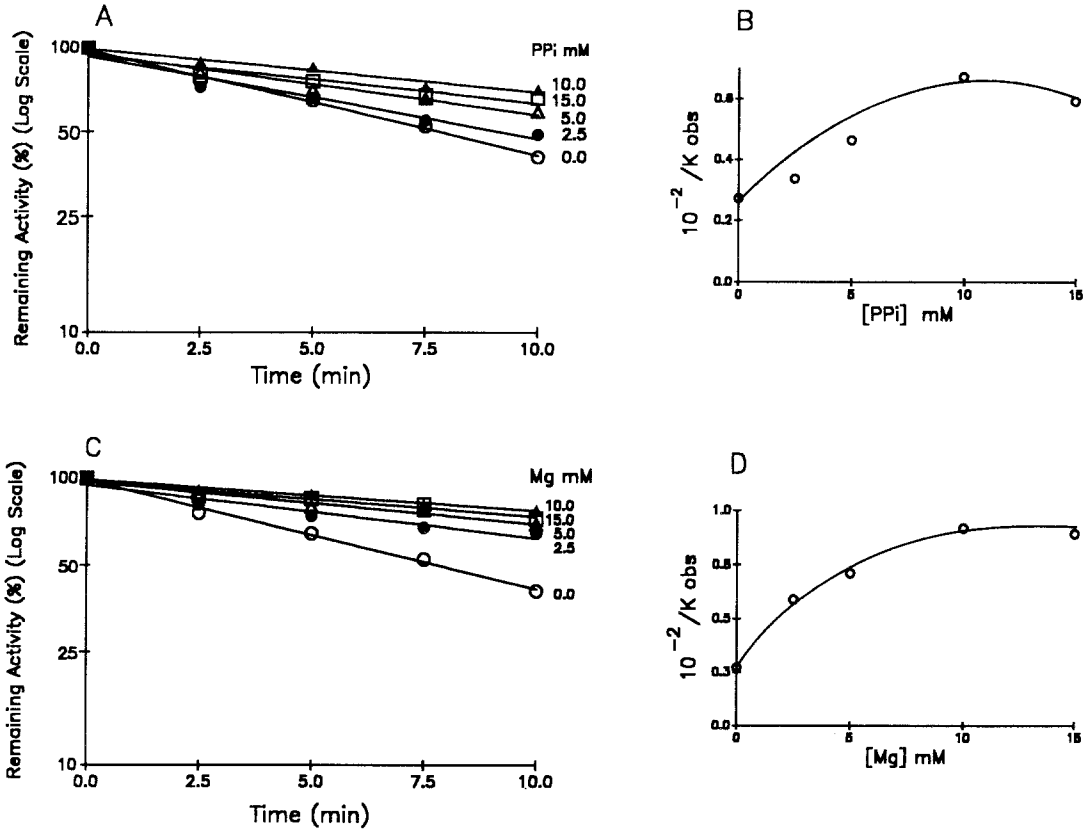


Fig. 4. Protection of PPi and Mg^{2+} against CMC inactivation. Chromatophores were incubated with 3 mM CMC as in Fig. 1, but in the presence of the indicated concentrations of PPi (A) or Mg^{2+} (C). (B, D) plots of $10^{-2}/K_{obs}$ vs. PPi or Mg^{2+} concentrations, respectively.

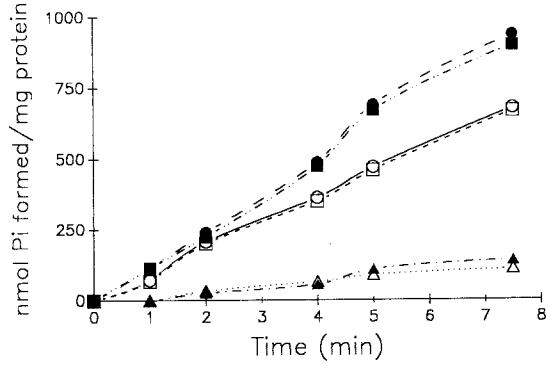


Fig. 5. Activation of membrane-bound pyrophosphatase by Mg^{2+}. Protection of EDC inactivation by PPi. The preincubation media contains 50 mM MES, 100 mM Taurine, pH 6, and 1 mg of protein of chromatophores: Control (○, ●), plus 3 mM EDC (△, ▲), plus 3 mM EDC and 10 mM PPi (□, ■). The hydrolytic activity was measured as described in Materials and Methods with Tris-maleate, pH 6.5, 1 mM Mg–PPi (○, △, □), and plus 1 mM free Mg^{2+} (●, ▲, ■).

been modified. F_1 indicates partial activity and F_2 complete inactivation. k_1 or k_2 are the modification constants for each amino acid. A/A_0 is equal to the fraction of the enzyme activity at time t.

In Fig. 6, the symbols represent the experimental data and the lines were drawn by the Simplex program (Cacesi and Cacheris, 1984) fed with Eq. (1). For EDC, concentrations higher than 1.25 mM fit better for straight lines, because they rapidly reach complete inactivation.

From the above experiments two possibilities emerge: first, that EDC and CMC, each one, modified two different carboxyl residues, and second, that only one of the modified carboxyls is shared by CMC and EDC; further work is needed to decide between these possibilities.

DISCUSSION

Several studies have been published aimed at exploring the effect of various reagents on the cyto-

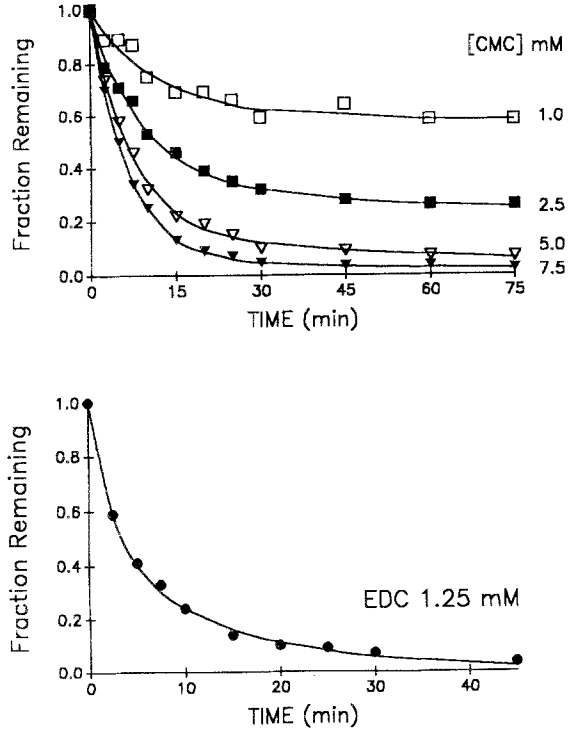


Fig. 6. Fractional pyrophosphatase activity remaining upon prolonged incubation with CMC and EDC. Chromatophores were incubated with CMC or EDC (at the indicated concentrations) as in Fig. 1, but at longer times. Symbols are from experimental data, and the lines are given by the Simplex program fed with Eq. (1).

plasmic pyrophosphatase in an effort to determine the identities and roles of essential amino acid residues at the active site (Cooperman and Chiu, 1973; Bond *et al.*, 1980; González and Cooperman, 1986; Komissarov *et al.*, 1987). Moreover, site-directed mutagenesis studies (Lathi *et al.*, 1990; 1991) have been developed in *Escherichia coli* cytoplasmic pyrophosphatase to determine some important residues for the structural integrity and for catalytic activity. Nevertheless, there are few studies of this type in membrane-bound pyrophosphatases (Baykov *et al.*, 1987 in mitochondria; Verstappen and Rausch, 1988 in microsomal membranes of plants). In the membrane-bound pyrophosphatase of *R. rubrum*, Randahl (1979) reported that *N*-ethylmaleimide and 4-chloro-7-nitrobenzofurazan inhibit the hydrolytic activity. The effect of both reagents is dependent on temperature. Mg^{2+} protects from this inhibition and PPi increases the rate of inhibition. These results suggest the presence of SH groups in or near the catalytic site.

The major conclusion that may be drawn from this work is that there is an essential carboxyl residue for membrane-bound pyrophosphatase activity. Two experimental results obtained herein support this conclusion: first, the complete inactivation of the enzyme by EDC; second, the enzyme is completely protected from EDC inactivation in the presence of Mg^{2+}, PPi (Fig. 3), or its substrate. It is possible that other carboxyls might be involved partially or indirectly in the activity of this enzyme, as reflected in the partial protection of Mg^{2+} or PPi against inactivation by CMC (Fig. 4) and in the curved lines obtained at longer times of preincubation with EDC and CMC (Fig. 6). Another conclusion of this work is that the activator site for Mg^{2+} is apparently part or near the active site of the enzyme. This conclusion is supported by the fact that PPi protects completely the activator effect of this divalent cation (Fig. 5). Nevertheless, another possibility is that other residues, different from carboxyls, could be involved in the binding of Mg^{2+}; however, this possibility is unlikely because the carboxyl moiety is the best candidate for the binding of this divalent cation.

Within their published 3 Å structure of yeast cytoplasmic pyrophosphatase, Kuranova *et al.* (1983) and Terzyan *et al.* (1984) have identified 17 polar residues, either as ligands for one of the four divalent metal ion binding sites per subunit or as potential sites of direct interaction with bound PPi in the putative active site cavity. From these polar residues required for Mg^{2+} binding, nine are carboxyl residues of aspartic or glutamic acids. Lahti *et al.*, (1990) also revealed the importance of Asp 97 and Asp 102 for the catalytic activity of *E. coli* cytoplasmic pyrophosphatase. Although membrane-bound and cytoplasmic enzymes can be very different, there might be similarities in their active sites, so it seems reasonable to conclude with the present data that, at least, one carboxyl is essential for the activity and for the activation by Mg^{2+}.

As a general working model for the function of the active site of membrane-bound pyrophosphatase of *R. rubrum*, we can propose that the carboxyl moiety is essential for the activity of the enzyme. Kinetic studies (Sosa *et al.*, 1992) strongly suggest that Mg^{2+} binds earlier than the substrate to the active site; consequently, it is possible that the negative charges of the carboxyls must be neutralized by Mg^{2+} before the entrance of the negative charged substrate. It is important to note that, in this work, we used the enzyme in the membrane; however, it remains for future studies with the solubilized and purified enzyme to determine

which amino acid(s) is modified at the active site. The experiments reported here give us an insight into the nature of the active site *in situ*, in the natural state of the membrane-bound pyrophosphatase.

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