INHIBITION OF D-RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE BY PYRIDOXAL 5'-PHOSPHATE[1]

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SUMMARY: Homogeneous D-ribulose 1,5-bisphosphate carboxylase from Rhodospirillum rubrum, Chlamydomonas reinhardtii, and Hydrogenomonas eutropha are inhibited by low concentrations of pyridoxal 5'-phosphate. In the case of the enzyme from Rhodospirillum rubrum, this inhibition is strongly antagonized by the substrate, D-ribulose 1,5-bisphosphate. These results suggest that pyridoxal 5'-phosphate may act close to or at the ribulose 1,5-bisphosphate binding site of the enzyme from Rhodospirillum rubrum.

The structural properties of the large catalytic-type subunit of ribulose 1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39) may conveniently be studied with the enzyme from the photosynthetic bacterium, Rhodospirillum rubrum. Unlike the enzymes from other procaryotes and eucaryotes, the R. rubrum enzyme is a dimer containing two large, catalytic-type subunits and lacks small subunits (1). Furthermore, this enzyme exhibits high specific activity which is retained upon storage when reactivated with dithiothreitol and EDTA (6). Yet, in other respects, the R. rubrum enzyme is very similar to the RuBP[2] carboxylases from higher plants. Like the higher plant enzymes, the R. rubrum enzyme also has a RuBP dependent oxygenase activity (2,3). For these reasons, the RuBP carboxylase from Rhodospirillum rubrum may provide a useful model system for the study of RuBP carboxylase.

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[2]Abbreviations used are: RuBP, D-ribulose 1,5-bisphosphate; MOPS, morpholinopropane sulfonic acid; PLP, pyridoxal 5'-phosphate.

PLP has been shown to be a potent inhibitor of enzymes that act on phosphorylated substrates (4,5). The mode of inhibition is usually by Schiff base formation at a lysyl residue at the active site. In this paper, we test this hypothesis with the RuBP carboxylase from Rhodospirillum rubrum and demonstrate the applicability of these results to RuBP carboxylases from other organisms.

MATERIALS AND METHODS

Rhodospirillum rubrum was grown on butyric acid as the electron donor and the RuBP carboxylase purified to electrophoretic homogeneity as previously described (6). Protein concentration was determined from absorbance at 280 nm of 0.974 cm^{-1} g^{-1} l (1). Homogeneous Hydrogenomonas eutropha RuBP carboxylase was prepared by the procedure of Kuehn and McFadden (7). Protein concentration was determined by the absorbance at 280 nm of 1.55 cm^{-1} g^{-1} l. Chlamydomonas reinhardtii WT, strain 4, was grown and the RuBP carboxylase purified to homogeneity as described by Iwanij, et al. (8) except that the resuspended 55 per cent saturated ammonium sulfate pellet was layered over a 0.1 - 0.3 M continuous sucrose gradient and centrifuged for 20 hours in the SW 25.1 rotor at 23,000 RPM at 0-4°C. The protein concentration was measured by the biuret method with crystalline bovine serum albumin as a standard (9). The absorption at 280 nm in 10 mM potassium phosphate, pH 7.5, and 1 mM EDTA was then determined to be 1.45 cm^{-1} g^{-1} l. Spinach RuBP carboxylase was isolated from spinach leaves obtained at the local market by the procedure of Paulsen and Lane (10).

The barium salt of RuBP was prepared by the enzymatic procedures described by Horecker, et al. (11), except that in the recrystallization step, equivalent amounts of acetic acid were substituted for hydrochloric acid. The barium salt was converted to the free acid with Dowex H^+ resin. RuBP obtained by this procedure was 80 to 90 percent pure with the major contaminants being inorganic phosphate and presumably ribulose 5-phosphate. This preparation was chloride free. Commercial preparations of RuBP were never as pure as the enzymatically generated substrate.

Prior to treatment with PLP, enzymes were incubated for 3 hours at 30°C in the presence of 10 mM dithiothreitol, 1 mM EDTA, and 10 mM MOPS, pH 7.5. This activated enzyme was dialyzed for at least 3 hours against a 500 fold or greater volume of the same buffer without dithiothreitol. Stock solutions of PLP were prepared prior to each experiment and the concentration determined by the absorbance at 388 nm of 6050 cm^{-1} M^{-1} in 0.1 N NaOH. Except where noted, incubations of the enzymes with PLP were for 60 minutes at 30°C. The incubation was terminated by the addition of the enzyme to the assay solution containing PLP at the same concentration as in the incubation, 0.8 mM RuBP, 20 mM $[^{14}C]NaHCO_3$, 20 mM Mg acetate, 1 mM EDTA, and 0.15 M MOPS-KOH, pH 7.8 in a 0.25 ml volume. After 10 minutes, 0.1 ml of 99 per cent propionic acid was added and the free $[^{14}C]CO_2$ allowed to dissipate. The amount of acid stable ^{14}C remaining was determined by counting 0.2 ml in 3 ml of liquid scintillation cocktail containing 930 ml toluene, 70 ml Biosolv BBS-3 (Beckman), and 4 g of 2,5-diphenyloxazole per liter in a Beckman LS-100C liquid scintillation counter.

RESULTS

A comparison of PLP inhibition of the R. rubrum RuBP carboxylase in a variety of buffers at pH 7.5 (Fig. 1) showed that inhibition was most pronounced in buffers lacking inorganic anions. In the presence of MOPS-NaOH, greater than

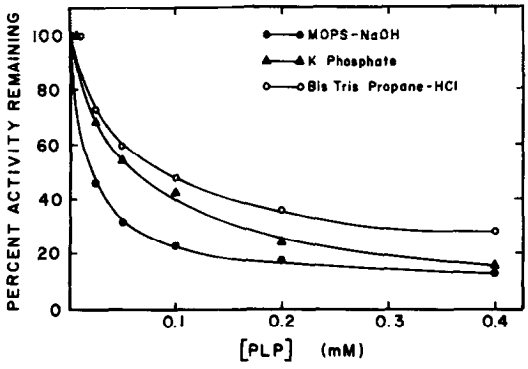

Fig. 1. Comparison of PLP inhibition of R. rubrum RuBP carboxylase in different buffers. 9×10^{-8} M R. rubrum RuBP carboxylase was incubated for 120 minutes in the presence and absence of PLP in 0.033 M potassium phosphate, bis tris propane-HCl, or MOPS-NaOH at pH 7.5. Assays were performed in the absence of PLP. Per cent activity remaining is expressed as (CPM in the presence of PLP/CPM in the absence of PLP) x 100.

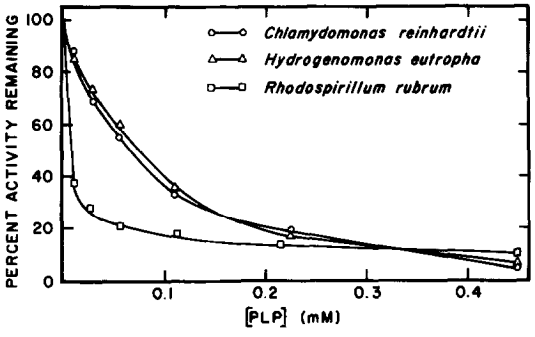

Fig. 2. Titration of RuBP carboxylase from three microorganisms with PLP. Incubations and assays were performed as described in Materials and Methods. The specific activity in micromoles CO_2 fixed/min/mg of the enzymes in the absence of PLP were: R. rubrum, 4.9; C. reinhardtii, 0.35; H. eutropha, 0.60.

50 per cent inhibition was obtained with 0.02 mM PLP. In the presence of potassium phosphate and bis tris propane-HCl greater than 50 per cent inhibition was obtained with 0.1 mM PLP. In a similar experiment, after incubation of the enzyme with MOPS-NaOH buffer, 0.1 mM PLP, and either 10 mM NaCl or 10 mM Na phosphate, 34 and 33 per cent activity remained while only 17 per cent activity remained in the absence of either anion. It was subsequently found that MOPS-KOH could be substituted for MOPS-NaOH with no effect on the inhibition by PLP.

When RuBP carboxylase activity of the Chlamydomonas reinhardtii and Hydrogenomonas eutropha enzymes was titrated with PLP (Fig. 2), substantial inhibition at low concentrations of PLP was evident. While these enzymes were titrated in a similar fashion, the R. rubrum RuBP carboxylase was more inhibited at low concentrations of PLP. In data not presented here, it was also found that spinach RuBP carboxylase activity was inhibited by PLP.

Table I: Protection of PLP[a] inhibition of R. rubrum RuBP carboxylase

Protector[b]	Per cent of activity remaining[c]
none	32
ribulose 1,5-bisphosphate	81
ribulose 5-phosphate	47
ribose 5-phosphate	45
3-phosphoglyceric acid	37
6-phosphogluconate	34
inorganic phosphate	34

[a]PLP concentration was 0.05 mM.
[b]The concentration of RuBP used was 0.8 mM; the other compounds were tested at 1.6 mM.
[c]Calculated from controls without PLP but with each potential protector compound present.

To determine more about the site of PLP interaction with RuBP carboxylase, the R. rubrum enzyme was treated with PLP in the presence of the substrate RuBP (Fig. 3) and other structurally related compounds (Table I). After a preincubation with PLP in the presence of RuBP, 82 per cent of the control activity without PLP remained as compared to 37 per cent in the absence of RuBP. Furthermore, assays were linear for at least 6 minutes after preincubation in

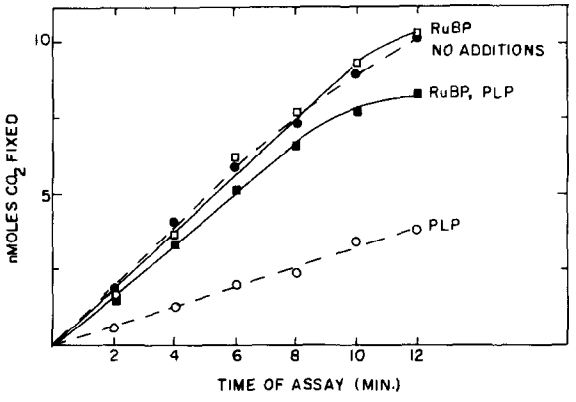

Fig. 3. Protection of PLP inhibition of R. rubrum RuBP carboxylase by RuBP. 4×10^{-7} M R. rubrum RuBP carboxylase was incubated in the presence and absence of 0.05 mM PLP, 0.8 mM RuBP, 1 mM EDTA, and 3.5 mM MOPS-KOH, pH 7.5 in a 0.06 ml volume. After 60 minutes, 0.05 ml was withdrawn and added to the complete assay mixture in a 0.75 ml volume. At 2 minute intervals, 0.1 ml of the assay mixture was added to 0.2 ml of propionic acid in a liquid scintillation vial and acid stable ^{14}C measured as described in Materials and Methods.

the presence or absence of PLP and RuBP (Fig. 3). Deviations from linearity observed for longer assay times could be abolished by lowering the enzyme concentration. Other phosphorylated compounds failed to protect against PLP inhibition to the extent of RuBP. The small amount of protection observed with inorganic phosphate and ribulose-5-phosphate indicate that these contaminants of the RuBP preparations cannot account for the extent of protection seen with RuBP (Table I). The failure of 6-phosphogluconate and low concentrations of 3-phosphoglyceric acid to protect is consistent with their lack of effect on enzyme activity (6,12).

DISCUSSION

Pyridoxal 5'-phosphate is a potent inhibitor of R. rubrum RuBP carboxylase. RuBP, structurally related compounds, and inorganic anions protect against this inhibition. However, both the extent and specificity of protection by RuBP suggests that PLP may act close to or at a RuBP binding site of the enzyme. Since allosterism has not been demonstrated with respect to RuBP for the enzyme from R. rubrum (1,6), the locus of PLP interaction may be at the active site of

this protein. The fact that RuBP carboxylase from diverse sources is also inhibited by low concentrations of PLP is expected if the mechanism of carboxylation is conserved throughout evolution.

PLP is a highly specific reagent for lysyl residues in proteins. Recently, the existence of two essential lysyl residues per large subunit has been proposed for the spinach RuBP carboxylase (13). The results reported here are consistent with the importance of lysyl residues. A subsequent report, in preparation, will discuss this aspect of PLP action in greater depth (14).

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