ACTIVATION OF SPINACH RIBULOSE BISPHOSPHATE CARBOXYLASE BY PYRIDOXAL PHOSPHATE

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1. Introduction

Ribulose 1,5-bisphosphate carboxylase catalyzes the primary step in carbon dioxide assimilation in most photosynthetic and chemosynthetic organisms. Pyridoxal 5′-phosphate (Pxl-P) is a potent inhibitor of the purified enzyme isolated from either procaryotes or eucaryotes [1]. The RuBP carboxylase from *Rhodospirillum rubrum,* a dimer of catalytic subunits, contains two binding sites for Pxl-P per enzyme. However, binding at either site is sufficient to inactivate both of the catalytic sites found in the *Rhs. rubrum* carboxylase [2]. Because both the substrate, RuBP, and an analog of the 6-carbon intermediate formed during the carboxylation reaction, carboxyribitol bisphosphate (CRBP), protect against Pxl-P inactivation and binding, Pxl-P is probably directed to the active site of the enzyme [3].

Like the *Rhs. rubrum* enzyme, Pxl-P has recently been found to modify lysyl residues on the spinach RuBP carboxylase [4]. Moreover, it was concluded that Pxl-P modified both the CO_2 activation site and the CO_2 catalytic site [4]. Here we show that Pxl-P can both activate and inhibit spinach RuBP carboxylase.

Abbreviations: bicine, *N,N*-bis (2-hydroxyethyl) glycine; CRBP, carboxyribitol bisphosphate; EDTA (ethylenedinitrilo) tetracetic acid; Pxl-P, pyridoxal 5′-phosphate; RuBP, ribulose 1,5-bisphosphate

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2. Materials and methods

2.1. *Materials*

RuBP carboxylase was prepared from spinach leaves essentially as in [5] and was highly purified by the criterion of polyacrylamide gel electrophoresis under non-denaturing [6] and denaturing conditions [7]. In the assay system [5], the freshly purified enzyme had a spec. act. 2.6 μmol CO_2 fixed/mg enzyme/min. The purified enzyme was taken to be $\epsilon_{280} = 1.64\ cm^{-1}\ .g^{-1}\ .l$ [5]. The enzyme was taken to be mol. wt 557×10^3 [5].

Pyridoxal 5′-phosphate and other biochemicals were obtained from Sigma Chemical Co., St Louis, MO. Tritiated sodium borohydride ($NaBT_4$) (186 Ci/mol) was obtained from New England Nuclear, Boston, MA. Carboxyribitol bisphosphate, CRBP, was prepared as in [8]. RuBP was prepared as in [1]. In some cases, RuBP was further purified as in [8] and used as the lithium salt.

2.2. *Methods*

RuBP carboxylase activity was measured as in [9] except that dithiothreitol was omitted from the assays and incubations because it was shown to have little effect. Otherwise, all incubations were performed in 0.1 M KOH–bicine buffer (pH 8.0) and 0.2 mM Na_2EDTA at 30°C with the noted additions. Incubations with magnesium, bicarbonate, or both were performed for ⩾ 30 min. When appropriate, the first incubation was followed by a second incubation with effectors or protectants for ⩾ 30 min prior to the addition of Pxl-P. Incubations with Pxl-P were performed for ⩾ 60 min and were protected from

light. Carboxylase assays at 'high bicarbonate' were initiated by the addition of preincubated and fully activated enzyme to a reaction mixture containing, in 0.25 ml final vol: 20 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$, 0.5 mM RuBP, 0.2 mM Na_2EDTA and 0.1 M KOH–bicine (pH 8.0). After 1 min at 30°C, assays were quenched by the addition of 0.1 ml propionic acid. Acid-stable radioactivity was determined as in [1]. Carboxylase assays at 'low bicarbonate' were performed as above except that $NaH^{14}CO_3$ was 1 mM and the assay was for 5 min. To determine the time course of the assay at low bicarbonate, the volume of the assay was increased 5-fold and 0.1 ml portions were quenched at 1 min intervals upon their addition to 0.1 ml propionic acid.

The Pxl-P–enzyme complex was reduced by the addition of $NaBT_4$ to 1 mM (18.6 Ci/mol). After 5 min, the enzyme was exhaustively dialyzed against 20 mM tris(hydroxymethyl) aminomethane (pH 7.5), 5 mM 2-mercaptoethanol and 0.1 mM Na_2EDTA. After dialysis, tritium incorporation was determined by liquid scintillation counting of 40–80 μg modified enzyme in 10 ml cocktail containing 5 g/l 2,5-diphenyloxazole and 100 g/l naphthalene in dioxane. The protein concentration was calculated from the A_{280} and ϵ of the native enzyme. At the levels of Pxl-P incorporated, it was not necessary to correct the extinction coefficient for the absorption of the phosphopyridoxylamino acid. When the reduction was performed in the presence of effectors, a separate control was performed where the enzyme was reduced in the presence of the effector without Pxl-P.

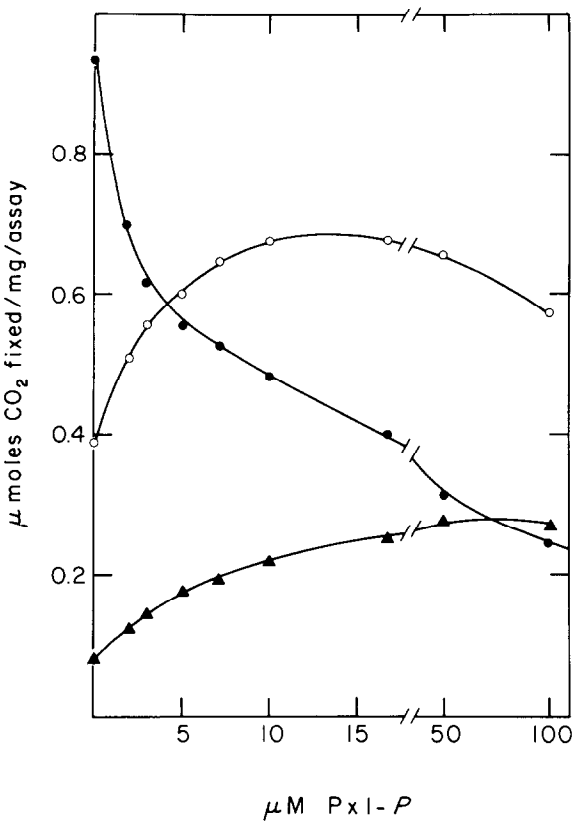

Fig.1. Titration of spinach RuBP carboxylase with pyridoxal phosphate. Enzyme was preincubated with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ (•), 10 mM $MgCl_2$ (○), or with no additions (▲) prior to the addition of Pxl-P. The enzyme was 1.4 μM. The incubation with bicarbonate and magnesium was assayed at high bicarbonate for 1 min. All other assays were performed at low bicarbonate for 5 min.

3. Results

Pyridoxal phosphate was found to be a potent inhibitor of the spinach RuBP carboxylase in the presence of 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ (fig.1). However, in the absence of exogenous bicarbonate or in the absence of both bicarbonate and magnesium, Pxl-P activated the spinach carboxylase 1.7- and 3.5-fold, respectively. Activation by Pxl-P was also observed when 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer was substituted for the bicine buffer and when the enzyme assays were performed at high bicarbonate for 1 min, although in the latter case the extent of activation was greatly reduced. The concentration of Pxl-P required for 50% of the maximal activation obtained in the presence or absence of magnesium was ~2.5 μM and 5 μM, respectively. In contrast, the concentration of Pxl-P required for 50% inactivation in the presence of 10 mM Mg^{2+} and 10 mM HCO_3^- was ~9 μM. Although the titration with Pxl-P under inactivation conditions appeared biphasic, this may indicate the accumulation of an unstable intermediate at high Pxl-P concentrations which dissociates upon dilution into the assay. Similar results were found with the Pxl-P inactivation of *Rhs. rubrum* RuBP carboxylase [2] and such a noncovalent intermediate has been

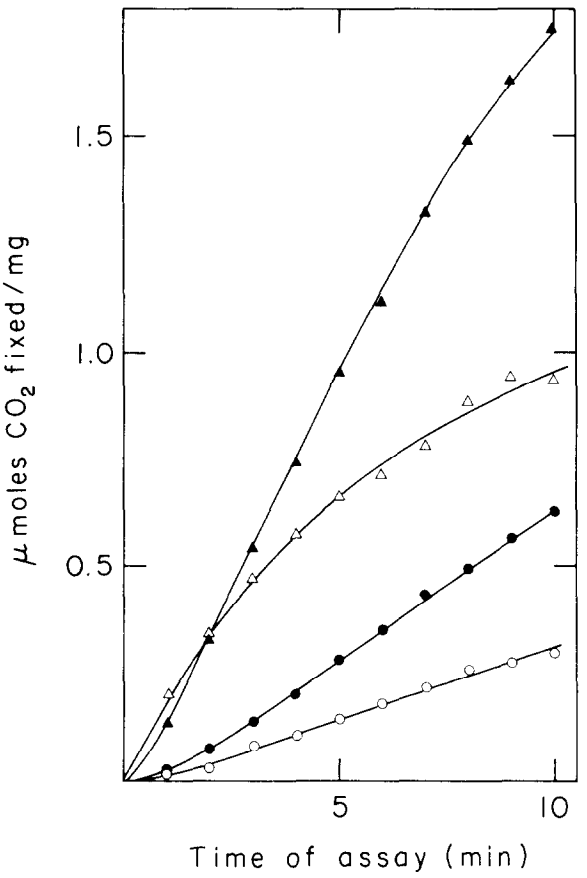

Fig.2. Time course of the assay at low bicarbonate. Spinach carboxylase, 1.3 μM, was incubated with 1 mM $NaHCO_3$ (●,○) and 1 mM $NaHCO_3$ and 10 mM $MgCl_2$ (▲,△) in the presence (●,▲) or absence (○,△) of 10 μM Pxl-P. Assays were performed at low (1 mM) bicarbonate as in section 2.

implicated in the interaction of PLP with spinach carboxylase [4].

Although activation by Pxl-P is most apparent when carboxylase activity is measured at low bicarbonate, Pxl-P does not appear to shorten the brief lag observed in the absence of Pxl-P (fig.2). Likewise, Pxl-P activation also differs from the 6-phosphogluconate or fructose bisphosphate-induced activation in the absence of a magnesium requirement (fig.1). A requirement for carbon dioxide was not tested because carbon dioxide is a component of all buffers prepared in the presence of air. However, the addition of 1 mM bicarbonate to the incubations with Pxl-P had little effect on the extent of activation.

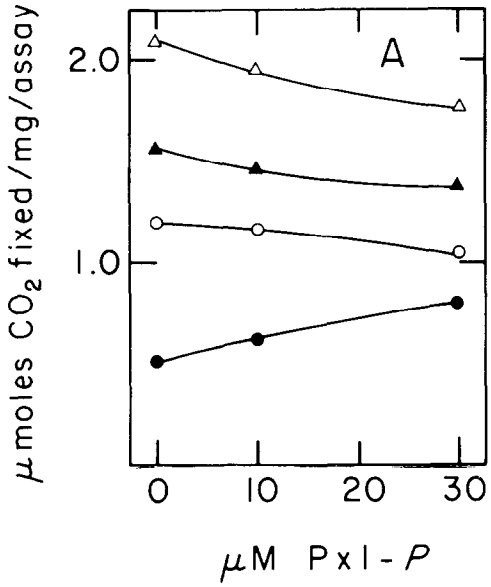

Fig.3. Influence of effectors on pyridoxal phosphate modification. Spinach RuBP carboxylase (4.5 μM) was incubated with 1 mM $NaHCO_3$, 10 mM $MgCl_2$ and 0.1 mM 6-phosphogluconate (△), 0.1 mM fructose bisphosphate (▲), 0.1 mM NADPH (○) or no additions (●). Enzymatic activity was measured at low bicarbonate for 5 min.

When the enzyme was activated with 6-phosphogluconate, fructose bisphosphate, or NADPH at 1 mM $NaHCO_3$ and 10 mM $MgCl_2$, slight inhibition was found upon the addition of Pxl-P (fig.3). However, NADPH gave almost no protection of Pxl-P binding measured after reduction with $NaBT_4$ and fructose bisphosphate and 6-phosphogluconate gave only partial protection (results not shown). In contrast, in the absence of bicarbonate and magnesium and the presence of 30 μM Pxl-P, 20 μM CRBP gave > 90% protection against Pxl-P binding. In the presence of 10 mM bicarbonate, 10 mM magnesium and 30 μM Pxl-P, 20 μM CRBP and 0.1 mM fructose bisphosphate gave 50% and 20% protection against Pxl-P binding, respectively.

The absorption spectra of the Pxl-P enzyme complex was determined in the presence and absence of 10 mM $NaHCO_3$ and 13 mM $MgCl_2$. In both cases a single $A_{410-412}$ max was observed at wavelengths > 300 nm. The $A_{410-412}$ max is indicative of an internally hydrogen-bonded Schiff base, which is reducible by sodium borohydride. In both cases, the

chromophore at 330 nm [3], which is not reducible by sodium borohydride, was absent in these experiments performed with spinach carboxylase. Moreover, after modification with $NaBT_4$ and Pxl-P and subsequent dissociation in sodium dodecyl sulfate, only large subunits were labeled under both inactivation [4] and activation conditions*.

4. Discussion

The spinach RuBP carboxylase is inactivated by low concentrations of Pxl-P in the presence of 10 mM $MgCl_2$ and 10 mM $NaHCO_3$. In contrast, Pxl-P, in the absence of high concentrations of bicarbonate, activates the spinach RuBP carboxylase. The mechanism of activation appears to differ from that of the activation by the effectors, fructose bisphosphate, phosphogluconate, and NADPH, because there is no magnesium requirement and because there is no effect on the lag observed when the enzyme is assayed at 1 mM bicarbonate. Furthermore, the effectors did not quantitatively protect against Pxl-P incorporation upon reduction with sodium borohydride. Therefore, the site of Pxl-P activation may differ from the site of action of these effectors.

Whether or not the site of Pxl-P activation is identical to the site of Pxl-P inactivation is not known. The absorption spectra of the Pxl-P–enzyme complexes under activating and inactivating conditions indicates that both should be susceptible to reduction with sodium borohydride and subsequent peptide analyses. Thus, the similarity or identity of these sites on the large subunit might be discerned. Current investigations are directed to this issue.

* Robison, P. D., and Tabita, F. R. (1978) unpublished observations

Lastly, it was proposed that CO_2 (when presented as HCO_3^-) protected the spinach enzyme from Pxl-P inactivation [4,9]. This effect was attributed to competition between CO_2 and Pxl-P for covalent bond formation of the ϵ-amino group of a lysyl residue. Nevertheless, these workers clearly show that potassium chloride is nearly as effective a protectant against Schiff base formation as HCO_3^- ([9], table 1). This latter result is consistent with our experience that inorganic anions protect against Pxl-P inactivation of the *Rhs. rubrum* carboxylase [1] and indicates that carbon dioxide has little or no specific interactions with the site of Pxl-P inactivation of the spinach RuBP carboxylase.

Acknowledgements

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