PHOSPHOENOLPYRUVATE, A NEW INHIBITOR OF PHOSPHORIBULOKINASE IN PSEUDOMONAS FACILIS

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SUMMARY

Using partially purified preparations of phosphoribulokinase from Pseudomonas facilis, a new inhibitor of the enzyme has been characterized. This inhibitor is phosphoenolypruvate. Phosphoenolpyruvate displays a K_i of 5.5×10^{-4} M and acts as a noncompetitive inhibitor of phosphoribulokinase with respect to ribulose-5-phosphate and ATP, the substrates of the enzyme. It is suggested that phosphoenolpyruvate may function as a general regulator of phosphoribulokinase and thus of ATP-dependent carbon dioxide fixation.

INTRODUCTION

The enzyme phosphoribulokinase (PRK) appears to play an important role in the regulation of ATP-dependent CO_2 fixation in autotrophic organisms. Phosphoribulokinase is an allosteric enzyme regulated by several effectors. One of these, NADH, acts as an activator of the enzyme from Pseudomonas facilis (MacElroy, Johnson and Johnson, 1969) and from Rhodopseudomonas spheroides and Rhodospirillum rubrum (Rindt and Ohmann, 1969). Another regulator of PRK, AMP, acts as an inhibitor, as demonstrated in Thiobacillus ferrooxidans (Gale and Beck, 1966), Thiobacillus thioparus, Thiobacillus neapolitanus, Chromatium D, and Spinach (Johnson and Peck, 1965; Johnson, 1966; MacElroy, Johnson and Johnson, 1968), R. spheroides and R. rubrum (Rindt and Ohmann, 1969) and Pseudomonas facilis (McFadden and Tu, 1965). The present communication describes a second inhibitor of phosphoribulokinase, phosphoenolpyruvate.

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METHODS

Pseudomonas facilis (Davis, et al., 1969), formerly assigned to the genus Hydrogenomonas, was maintained and grown according to the methods described by MacElroy, et al. (1969). The enzyme phosphoribulokinase (PRK) was obtained from cells at 0°C, harvested by centrifugation, washed, and resuspended in 0.05 M imidazole buffer, pH 7.4, to a concentration of 20% (wet weight/volume). Cell-free extracts were obtained by passing the cell suspension through a French pressure cell at 20,000 lb/in^2, and centrifuging 30 min at 100,000 × g. The protein concentration of the supernatant fraction was then adjusted to 6.5 mg/ml by the addition of 0.05 M imidazole-HCl buffer, pH 7.4, and then slowly brought to 55% saturation with solid $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation at 10,000 × g for 15 min. and resuspended in 0.01 M phosphate buffer, pH 6.8. The enzyme preparation was then passed through a column (2.5 × 38 cm) of Sephadex G-25 by elution with 0.01 M phosphate buffer, pH 6.8. The eluant was collected and passed through a column (2.5 × 38 cm) of DEAE Sephadex A-25 by elution with 500 ml of a gradient of 0.01 to 0.4 M phosphate buffer, pH 6.8. The eluted activity peak was collected and condensed by ultrafiltration using a Diaflo Cell with a PM 30 membrane filter (Amicon Corp., Lexington, Mass.). The resulting enzyme preparation had a specific activity of 3.6 μmoles/min/mg protein, a level about 350-fold greater than the specific activity of the starting crude cell-free extract.

The activity of PRK was assayed according to the method described by MacElroy et al. (1968) which employs ribulose -1,5-diphosphate carboxylase to couple the production of ribulose 1,5-diphosphate to the incorporation of $^{14}CO_2$. The final concentrations of the components in the assay mixture were 50 mM Tris-HCl buffer, pH 7.5, 3.3 mM ribulose-5-phosphate (Sigma Chemical Co., St Louis), 3.3 mM ATP, 15 mM $MgCl_2$, 10 mM $Na_2{}^{14}CO_3$ (specific activity 0.23 μc/μM), 2 mM NADH and excess ribulose-1,5-diphosphate carboxylase in a total volume of 0.2 ml.

Ribulose-1,5-diphosphate carboxylase was obtained from P. facilis cells by following the PRK purification procedure through the precipitation with $(NH_4)_2SO_4$ at 55% saturation. The supernatant fraction that contained the carboxylase was further saturated with $(NH_4)_2SO_4$ to a level of 75%, centrifuged 15 min. at 10,000 × g and the pellet resuspended in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M $MgCl_2$, to a concentration of 6.5 mg protein/ml. The carboxylase was then reprecipitated with $(NH_4)_2SO_4$ by bringing the solution to 65% saturation, centrifuging, discarding the resultant pellet, and then further saturating to 75%, centrifuging and resuspending the pellet in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.01 M $MgCl_2$. The second precipitation was required to remove the enzyme enolase, which would make kinetic experiments involving phosphoenolpyruvate (PEP) difficult.

Protein determinations on the cell-free extracts were done by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

In the presence of saturating concentrations (8 × K_m) of ribulose-5-phosphate (Ru-5-P) and ATP, PEP inhibited the activity of PRK and exhibited an apparent K_i of

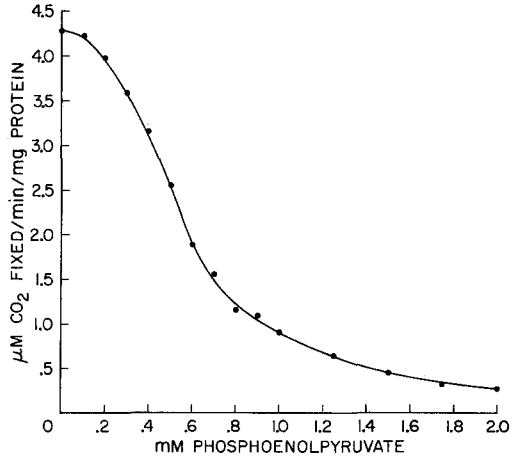

Fig. 1 The response of phosphoribulokinase, at saturating concentrations of Ru-5-P and ATP, to increasing concentrations of PEP. The activity of phosphoribulokinase was assayed as described in the text.

5.5×10^{-4} M (Fig. 1). The low response of the enzyme to the inhibitor at low inhibitor concentrations, as shown in Figure 1, suggests that the inhibitor molecules are acting cooperatively to produce the inhibition. The ribulose 1,5-diphosphate carboxylase used in the assay was not affected by PEP. This was demonstrated by assaying the ability of the carboxylase to fix $^{14}CO_2$ when using ribulose-1,5-diphosphate as the sole substrate in the presence and absence of PEP.

The Km for Ru-5-P, determined in the presence of excess ATP and NADH, was found to be 2.4×10^{-4} M (Fig. 2). The effect of two different concentrations of PEP on the response of the enzyme to Ru-5-P is also shown in the double reciprocal plots of Figure 2. It can be seen that while the K_m for the substrate is not altered, the V_{max} is decreased. This indicates that PEP acts as a noncompetitive inhibitor of PRK with respect to Ru-5-P. The inhibition caused by PEP could not be reversed by the addition of excess amounts of ATP, NADH or $MgCl_2$, which suggests that PEP is also noncompetitive with respect to these compounds in the assay reaction mixture.

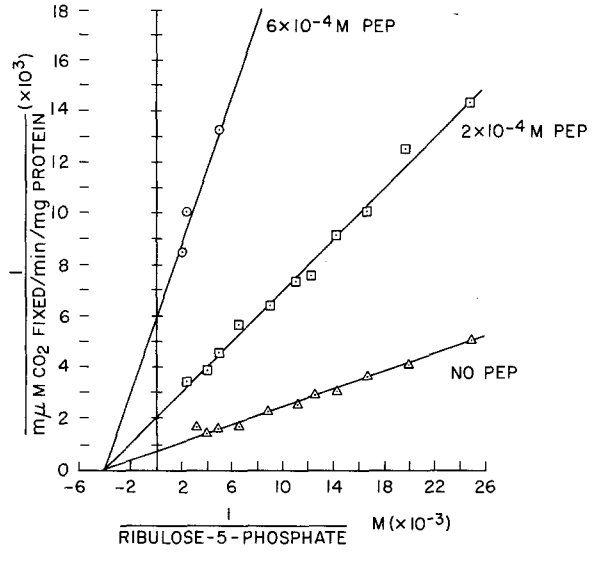

Fig. 2. Reciprocal plots of increasing concentrations of ribulose-5-phosphate in the presence and absence of PEP. The activity of PRK was assayed as described in the text, except that the ribulose-5-phosphate concentration was varied as indicated.

A number of compounds which possess varying degrees of structural similarity to PEP were tested in order to determine if any of them could also inhibit PRK. The compounds tested were pyruvate, 2-phosphoglycerate, 3-phosphoglycerate, glyceraldehyde-3-phosphate, DL-α-glycerophosphate, dihydroxyacetone phosphate, L-alanine, L-serine, acetylphosphate, 6-phosphogluconate, D-glucose-6-phosphate, D-fructose-6-phosphate and glyoxylate. None of these compounds exhibited any inhibition of PRK, indicating that PRK possesses a very high degree of specificity for PEP as an inhibitor.

The inhibitory effect of PEP has also been observed in partially purified preparations of phosphoribulokinase from Thiobacillus thioparus and Thiobacillus neapolitanus (MacElroy and Mack, personal communication) suggesting that PEP may function as a general regulator of PRK. This reinforces the supposition that this enzyme occupies a central position in the control of ATP-dependent carbon dioxide fixation in autotrophic metabolism.

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