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EVIDENCE FOR AN ESSENTIAL LYSINE RESIDUE ON THE PHOSPHORIBOSYL TRANSFERASE SUBUNIT OF THE ANTHRANILATE SYNTHETASE-ANTHRANILATE 5-PHOSPHORIBOSYL-PYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE ENZYME COMPLEX FROM SALMONELLA TYPHIMURIUM

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SUMMARY

Pyridoxal 5'-phosphate reacts with the anthranilate synthetase-phosphoribosyltransferase enzyme complex of Salmonella typhimurium to inhibit PR transferase activity. Glutamine-dependent anthranilate synthetase is not affected. Spectral and kinetic data suggest that the inactivation results from the modification of an essential lysine residue which interacts with 5-phosphoribosyl 1-pyrophosphate.

The anthranilate synthetase† enzyme complex from Salmonella typhimurium catalyzes the first two reactions of tryptophan biosynthesis. The two subunits each of AS and PRT which comprise the complex carry specific binding sites for chorismate, ammonia, a divalent metal ion, and tryptophan (on AS); glutamine (on the amino-terminal portion of PRT); and anthranilate and PRPP (on the carboxy-terminal portion of PRT). Little is known about the amino acids concerned with binding these ligands at their specific sites except for evidence that a cysteine residue is involved in binding of glutamine to AS-PRT from S. typhimurium (1) and to other, analogous glutamine amidotransferases (2, and references therein). The present study provides evidence that an essential lysine residue is present at the active site on

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†The abbreviations used are: AS = anthranilate synthetase; PRT = anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase; PRPP = 5-phosphoribosyl 1-pyrophosphate; PLP = pyridoxal 5'-phosphate; PMP = pyridoxamine 5'-phosphate.

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the carboxy-terminal portion of PRT and that it probably functions to bind PRPP or Mg^{+2}-PRPP.

MATERIALS AND METHODS

The preparation of the enzyme complex from S. typhimurium has been described (3). Glutamine-dependent AS activity was measured by the method of Henderson et al (4). PRT was assayed spectrophotometrically (3) on a Gilford 240 spectrophotometer at 320 nm in 20 mM barbitol, pH 8.9. Protein concentration was determined using the Lowry procedure. Incubations with PLP were carried out at 0-4° in the dark in 50 mM barbitol, pH 8.7. Control incubations received water instead of PLP. There was a 5-15% loss of PRT activity in 25-30 min. in these control incubations; assays of PLP-treated enzyme were corrected for these non-specific activity losses. A buffer used extensively in these studies, referred to as Buffer 1 in the text, had the following composition: 0.05 M potassium phosphate, 0.1 mM EDTA, 0.4 mM 2-mercaptoethanol, 30% (v/v) glycerol, pH 7.4.

Barium chorismate, PRPP and L-glutamine were obtained from Sigma Chemical Company; chorismate was converted to the potassium salt before use. Anthranilate (once recrystallized) came from Eastman Chemical Company and PLP from Nutritional Biochemicals Corporation. All other chemicals were of reagent grade.

RESULTS

When enzyme complex was incubated with PLP, there was a pseudo-first order, time-dependent loss of PRT activity (Fig. 1). Glutamine-dependent AS activity was not affected. At 1.89 mM PLP, PRT activity declined after 26 min to 35% of the initial activity. This leveling off of activity reflects a reversible equilibrium between covalently and non-covalently modified enzyme complex-PLP adducts (5). The pseudo-first order rate constants, k', were determined from the data in Fig. 1 and the half-times ($t_{0.5}$) were calculated using the relationship $t_{0.5} = 0.693/k'$. Plotting log $(t_{0.5})^{-1}$ against log (PLP) (6) gave a straight line with a slope of 0.87 (Fig. 2). A plot of the reciprocal of k' against the reciprocal concentration of PLP (7) gave a straight line with a y-intercept of 0.07 min^{-1} and an x-intercept from which a K_i of 1.17 mM was calculated (Fig. 3).

Inhibition studies demonstrated that PLP inhibited competitively with respect to PRPP at saturating Mg^{++} and anthranilate (K_i = 0.44 mM), and non-competitively with respect to anthranilate at saturating Mg^{++} and non-

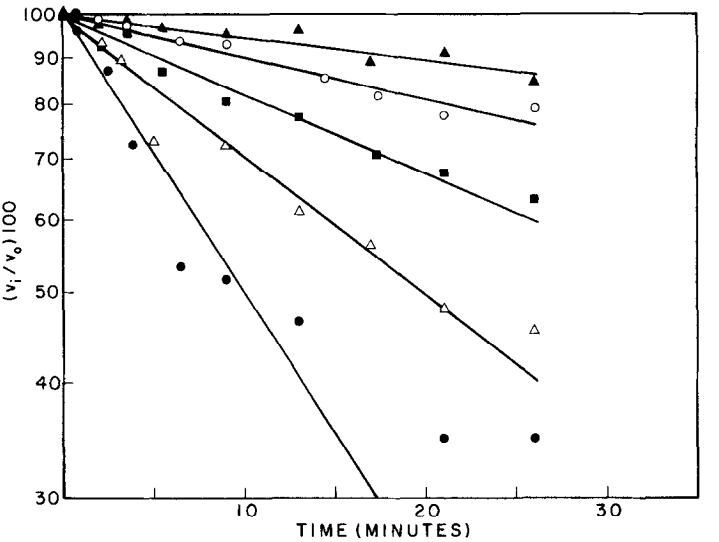

Fig. 1 Rate of inactivation of PRT activity with various concentrations of PLP. Enzyme complex (206 μg/ml) was incubated in 50 mM barbitol, pH 8.7, with PLP at 0.1 mM (▲), 0.2 mM (○), 0.4 mM (■), 0.94 mM (△) and 1.89 mM (●). Aliquots were removed at the times indicated and assayed as described in Methods. V_o is the initial velocity of the control at zero time, V_i represents the initial velocity of the test sample at various times during the incubation.

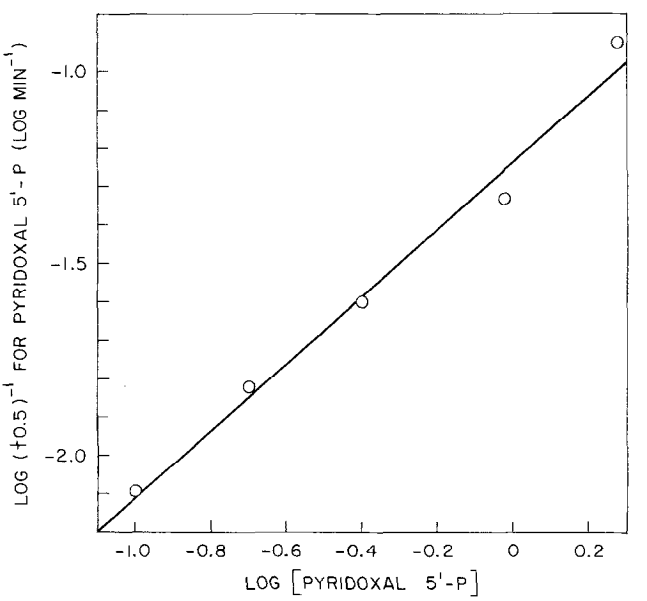

Fig. 2 Determination of the order of PLP inhibition of PRT activity. Data for PLP were taken from Fig. 1 and plotted as indicated in the text. Concentrations are molar; half-times are in min.

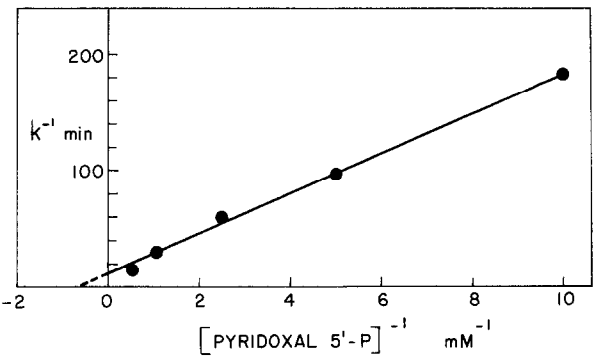

Fig. 3 Double reciprocal plot of the pseudo-first order rate constant for PLP inhibition of PRT activity versus concentration of PLP. Data for PLP were taken from Fig. 1 and plotted as indicated in the text.

Table I

EFFECT OF SUBSTRATES ON PLP INHIBITION OF PRT ACTIVITY.[a]

Additions	% Activity Remaining
Control	100
None	42
PRPP	43
Anthranilate	48
Anthranilate + PRPP + EDTA	59
Mg^{+2}	34
Mg^{+2} + anthranilate	39
Mg^{+2} + PRPP	74

[a]Enzyme complex (144 µg/ml) was incubated in 50 mM barbitol, pH 8.7, at 0-4°C in the dark. PLP was present at 1.89 mM in all of the incubations except for the control which contained none. The concentrations of Mg^{+2}, PRPP, anthranilate and EDTA were 5 mM (38 K_m), 1.26 mM (42 K_m), 1.40 mM (117 K_m) and 2 mM, respectively. The % activity remaining after 26 min. is expressed relative to the control.

saturating PRPP. Studies on the protection of PRT activity by substrates against PLP inhibition showed that only the combination of Mg^{++} and PRPP afforded any significant protection (Table 1). Mg^{++} alone enhanced PLP inhibition slightly. Free anthranilate was found not to react with PLP.

Incubation of PLP-inhibited enzyme complex in 50 mM Tris reversed inhibition by 60% after incubation with 0.98 mM PLP and by 45% after incubation with 1.96 mM PLP. Dialysis for 20 hours against Buffer 1 completely

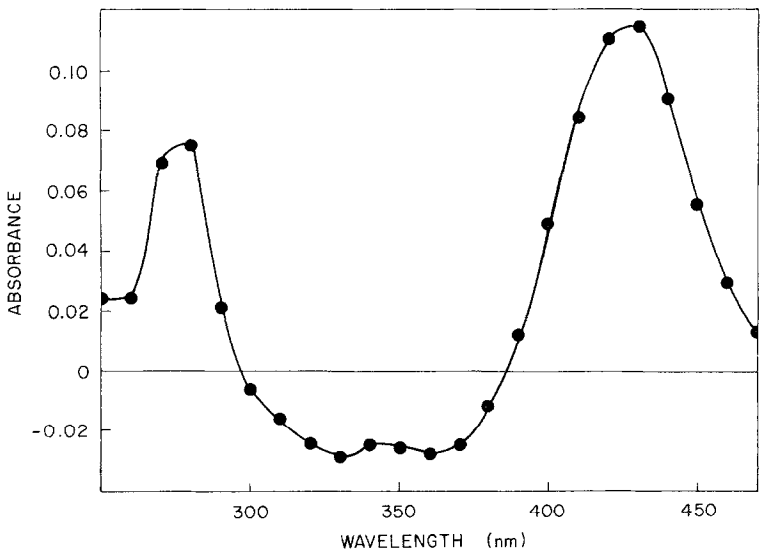

Fig. 4 Difference spectrum of the enzyme complex-PLP reversible adduct. The buffer used for all spectra was 0.05 M potassium phosphate, pH 7.4. The spectrum of enzyme complex (515 μg/ml) in the presence of 0.4 mM PLP was measured 26 min after the addition of PLP. The blank was buffer containing 0.4 mM PLP. The difference spectrum was generated by subtracting from the above spectrum the spectrum of 515 μg/ml enzyme complex; the blank for this spectrum was buffer. Spectra were determined with a Cary 15 spectrophotometer.

reversed inhibition. This reversibility is consistent with PLP modification of a primary amine (5,8), but not a cysteine or histidine residue (9,10). The difference spectrum between enzyme complex and PLP-treated enzyme complex is shown in Fig. 4.

In order to determine the stoichiometry of PMP groups bound the enzyme complex was incubated with 1.85 mM PLP for 26 min., reduced with $NaBH_4$ and dialyzed for 20 hours in the dark against 4000 volumes of Buffer 1. The molar extinction coefficients of 8700 M^{-1} cm^{-1} at 316 nm (11) and 9700 $M^{-1}cm^{-1}$ at 324 nm (12) for N^6-phosphopyridoxyllysine were used to determine the number of moles of PMP bound per mole of enzyme complex. When 70% of the activity was inactivated, 11 to 12 moles of PMP were bound per mole of enzyme complex.

DISCUSSION

PLP, a chemical modification reagent which reacts with nucleophiles and shows high selectivity for the ε-amino group of specific lysine residues (13), caused rapid inhibition of PRT activity. The reversible inhibition by PLP, which became irreversible after reduction with $NaBH_4$, and the difference spectrum of the PLP-enzyme complex adduct provide evidence that PLP is binding principally to primary amino groups. In neutral aqueous solution, conjugates of PLP and cysteine, histidine or tryptophan have an absorption maximum around 330 nm (14,15). PLP conjugates of primary amines exist in two tautomeric forms with characteristic absorption maxima (16-18),-330 nm for the enol-imine form and 430 nm for the keto-enamine form. The difference spectrum (Fig. 4) of the PLP-enzyme complex adduct suggests that the majority of PLP was bound to primary amino groups as the keto-enamine form. It is similar to the difference spectra found when PLP is bound reversibly to a lysine ε-amino group in glucose 6-phosphate dehydrogenases from Leuconostoc mesenteroides (19) and Candida utilis (20) and in rabbit muscle phosphoglucose isomerase (21).

An examination of several aspects of the interaction between PLP and AS-PRT provides evidence that PLP binds to that portion of the PRT active site which reacts with PRPP or Mg^{+2}-PRPP. In Fig. 2, the slope of the line is equal to the average order of the reaction with respect to the concentration of PLP (6). The slope of 0.87 suggests that one molecule of PLP is bound per active unit of PRT when the enzyme is inactivated. The data for the rate of inactivation were analyzed in Fig. 3 by the method of Kitz and Wilson (7). Inactivation by PLP appears to be a saturation phenomenon suggesting a discrete binding site for PLP. No subunit interaction is detectable. Since the extrapolation to infinite PLP concentration does not pass through the origin, the y-intercept indicates a limiting rate of inactivation, $k = (0.07\ min^{-1})$, owing to the conversion of all free enzyme to an intermediate complex with PLP. This suggests that PLP is substrate-like and is

not inactivating by bimolecular kinetics. A K_i of 1.17 mM is calculated from these data. Inhibition studies demonstrate PLP is competitive with respect to PRPP and non-competitive with respect to anthranilate. A K_i of 0.44 mM is calculated from these data.

The experiments in Table 1 show that only the combination of Mg^{+2} and PRPP afforded significant protection. This suggests that the substrate for PRT is Mg^{+2}-PRPP and not PRPP alone, and that the metal ion requirement for PRT activity may actually be with respect to the substrate PRPP. Electron paramagnetic resonance studies have shown that Mn^{+2} is bound tightly to PRPP, with K_d = 12 μM (22).

In summary, PLP apparently reacts with PRT by binding to that portion of the active site which reacts with PRPP or Mg^{+2}-PRPP. PLP is also reacting with additional groups on the AS-PRT enzyme complex as 11 to 12 moles of PMP are bound per mole of enzyme even at a 70% level of inactivation. The identity of the essential residues still remains uncertain. The primary amino groups, essential for PRT activity, could be N-terminal amino acids and/or ε-amino groups of lysine. There are 4 N-terminal amino acids and 72 lysine residues present in the enzyme complex (23). The close similarity between the spectrum of the PLP-modified enzyme complex and the spectra of other PLP-modified enzymes in which PLP was shown to react with the ε-amino group of lysine residues, and the remarkable selectivity of PLP in reacting with lysine residues which bind sugar phosphate (19), suggests strongly that the essential residue modified is a lysine residue.

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