Specific Modification of Isoleucyl Transfer Ribonucleic Acid Synthetase by Pyridoxal 5′-Phosphate†

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ABSTRACT: Pyridoxal 5′-phosphate rapidly abolished the abilities of isoleucyl-, leucyl-, and valyl-tRNA synthetases to esterify amino acids to tRNA. Pyridoxal had no significant effect on the activities of these enzymes. In all three cases, inactivation was partially reversed by dilution indicating an equilibrium between enzyme and reagent and the inactive adduct. Reversal of inactivations was prevented by reduction with sodium borohydride prior to dilution. Inactivation of isoleucyl-tRNA synthetase was studied in detail. Pyridoxal 5′-phosphate rapidly and completely abolished the abilities of this enzyme to esterify isoleucine to tRNA, to catalyze the exchange of isotope from [^{32}P]pyrophosphate to [^{32}P]ATP, and to hydrolyze isoleucyl-tRNA. The presence of isoleucine or Mg-ATP had no effect on the rate or extent of inactivation; however, isoleucyl-AMP partially protected and tRNA completely protected the tRNA esterification activity. Incorporation of one molecule of reagent per molecule of enzyme was required to inactivate the synthetase. The modified residue was identified as a lysine by the absorbance spectrum of the product and the chromatographic mobility of the product after acid hydrolysis. The inactivation was a second-order reaction; the kinetic data did not indicate formation of a noncovalent complex of enzyme and pyridoxal 5′-phosphate prior to inactivation. The rate of inactivation was dependent upon an apparent basic ionization, presumably that of the reactive amino group, which had a pK_{app} of 8.0.

The aminoacyl-tRNA synthetases esterify the 20 amino acids to their cognate tRNAs prior to the process of protein biosynthesis (Söll and Schimmel, 1974; Kisselev and Favorova, 1974). The substrates for these enzymes, amino acids, ATP, and tRNA, all have negative charges at neutrality. Therefore, it is likely that their binding to the enzyme is stabilized by interactions with the positively charged side-chain functional groups of lysyl and/or arginyl residues.

In an attempt to identify substrate binding or catalytic groups at the active sites of these enzymes, we have begun studies of reactions of their amino groups with pyridoxal 5′-phosphate. This reagent has been shown to react specifically with amino groups essential for the activities of numerous enzymes (Means and Feeney, 1971; Glazer et al., 1975) and its negatively charged phosphate group would be expected to attract pyridoxal 5′-phosphate to the active sites of the aminoacyl-tRNA synthetases. In this paper we report that isoleucyl-, valyl-, and leucyl-tRNA synthetases of *Escherichia coli* react with pyridoxal 5′-phosphate, and we present a detailed study of the reaction of isoleucyl-tRNA synthetase with this reagent.

Experimental Procedures

Materials. Isoleucyl-tRNA synthetase was prepared from *E. coli* B (obtained from Grain Processing, Muscatine, Iowa) by the method of Baldwin and Berg (1966a) as modified by Eldred and Schimmel (1972). Isoleucyl-tRNA synthetase was homogeneous by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels (Weber and Osborn, 1969), and it had a specific activity of 553 nmol of tRNA esterified per mg of protein in 10 min in the tRNA esterification assay (Muench and Berg, 1966). The freshly prepared enzyme was used in determination of reaction stoichiometry. The purified enzyme lost some activity upon storage; however, the results of kinetic studies for which it was used were independent of the specific activity. Valyl- and leucyl-tRNA synthetases were prepared by column chromatography on Sephadex G-150 followed by column chromatography on DEAE-cellulose as in the isoleucyl-tRNA synthetase preparation (Eldred and Schimmel, 1972). Both were brought to near-homogeneity by affinity chromatography on blue dextran–Sepharose (Moe and Piszkiewicz, 1976). The valyl-tRNA synthetase had a specific activity of 330 nmol of tRNA esterified per mg of protein in 10 min, and the leucyl-tRNA synthetase had a specific activity of 906 nmol of tRNA esterified per mg of protein in 10 min in the tRNA esterification assay (Muench and Berg, 1966). The purified enzymes were concentrated in an Amicon diaflow apparatus using an XM-50 membrane. They were stored in the freezer at a concentration of 1 mg/mL in 50% 0.02 M potassium phosphate buffer at pH 7.5 containing 0.01 M 2-mercaptoethanol and 50% glycerol. Before use, aliquots of these stock synthetase solutions were dialyzed against 0.1 M sodium phosphate buffer at pH 7.05 containing 0.01 M 2-mercaptoethanol.

E. coli B tRNA was obtained from Schwarz/Mann. Pyridoxal, pyridoxal 5′-phosphate, and ATP were obtained from Calbiochem. Sodium [^{3}H]borohydride was from Amersham/Searle, and [^{3}H]isoleucine, [^{3}H]valine, [^{3}H]leucine, and [^{32}P]pyrophosphate were from ICN. [^{3}H]Isoleucyl-tRNA was prepared from unfractionated tRNA by the method of Schreier and Schimmel (1972); it was stored in the freezer in 0.1 mM cacodylate buffer containing 0.1 mM $MgCl_2$ at pH 5.5.

Standard ε-pyridoxyllysine was prepared by reacting hippuryl-L-lysine with a 100-fold molar excess of pyridoxal 5′-phosphate according to the method of Shapiro et al. (1968); the adduct was then reduced with sodium [^{3}H]borohydride and hydrolyzed in vacuo with 6 N HCl for 24 h at 110 °C. Standard α-pyridoxylthreonine was prepared by substituting threonine for hippuryl-L-lysine in this procedure. The pyridoxal derivatives were located on chromatograms by their radioactivity.

Assays of Catalytic Activity. Three methods were used to

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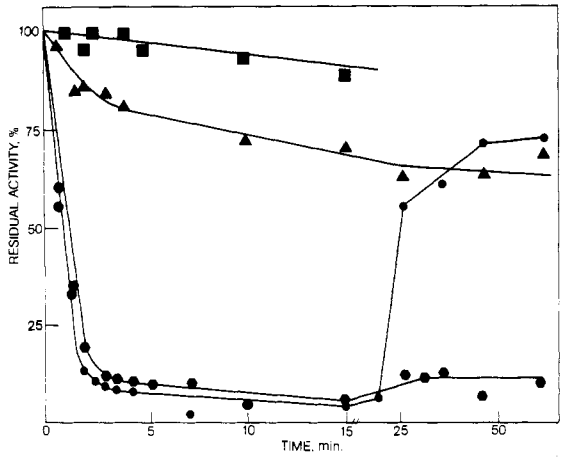

FIGURE 1: Curve A (●): Inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate; reactivation after 15 min by 50-fold dilution. Curve B (▲): Inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate, both at 0.02 the concentration of the experiment shown in curve A. Curve C (●): Inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate followed by reduction with sodium borohydride before 50-fold dilution. Curve D (■): Effect of pyridoxal on the activity of isoleucyl-tRNA synthetase.

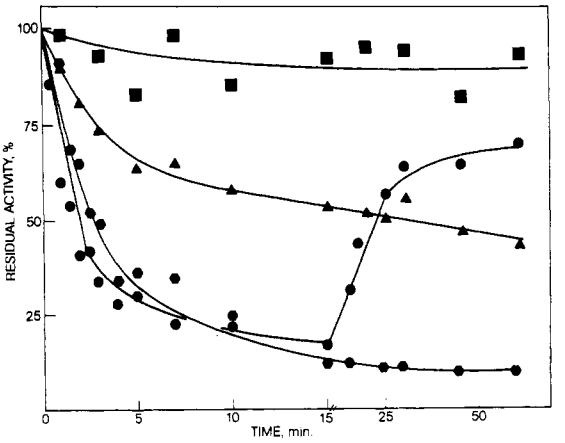

FIGURE 2: Curve A (●): Inactivation of valyl-tRNA synthetase by pyridoxal 5′-phosphate; reactivation after 15 min by tenfold dilution. Curve B (▲): Inactivation of valyl-tRNA synthetase by pyridoxal 5′-phosphate, both at 0.1 the concentration of the experiment shown in curve A. Curve C (●): Inactivation of valyl-tRNA synthetase by pyridoxal 5′-phosphate followed by reduction with sodium borohydride before tenfold dilution. Curve D (■): Effect of pyridoxal on the activity of valyl-tRNA synthetase.

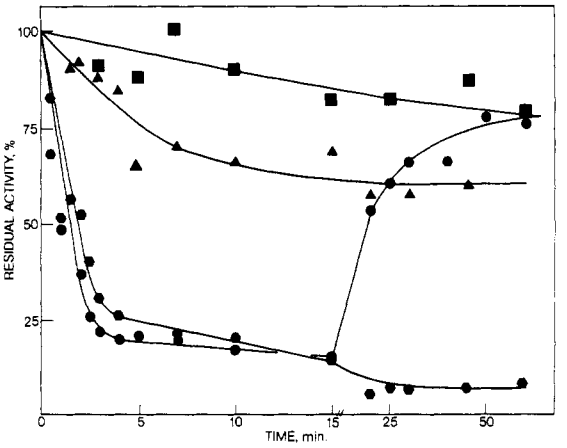

FIGURE 3: Curve A (●): Inactivation of leucyl-tRNA synthetase by pyridoxal 5′-phosphate; reactivation after 15 min by 50-fold dilution. Curve B (▲): Inactivation of leucyl-tRNA synthetase by pyridoxal 5′-phosphate, both at 0.02 the concentration of the experiment shown in curve A. Curve C (●): Inactivation of leucyl-tRNA synthetase by pyridoxal 5′-phosphate followed by reduction with sodium borohydride before 50-fold dilution. Curve D (■): Effect of pyridoxal on the activity of leucyl-tRNA synthetase.

measure the catalytic activities of the aminoacyl-tRNA synthetases. In all three assay procedures enzyme concentrations and times of incubation were adjusted and selected so that the measured activity was proportional to the concentration of active synthetase.

The abilities of the enzymes to esterify [^{3}H]isoleucine, [^{3}H]valine, or [^{3}H]leucine to tRNA were measured in 0.05 M Tris-HCl (pH 7.6) at 30 °C according to the method of Muench and Berg (1966).

The ATP-[^{32}P]pyrophosphate exchange assay was carried out according to Baldwin and Berg (1966b) with the exception that the charcoal sample was filtered on a glass fiber filter which was presoaked in 0.1 M neutral pyrophosphate. The glass fiber filters were then washed with the same buffer, dried, and counted with a planchet counter.

The deacylation of [^{3}H]isoleucyl-tRNA in the absence of AMP and pyrophosphate generally followed the method of Schreier and Schimmel (1972). Aliquots of synthetase which had been reacted with pyridoxal 5′-phosphate for various times were incubated with [^{3}H]isoleucyl-tRNA in 0.1 M cacodylate buffer at pH 7.0 and 37 °C for 3 min. The samples were then precipitated, filtered, washed, and counted as described by Schreier and Schimmel (1972).

Measurements of Concentrations. Concentrations of isoleucyl-tRNA synthetase were determined spectrophotometrically at 280 nm using a molar extinction coefficient of 1.909×10^5 (Iaccarino and Berg, 1969) and by the method of Lowry as modified by Ross and Schatz (1973). The molecular weight of the single polypeptide chain of the synthetase was taken to be 114 000 (Arndt and Berg, 1970). The concentration of the adduct of pyridoxal 5′-phosphate to lysine was determined spectrophotometrically at 325 nm using a molar extinction coefficient of 1.015×10^4 (Fischer et al., 1963).

Inactivation of Aminoacyl-tRNA Synthetases. Enzymes (0.1 mg/mL) were reacted with pyridoxal 5′-phosphate at 30 °C in 0.1 M phosphate buffer at pH 7.05 containing 0.01 M 2-mercaptoethanol except where other conditions are specified. Concentrations of pyridoxal 5′-phosphate range from 3×10^{-4} to 4×10^{-3} M. Aliquots of the reaction mixture were withdrawn at timed intervals, and either assayed directly or added to an excess of sodium borohydride and then assayed when convenient after appropriate dilution.

Kinetic Analyses. Kinetic experiments were generally performed with concentrations of pyridoxal 5′-phosphate which resulted in more than 80% inactivation as measured by the ability of the enzyme to esterify [^{3}H]isoleucine to tRNA. Therefore, the observed pseudo-first-order rate constants (k_{obsd}) were reasonable approximations of the actual rate constants in the forward direction. Pseudo-first-order rate constants were obtained by multiplying the slopes of plots of $\log [(Act_\infty - Act_0)/(Act_\infty - Act_t)]$ vs. time by 2.303.

Results

Initial experiments to characterize the reaction of aminoacyl-tRNA synthetases with pyridoxal 5′-phosphate used the tRNA esterification assay (Muench and Berg, 1966) because of its simplicity and the fact that it measures the biologically significant reaction. Isoleucyl-, valyl-, and leucyl-tRNA synthetases gave qualitatively identical results when reacted with pyridoxal 5′-phosphate (Figures 1, 2, and 3). Isoleucyl-, valyl-, and leucyl-tRNA synthetases (0.1 mg/mL) rapidly lost their

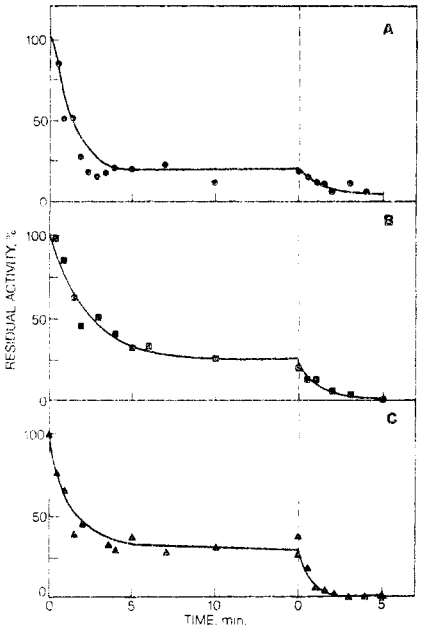

FIGURE 4: The effect of pyridoxal 5′-phosphate on the activities of isoleucyl-tRNA synthetase as a function of time: (A) residual ability to esterify [^{3}H]isoleucine to tRNA; (B) residual ability to catalyze the ATP-[^{32}P]pyrophosphate exchange reaction; (C) residual ability to hydrolyze [^{3}H]isoleucyl-tRNA. At timed intervals aliquots were withdrawn, reaction stopped by addition of sodium borohydride, and residual catalytic activities of the reduced aliquots were determined. After 15 min of reaction of synthetase with pyridoxal 5′-phosphate, the reaction mixture was reduced with sodium borohydride, dialyzed against buffer for 3 h, and then additional pyridoxal 5′-phosphate (10^{-3} M) was added.

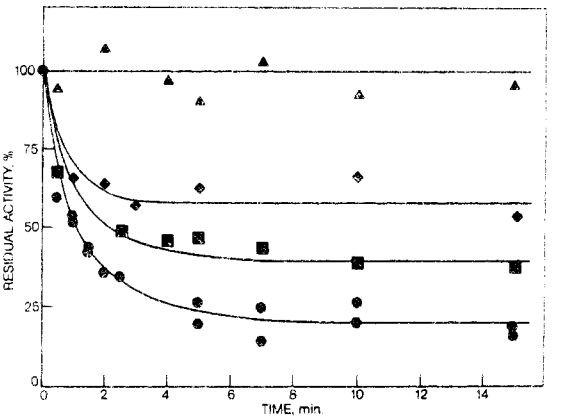

FIGURE 5: Time course of the inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate in 0.005 M phosphate buffer at pH 7.05 and 30 °C: (●) no added substrate; (●) with 10^{-2} M ATP and 10^{-2} M Mg^{2+}; (■) with 10^{-2} M isoleucine; (♦) with 10^{-3} M isoleucine, 10^{-3} M ATP, and 10^{-3} M Mg^{2+}; (▲) with 10 mg/mL unfractionated tRNA.

abilities to esterify ^{3}H-labeled amino acid to tRNA when reacted with pyridoxal 5′-phosphate (10^{-3} M) (curve A in Figures 1, 2, and 3). After 15 min, when the synthetases had reached their greatest degree of inactivation, they were diluted with buffer. All three synthetases then regained a substantial portion of their activity (curve A in Figures 1, 2, and 3), presumably due to a displacement of an equilibrium from the inactive pyridoxal-5′-phosphate synthetase adducts back to the active free enzymes and reagent. The levels of residual activity obtained upon reactivation after dilution (curve A in Figures 1, 2, and 3) were approximately the same as those obtained when the initial concentrations of synthetase and pyridoxal 5′-phosphate were identical with those produced by these dilutions (curve B in Figures 1, 2, and 3). When the inactivated synthetases were reduced with sodium borohydride prior to dilution, no reactivation took place (curve C in Figures 1, 2, and 3). Lastly, pyridoxal had no significant effect on the activities of any of the three synthetases (curve D in Figures 1, 2, and 3). In control experiments conducted in the absence of pyridoxal 5′-phosphate or pyridoxal, isoleucyl-, valyl-, and leucyl-tRNA synthetases retained 95, 80, and 90%, respectively, of their activities over the 60-min time period of these experiments.

Since, in these initial experiments, inactivation reactions of isoleucyl-, valyl-, and leucyl-tRNA synthetases by pyridoxal 5′-phosphate followed identical patterns, it appeared probable that the mechanisms of all three reactions were identical. Therefore, we decided to limit a thorough study of the inactivation by pyridoxal 5′-phosphate to only one synthetase, namely isoleucyl-tRNA synthetase.

The time courses of the inactivation of isoleucyl-tRNA synthetase (0.1 mg/mL) by pyridoxal 5′-phosphate (10^{-3} M) as measured by the three assay methods described under Experimental Procedures are given in Figures 4A, B, and C. By all three assay procedures activity was lost rapidly, and reached a minimum of approximately 20% residual activity. This residual activity might be attributable to an enzyme-inactivator complex which is partially active; alternatively, it might be due to partial reaction of synthetase to an equilibrium position with complete inactivation of those molecules which complexed to pyridoxal 5′-phosphate. To distinguish between these two possibilities, the enzyme was subjected to a second cycle of inactivation. After 15 min in the presence of pyridoxal 5′-phosphate the enzyme solution was treated with sodium borohydride and dialyzed against buffer for 3 h; then additional pyridoxal 5′-phosphate (10^{-3} M) was added. By all three assay procedures (Figures 4A, B, and C), the residual activity then dropped to near background levels, indicating that complete inactivation of the enzyme by pyridoxal 5′-phosphate could be effected. In control experiments, the synthetase retained full activities in the absence of pyridoxal 5′-phosphate and in the presence of sodium borohydride.

If the activity of isoleucyl-tRNA synthetase was lost as a result of modification of a residue which was essential for either catalysis or substrate binding, one would expect substrate to protect the enzyme from inactivation. The time course of the inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate was determined in the presence of saturating concentrations of substrates. Residual synthetase activities were determined by the tRNA esterification assay in all cases. When experiments were carried out in 0.1 M phosphate buffer at pH 7.05, substrates had no significant effect on either the extent or rate of inactivation. However, in 0.005 M phosphate buffer at pH 7.05, significant effects were seen (Figure 5). As before, pyridoxal 5′-phosphate caused a rapid loss of synthetase activity with some residual activity presumably due to achievement of an equilibrium. When 10^{-2} M ATP and 10^{-2} M Mg^{2+} were included at concentrations which should have saturated the synthetase ($K_d = 1.76 \times 10^{-4}$ M) (Holler and Calvin, 1972), no decrease in rate or degree of inactivation was found (Figure 5). Isoleucine (10^{-2} M) at a concentration which would have saturated the synthetase ($K_d = 5.8 \times 10^{-6}$ M) (Holler and Calvin, 1972) appeared to afford a small amount of protection from inactivation by pyridoxal 5′-phosphate. However, this apparent protection was probably the result of direct reaction of the amino acid with pyridoxal 5′-phosphate, thereby lowering the pool of reagent and the apparent degree of synthetase inactivation. The effect of isoleucyl-AMP on inactivation was also determined. The synthetase was incu-

bated with 10^{-3} M isoleucine, 10^{-3} M ATP, and 10^{-3} M Mg^{2+} for 10 min at pH 7.05 and 30 °C prior to addition of pyridoxal 5′-phosphate. Similar conditions have been used to saturate this enzyme with isoleucyl-AMP by in situ synthesis (Yarus and Berg, 1970). A significant, though not complete, protection of synthetase activity was obtained (Figure 5). The presence of unfractionated tRNA (10 mg/mL) brought about protection from inactivation. If one assumes that $tRNA^{Ile}$ is 5% of the unfractionated material, and that tRNAs have molecular weights of 25 000 to 30 000 (RajBhandary and Stuart, 1966), one can calculate that 10 mg/mL represents a $tRNA^{Ile}$ concentration of approximately 2×10^{-5} M. Thus, a saturating concentration of $tRNA^{Ile}$ ($K_d = 5.9 \times 10^{-7}$ M) (Yarus and Berg, 1969) effected complete protection of the synthetase from inactivation by pyridoxal 5′-phosphate (Figure 5).

To characterize the chemical nature of the inactive synthetase–pyridoxal 5′-phosphate adduct, the enzyme was reacted with reagent, then reduced with sodium borohydride. The inactivated, reduced protein sample was dialyzed exhaustively to remove excess reagents and side products, and its absorption spectrum was determined (Figure 6). Both the native and modified enzymes had an absorbance maximum at 280 nm which is characteristic of proteins. Synthetase which had been modified with pyridoxal 5′-phosphate had an additional peak at 325 nm; the height of this peak increased as the concentration of pyridoxal 5′-phosphate to which the enzyme was exposed increased. The reduced adduct of pyridoxal 5′-phosphate and lysine has an absorbance maximum at this wavelength (Fischer et al., 1963). In view of the reversibility of the reaction before reduction, its irreversibility after reduction, and the absorption spectrum of the product, it is probable that inactivation of the synthetase by pyridoxal 5′-phosphate is the result of reversible formation of an imine. However, it should be noted that the thiazolidine-like complex formed between pyridoxal 5′-phosphate and L-cysteine has a similar absorption spectrum with a maximum at 330 nm (Coombs et al., 1964). The spectrum of the product (Figure 6) is consistent with imine formation followed by addition of the thiol of a cysteinyl residue across the double bond. However, this possibility is unlikely in view of the reversibility of the inactivation reaction (Figure 1, curve A) and its irreversibility after reduction (Figure 1, curve C).

The amino acid residue of isoleucyl-tRNA synthetase modified by pyridoxal 5′-phosphate was identified as a lysine by electophoretic and chromatographic methods. An enzyme sample which had been reacted with pyridoxal 5′-phosphate, reduced with sodium [^{3}H]borohydride, exhaustively dialyzed, and found by a spectrophotometric method (see below) to contain 0.8 modified residue per enzyme molecule was hydrolyzed in 6 N HCl for 24 h at 110 °C. The hydrolysate was then subjected to high voltage paper electrophoresis at pH 1.9, and descending paper chromatography in 1-butanol–acetic acid–water (200:30:75). By both methods a spot was found which had a mobility identical with that of ε-pyridoxallysine. No spot was found by either method which corresponded to α-pyridoxylthreonine which would have been formed if reaction took place at the amino terminus of the polypeptide chain (Arndt and Berg, 1970).

The number of residues needed to be modified to completely inactivate the synthetase was determined by first reacting several samples of the enzyme with varying concentrations of pyridoxal 5′-phosphate. These samples were then reduced with sodium borohydride and dialyzed to remove excess reagent. The residual catalytic activity and the number of moles of reagent incorporated per mole of synthetase were determined

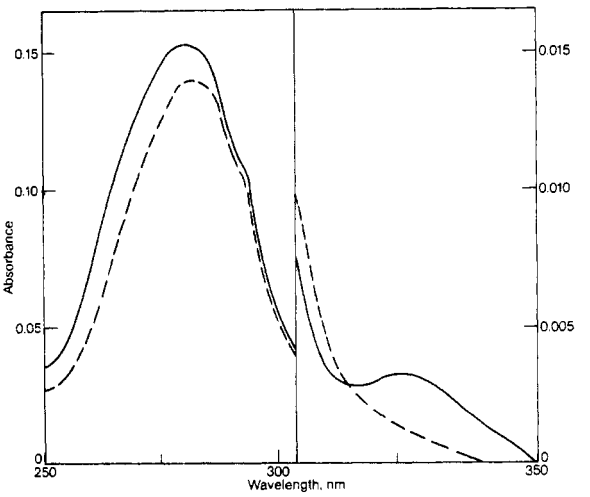

FIGURE 6: Absorption spectrum of native isoleucyl-tRNA synthetase (broken line) and absorption spectrum of isoleucyl-tRNA synthetase after modification with pyridoxal 5′-phosphate, reduction by sodium borohydride, and dialysis (solid line); modified enzyme contained 0.8 mol of reagent per mol of enzyme.

for each sample. The percent of the residual catalytic activity when plotted as a function of the number of moles of reagent incorporated gave a linear graph (figure not shown). When the line was extrapolated to zero activity, 1.2 residues of pyridoxal 5′-phosphate were found to be incorporated per enzyme molecule. Thus, loss of catalytic activity can be correlated with modification of approximately one lysyl residue per enzyme molecule of molecular weight 114 000 (Arndt and Berg, 1970).

Quantitative kinetic experiments were performed to characterize the inactivation reaction. Rates of inactivation of isoleucyl-tRNA synthetase were determined at varying concentrations of pyridoxal 5′-phosphate and at several pH values. Plots of log $[(Act_\infty - Act_0)/(Act_\infty - Act_t)]$ vs. time were generally found to be linear (figure not shown) indicating that the reactions were first order with respect to enzyme concentration. Furthermore, a plot of the observed pseudo-first-order rate constants as a function of pyridoxal 5′-phosphate concentrations at pH 7.05 was linear up to a concentration of 4×10^{-3} M (figure not shown). The rate of inactivation did not become independent of pyridoxal 5′-phosphate at high concentrations, which would have indicated a rapid noncovalent association of inactivator with enzyme prior to covalent bond formation.

The pH dependence of the second-order rate constant of inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate is shown in Figure 7. Accurate rate constants could not be determined below pH 5.5 or above pH 9.2 because of the instability of the enzyme under these conditions. The pH dependence described a sigmoid-shaped curve with the observed second-order rate constant, k_0, increasing at higher pH values and with an apparently pH-independent rate constant at lower pH values. A simple theoretical expression which fit the data is

$$k_0 = k_i + k_2 \left(\frac{K_{app}}{K_{app} + [H^+]} \right) \quad (1)$$

where k_i is a pH-independent second-order rate constant and k_2 is a second-order rate constant of inactivation which is dependent upon an apparent basic ionization which has a dissociation constant of K_{app}. A theoretical curve which fits the data is generated when $k_i = 400\ M^{-1}\ min^{-1}$, $k_2 = 2600\ M^{-1}\ min^{-1}$, and $pK_{app} = 8.0$ (Figure 7). If, as appears probable, inactiva-

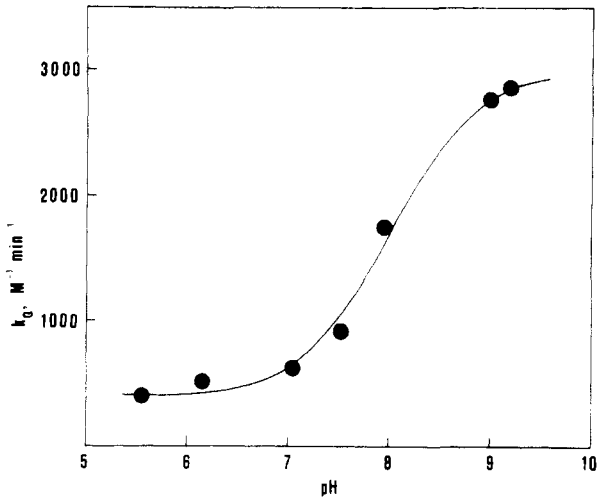

FIGURE 7: Second-order rate constants of inactivation of isoleucyl-tRNA synthetase by pyridoxal 5′-phosphate plotted as a function of pH. The points were determined experimentally, and the curve was calculated from eq 1.

tion is the result of imine formation, pK_{app} describes the dissociation of the protonated form of the reactive lysyl ϵ-amino group, k_2 is the second-order rate constant of reaction of this form. One possible interpretation of the term k_i is that it reflects the rate of reaction of the protonated amine to give a protonated Schiff base. Examples of such a mechanism have been discussed by Bruice and Benkovic (1966).

Discussion

The interactions of pyridoxal 5′-phosphate with three aminoacyl-tRNA synthetases were studied in an attempt to locate amino groups at the active sites of these enzymes which might be essential for substrate binding or catalysis. Isoleucyl-, valyl-, and leucyl-tRNA synthetases were all rapidly and reversibly inactivated by pyridoxal 5′-phosphate; pyridoxal had only a minor effect on the activities of these enzymes. The failure of pyridoxal to inactivate these enzymes might be attributed to the fact that a large part of it is present in solution as the unreactive, internal hemiacetal (Metzler and Snell, 1955). Pyridoxal 5′-phosphate reacted with isoleucyl-tRNA synthetase to form a 1:1 complex; the product apparently was an imine formed between the reagent and the ϵ-amino group of a lysyl residue of the enzyme. The modified enzyme lacked the ability to esterify isoleucine to tRNA, to catalyze the ATP-[^{32}P]pyrophosphate exchange reaction, and to deacylate isoleucyl-tRNA.

This study was based on the hypothesis that pyridoxal 5′-phosphate is an active-site-directed reagent for aminoacyl-tRNA synthetases. This hypothesis was supported by the fact that tRNA completely protected the synthetase from inactivation. This protection was observed in 0.005 M phosphate buffer at pH 7.05 but not in 0.1 M buffer. The fact that protection was observed only at low salt concentration should not be surprising since the binding of $tRNA^{Ile}$ to its synthetase is very sensitive to salt, and probably reflects a strong electrostatic component in their association (Lam and Schimmel, 1975). The protection of the synthetase from inactivation by pyridoxal 5′-phosphate in the presence of tRNA strongly suggests that inactivation is the result of the inactivator binding to a portion of the tRNA binding site and thereby preventing tRNA binding to the enzyme surface.

The presence of isoleucyl-AMP in the reaction mixture effected some protection of synthetase activity. This partial protection may reflect a decrease in rate of imine formation and the final equilibrium position due to conformational constraints imposed on the enzyme by the presence of the isoleucyl-AMP intermediate. This effect appears to be reciprocal since formation of the pyridoxal 5′-phosphate adduct prevents the exchange of ^{32}P between pyrophosphate and ATP (Figure 4B) which requires formation of isoleucyl-AMP but does not directly involve tRNA.

Inactivation of the synthetase was first order in both enzyme and reagent. No kinetic evidence for formation of a noncovalent complex of pyridoxal 5′-phosphate and synthetase prior to covalent bond formation similar to Michaelis–Menten complex formation could be detected. It is possible that noncovalent complex formation was not detected because the concentration of reagent never became great enough to saturate the enzyme; that is, reagent concentration never exceeded the dissociation constant of the noncovalent complex.

Kinetic analysis of the inactivation of isoleucyl-tRNA synthetase has shown that the reaction is apparently a second-order process with the rate of reaction being in part proportional to the mole fraction of a basic group with a pK_{app} of 8.0 (eq 1). The most reasonable interpretation of pK_{app} is that it describes the ionization of the ϵ-amino group of the reactive lysyl residue, and the rate-limiting step is nucleophilic addition of this group to the aldehyde. The value of pK_{app} is 1.4 to 2.6 units lower than is normally found for an ϵ-amino group of a lysyl residue in a protein (Edsall, 1943), and it is likely that this abnormally low pK_{app} accounts for its reactivity. The abnormally low pK_{app} of the amino group would result in a greater mole fraction in the reactive, unprotonated form at neutral pH values, and consequently a greater rate of reaction than any of the other amino groups in the protein. Other proteins whose reactivity with pyridoxal 5′-phosphate has been attributed to an amino group with an abnormally low pK_a include bovine liver glutamate dehydrogenase (Piszkiewicz and Smith, 1971) and *E. coli* RNA polymerase (Bull et al., 1975).

We have found that valyl- and leucyl-tRNA synthetases of *E. coli* are also inactivated by pyridoxal 5′-phosphate at rates comparable with that for inactivation of isoleucyl-tRNA synthetase. The nature of the reagent (Means and Feeney, 1971) indicates that the site of reaction is an amino group, probably that of a lysyl residue as is the case for isoleucyl-tRNA synthetase. Bruton and Hartley (1970) have found that a lysyl residue of methionyl-tRNA synthetase of *E. coli* is affinity labeled and the enzyme inactivated by *p*-nitrophenylcarbamoyl methionyl-tRNA. Thus, four aminoacyl-tRNA synthetases now appear to have lysyl residues which are essential for activity. It is conceivable that all of the aminoacyl-tRNA synthetases have lysyl residues which are essential for activity, as well as cysteinyl residues which have been proposed as being essential for activity (Söll and Schimmel, 1974; Kisselev and Favorova, 1974).

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Pre-Steady-State Kinetics of Intermediate Formation in the Deuteroferriheme–Hydrogen Peroxide System†

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ABSTRACT: The pH dependence of formation of a peroxidatic intermediate from the reaction of deuteroferriheme with hydrogen peroxide has been determined for the region pH 8.7–10.1 from stopped-flow kinetic studies in which absorbancy changes are observed at heme monomer–dimer isosbestic points. Results are interpreted primarily in terms of the attainment of double "steady-state" concentrations of Michaelis–Menten complex I and peroxidatic intermediate I′. A linear correlation of observed first-order rate constants with α, the degree of dissociation of heme dimer, has been demonstrated and nonzero intercepts are obtained. Slopes and intercepts show a linear logarithmic dependence on pH which is interpreted in terms of HO_2^- participation both in the formation and subsequent (catalatic) decomposition of a peroxidatically active intermediate. General acid catalysis of intermediate formation is indicated from studies in phosphate, arsenate, and citrate buffer at pH 7.4–9.3. It is suggested that such catalysis may be responsible for anomalously high rates of H_2O_2 decomposition previously observed in phosphate buffer solution.

In a previous paper (Jones et al., 1974a), we reported on the kinetics of formation of the peroxidatic intermediate from the reaction of deuteroferriheme [chloro(dihydrogen 3,7,12,17-tetramethyl-2,18-porphinedipropionato(2$^-$))iron(III)] and hydrogen peroxide at pH 7.75 and 8.5 at 25 °C. The results obtained from stopped-flow spectrophotometric studies were interpreted in terms of the mechanistic model depicted in reaction 1,

$$M + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} I' \qquad (1)$$

wherein M denotes monomeric deuteroferriheme; I, the Michaelis–Menten complex; and I′, the peroxidatic intermediate analogous to compounds I of various oxidase enzymes, e.g., catalase (EC 1.11.1.6) reported by Chance et al. (1947, 1952). Values of kinetic parameters k_3 and $K_m(= (k_2 + k_3)/k_1)$ were calculated for reaction 1, employing data of Prudhoe (1971) for the pH and (heme) concentration dependence of the equilibrium concentrations of monomeric and dimeric heme at 25 °C.

Although this model modifies an earlier mechanism proposed by Portsmouth and Beal (1971) in recognizing mono-

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