A New Thiol-Dependent Transamination Reaction Catalyzed by the B Protein of *Escherichia coli* Tryptophan Synthetase*

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ABSTRACT: Pyridoxal phosphate, the cofactor for the B protein of tryptophan synthetase, is rapidly lost during dialysis against buffers containing L-serine and certain thiols such as mercaptoethanol. This resolution of the B protein is a result of a thiol-dependent transamination reaction which is described here for the first time; in the course of this reaction pyridoxal phosphate is converted into pyridoxamine phosphate, which dissociates readily from the enzyme. Spectrophotometric observations during the reaction and the isolation and identification of products are described. The predominant reaction of the B protein in the presence of mercaptoethanol is a β-addition reaction (previously observed with the AB complex) producing *S*-hydroxyethyl-L-cysteine. An analogous reaction occurs in the presence of dithioerythritol. The α-keto acid products of the two thiol-dependent transamination reactions studied are the corresponding S-substituted derivatives of mercaptopyruvate.

The relative rates of the several reactions of the B protein and the AB complex involving L-serine in the presence and absence of thiols and indole were determined. Enzyme-bound phosphopyridoxylaminoacrylic acid appears to be a common intermediate in all the reactions of tryptophan synthetase. The A protein is not required for the formation of this intermediate but does have a strong influence on its fate. These results demonstrate the usefulness of this system for the study of the specificity and control of the catalytic site of a pyridoxal phosphate enzyme.

Reactions 1–3 are catalyzed by *Escherichia coli* tryptophan synthetase (Crawford and Yanofsky, 1958). The enzyme is composed of two nonidentical, readily separable subunits, the A and B proteins. Each subunit alone has a small amount of activity in one of the reactions, the A protein in reaction 2 and the B protein in reaction 1. Reaction 3 occurs only in the presence of the AB complex. The B protein carries out another reaction (4), serine deamination (Crawford and Ito, 1964). Recently Goldberg and Baldwin (1967) reported that in the presence of mercaptoethanol the AB complex carries out a β-addition reaction, adding the thiol to L-serine to form *S*-hydroxyethyl-L-cysteine (reaction 5). These authors also reported that in the presence of pyridoxal phosphate, L-serine, and mercaptoethanol, the AB complex exhibits a new absorption band centered at 468 mμ.

$$\text{indole} + \text{L-serine} \xrightarrow{\text{PLP}^1} \text{L-tryptophan} \quad (1)$$

$$\text{indole-3-glycerol phosphate} \rightleftharpoons \text{indole} + \text{D-glyceraldehyde 3-phosphate} \quad (2)$$

$$\text{indole-3-glycerol phosphate} + \text{L-serine} \xrightarrow{\text{PLP}} \text{L-tryptophan} + \text{D-glyceraldehyde 3-phosphate} \quad (3)$$

$$\text{L-serine} \xrightarrow{\text{PLP}} \text{pyruvate} + \text{ammonia} \quad (4)$$

$$\text{mercaptoethanol} + \text{L-serine} \xrightarrow{\text{PLP}} S\text{-hydroxyethyl-L-cysteine} + H_2O \quad (5)$$

The B protein contains bound pyridoxal phosphate (Wilson and Crawford, 1965). Reactions 1 and 3–5 all require this cofactor and are probably carried out through a series of Schiff base intermediates formed between enzyme-bound pyridoxal phosphate and L-serine (Figure 1) consistent with the general mechanism for pyridoxal-catalyzed reactions (Metzler *et al.*, 1954). Formation of enzyme-bound α-aminoacrylic acid (V) is the key reaction in this series, for this would appear to be a common intermediate in reactions 1 and 3–5.

This paper reports that the B protein alone can catalyze reaction 5 with mercaptoethanol, and that both the B protein and the AB complex carry out an analogous reaction with dithioerythritol. It also demonstrates

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; β-ME, mercaptoethanol; HEC, *S*-hydroxyethyl-L-cysteine; DTE, dithioerythritol; RSH, mercaptoethanol or dithioerythritol; R-S-Pyr, *S*-pyruvylmercaptoethanol or *S*-pyruvyldithioerythritol; R-S-Cys, *S*-alanylmercaptoethanol or *S*-alanyldithioerythritol.

the occurrence of a new thiol-addition reaction which is carried out only by the B protein (reaction 6). The

$$\text{RSH} + \text{L-serine} + \text{PLP} \rightleftharpoons \text{R-S-mercaptopyruvate} + \text{PMP} + \text{H}_2\text{O} \quad (6)$$

mechanism proposed for this reaction includes the aminoacrylic intermediate, V, and another intermediate, VIII, which is shared with reaction 5 (Figure 1).

Experimental Section

Materials

The B protein of tryptophan synthetase was purified from extracts of the A2/F′A2 strain of *E. coli* (Baker and Crawford, 1966) according to the method of Wilson and Crawford (1965). The A protein of tryptophan synthetase was purified from extracts of the B-8 strain of *E. coli* by a modification (Hatanaka *et al.*, 1962) of the method of Henning *et al.* (1962). Lactic dehydrogenase (rabbit muscle), malic dehydrogenase (pig heart), catalase (beef liver), and L-amino acid oxidase (snake venom) were purchased from Worthington Biochemical Corp., Freehold, N. J. Glutamic–oxalacetic transaminase (pig heart) was purchased from California Corp. for Biochemical Research, Los Angeles, Calif., as were dithiothreitol and 2,3-dimercaptopropanol. Hydroxyethyl-L-cysteine was synthesized by the method of Verderame (1961). Bromopyruvate was a gift of Dr. Paul Meloche of the Institute for Cancer Research (Philadelphia, Pa.) and was synthesized by the method of Dickens (1962). Pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., as were DPNH, dithioerythritol, pyruvic acid, α-ketoglutaric acid, and glycolaldehyde. L-Serine, L-aspartic acid, L-cysteine, and lithium hydroxypyruvate were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Uniformly labeled L-[^{14}C]serine (12.2 Ci/mole) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill.

Methods

Assays for Tryptophan Synthetase Activities and Protein. The standard assay for the activity of the A or B protein in reaction 1 has been described by Crawford and Yanofsky (1958). Serine deamination (reaction 4) was measured by coupling the pyruvate produced with lactic dehydrogenase as described by Crawford and Ito (1964). Pyruvate was also determined on aliquots of deproteinized reaction mixtures with lactic dehydrogenase. A suspension of crystals of lactic dehydrogenase in 50% ammonium sulfate (0.001 ml) was added to 0.5 ml of solution containing the material to be tested, Tris-HCl buffer (pH 7.8, 50 μmoles) and DPNH (0.05 μmole). The decrease in optical density at 340 mμ, which was completed in approximately 1 min, was observed. The rate of synthesis of tryptophan (reaction 1), of pyruvate (reaction 4), or of S-substituted L-cysteine (reaction 5) was measured by determining the proportion of radioactivity in the compound after chromatographic separation of reaction mixtures.

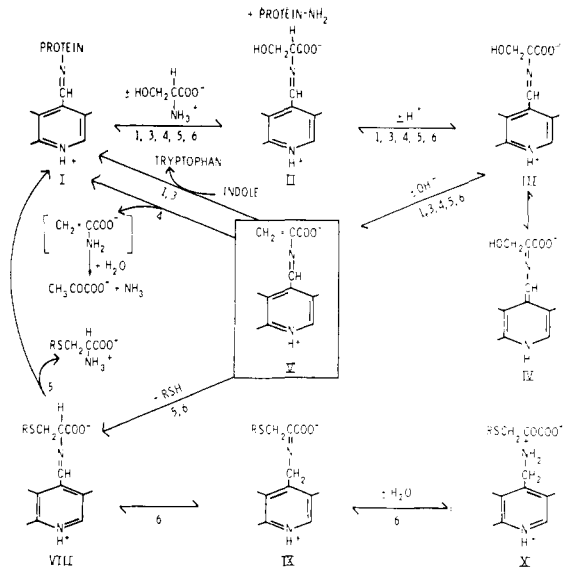


FIGURE 1: Scheme for the various reactions catalyzed by tryptophan synthetase. The roman numerals refer to proposed intermediate pyridoxal phosphate complexes discussed in the text. The complex with aminoacrylate (V) is the key intermediate common to all of the reactions. The arabic numerals indicate steps in the corresponding reactions given in the text.

Pyridoxal phosphate disappearance (reaction 6) was determined on aliquots of reaction mixtures by the method of Wada and Snell (1961) scaled down to a 0.5-ml reaction volume. Pyridoxamine phosphate formation (reaction 6) was followed continuously in a spectrofluorimeter similar to that described by Weber and Young (1964). This instrument records the ratio of incident to emitted light, collecting the fluorescence from the illuminated face of a 1-cm quartz cuvet at a controlled temperature. Incident light intensity was kept very low to avoid photodecomposition (Chen, 1965). Although excitation was maximal at 325 mμ, the 313-mμ mercury line gave adequate sensitivity and was used for excitation in most of these studies. The instrument could detect 0.2–10 mμmoles of pyridoxamine phosphate; other reaction components neither quenched nor interfered with its estimation.

All reactions were performed at 37° except where noted. A unit of activity in any reaction (1–6) is the disappearance of 0.1 μmole of substrate or the appearance of 0.1 μmole of product in 20 min. Protein was determined by the method of Lowry *et al.* (1951). Spectra and absorption measurements were made in Cary Model 11 and 14 spectrophotometers.

Assay for Pyridoxamine Phosphate with Glutamic–Oxalacetic Transaminase. The pyridoxal phosphate form of glutamic–oxalacetic transaminase was resolved by a modification of the method of Wada and Snell (1962). A 1% suspension of the holoenzyme (1 ml) was dialyzed for 16 hr at 4° against a solution 60% saturated with ammonium sulfate at pH 3.0. The suspension in the dialysis bag was centrifuged, and the pellet was dissolved in 1 ml of 0.1 M K_2HPO_4 and dialyzed against 0.1 M potassium phosphate at pH 7.8. Activity was determined by a modification of the

method of Sizer and Jenkins (1962) in a coupled assay with malic dehydrogenase. Since Meister *et al.* (1954) observed that lengthy preincubation with pyridoxamine phosphate was necessary for maximal activation of glutamic–oxalacetic transaminase, apoenzyme (0.05 μg) was incubated with six different concentrations of pyridoxal phosphate (1–25 mμmoles) or pyridoxamine phosphate (2–100 mμmoles) in 0.5 ml of 0.20 M potassium phosphate buffer (pH 7.5) for 1 hr at 37°. A reaction mixture containing DPNH (0.1 μmole), L-aspartate (30 μmoles), and α-ketoglutarate (5 μmoles) in 0.5 ml was added and the decrease in absorbancy at 340 mμ was followed. The reciprocal of each observed initial rate was plotted against the reciprocal of the cofactor concentration (Lineweaver and Burk, 1934). Under these conditions of assay the same V_{max} was obtained with either pyridoxamine phosphate or pyridoxal phosphate. The apparent K_m for pyridoxamine phosphate was 10^{-5} M; the apparent K_m for pyridoxal phosphate was 2.7×10^{-6} M. The concentration of pyridoxamine phosphate in a solution of unknown concentration was determined from assays on several aliquots, using the reciprocal plot for pyridoxamine phosphate as a standard curve.

Electrophoresis of Pyridoxamine Phosphate. Samples were applied 5 cm from one end of 3 × 30.5 cm strips of Whatman No. 3MM paper and subjected to electrophoresis for 2 hr at 131 V/cm. The buffer used was 0.02 M potassium phosphate (pH 7.5). Pyridoxamine phosphate migrated toward the anode and could be detected as either a fluorescent band or an orange band after spraying with ninhydrin and heating to 100° for 10 min.

Chromatography and Determination of Radioactivity. Whatman No. 3MM paper was used in solvent systems I and II. Solvent I (80% pyridine–20% water (v/v)) was used for ascending or descending chromatography for 15 hr. This system gave a good separation of serine and S-substituted cysteine derivatives. In solvent system II, serine was first separated from other, more rapidly moving compounds by ascending chromatography in solvent I for 3 hr. The chromatogram was then cut horizontally above the serine area (determined by a control strip bearing a serine standard) and subjected to a second ascending chromatographic development in 1-butanol–formic acid–water (70:15:15, v/v) for 5 hr.

2,4-Dinitrophenylhydrazones of α-keto acids were prepared by treating samples or standards with less than a twofold excess of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl for 15 min at 25°. The resulting derivatives were extracted three times with one volume of ethyl acetate per volume of aqueous solution. The extracts were concentrated in a stream of air and subjected to chromatography on Whatman No. 1 paper in one or more of several solvents in the descending direction for 15 hr. Solvent III was the organic phase of 1-butanol saturated with an equal volume of 1.8 M ammonium hydroxide. It was used with the aqueous layer in the bottom of the tank. Solvent IV was 2-propanol–H_2O–NH_4OH (200:20:10, v/v). Solvent V was 1-butanol–ethanol–0.5 N NH_4OH (70:10:20, v/v). Solvent VI was 1-butanol–ethanol–H_2O (70:10:20, v/v). The dried papers were dipped in 2% NaOH in 90% ethanol. The radioactive areas on paper strips were located with a Vanguard Autoscanner 880. The areas of interest (usually 2.5 × 4–6 cm) were cut out and counted in a Packard liquid scintillation counter in the scintillation solution of Wang and Jones (1959).

Amino acids and pyridoxamine phosphate were located on paper by dipping in 0.5% ninhydrin in acetone and heating 10 min at 100°; sulfhydryl compounds were located by the nitroprusside dip of Toennies and Kalb (1951); sulfur-containing compounds were located with the azide–iodine dip of Smith and Tuller (1954); α-keto acids were located by dipping in 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl; *cis*-glycols were detected with the silver nitrate dip of Anet and Reynolds (1954).

Amino Acid Analysis. In conventional analysis with the Beckman-Spinco Model 120C amino acid analyzer, *S*-hydroxyethyl-L-cysteine is eluted just before threonine (Goldberg and Baldwin, 1967). It is completely separated from serine, which elutes just after threonine; threonine, of course, was absent from our samples. Using long-path cuvets, we were able to determine 5–30 mμmoles of hydroxyethylcysteine.

Determination of Hydroxypyruvate. Hydroxypyruvate was determined by the absorbancy of the bis-2,4-dinitrophenylosazone of its decarboxylation product, glycolaldehyde, which was extracted by isoamyl alcohol from alkaline solution. Bis-2,4-dinitrophenylosazones of glycolaldehyde and similar compounds have a characteristic purple color and absorbancy at 560 mμ when prepared and extracted under the conditions used (Friedemann and Haugen, 1943; Miles and Meister, 1967). A sample containing 0.01–0.04 μmole of hydroxypyruvate or glycolaldehyde in 0.05 ml was treated with 0.05 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl at 100° for 60 min. The solution was cooled, made alkaline with 0.9 ml of 1.4 N NaOH, and extracted with 1 ml of isoamyl alcohol. The absorbancy of the extract at 560 mμ was determined. The derivatives prepared from either hydroxypyruvate or glycolaldehyde under these conditions had an extinction coefficient of 25,000 at 560 mμ. Pyruvate (1–5 μmoles) gave no absorbancy at 560 mμ when treated under the same conditions. The method is therefore sensitive and specific for the determination of hydroxypyruvate in the presence of excess pyruvate.

Synthesis of Sulfur-Containing α-Keto Acids. Aliquots (0.1 ml) of 0.1 M bromopyruvate in 0.1 M sodium acetate buffer (pH 5.5) were mixed with 0.1 ml of 0.1 M mercaptoethanol (A) or 0.1 M dithioerythritol (B) and incubated 10 min at 25°. 2,4-Dinitrophenylhydrazone derivatives were prepared from aliquots of each reaction mixture and chromatographed in solvents III–VI. The derivatives of the product of reaction mixture A, *S*-pyruvylmercaptoethanol, gave two spots and that of B, *S*-pyruvyldithioerythritol, gave one long spot in each solvent. The R_F values of the 2,4-dinitrophenylhydrazones of the products differed from those of the derivatives of bromopyruvate and hydroxypyruvate (see Results). The free acids were also chromatographed. Synthetic *S*-pyruvylmercaptoethanol and *S*-pyruvyl-

mercaptoethanol prepared with L-amino acid oxidase (see next paragraph) each gave a spot in solvent I (R_F 0.67) which ran close to pyruvate and which gave a positive reaction with sodium azide–iodine for a sulfur-containing compound. *S*-Pyruvyldithioerythritol gave a spot (R_F 0.79 in solvent I) which ran identically with pyruvate in solvent system II and which gave a positive reaction with nitroprusside for a free SH group and with silver nitrate for vicinal hydroxyls. This product was labile or impure and gave at least one other spot which reacted with silver nitrate but not with nitroprusside.

Reaction of Hydroxyethylcysteine with L-Amino Acid Oxidase. A reaction mixture containing hydroxyethyl-L-cysteine (50 μmoles), potassium chloride (120 μmoles), Tris-HCl buffer (pH 7.8, 200 μmoles), catalase (2 mg), and L-amino acid oxidase (2 mg) in a final volume of 2.5 ml was incubated for 2 hr at 37°. Aliquots (0.005 ml) were removed at 30-min time intervals, added to ethanol (0.025 ml) to stop the reaction, and subjected to chromatography in solvent I. Development with ninhydrin showed that only a small proportion of the amino acid remained after 1 hr and that none remained after 90 min. The 2,4-dinitrophenylhydrazone of the product was prepared and chromatographed in solvents III–V. The two spots observed in each case had the same R_F values and colors after an alkaline dip as the derivatives synthetic *S*-pyruvylmercaptoethanol. Hydroxyethyl-L-cysteine was treated with L-amino acid oxidase on a larger scale to permit isolation of the product, *S*-pyruvylmercaptoethanol. A reaction mixture containing hydroxyethyl-L-cysteine (1 mmole), L-amino acid oxidase (3 mg), and catalase (3 mg) in 10 ml was adjusted to pH 7.6 with 1 N potassium hydroxide. A little Dow antifoam was added and the solution was incubated at 37° for 15 hr with air bubbled through. A test with ninhydrin showed no residual amino acid in an aliquot after this period. The solution, reduced to a volume of 2 ml, was placed on a 10-ml column of Sephadex G-25 fine and eluted with water. Fractions giving a positive reaction with 2,4-dinitrophenylhydrazine were combined, treated with charcoal, and passed through a 10-ml column of Dowex 50-H^+. The first 24 ml of aqueous eluate was adjusted to pH 5.0 with NaOH and taken to dryness by lyophilization. The material was recrystallized two times from 90% ethanol; yield, 36 mg. *Anal.* Calcd for $C_5H_7NaO_4S$ (186.2): C, 32.2; H, 3.8; S, 17.2. Found: C, 32.3; H, 4.15; S, 17.05.

The rates of reaction of lactic dehydrogenase at 25° with four concentrations of *S*-pyruvylmercaptoethanol (0.002–0.02 M) were determined by the assay for pyruvate described above using 0.01 ml of enzyme (38 mg/ml). The data plotted according to the method of Lineweaver and Burk (1934) fell on a straight line from which a K_m of 0.02 M was calculated. The V_{max} was 1.3×10^5 times lower than that with pyruvate as a substrate (measured with a 10^5-fold dilution of enzyme).

Results

Removal of Pyridoxal Phosphate from the B Protein of Tryptophan Synthetase. Bound pyridoxal phosphate is removed from the B protein of tryptophan synthetase by dialysis for 72 hr against 0.1 M Tris buffer (pH 7.8) containing 10 mM mercaptoethanol (Wilson and Crawford, 1965). More rapid resolution of the enzyme is obtained by dialysis for 24 hr against 0.1 M L-serine in 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM mercaptoethanol (unpublished observation of M. Hatanaka and I. P. Crawford). The recent report of Goldberg and Baldwin (1967) on the interaction of mercaptoethanol with the AB complex of tryptophan synthetase led us to examine its effect on the B subunit. When dialyzed against 10 mM mercaptoethanol and 0.1 M L-serine in 0.1 M potassium phosphate (pH 7.8) the B protein rapidly loses the ability to catalyze the serine deaminase reaction in the absence of added pyridoxal phosphate (Figure 2, curve A). Omitting mercaptoethanol from the buffer abolishes the effect (curve B). Undialyzed and dialyzed enzymes have identical enzymatic activity when pyridoxal phosphate is added back to the reaction mixture, indicating that the mercaptoethanol-dependent loss of activity is due to resolution, not inactivation. The resolution shown in Figure 2 was more rapid than that attained previously with 1 mM mercaptoethanol; resolution can be even more rapid at higher thiol concentrations (0.05 M).

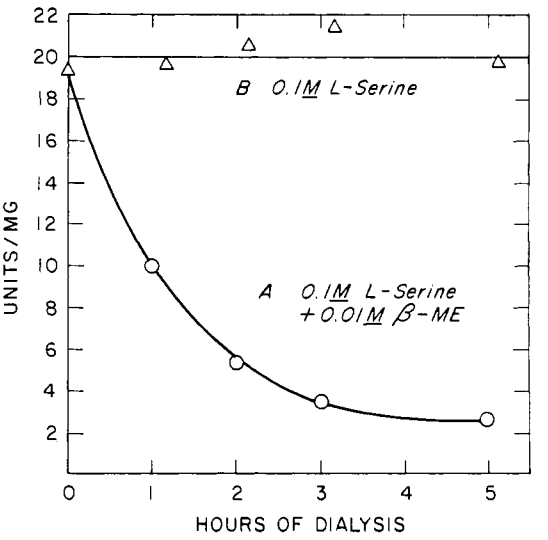


FIGURE 2: Effect of dialysis against L-serine in the presence (A) or absence (B) of mercaptoethanol on the L-serine deaminase activity of the B protein. Aliquots of the B protein (0.2 ml containing 9 mg/ml) were dialyzed at 4° against 0.1 M L-serine in 0.1 M potassium phosphate (pH 7.8) containing 0.01 M mercaptoethanol (A) and against the same solution without mercaptoethanol (B). Aliquots (0.005 ml) of each enzyme solution were removed at the indicated times and assayed for serine deaminase activity at 25° in the absence of added pyridoxal phosphate. Equal aliquots removed at the same time and assayed in the presence of pyridoxal phosphate exhibited a constant activity (not shown).

Effects of L-Serine and Mercaptoethanol on the Absorption Spectrum and Products of the B Protein. Figure 3A shows spectra of the B protein in 0.1 M potassium phosphate buffer (pH 7.8) before and at the indicated number of minutes after the addition of L-[^{14}C]serine to a final concentration of 0.05 M. The absorption peak in the visible region initially increases in amplitude and shifts to longer wavelengths (from 410 to 425 mμ). Then

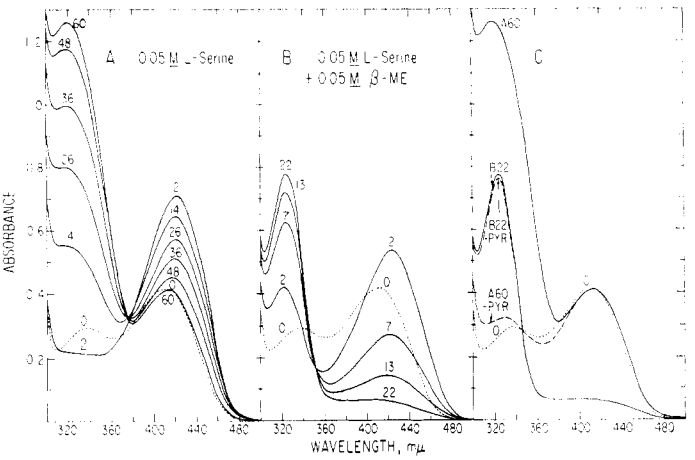


FIGURE 3: Effects of L-serine and mercaptoethanol on the absorption spectrum of the B protein. Spectra of the B protein (5 mg/ml in 0.1 M potassium phosphate, pH 7.8) were recorded at 25° (A–C, – – – –) and at the indicated number of minutes after the addition of L-serine to a final concentration of 0.05 M (A) or of L-serine and mercaptoethanol, each to a final concentration of 0.05 M (B). The final spectra recorded in A after 60 min (A 60) and in B after 22 min (B22) did not change after an additional 15-min incubation and are recorded in C. The concentration of pyruvate in these solutions was determined from assays of aliquots with lactic dehydrogenase to be 0.0425 M in A and 0.0005 M in B. The absorbancy of pyruvate at 300–400 mμ at these concentrations in 0.1 M potassium phosphate was determined and subtracted from the corresponding solutions to give difference curves (A 60-Pyr) and (B 22-Pyr).

this peak gradually returns to its original shape and a concomitant increase in absorbancy at 320 mμ is observed. No further change in absorbancy occurs after 60 min. After the reaction, it can be shown that virtually all of the L-[^{14}C]serine has been converted into [^{14}C]pyruvate (Figure 4A). The final concentration of pyruvate in the experiment of Figure 3A was determined with lactic dehydrogenase and the spectrum observed after 60 min was corrected for the adsorbancy of the pyruvate (E_{max} at 322 mμ) in the solution (Figure 3C). The difference spectrum is similar to that of the enzyme before the addition of L-serine. These results indicate that there is no net change in the enzyme-bound pyridoxal phosphate after reaction with L-serine. The observed absorbancy at 425 mμ is probably due to the Schiff base shown as structure II in Figure 1. The gradual reversal of the absorption change at 425 mμ is probably due to a decrease in the proportion of pyridoxal phosphate complexed with L-serine as the concentration of L-serine decreases. The K_m for L-serine is sufficiently high (about 0.01 M) that the enzyme would not be fully saturated under these conditions.

Figure 3B shows absorption spectra of the B protein in 0.1 M potassium phosphate buffer (pH 7.8) containing 0.05 M mercaptoethanol, before and at the indicated number of minutes after the addition of 0.05 M L-[^{14}C]serine. Although the absorption peak at 410 mμ initially increases and shifts to 425 mμ, as observed in the absence of mercaptoethanol, it then rapidly decreases until it reaches a low final value at 22 min. A concomitant increase in absorption at 325 mμ is observed. Chromatography of an aliquot of the reaction mixture (Figure 4) shows that only a small fraction of the L-serine reacts, and that very little (1–2%) is converted into pyruvate. A pyruvate assay with lactic dehydrogenase confirms this (Figure 3C) and indicates that the absorbancy at 325 mμ is not due to pyruvate, but more likely to an inactive form of the cofactor such as pyridoxamine phosphate, which absorbs maximally at this wavelength. The only radioactive product observed in this experiment has the same chromatographic mobility as *S*-hydroxyethylcysteine (Figure 4). Subsequent results (see next paragraph) demonstrate that when excess pyridoxal phosphate is added to the enzyme under conditions similar to those of Figure 3B, most of the L-serine is converted into *S*-hydroxyethyl-L-cysteine. This amino acid has been identified by comparison with the synthetic product; both are identical in R_F values on paper chromatograms developed in solvent I, in reacting on paper chromatograms with azide–iodine and ninhydrin, and in chromatography on the amino acid analyzer. Thus, reaction 5, previously observed by Goldberg and Baldwin (1967) with the AB complex of tryptophan synthetase, is also the major reaction of the B protein in the presence of L-serine and mercaptoethanol.

Isolation and Identification of Pyridoxamine Phosphate. The following experiment was performed to demonstrate the occurrence of pyridoxamine phosphate in the reaction just described. B protein (1.6 mg) was incubated with 25 μmoles of L-serine and 25 μmoles of mercaptoethanol in 0.55 ml of 0.05 M potassium phosphate (pH 7.8) at 37° for 40 min. A total of 0.4 μmole of 0.01 M pyridoxal phosphate was added in 0.005-ml aliquots at eight 5-min intervals as the absorbancy at 410 mμ was followed. The absorbancy at 410 mμ disappeared almost completely after each addition of pyridoxal phosphate except the last one. The rate of disappearance of added pyridoxal phosphate, estimated from the decrease in 410-mμ absorption, was about the same as that of the pyridoxal phosphate initially bound to the enzyme. This observation indicates that the product (presumed to be pyridoxamine phosphate) is bound to the enzyme much less strongly than pyridoxal phosphate and dissociates readily, so that the enzyme can bind and react further with pyridoxal phosphate. Both free and enzyme-bound pyridoxamine phosphate would be expected to absorb maximally at 325 mμ. Both free and enzyme-bound pyridoxal phosphate under these conditions form a Schiff base with L-serine absorbing maximally at 410–425 mμ. The reaction mixture was then placed on a 10-ml column of Sephadex G-25 fine and eluted with water. Pooled fractions with absorbancy at 325 mμ (equivalent to about 0.4 μmole of pyridoxamine phosphate) were separated from the protein peak and were applied to a 1-ml column of Dowex 1-formate. The column was washed with 10 ml of water and eluted with a linear formic acid gradient (25 ml of water in the mixing chamber and 25 ml of 1 M formic acid in the reservoir). The first tubes which were faintly acid contained all of the fluorescent material which absorbed at 325 mμ in neutral solution. (Authentic pyridoxamine phosphate was eluted at the same position in this system and was separated from α-keto acids, pyridoxal phosphate, and amino acids.)

TABLE I: Effect of Thiols on Pyridoxamine Phosphate Formation.[a]

Sulfur Compound	Concn (M)	PMP Formation (units/mg)
None		0
Mercaptoethanol	0.05	2.2
Dithioerythritol	0.05	7.8
Dithiothreitol	0.025	5.0
2,3-Dimercaptopropanol	0.01	2.2
Cysteine or glutathione	0.01	0
Dithioerythritol (−B protein)	0.05	0
Dithioerythritol (+A protein)	0.05	0
Dithioerythritol (−PLP)	0.05	0
Dithioerythritol (−serine)	0.05	0
S-Ethyl-L-cysteine (−serine)	0.01	0
S-Hydroxyethyl-L-cysteine (−serine)	0.01	0

[a] The complete reaction mixture contained tryptophan synthetase B protein (0.08 mg), A protein (0.08 mg) where indicated, L-serine (50 μmoles), pyridoxal phosphate (0.02 μmole), potassium phosphate (pH 7.8) (66–88 μmoles), and the indicated sulfur compound in 1.0 ml. Reactions were observed at 37° in the fluorimeter (excitation 313 mμ, emission 395 mμ) for at least 10 min.

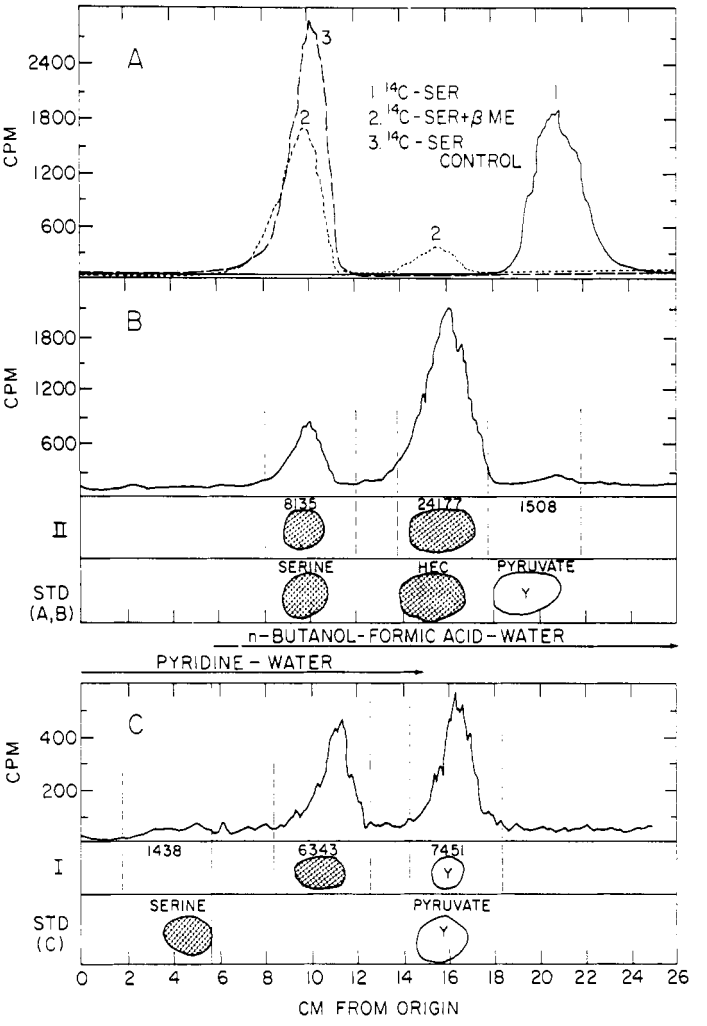


FIGURE 4: Chromatography of products of the reaction of the B protein with L-[^{14}C]serine in the presence and absence of thiols. (A) Scan of chromatograms of aliquots (0.005 ml) of reaction mixtures described in Figure 3 after the recording of the final spectra. Curve 1: reaction A with L-[^{14}C]serine. Curve 2: reaction B with L-[^{14}C]serine + mercaptoethanol. Curve 3: L-[^{14}C]serine control. The chromatogram was developed in solvent I; standards are shown below in B. (B) Chromatogram and scan of products of the mercaptoethanol experiment of Table II. The chromatogram was developed in solvent I. (C) Chromatogram and scan of products of the dithioerythritol experiment of Table II. The chromatogram was developed in solvent system II as described in Methods. The distance each of the two solvents used in system II moved is shown above. Strips with standards and duplicates of the reaction mixtures were treated with ninhydrin or 2,4-dinitrophenylhydrazine reagent for identification of spots. Ninhydrin-positive areas are indicated by cross hatching. Areas reacting with 2,4-dinitrophenylhydrazine are indicated by Y. Synthetic *S*-pyruvylmercaptoethanol (IV) had the same R_F as pyruvate in solvent I. Synthetic *S*-pyruvyldithioerythritol had the same R_F as pyruvate in solvent II. Broken vertical lines indicate the areas cut out for counting. The numbers between these lines indicate the counts per minute obtained.

These fractions were pooled and lyophilized for further study.

Chromatographic examination of the amino acid fraction eluted from the Dowex 1-formate column by water showed that hydroxyethylcysteine was the only amino acid present. The failure of pyridoxal phosphate to undergo further reaction after 40 min was evidently due to the disappearance of the L-serine. This was confirmed by other experiments in which the addition of L-serine restored the reaction with pyridoxal phosphate. The ratio of L-serine to pyridoxal phosphate disappearance in the presence of mercaptoethanol was about 60:1, indicating that reaction 5 occurred much more rapidly than transamination, reaction 6.

The lyophilized reaction product was dissolved in water and its pyridoxamine phosphate content was determined by two quantitative methods. Aliquots (0.005, 0.010, and 0.025 ml) were assayed with apoglutamic–oxalacetic transaminase. The concentration of pyridoxamine phosphate in the unknown solution was calculated from the reaction rates observed to be $6.2 \pm 0.6 \times 10^{-4}$ M. Spectra recorded from 280 to 400 mμ on 1:10 dilutions of the same solution in three solvents (0.1 M sodium phosphate (pH 7.0), 0.1 N NaOH, and 0.1 N HCl) were identical with those of a known sample of pyridoxamine phosphate and with those reported by Peterson and Sober (1954) under the same conditions. The concentration of pyridoxamine phosphate in the unknown solution calculated from the three spectra was $7.1 \pm 0.1 \times 10^{-4}$ M, which agrees fairly well with the value determined enzymatically. Electrophoresis and chromatography (solvent I) of aliquots of the solution gave single, fluorescent areas in the same position as authentic pyridoxamine phosphate. These areas gave an orange color

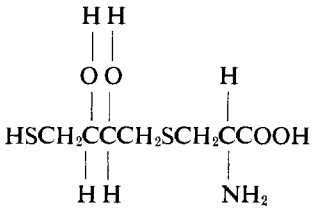
TABLE II: Stoichiometry of the Reactions Catalyzed by the B Protein in the Presence of Thiols.

Product	Dithioerythritol Experiment[a] μmoles	% Initial Serine	Molar Ratio	Mercaptoethanol Experiment[b] μmoles	% Initial Serine	Molar Ratio
A. Direct analysis[c]						
Pyruvate	10	40		1.5	3.0	
PMP	2.9	12		0.46	0.9	
Hydroxypyruvate	0 (<0.3)	0		0 (<0.05)	0	
Pyruvate + PMP	12.9	52		2.0	3.9	
Pyruvate:PMP			3.5			3.3
B. Paper chromatography[d]						
Serine	2.35	9.4		12.0	24.0	
R-S-cysteine	10.4	41.5		35.6	71.3	
Pyruvate + R-S-pyruvate	12.2	49.0		2.2	4.4	
C. Dowex 50 fractionation[e]						
α-Keto acids	13.7	55		2.4	4.8	
Amino acids	11.3	45		47.6	95.2	
D. Chromatography of 2,4-dinitrophenylhydrazones						
Pyruvate:R-S-pyruvate			3.6[f]			3.0[g]

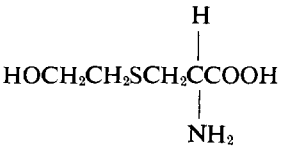
[a] Reactants in 1.0 ml: B protein (1.0 mg), L-[^{14}C]serine (25 μmoles, 2.6×10^6 cpm), dithioerythritol (50 μmoles), pyridoxal phosphate (4 μmole), and potassium phosphate (pH 7.8) (50 μmoles). Reaction time = 70 min at 37°. [b] Reactants in 1.0 ml: B protein (1.0 mg), L-[^{14}C]serine (50 μmoles, 5.2×10^6 cpm), mercaptoethanol (100 μmoles), pyridoxal phosphate (0.5 μmole), and potassium phosphate (pH 7.8) (50 μmoles). Reaction time = 30 min at 37°. [c] Reaction mixtures were chilled, applied to columns (1 × 12 cm) of Sephadex G-25 fine, and eluted with water. Radioactivity appeared after the protein. Pyridoxal phosphate, pyruvate, and hydroxypyruvate were determined in the combined radioactive fractions (3 ml) (see Methods). The concentration of pyridoxamine phosphate was calculated from the decrease in pyridoxal phosphate. [d] Aliquots (15 μl) of the Sephadex fraction were subjected to paper chromatography in solvent I (mercaptoethanol experiment) or in solvent system II (dithioerythritol experiment). Chromatographic strips were scanned, cut out, and counted (see Methods and Figure 4). [e] Each Sephadex fraction (1 ml of each) was placed on a column (0.6 × 3.8 cm) of Dowex 50 H^+, washed with 4 ml of water, and then with 4 ml of 2 M NH_4OH. Aliquots of each 1-ml fraction were counted, and the per cent of counts in the α-keto acid fraction (eluted with water) and the amino acid fraction (eluted with NH_4OH) calculated. [f] The acid fraction was divided into two 1-ml portions and each was made 0.1 M with Tris-HCl buffer (pH 7.8). Lactic dehydrogenase (2 μl) and DPNH (3 mM) were added to one-half to reduce the pyruvate. Dinitrophenylhydrazones were made and extracted into three 1-ml portions of ethyl acetate. The extracts were taken to dryness, redissolved in 0.1 ml of ethyl acetate, and chromatographed in solvent III (Figure 5). Areas containing the pyruvate derivatives and the more slowly moving derivative of α-keto acid III from the reaction mixture not treated with lactic dehydrogenase were cut out and counted. [g] Operations were carried out analogous to those of footnote *f*. The derivative of the α-keto acid not destroyed by lactic dehydrogenase IV was not cleanly separated from that of pyruvate in solvents III–VI. The counts in the radioactive region on paper chromatograms in solvent III of derivatives after lactic dehydrogenase treatment were 25% of those found before lactic dehydrogenase treatment.

when treated with ninhydrin and heated to 100°, a characteristic reaction of pyridoxamine phosphate. Moreover, the reaction product showed the same fluorescence excitation and emission spectra reported by Chen (1965) and others and observed with authentic pyridoxamine phosphate.

Fluorimetric Assay of Pyridoxamine Phosphate Formation and the Effect of Various Thiols on This Reaction. The fluorimetric assay for pyridoxamine phosphate (see Methods) is much more sensitive and specific than the spectrophotometric determination at 325 mμ and was used to follow the rate of transamination (Table I). Mercaptoethanol, dithioerythritol, dimercaptopropanol, and dithiothreitol were all found to bring about pyridoxamine phosphate formation at rates which were linear and proportional to enzyme concentration. Dithioerythriol was the most effective thiol and was found to have an apparent K_m = 0.006 M. Mercaptoethanol evoked a lower reaction rate but had a similar apparent K_m = 0.005 M. The reaction was absolutely dependent



I, *S*-alanyldithioerythritol



II, *S*-hydroxyethyl-L-cysteine or *S*-alanylmercaptoethanol

upon the addition of the B protein, a thiol, and serine (K_m = 0.009 M) and was inhibited by the addition of the A protein. Glutathione and cysteine did not cause pyridoxamine phosphate formation; cysteine, which is known to be an inhibitor of other reactions of the B protein, inhibited the reaction in the presence of dithioerythritol. In contrast to other reactions of the B protein, the rate was decreased in the presence of a high concentration of ammonium ions. These results indicate that the B protein carries out a transamination reaction which is dependent upon the addition of L-serine and one of several thiols. Sulfur-substituted L-cysteine derivatives did not replace this double requirement.

Products and Stoichiometry of Reactions in the Presence of Thiols. Table II describes experiments carried out in order to identify and determine quantitatively the products of reaction of the B protein with L-[^{14}C]serine in the presence of dithioerythritol or mercaptoethanol. Although thiols strongly inhibit the formation of pyruvate from L-serine (see Figures 3C and 4A), three to four times as much pyruvate was formed as pyridoxamine phosphate in both experiments (Table IIA). The total amounts of these two compounds were much higher in the presence of dithioerythritol. Although the lactic dehydrogenase assay used would not distinguish between pyruvate and hydroxypyruvate, direct analysis for hydroxypyruvate showed it to be absent.

Chromatography of the entire reaction mixtures (Table IIB and Figure 4B,C) showed that the main product in the presence of mercaptoethanol was *S*-hydroxyethyl-L-cysteine, and that dithioerythritol evoked the production of appreciable amounts of a different amino acid. This new amino acid moved close to pyruvate in solvent I so solvent system II was used to separate it from pyruvate. This product gave positive reactions with ninhydrin, with nitroprusside indicating a free sulfhydryl, and with a silver nitrate dip for vicinal hydroxyls. These properties suggest that it is I, *S*-alanyldithioerythritol, formed by an addition of dithioerythritol to L-serine according to reaction 5. The structure of *S*-hydroxyethylcysteine (II) is shown for comparison and might more consistently be termed *S*-alanylmercaptoethanol.

Fractionation of the reaction mixtures on Dowex 50 gave an acid and an amino acid fraction (Table IIC). The material in the acid fraction in both experiments was close to the sum of the pyruvate and pyridoxamine phosphate found (Table IIA) as would be expected if this fraction contained pyruvate plus an α-keto acid formed by transamination in an amount equivalent to the pyridoxamine phosphate. Treatment of an aliquot of the acid fraction with 2,4-dinitrophenylhydrazine followed by extraction with ethyl acetate resulted in the recovery of all the radioactivity in a fraction containing α-keto acid derivatives. A portion of this radioactivity was also extracted in each experiment even after the acid fraction was first treated with lactic dehydrogenase and DPNH (see Table II, footnotes *f* and *g*). These results indicated that an α-keto acid which did not react with lactic dehydrogenase under these conditions (and was therefore not pyruvate or hydroxypyruvate) was present.

The results of chromatography of the 2,4-dinitrophenylhydrazones prepared before and after lactic dehydrogenase treatment in the dithioerythritol experiment are shown in Figure 5B.[2] The pyruvate derivative was absent after the lactic dehydrogenase treatment, but a slower moving derivative of a new α-keto acid, III, was present. The ratio of radioactivity in the areas of the pyruvate derivative to those in the derivative of III was 3.6 (Table II, footnote *f*) which is close to the ratio of pyruvate to pyridoxamine phosphate calculated from Table IIA to be 3.5. Similarly, in the mercaptoethanol experiment the ratio of pyruvate to the α-keto acid IV not destroyed by lactic dehydrogenase is 3.0 (Table II, footnote *g*), which is close to the ratio of pyruvate to pyridoxamine phosphate calculated from Table IIA to be 3.3 in this experiment. These data indicate that the α-keto acids III and IV, which do not react with lactic dehydrogenase in the two experiments, are formed stoichiometrically with pyridoxamine phosphate and are the likely products of transamination. The 2,4-dinitrophenylhydrazones of α-keto acids III and IV have the same R_F values as the derivatives of the α-keto acids synthesized from bromopyruvate and dithioerythritol or mercaptoethanol, respectively (see Methods and Figure 5). The most probable structure for III is therefore *S*-pyruvyldithioerythritol and for IV is *S*-pyruvylmercaptoethanol. These new α-keto acids are the α-keto acid analogs of the amino acids I and II. Since III and IV move near pyruvate in the solvent systems used in Table IIB, their presence in the reaction mixtures would be consistent with the observation that the amount of product in the pyruvate region (Table IIB) was closer to the sum of pyruvate plus pyridoxamine phosphate than to the amount of pyruvate found by direct analysis (Table IIA).

[2] The sharp peak marked by the arrow in Figure 5B is probably lactate which was not completely retained in the aqueous fraction. On some chromatograms, it has been separated from the dinitrophenylhydrazone, which retained the radioactivity observed before treatment.

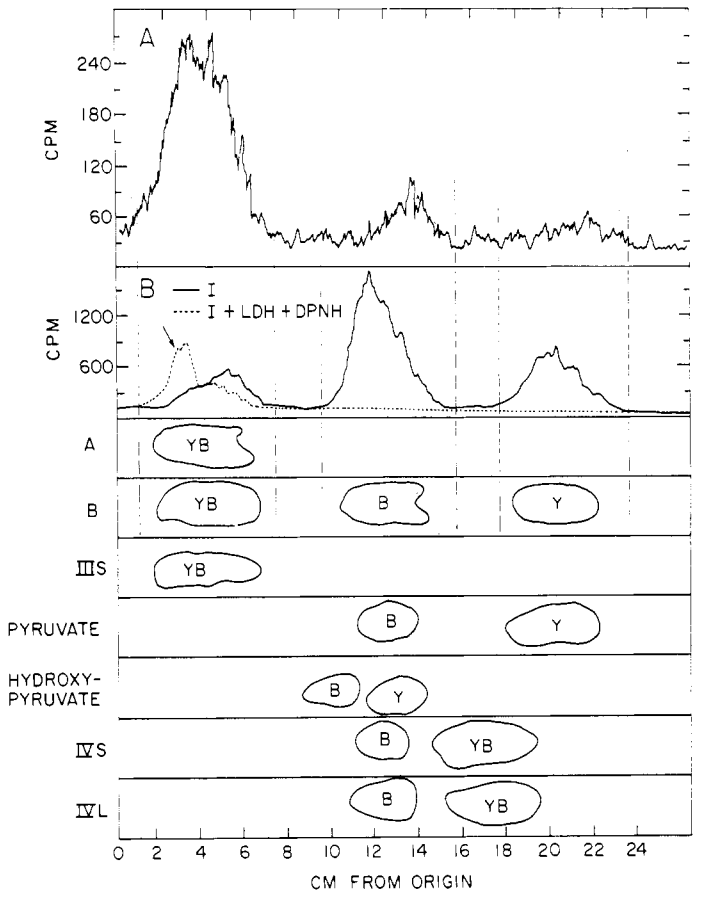
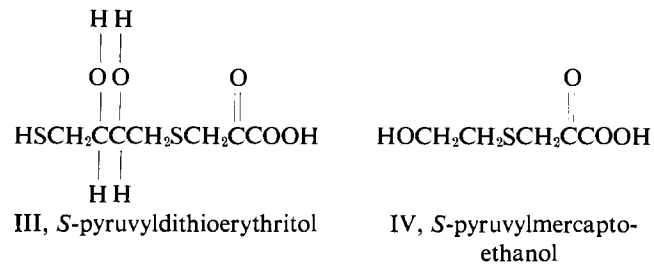


FIGURE 5: Chromatography of 2,4-dinitrophenylhydrazones of α-keto acids. (A) Chromatogram and scan of derivatives of α-keto acids produced by the action of L-amino acid oxidase on the amino acid fraction produced from L-[^{14}C]serine + dithioerythritol (see Table II and Results). (B) Chromatogram and scan of derivatives of α-keto acids produced from L-[^{14}C]serine + dithioerythriol (Table II). The dashed curve shows a scan of the derivatives prepared and chromatographed after the reaction mixture was treated with lactic dehydrogenase and DPNH. The chromatogram strips below A and B are of derivatives of standard pyruvate and hydroxypyruvate, III S (synthetic *S*-pyruvyldithioerythritol), IV S (synthetic *S*-pyruvylmercaptoethanol), and IV L (*S*-pyruvylmercaptoethanol prepared by the action of L-amino acid oxidase on hydroxyethyl-L-cysteine). The chromatograms were developed in solvent III. The letters refer to the colors of the spots after an alkaline dip: Y = yellow, YB = yellow-brown, and B = brown. Broken vertical lines indicate the areas cut out for counting.



III, *S*-pyruvyldithioerythritol

IV, *S*-pyruvylmercaptoethanol

S-Pyruvylmercaptoethanol (IV) was also synthesized by L-amino acid oxidase treatment of hydroxyethyl-L-cysteine and found to be a very poor substrate for lactic dehydrogenase (see Methods). This is consistent with the proposed structure IV for the product of transamination in the mercaptoethanol experiment (Table II) since this product was present at too low a concentration to be appreciably reduced by the amount of lactic dehydrogenase added.

Treatment of I with L-*Amino Acid Oxidase.* The amino acids in the dithioerythritol experiment (Table II) were isolated by passing 0.5 ml of the Sephadex fraction over a column (0.6 × 1.8 cm) of Dowex 1-formate and washing with water. The radioactive fraction (0.5 ml) was incubated with Tris buffer (pH 7.8, 50 μmoles), catalase (0.5 mg), potassium chloride (25 μmoles), and L-amino acid oxidase (0.2 mg) in a total volume of 0.6 ml for 90 min at 37°. The solution was treated with 0.3 ml of 2,4-dinitrophenylhydrazine reagent for 30 min at 25° and extracted with three 1-ml aliquots of ethyl acetate. The results of chromatography of the 2,4-dinitrophenylhydrazones prepared from this reaction are shown in Figure 5A. All of the detectable color and 76% of the radioactivity were found in the same region where the derivatives of the synthetic compound III and the enzymatic product moved (Figure 5B). The remaining counts were located in the area of the pyruvate derivative as would be expected since the products of the experiment included some unreacted L-serine (Table IIB). These results support the proposed structure for III. Figure 5 also shows that the 2,4-dinitrophenylhydrazones of IV prepared either synthetically or by L-amino acid oxidase have the same mobility but are not well separated from the derivative of pyruvate, as was true of the derivative of the enzymatic product.

Reaction of Apoenzyme with Pyridoxamine Phosphate and S-Pyruvylmercaptoethanol. Figure 6A shows the spectra of the B protein before (curve 1) and after (curve 2) resolution. Figure 6B shows spectra of the enzyme at the indicated number of minutes after the addition of pyridoxamine phosphate and *S*-pyruvylmercaptoethanol, each to a final concentration of 0.01 M. No reaction occurred if the α-keto acid was omitted. A control solution containing no enzyme showed no increase in the visible region during the 215-min incubation period. The absorbance of this control solution (dashed curve, Figure 6B) was subtracted from the absorbance of the enzyme at 215 min to give a difference spectrum (curve 3, Figure 6A). The observed maximum absorbance at 410 mμ indicates that enzyme-bound pyridoxal phosphate has been formed by the reversal of transamination reaction 6. The spectrum of the enzyme after dialysis (curve 4, Figure 6A) has a shape similar to that of the holoenzyme (curve 1) although the peak at 410 mμ is considerably reduced. The differences in absorbance at 410 mμ between curves 3, 1, and 4 can be explained by partial resolution of the enzyme during dialysis. The B protein appears to bind pyridoxal phosphate less tightly after resolution (unpublished results). The pyridoxal phosphate content of the dialyzed solution (curve 4) was determined by the phenylhydrazine method to be 1.0 mole/mole of the B protein, or approximately one-half the content of the fully reconstituted holoenzyme. The demonstration of reaction 6 in the reverse direction provides additional evidence for the identity of the products.

Rates of Reaction of the B Protein and the AB Complex of Tryptophan Synthetase with L-*Serine.* Table III gives data for the initial rates of reactions 1 and 4–6 with the B protein in the presence or absence of the A protein. The reactions were carried out under similar conditions so that the rates are comparable. Reactions 4 and 6 occurred only in the absence of A. In the presence of thiols, reactions 4–6 occur concomitantly, and their sum, expressed as total serine disappearance, is given. Although the rates obtained were linear and reproducible with a particular solution of the B protein, somewhat variable rates, particularly for reaction 5, have been found when the experiment was repeated with different enzyme solutions. The rate of reaction 5 with dithioerythritol was about two times greater with the enzyme used in Table II than reported in Table III. The A protein stimulates the rate of the β addition of thiols (reaction 5) much less than the rate of the β addition of indole (reaction 1).

Effects of Thiols on Other Reactions of Tryptophan Synthetase. Goldberg and Baldwin (1967) reported that mercaptoethanol produced no inhibition of reaction 1 of the AB complex at concentrations at which it was a good substrate for reaction 5 in the absence of indole. We have confirmed this observation with both mercaptoethanol and dithioerythritol. The thiols also cause no inhibition of reaction 1 catalyzed by the B protein under optimal assay conditions (2 M ammonium ion; Hatanaka *et al.*, 1962). Mercaptoethanol and dithioerythritol at high concentrations (0.05 M) do cause 50% inhibition of the activity of the B protein in reaction 1 in 0.1 M potassium phosphate (pH 7.8) in the absence of added ammonium ions. Mercaptoethanol and dithioerythritol cause 50% inhibition of the serine deaminase activity of the B protein at lower concentrations (about 0.01 M).

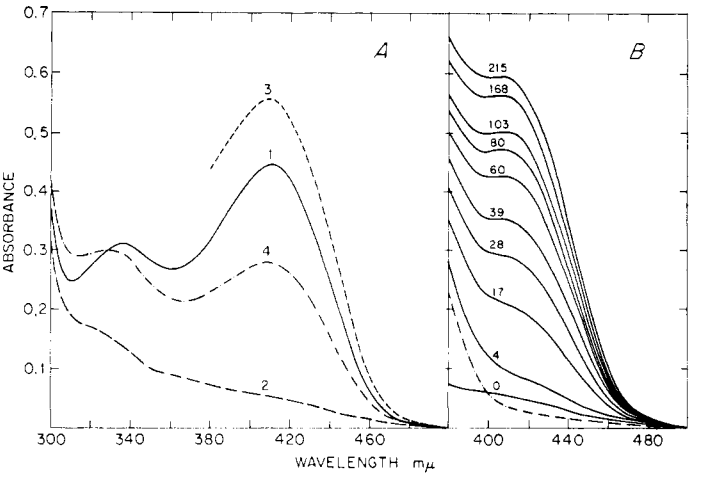


FIGURE 6: Effect of pyridoxamine phosphate and *S*-hydroxyethylmercaptopyruvate on the spectra of the B protein. Spectra of the B protein (5 mg/ml in 0.1 M potassium phosphate, pH 7.8) were recorded before (A, curve 1) and after (A, curve 2) resolution. The B protein (5 mg/ml) was resolved by dialysis for 150 min at 4° against 0.1 M potassium phosphate (pH 7.8) containing 0.05 M L-serine and 0.05 M β-ME followed by dialysis for 15 hr against 0.1 M potassium phosphate (pH 7.8). The resolved enzyme (0.4 ml) was treated with 0.05 ml of 0.1 M pyridoxamine phosphate and 0.05 ml of 0.1 M *S*-hydroxyethylmercaptopyruvate; spectra were recorded at 25° at the indicated number of minutes after treatment B. The absorbance of a solution in which the enzyme was replaced by an equal volume of 0.1 M potassium phosphate (pH 7.8) was recorded at the same time intervals and showed no change (dashed curve in B). The absorbance of this solution was subtracted from that of the enzyme after 215 min to give curve 3 (A). The enzyme solution was dialyzed 18 hr against two 1-l. changes of 0.1 M potassium phosphate buffer (pH 7.8) before recording spectrum 4. Curves 3 and 4 in A and curves in B were not corrected for dilution.

Discussion

General Mechanism of Reactions of Tryptophan Synthetase. The various reactions of tryptophan synthetase and the roles of thiols in these reactions can best be discussed in relation to the scheme for pyridoxal phosphate catalysis shown in Figure 1. Similar schemes have been proposed for related enzymes such as tryptophanase (Morino and Snell, 1967). Wilson and Crawford (1965) found that the holoenzyme form of the B protein was reduced and inactivated by sodium borohydride to yield a covalently bound derivative of pyridoxal phosphate. Studies of a number of other pyridoxal phosphate enzymes which are reduced by sodium borohydride have led to the conclusion that the pyridoxal phosphate is bound in a Schiff base linkage to an ϵ-amino group of a protein lysine before reduction, as shown schematically in I (Figure 1) (Fasella, 1967). The first step in the reaction of all pyridoxal phosphate enzymes is thought to be a transaldimination step in which the amino acid substrate displaces the amino group of the protein to form a Schiff base linkage between the amino acid and pyridoxal-phosphate (II, Figure 1). Spectral evidence for this step was obtained in Figure 3. The largest class of pyridoxal-catalyzed reactions requires the labilization of the α-hydrogen to form an intermediate such as III (Figure 1) (Metzler *et al.*, 1954). These reactions include transamination, β elimination and addition, racemization, β decarboxylation, and others. The β-elimination and β-addition reactions share a further key intermediate resembling V, the Schiff base of amino acrylic acid. This intermediate can be hydrolyzed to pyruvate and ammonia (β elimination, reaction 4) or can undergo a β-addition reaction with indole to form tryptophan (reaction 1) or with mercaptoethanol to form hydroxyethylcysteine (reaction 5). Reaction 5 occurs through intermediate VIII which is the enamine form of the Schiff base of hydroxyethyl-L-cysteine. The transamination reaction 6 can be envisaged as a result of the tautomerization of VIII to yield the ketamine form of the Schiff base IX which is then hydrolyzed to pyridoxamine phosphate X and the α-keto acid. The tautomerization step is the key step in all transaminase reactions and has been found to be the rate-limiting step (Hammes and Fasella, 1962).

A number of other multifunctional pyridoxal phosphate enzymes have been found and their study has given important information concerning the mechanism and intermediate steps in pyridoxal-catalyzed reactions. An enzyme closely related to tryptophan synthetase is tryptophanase (Morino and Snell, 1967) which catalyzes a large number of reactions involving the common intermediate V (Figure 1). These reactions include re-

TABLE III: Rates of Reactions of Tryptophan Synthetase.[a]

Expt	Compd	TSase	Max Time (min)	Reaction[b] and Product (units/mg) Trp 1	Pyr 4	R-S-Cys 5	PMP 6	ΔSer
I	None	B	25		120		0	120
		AB	25		5		0	5
II	β-ME	B	25		10	140	3.5	153
		AB	15		0	440	0	440
III	DTE	B	50		27	25	14	66
		AB	50		0	56	0	56
IV	Indole	B	10	10	0		0	10
		AB	10	600	0		0	600

[a]The reaction mixtures consisted of the B protein (0.025 mg), the A protein (0.025 mg) where indicated, L-[^{14}C]-serine (2.5 μmoles, 2.6 × 10^5 cpm), β-ME or DTE (5 μmoles) where indicated or indole (2.4 μmoles) where indicated, pyridoxal phosphate (0.02 μmole in I, II, and IV and 0.10 μmole in III), and potassium phosphate buffer (pH 7.8, 10 μmoles) in a final volume of 0.10 ml. Aliquots (0.01 ml) of each reaction mixture were removed at five equal time intervals during the period indicated and added to ethanol (0.03 ml) for later chromatographic separation and estimation of amino acids and pyruvate as an assay of reactions 1, 4, and 5 (see Methods and Figure 2). Entire 0.1-ml reaction mixtures (I, II, and IV) and 0.01-ml aliquots (III) were treated with phenylhydrazine at the same five time intervals to determine the disappearance of pyridoxal phosphate as an assay of reaction 4 (see Methods). Time intervals were chosen for each experiment so that the maximum serine disappearance was 20%. These conditions were found to give linear rates of reaction. [b]The reaction numbers refer to reactions given in the text.

actions 1 and 4 as well as a number of other β-elimination reactions and α,β-addition reactions.

Effects of Thiols on the Course of Reactions. Three different effects of thiols have been observed by Goldberg and Baldwin (1967) and in the present paper: promotion of a form of the AB complex with an intense absorption maximum at 468 mμ; β-addition reactions, forming substituted cysteines (reaction 5); and β addition followed by transamination (reaction 6). Our finding that reactions 5 and 6 occur with either mercaptoethanol or dithioerythritol distinguishes them from the formation of the complex absorbing at 468 mμ since the accumulation of this intermediate is promoted by mercaptoethanol but not by any other thiol tested including dithioerythritol (Goldberg and Baldwin, 1967). These authors suggested that the complex absorbing at 468 mμ had structure V (Figure 1). Enzyme–substrate intermediates absorbing intensely near 500 mμ have been described in studies of a number of other pyridoxal phosphate enzymes including tryptophanase (Morino and Snell, 1967) and serine transhydroxymethylase (Schirch and Jenkins, 1964b), and there is good evidence that they have a semiquinoid structure similar to IV (Figure 1). Both tryptophanase and serine transhydroxymethylase form this complex with alanine, which cannot undergo β elimination to form a structure of type V (Figure 1). Complexes III and IV are resonant forms of the same compound. It is not yet possible to determine which of these or other possible resonant forms is responsible for the absorbance at 468 mμ. Further evidence for the semiquinoid structure has come from model chemical studies of Schirch and Slotter (1966).

The β addition of a thiol to complex V (Figure 1) is strictly analogous to the β addition of indole. The reaction requires high concentrations of thiol, and therefore is probably nonphysiological. But because it represents an attack on an active intermediate by SH groups present on certain favorable molecules, it may be used to probe the mechanism and specificity of the catalytic functions of tryptophan synthetase.

Mechanism of the Transamination Reaction. The transamination reaction 6 is actually the sum of two reactions, 6a and 6b, in which the pyridoxamine phosphate formed rapidly dissociates from the enzyme to yield apoenzyme which is then reactivated by pyridoxal phosphate.

$$\text{holoenzyme} + \text{L-serine} + \text{RSH} \rightleftharpoons \text{R-S-mercaptopyruvate} + \text{PMP} + \text{apoenzyme} + \text{H}_2\text{O} \quad (6a)$$

$$\text{apoenzyme} + \text{PLP} \rightleftharpoons \text{holoenzyme} \quad (6b)$$

$$\text{PLP} + \text{L-serine} + \text{RSH} \rightleftharpoons \text{R-S-mercaptopyruvate} + \text{PMP} + \text{H}_2\text{O} \quad (6)$$

Thus the apoenzyme catalyzes the net conversion of pyridoxal phosphate into pyridoxamine phosphate by reaction 6. Several other pyridoxal phosphate enzymes have been found to possess similar minor transamination reactions which result in resolution and inactivation. Schirch and Jenkins (1964a) reported that serine transhydroxymethylase would catalyze reaction 7 at a rate about three orders of magnitude slower than the rate of its physiological reaction.

$$\text{D-alanine} + \text{PLP} \rightleftharpoons \text{pyruvate} + \text{PMP} \quad (7)$$

Novogrodsky and Meister (1964) reported that aspartate β-decarboxylase catalyzes a similar transamination reaction with L-aspartate at a rate about three orders of magnitude slower than the β-decarboxylation reaction and also acts as a rather nonspecific L-amino acid transaminase. The rate of transamination by the B protein is only one or two orders of magnitude lower than other reactions catalyzed by the B protein or AB complex and is thus greater than the minor rates of transamination catalyzed by aspartate β-decarboxylase and serine transhydroxymethylase. The unique thiol dependence of the transaminase reaction of the B protein of tryptophan synthetase is explained by the finding that the α-keto acid product is the α-keto acid analog of the amino acid produced by reaction 5.

Role of the A Protein in Controlling the Reactions of Tryptophan Synthetase. The present report and previous studies have shown that the A protein completely inhibits transamination (6) and deamination (4), slightly stimulates the thiol β-addition reaction (5), and greatly stimulates the indole β-addition reaction (1). Since all of these reactions probably have a common intermediate, enzyme-bound phosphopyridoxylaminoacrylic acid (V, Figure 1), the results suggest that the A protein is not required for the formation of this intermediate but does greatly affect its subsequent reactions. The effect of the A protein on the β-addition reactions might result from a conformational change in the active site due to an interaction of the A and B subunits. Of course, the A protein has another vital role to play in the binding and activation of indoleglycerol phosphate in reactions 2 and 3. This may be related to its large influence on reaction 1 with indole.

It appears that the influence of the A protein on the pyridoxal phosphate active site may be twofold: (1) to promote the binding of indole and/or its β addition to the amino acrylate intermediate V (Figure 1) and (2) to prevent complex V from undergoing further enzymatic reactions in the absence of indole. The latter role might be mediated through the formation of a stable complex with intermediate V. Evidence has been obtained in this laboratory for a new intermediate formed by serine with the AB complex in the absence of indole or mercaptoethanol which has an absorption maximum at 330–340 mμ and which may be related to this second function. This work will be presented in a subsequent communication.

The studies presented here have given further information on the mechanism, specificity, and control of the multiple reactions of tryptophan synthetase.

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