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Stereochemistry and Mechanism of Reactions Catalyzed by Tryptophan Synthetase and Its β_2 Subunit*

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The synthesis of tryptophan from serine and indole or indoleglycerol phosphate catalyzed by native tryptophan synthetase or β_2 protein is shown to proceed stereospecifically with retention of configuration at C_β. In the α,β elimination reaction of serine to give pyruvate and ammonia catalyzed by the β_2 protein, the hydrogen from C_α is transferred intramolecularly and without exchange with solvent protons to C_β, where it replaces the OH group with net retention of configuration. In a competing reaction, an abortive transamination in the presence of mercaptoethanol to give pyridoxamine and *S*-pyruvyl mercaptoethanol, the same proton is transferred to the extent of 70% to C-4′ of the cofactor when the reaction is carried out in D_2O. Together with the finding of Dunathan and Voet (Dunathan, H. G. and Voet, J. G. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 3888) that the cofactor is protonated from the *si* face, these data fully define the geometry of the substrate•coenzyme complex and the position of an essential base relative to it. Since no isotope effect was observed in the protonation of C_α of tryptophan synthesized from indole and serine in 50% D_2O, the base must be monoprotic. The known inability of the enzyme to degrade tryptophan by α,β elimination is not due to inability to remove H_α of tryptophan; native enzyme and β_2 protein catalyze α hydrogen exchange of tryptophan at a rate of 20% and 14%, respectively, of that of tryptophan synthesis.

Tryptophan synthetase (EC 4.1.2.20) (1) is a tetrameric enzyme containing two pairs of identical subunits, which normally catalyzes the reaction:

Indole-3-glycerol phosphate + L-serine
→ L-tryptophan + D-glyceraldehyde 3-phosphate (1)

Alternatively, the enzyme can also synthesize tryptophan from L-serine and indole according to Equation 2:

Indole + L-serine → L-tryptophan (2)

Its β_2 subunit which contains pyridoxal phosphate as the prosthetic group is unable to catalyze reaction 1, but carries out reaction 2 in addition to a number of other reactions, *e.g.* (1):

L-Serine → pyruvate + ammonia (3)

β-Mercaptoethanol + L-serine → *S*-hydroxyethyl-L-cysteine (4)

β-Mercaptoethanol + L-serine + pyridoxal phosphate
→ *S*-pyruvyl mercaptoethanol + pyridoxamine phosphate (5)

As in the case of other pyridoxal phosphate-containing enzymes which carry out β replacement and/or α,β elimination reactions of amino acids, the reaction sequence leads via a series of aldimine and ketimine complexes between the substrate amino acid and the cofactor to an enzyme-bound Schiff's base between pyridoxal phosphate and α-aminoacrylic acid as a universal intermediate. This intermediate can follow various reaction paths, giving the different observed products.

In this paper we report results which clarify various stereochemical aspects of these reactions, define the geometry of the coenzyme·substrate complex in the active site and throw some light on several of the protonation steps involved in these reactions. Some of the results have been communicated in preliminary form (2, 3).

EXPERIMENTAL PROCEDURES

Materials—The chemicals used were of reagent grade or of the highest purity commercially available and they were used without further purification. All enzymes obtained commercially were purchased from Sigma. L-[*U*-^{14}C]Serine (159 mCi/mmol), L-[3-^{14}C]tryptophan (~45 mCi/mmol), and [2-^{14}C]acetate (~50 mCi/mmol) were purchased from Amersham/Searle. Reference indolmycin was a gift from Charles Pfizer and Co. (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{3}H]serine were prepared as described previously (4) whereas (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{2}H,3-^{3}H]serine were prepared analogously using D_2O as the solvent in the first step. DL-[2-^{2}H]Serine and DL-(3*R*)-[2-^{2}H, 3-^{3}H]serine were synthesized from DL-serine and (2*S*,3*R*)-[3-^{3}H]serine, respectively, according to the procedures of Miles and McPhie (5). Double-labeled samples were prepared by mixing the appropriate single-labeled species and constancy of their ^{3}H/^{14}C ratio was usually ascertained by rechromatography of an aliquot in at least one solvent system.

Streptomyces griseus ATCC 12648 was obtained from the American Type Culture Collection and was maintained on slants of Emerson's agar at 24°C.

Enzymes and Assay Procedures—The native tryptophan synthetase was purified from *Neurospora crassa* according to the procedure of Meyer *et al.* (6) and Yanofsky (7). Tryptophan synthetase α protein was partially purified from *Escherichia coli* B_8 (8), omitting the final DEAE-cellulose column step, to give preparations of 4.7 IU/mg of protein. Tryptophan synthetase β_2 protein was purified from *E. coli* $A_2/F'A_2$ according to the procedure of Adachi and Miles (9) and had a specific activity of 14.6 IU/mg of protein. Both *E. coli* mutants were kindly provided by Dr. R. Somerville. Protein determinations were carried out by Lowry's method (10) using serum albumin as standard.

Tryptophan synthetase activity was measured by following indole disappearance colorimetrically in the reaction between indole and serine to form tryptophan (11).

Chromatography—Paper chromatography was carried out on Whatman No. 3 MM paper using the following solvent systems: System A, propanol-1/concentrated NH_4OH/water, 6:3:1 (descending, R_F values: tryptophan 0.70, serine 0.46); System B, butanol-1/88% formic acid/water, 14:3:3 (descending, tryptophan 0.54, serine 0.20); System C, 95% ethanol/concentrated NH_4OH/water, 80:4:16 (descending, serine 0.61, lactic acid 0.87); and System D, butanol-1/

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glacial acetic acid/water, 2:1:1 (descending, serine 0.38, *S*-hydroxyethyl cysteine 0.51). Amino acids were visualized with ninhydrin spray reagent (5% ninhydrin in ethanol) and lactic acid was located by comparison with [*U*-^{14}C]lactic acid as reference.

Radioactivity Determinations—Radioactivity on chromatograms was located using a Packard model 7201 radiochromatogram scanner. The radioactivity of compounds in solution was determined in a Beckman LS 100 or LS 250 liquid scintillation counter using 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(methyl-5-phenyloxazolyl)]benzene (dimethyl POPOP) in toluene or toluene/ethanol as scintillator solution. Counting efficiencies and the spillover of ^{14}C into the tritium channel were determined by internal standardization with [^{14}C]toluene and [^{3}H]toluene.

Conversion of Serine into Tryptophan—The serine samples stereospecifically tritiated at the β position were mixed with L-[*U*-^{14}C]-serine and converted into tryptophan with the native tryptophan synthetase from *Neurospora crassa*, the mixture of α and β_2 protein isolated separately from *E. coli*, or the β_2 protein of tryptophan synthetase from *E. coli*. The incubation mixture contained in a volume of 0.2 ml: Tris/HCl, pH 7.8, 10 μmol; 0.1 μmol or less of serine; 0.2 μmol of indole (or indoleglycerol phosphate); and 0.25 IU of enzyme. In cases where a mixture of α and β_2 subunits was used, a 2-fold excess of α-protein was added and parallel reactions were carried out in the presence of 0.2 M NaCl. For the experiments with the β_2 protein of tryptophan synthetase, parallel incubations were carried out in the presence of 0.2 M NH_4^+ ion. After incubation for 4 h at 37°C, the reaction mixtures were boiled for 2 min and the tryptophan was isolated by successive chromatography in Systems A and B.

In the stable isotope experiments, the incubation mixture contained in 4 ml of potassium phosphate buffer (pH 7.8): 0.4 mmol of indole, 0.4 mmol of serine, 10 mM glutathione, 0.2 mM pyridoxal phosphate, and 2 IU of the β_2 protein mixed with 2-fold excess of the α protein of tryptophan synthetase from *E. coli*. In order to minimize exchange of the α-hydrogen of tryptophan after it has been formed, the incubation was carried out at 37°C for only 20 min; under these conditions the yield was not more than 10%. The deuterium incorporation at the α position of the isolated tryptophan samples was analyzed by proton NMR and mass spectroscopy (chemical ionization, isobutane).

Conversion of Tryptophan into Indolmycin—The tryptophan samples were converted into indolmycin by incubation with 25-ml shake cultures of *Streptomyces griseus* ATCC 12648 in peptone/yeast medium. The fermentation as well as the isolation of indolmycin were carried out as described earlier (12). The indolmycin was assayed for its ^{3}H/^{14}C ratio.

Degradation of Stereospecifically Tritiated Tryptophan—The tryptophan samples (5 μCi) obtained from (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{3}H]serine with the native tryptophan synthetase from *N. crassa* were mixed with L-[3-^{14}C]tryptophan and diluted with 5 mg of carrier L-tryptophan. A small aliquot was assayed for its ^{3}H/^{14}C ratio. Birch reductions of these samples were carried out at −70°C with 30 ml of liquid NH_3 (dried over sodium) and 20 mg of lithium (13). After stirring for 1 h, 1 ml of MeOH was added dropwise followed by 220 mg of NH_4OAc, and the solvent was allowed to evaporate. The dried reaction mixtures were then dissolved in 2 ml of H_2O + 2 ml of MeOH and ozonized for 5 min at 0°C followed by addition of 1 ml of 30% H_2O_2, and left at room temperature for 3 h. After evaporation of solvents, the crude products were placed on columns of 12 ml of Dowex 50 H^+, washed with 200 ml of H_2O, and eluted with 1.5 N NH_4OH to give aspartate containing 10 to 15% of the starting radioactivity. The aspartate samples were further purified by chromatography on 0.5-mm silica gel plates in *n*-PrOH/H_2O, 7:3 and eluted with NH_4OH/H_2O. The eluents were washed through Dowex 50 H^+ columns and eluted with 1.5 N NH_4OH. The purity of the aspartate samples was checked by TLC in EtOH/H_2O, 7:3; each gave a single radioactive peak coinciding with reference aspartate. A small aliquot of each was mixed with 20 mg of L-aspartate, recrystallized from H_2O and analyzed for its ^{3}H/^{14}C ratio.

Aliquots of these aspartate samples (7.5 μmol) were then incubated with α-ketoglutarate (40 μmol), NADH (20 μmol), glutamic-oxalacetic acid transaminase (5.2 IU), malate dehydrogenase (160 IU), and fumarase (36 IU) in 3 ml of 0.1 M potassium phosphate buffer (pH 7.8) at 25°C for 4 h. The reaction was followed spectrophotometrically at 340 nm. The reaction mixtures were then washed through columns of Dowex 50 H^+ and the effluents were evaporated to dryness. The residues were counted to determine the ^{3}H/^{14}C ratios of the mixtures of fumarate plus malate after equilibration with fumarase. Parallel incubations from which fumarase had been omitted were carried out to give malate.

Deamination of Serine—The incubation mixture contained in 1 ml of 0.1 M potassium phosphate buffer, pH 7.8: 40 μmol of L-serine samples (0.5 μCi), 10 mM glutathione, 0.2 mM pyridoxal phosphate, 80 μmol of NADH, 5 IU of the β_2 protein of tryptophan synthetase from *E. coli*, and excess lactate dehydrogenase from pig heart. For the experiments in columns 1 to 4 of Table II, the ^{3}H-labeled serine (100 μCi/μmol) was diluted with nonlabeled L-serine. For the experiment of column 5, Table II, the (2*S*,3*R*)-[3-^{3}H]serine was diluted with L-serine before labeling at the α position with deuterium and the resulting DL mixture was used directly, whereas in the experiment of column 6 the carrier serine consisted of a 64-fold excess of DL-[2-^{2}H]serine. The incubation was carried out at 37°C for 4 h and stopped by boiling for 3 min. The decrease in the absorbance at 340 nm was followed during the incubation. The proteins were separated from the small molecules by dialysis. The lactate samples were isolated by paper chromatography in System C and further purified by acidification with HCl and extraction with ether, with the yields varying from 20% to 50%. The resulting lactate samples were then oxidized to acetate by heating with 12 ml of oxidation mixture (153 mg of $K_2Cr_2O_7$; 24 ml of concentrated H_2SO_4; made up to 100 ml with water) on a steam bath for 20 min under an argon atmosphere (14). The acetate samples were then isolated by steam distillation and analyzed for chirality by the method of Cornforth *et al.* (15) and Arigoni and co-workers (16).

The stable isotope experiments were carried out under the same conditions and the lactate samples were analyzed for deuterium distribution by proton NMR.

Abortive Transamination of Serine—300 IU of the β_2 protein of tryptophan synthetase from *E. coli* were first dialyzed against 0.1 M potassium phosphate buffer (pH 7.8) containing 50 mM β-mercaptoethanol to remove the pyridoxal phosphate. The apoenzyme was then dialyzed against 0.1 M potassium phosphate buffer in D_2O (pD 7.8) containing 50 mM serine and 50 mM β-mercaptoethanol. NMR analysis of the final enzyme solution (in a volume of 50 ml) indicated >98.5% of D_2O content. The reaction was started by addition of 10 μmol of pyridoxal phosphate followed by incubation at 37°C for 2 h, and was stopped by boiling for 3 min. Transamination was observed spectrally by the decrease in the absorption at 410 nm and the increase at 325 nm during incubation. The substrates and products were separated from the enzyme by dialysis and then passed onto the top of an anion exchange column (Dowex AG-1-X8, formate form, 200 to 400 mesh, 1.8 × 34 cm). Elution with a formic acid gradient (500 ml of 1 N formic acid and 500 ml of H_2O) (17) gave a fraction containing 1.5 μmol of pyridoxamine phosphate which was found to be pure by UV spectroscopy. After the solvent had been removed by evaporation under reduced pressure, the product was dissolved in MeOH saturated with HCl, transferred to capillary tubes, evaporated to dryness, and analyzed by mass spectroscopy. From the spectra obtained with chemical ionization (isobutane), the deuterium content was determined as 25% by comparing the ratio of the parent peaks $M + 2/M + 1$ (m/e 170/169) with that of authentic nonlabeled pyridoxamine phosphate·HCl. From the spectra obtained with electron impact ionization the same deuterium content of the pyridoxamine phosphate (26%) was determined by comparing the ratio of the parent peaks $M + 1/M$ (m/e 169/168) with that of the authentic sample; the fragment ions showed patterns consistent with this isotopic ratio.

α-Hydrogen Exchange of Tryptophan in D_2O—The reaction mixture in a volume of 0.75 ml of D_2O contained: 0.1 M potassium phosphate, pD 7.8; L-tryptophan, 1.5 mg; pyridoxal phosphate, 0.2 mM; β-mercaptoethanol, 10 mM; phenylmethylsulfonylfluoride, 0.5 mM; and the native tryptophan synthetase (mixture of β_2 protein and excess α protein) or its β_2 protein. The incubation was carried out at 37°C for 20 min and stopped by boiling for 3 min. The enzyme activity for each experiment was assayed in a parallel incubation. The protein was removed by centrifugation and the tryptophan was isolated by paper chromatography (System A). The percentage of deuteration was determined by the ratio $M + 2/M + 1$ in the mass spectra (chemical ionization, isobutane). The location of deuterium at the α position was confirmed by NMR for one of the tryptophan samples obtained and a control experiment from which enzyme was omitted indicated no incorporation of deuterium. The numbers of micromoles of tryptophan in which the α-carbon was deuterated in each experiment were: 0.88/0.225 IU of $\alpha_2\beta_2$; 1.54/0.35 IU of $\alpha_2\beta_2$; 1.84/0.45 IU of $\alpha_2\beta_2$; 0.85/0.35 IU of β_2; 1.31/0.45 IU of β_2; and 1.91/0.625 IU of β_2.

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TABLE I

Stereochemistry of tryptophan synthesis with different preparations and substrates of tryptophan synthetase

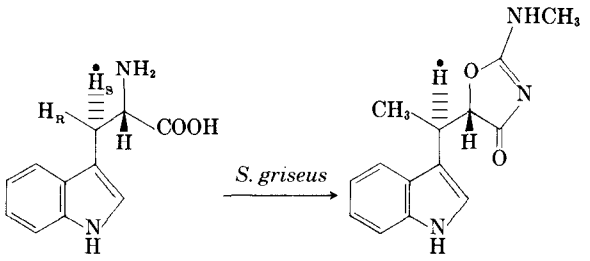
T/^{14}C of	Native enzyme from *N. crassa* L-[3-^{3}H]-serine + indole		Native enzyme from *N. crassa* L-[3-^{3}H]-serine + indole		Native enzyme from *E. coli* L-[3-^{3}H]serine + indoleglycerol phosphate		Native enzyme from *E. coli* L-[3-^{3}H]serine + indole		β_2 protein from *E. coli* L-[3-^{3}H]serine + indole	
	3*R*	3*S*	3*R*	3*S*	3*R*	3*S*	3*R*	3*S*	3*R*	3*S*
Serine					3.29	3.22	3.29	3.22	3.29	3.22
Tryptophan	2.10	2.06	2.52	3.85	3.33 (3.21)[a]	3.16 (3.16)[a]	3.21 (2.94)[a]	3.13 (2.90)[a]		
Aspartate	1.86	1.63								
Malate	2.28	2.17								
Fumarate + Malate	0.33	1.68								
Indolmycin			2.64	0.086	3.00 (2.99)[a]	0.14 (0.07)[a]	2.82 (3.11)[a]	0.14 (0.09)[a]	3.23 (3.21)[b]	0.28 (0.09)[b]

[a] Figures in parentheses are the results from the parallel experiments in the presence of 0.2 M NaCl.
[b] Figures in parentheses are the results from the parallel experiments in the presence of 0.2 M ammonium ion.

RESULTS

Stereochemistry of the Replacement Reaction at the β-Carbon Atom—The replacement of the hydroxyl group at C_β of serine by the indolyl group may proceed either with retention or with inversion of configuration at C_β. To distinguish between these alternatives, we converted (2*S*,3*R*)- and (2*S*,3*S*)-[*U*-^{14}C, 3-^{3}H]serine, prepared as described earlier (4), into L-tryptophan using tryptophan synthetase purified from *Neurospora crassa* by the method of Meyer *et al.* (6) or from *Escherichia coli* (8, 9). As shown in Table I, the ^{3}H/^{14}C ratios of the substrates do not change during this transformation, indicating complete retention of the tritium. Samples of tryptophan prepared in this way with the *Neurospora* enzyme were then degraded according to the scheme in Fig. 1 to determine their configuration at the β-carbon atom of the side chain. Initial attempts to carry out direct ozonolysis of *N*-acetyltryptophan led to extensive tritium exchange and racemization at C_β, presumably due to formation of a readily enolizable carbonyl function adjacent to the benzene ring and the methylene group. This problem was largely circumvented by Birch reduction of tryptophan to give the 4,7-dihydro derivative (13), which was then subjected to oxidation. The results of the degradation show that the tryptophan sample obtained from (3*R*)-[3-^{3}H]serine gave malate which lost most of its tritium in the fumarase reaction. Since fumarase removes the *pro*-3*R* hydrogen of L-malate (18, 19), it follows that this tryptophan sample had 3*S* configuration. Conversely, the tryptophan sample from (3*S*)-[3-^{3}H]serine was found to have 3*R* configuration. The replacement reaction therefore proceeds with retention of configuration at the β-carbon atom.[1]

We next used these tryptophan samples to calibrate a simple system with which the configuration at C_β of tritiated tryptophan could be analyzed more readily. For this we chose the conversion of tryptophan to the antibiotic indolmycin by intact cells of *Streptomyces griseus*, which proceeds with loss of one of the two β hydrogens (12):



Conversion of the two tryptophan samples of established configuration into indolmycin (Table I) showed that these were, within the limits of error, completely stereospecifically labeled and that the *pro*-3*R* hydrogen is eliminated in indolmycin formation (20). Using this analytical system we then determined the stereochemical course of tryptophan formation with the native tryptophan synthetase isolated from *E. coli*, using either indole or indole-3-glycerol-P as the second substrate, and with tryptophan synthetase β_2 protein from *E. coli* in the presence of excess indole. As shown in Table I, the reaction in each case is completely stereospecific and occurs with retention of configuration.

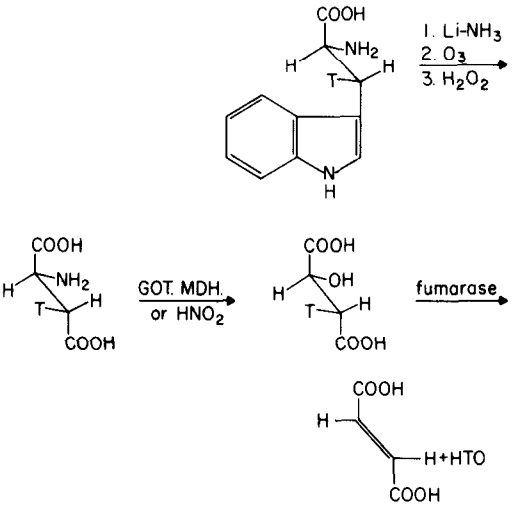


FIG. 1. Degradation of tryptophan to determine the configuration at the stereospecifically tritiated β-carbon atom.

Stereochemistry of the Replacement of —OH by —H at C_β in the α,β Elimination Reaction—In the α,β elimination reaction catalyzed by tryptophan synthetase β_2 protein, a hydrogen is added at the β carbon atom of serine, replacing the OH— group, and one can ask whether this protonation step is stereospecific. To examine this question, (2*S*,3*R*)- and (2*S*,3*S*)-[*U*-^{14}C, 3-^{3}H]serine were incubated with tryptophan synthetase β_2 protein in D_2O and the resulting pyruvate was trapped *in situ* as lactate by reduction with excess lactate dehydrogenase and NADH. The lactate samples were isolated and oxidized to acetate and the chirality of the methyl group in the acetate samples was determined by the method of Cornforth *et al.* (15) and Arigoni and co-workers (16). In this analysis procedure, which involves conversion to malate with malate synthetase followed by reaction with fumarase, (*R*)-[2-^{2}H, 2-^{3}H]acetate gives rise to malate which retains more than half of its tritium in the fumarase reaction, whereas the *S* isomer produces malate which retains less than half of its

[1] Note the change in the sequence rule priorities of the substituents in going from serine to tryptophan.

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TABLE II

Stereochemistry of the α,β elimination/deamination of serine catalyzed by tryptophan synthetase β_2 protein

$^3H/^{14}C$ of	L-[*U*-^{14}C,3-^{3}H]Serine in D_2O		L-[3-^{2}H,3-^{3}H]Serine in H_2O		DL-[2-^{2}H,3-^{3}H]Serine in D_2O	L-[3-^{2}H,3-^{3}H]Serine + excess DL-[2-^{2}H]-serine in D_2O	[2-^{14}C,2-^{2}H,2-^{3}H]Acetate (control)	
	3*R*	3*S*	3*R*	3*S*	3*R*	3*S*	2*R*	2*S*
Substrate	2.00	2.00						
Lactate	2.09	2.04						
Acetate	3.02	2.90	8.50[a]	1.94[a]	4.22[a]	2.57[a]	7.60	8.15
Malate	2.56	2.47	6.30	1.52	3.67	1.91	6.12	6.68
Fumarate	1.31	1.27	2.35	1.08	2.38	1.22	4.42	2.25
% ^{3}H retention in fumarase reaction	51.1	51.4	37.3	71.1	64.8	63.9	72.2	33.7

[a] [2-^{14}C]Acetate was added as reference.

tritium in the fumarase reaction. The results which are shown in Table II (Columns 1 and 2) indicated that the methyl group in both samples was achiral or racemic. This is in contrast to the same reaction catalyzed by tryptophanase (3) and by tyrosine phenol-lyase (21) and to the deamination of D-serine by D-serine dehydratase (22), all of which are stereospecific and occur with retention of configuration. A conclusion that the protonation at C_β in the α,β elimination reaction catalyzed by tryptophan synthetase β_2 is nonstereospecific (3) is predicated on the assumption that one, and only one, atom of D is incorporated into the methyl group from the D_2O medium. This assumption was subsequently shown to be incorrect. NMR analysis of a sample of lactate prepared from nonlabeled serine in D_2O by the same reaction showed the presence of no deuterium within the limits of detection. Thus, the third hydrogen of the methyl group must originate from within the enzyme·substrate complex. We therefore prepared (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{2}H,3-^{3}H]serine[2] and subjected it to the α,β elimination reaction with tryptophan synthetase β_2 protein in H_2O. Chirality analysis (Table II, columns 3 and 4) showed that in both cases the methyl group was chiral. 3*S*-Serine had produced *R*-acetate and 3*R*-serine had given *S*-acetate, indicating that the protonation at C_β does occur stereospecifically from the same side at which OH has been removed.

Origin of the Third Hydrogen of the Methyl Group of Pyruvate Formed by α,β Elimination—The data presented show that the hydrogen which is added at C-3 of serine to give the pyruvate methyl group must originate from within the enzyme substrate complex and must be transferred without appreciable exchange with solvent protons. The only other nonexchangeable hydrogen in the substrate is H_α, which must therefore be the source of the third methyl hydrogen. This was demonstrated to be true by repeating the experiment in column 1, Table II, with substrate carrying deuterium in position 2, in addition to the stereospecific tritium label at C-3. DL-(3*R*)-[2-^{2}H, 3-^{3}H]serine, in contrast to the undeuterated sample, gave pyruvate containing a chiral methyl group (Table II, Column 5). In a second experiment, it was shown that the transfer of the α-hydrogen to C_β is intramolecular. For this experiment, (2*S*,3*S*)-[3-^{2}H, 3-^{3}H]serine was mixed with a large (64-fold) excess of [2-^{2}H]serine and converted into lactate. Chirality analysis (Table II, Column 6) showed that the methyl group was chiral and had *R* configuration. Had intermolecular transfer of the α-hydrogen occurred to even a small extent, the majority of tritiated methyl groups would have contained two atoms of deuterium and would thus have been achiral. The difference in apparent chiral purity of the acetate samples reported in Columns 4, 5, and 6 of Table II, although appreciable, is not statistically significant since the error of the chirality analysis is approximately ±5% absolute.

Transfer of H_α to C-4′ of Pyridoxal Phosphate in the Abortive Transamination Reaction—In order to obtain further information on the geometry of the enzyme substrate complex we determined the origin of the hydrogen which is added at C-4′ of the cofactor in the abortive transamination reaction to give pyridoxamine phosphate (Reaction 5). Unlabeled serine and mercaptoethanol were incubated with tryptophan synthetase β_2 protein in D_2O and the pyridoxamine phosphate formed was isolated from the reaction mixture.[3] After purification it was analyzed for its deuterium content by chemical ionization and electron impact mass spectrometry. The data showed the presence of only 0.25 and 0.26 atom of deuterium per mol, respectively, in the two analyses, implying that about 75% of the hydrogen added at C-4′ must have originated from within the enzyme·substrate complex, most likely from the α position of serine. Such a 1 → 3 hydrogen shift from C_α to C-4′ has been observed in pyridoxamine-pyruvate transaminase (23). Since the hydrogen being transferred undergoes little exchange with the medium, the transfer can be assumed to be intramolecular. However, since the experiment requires a very large amount of enzyme, no attempt was made to further examine this point.

Nature of the Base Group Catalyzing Protonation/Deprotonation at C_α—An attempt was made to establish the origin of the hydrogen which is added at C_α of the tryptophan formed in the tryptophan synthetase reaction. Incubation of the native enzyme with nonlabeled indole and serine in D_2O gave tryptophan which was analyzed for deuterium content by mass spectrometry and by proton NMR. Mass spectrometry indicated the presence of 0.86 atom of deuterium, whereas integration of the signal for H_α in the proton NMR spectrum showed the presence of 0.09 atom of ^{1}H. The α-hydrogen of tryptophan thus originates predominantly from the solvent, but the presence of a small amount of ^{1}H is consistent with some internal transfer of a proton within the enzyme substrate complex, presumably from C_α of serine. Repetition of the above experiment in a medium of 50% D_2O in H_2O gave tryptophan which by mass spectral analysis was shown to contain 49.6% deuterium in the α position. Thus, the protonation of this site proceeds without an appreciable isotope effect. Based on the arguments put forth by O'Leary (24), these data indicate that the proton is transferred to C_α by a monoprotic base group in the enzyme, consistent with the

[2] These samples were labeled in such a way that essentially every tritiated molecule also contained deuterium.

[3] *S*-Hydroxyethyl-L-cysteine, the product of Reaction 4, was also isolated from the incubation and found to be completely deuterated at C_α by NMR analysis. However, this finding is probably not mechanistically significant since this amino acid undergoes hydrogen exchange at C_α under the conditions of the workup and analysis.

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results of Miles and Kumagai (25) implicating a histidyl residue in the abstraction of the α-proton of serine.

Rate of α-Hydrogen Exchange of Tryptophan—One of the perplexing features of tryptophan synthetase β_2 protein is the fact that it can deaminate serine, but not tryptophan.[4] As one conceivable explanation, we considered the possibility that tryptophan cannot undergo α-hydrogen cleavage, thus preventing an essential step of the reaction sequence. To examine this question we measured the exchange of the α-hydrogen of tryptophan against deuterium catalyzed by β_2 protein and native tryptophan synthetase in D_2O. Since the enzyme used was not homogeneous, the rates were related to the overall rate of tryptophan synthesis by the native enzyme. It was found that the native enzyme catalyzes α-hydrogen exchange of tryptophan at 20% of the rate of tryptophan synthesis. The β_2 protein catalyzes α-hydrogen exchange of tryptophan at 70% of the rate observed for the native enzyme. Thus, inability to catalyze C_α-H bond cleavage in tryptophan does not account for the inability of tryptophan synthetase β_2 protein to carry out the α,β elimination reaction with tryptophan as substrate.

DISCUSSION

The stereochemistry observed for the β replacement reaction catalyzed by tryptophan synthetase, which was independently confirmed by Fuganti's group (26), conforms to that seen in all the pyridoxal phosphate-catalyzed β replacement reactions studied, *i.e.* in the replacement reactions catalyzed by tryptophanase (3, 27), tyrosine phenol-lyase (28, 29), *O*-acetylserine sulfhydrase (4), and β-cyanoalanine synthetase.[5] This may have an implication for the reaction mechanism. As was discussed in a previous paper (4), formation and cleavage of a bond at C_β is expected to require an orthogonal orientation of this bond relative to the π plane and a corresponding alignment of the incoming substituent and the leaving group. Retention of configuration requires orthogonal alignment of these two groups on the same side of the π plane implying, since two objects cannot be in the same place at the same time, that the reaction either proceeds by a ping-pong mechanism or involves a conformational change of the enzyme during the reaction which reorients the incoming and the leaving group relative to the π plane. For *O*-acetylserine sulfhydrase a ping-pong mechanism has recently been demonstrated (30).

The stereochemical data reported define the geometry of the substrate cofactor complex and of the reaction intermediates in the active site and the position of an essential base relative to these. These relationships are summarized in Fig. 2. A monoprotic base, presumably a histidine residue, catalyzes the abstraction of the α-hydrogen (H_c) and a completely intramolecular transfer of this hydrogen to C_β. Since this 1,2-hydrogen shift is intramolecular it must be suprafacial, and since the replacement of the OH group at C_β by H_c occurs with retention of configuration, the conformation around the C_α-C_β bond must be as shown in Fig. 2, *i.e.* H_c and OH are *syn*. This defines the configuration of the α,β-double bond in the intermediate aminoacrylate Schiff's base; in the case of (3*R*)-[3-^{3}H]serine as substrate it is *E*, with the 3*S* isomer it is Z. Presumably catalyzed by the same base, H_c can also undergo a 1,3-azaallylic shift to C-4′ in the abortive transamination reaction (Reaction 5). Although this has not been strictly proven, this shift can also be expected to be intramolecular and therefore suprafacial. Since Dunathan has shown that the hydrogen is added on the *si* face of C-4′ (31), the

[4] Likewise, the enzyme does not catalyze ^{14}C exchange between tryptophan and [^{14}C]indole (R. Potts and H. G. Floss, unpublished results).

[5] E. E. Conn, M.-D. Tsai, and H. G. Floss, unpublished results.

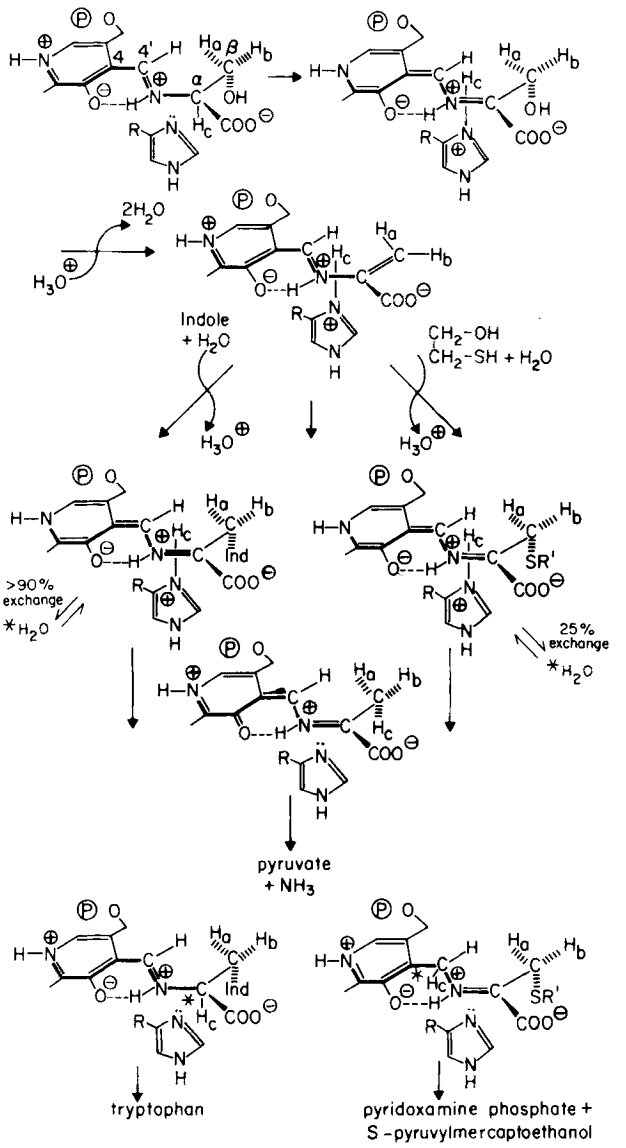

FIG. 2. Stereochemical course of reactions catalyzed by tryptophan synthetase.

conformation around the C_α-N bond must be as shown in Fig. 2, *i.e.* H_c is displayed orthogonal to the π plane on the side which corresponds to the *si* face at C-4′. This conclusion is predicated on the widely accepted assumption (32) that the conformation of the Schiff's base around the C_4-$C_{4'}$ bond is *syn*, allowing hydrogen bonding between the nitrogen and the phenolic hydroxyl group. The atoms C_4—N—C_α—C_β thus lie in a plane, underneath which the essential base is situated which functions in the abstraction of the α-hydrogen of the substrate and the protonation of C_α to give the product. Under certain conditions this base can also donate the proton to sites other than C_α.

The finding that H_α is transferred completely to C_β in the α,β elimination reaction, but only to the extent of 75% to $C_{4'}$ in the abortive transamination reaction, requires some comment. Reaction 5 is much slower than Reaction 3, and Reaction 5 is at least partially reversible (17) whereas Reaction 3 is not. After formation of the aminoacrylate Schiff's base, protonation at C_β by transfer of H_α from the protonated base and addition of $RS^\ominus$ at C_β are competing reactions. Following addition of $RS^\ominus$, protonation at C-4′ may be a slower process, allowing for some exchange of the hydrogen on the base with solvent protons in this species.

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It has been shown (33, 34) that the tryptophan synthetase β_2 protein upon binding of the α subunit undergoes a conformational change, which increases its catalytic efficiency. The small difference in the rates of α-hydrogen abstraction from tryptophan seen with these two forms of the enzyme suggests that this conformational change does not involve any major change in the position of the essential base relative to C_α of the tryptophan·enzyme complex.

It is interesting to compare tryptophan synthetase β_2 protein with the enzyme tryptophanase, with which it shares many catalytic capabilities. As has been pointed out (1), there are many similarities between these two enzymes. The reactions seems to involve essentially the same intermediates and, as shown in this and the following paper (27), the overall geometry of the coenzyme·substrate complexes in the two enzymes as well as the steric course of the reactions catalyzed are very similar or identical. A major difference is the fate of the hydrogen abstracted from the α position. In tryptophanase it is used to protonate the leaving group, whereas in tryptophan synthetase β_2 protein it can be transferred quantitatively to C_β, implying that it is not used to protonate the leaving group. Another difference is the inability of tryptophan synthetase β_2 protein to catalyze α,β elimination of tryptophan, and, as the data show, this cannot be explained by inability to cleave the C_α—H bond in this substrate. Perhaps related to this may be the inability of tryptophan synthetase to catalyze synthesis of tryptophan from indole, pyruvate, and ammonia,[6] a reaction readily catalyzed by tryptophanase (35, 36). Since this presumably reflects the ability or inability of these enzymes to abstract a hydrogen from the methyl group of pyruvate it is particularly perplexing, because both enzymes can stereospecifically protonate C_β to generate the methyl group and both must thus have a base group proximal to this site. It is possible that the difference lies in the nature of the base groups in the two enzymes and/or in the exact position of the bases relative to the pyridoxal phosphate amino acid complex.

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