Tryptophan Synthase Mutations That Alter Cofactor Chemistry Lead to Mechanism-Based Inactivation

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ABSTRACT: Mutations in the pyridoxal phosphate binding site of the tryptophan synthase β subunit (S377D and S377E) alter cofactor chemistry [Jhee, K.-H., et al. (1998) *J. Biol. Chem. 273*, 11417−11422]. We now report that the S377D, S377E, and S377A β_2 subunits form $\alpha_2\beta_2$ complexes with the α subunit and activate the α subunit-catalyzed cleavage of indole 3-glycerol phosphate. The apparent K_d for dissociation of the α and β subunits is unaffected by the S377A mutation but is increased up to 500-fold by the S377D and S377E mutations. Although the three mutant $\alpha_2\beta_2$ complexes exhibit very low activities in β elimination and β replacement reactions catalyzed at the β site in the presence of Na^+, the activities and spectroscopic properties of the S377A $\alpha_2\beta_2$ complex are partially repaired by addition of Cs^+. The S377D and S377E $\alpha_2\beta_2$ complexes, unlike the wild-type and S377A $\alpha_2\beta_2$ complexes and the mutant β_2 subunits, undergo irreversible substrate-induced inactivation by L-serine or by β-chloro-L-alanine. The rates of inactivation (k_{inact}) are similar to the rates of catalysis (k_{cat}). The partition ratios are very low ($k_{cat}/k_{inact} = 0.25-3$) and are affected by α subunit ligands and monovalent cations. The inactivation product released by alkali was shown by HPLC and by fluorescence, absorption, and mass spectroscopy to be identical to a compound previously synthesized from pyridoxal phosphate and pyruvate. We suggest that alterations in the cofactor chemistry that result from the engineered Asp377 in the active site of the β subunit may promote the mechanism-based inactivation.

Our investigations of tryptophan synthase are aimed at increasing the understanding of how the enzyme architecture tailors its function. The bacterial tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.2.1.20) catalyzes the last two steps in the biosynthesis of L-tryptophan. The α subunit cleaves indole 3-glycerol phosphate reversibly to D-glyceraldehyde 3-phosphate and indole (α reaction). Indole is transferred through a unique 25 Å tunnel to the active site of the β subunit[1] where it undergoes a pyridoxal phosphate (PLP[2])-dependent β replacement reaction with L-serine to form L-tryptophan (β reaction).

α reaction: indole 3-glycerol-P $\leftrightarrow$ indole + D-glyceraldehyde 3-P

β reaction: L-serine + indole $\rightarrow$ L-tryptophan + H_2O

The β_2 subunit and the $\alpha_2\beta_2$ complex also catalyze β replacement and β elimination reactions with a variety of other amino acids, including β-chloro-L-alanine. These reactions involve a key, common intermediate, the external aldimine of aminoacrylate (E-AA, Scheme 1 in Results).

Table 1: Families of PLP Enzymes

fold[a]	prototype	no. of structures	N-1 (PLP)[f]
I	L-aspartate aminotransferase	9[b]	Asp
II	tryptophan synthase β subunit	3[c]	Ser
III	alanine racemase	1[d]	Arg
IV	D-amino acid aminotransferase	2[e]	Glu

[a] Folds of PLP enzymes as defined by ref *2*. [b] L-Aspartate aminotransferase (*45*), ω-amino acid:pyruvate aminotransferase (*64*), dialkylglycine decarboxylase (*65*), ornithine aminotransferase (*60*), prokaryotic ornithine decarboxylase (*66*), tyrosine phenol-lyase (*67*), tryptophanase (*68*), glutamate-1-semialdehyde aminomutase (*69*), and cystathionine β-lyase (*55*). [c] Tryptophan synthase β subunit (*3*), threonine deaminase (*4*), and L-cysteine synthase (*5*). [d] Alanine racemase (*6*). [e] D-Amino acid aminotransferase (*70*) and branched-chain L-amino acid aminotransferase (*71*). [f] Residue that interacts with N-1 of PLP in enzymes in each fold.

It is important to understand how tryptophan synthase and other PLP-dependent enzymes control their reaction and substrate specificities. Sequence comparisons designed to establish evolutionary relationships (*1*, *2*) have resulted in PLP-dependent enzymes being classified in at least four folds (Table 1). The available crystal structures of enzymes within each fold are structurally similar. The three-dimensional structure of the β subunit in the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* (*3*) has served as the

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[1] The term β_2 subunit is used for the isolated enzyme in solution; β subunit is used for the enzyme in the $\alpha_2\beta_2$ complex, to describe a specific residue in the β subunit, or in titrations to determine subunit interaction.

[2] Abbreviations: PLP, pyridoxal phosphate; GP, DL-α-glycerol 3-phosphate; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; X, 4-[2-methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid; MBP, morpholinopropanesulfonic acid/bicine/proline; TEA, triethanolamine; TFA, trifluoroacetic acid; FAB, fast atom bombardment.

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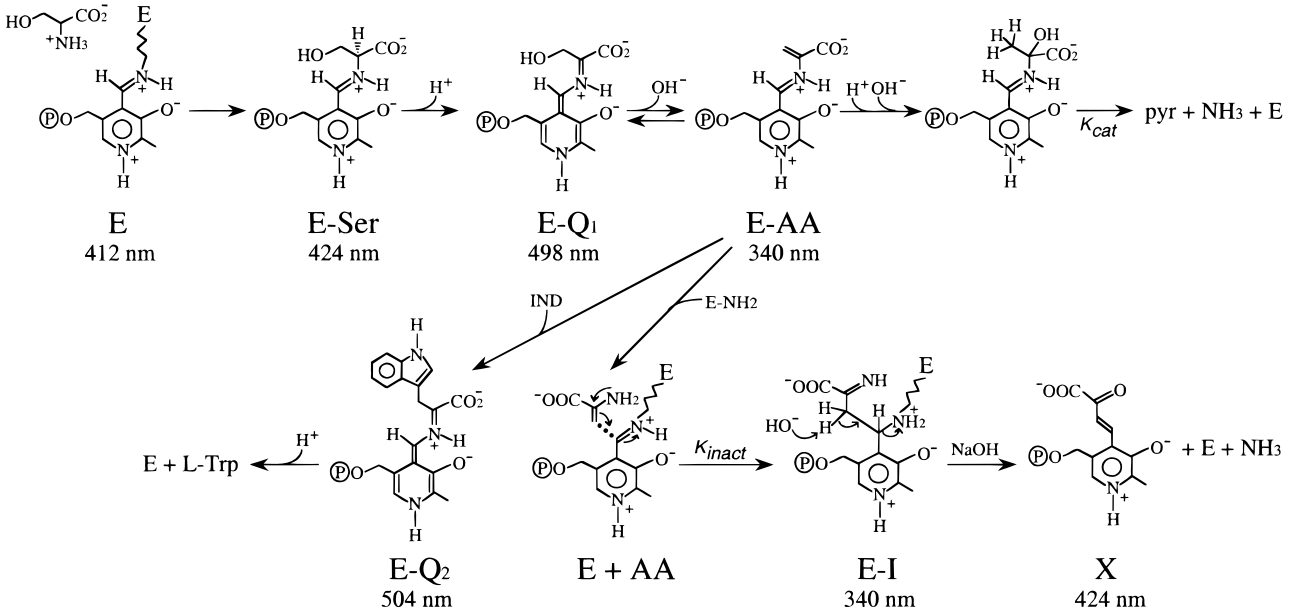
Scheme 1: Mechanisms of β Elimination and β Replacement Reactions and of Mechanism-Based Inactivation



prototype for other PLP enzymes in fold II. Structures of two other enzymes in this fold, threonine deaminase (*4*) and *O*-acetylserine sulfhydrylase (L-cysteine synthase) (*5*), have been determined recently.

One of the most important and best studied interactions between the cofactor and the active site of all PLP enzymes is that between the pyridine nitrogen of PLP and an amino acid side chain (Table 1) (*6*). This interaction is a hydrogen bond/salt bridge between the N-1 proton of PLP and a negatively charged aspartate or glutamate side chain in members of folds I or IV, respectively. The PLP-binding site of the tryptophan synthase β subunit (fold II) has the neutral hydroxyl of Ser377 interacting with PLP N-1 (Figure 1) (*3*, *7*). Alanine racemase (EC 5.1.1.1) (fold III) is unique in having a positively charged arginine near N-1 of PLP (*6*).

We have reported recently (*8*) that changing the tryptophan synthase β subunit Ser377 to Asp (S377D) or Glu (S377E) alters the effects of pH on the absorption spectra of the enzymes and results in the accumulation of quinonoid intermediates in the reactions with substrates. We proposed that the engineered Asp or Glu residue changes the cofactor chemistry by stabilizing the protonated pyridine nitrogen of pyridoxal phosphate, reducing the p*K* of the internal aldimine nitrogen, and promoting formation of quinonoid intermediates. We have also reported briefly (*9*) that mutation of βSer377 to Ala, Asp, or Glu results in a >100-fold decrease in the rate of conversion of L-serine and indole to tryptophan.

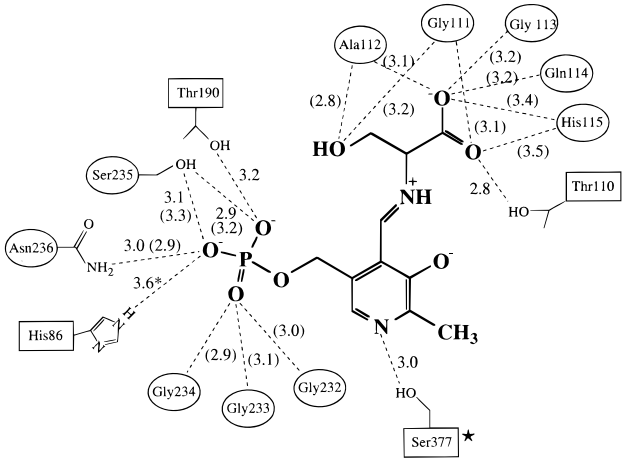


FIGURE 1: Active site of the tryptophan synthase β subunit, showing residues that interact with the external aldimine between PLP and L-serine. This structure was taken from the βK87T-Ser-IPP structure in ref *7*.

In this paper, we examine the effects of mutational alteration of Ser377 on the activities and spectroscopic properties of the β_2 subunit and $\alpha_2\beta_2$ complex. An important finding is that the S377D and S377E $\alpha_2\beta_2$ complexes undergo irreversible substrate-induced inactivation by a mechanism first elucidated by Metzler et al. (*10*, *11*) and later found with different mutant forms of the tryptophan synthase $\alpha_2\beta_2$ complex and with the wild-type β_2 subunit (*12*, *13*). This inactivation results from displacement of aminoacrylate from the key E-AA intermediate followed by reaction of aminoacrylate with the methylene carbon of enzyme-bound PLP which forms a covalent adduct (E−I, Scheme 1). Alkali treatment of E−I yields the PLP derivative, X. We suggest that the altered cofactor chemistry may promote this inactivation.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. Pyridoxal phosphate, pyridoxamine phosphate, L-serine, DL-α-glycerol phosphate (GP), β-chloro-L-alanine hydrochloride, D-glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase were from Sigma. Solutions of β-chloro-L-alanine hydrochloride were freshly prepared and adjusted to pH 7.8 with sodium hydroxide immediately before use. Indole 3-glycerol phosphate was prepared enzymatically (*14*). Oligonucleotides were supplied by Integrated DNA Technologies. Na^+/bicine (50 mM, pH 7.8) (buffer B), Cs^+/bicine (50 mM, pH 7.8), TEA-bicine (50 mM, pH 7.8), and MBP (*15*) buffers were used for spectroscopic studies with additional NaCl or CsCl where indicated. MBP buffer contains 50 mM morpholinopropanesulfonic acid, 50 mM bicine, and 50 mM proline. The pH was raised with sodium hydroxide to 11.2; the

solution was then back-titrated with HCl to pH 7.8. Compound X [4-[2-methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid] (see Scheme 1 for structure) was synthesized by a modification of the method of Schnackerz (*16*). Pyridoxal phosphate (1.5 mmol) and sodium pyruvate (6 mmol) were dissolved in 2 mL of 0.5 M KOH. The solution was stirred for 24 h in the dark at room temperature. The reddish reaction mixture was adjusted to pH 7.0 with 5 N HCl. X was subjected to HPLC as described above to remove unreacted PLP and pyruvate. X was also isolated from the inactivated enzyme. The S377D β_2 subunit (8 mg/mL) was incubated with the α subunit (16 mg/mL, 3-fold excess) and 50 mM L-serine in the presence of 0.2 M NaCl for 4 h at 25 °C in a total volume of 4 mL. X was dissociated from the protein by addition of NaOH to 0.5 N followed by addition of HCl to a final concentration of 0.5 N to precipitate the protein. The denatured protein was removed by centrifugation, and the yellow supernatant solution containing X was lyophilized. The lyophilized X was dissolved in 500 μL of 0.1% TFA and was subjected to HPLC as described below to remove other compounds (L-serine, pyruvate, and PLP) and excess salt. The eluted X was lyophilized, dissolved in 100 μL of 0.1% TFA, and rechromatographed to remove residual salt. After the final lyophilization, the dry sample was stored at −20 °C. The lyophilized sample was dissolved in water just prior to analysis for mass spectrometry or absorption spectroscopy.

Bacterial Strains, Plasmids, and Enzymes. The plasmid pEBA-10 (*17*) was used to express wild-type and mutant β subunit forms (S377A, S377D, and S377E) of the *S. typhimurium* tryptophan synthase $\alpha_2\beta_2$ complex in *Escherichia coli* CB 149 (*14*), which lacks the trp operon. The construction of two of the mutant β subunit forms (S377D and S377E) has been reported (*8*). The S377A mutant form was constructed in an analogous way using the mutagenic primer 5′-GTC-AAT-CTC-GCT-GGC-CGC-GGA-GT-3′, where the base changes are underlined. The mutant enzyme was expressed and purified to homogeneity in high yield (∼1 g/liter of culture) as the $\alpha_2\beta_2$ complex by a method that utilizes crystallization from crude extracts (*18*). The wild-type, S377A, and S377D β_2 subunits were obtained by heat precipitation of the α subunit from the $\alpha_2\beta_2$ complex (*19*). The α subunit was purified as described previously (*8*). Protein concentrations were determined from the specific absorbance at 278 nm using an $A^{1\%}_{1cm}$ of 6.0 for the $\alpha_2\beta_2$ complex, an $A^{1\%}_{1cm}$ of 6.5 for the holo β_2 subunit, and an $A^{1\%}_{1cm}$ of 4.4 for the α subunit (*19*).

Enzyme Assays. One unit of activity in any reaction is the formation of 0.1 μmol of product in 20 min at 37 °C. Activities of $\alpha_2\beta_2$ complexes in β replacement reactions [reaction of indole (0.2 mM) and L-serine (40 mM) or β-chloro-L-alanine (10 mM) to form L-tryptophan] were determined by a spectrophotometric assay (*19*) in the presence of a 3-fold molar excess of the α subunit. CsCl (0.2 M) and DL-α-glycerol 3-phosphate (10 mM) were added in assays where indicated. Activities in β elimination reactions with L-serine and β-chloro-L-alanine were measured by spectrophotometric assays coupled with lactate dehydrogenase (*20*) with modified components (40 mM L-serine or 10 mM β-chloro-L-alanine, 0.16 mM NADH, and excess lactate dehydrogenase in buffer B). The rate of cleavage of indole 3-glycerol phosphate in the presence or absence of L-serine was measured by a spectrophotometric assay coupled with D-glyceraldehyde 3-phosphate dehydrogenase (*21*). The extent of formation of indole in reactions with indole 3-glycerol phosphate was determined by a colorimetric assay with *p*-dimethylaminobenzaldehyde (*22*). Indole 3-glycerol phosphate (0.6 mM) was incubated at 37 °C in the presence or absence of 50 mM L-serine with the wild-type α subunit (10 μM) and either a 3-fold excess of the wild-type or S377A β subunit or a 10-fold excess of the S377D or S377E β subunit. After 40 min, the indole reagent was added, and the absorbance at 540 nm was determined after 5 min. The amount of indole found is expressed as the percentage of the initial amount of indole 3-glycerol phosphate added.

Spectroscopic Methods. Absorption spectra and assays of enzyme activities at single wavelengths were made using a Hewlett-Packard 8452 diode array spectrophotometer thermostated at 25 °C. Fluorescence measurements were made using a Photon Technology International (PTI) dual excitation spectrofluorimeter thermostated at 25 °C. The change of fluorescence emission at 510 nm (with excitation at 420 nm) due to formation of the external aldimine of L-serine (E-Ser) was measured as described previously (*23*−*25*). Circular dichroism measurements (mean residue ellipticity in deg cm^2 dmol^{-1}) were taken in a Jasco J-500C spectrophotometer, equipped with a DP-500N data processor (Japan Spectroscopic Co., Easton, MD) at 25 °C. Mean residue ellipticities were converted to molar ellipticities (θ) by multiplication by the number of amino acids (e.g., 397 for the β subunit and 665 for an $\alpha\beta$ pair). Molar circular dichroism values were calculated using the relationship $\Delta\epsilon = \theta/3298$. Negative ion FAB spectra were obtained on a JEOL SX102 mass spectrometer operated at an accelerating voltage of −10 kV. Samples were desorbed from a magic bullet matrix. Linked scan analyses were run on the parent ion.[3]

Analysis of Pyruvate Formation. The extent of pyruvate formation was measured in the reaction of the S377D β subunit (3 mg/mL, 70 μM) in the presence of 5-fold excess of the α subunit (10 mg/mL, 350 μM) with 50 mM L-serine in 50 mM Cs^+/bicine (pH 7.8) containing 0.2 M CsCl or in 50 mM Na^+/bicine (pH 7.8) containing 0.2 M NaCl and 10 mM DL-α-glycerol 3-phosphate. Reactions were terminated at time intervals by addition of HCl to 0.5 N followed by centrifugation to remove the precipitated protein. The supernatants were adjusted to pH 7 by addition of NaOH, and the amount of pyruvate formed was determined by an assay with NADH and lactate dehydrogenase (*20*).

Analysis of PLP and PLP Derivatives. The PLP content of wild-type and mutant enzymes was determined by a fluorometric method (*26*). PLP and its derivatives (PMP and X) were also detected by an HPLC method. The reaction of the S377D $\alpha_2\beta_2$ complex with L-serine was carried out as described above for measurement of the extent of pyruvate formation. Aliquots of the reaction mixture (50 μL) were removed at time intervals, treated with NaOH (final concentration of 0.5 N) to dissociate X from the protein, and acidified with HCl to 0.5 N to precipitate the protein.

[3] We thank L. K. Pannell of NIDDK for these analyses.

Aliquots of the supernatants (50 μL) obtained after centrifugation were diluted with equal volumes of 0.1% TFA to give a final pH of 2.3. Samples (60 μL, corresponding to 2 nmol of cofactor) were injected into a Waters Puresil C18 column (4.6 mm × 150 mm), a reversed-phase silica gel column, connected to an Altex (model 332) gradient liquid chromatograph HPLC control system. The eluent was 0.1% TFA (pH 2.3), at a flow rate of 1.0 mL/min. The peak detector (Kratos, Spectroflow 773) was set at 295 nm, and peaks were integrated with a Shimadzu C-R1A Chromatopac. Under these conditions, the retention times were 2.2 min for PMP, 4.1 min for PLP, and 7 min for X.

Data Analysis. Rate constants for the reactions of the S377D $\alpha_2\beta_2$ complex with L-serine (see Scheme 1) were computed by direct fits of the data to eq 3 or 4 using a nonlinear least-squares analysis computer program. Measurements of the absorbance due to E−Q formation and decay showed an initial rapid increase followed by a slow decrease consistent with a two-step reaction:

$$\text{E-Ser} \xrightarrow{k_1} \text{E}-\text{Q} \xrightarrow{k_2} \text{E}-\text{I}$$

Measurements of fluorescence at 384 nm due to E−I formation showed a short lag, consistent with the two reactions above. In the presence of serine at high concentrations, these reactions will be irreversible and

$$[\text{E}-\text{I}] = \{[\text{E-Ser}]_0/(k_2 - k_1)\}[k_1 \exp(-k_2 t) - k_2 \exp(-k_1 t)] + [\text{E-Ser}]_0 \quad (1)$$

The short lag time indicates that $k_1 \gg k_2$; after 2 min, this expression simplifies to eq 2

$$[\text{E}-\text{I}] = [\text{E-Ser}]_0[1 - \exp(-k_2 t)] \quad (2)$$

A linear term was added to eq 2, to account for a subsequent slow increase in fluorescence.

$$[\text{E}-\text{I}] = [\text{E-Ser}]_0[1 - \exp(-k_2 t)] + mt \quad (3)$$

Rate constants were estimated by nonlinear least-squares fit of fluorescence changes or absorbance changes to eq 3.

Measurements of the decrease in the extent of pyruvate formation or in PLP content or of the increase in the X content did not exhibit a lag phase because fewer points were analyzed at early times. These data were analyzed by direct fits of the data to eq 4.

$$Y = (Y_{max} - Y_{min}) \exp(-k_2 t) + \underline{mt + c} \quad (4)$$

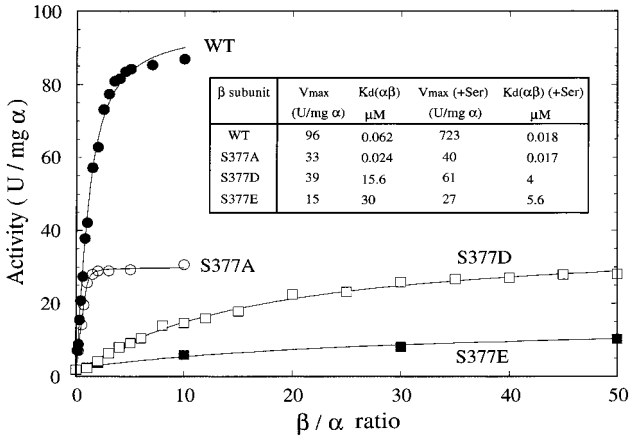
where Y is the concentration of PLP, X, or pyruvate, Y_{max} (Y_{min}) is the maximum (minimum) value of Y of the exponential phase, k_2 is the rate constant of the fast phase, m is a linear kinetic parameter, and c is a constant. Because the semilogarithmic plot of Y versus time shows a fast phase and a much slower phase, the slow phase can be approximated as the underlined part of eq 4. From the data fitting to eq 4, the inactivation rate (k_{inact}) was obtained as k_2 and the k_{cat} was calculated from the initial rate.

The apparent dissociation constant for the α and β subunits K_d ($\alpha\beta$) was analyzed by measuring the rate of indole 3-glycerol phosphate cleavage in the presence or absence of 50 mM L-serine as a function of β subunit concentration or by measuring the rate of formation of X as a function of α subunit concentration in the presence of 50 mM L-serine. The data were modeled assuming that the α and β subunits associate in a noncooperative fashion. Under this assumption, the α−β interaction can be simply modeled according to eq 5:

$$K_d = [\alpha][\beta]/[\alpha\beta] \quad (5)$$

K_d($\alpha\beta$) was obtained from eq 6 (*8*):

$$Y = Y_0 + (Y_{max} - Y_0) \times \frac{([\beta]_{tot} + f_\alpha[\beta]_{tot} + K_d) - \sqrt{([\beta]_{tot} + f_\alpha[\beta]_{tot} + K_d)^2 - 4f_\alpha[\beta]_{tot}^2}}{2[\beta]_{tot}} \quad (6)$$



β subunit	Vmax (U/mg α)	Kd(αβ) μM	Vmax (+Ser) (U/mg α)	Kd(αβ) (+Ser) μM
WT	96	0.062	723	0.018
S377A	33	0.024	40	0.017
S377D	39	15.6	61	4
S377E	15	30	27	5.6

FIGURE 2: Titration of the wild-type α subunit with wild-type and mutant β subunits. Activities of the wild-type α subunit (10 μg/mL) in the α reaction were determined in the presence of increasing amounts of the wild-type (●), S377A (○), S377D (□), and S377E (■) β subunit as described in Experimental Procedures. Values of V_{max}, the maximal specific activity of the reconstituted $\alpha_2\beta_2$ complex expressed in units per milligram of α subunit, and of K_d($\alpha\beta$), the apparent dissociation constant of the α and β subunits, in the presence or absence of L-serine, were obtained as described in Experimental Procedures. These results are given in the inset along with additional data obtained from assays of the reaction in the presence of 50 mM L-serine.

RESULTS

Wild-Type and Mutant β Subunits Stimulate the Activity of the α Subunit. Interaction of the α and β subunits was characterized by measuring the activity in the α reaction as a function of β subunit concentration (Figure 2). The results show clearly that interaction of the wild-type α subunit with wild-type and S377A β subunits is strong whereas interaction with S377D and S377E β subunits is weak. Nonlinear least-squares analysis of the data in Figure 2 as described in Experimental Procedures gave the parameters compiled in the inset. On the basis of these data, the apparent dissociation constants, K_d($\alpha\beta$), for the S377D and S377E $\alpha_2\beta_2$ complexes are ~250- and 500-fold larger, respectively, than the K_d-($\alpha\beta$) for the wild-type enzyme. The fully saturated S377A, S377D, and S377E $\alpha_2\beta_2$ complexes are 34, 40, and 16% as active (V_{max}) as the wild-type $\alpha_2\beta_2$ complex. The finding that the mutant β subunits all stimulate the intrinsic activity of the α subunit provides partial evidence for the structural integrity of the mutant β subunits. However, changing

Table 2: Specific Activities of Wild-Type and Mutant $\alpha_2\beta_2$ Complexes[a]

reaction	addition	specific activity (units/mg of $\alpha_2\beta_2$) WT $\alpha_2\beta_2$	S377A $\alpha_2\beta_2$	S377D $\alpha_2\beta_2$	S377E $\alpha_2\beta_2$
(1) Ind + Ser → Trp + H_2O	Na^+	883 (100%)	3 (0.3%)	2 (0.2%)	1 (0.1%)
(2) Ind + Ser → Trp + H_2O	Na^+/GP	364 (41%)	21 (2.4%)	2 (0.2%)	<1 (<0.1%)
(3) Ind + Ser → Trp + H_2O	Cs^+	907 (103%)	221 (25%)	4 (0.4%)	37 (4%)
(4) Ind + ClAla → Trp + HCl	Na^+	171 (19%)	55 (6%)	3 (0.3%)	2 (0.2%)
(5) Ser → Pyr + NH_3	Na^+	12 (1%)	7 (1%)	1.5 (0.2%)	2.6 (0.3%)
(6) ClAla → Pyr + NH_3 + HCl	Na^+	30 (3%)	16 (2%)	1 (0.1%)	1 (0.1%)

[a] Specific activities were determined by assays as described in Experimental Procedures with the indicated additions: 0.2 M CsCl or NaCl, 10 mM DL-α-glycerol 3-phosphate, or 50 mM L-serine. Values in parentheses show the activity of the mutant $\alpha_2\beta_2$ complex as a percentage of the activity of the wild-type $\alpha_2\beta_2$ complex in the β reaction (Ind + Ser → Trp + H_2O) in the presence of Na^+.

Ser377 to a negatively charged residue dramatically reduces the strength of subunit interaction, $K_d(\alpha\beta)$.

The wild-type $\alpha_2\beta_2$ complex reacts with indole 3-glycerol phosphate in the presence of L-serine to form D-glyceraldehyde 3-phosphate and L-tryptophan by the $\alpha\beta$ reaction, which is essentially the sum of the α and β reactions and involves sequential catalysis at the α and β sites.

$\alpha\beta$ reaction: L-serine + indole 3-glycerol phosphate → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O

The rates of cleavage of indole 3-glycerol phosphate by the α subunit in the presence of L-serine were determined as a function of β subunit concentration by titrations analogous to those shown in Figure 2. The parameters obtained are compiled in the inset in Figure 2. Addition of L-serine strongly stimulates the maximal rate of cleavage of indole 3-glycerol phosphate by the wild-type $\alpha_2\beta_2$ complex, as observed previously, but has very small effects on the maximal rates of the mutant enzymes.

Because the mutant enzymes have very low activities in reactions catalyzed at the β site (see the text and Table 2 below), we determined whether the product of the reactions of indole 3-glycerol phosphate in the presence or absence of L-serine was indole or L-tryptophan as described in Experimental Procedures. In the absence of L-serine, the wild-type, S377A, S377D, and S377E $\alpha_2\beta_2$ complexes all accumulate an amount of indole equivalent to ~60% of the amount of indole 3-glycerol phosphate initially added. In the presence of L-serine, the wild-type and S377A $\alpha_2\beta_2$ complexes accumulate no indole whereas the S377D and S377E $\alpha_2\beta_2$ complexes accumulate an amount of indole equivalent to 11 and 56%, respectively, of the amount of indole 3-glycerol phosphate initially added. Thus, the β site activities of the wild-type and S377A enzymes are sufficient, but the β site activities of the S377D and S377E enzymes insufficient, to convert all of the indole intermediate to L-tryptophan in the presence of L-serine. Nevertheless, addition of L-serine to each of the mutant enzymes decreases the $K_d(\alpha\beta)$, as observed for the wild-type $\alpha_2\beta_2$ complex. This result indicates that formation of an enzyme−substrate intermediate at the active site of each of the three mutant β subunits promotes subunit association, as is well established for the wild-type β subunit (*27*).

Effects of the Ser377 Mutations on Specific Activities of Reactions Catalyzed at the β Site. The wild-type $\alpha_2\beta_2$ complex catalyzes β replacement and β elimination reactions with L-serine (Scheme 1) and analogous reactions with β-chloro-L-alanine. The specific activities are modulated by DL-α-glycerol 3-phosphate, an α subunit ligand, and by exchange of Cs^+ for Na^+ (Table 2). The three mutant $\alpha_2\beta_2$ complexes exhibit very low activities (≤0.3% of that of the wild type) in the conversion of L-serine to L-tryptophan in the presence of Na^+ (reaction 1). However, the specific activities of the S377A $\alpha_2\beta_2$ complex are significantly higher in the presence of DL-α-glycerol 3-phosphate (reaction 2) or of Cs^+ (reaction 3) and in reaction 4 with β-chloro-L-alanine and indole. In contrast, the S377D and S377E complexes exhibit very low activities under most assay conditions. The S377E complex shows low but significant activity in the presence of Cs^+.

Effects of the S377A Mutation on Spectroscopic Properties. The absorption spectra of PLP bound to the S377A β_2 subunit and $\alpha_2\beta_2$ complex (Figure 3B,D) are similar to the spectra of the wild-type enzymes (Figure 3A,C). Substitution of Cs^+ for Na^+ has very small effects on the spectra. The reactions of the wild-type β_2 subunit and of the S377A β_2 subunit with L-serine in the presence of Na^+ give a predominant intermediate with maximum absorbance at 424 nm (Figure 3A,B), which is ascribed to the external aldimine of PLP with L-serine (E-Ser in Scheme 1). The reaction of the wild-type $\alpha_2\beta_2$ complex with L-serine in the presence of Na^+ yields an equilibrium mixture of E-Ser and of E-AA, the aldimine of aminoacrylate, which has maximum absorbance at 340 nm (Figure 3C). In contrast, the S377A $\alpha_2\beta_2$ complex forms E-Ser predominantly under these conditions (Figure 3D). Thus, the main effect of the S377A mutation is to shift the equilibrium distribution of L-serine intermediates formed by the $\alpha_2\beta_2$ complex in the presence of Na^+ from predominantly E-AA to predominantly E-Ser. Exchange of Cs^+ for Na^+ shifts the distribution of L-serine intermediates formed by the S377A $\alpha_2\beta_2$ complex toward E-AA.

The E-Ser intermediate formed by the S377A β_2 subunit and $\alpha_2\beta_2$ complex exhibits the same fluorescence properties as the intermediate formed by the wild-type β_2 subunit (*23*). Fluorescence titrations of the S377A β_2 subunit and $\alpha_2\beta_2$ complex with L-serine in the presence of Na^+ give values for K_d(L-Ser) of 6.98 ± 0.29 and 1.81 ± 0.04 mM, respectively; the value for the wild-type β_2 subunit is 1.48 ± 0.04 mM (data not shown).

The CD spectra of the wild-type and S377A $\alpha_2\beta_2$ complexes are similar (Figure 3E,F) and exhibit positive ellipticity bands at 336 and 412 nm due to bound PLP as reported previously for the wild-type enzyme (*28*−*31*). Addition of L-serine in the presence of Cs^+ to the S377A $\alpha_2\beta_2$ complex results in a decrease in ellipticity at 420 nm

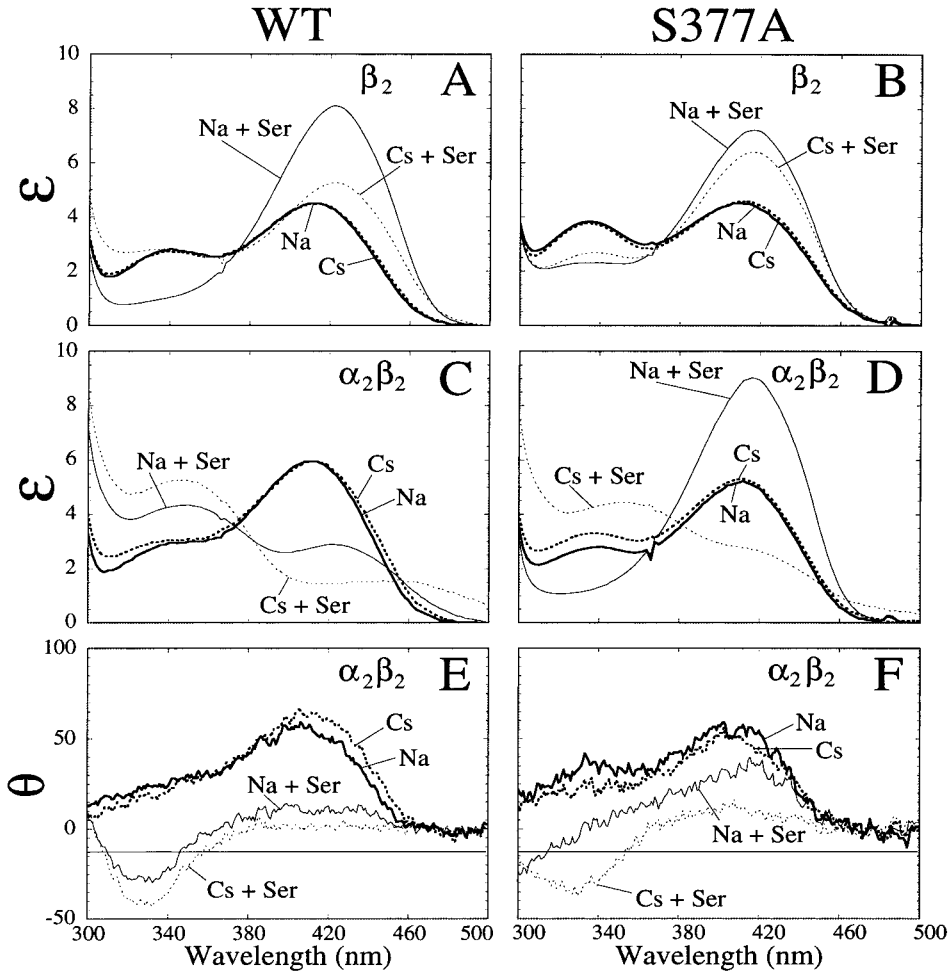


FIGURE 3: Effects of monovalent cations and of L-serine on the absorption and CD spectra of wild-type and S377A enzymes. Absorption spectra of the wild-type and the S377A β_2 subunits (A and B) and $\alpha_2\beta_2$ complexes (C and D) in 50 mM TEA-bicine buffer at pH 7.8 and 25 °C in the presence of 0.2 M CsCl or 0.2 NaCl and in the presence or absence of 50 mM L-serine. CD spectra of the wild-type $\alpha_2\beta_2$ complexes (E) in MBP buffer (pH 8.1) containing 0.1 M Na^+ or Cs^+ and of the S377A $\alpha_2\beta_2$ complex in Na^+/bicine or Cs^+/bicine buffer (pH 7.8) containing 0.2 M NaCl or CsCl, respectively, and in the presence or absence of 50 mM L-serine as described in Experimental Procedures. All spectra were recorded on enzymes at 1 mg/mL; results are shown as millimolar absorptivity (ϵ in mM^{-1} cm^{-1}) or as mean residue ellipticity (θ in deg cm^2 $dmol^{-1}$).

with formation of a strong negative ellipticity band at 330 nm as observed with the wild-type $\alpha_2\beta_2$ complex (*31*, *32*). Addition of L-serine to the S377A $\alpha_2\beta_2$ complex in the presence of Na^+ results in smaller changes.

Molar circular dichroism values ($\Delta\epsilon$) and molar absorptivity values (ϵ) at λ_{max} (412−424 nm) derived from the spectra of the wild-type and S377A enzymes in Figure 3, from CD spectra of the wild-type β_2 subunit (spectra not shown), and from the S377D $\alpha_2\beta_2$ complex in Figure 4D (see below) are compiled in Table 3. Ratios of $\Delta\epsilon/\epsilon$ reflect the asymmetric orientation of the bound PLP in different enzymes and enzyme−substrate complexes (see Discussion).

Reactions of the S377D $\alpha_2\beta_2$ Complex with L-Serine. Figure 4 shows the spectroscopic changes observed upon reaction of the S377D $\alpha_2\beta_2$ complex with L-serine in the presence of Na^+ (A) or Cs^+ (B). The reaction in the presence of Na^+ results in a rapid increase in absorbance at 416 nm followed by a decrease at 416 nm concomitant with an increase at 340 nm. The time courses at 416 and 340 nm are shown in the inset. The reaction in the presence of Cs^+ results in formation of a pronounced absorbance band at 498 nm (E_{max} = 17.5 mM^{-1} cm^{-1}) that can be attributed to the quinonoid E−Q1 (Scheme 1) as reported previously (*8*). This species decays with a concomitant increase at 340 nm (see the inset). The reaction with L-serine and indole in the presence of Na^+ (Figure 4C) results in a rapid increase in absorbance at 504 nm followed by a decrease concomitant with an increase at 338 nm. The band at 504 nm can be ascribed to E−Q2, the quinonoid of L-tryptophan that is formed by the reaction of indole with E-AA (see Scheme 1). A similar band was observed in the reaction of the S377D $\alpha_2\beta_2$ complex with L-tryptophan (*8*). The calculated rates of change at specific wavelengths are compiled in Table 4. The CD spectrum of the S377D $\alpha_2\beta_2$ complex (Figure 4D) exhibits a positive ellipticity band at 414 nm, whereas the spectrum of the inactivated enzyme (E−I) exhibits a positive ellipticity band at 340 nm. Derived values of $\Delta\epsilon$ and ϵ at 414 nm and ratios of $\Delta\epsilon/\epsilon$ are compiled in Table 3.

Substrate-Induced Inactivation of the S377D $\alpha_2\beta_2$ Complex. To determine whether the S377D $\alpha_2\beta_2$ complex is inactivated by reaction with L-serine, we determined the kinetics of pyruvate formation (Figure 5A) under the conditions used in Figure 4A−C. The rate of pyruvate formation decreases rapidly and stops after formation of 1.4 nmol of pyruvate/nmol of β site in the presence of Na^+, 4

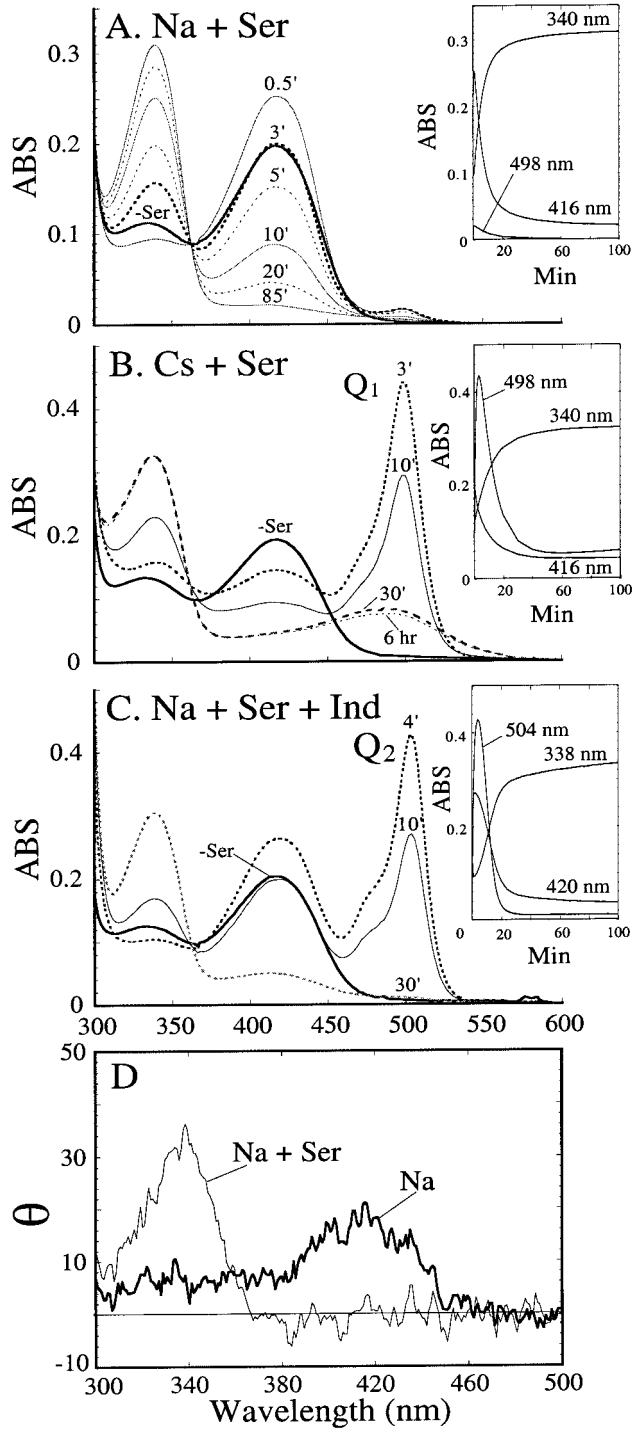


FIGURE 4: Reactions of the S377D $\alpha_2\beta_2$ complex with L-serine in the presence of Na^+ or Cs^+ alter spectral properties. Absorption spectra of the S377D $\alpha_2\beta_2$ complex [7 μM β subunit and 35 μM α subunit in 50 mM Na^+/bicine (pH 7.8) containing 0.2 M NaCl (A) or 50 mM Cs^+/bicine (pH 7.8) containing 0.2 M CsCl (B)] were recorded at 25 °C before and at the indicated times after addition of 50 mM L-serine. The insets show time courses at single wavelengths. (C) Spectra as in panel A with the complex in the presence of 50 mM L-serine and 0.2 mM indole. (D) CD spectra of the S377D $\alpha_2\beta_2$ complex (1 mg/mL in Na^+/bicine or Cs^+/bicine buffer containing 0.2 M NaCl or CsCl, respectively, and in the absence of L-serine or 2 h after addition of 50 mM L-serine).

nmol of pyruvate/nmol of β site in the presence of Cs^+, and after 0.5 nmol of pyruvate/nmol of β site in the presence of Na^+ and DL-α-glycerol 3-phosphate. The initial rates of pyruvate formation (k_{cat}) and rates of decrease in pyruvate formation derived from analyses of these curves are compiled in Table 4.

Detection of the Product of Inactivation of the S377D $\alpha_2\beta_2$ Complex in the Reaction with L-Serine. Because the peak at 340 nm observed in absorption spectra in Figure 4A,B could result from pyruvate or from one of several possible PLP derivatives or both, we sought a more sensitive and specific way to detect and monitor formation of the product of inactivation of the S377D $\alpha_2\beta_2$ complex. Figure 5B shows that the reaction of the S377D $\alpha_2\beta_2$ complex with L-serine in the presence of Na^+ produces a new fluorescent species with maximal emission at 384 nm upon excitation at 340 nm. Figure 5C shows the time course of formation of the fluorescent product from L-serine in the presence of Na^+. Addition of indole or β-mercaptoethanol decreases the rates of formation of the fluorescent species. No increase in fluorescence is observed when 100 mM pyruvate is substituted for L-serine under these conditions (data not shown). The rates of increase in fluorescence in the presence of L-serine and Na^+ or Cs^+ closely parallel the rates of disappearance of PLP under the same conditions (Figure 5D). The calculated rates of these changes are compiled in Table 4.

Effects of α Subunit and of L-Serine Concentrations on Rates of Formation of the Fluorescent Product. The rate of formation of the fluorescent product depends on the concentration of the α subunit (Figure 6A,B). Nonlinear least-squares analysis of the data in Figure 6B gives an apparent dissociation constant $K_d(\alpha\beta)$ of 9.35 ± 1.13 μM. The rate of formation of the fluorescent product also depends on the concentration of L-serine (Figure 6C,D). Analysis of the data in Figure 6D gives an apparent dissociation constant K_d(L-Ser) of 1.24 ± 0.09 mM.

Isolation and Identification of the Product of Inactivation of the S377D $\alpha_2\beta_2$ Complex with L-Serine. Several experiments outlined in Scheme 2 provide evidence that the inhibited form of the S377D $\alpha_2\beta_2$ complex has the structure E−I shown in Scheme 1 and demonstrate that the product X is released by NaOH. The S377D $\alpha_2\beta_2$ complex used for these experiments (E−I) was prepared by reaction for 4 h with L-serine in the presence of Na^+ under the conditions described in Figure 4A. The spectrum exhibited maximum absorbance at 340 nm and was not altered by overnight dialysis [Scheme 2 (A)]. Our finding that no chromophoric material was released by acid precipitation of the protein provides evidence that E−I contains a PLP derivative that is covalently attached in an acid stable linkage [Scheme 2 (B)]. Addition of 5 M guanidine hydrochloride to dissolve the acid protein precipitate followed by addition of NaOH to pH 12 yielded a solution with maximum absorbance at 424 nm that can be ascribed to the release of X by NaOH [Scheme 2 (B)]. Direct treatment of E−I with NaOH at pH 12 also gave a peak with maximum absorbance at 424 nm, indicating release of X from the protein [Scheme 2 (C)]. The stoichiometry of X formation relative to initial PLP content was calculated to be 0.69 (Table 5). Similar results were obtained after reactions with L-serine or with β-chloro-L-alanine in the presence of Na^+, Cs^+, or DL-α-glycerol 3-phosphate (Table 5). Treatment of E−I with sodium borohydride followed by NaOH resulted in no increase in absorbance at 424 nm [Scheme 2 (D)], indicating that E−I was reduced by sodium borohydride to E−I′. HPLC analysis

Table 3: Values of Molar Circular Dichroism ($\Delta\epsilon$), Molar Absorptivity (ϵ), and Dissymmetry Factors ($\Delta\epsilon/\epsilon$) of PLP Bound to Wild-Type and Mutant Forms of Tryptophan Synthase as Internal Aldimines (without Ser) or External Aldimines (with Ser)[a]

enzyme	cation	ϵ without Ser (M^{-1} cm^{-1})	ϵ with Ser (M^{-1} cm^{-1})	$\Delta\epsilon$ without Ser (M^{-1} cm^{-1})	$\Delta\epsilon$ with Ser (M^{-1} cm^{-1})	$\Delta\epsilon/\epsilon$ without Ser	$\Delta\epsilon/\epsilon$ with Ser
WT β_2	Na^+	4500	8090	11.5	10.6	0.0026	0.0013
	Cs^+	4460	5250	12.4	7.88	0.0028	0.0015
WT $\alpha_2\beta_2$	Na^+	5940	2880	17.9	3.64	0.0030	NA
	Cs^+	5950	1450	20.0	0.30	0.0034	NA
S377A $\alpha_2\beta_2$	Na^+	5230	8980	12.1	7.58	0.0023	0.0008
	Cs^+	5320	2770	10.6	2.12	0.0020	NA
S377D $\alpha_2\beta_2$	Na^+	2690	520	6.06	0.15	0.0023	NA

[a] Molar ellipticities (θ) and molar absorptivities (ϵ) were determined (see Experimental Procedures and Figures 3 and 4) in the absence of L-serine for the wild-type (WT) β_2 subunit and $\alpha_2\beta_2$ complex at 412 nm, for the S377D $\alpha_2\beta_2$ complex at 414 nm, for the S377A $\alpha_2\beta_2$ complex at 416 nm, and for enzymes in the presence of L-serine at 424 nm. Molar circular dichroism values were calculated using the relationship $\Delta\epsilon = \theta/3298$. NA (not applicable) is given when E-Ser (see Scheme 1) is not the predominant species formed (see the text).

Table 4: Inactivation Parameters for the S377D $\alpha_2\beta_2$ Complexes[a]

		reaction rate (min^{-1})				
rate monitored	Figure	Na^+	Cs^+	Na^+/GP	Na^+/2-ME	Na^+/Ind
decrease in A_{416nm}[b]	4A−C	0.143 ± 0.003	0.107 ± 0.003	0.539 ± 0.013	0.024 ± 0.001	0.102 ± 0.002
increase in A_{340nm}[c]	4A−C	0.156 ± 0.002	0.083 ± 0.001	0.541 ± 0.014	0.023 ± 0.001	0.096 ± 0.001
decrease in A_{498nm}[d]	4A−C	0.094 ± 0.004	0.089 ± 0.002	0.422 ± 0.012	0.025 ± 0.001	0.152 ± 0.003
increase in fluorescence	5C,D	0.153 ± 0.004	0.060 ± 0.002	0.615 ± 0.044	0.019 ± 0.001	0.038 ± 0.001
decrease in pyruvate formation rate	5A	0.113 ± 0.016	0.046 ± 0.009	0.426 ± 0.061		
decrease in PLP level	5D	0.153 ± 0.011	0.059 ± 0.012			
increase in X level (HPLC)	7	0.150 ± 0.010	0.061 ± 0.014			
average k_{inact}		0.137	0.072	0.509	0.023	0.097
k_{cat}	5A	0.137	0.216	0.129		
k_{cat}/k_{inact}		1.0	3.0	0.25		

[a] Rates of k_{cat} and k_{inact} were determined from results of experiments depicted in the indicated figures as described in Experimental Procedures. [b] A value of 420 nm was used for β-mercaptoethanol (2-ME)/Na^+. [c] Values of 334 and 338 nm were used for 2-ME/Na^+ and indole (IND)/Na^+, respectively. [d] Values of 508 and 504 nm were used for 2-ME/Na^+ and indole (IND)/Na^+, respectively.

of aliquots of the enzyme that were treated with NaOH before (A) or 1 h after (B) reaction with L-serine (Figure 7) demonstrates the conversion of PLP to X and the absence of PMP formation. The spectra of X purified by HPLC after isolation from the enzyme and from the synthetic compound were identical at three pH values (data not shown) and were closely similar to those reported for the three ionic forms of X (*11*). Mass spectra were recorded as described in Experimental Procedures on HPLC-purified samples of the enzyme-derived X and of the synthetic compound. The values for M − 1 were 316.0224 for the enzyme-derived sample and 316.0228 for the synthetic compound; the theoretical value for $C_{11}H_{11}O_8NP$ was 316.0222.[3] The fragmentation patterns for the two samples were identical.

DISCUSSION

The PLP binding sites of the tryptophan synthase β subunit (Figure 1) and of other PLP enzymes in fold II of the known structure (Table 1) have the neutral hydroxyl of a serine residue interacting with N-1 of PLP. To investigate the importance of this interaction, we have changed Ser377 of the tryptophan synthase β subunit to Asp, the residue found at the equivalent position in enzymes in fold I, or to Glu or Ala. Although Asp and Glu are larger residues than Ser and are not isosteric, modeling of the S377D and S377E mutations indicates that either mutated residue could form a hydrogen bond with PLP N-1. This modeling shows only slightly unfavorable van der Waals interactions with nearby residues, which might be relieved by small changes in the conformation of the protein.[4] It seems unlikely that additional steric clashes would occur after formation of reaction intermediates, because the position of the PLP N-1 is not changed in reaction intermediates of known structure (*7*, *33*).

Our finding that the S377A $\alpha_2\beta_2$ complex has greatly reduced activity in the presence of Na^+ (Table 2), but much higher activity in the presence of Cs^+ (24% of that of the wild type), indicates that the hydrogen bond between Ser377 and PLP N-1 is not necessary for enzymatic activity or for stabilization of PLP in the correct orientation. It is possible that the activity of the S377A enzyme results from "chemical rescue" by a water molecule filling in for the missing serine hydroxyl.

Substitution of Cs^+ for Na^+ also changes the equilibrium distribution of intermediates in the reaction of the S377A $\alpha_2\beta_2$ complex with L-serine to that observed with the wild-type $\alpha_2\beta_2$ complex (Figure 3D). In contrast, the S377D $\alpha_2\beta_2$ complex is not significantly activated by Cs^+ or by DL-α-glycerol 3-phosphate. The occurrence of the partial repair of activity by Cs^+ ion (*17*, *34*, *35*), NH_4^+ ion (*20*, *36*, *37*), or DL-α-glycerol 3-phosphate (*38*−*40*) has been observed with several other mutant forms of tryptophan synthase. We have suggested that these ligands activate the mutant enzymes by preferentially binding to and stabilizing a higher-activity, closed conformer of the enzyme that is in equilibrium with a low-activity, open conformer of the enzyme (*12*, *34*, *41*). The repairable mutants investigated previously all exhibited rather high activities in reactions with β-chloro-

[4] We thank S. Rhee of NIDDK for this analysis.

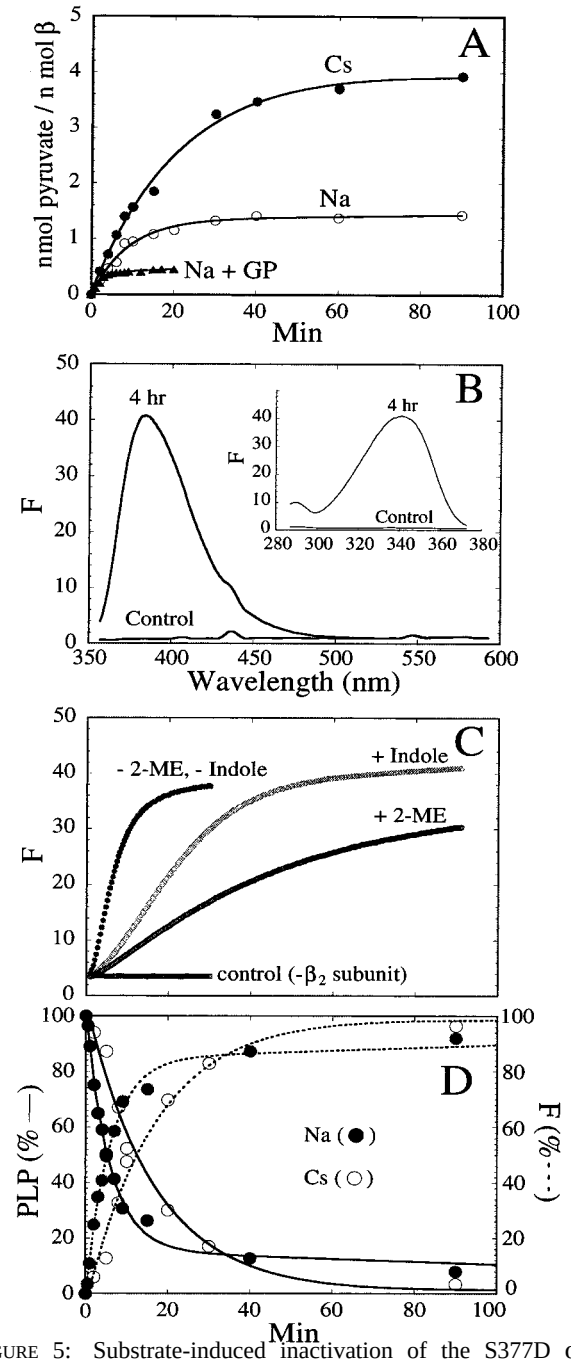


FIGURE 5: Substrate-induced inactivation of the S377D $\alpha_2\beta_2$ complex and formation of a fluorescent product of inactivation. (A) The time course of pyruvate formation was determined by analysis of the amount of pyruvate in aliquots of reaction mixtures identical to those described panels A (Na^+) and B (Cs^+) of Figure 4 and also in the presence of Na^+ and DL-α-glycerol 3-phosphate (10 mM) (see Experimental Procedures). (B) Fluorescence emission spectrum of the S377D $\alpha_2\beta_2$ complex [3 mg/mL β subunit and 10 mg/mL α subunit (5-fold molar excess) in 50 mM Na^+/bicine (pH 7.8) containing 0.2 M NaCl] were recorded with excitation at 340 nm at 25 °C before (control) and 4 h after addition of 50 mM L-serine. The inset shows the fluorescence excitation spectrum with emission at 384 nm under the same conditions. (C) Time course of fluorescence emission at 384 nm upon excitation at 340 nm after addition of 50 mM L-serine under the conditions described in the legend of in Figure 4A in the presence or absence of 50 mM β-mercaptoethanol (2-ME) or 0.2 mM indole. No increase in fluorescence is observed when 100 mM pyruvate is substituted for L-serine under these conditions (data not shown). (D) The time courses of changes in PLP content and of increases in fluorescence were determined under the conditions used in the experiments whose results are shown in Figure 4A,B.

L-alanine and had approximately equal activities in β replacement and β elimination reactions with this substrate. That is, the activities in reactions 4 and 6 (described in Table 2) were ~50% of that of the activity of the wild-type enzyme in reaction 1. In contrast, the activities of the S377A $\alpha_2\beta_2$ complex in these two reactions are 6 and 2% of that of the wild type, respectively (Table 2), whereas the activity in the presence of Cs^+ was higher (25% of that of the wild type). These results suggest that Cs^+ may repair the S377A $\alpha_2\beta_2$ complex by a mechanism different than that postulated for other mutant enzymes. A possible mechanism is discussed below.

Effects of Ser377 on PLP Orientation. Formation of a hydrogen bond between Ser377 and N-1 of PLP (Figure 1) may stabilize the PLP ring in the optimal orientation for catalysis. The absence of this interaction in the S377A mutant $\alpha_2\beta_2$ complex could result in an incorrect orientation of the PLP ring or allow the ring to be more mobile. Monovalent cations bind to a site in the β subunit about 8 Å from the phosphate of PLP (*42*). Exchange of Cs^+ for Na^+ at this site induces local and long-range changes in the structure of the wild-type $\alpha_2\beta_2$ complex (*42*). We can speculate that exchange of Cs^+ for Na^+ induces a conformational change that stabilizes a more correct or more rigid orientation of the PLP ring in the S377A mutant enzyme. Alternatively, the repair by Cs^+ may be related to binding of Cs^+ at a second site located in the interaction site between the two β subunits (*42*). Ligand-induced conformational changes have also been observed in the structure of a mutant $\alpha_2\beta_2$ complex (βK87T-Ser-GP) having DL-α-glycerol 3-phosphate bound at the α site and L-serine bound to the β site (*7*). Fluorescence and phosphorescence studies provide direct evidence that α subunit ligands make the structure of each subunit of the wild-type $\alpha_2\beta_2$ complex more rigid (*43*). Thus, DL-α-glycerol 3-phosphate may stabilize a more rigid, closed structure of the S377A $\alpha_2\beta_2$ complex and thereby partially overcome the destabilizing effects of the mutation.

Although free PLP is symmetrical and therefore an optically inactive chromophore, enzyme-bound PLP usually exhibits induced Cotton effects in the absorption bands of the coenzyme (*44*). The induced optical activity is conveniently measured by the dissymmetry factor, which can be expressed as the ratio ($\Delta\epsilon/\epsilon$) of the molar circular dichroism ($\Delta\epsilon$) to the molar absorptivity (ϵ) at a single wavelength. The positive ellipticity band exhibited by the internal aldimine of aspartate aminotransferase and the dissymmetry factor of this band are greatly reduced upon cleavage of the internal aldimine by reaction with substrates or substrate analogues (reviewed in ref *44*). These observations provided the first indication that conformational changes are associated with catalysis by aspartate aminotransferase. Later crystallographic studies revealed that formation of the external aldimine is coupled with tilting of the PLP ring by ~30° relative to its orientation in the internal aldimine, whereas the position of the phosphorus atom remains unchanged (*45*). Conformational changes in the active site of tryptophanase have also been revealed by CD studies (*46*, *47*).

The absorption and CD spectra of the wild-type and S377A $\alpha_2\beta_2$ enzymes (Figure 3 and Table 3) provide information on the interaction of PLP with the active site of the β subunit, although it is difficult to interpret these effects unequivocally (*29*, *30*). The wild-type $\alpha_2\beta_2$ complex exhibits two ellipticity

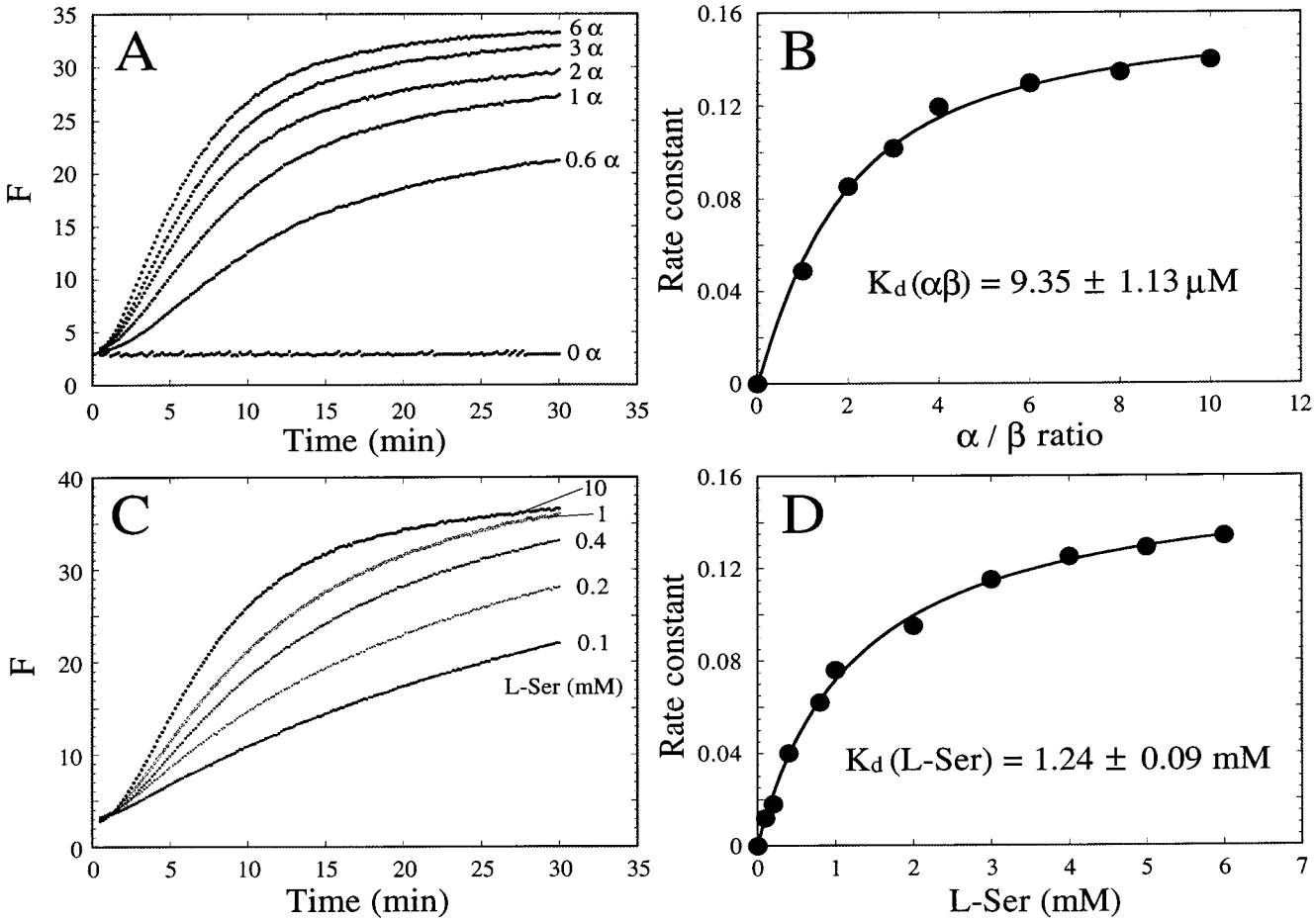
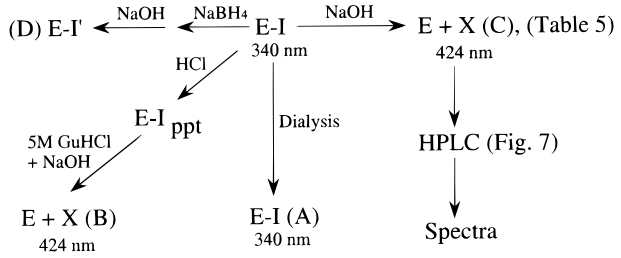


FIGURE 6: Effects of the α subunit and L-serine concentration on formation of the fluorescent product. (A) Time courses of fluorescence emission at 384 nm of S377D β_2 subunit [7 μM in 50 mM Na^+/bicine buffer (pH 7.8) containing 0.2 M NaCl and 50 mM L-serine] were determined at 25 °C in the presence of the indicated molar ratio of α subunit. (B) Plots of the rate of product formation vs the α/β ratio. Data analysis gave a value for $K_d(\alpha\beta)$ of 9.35 ± 1.13 μM. (C) Time courses of fluorescence emission at 384 nm of S377D β_2 subunit [7 μM in 50 mM Na^+/bicine buffer (pH 7.8) containing 0.2 M NaCl and 35 μM α subunit] were determined at 25 °C in the presence of the indicated concentration of L-serine. (D) Plots of the rate of product formation in the reaction with L-serine (see Experimental Procedures) vs L-serine concentration. Data analysis gave a value for K_d(L-Ser) of 1.24 ± 0.09 mM.

Scheme 2: Isolation and Identification of the Product of Inactivation of the S377D $\alpha_2\beta_2$ Complex with L-Serine



bands (Figure 3E) at 336 and 412 nm. The wild-type β_2 subunit and $\alpha_2\beta_2$ complex have absorptivity peaks at corresponding wavelengths (Figure 3A,C), which have been attributed to enolimine and ketoenamine tautomers of the internal aldimine, respectively (*48*, *49*).

In the absence of L-serine, exchange of Cs^+ for Na^+ has little effect on absorptivity at 412 nm but increases the ellipticity at 412 nm and the dissymmetry factor ($\Delta\epsilon/\epsilon$) of both the wild-type β_2 subunit and the $\alpha_2\beta_2$ complex 1.1−1.2-fold (i.e., 0.0028/0.0026 and 0.0034/0.0030 in Table 3). These results imply that the two monovalent cations have different effects on the environment of the bound PLP. Exchange of Cs^+ for Na^+ also induces local and long-range changes in the three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex (*42*). The dissymmetry factor ($\Delta\epsilon/\epsilon$) for the wild-type $\alpha_2\beta_2$ complex is 1.2-fold greater than that of the wild-type β_2 subunit in the presence of either Na^+ or Cs^+ (i.e., 0.0030/0.0026 or 0.0034/0.0028 in Table 3), suggesting that the orientation of PLP at the active site of the β subunit is more asymmetric or more rigid in the

Table 5: PLP Content and Stoichiometry of Inactivation of the S377D $\alpha_2\beta_2$ Complex[a]

substrate	addition	PLP/β	X/PLP
L-serine	Na^+	0.85	0.69
L-serine	Cs^+	0.83	0.69
L-serine	Na^+/GP	0.85	0.68
β-Cl-Ala	Na^+	0.85	0.65
β-Cl-Ala	Cs^+	0.83	0.62
β-Cl-Ala	Na^+/GP	0.85	0.60

[a] The S377D $\alpha_2\beta_2$ complex was incubated for 4 h at 25 °C and pH 7.8 with 50 mM L-serine or 10 mM β-chloro-L-alanine (β-Cl-Ala) as described in the legend of Figure 4 in the presence of the indicated additions: 0.2 M NaCl or CsCl and 10 mM DL-α-glycerol 3-phosphate (GP). The enzyme was adjusted to pH 11.5 with NaOH after 4 h, and the concentration of X was determined from the absorbance at 424 nm using the relationship $E_{424nm} = 8\ mM^{-1}\ cm^{-1}$ (*10*, *11*). The PLP content before reaction was determined by the KCN method (26) or the phenylhydrazine method (72) as described in Experimental Procedures.

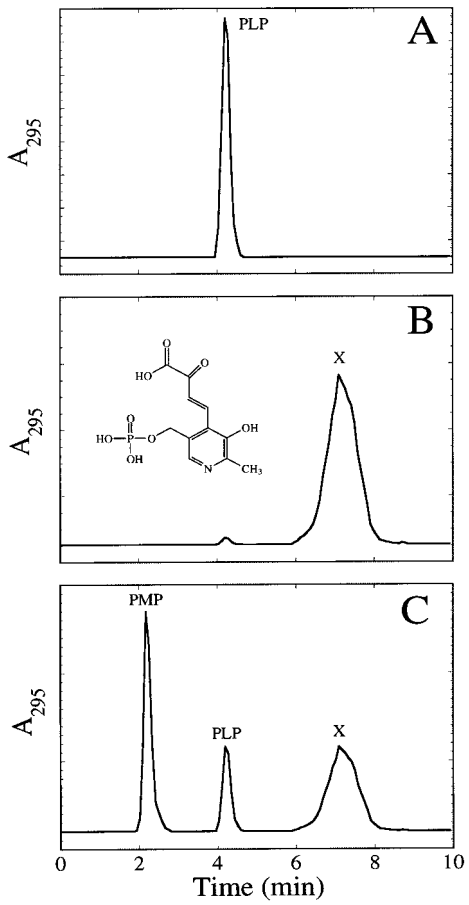


FIGURE 7: HPLC analysis of PLP and its derivatives from the S377D $\alpha_2\beta_2$ complex and its inactivation product. HPLC analysis of aliquots of the S377D $\alpha_2\beta_2$ complex that were treated with NaOH before (A) or 1 h after reaction with L-serine (B). Standards of PLP, PMP, and X are shown in panel C. The synthetic X and X isolated from the enzyme elute identically.

presence of the α subunit. Other evidence for reduced flexibility of the β subunit in the presence of the α subunit is provided by measurements of the lifetimes of fluorescence decay and of phosphorescence decay of Trp177 in the $\alpha_2\beta_2$ complex (*50*). The dissymmetry factor ($\Delta\epsilon/\epsilon$) for the S377A $\alpha_2\beta_2$ complex in the presence of Na^+ or Cs^+ is 77 or 60% of that of the wild-type $\alpha_2\beta_2$ complex, respectively (i.e., 0.0023/0.0030 or 0.0020/0.0034 in Table 3). These results suggest that the absence of hydrogen bonding interaction between Ser377 and N-1 of PLP reduces the asymmetry or rigidity of the PLP internal aldimine. The molar absorptivity and the molar circular dichroism of the S377D $\alpha_2\beta_2$ complex are both low compared to those of the wild-type and S377A $\alpha_2\beta_2$ complexes for unknown reasons. However, the dissymmetry factors for the two mutant $\alpha_2\beta_2$ complexes are similar, indicating that PLP has similar orientations in both mutant enzymes.

The reactions of L-serine with the wild-type β_2 subunit and S377A $\alpha_2\beta_2$ complex result in the predominant accumulation of E-Ser. The dissymmetry factor ($\Delta\epsilon/\epsilon$) for E-Ser is half that of the internal aldimine (E) for the wild-type β_2 subunit in the presence of Na^+ (i.e., 0.0013/0.0026 in Table 3), suggesting that the environment of E-Ser is less asymmetric or rigid than that of the internal aldimine. Recent crystallographic studies of the tryptophan synthase $\alpha_2\beta_2$ complex revealed that formation of the E-Ser intermediate by a mutant $\alpha_2\beta_2$ complex (βK87T) is coupled with tilting of the PLP ring by ~10° relative to its orientation in the internal aldimine (*7*). The dissymmetry factor ($\Delta\epsilon/\epsilon$) for E-Ser in the presence of Na^+ is about $^1/_3$ of that of the internal aldimine (E) for the S377A $\alpha_2\beta_2$ complex (i.e., 0.0008/0.0023 in Table 3), indicating that the absence of the hydrogen bonding interaction between Ser377 and N-1 of PLP reduces the asymmetric orientation of PLP in the external aldimine.

Mechanism-Based Inactivation of the S377D $\alpha_2\beta_2$ Complex. Our results support a mechanism of inactivation shown in Scheme 1 in which an enzyme substrate intermediate (E-AA in Scheme 1) partitions between formation of products (pyruvate and NH_3) with a rate k_{cat} and formation of a covalently inactivated enzyme (E−I) with a rate k_{inact}. This mechanism was first elucidated by Metzler et al. (*10*, *11*) for the reactions of aspartate aminotransferase and glutamate decarboxylase with a quasisubstrate, L-serine-*O*-sulfate. We demonstrated this type of inactivation in the reactions of several mutant tryptophan synthase $\alpha_2\beta_2$ complexes and of the wild-type β_2 subunit with β-chloro-L-alanine (*12*, *13*). Inactivation results from displacement of aminoacrylate from the key E-AA intermediate followed by nucleophilic attack by the β-carbon of aminoacrylate on the internal aldimine which forms a covalent adduct (E−I, Scheme 1). The aminoacrylate formed need not leave the active site but may simply rotate around the bond to the carboxylate group, the latter remaining bound in the active site (*11*). Alkali treatment of E−I yields the PLP derivative, X.

Our new results are of special interest because inactivation takes place with the natural substrate, L-serine, and because inactivation occurs with a very low partition ratio (k_{cat}/k_{inact} = 0.25−3 in Table 4). The value of k_{cat} is determined from the initial rate of pyruvate formation (Figure 5A). The value of k_{inact} (Table 4) is the average of rates determined for changes in absorbance at certain wavelengths in Figure 4, the increase in fluorescence of E−I (Figure 5C), the increase in the amount of product X determined by HPLC (Figure 7), the decrease in PLP content (Figure 5D), and the decrease in the extent of pyruvate formation (Figure 5A). The reasonable agreement between these various rates indicates that the various parameters measured all result from the same inactivation event. Although the ratio of X/PLP obtained in Table 5 ranges from 0.6 to 0.69 under different conditions and is lower than the expected 1.0, the close parallel between the rates of PLP disappearance and increase in fluorescence in Figure 5D provides evidence that E−I is indeed the primary product.

Since thiols readily react with $\alpha\beta$-unsaturated compounds (*51*, *52*), we added β-mercaptoethanol to the mixture for the reaction of S377D with L-serine to determine whether this thiol could trap free aminoacrylate and thereby decrease the rate of inactivation (Figure 5C). Although the results show that addition of either β-mercaptoethanol or indole does decrease the rate of increase in fluorescence (Figure 5C and Table 4), absorption spectra recorded under the same conditions reveal the transient formation of quinonoids with maximum absorbance at 504 nm with indole (Figure 4C) and at 510 nm with β-mercaptoethanol as observed previously (*8*). These results indicate that indole or β-mercaptoethanol reacts with E-AA to form the quinonoid of L-tryptophan (E−Q2 in Scheme 1) or of *S*-(hydroxyethyl)-L-cysteine (*8*). The quinonoid of L-tryptophan is also formed

from L-tryptophan by the reverse reaction (*8*). We conclude that β-mercaptoethanol acts as a cosubstrate, not as a chemical trapping agent.

The ratio of the turnover number to the rate of inactivation (k_{cat}/k_{inact}) is the partition ratio, that is, the catalytic turnovers (conversion of substrate to product) per inactivation event (conversion of E to E−I). The experimental determination of the partition ratio for a mechanism-based inactivator is useful both as a clue to the chemical nature of the partitioning and as a key indicator of potential in vivo specificity and utility (*53*). The low partition ratios determined here (0.25−3 in Table 4) result at least in part from the very low activity of the S377D $\alpha_2\beta_2$ complex (Tables 2 and 4 and Figure 5A). Our prior finding that the S377D $\alpha_2\beta_2$ complex accumulates a quinonoid intermediate in the reaction of L-serine in the presence of Cs^+ but not in the presence of Na^+ indicates that Cs^+ stabilizes a somewhat different conformation of the S377D $\alpha_2\beta_2$ complex (*8*). This conformation may be more active or may undergo inactivation less readily than the conformation that is stabilized by Na^+. Different cations affect the extent of inactivation of the wild-type β_2 subunit by β-chloro-L-alanine (*13*). DL-α-Glycerol 3-phosphate may reduce the rate of formation of pyruvate and NH_3 by stabilizing the closed conformation that disfavors the β elimination reaction (*12*, *34*).

We now ask how the S377D mutation results in inactivation with a low partition ratio. One possibility is that the mutation reduces k_{cat} by hindering one of the steps in the conversion of aminoacrylate to pyruvate and NH_3 (Scheme 1): either the intramolecular transfer of the hydrogen from C-α to C-β (*54*) or the subsequent addition of OH^-. Another possibility is that a changed conformation in the S377D $\alpha_2\beta_2$ complex, as evidenced by the decreased dissymmetry factor of E-Ser (Table 3), promotes release of aminoacrylate from E-AA. It seems likely that the S377D mutation leads to mechanism-based inactivation by both reducing k_{cat} and increasing the rate of release of aminoacrylate.

Comparisons of Different Modes of Inactivation of PLP Enzymes. PLP enzymes have been prime targets for the design and testing of inhibitors, which operate by several different types of mechanism-based inhibition (*53*). The first type of inhibition results in covalent modification of a protein residue by a metabolite of the inhibitor. Crystallographic proof for this sort of inhibition is provided by the structure of trifluoroalanine-inactivated cystathionine β-lyase (*55*). Solution studies provide evidence that halovinyl glycines inactivate tryptophan synthase (*56*) and alanine racemase (*57*) by a covalent mechanism. A second type of inhibition results in the formation of a stable PLP derivative without covalent modification of the enzyme. Recent investigations include those of the inactivation of D-amino acid aminotransferase by D-cycloserine (*58*), γ-aminobutyric acid aminotransferase by L-cycloserine (*59*), human ornithine aminotransferase by L-canaline and gabaculine (*60*), cystathionine β-lyase by L-aminoethoxyvinylglycine (*61*), and D-amino acid aminotransferase by its natural substrate, D-alanine (*62*). In the third type of inactivation, PLP is alkylated by the three-carbon suicide substrate (Scheme 1) (*10*, *11*). In this case, the PLP remains covalently linked to the protein via the lysyl residue that forms the internal aldimine in the unmodified enzyme. The identification of the product X released by alkali in this report and in refs *10*−*13* provides strong evidence for the proposed mechanism. Unsettled points include whether the attack is by aminoacrylate, as shown, or by pyruvate, and whether the nitrogen from aminoacrylate is present in E−I, as shown. Our results (see the text and the legend of Figure 5C) show that the enzyme is not inactivated by added pyruvate. It would be desirable to have additional support for the identity of E−I and the borohydride reduction product E−I′ (see Scheme 2) from crystallographic or mass spectroscopic data. Unfortunately, we have been unable to obtain suitable crystals of the S377D $\alpha_2\beta_2$ complex. Other mutant forms of the tryptophan synthase $\alpha_2\beta_2$ complex (βE109A, βD305N, βF306A, and βE350A), which also undergo inactivation by this mechanism (*12*), may prove more suitable for crystallography.

The S377D Mutation Weakens Subunit Interaction. Why do the S377D and S377E mutations dramatically reduce the strength of interaction between the α and β subunits whereas the S377A mutation does not? The values of $K_d(\alpha\beta)$ obtained for the S377D and S377E $\alpha_2\beta_2$ complexes from measurements of activity in the cleavage of indole 3-glycerol phosphate (α reaction) (15.6 and 30 μM, respectively, in the inset in Figure 2) are close to those obtained (7 and 32 μM, respectively) from measurements of the effect of α subunit concentration on the rate of formation of a tryptophan quinonoid (*8*). The $K_d(\alpha\beta)$ values of the two mutant enzymes are 250−500 times greater than the $K_d(\alpha\beta)$ (0.062 μM) of the wild-type holo β_2 subunit and 5−9 times greater than the value of 3.4 μM[5] of the apo β_2 subunit (*63*). The $K_d(\alpha\beta)$ value for the wild-type holo β_2 subunit obtained here (0.062 μM) is in close agreement with the value of 0.07 μM[5] from ref *63*.

Three-dimensional structures of the tryptophan synthase $\alpha_2\beta_2$ complex reveal that PLP is located at the interface between two structural domains of the β subunit and interacts with residues from each domain (*3*, *7*). The carbonyl group of PLP forms an internal aldimine with Lys87 in the N domain, whereas the phosphate group of the coenzyme forms hydrogen bonds with several residues in the C domain. The N-1 of PLP also forms a hydrogen bond with Ser377 in the C domain (Figure 1). Thus, PLP forms a bridge between the two domains. Our proposal (*63*) that this interdomain bridge stabilizes the interaction of the β subunit with the α subunit was supported by the results of investigations of a mutant β subunit (K87T) that is unable to form an internal aldimine with Lys87. The observation that the S377A mutation does not weaken interaction between the α and β subunits (Figure 2 and inset) indicates that hydrogen bonding between the N-1 of PLP and Ser377 is not needed to stabilize subunit interaction. Thus, the introduction of a negatively charged group at position 377 must destabilize subunit interaction. The finding that the absorption spectra of the S377D β_2 subunit and $\alpha_2\beta_2$ complex undergo pH-dependent changes provided evidence that the mutant enzymes do form a hydrogen bond/salt bridge between the N-1 proton of PLP and Asp377 that alters cofactor chemistry (*8*). This salt bridge interaction may change the orientation of the PLP and result in a conformation of the β subunit that does not interact well with the α subunit. However, comparisons of the dissymmetry factors ($\Delta\epsilon/\epsilon$) (Table 3) discussed above

[5] Values of $K_d(\alpha\beta)$ were recalculated from the original data to fit the model in eq 6.

do not support a difference between the orientations of PLP in the two mutant enzymes. Consequently, a negatively charged residue at position 377 may have other effects on the conformation of the β subunit that weaken interaction with the α subunit. This change in the conformation as well as the altered cofactor chemistry may also promote mechanism-based inactivation by reducing k_{cat} and promoting release of aminoacrylate.

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