Guanidine Hydrochloride Exerts Dual Effects on the Tryptophan Synthase $\alpha_2\beta_2$ Complex as a Cation Activator and as a Modulator of the Active Site Conformation‡

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Received February 9, 1999; Revised Manuscript Received April 21, 1999

ABSTRACT: To characterize the conformational transitions that regulate the activity and specificity of the tryptophan synthase $\alpha_2\beta_2$ complex, we have determined some effects of low concentrations of guanidine hydrochloride (GuHCl) and of urea on functional properties. We report the novel finding that GuHCl at low concentrations (0.02−0.08 M) is a cation activator of the tryptophan synthase $\alpha_2\beta_2$ complex. Molecular modeling studies show that GuH^+ could bind at a previously identified cation binding site in the tryptophan synthase β subunit. Addition of increasing concentrations of GuHCl has strikingly different effects on the rates of different reactions with L-serine or β-chloro-L-alanine in the presence or absence of indole. Spectroscopic studies demonstrate that GuHCl alters the equilibrium distribution of pyridoxal 5′-phosphate intermediates formed in reactions at the active site of the β subunit. Data analysis shows that GuHCl binds preferentially with the conformer of the enzyme that predominates when the aldimine of L-serine is formed and shifts the equilibrium in favor of this conformer. These results provide evidence that GuHCl exerts dual effects on tryptophan synthase as a cation, stimulating activity, and as a chaotropic agent, altering the distribution of conformational states that exhibit different reaction specificities. Our finding that the nonionic urea stabilizes the aldimine of L-serine in the presence, but not in the absence, of NaCl shows that cation binding plays an important role in the conformational transitions that regulate activity and the transmission of allosteric signals between the α and β sites.

Enzymes must have flexible active sites that undergo rapid conformational transitions to catalyze sequential chemical transformations and to accommodate different chemical intermediates. One approach to investigating the role of active site flexibility in enzyme catalysis is to determine the effects of chaotropic agents, such as urea and guanidine hydrochloride, that may stabilize alternative enzyme conformational states. Many enzymes are either activated or inactivated by concentrations of GuHCl or urea that are lower than those needed to cause demonstrable structural changes (for reviews, see refs *1*−*3*).

The tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.1.2.20) is a useful system for investigating the relationships between protein structure and function (for reviews, see refs *4*−*7*). The three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* (*8*) revealed that the α and β subunits[1] are arranged in a nearly linear $\alpha\beta\beta\alpha$ order. The active sites of the α and β subunits are ∼25 Å apart and are connected by a largely hydrophobic intramolecular tunnel. The β subunit contains one pyridoxal 5′-phosphate (PLP)[2] coenzyme at each active site that provides a spectroscopic probe for detecting enzyme−substrate intermediates and monitoring conformational changes at the active site of the β subunit.

The individual tryptophan synthase α and β_2 subunits catalyze the α reaction (reaction 1) and the β reaction (reaction 2), respectively. Subunit association stimulates the rates of these reactions 10−100-fold.

$$\text{indole-3-glycerol phosphate} \leftrightarrow \text{indole} + \text{D-glyceraldehyde 3-phosphate} \quad (1)$$

$$\text{L-serine} + \text{indole} \rightarrow \text{L-tryptophan} + H_2O \quad (2)$$

The $\alpha_2\beta_2$ complex and β_2 subunit also catalyze an analogous β-replacement reaction with β-Cl-Ala and indole (reaction 3) and β-elimination reactions with L-serine and β-Cl-Ala (reactions 4 and 5, respectively) (*9*, *10*).

$$\beta\text{-Cl-Ala} + \text{indole} \rightarrow \text{L-tryptophan} + \text{HCl} \quad (3)$$

$$\text{L-serine} \rightarrow \text{pyruvate} + NH_3 \quad (4)$$

$$\beta\text{-Cl-Ala} \rightarrow \text{pyruvate} + NH_3 + \text{HCl} \quad (5)$$

Reactions at the β site proceed through a series of PLP−substrate intermediates that have characteristic spectroscopic properties (Scheme 1) (*11*−*14*). The reaction of the $\alpha_2\beta_2$

‡ The coordinates of the wild type tryptophan synthase $\alpha_2\beta_2$ complex showing the Na^+ binding site are available in the Brookhaven Protein Data Bank under file name 1bks.

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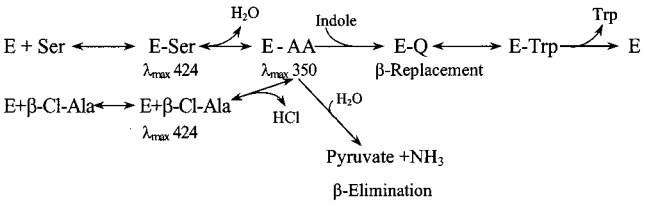
[1] The term β_2 subunit is used for the isolated enzyme in solution; β subunit is used for the enzyme in the $\alpha_2\beta_2$ complex, for a specific residue in the β subunit, or for the dissociation of the α and β subunits.

10.1021/bi990307e
Published on Web 05/26/1999

Table 1: Conformational States of the β_2 Subunit and $\alpha_2\beta_2$ Complex

	open	closed	ref
primary intermediate	E−Ser	E−AA	*16, 18*
activity in reaction 2 (Ser + Ind → Trp)	low	high	*10*
activity in reaction 3 (β-Cl-Ala + Ind → Trp)	high	high	*10*
activity in reaction 4 (β−Cl-Ala → Pyr)	high	low	*10*
stabilizing factors	lower temperature	higher temperature	*18*
	higher pH	lower pH	*18*
		α subunit and α subunit ligands	*10, 18*
	Na^+ and K^+	Cs^+ and NH_4^+	*19−21*
	mutations		*10*
	solvents		*22, 23*
	GuHCl and urea		this work

Scheme 1: Intermediates in Reactions of L-Ser and β-Cl-Ala



complex with L-serine yields the PLP aldimine of L-serine (E−Ser) which eliminates H_2O to form the aldimine of aminoacrylate (E−AA). In the absence of indole, an equilibrium mixture of E−Ser and E−AA accumulates. E−Ser absorbs maximally at 420 nm and exhibits an intense fluorescence at 520 nm upon excitation at 420 nm. E−AA absorbs maximally at 350 nm and is not fluorescent.

It has been proposed that allosteric signals derived from reactions at the two active sites switch the $\alpha_2\beta_2$ complex from an open, low-activity[3] state to a closed, high-activity state (Table 1). E−AA has been identified as the key intermediate that triggers the activation of indole-3-glycerol phosphate cleavage at the α site (*15−17*). Thus, formation of E−AA is associated with conversion of the $\alpha_2\beta_2$ complex from the open to the closed conformation (Table 1). The equilibrium distribution of the E−Ser and E−AA intermediates is altered by a number of factors, including pH, temperature, and α subunit ligands (*18*) and different cations (*19−21*) (Table 1).

Association of the β_2 subunit with the α subunit increases the activity in the β-replacement of L-serine and indole (reaction 2) and decreases the activities in β-elimination reactions with L-serine and β-Cl-Ala (reactions 4 and 5) (*10*). Thus, association with the α subunit alters the substrate and reaction specificity of the β subunit. Several $\alpha_2\beta_2$ complexes having single amino acid replacements in the β subunit have substrate and reaction specificities similar to those of the β subunit (*10*). This finding led to the suggestion that the wild type β_2 subunit and these mutant $\alpha_2\beta_2$ complexes exist in an open conformation, whereas the wild type $\alpha_2\beta_2$ complex exists in a closed conformation (*10*) (Table 1). We have postulated that the open conformation results in a poor alignment of the weak hydroxyl leaving group of L-serine for protonation and β-elimination. Enzymes in the open conformation may have much higher activities with β-Cl-Ala because this substrate has a strong chloride leaving group that does not require protonation. The greater solvent accessibility of the open form in the mutant β subunits may promote hydrolysis of the E−AA intermediate in the β-elimination reactions (reactions 4 and 5). E−Ser is the predominant intermediate that accumulates in the reactions of L-serine with the separate β_2 subunit and the mutant $\alpha_2\beta_2$ complexes.

We have reported previously (*22*, *23*) that solvents, including ethanol and β-mercaptoethanol, stabilize an alternative conformation of the wild type $\alpha_2\beta_2$ complex that exhibits characteristics that are attributed to the open conformation (Table 1). The altered characteristics (i.e., altered substrate and reaction specificities and spectroscopic properties) are similar to those of the separate β_2 subunit in aqueous solution.

In this paper, we provide evidence that GuHCl exerts dual effects on tryptophan synthase as a cation and as a chaotropic agent. Our results also show that cation binding plays an important role in the conformational transitions that regulate activity and allosteric communication.

MATERIALS AND METHODS

Chemicals and Buffers. L-Serine, β-chloro-L-alanine (hydrochloride), DL-α-glycerophosphate disodium salt (GP), pyridoxal 5′-phosphate (PLP), Bis-Tris propane, and tetraethylammonium chloride were from Sigma. GuHCl was from Gibco-BRL, and urea was from Mallinckrodt. All experiments were carried out in 50 mM Bis-Tris propane buffer containing 2 mM dithiothreitol. The pH of this buffer was adjusted to 7.8 by addition of HCl. Sodium-free GP was prepared by repeated passage of the solution of the disodium salt over an ion exchange column (DW-50 in the H^+ form, Sigma) (*19*); the pH of the final eluate was adjusted to 7.8 with Bis-Tris propane.

Bacterial Strains, Plasmids, and Enzymes. Plasmids pEBA-10 and pEBA-8 (*24*) were used to express the wild type tryptophan synthase $\alpha_2\beta_2$ complex and β_2 subunit from *S. typhimurium*, respectively, in *Escherichia coli* CB 149 (*25*), which lacks the trp operon. The enzymes were purified to

[2] Abbreviations: GuHCl, guanidine hydrochloride; PLP, pyridoxal phosphate; GP, DL-α-glycerol 3-phosphate; Bis-Tris propane, 1,3-bis-[tris(hydroxymethyl)methylamino]propane; E−Ser, external aldimine of L-serine; E−AA, external aldimine of aminoacrylate; E−Trp, external aldimine of L-tryptophan; E−Q, quinonoid intermediate; β-Cl-Ala, β-chloro-L-alanine; TEAC, tetraethylammonium chloride.

[3] Activity in reaction 2 (L-serine + indole → L-tryptophan + H_2O).

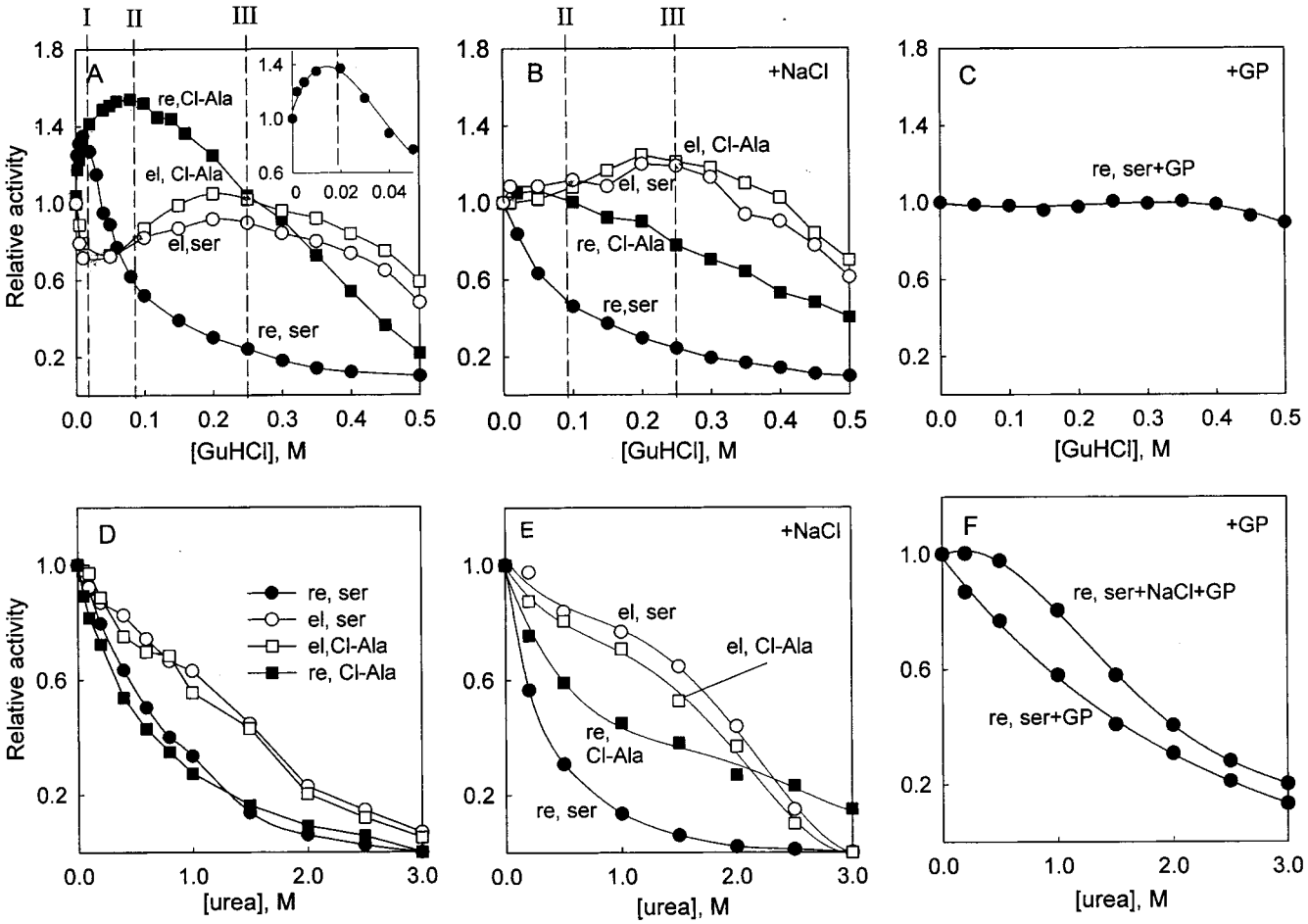


FIGURE 1: Effects of GuHCl (A−C) and urea (D−F) concentration on the relative activities of the $\alpha_2\beta_2$ complex. Activities in reactions 2−5 were measured in the absence of NaCl (A and C), in the presence of 0.1 M NaCl (B and E), and in the presence of 50 mM GP in the absence of NaCl (C) or in the presence or absence of 0.1 M NaCl (F) over a range of urea and GuHCl concentrations and were normalized to the activity in the absence of urea or GuHCl (relative activity of 1). The inset in A shows partial data for the β-replacement reaction of L-serine with indole on an expanded scale. Symbols I−III and the vertical dashed lines at 0.02, 0.08, and 0.25 M GuHCl denote concentrations of GuHCl that give maximal activity in reactions 2, 3, and 4 or 5, respectively. I is not shown in panel B because GuHCl does not stimulate activity in reaction 2 in the presence of NaCl. Different conformational states of the enzyme (I−III) may accumulate maximally at these three GuHCl concentrations (see the Discussion). Table 2 gives the specific activities for each reaction whose data are shown in panels A and B in the presence and absence of 0.02, 0.08, and 0.25 M GuHCl. The specific activities in the absence of urea or GuHCl and the abbreviations used are as follows: β-replacement reaction 2 with L-serine and indole (re, ser), 122 units/mg (without additives), 394 units/mg (with 0.1 M NaCl), 30 units/mg (with 50 mM GP), and 97 units/mg (with 50 mM GP and 0.1 M NaCl); β-replacement reaction with β-Cl-Ala (re, Cl-Ala), 33 and 105 units/mg in the presence and absence of 0.1 M NaCl; β-elimination reaction with L-serine (el, ser), 4 and 14 units/mg in the presence and absence of 0.1 M NaCl; β-elimination reaction with β-Cl-Ala (el, Cl-Ala), 7 and 15 units/mg in the presence and absence of 0.1 M NaCl.

homogeneity as described previously (*26*, *27*) and were dialyzed against monovalent cation-free Bis-Tris propane buffer (pH 7.8) before use. Protein concentrations were determined from the specific absorbance at 278 nm of the $\alpha_2\beta_2$ complex ($E^{1\%} = 6.0$) or of the β_2 subunit ($E^{1\%} = 6.5$) (*28*). All experiments and assays were carried out at 25 °C to avoid possible aggregation in the presence of GuHCl or urea at 37 °C.

Enzyme Assays. One unit of activity in any reaction is the formation of 0.1 μmol of product in 20 min at 25 °C. Replacement reactions with L-serine or β-Cl-Ala and indole were assessed by a direct spectrophotometric assay (*28*) containing modified components (50 mM L-serine or 40 mM β-Cl-Ala, respectively, and 0.4 mM indole). Activities in β-elimination reactions with L-serine and β-Cl-Ala were measured by spectrophotometric assays coupled with lactate dehydrogenase with modified components (*21*) [50 mM L-serine or β-Cl-Ala, 0.2 mM NADH, and excess lactate dehydrogenase, which was dialyzed against 50 mM Bis-Tris propane buffer (pH 7.8) to remove ammonium sulfate].

Spectroscopic and Analytical Methods. Absorption spectra were measured using a Hewlett-Packard 8452 diode array spectrophotometer. The fluorescence emission of the L-serine external aldimine was assessed at 510 nm with excitation at 420 nm using a PTI dual excitation spectrofluorimeter. All spectra were obtained within 30 s after mixing the reactants to avoid subsequent slow spectroscopic changes that might result from pyruvate formation or aggregation. Size-exclusion chromatography was carried out with a Superose 12 HR 10/30 column on a FPLC system (Pharmacia).

RESULTS

Effects of GuHCl on the Activity of the $\alpha_2\beta_2$ Complex. Panels A and B of Figure 1 show the effects of GuHCl concentration on the relative activities of the $\alpha_2\beta_2$ complex in β-replacement and β-elimination reactions 2−5 with

Table 2: Specific Activities and Absorbance at 424 nm of the $\alpha_2\beta_2$ Complex at Selected GuHCl Concentrations[a]

reaction	addition	figure	specific activity (units/mg of $\alpha_2\beta_2$) 0 M GuHCl	0.02 M GuHCl	0.08 M GuHCl	0.25 M GuHCl
(2) Ind + Ser → Trp + H_2O	none	1A	122	167	60	31
(2) Ind + Ser → Trp + H_2O	NaCl	1B	394		177	99
(3) Ind + Cl-Ala → Trp + HCl	none	1A	33	46	53	32
(3) Ind + Cl-Ala → Trp + HCl	NaCl	1B	105		103	84
(4) Ser → Pyr + NH_3	none	1A	4	2.8	3.2	3.8
(4) Ser → Pyr + NH_3	NaCl	1B	14		15	17
(5) Cl-Ala → Pyr + NH_3 + HCl	none	1A	7	5	5.8	7.7
(5) Cl-Ala → Pyr + NH_3 + HCl	NaCl	1B	15		16	18

reaction	addition	figure	absorbance at 424 nm 0 M GuHCl	0.02 M GuHCl	0.08 M GuHCl	0.25 M GuHCl
$\alpha_2\beta_2$ complex + Ser	none	2B	0.022	0.027	0.071	0.116
$\alpha_2\beta_2$ complex + Ser	NaCl	2B	0.038	0.043	0.071	0.107

[a] Values of specific activity and absorbance at 424 nm are taken from the experiments described in Figures 1A,B and 2B in the absence of GuHCl (0) and at 0.02, 0.08, and 0.25 M GuHCl as indicated by I−III, respectively, and by vertical dashed lines in Figure 1A,B. These concentrations of GuHCl give maximal activity in reactions 2, 3, and 4 or 5, respectively. Data are not given for 0.02 M GuHCl in the presence of NaCl, because reaction 2 is not activated under these conditions (Figure 1B). Different conformational states of the enzyme (I−III) may accumulate maximally at these three GuHCl concentrations (see the Discussion).

L-serine and β-Cl-Ala in the absence or presence of 0.1 M NaCl, respectively. The activities in β-replacement reactions with indole and either L-serine or β-Cl-Ala increase at low GuHCl concentrations in the absence of 0.1 M NaCl (Figure 1A). In the reaction with L-serine and indole, maximum activation (1.4-fold) occurs at 0.02 M GuHCl and the activity decreases sharply at higher GuHCl concentrations (see the inset of Figure 1A for an expanded view of these data). The symbol I at the top of Figure 1A and the vertical dashed line beneath denote data at 0.02 M GuHCl. The specific activities in reactions 2−5 at 0.02 M GuHCl are listed in Table 2. In the reaction with β-Cl-Ala and indole, maximum activation (1.6-fold) occurs at 0.08 M GuHCl, denoted by the symbol II and the vertical dashed line in Figure 1A. The decrease in activity at higher GuHCl concentrations is much less sharp than that in the reaction with L-serine and indole. The activities in β-elimination reactions with either L-serine or β-Cl-Ala decrease at GuHCl concentrations of <0.02 M, increase at GuHCl concentrations between 0.05 and 0.2 M, and then decrease at higher GuHCl concentrations. The symbol III at the top of Figure 1A and the vertical dashed line at 0.25 M GuHCl denote data at this concentration. Table 2 gives the specific activities in reactions 2−5 in the presence or absence of 0.02, 0.08, and 0.25 M GuHCl. These are the concentrations denoted by I−III that yield maximal activity in reaction 2, reaction 3, and reaction 4 or 5, respectively (see the footnote of Table 2 and the Discussion).

The specific activities of the $\alpha_2\beta_2$ complex in the β-replacement reactions with L-serine or β-Cl-Ala and indole are about 3.2-fold higher in the presence of 0.1 M NaCl than its absence (legend of Figure 1 and Table 2). Addition of low GuHCl concentrations in the presence of 0.1 M NaCl results in no further activation of the β-replacement reactions (Figure 1B). Activity decreases at higher concentrations of GuHCl are similar to those in the absence of NaCl (Figure 1A). The changes in the activities of the β-elimination reactions with increasing GuHCl concentrations in the presence of 0.1 M NaCl are similar to the changes in the absence of NaCl, except that inhibition at GuHCl concentrations of <0.02 M was not found. The noncoincidence of the effects of GuHCl concentration on the activities in different reactions shows that GuHCl alters both the substrate and reaction specificity of the $\alpha_2\beta_2$ complex. The symbol II at the top of Figure 1B denotes a concentration of GuHCl (0.08 M) that gives a significantly higher activity in reaction 3 than in reaction 2. Similarly, III denotes a concentration of GuHCl (0.25 M) that gives a significantly higher activity in reaction 4 or 5 than in reaction 3. Specific activities at these two concentrations are given in Table 2.

Effects of Urea on Activity. Although urea and GuHCl are believed to have similar modes of action (*29*), GuHCl is a monovalent salt that has both ionic and chaotropic effects (*30*−*32*), whereas urea has only chaotropic effects. Thus, urea is an ideal control agent for distinguishing between the ionic and chaotropic effects of GuHCl. The β-elimination activities of the $\alpha_2\beta_2$ complex decrease less sharply than β-replacement activities with increasing urea concentrations in the absence of NaCl (Figure 1D). The effects of urea concentration on the activities are somewhat altered in the presence of 0.1 M NaCl (Figure 1E).

Effect of GuHCl on Absorption and Fluorescence Spectra. The spectrum observed upon addition of L-serine to the $\alpha_2\beta_2$ complex in the absence of GuHCl (Figure 2A) exhibits an absorption maximum near 350 nm and a broad envelope of absorbance that extends out to 525 nm as described previously (*11*). The band at 350 nm has been attributed to E−AA (Scheme 1). Addition of increasing GuHCl concentrations in the presence of L-serine, but in the absence of NaCl, results in decreased absorbance at 350 nm and increased absorbance at 424 nm (Figure 2A,B). The presence of the isosbestic point in the spectra in Figure 2A from 0−0.3 M GuHCl indicates that GuHCl shifts the equilibrium distribution of the two species from E−AA to E−Ser. Similar results were obtained in the presence of 0.1 M NaCl (Figure 2B). Table 2 lists the absorbance at 424 nm from Figure 2B in the presence or absence of NaCl and in the presence or absence of 0.02, 0.08, and 0.25 M GuHCl. A comparison of the absorbance and activity data in Table 2 shows that only a small change

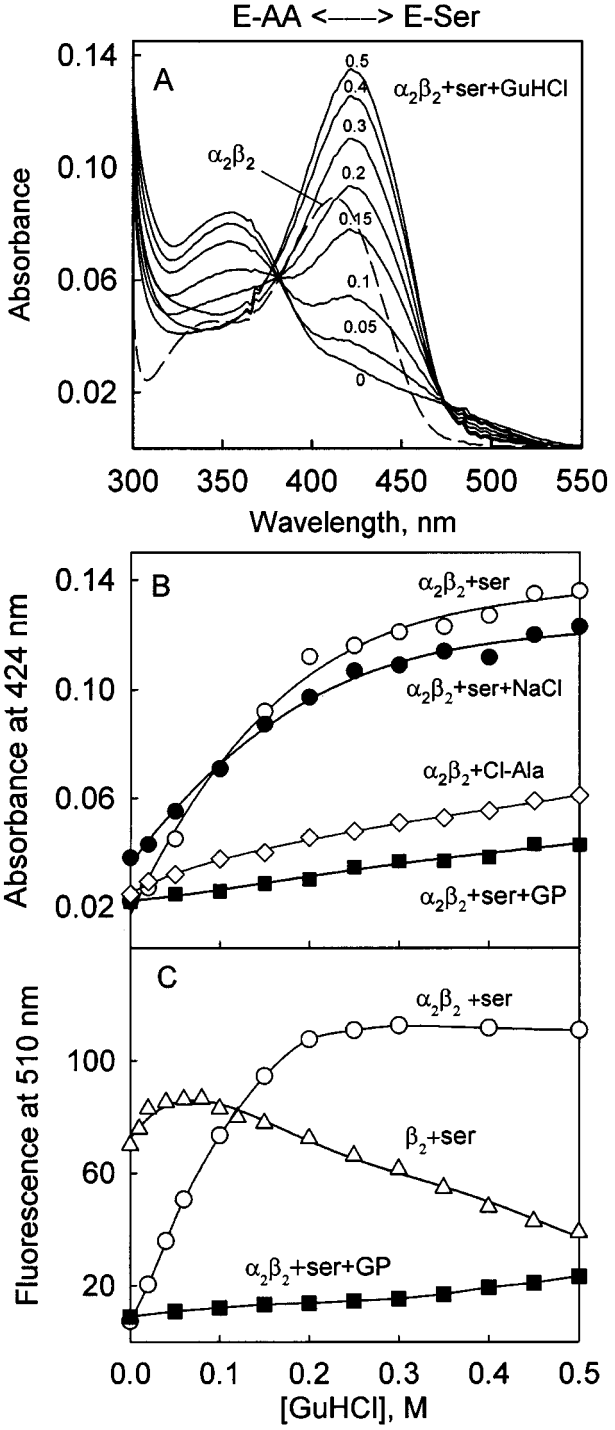


FIGURE 2: Effects of GuHCl concentration on the absorption and fluorescence properties of the $\alpha_2\beta_2$ complex. Absorption spectra in the presence of 50 mM L-serine (A), the absorbance at 424 nm (B), and the fluorescence intensity at 510 nm (C) were measured at 25 °C immediately after addition of 50 mM L-serine or 50 mM β-Cl-Ala to the enzyme in Bis-Tris propane buffer (pH 7.8) containing the indicated molar concentration of GuHCl and 0.1 M NaCl or 50 mM ion-free GP as indicated in panels B and C. The concentration of the $\alpha_2\beta_2$ complex was 7 μM in the experiments whose results are depicted in panels A and B and 1.4 μM in panel C. The β_2 subunit concentration was 1.4 μM in the experiments whose results are depicted in panel C. The solutions used in the experiments whose results are depicted in panels A−C contained no monovalent cations other than GuH^+ with the exception of the solution denoted in panel B (●) which contained 0.1 M NaCl. Table 2 shows the absorbance at 424 nm from panel B in the presence or absence of NaCl and in the presence or absence of 0.02, 0.08, and 0.25 M GuHCl.

in absorbance at 424 nm occurs at a low GuHCl concentration (0.02 M) which results in a 1.4-fold stimulation of the activity in reaction 2 in the absence of NaCl.

As shown in Figure 2C, the fluorescence of the $\alpha_2\beta_2$ complex in the presence of L-serine increases with increasing GuHCl concentrations up to 0.2 M and then remains constant up to 0.5 M GuHCl. This result also shows that GuHCl shifts the equilibrium distribution of enzyme−substrate intermediates from E−AA to E−Ser. The fluorescence of the E−Ser intermediate formed by the reaction of the β_2 subunit with L-serine increases at very low GuHCl concentrations, but decreases at GuHCl concentrations of >0.05 M (Figure 2C). In contrast, the absorption spectra of the β_2 subunit in the presence of L-serine do not change in 0−0.5 M GuHCl (data not shown). These results indicate that GuHCl alters the conformation of the β_2 subunit to some extent, and that fluorescence is a more discriminatory method than absorbance for monitoring subtle conformational changes of the enzyme.

The reaction of β-Cl-Ala with the $\alpha_2\beta_2$ complex also yields E−AA by elimination of HCl (Scheme 1). Although addition of increasing GuHCl concentrations increases the absorbance at 424 nm and decreases the absorbance at 350 nm, the changes are much smaller than in the case of the reaction of L-serine; higher concentrations of GuHCl are needed to produce a half-maximal increase (Figure 2B).

Effects of Urea Concentration on the Absorption and Fluorescence Spectra in the Presence of L*-Serine.* Addition of increasing concentrations of urea to the $\alpha_2\beta_2$ complex in the presence of L-serine has much larger effects on the absorption and fluorescence spectra in the presence of NaCl than in the absence of NaCl (Figure 3A−D). The effects of urea in the presence of L-serine and NaCl are very similar to those of GuHCl. These results provide direct evidence that the cation GuH^+, like Na^+ (*19*), plays an important role in stabilizing E−Ser.

An Allosteric Ligand Stabilizes E−AA. An allosteric ligand, GP, which binds to the active site of the α subunit, stabilizes the closed form of the $\alpha_2\beta_2$ complex (*18*) (Table 1). In the presence of 50 mM GP, the activity of the $\alpha_2\beta_2$ complex in the β-replacement reaction with L-serine and indole is essentially constant up to 0.5 M GuHCl (Figure 1C). Both absorption (Figure 2B) and fluorescence (Figure 2C) experiments show that GP stabilizes E−AA in GuHCl concentrations of up to 0.5 M. The protection of the $\alpha_2\beta_2$ complex by GP from inhibition by urea is much less pronounced, but becomes stronger in the presence of 0.1 M NaCl (Figure 1F). These results provide additional evidence that monovalent cation binding is important for the ligand-dependent communication between the α and β subunits that stabilizes the active, closed form of the subunit as established previously (*7*, *19*−*21*, *33*).

Analysis of Spectroscopic Data. To account for the results in Figures 2 and 3, we postulate a simple model (Scheme 2) in which the enzyme shows weak "binding"[4] to E−Ser of up to *N* moles of GuHCl per mole of α/β pairs or *N*′ moles of urea per mole α/β pairs and to E−AA of up to *M* moles of GuHCl per mole of α/β pairs or up to *M*′ moles of urea per mole of α/β pairs. A similar model has been used to

[4] See the Discussion for consideration of the mechanism of GuHCl interaction or binding to tryptophan synthase.

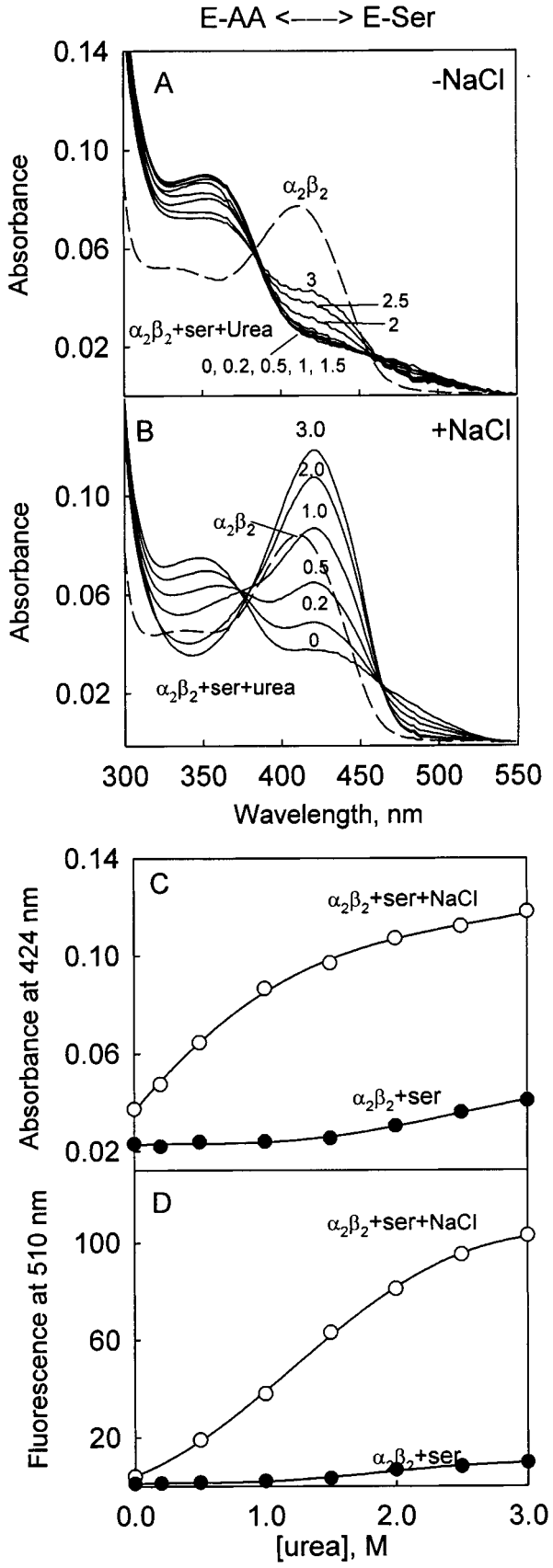


FIGURE 3: Effects of urea concentration on the absorption and fluorescence properties of the $\alpha_2\beta_2$ complex in the presence of 50 mM L-serine. Absorption spectra in the absence (A) and presence (B) of 0.1 M NaCl, the absorbance at 424 nm (C), and the fluorescence intensity at 510 nm (D) were measured as described in the legend of Figure 2 in the presence of the indicated urea concentrations.

Table 3: Effects of GuHCl and Urea Concentration on the Equilibrium Distribution of Reaction Intermediates[a]

enzyme	agent	cation	signal	figure	$N - M$ or $N' - M'$
$\alpha_2\beta_2$	GuHCl	none	A_{424nm}	2B	2.22 ± 0.09
$\alpha_2\beta_2$	GuHCl	NaCl	A_{424nm}	2B	1.0 ± 0.07
$\alpha_2\beta_2$ + GP	GuHCl	none	A_{424nm}	2B	0
$\alpha_2\beta_2$	GuHCl	none	F_{510nm}	2C	2.29 ± 0.25
$\alpha_2\beta_2$ + GP	GuHCl	none	F_{510nm}	2C	0
$\alpha_2\beta_2$	urea	none	A_{424nm}	3C	0
$\alpha_2\beta_2$	urea	NaCl	A_{424nm}	3C	1.41 ± 0.015
$\alpha_2\beta_2$	urea	none	F_{510nm}	3D	0
$\alpha_2\beta_2$	urea	NaCl	F_{510nm}	3D	2.39 ± 0.035

[a] Spectroscopic data from Figures 2 and 3 were analyzed using the PC-MLAB program (Civilized Software, Bethesda, MD) and fit to the model in Scheme 2 using the equation $d[\log K(L)]/d(\log L) = M - N$. The model assumes that the enzyme shows weak "binding"[4] to E−Ser of up to N moles of GuHCl per mole of α/β pairs or N' moles of urea per mole of α/β pairs and to E−AA of up to M moles of GuHCl per mole of α/β pairs or up to M' moles of urea per mole of α/β pairs. Positive values of $N - M$ or $N' - M'$ show that GuHCl or urea perturbs the equilibrium by preferentially binding to E−Ser. It was assumed that $f(L)$, the fraction of enzyme converted from the closed to open state, at denaturant concentration L, was proportional to the fractional change in absorbance or fluorescence at that concentration L. Then, $K(L)$, the equilibrium constant for the conformational change, $= f(L)/[1 - f(L)]$.

account for the effects of solvents on tryptophan synthase (*23*). If $N > M$ or $N' > M'$, then the theory of linked functions (*34*) predicts that increasing concentrations of GuHCl or urea will shift the equilibrium from E−AA to E−Ser, as observed. The data in Figures 2 and 3 gave good fits to the equation in Table 3 which yields values of $N - M$ or $N' - M'$ (Table 3). The results show that urea in the presence of NaCl and that GuHCl shift the equilibrium from E−AA toward E−Ser, whereas urea in the absence of NaCl does not (see the Discussion).

Effect of GuHCl on Subunit Association. Gel filtration was carried out to determine the effects of GuHCl on subunit association. Gel filtration of the $\alpha_2\beta_2$ complex in the absence of GuHCl yields a major peak 1 at 11.8 mL (the position expected for the $\alpha_2\beta_2$ or $\alpha\beta_2$ complex) and a second small peak 2 at 13.7 mL (the position found for the isolated α subunit) (bottom panel in Figure 4A) (*35*). Gel filtration of the isolated β_2 subunit yields a single peak at 12.1 mL. The relative positions of peak 2 and of the β_2 subunit peak indicate that $\alpha\beta_2$ is the dominant form in peak 1 (*35*). The fraction of free α subunit estimated from the heights of the two peaks is about 0.5 (Figure 4C). This result also indicates that $\alpha\beta_2$ is the dominant state in the absence of GuHCl or other monovalent cations. Peak 2 becomes smaller at GuHCl concentrations between 0.02 and 0.4 M, while peak 1 shifts further from the peak of the isolated β_2 subunit. These results show that low concentrations of GuHCl promote association of the α and β subunits and that the dominant state becomes $\alpha_2\beta_2$. NaCl also promotes association of the α and β subunits (*36*). The extent of subunit dissociation increases at GuHCl concentrations of >0.4 M (Figure 4C).

Gel filtration of the $\alpha_2\beta_2$ complex in the presence of 50 mM L-serine yields a single peak at 11.2 mL, whereas the isolated β_2 and α subunits chromatographed separately elute at the same position as in the absence of L-serine (Figure 4B,C). Thus, the enzyme is mainly the $\alpha_2\beta_2$ complex in the presence of 50 mM L-serine. L-Serine largely prevents dissociation of the wild type (*36*) and mutant (βK87T) holo-

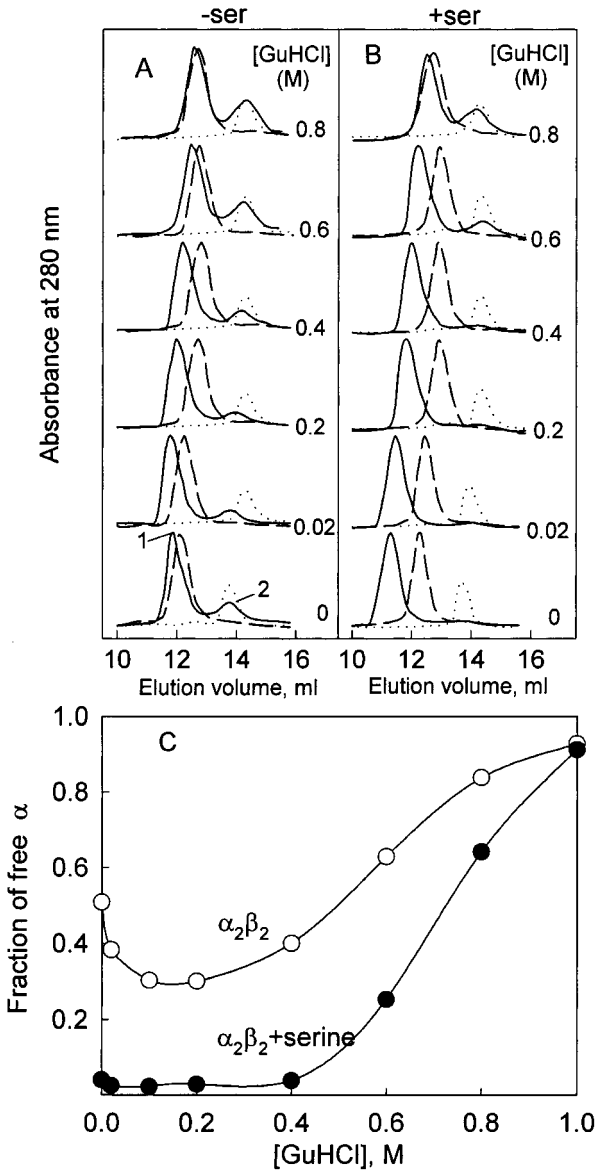
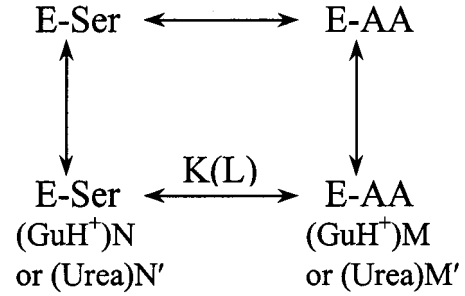


FIGURE 4: Effects of GuHCl on gel filtration of the tryptophan synthase $\alpha_2\beta_2$ complex. (A and B) Elution profiles of the $\alpha_2\beta_2$ complex (—), the β_2 subunit (- - -), and the α subunit (•••) in the presence of the indicated GuHCl concentrations in the absence (A) and presence (B) of 50 mM L-serine in both samples and elution buffers. For all samples, 100 μL of 6 μM protein was applied. The elution buffer was 50 mM Bis-Tris propane containing 1 mM PLP and the indicated GuHCl concentrations. The elution buffer used in the experiments whose results are depicted in panel B contained 50 mM L-serine. TEAC was added to maintain the ionic strength of the buffer. (C) The fraction of free α subunit dissociated from the $\alpha_2\beta_2$ complex at different GuHCl concentrations. The fraction of free α was estimated by $H_\alpha\epsilon_\alpha/(H_{\alpha_2\beta_2}\epsilon_\alpha + \epsilon_{\alpha_2\beta_2})$, where H_α and $H_{\alpha_2\beta_2}$ are the height of the α subunit and $\alpha_2\beta_2$ complex peaks, respectively, and ϵ_α (12 600) and $\epsilon_{\alpha_2\beta_2}$ (86 000) are the related molar extinction coefficients. The results show that L-serine or low concentrations of GuHCl increase the extent of association between the α and β subunits. It should be pointed out that the elution volumes of peaks of the $\alpha_2\beta_2$ complex and of the isolated α and β_2 subunits become greater with increasing GuHCl concentrations (A and B). This can be attributed to ion exclusion interactions between the proteins and the gel matrix at low ionic strengths when the pH of the elution buffer (pH 7.8) is higher than the pI values of the proteins (pI < 5) (*49*). To increase the ionic strength of the elution buffer and to thereby eliminate protein resin interactions, we have added TEAC in all experiments. Addition of TEAC decreases but cannot eliminate the ion exclusion interaction. TEAC may have other ion effects on tryptophan synthase.

Scheme 2



$\alpha_2\beta_2$ complexes (*35*). Our finding that the $\alpha_2\beta_2$ complex is stable in up to 0.4 M GuHCl in the presence of L-serine demonstrates that the activity and spectral changes of the $\alpha_2\beta_2$ complex are due not to the subunit dissociation but to conformational changes. The E−Ser complex dissociates at GuHCl concentrations of >0.4 M (Figure 4B,C).

DISCUSSION

An unexpected and novel finding of this work is that low concentrations of GuHCl activate the tryptophan synthase $\alpha_2\beta_2$ complex in the absence of added NaCl whereas urea does not. Another important observation is that addition of increasing concentrations of GuHCl or urea results in differential effects on several activities of the $\alpha_2\beta_2$ complex. These results and others provide evidence that GuHCl exerts dual effects as a cation activator and as a conformational modifier.

Roles of GuH^+ as a Cation. Monovalent cations activate the tryptophan synthase $\alpha_2\beta_2$ complex (*19*−*21*). We find that low GuHCl concentrations (0.02−0.08 M) stimulate the activities of the $\alpha_2\beta_2$ complex in the two β-replacement reactions in the absence of NaCl (Figure 1A), but not in the presence of NaCl (Figure 1B). The observation that the effects of GuHCl and NaCl are not additive strongly suggests a common mechanism for the activation by monovalent cations and by low GuHCl concentrations. This suggestion is supported by our finding that urea, which is a nonionic denaturant, does not activate the enzyme in either the presence or absence of NaCl (Figure 1D,E).

Monovalent cations also alter the equilibrium distribution of E−Ser and E−AA intermediates in the reaction of the $\alpha_2\beta_2$ complex with L-serine (Schemes 1 and 2) (*19*−*21*, *37*, *38*). Na^+ and K^+ stabilize E−Ser, whereas Cs^+ and NH_4^+ stabilize E−AA. We find that increasing concentrations of GuHCl promote formation of E−Ser in either the absence or the presence of NaCl (Figure 2A,B and Table 2), whereas urea promotes formation of much more E−Ser in the presence of NaCl than in the absence of NaCl (Figure 3A,B). These results provide evidence that GuH^+ substitutes for Na^+ in stabilizing E−Ser. In the absence of any added cation, the E−AA intermediate accumulates (Figures 2A and 3A) as reported previously (*19*). Our finding that the nonionic urea stabilizes the aldimine of L-serine in the presence, but not in the absence, of NaCl provides further evidence that cation binding plays an important role in the conformational transitions that regulate activity and allosteric communication as proposed by Dunn and co-workers (*7*, *38*).

Finally, monovalent cations are known to promote association of the α and β subunits (S. A. Ahmed, unpublished

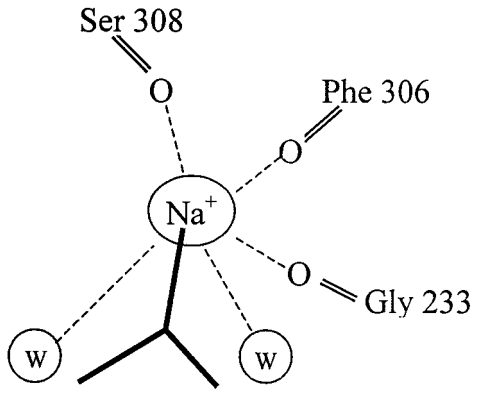


FIGURE 5: Cartoon of the Na^+ binding site in tryptophan synthase showing a GuH^+ molecule (solid line) positioned on the basis of computer graphics modeling as described in the text (W is H_2O). Each nitrogen of GuH^+ in the model forms favorable hydrogen bonds (2.4−3.2 Å) with the carbonyl oxygens of βGly-232, βPhe-306, and βSer-308, which are residues that coordinate with Na^+, and also with the carbonyl oxygens of βVal-231, βGly-268, and βLeu-304, which are residues that form additional interactions with Cs^+ (details of the model not shown).

results; *36*). We find that L-serine alone or low concentrations of GuHCl (0.08−0.25 M) in the absence of L-serine promote association of the α and β subunits (Figure 4C). These results suggest that L-serine alone or cations (GuH^+ or Na^+) stabilize a conformation of the β subunit that interacts more tightly with the α subunit.

Observed differences between the effects of neutral urea and cationic GuHCl with several other proteins have provided evidence that GuHCl can have a cation role. An example most closely related to our results is that of spinach chloroplast fructose 1,6-bisphosphatase, which is activated by low GuHCl concentrations, but not by urea (*39*). The observation that the effects of GuH^+ and K^+ are not additive strongly suggests a common mechanism for the activation by monovalent cations and by low GuHCl concentrations.

A cation such as GuH^+ may also shield negative charges. Hodges and co-workers have found that protein denaturation with urea or GuHCl provides a different estimate of stability depending on the contributions of electrostatic interactions (*30*−*32*). The results show that GuHCl masks the effects of electrostatic interactions during protein denaturation whereas urea does not. A role for GuHCl in shielding the anionic C-termini of tubulin has been proposed to account for the ability of low concentrations of GuHCl to increase the rate of polymerization of tubulin (*40*). GuHCl (0.1 M) slightly stabilizes RNase T1 from thermal denaturation, whereas urea does not (*41*). The suppression of the stabilizing effect of GuHCl by NaCl provides evidence that stabilization by GuHCl results from a cation effect.

A Possible Binding Site for GuH^+. Crystallographic studies have identified a Na^+ binding site in the structure of the wild type $\alpha_2\beta_2$ complex (PDB 1bks) and in a mutant (βK87T) $\alpha_2\beta_2$ complex (*42*). K^+ or Cs^+ can be exchanged for Na^+ at the same site (*33*). The cation binding site is located in the β subunit about 8 Å from the phosphate of PLP. Na^+ is coordinated to the carbonyl oxygens of three enzyme residues (βGly-232, βPhe-306, and βSer-308) and two water molecules (see the cartoon in Figure 5). Molecular modeling of GuH^+ into the Na^+ site by placement of one nitrogen in the position of Na^+ and the other two nitrogens near the two waters shows that GuH^+ could be very well accommodated if the two waters were displaced. Each nitrogen of GuH^+ in the model forms favorable hydrogen bonds (2.4−3.2 Å) with the carbonyl oxygens of βGly-232, βPhe-306, and βSer-308, which are residues that coordinate with Na^+, and also with the carbonyl oxygens of βVal-231, βGly-268, and βLeu-304, which are residues that form additional interactions with Cs^+ (details of the model not shown). Furthermore, GuH^+ is not too close (<2.4 Å) to any other residues in the model. Thus, GuH^+, if bound at this site, may act both as a cation and as a noncovalent cross-linking agent by forming hydrogen bonds between different portions of the protein. A crystal structure of ribonuclease A with GuHCl has identified binding sites for four GuH^+ ions (*43*). Each GuH^+ ion interacts with several protein residues.

Roles of GuHCl and Urea as Conformational Modifiers. Addition of increasing GuHCl concentrations has strikingly different effects on the rates of reactions 2−5 in the absence of NaCl (Figure 1A and Table 2). Symbols I−III and vertical dashed lines in Figure 1A mark three GuHCl concentrations, 0.02, 0.08, and 0.25 M, that give maximal activity in reactions 2, 3, and 4 or 5, respectively. Increasing the GuHCl concentration from 0.02 to 0.08 M further stimulates the activity in reaction 3 but results in a large reduction in the rate of reaction 2. Activities in the two β-elimination reactions (reactions 4 and 5) increase with increasing GuHCl concentrations to a maximum at about 0.25 M and then decrease.

We propose that different conformational states designated I−III accumulate maximally at 0.02, 0.08, and 0.25 M GuHCl, respectively. Conformational state I is the active, closed form of the enzyme that is stabilized by GuH^+ binding to the cation binding site. This conformer can efficiently catalyze the β-elimination of the hydroxyl group of L-serine and the β-replacement with indole (Scheme 1). Conformational state II is an open form that efficiently catalyzes the β-elimination of the chloride group of β-Cl-Ala and β-replacement with indole (Scheme 1). The conversion of I to II may disrupt the enzyme architecture needed to protonate the hydroxyl leaving group of L-serine. Similar effects on reaction specificity result from certain mutations (*10*) and cosolvents (*22*, *23*) (see the introductory section and Table 1). Conformer III can catalyze β-elimination reactions but has reduced activity in β-replacement reactions. This conformer may be unable to activate indole for β-replacement (Scheme 1). Thus, GuHCl acts as a cation at low concentrations (0.02 M) but as a conformational modifier at higher concentrations.

Addition of increasing urea concentrations in the presence of NaCl (Figure 1E) results in differential decreases in the rates of reactions 2, 3, and 4 or 5, similar to those observed with GuHCl in the presence or absence of NaCl (Figure 1A,B). In the absence of NaCl (Figure 1D), all activities decrease at lower urea concentrations and the rates of reactions 2 and 3 decrease equally with increasing urea concentrations. The results show that cation binding is important for stabilizing conformational states II and III that have partial activity in reaction 3 and in reaction 4 or 5, respectively.

The reaction of the $\alpha_2\beta_2$ complex with L-serine in the absence of indole results in a mixture of E−Ser and E−AA intermediates which have been associated with open and

closed conformations of the enzyme, respectively (see the introductory section and Table 1). The finding that increasing concentrations of GuHCl decrease the amount of E−AA and increase the amount of E−Ser (Figure 2) provides additional evidence that GuHCl induces or stabilizes the open form of the enzyme. Similar results are obtained with urea in the presence of NaCl (Figure 3). Higher GuHCl concentrations are required to reduce the amount of E-AA formed in the reaction with β-Cl-Ala (Figure 2B). This result is consistent with the activity results (Figure 1A,B and Table 1) which indicate that the open form of the enzyme (conformer II) maintains the ability to react with β-Cl-Ala.

The α subunit ligand GP almost completely protects the enzyme from inactivation by GuHCl (Figure 1C) and strongly reduces the increase in the level of the E−Ser intermediate observed with increasing GuHCl concentrations (Figure 2B,C). GP has been shown to stabilize the active, closed conformation of the $\alpha_2\beta_2$ complex associated with E−AA (*18*) (Table 1). Our results show that this stabilization is strong enough to prevent modification of the conformation by GuHCl concentrations of up to 0.5 M. Interestingly, GP exerts much weaker protection against the effects of urea on activity (Figure 1C), implying that GuHCl and urea differ somewhat in their mechanism of conformational perturbation.

GuHCl Does Not Alter the Association State or Tertiary Structure. Because the functional properties of the $\alpha_2\beta_2$ complex in the presence of GuHCl (Figures 1A−C and 2) are similar to those of the isolated β_2 subunit, it is important to exclude the possibility that GuHCl induces dissociation of the $\alpha_2\beta_2$ complex. Our finding that GuHCl at >0.1 M reduces the fluorescence of the E−Ser intermediate formed by the β_2 subunit but not that formed by the $\alpha_2\beta_2$ complex (Figure 2C) provides the first evidence that the $\alpha_2\beta_2$ complex remains associated in up to 0.5 M GuHCl in the presence of L-serine. This conclusion is supported by gel filtration experiments carried out in the presence of L-serine (Figure 4). GuHCl concentrations of up to 0.5 M have little or no effect on the far-UV CD spectra of the $\alpha_2\beta_2$ complex or on the intrinsic fluorescence of tyrosine and tryptophan (unpublished results), indicating that these low GuHCl concentrations have minimal effects on the tertiary conformation despite their striking effects on active site properties.

Mechanism of Perturbation by GuHCl and Urea. The good fits of our data from Figures 2 and 3 (Table 3) to the model in Scheme 2 provide evidence that urea in the presence of NaCl and that GuHCl interact preferentially with the conformer of the enzyme that predominates when E−Ser is formed and that this interaction shifts the equilibrium from E−AA to E−Ser (Table 1). We now ask about the mechanism of interaction of these chaotrophic agents. Crystallographic studies have shown that GuHCl and urea bind to specific sites on ribonuclease A and dihydrofolate reductase, respectively, and reduce the overall thermal factor (*43*). NMR studies have also shown that urea interacts directly with proteins (*44*). GuHCl and urea can also alter the behavior of the solvent by disrupting the dynamic network of hydrogen bonds that exists in pure water (*45*). Alternatively, GuHCl and urea may displace solvent molecules bound to the surface of the protein (*46*).

Our experimental results do not directly distinguish between binding of chaotrophs to tryptophan synthase or effects of the chaotrophs on the solvent. However, the finding that urea has very little effect on the spectroscopic properties in the absence of NaCl (Figure 3 and Table 3) indicates that urea does not affect the solvent and only binds to the form of the $\alpha_2\beta_2$ complex containing bound Na^+. Table 3 shows that the calculated $N - M$ for GuHCl in the absence of NaCl is greater (2.22) than that in the presence of NaCl (1.0), suggesting that GuHCl and NaCl compete for two or more sites per α/β pair. Our combined results indicate that GuHCl and urea bind directly to sites in tryptophan synthase and that about two more sites are available in E−Ser than in E−AA.

Recent crystallographic results have provided structural information about ligand-induced conformational changes in the $\alpha_2\beta_2$ complex that may help in interpreting the results with urea, GuHCl, and solvents. The results show that the reaction of L-serine results in movement of a mobile region (β subunit residues 93−183) toward the rest of the β subunit (*42*, *47*). The observed ligand-induced domain movement excludes water from the active site and increases the hydrophobicity of the β site. The interactions between the mobile region and the rest of the β subunit are about half-hydrophobic and half-hydrophilic. Because GuHCl and urea are predicted to cause unfolding by solvating nonpolar groups better than water does (*48*), GuHCl and urea may shift the equilibrium from the closed to the open conformation by disrupting the hydrophobic interaction site between the mobile region and the rest of the β subunit in the closed form.

ACKNOWLEDGMENT

We thank Dr. Sangkee Rhee of the National Institute of Diabetes and Digestive and Kidney Diseases for carrying out molecular modeling of the putative GuH^+ binding site using the coordinates (1bks) for the wild type $\alpha_2\beta_2$ complex.

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BI990307E