Mutations in the Contact Region between the α and β Subunits of Tryptophan Synthase Alter Subunit Interaction and Intersubunit Communication

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ABSTRACT: Interaction between the α and β subunits of tryptophan synthase leads to mutual stabilization of the active conformations and to coordinated control of the activities of the two subunits. To elucidate the roles of specific residues in the interaction site between the α and β subunits, mutant α and β subunits were constructed, and the effects of mutation on subunit interaction and intersubunit communication were determined. Mutation of either α subunit Asp56 (αD56A) or β subunit Lys167 (βK167T), residues that interact in some crystal structures of the tryptophan synthase $\alpha_2\beta_2$ complex, decreases the ability of the α subunit to activate the β subunit and alters the reaction and substrate specificity of the β subunit. Partial conformational repair is provided by α-glycerol 3-phosphate, a ligand that binds to the α subunit, or by Cs^+ or NH_4^+, ligands that bind to the β subunit. Mutation of β subunit Arg175 (βR175A), a residue that interacts with α subunit Pro57 in some structures, has much smaller effects on activity but results in a 15-fold increase in the apparent K_d for dissociation of the α and β subunits. Replacement of the single tryptophan in the β subunit by phenylalanine (W177F) has only small effects on activity but increases the apparent subunit dissociation constant ∼10-fold. The most important conclusions of this investigation are that interaction between αAsp56 and βLys167 is important for intersubunit communication and that mutual stabilization of the active conformations of the two subunits is impaired by mutation of either residue.

The tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.2.1.20) is a useful model system for investigating subunit interaction and intersubunit communication [for reviews, see (*1*−*6*)]. The α and β_2[1] subunits can be separated and shown to catalyze two distinct reactions, termed the α and β reactions, eqs 1 and 2, respectively. The $\alpha_2\beta_2$ complex catalyzes the α and β reactions and the αβ reaction, eq 3, which is essentially the sum of the α and β reactions:

indole-3-glycerol phosphate ⇌ indole + D-glyceraldehyde 3-phosphate (α reaction) (1)

L-serine + indole → L-tryptophan + H_2O (β reaction) (2)

L-serine + indole-3-glycerol phosphate → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O (αβ reaction) (3)

The separate β_2 subunit catalyzes the deamination of L-serine, eq 4, whereas the $\alpha_2\beta_2$ complex has a very low activity in this reaction.

L-serine → pyruvate + NH_3 (deaminase reaction) (4)

Thus, association of the β subunit with the α subunit alters the reaction specificity of the β subunit. β-Chloro-L-alanine is an alternative substrate in a β-elimination reaction analogous to eq 4 and in a β-replacement reaction analogous to eq 2.

Three-dimensional structures of wild-type and mutant forms of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* have revealed many features of the multienzyme complex, including the arrangement of the α and β subunits in the complex and the location of residues in the interface between the α and β subunits (*7*−*9*). Crystal structures of the $\alpha_2\beta_2$ complex in the presence of Na^+, K^+, or Cs^+ show that all three cations bind to the same site located in the β subunit, but that differences in coordination give rise to two distinctly different protein conformations (*8*) (reviewed in ref *6*). In the conformation favored by Na^+, the carboxylate of βAsp305 forms a salt bridge with the ϵ-amino group of βLys167 as shown in Figure 1A. In the conformation favored by K^+ or Cs^+, βAsp305 is in an alternative conformation and βLys167 makes a salt bridge

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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.

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Table 1: Primers Used To Construct Mutant Subunits of Tryptophan Synthase[a]

primer	sequence (5′→3′)
αD56A[b] Pm$_2$	GCTCTAGAACTGGGGGTTCCCTTCTCCGCTCCGCTGGCC
βR175A (anti)	GACCAGTCGGCCAGCGCCTCGT
βW177F (anti)	CTACCGGAGAAGTCGCGCAGCG

[a] Underlined bases are altered; mutagenesis utilized a PCR method (*11*) (see Experimental Procedures). [b] TCTAGA introduces an *Xba*I site. Because the mutagenesis point in αD56 is close to the primer PE3, the mutagenic primer and PE3 were synthesized as one primer, Pm$_2$.

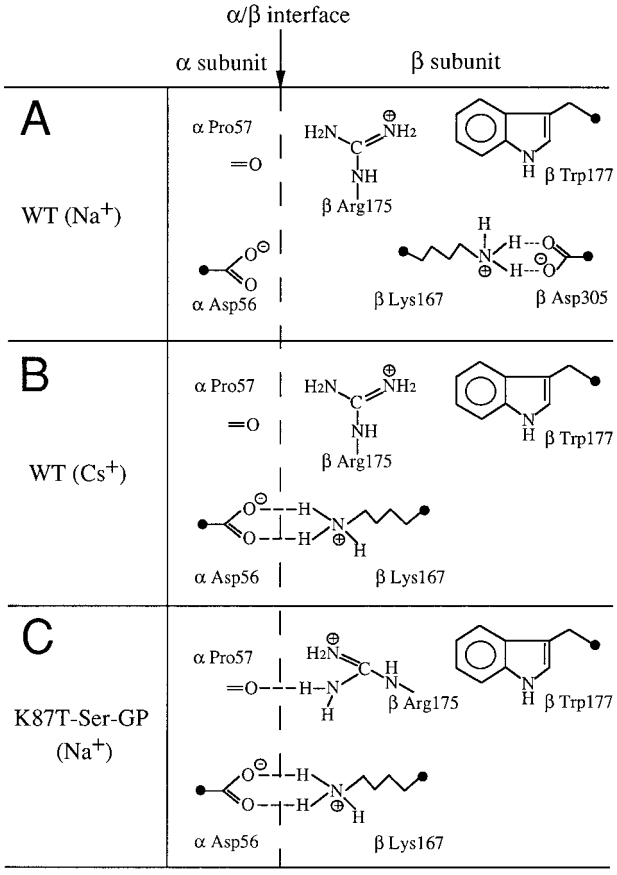

FIGURE 1: Scheme showing residues in the interface between the tryptophan synthase α and β subunits that exhibit altered interactions in different three-dimensional structures of tryptophan synthase (see the introduction). Contact residues in the X-ray structures of the wild-type $\alpha_2\beta_2$ complex in the presence of Na^+ (*7*) (A) or Cs^+ (*8*) (B) or in the structure of the mutant $\alpha_2\beta_2$ complex (βK87T-Ser-GP) in the presence of serine, DL-α-glycerol 3-phosphate, and Na^+ (*9*) (C). The indole ring of βTrp177 is ~10 Å from the subunit interface. The coordinates of the structures are in the Brookhaven Protein Data Bank with names 1WSY for the Na^+ complex (A), 1TTP for the Cs^+ complex (B), and 2TSY for the βK87T-Ser-GP in the presence of Na^+ complex (C). The EMBL/SWISSPROT identifier of the α subunit is trpa_salty and of the β subunit is trpb_salty.

across the subunit interface with the carboxylate of αAsp56 as shown in Figure 1B. Other conformational differences in subunit interface residues have been observed in the crystal structure of a mutant (βK87T) $\alpha_2\beta_2$ complex having L-serine bound to the active site of the β subunit and α-glycerol 3-phosphate bound to the active site of the α subunit (βK87T-Ser-GP)[2] (*9*). The structure of βK87T-Ser-GP in the presence of Na^+ (see Figure 1C) shows a salt bridge interaction between βLys167 and αAsp56 that is not seen in the wild-type complex in the presence of Na^+ (Figure 1A). An additional interaction between βArg175 and the backbone oxygen of α subunit Pro57 is also seen in βK87T-Ser-GP in the presence of Na^+ (see Figure 1C).

Because the structural studies indicate that the side chains of βLys167 and βArg175 are affected by ligand binding and because ligand binding affects allosteric communication between the α and β subunits, we report here some effects of mutagenesis of βLys167 and βArg175 and of αAsp56, the residue that interacts with βLys167. Because βLys167 and βArg175 are located on the surface of a subdomain that contains the single invariant tryptophan in the β subunit (βTrp177), we also report some effects of replacement of this tryptophan by phenylalanine (βW177F).

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. Pyridoxal phosphate (PLP), DL-α-glycerol phosphate (GP), D-glyceraldehyde-3-phosphate dehydrogenase, β-chloro-L-alanine (hydrochloride), and other chemicals 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 (*10*). Buffer B [50 mM sodium *N*,*N*-bis(2-hydroxyethyl)glycine containing 1 mM EDTA at pH 7.8] was used for spectroscopic studies unless otherwise specified. Oligonucleotides were supplied by Integrated DNA Technologies. Restriction endonucleases, T4 DNA ligase, and DNA polymerase I large fragment (Klenow) were purchased from Promega, New England Biolabs, Boehringer-Mannheim, or GibcoBRL. Pfu DNA polymerase and Taq DNA polymerase were from Stratagene. Sequenase version 2.0 DNA sequencing kit was from United States Biochemical. Deoxyadenosine 5′-[α-^{35}S]-thiotriphosphate (3000 Ci/mmol) was from Amersham Life Science.

Bacterial Strains and Plasmids. Growth of the *Escherichia coli* host strain CB149 (*10*) harboring wild-type and mutant forms of plasmids pEBA-10, pEBA-6, and pEBA-4A8 that express the *S. typhimurium* $\alpha_2\beta_2$ complex, β_2 subunit, and α subunit of tryptophan synthase was as described (*11*, *12*). Purification of the wild-type and mutant $\alpha_2\beta_2$ complexes (*13*) and β_2 subunits (*14*) utilized crystallization from crude extracts followed by recrystallization. Some mutant β_2 subunits were obtained from the corresponding $\alpha_2\beta_2$ complexes by heat denaturation of the α subunit (*15*). The α subunit was purified from extracts containing α subunit alone (*14*) or separated from the apo $\alpha_2\beta_2$ complex (*16*). The latter method was used for

[2] Abbreviations: PLP, pyridoxal phosphate; GP, DL-α-glycerol 3-phosphate.

preparation of the αD56A α subunit. The βK167T $\alpha_2\beta_2$ complex was obtained as described (*17*). Protein concentrations were determined from the specific absorbance at 278 nm using $A^{1\%}_{1cm} = 6.0$ for holo $\alpha_2\beta_2$ complex, $A^{1\%}_{1cm} = 6.5$ for holo β_2 subunit, and $A^{1\%}_{1cm} = 4.4$ for the α subunit (*15*). The protein concentrations of the βW177F β_2 subunit and $\alpha_2\beta_2$ complex were normalized to those of the corresponding wild-type enzymes on the basis of determinations of the PLP concentrations by a fluorometric method (*18*).

PCR-Based Mutagenesis. The expression vector pEBA-10 was used as the template for quick and convenient mutagenesis by megaprimer PCR (*11*) (see this reference for the sequences of primers PE7, PE6, PE3, and PE4 and descriptions of templates used below).

βR175A and βW177F Mutations. Each mutagenic primer (see Table 1) and PE7, which contains a *Bgl*II restriction site, were used to amplify the first round of PCR with the pEBA-10 template plasmid using Pfu DNA polymerase. The first-round PCR fragments were purified and used directly as primers together with the alternate primer PE6, which contains a *Sph*I restriction site, to amplify a second round of DNA synthesis. Deoxyadenosine (A) was added to the newly amplified second-round PCR products by the non-template-dependent activity of Taq polymerase. The second-round PCR fragments were purified and directly inserted to the linearized pCR II sequencing plasmid (Invitrogen) which has single 3′-deoxythymidine (T) residues. After confirmation of the mutation by DNA sequencing, the inserted DNA fragment was restricted with *Bgl*II and *Sph*I and ligated into the original parent plasmid (pEBA-10) which had been digested with *Bgl*II and *Sph*I.

αD56A Mutation. Because the mutagenesis point in αD56 is close to the primer PE3, the mutagenic primer and PE3 were synthesized as one primer, Pm_2 (Table 1). PE4, which contains a *Pst*I site, and Pm_2, which contains an *Xba*I site, were phosphorylated with T_4 polynucleotide kinase and then used as primers for one round of PCR with the pEBA-10 template plasmid. The PCR fragment was filled in by DNA polymerase I large fragment (Klenow), purified, inserted into the two blunt ends of the linearized pGEM-5zf(+) vector,[3] which was restricted with *Eco*RV restriction enzyme to produce the two blunt ends, and then dephosphorylated with alkaline phosphatase to suppress self-ligation and circularization. After confirmation of mutation by DNA sequencing, the mutated DNA fragment was restricted with *Xba*I and *Pst*I and ligated into the original parent plasmid (pEBA-10), which had also been digested with *Xba*I and *Pst*I.

Enzyme Assays. One unit of enzyme activity 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 (40 mM) to form L-tryptophan] were determined by a spectrophotometric assay (*15*) in the presence of a 3-fold molar excess of α subunit. CsCl or NH_4Cl or both (0.18 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 (*19*) with modified components (40 mM L-serine or β-chloro-L-alanine, 0.14−0.28 mM NADH, and excess lactate dehydrogenase in 100 mM Tris-HCl buffer, pH 7.8). The activity of the α subunit or the $\alpha_2\beta_2$ complex in the α reaction or αβ reaction was measured by a spectrophotometric assay coupled with D-glyceraldehyde-3-phosphate dehydrogenase in the presence or absence of L-serine (*20*).

Spectroscopic Methods. Absorption spectra were measured in a Hewlett-Packard 8452 diode array spectrophotometer thermostated at 25 °C. Measurements of enzyme activities at single wavelengths were made using a Cary 118 spectrophotometer or a Hewlett-Packard 8452 diode array spectrophotometer at 37 °C. Fluorescence measurements were made using a Photon Technologies spectrofluorometer. 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 before (*21*−*23*) with a sample of $\alpha_2\beta_2$ complex in buffer B in the presence of a 5−10-fold excess of α subunit at 37 °C. Circular dichroism measurements (mean residue ellipticity in degrees centimeter squared per decimole) were made in a Jasco J-500C spectrophotometer, equipped with a DP-500N data processor (Japan Spectroscopic Co., Easton, MD) at 25 °C.

Subunit Association. The interaction of the α and β subunits was characterized by measuring the activity of the β subunit as a function of α subunit concentration. 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:

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

for the reaction $\alpha + \beta \rightleftharpoons \alpha\beta$. The position of the equilibrium of eq 5 can be monitored by activity measurements, where the specific activity $S = S_o + (S_{max} - S_o)f_{\alpha\beta}$ where $f_{\alpha\beta} = [\alpha\beta]/[\beta]_{tot}$; S_o is the specific activity of the β_2 subunit alone and S_{max} is the intrinsic specific activity of the $\alpha_2\beta_2$ complex. The concentration of $[\alpha\beta]$ at any given $[\alpha]_{tot}/[\beta]_{tot}$ ratio can be solved explicitly from eq 6, where $f_\alpha = [\alpha]_{tot}/[\beta]_{tot}$:

$$K_d = \frac{(f_\alpha[\beta]_{tot} - [\alpha\beta])([\beta]_{tot} - [\alpha\beta])}{[\alpha\beta]} \quad (6)$$

The unique solution for $[\alpha\beta]$ is described by eq 7:

$$[\alpha\beta] = [([\beta]_{tot} + f_\alpha[\beta]_{tot} + K_d) - \sqrt{([\beta]_{tot} + f_\alpha[\beta]_{tot} + K_d)^2 - 4f_\alpha[\beta]^2_{tot}}]/2 \quad (7)$$

and the variation of specific activity, S, with the fraction of added α subunit, f_α, is given by eq 8

$$S = S_0 + (S_{max} - S_0)[([\beta]_{tot} + f_\alpha[\beta]_{tot} + K_d) - \sqrt{([\beta]_{tot} + f_\alpha[\beta]_{tot} + K_d)^2 - 4f_\alpha[\beta]^2_{tot}}]/2[\beta]_{tot} \quad (8)$$

The apparent dissociation constant of α with β subunit as measured by activity in the reaction of L-serine with indole to form L-tryptophan, $K_d(\alpha\beta)$, was obtained from eq 8. Using

[3] Alternatively, pCR II could be used for this step.

eq 9, it is possible to estimate the free energy of dissociation of the α and β subunits:

$$\Delta G^\circ_d = -RT \ln K_d(\alpha\beta) \quad (9)$$

RESULTS

To investigate the roles of residues in the interaction site between the α and β subunits of tryptophan synthase, we have replaced two interaction site residues, αAsp56 and βArg175 (Figure 1), by alanine. The single tryptophan in the β subunit (βTrp177) was replaced by phenylalanine. This residue is invariant in the aligned sequences of the β subunit from many species and is two residues from βArg175 in the sequence. The indole ring of βTrp177 is located ~10 Å from the α−β interaction site and is buried in a subdomain (residues 93−189), termed the "mobile region", that undergoes conformational changes in some structures (*9*). The three mutations (βR175A, βW177F, and αD56A) were successfully constructed and verified by DNA sequencing of the cloned PCR product and of the expression vector. The mutant α and β subunits were all expressed and purified in high yield (~100 mg of enzyme per 100 mL of culture) as $\alpha_2\beta_2$ complexes. The ability of these mutant enzymes to form stable $\alpha_2\beta_2$ complexes provides partial evidence for their structural integrity and indicates that subunit association has not been greatly weakened. We also compare some properties of the newly prepared enzymes with those of interaction site enzymes engineered previously: βK167T (*17*) and αP57A (*24*).

Initial Characterization of the βW177F $\alpha_2\beta_2$ Complex. The βW177F $\alpha_2\beta_2$ complex was characterized by fluorescence and circular dichroism spectroscopy to provide additional supportive data for the mutation of the single tryptophan in the $\alpha_2\beta_2$ complex to phenylalanine. The fluorescence emission intensity at 337 nm (Figure 2A) and excitation at 285 nm (Figure 2B) of the βW177F $\alpha_2\beta_2$ complex are about half those of the wild-type enzyme. The difference excitation spectrum (Figure 2B) clearly resembles the absorption spectrum of tryptophan, with a broad peak centered around 283 nm, and a sharper, narrower peak at 290 nm. The circular dichroism spectra of the wild-type and βW177F β_2 subunits (Figure 2C) are almost identical in the region ascribed to the bound PLP (300−500 nm), but differ in the region ascribed to tryptophan (260−300 nm). The difference circular dichroism spectrum clearly resembles that of tryptophan.

Effects of the αD56A Mutation on Specific Activities in the α, β, and αβ Reactions. The isolated wild-type and αD56A α subunits both have very low specific activities in the α reaction (Table 2). Addition of excess β_2 subunit stimulates the activities of the wild-type and αD56A α subunits 60- and 30-fold, respectively. Thus, the αD56A α subunit is activated by association with the β subunit. The αD56A $\alpha_2\beta_2$ complex has 32% of the activity of the wild-type $\alpha_2\beta_2$ complex in this reaction. The activity of the αD56A $\alpha_2\beta_2$ complex in the β reaction is drastically reduced (3% of wild type) but is significantly higher in the αβ reaction (15% of wild type) or in the β reaction in the presence of DL-α-glycerol 3-phosphate (52% of wild type), an α site ligand. A similar behavior has been reported for the βK167T $\alpha_2\beta_2$ complex (*17*), as shown in Table 2. We conclude that the presence of indole-3-glycerol phosphate or of DL-α-glycerol 3-phosphate at the α site increases the activities of the two mutant $\alpha_2\beta_2$ complexes in the reaction with L-serine at the β site. Thus, a mutation in either member of this pair of interacting residues reduces the activation of the β subunit by the α subunit. The striking increase in β reaction activity provided by DL-α-glycerol 3-phosphate or indole-3-glycerol phosphate could result from either conformational correction or increased subunit interaction (see below).

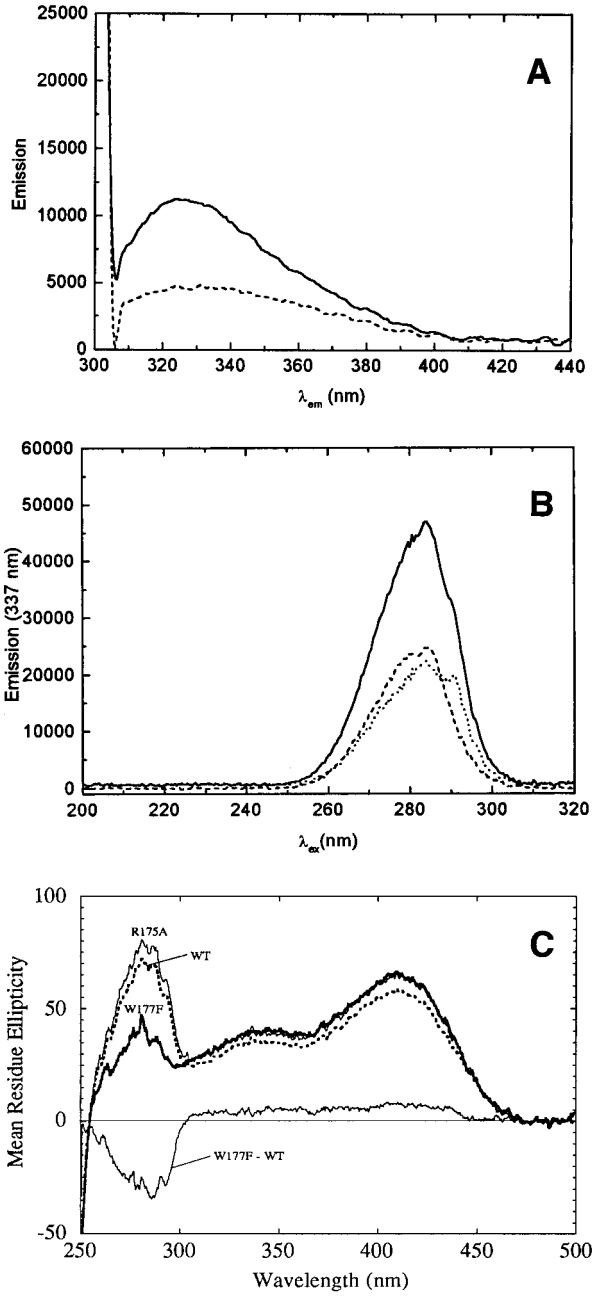

FIGURE 2: Fluorescence and circular dichroism spectra for the wild-type and mutant enzymes. Fluorescence emission (A) and excitation (B) spectra for the wild type (—) and βW177F (---) $\alpha_2\beta_2$ complexes. The difference spectrum (wild type − βW177F) is shown in (B) (•••). (C) Circular dichroism spectra of the wild-type, βR175A, and βW177F β subunits and the difference spectrum between the βW177F and wild-type β subunits. (A) 2.8 μM $\alpha_2\beta_2$ complex in 50 mM Bicine−1 mM EDTA, 25 °C, pH 7.80; λ_{ex} = 295 nm. (B) 1.4 μM $\alpha_2\beta_2$ in buffer B, 25 °C, pH 7.80; λ_{em} = 337 nm. (C) β subunits at 1 mg/mL in 10 mM sodium phosphate buffer, pH 7.8.

Table 2: Specific Activities of Wild-Type and Mutant $\alpha_2\beta_2$ Complexes or α Subunits in the α, β, and αβ Reactions[a]

reaction	addition	sp act. (units/nmol of αβ or α) WT	αD56A	βK167T
α: IGP ⇌ Ind + G3P (−β)	none	0.03	0.02 (67%)	0.03
α: IGP ⇌ Ind + G3P (+β)	none	1.9	0.6 (32%)	(88%)[b]
β: Ind + Ser → Trp + H_2O	none	108	3.2 (3%)	(4%)[b,c]
β: Ind + Ser → Trp + H_2O	GP	53	27 (52%)	(28%)[c]
αβ: IGP + Ser → Trp + G3P + H_2O	none	21.4	3.1 (15%)	(31%)[b]

[a] Assays as described under Experimental Procedures plus or minus 10 mM DL-α-glycerol 3-phosphate. Specific activities were measured on the α subunit alone (−β) or on $\alpha_2\beta_2$ complexes (+β) in the presence of a 3-fold excess of wild-type α subunit (for wild-type or βK167T enzyme) or a 3-fold excess of αD56A α subunit (enzyme). Values in parentheses show the activity of the mutant $\alpha_2\beta_2$ complex as percent of activity of the wild-type (WT) $\alpha_2\beta_2$ complex under the same conditions. IGP, indole-3-glycerol phosphate; Ind, indole; G3P, D-glyceraldehyde 3-phosphate; GP, DL-α-glycerol 3-phosphate. [b] (17); 80 mM DL-α-glycerol 3-phosphate was added where indicated. [c] This work; see Figure 4.

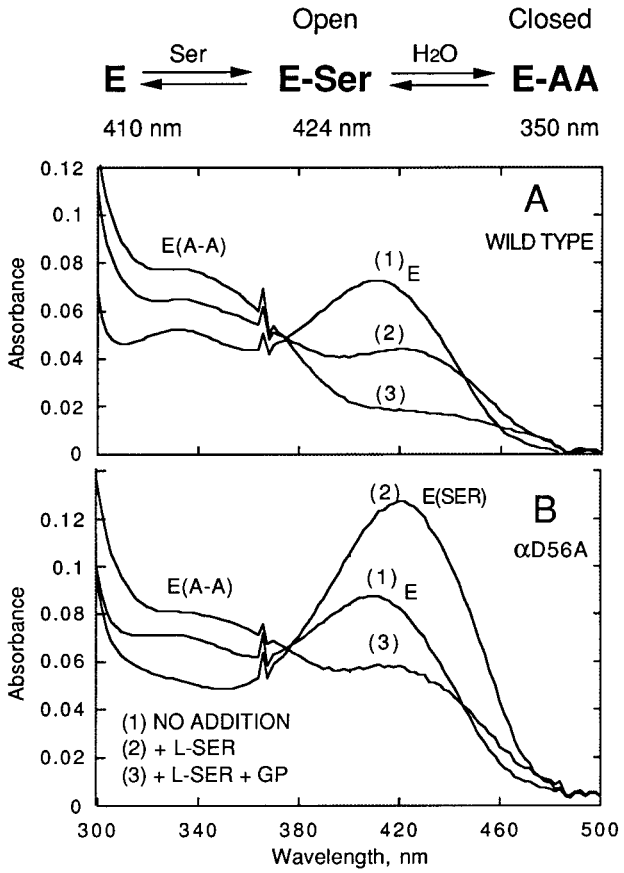

FIGURE 3: Effects of ligands on the absorption spectra of the wild-type and αD56A $\alpha_2\beta_2$ complexes. Absorption spectra of the wild-type (A) and αD56A $\alpha_2\beta_2$ complex in the presence or absence of 40 mM L-serine or 40 mM L-serine plus 10 mM DL-α-glycerol 3-phosphate (GP).

Effects of Mutations and of Ligands on Spectroscopic Properties. The absorption spectra of the wild-type and mutant $\alpha_2\beta_2$ complexes are essentially identical in the absence of ligands (see curves 1 in Figure 3A,B and in Figure 3A,B in ref *17*). The reaction of tryptophan synthase with L-serine yields an equilibrium mixture of the external aldimine of L-serine (E-Ser), which has maximum absorbance at 420 nm, and the aldimine of aminoacrylate (E-AA), which has maximum absorbance at 340 nm. E-Ser is a fluorescent species with maximal emission at 510 nm (*21*); E-AA is nonfluorescent. E-AA is the main L-serine intermediate that accumulates with the wild-type $\alpha_2\beta_2$ complex (Figure 3A) and with the βW177F and βR175A $\alpha_2\beta_2$ complexes (data not shown). The change in fluorescence emission at 510 nm due to formation of E-Ser (see Experimental Procedures) for these three enzymes in the presence of L-serine is very small. In contrast, E-Ser is the main L-serine intermediate that accumulates with the αD56A $\alpha_2\beta_2$ complex (curves 2, Figure 3A,B) and with the βK167T $\alpha_2\beta_2$ complex (*17*). The change in fluorescence emission for the αD56A and βK167T $\alpha_2\beta_2$ complex in the presence of L-serine is approximately 25 times larger than that for the wild-type, βW177F, and βR175A $\alpha_2\beta_2$ complexes under the same experimental conditions.

The α subunit ligand, DL-α-glycerol 3-phosphate, alters the equilibrium distribution of the L-serine intermediates and stabilizes the E-AA intermediate (*25*). The spectra of the αD56A $\alpha_2\beta_2$ complex (curve 3, Figure 3B) and of the βK167T $\alpha_2\beta_2$ complex (*17*) in the presence of L-serine are dramatically affected by addition of DL-α-glycerol 3-phosphate and become closely similar to that of the wild-type $\alpha_2\beta_2$ complex under the same conditions.

Mutations Alter Substrate and Reaction Specificity. The wild-type β_2 subunit and $\alpha_2\beta_2$ complex differ greatly in substrate and reaction specificity (*26*), as illustrated in Figure 4. We have defined substrate specificity as the relative activity with L-serine and with β-chloro-L-alanine in β-replacement reactions with indole (compare assays 1 and 4 in Figure 4). The wild-type $\alpha_2\beta_2$ complex has much greater activity with L-serine than with β-chloro-L-alanine, whereas the opposite is true with the β_2 subunit. Reaction specificity is defined as the relative activity in β-replacement and β-elimination reactions with the same substrate (compare assays 4 and 5 in Figure 4). The wild-type $\alpha_2\beta_2$ complex has greater activity in β-replacement reactions than in β-elimination reactions, whereas the β_2 subunit has similar activities in both reactions. Figure 4 shows the assays described above and the effects of Cs^+ (assay 3) and of DL-α-glycerol 3-phosphate (assay 2) on the activities of wild-type and mutant enzymes. The βW177F and βR175A $\alpha_2\beta_2$ complexes exhibit activity profiles that are similar to that of the wild-type $\alpha_2\beta_2$ complex. However, the βR175A enzyme has reduced activity in the β reaction plus Na^+ (assay 1), which is activated by DL-α-glycerol 3-phosphate or Cs^+. The wild-type β_2 subunit exhibits a very different activity profile (Figure 4B) with low activity in the β reaction plus Na^+ (assay 1) and approximately equal activities in assays 3−5. The activity profile of the αD56A $\alpha_2\beta_2$ complex is very similar to that of the βK167T $\alpha_2\beta_2$ complex. The profiles of these two mutant enzymes are more similar to that of the wild-type β_2 subunit than to that of the wild-type $\alpha_2\beta_2$ complex. That is, these mutant enzymes and the wild-type β_2 subunit have significant activities in the β-elimination

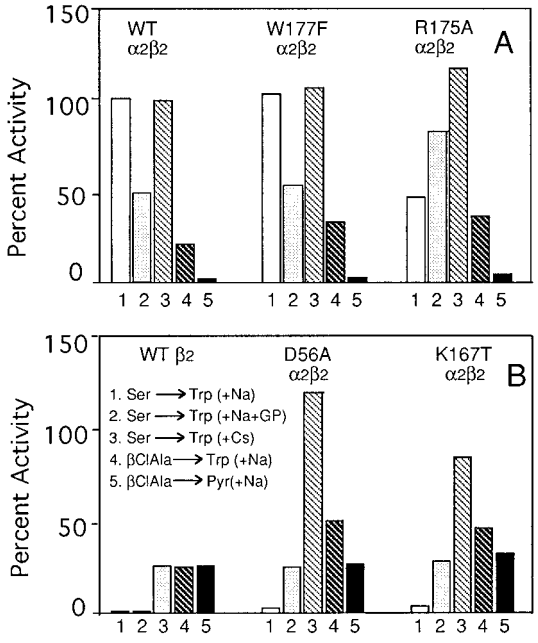

FIGURE 4: Reaction and substrate specificities of wild-type and mutant enzymes: effects of DL-α-glycerol 3-phosphate and Cs^+. Activities were determined in the β-replacement reaction with L-serine and indole in the presence of Na^+ (1), Na^+ + DL-α-glycerol 3-phosphate (2), or Cs^+ (3), in the β-replacement reaction with β-chloro-L-alanine and indole in the presence of Na^+ (4), and in the β-elimination reaction with β-chloro-L-alanine in the presence of Na^+ (5). Activities are expressed as a percent of the activity of the wild-type $\alpha_2\beta_2$ complex in (1). (A) Wild-type, βW177F, and βR175A $\alpha_2\beta_2$ complexes. (B) Wild-type β subunit and αD56A and βK167T $\alpha_2\beta_2$ complexes.

Table 3: Effects of Cations on Activity in the β Reaction[a]

	cation				
enzyme (α/β)	Na^+	Cs^+	NH_4^+	$Cs^+ + NH_4^+$	avg Cs^+ and NH_4^+
WTα/WTβ	[100]	99	144	119	121
αD56A/WTβ	3	120	52	91	86
WTα/βK167T	4	84	92	**103**	88
αD56A/βK167T	2.6	68	37	49	52

[a] Activities of enzymes in the β reaction (L-serine + indole → L-tryptophan + H_2O) (see Experimental Procedures) in the presence of 0.20 M NaCl, CsCl, or NH_4Cl or both CsCl and NH_4Cl (Cs^+ + NH_4^+) are expressed as a percent of the activity of the wild-type $\alpha_2\beta_2$ complex in the presence of NaCl. The column on the right gives the calculated average of the activities with CsCl alone and with NH_4Cl alone. Activities of $\alpha_2\beta_2$ complexes were determined in the presence of a 3-fold excess of wild-type α subunit or of αD56A α subunit.

reaction with β-chloro-L-alanine (assay 5) and higher activities in the β-replacement reactions with β-chloro-L-alanine than with L-serine in the presence of Na^+ and are activated by Cs^+ in the β-replacement reaction with L-serine (assay 3). It is noteworthy that the activities of the αD56A and βK167T $\alpha_2\beta_2$ complexes in the presence of Cs^+ (assay 3) are much higher than the corresponding activity of the wild-type β_2 subunit and are similar to those of the wild-type $\alpha_2\beta_2$ complex.

Cation Activation. Table 3 shows the effects of different cations on the activities of the wild-type and mutant $\alpha_2\beta_2$ complexes in the β reaction. K^+ was not tested here because K^+ is much less effective than Cs^+ and NH_4^+ as an activator of the wild-type β_2 subunit (*25*) and of many mutant forms of the $\alpha_2\beta_2$ complex. Whereas the activities of the wild-type enzyme are similar in the presence of Na^+, Cs^+, or NH_4^+ ions, the activities of the αAsp56 or βLys167 mutant enzymes are much lower in the presence of Na^+ than in the presence of Cs^+ or NH_4^+ ions. The effects of Cs^+ ion on the activities of $\alpha_2\beta_2$ complexes with mutations in either the α subunit (αD56A) or the β subunit (βK167T) or in both positions (αD56A/βK167T) differ from the effects of NH_4^+ ion. These results are relevant to the proposed roles of NH_4^+ in conformational correction or in substituting for the deleted side chain of βLys167 (see Discussion).

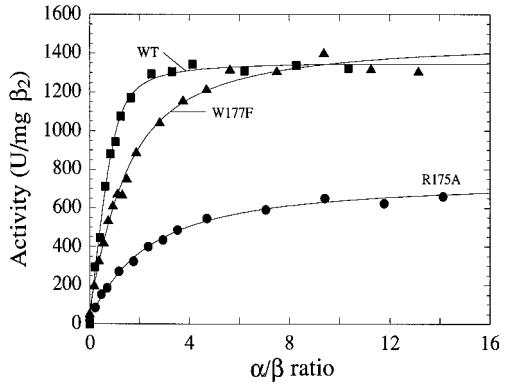

FIGURE 5: Titration of wild-type and mutant β subunits with wild-type α subunit. Activities of the wild-type (■), W177F (▲), and R175A (●) β subunits in the β reaction were determined in the presence of increasing amounts of the wild-type α subunit as described under Experimental Procedures. The concentrations of β_2 subunits used were as follows: wild type, 12.5 μg/mL = 0.146 μM β; R175A, 13.3 μg/mL = 0.154 μM β; W177F, 16.7 μg/mL = 0.194 μM β. Results of data analysis are presented in Table 4.

Titration of β Subunit with α Subunit. The interaction of the α and β subunits was characterized by measuring the activity of the β subunit in the β reaction as a function of α subunit concentration (Figure 5). Figure 5 clearly shows that the α subunit interacts more weakly with the βR175A β subunit and with the βW177F β subunit than with the wild-type β subunit. Nonlinear least-squares analysis of the data in Figure 5 and additional data obtained in the same way using eq 8 (see Experimental Procedures) gave the parameters compiled in Table 4. Based on these data, the apparent dissociation constants, $K_d(\alpha\beta)$, for the βR175A $\alpha_2\beta_2$ complex and for the βW177F $\alpha_2\beta_2$ complex in the presence of Na^+ are ~15-fold and 10-fold larger, respectively, than that for the wild-type enzyme. The fully saturated βR175A $\alpha_2\beta_2$ complex is about 50% as active as the wild type whereas the fully saturated βW177F $\alpha_2\beta_2$ complex has an activity close to that of the wild-type $\alpha_2\beta_2$ complex. Using eq 9, it is possible to estimate the free energy of dissociation, $\Delta G°_d$, of the α and β subunits (Table 4). Comparing the $\Delta G°_d$ values of the wild-type and βR175A β subunits in the presence of Na^+, we arrive at a $\Delta\Delta G°$ value of 1.7 kcal/mol; that is, the βR175A β subunit associates with the α subunit with an energy that is 1.7 kcal/mol less than the wild-type β subunit. This value falls within the range of a single hydrogen bond interaction, which is typically 1−5 kcal/mol in aqueous solution (*27*). Comparing the wild-type and βW177F β subunits gives a $\Delta\Delta G°$ of 1.5 kcal/mol.

Additional data collected for the titration of the wild-type and mutant α and β subunits in the presence of various effectors are summarized in Table 4. For wild-type β subunit, DL-α-glycerol 3-phosphate reduces the maximal

Table 4: Apparent Dissociation Constants of the Wild-Type and Mutant α and β Subunits[a]

enzyme	cation	effector	V_{max} (units/mg) $\times 10^{-3}$	$K_d(\alpha\beta)$ (μM)	$\Delta G°_d$ (kcal/mol)
wild type	Na^+	none	1.36 ± 0.02	0.018 ± 0.002	11.1 ± 0.1
wild type	Na^+	GP	0.70 ± 0.02	0.045 ± 0.010	10.5 ± 0.1
wild type	Cs^+	none	1.58 ± 0.02	0.051 ± 0.005	10.4 ± 0.1
αD56A	Na^+	none	0.035 ± 0.001	0.022 ± 0.011	10.9 ± 0.3
βK167T[b]	Na^+	none	0.050 ± 0.001	0.16 ± 0.032	9.70 ± 0.11
βK167T[b]	Na^+	GP	0.40 ± 0.007	0.043 ± 0.006	10.5 ± 0.1
βR175A	Na^+	none	0.75 ± 0.01	0.27 ± 0.02	9.38 ± 0.04
βR175A	Na^+	GP	1.30 ± 0.02	0.21 ± 0.01	9.53 ± 0.03
βR175A	Cs^+	none	2.49 ± 0.02	0.11 ± 0.005	9.93 ± 0.03
βW177F	Na^+	none	1.48 ± 0.03	0.181 ± 0.019	9.63 ± 0.07
βW177F	Na^+	GP	0.82 ± 0.02	0.10 ± 0.018	9.99 ± 0.10
βW177F	Cs^+	none	1.47 ± 0.02	0.12 ± 0.018	9.88 ± 0.09

[a] Activities of the wild-type or mutant β subunit in the β reaction were determined (see Figure 5) in the presence of increasing amounts of the wild-type or mutant α subunit and in the presence of 0.18 M NaCl or CsCl and of DL-α-glycerol 3-phosphate (10 mM) where indicated. Values of V_{max}, the maximal specific activity of the reconstituted $\alpha_2\beta_2$ complex expressed in units per milligram of β subunit, and of $K_d(\alpha\beta)$, the apparent dissociation constant of the α subunit with the β subunit in the presence of L-serine, were obtained by nonlinear least-squares analysis using eq 8. The free energy of dissociation of the α and β subunits, $\Delta G°_d$, was estimated by eq 9. [b] Analysis of published data (*17*).

specific activity, and slightly increases the $K_d(\alpha\beta)$. Cesium ion has only a modest effect on V_{max}, and also increases the $K_d(\alpha\beta)$ slightly. The V_{max} of the βW177F $\alpha_2\beta_2$ complex, like that of wild type, is relatively insensitive to Cs^+ and is decreased by DL-α-glycerol 3-phosphate; the presence of effectors has a minimal effect on $K_d(\alpha\beta)$. DL-α-Glycerol 3-phosphate and Cs^+ increase the maximal specific activity of βR175A, by 73% and 232%, respectively. However, only Cs^+ significantly lowers the $K_d(\alpha\beta)$, bringing it close to the value for the wild-type β subunit. Interestingly, the αD56A α subunit has a $K_d(\alpha\beta)$ similar to that of wild-type α subunit whereas that of βK167T, its interaction partner, is 10-fold higher than that of wild type, but is reduced in the presence of DL-α-glycerol 3-phosphate (Table 4).

DISCUSSION

The experiments described above were aimed at assessing the functional roles of several residues in the interaction site between the tryptophan synthase α and β subunits that have been observed to undergo ligand-induced conformational changes in several crystal structures (Figure 1). Residues that interact in the βK87T-Ser-GP (Na^+) structure (Figure 1C) but not in the wild-type (Na^+) structure (Figure 1A) are in two pairs: αPro57−βArg175 and αAsp56−βLys167. The properties of two new mutant enzymes (αD56A and βR175A) are reported and compared with those of two mutant enzymes engineered before [αP57A (*24*) and βK167T (*17*)]. Our results show how mutations of these residues affect interaction between the α and β subunits and mutual stabilization of the active conformations.

Effects of Mutations on Subunit Interaction. None of the mutations examined here had a large effect on the apparent dissociation constants $K_d(\alpha\beta)$ (Table 4) determined from measurements of activity in the β reaction (Figure 5). A 15-fold increase in $K_d(\alpha\beta)$ was observed for the βR175A β subunit. Thus, disruption of interaction between αPro57 and βArg175 results in a small decrease in subunit affinity in the range expected for the loss of one hydrogen bond. Although the βK167T mutation also increases the $K_d(\alpha\beta)$ about 10-fold, the αD56A mutation has little effect on $K_d(\alpha\beta)$.[4] Thus, subunit affinity is not affected by interaction of this pair of residues. The βK167T mutation may affect the $K_d(\alpha\beta)$ by altering the conformation of the β subunit. The reasons why the βW177F mutation also increases the $K_d(\alpha\beta)$ about 10-fold are not clear. βTrp177 is buried in the hydrophobic interior of a subdomain (residues 93−189), termed the "mobile region", which exists in different conformations in the wild-type $\alpha_2\beta_2$ and in the βK87T-Ser-GP $\alpha_2\beta_2$ complexes (*9*). Substitution of βTrp177 by Phe may cause a partial collapse of this subdomain. The putative collapse may alter the position of βArg175 or make its hydrogen bond contact with the αPro57 backbone carbonyl less favorable. Cations (Cs^+) or α subunit ligands (DL-α-glycerol 3-phosphate), which stabilize the active conformation of mutant β subunits (see below), have only small effects on $K_d(\alpha\beta)$. DL-α-Glycerol 3-phosphate does decrease the $K_d(\alpha\beta)$ for the βK167T β subunit ∼4-fold.

Effects of Mutations on Mutual Subunit Activation. The activation of the wild-type β subunit by interaction with the α subunit alters the reaction specificity and substrate specificity (Figure 4). We have suggested that the wild-type β_2 subunit and certain mutant $\alpha_2\beta_2$ complexes, which have substrate and reaction specificities similar to those of the wild-type β_2 subunit, exist predominantly in a low-activity, "open" form, whereas the wild-type $\alpha_2\beta_2$ complex exists predominantly in a high-activity, "closed" form (*11*, *12*, *25*, *26*). The conformational states of the β_2 subunit and $\alpha_2\beta_2$ complex also affect the equilibrium distribution of catalytic intermediates, E-Ser and E-AA, formed in the reaction with L-serine (see equation at top of Figure 3). Enzymes in the open form (β_2 subunit and certain mutant $\alpha_2\beta_2$ complexes) predominantly accumulate E-Ser whereas enzymes in the closed form (wild-type $\alpha_2\beta_2$ complex) predominantly accumulate E-AA. Certain ligands (e.g., DL-α-glycerol 3-phosphate and Cs^+ or NH_4^+) stabilize the closed form.

Our present results provide evidence that the αD56A α subunit fails to stabilize the active, closed conformation of the wild-type β subunit. The αD56A $\alpha_2\beta_2$ complex accumulates the E-Ser intermediate (Figure 3) and exhibits altered substrate and reaction specificities (Figure 4). A different mutant form of αAsp56 (αD56G) also has very low activities in the αβ and β reactions (*28*). Interestingly, mutation of βLys167, which interacts with αAsp56 in some structures (Figure 1B,C), has similar effects on the spectroscopic properties (*17*) and substrate and reaction specificities (Figure 4).

Addition of the α subunit ligand, DL-α-glycerol 3-phosphate, results in partial activation of the βK167T $\alpha_2\beta_2$ complex [Figure 4 and (*17*, *29*)[5]] and of the αD56A $\alpha_2\beta_2$ complex (Figure 4). DL-α-glycerol 3-phosphate may partially repair alterations in the conformations of the mutant α and β subunits that result from the specific mutations or from

[4] A mutant form of αAsp56 (αD56G) has reduced affinity for the β subunit (personal communication from J. K. Hardman).

[5] E. U. Woehl, C. Meador, U. Banik, E. W. Miles, and M. F. Dunn, in preparation.

the disruption of the interaction between these residues. The close parallel between the effects of mutation of either βLys167 or αAsp56 argues that interaction between these two residues is important for stabilization of the active, closed conformation of the β subunit. The αD56A $\alpha_2\beta_2$ complex exhibits a lower activity (32% of wild type) in the α reaction than the αD56A α subunit alone (67% of wild type) (Table 2). These results suggest that activation by the wild-type β subunit is incomplete and has been compromised by disruption of the interaction between these two residues.

Substitution of Cs^+ or NH_4^+ for Na^+ greatly stimulates the activity of the αD56A $\alpha_2\beta_2$ complex or of the βK167T $\alpha_2\beta_2$ complex in the β reaction (Figure 4 and Table 3) or the $\alpha_2\beta_2$ complex composed of both mutant subunits (Table 3). We have suggested that Cs^+ and NH_4^+ activate a number of mutant β subunits by stabilizing the active, closed conformation of the β subunit (*25*). A binding site for Na^+, K^+, and Cs^+ in the β subunit has been identified by X-ray crystallography (*8*) and may also bind NH_4^+ ion. Woehl (*29*)[5] has also observed activation of the βK167T $\alpha_2\beta_2$ complex by NH_4^+ and has suggested that NH_4^+ may activate by binding at the location normally occupied by the ϵ-amino group of βLys167 in the wild-type or βK87T structure (Figure 1B,C). The results in Table 3 provide partial support for the latter hypothesis. Whereas Cs^+ is more effective than NH_4^+ as an activator of the αD56A $\alpha_2\beta_2$ complex, NH_4^+ is somewhat more effective than Cs^+ as an activator of the βK167T $\alpha_2\beta_2$ complex. In contrast, NH_4^+ is less effective than Cs^+ as an activator of the $\alpha_2\beta_2$ complex composed of both mutant subunits. One interpretation of this result is that NH_4^+ can only bind at the site of the deleted ϵ-amino group of βLys167 and activate the α subunit when NH_4^+ can interact with the carboxylate of αAsp56. The activities in the presence of a mixture of equal amounts of Cs^+ and NH_4^+ are approximately the average of activities for each separate ion with one exception: the βK167T $\alpha_2\beta_2$ complex. In this case, the activity with the mixture is greater than the activity with either ion alone, suggesting that NH_4^+ binds at a site different from the Cs^+ site and acts by a different mechanism, as proposed by Woehl (*29*).[5]

The two other new mutant $\alpha_2\beta_2$ complexes examined (βR175A and βW177F) are similar to the wild-type $\alpha_2\beta_2$ complex in substrate and reaction specificity (Figure 4) and in the reaction with L-serine. βArg175 interacts with the backbone oxygen of α subunit Pro57 under some conditions (see Figure 1C). The βR175A $\alpha_2\beta_2$ complex (Figure 4), like the αP57A $\alpha_2\beta_2$ complex reported earlier (*24*), exhibits about 50% of wild-type activity in the β reaction in the presence of Na^+. The activity of either the βR175A or the βW177F $\alpha_2\beta_2$ complex in the presence of DL-α-glycerol 3-phosphate is greater than that of the wild-type enzyme in the presence of DL-α-glycerol 3-phosphate. Cs^+ ion stimulates the activity of βR175A more than 2-fold (Figure 4). The results imply that DL-α-glycerol 3-phosphate and Cs^+ repair the active conformation of both mutant $\alpha_2\beta_2$ complexes as found to a greater extent with the αD56A and βK167T mutant enzymes.

In a recent review, Dunn concludes that "αAsp56 and βLys167 likely are part of the allosteric linkage between the α and β subunits, and the cation plays a pivotal role in intersubunit communication" (*6*). Our results support and extend this conclusion and give new information about the effects of mutations at four positions on subunit interaction and on intersubunit communication.

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