Structural Basis for the Impaired Channeling and AllostERIC
Inter-subunit Communication in the βA169L/βC170W Mutant of
Tryptophan Synthase*

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We determined the 2.25 Å resolution crystal structure of the βA169L/βC170W mutant form of the tryptophan synthase αββα complex from Salmonella typhimurium complexed with the α-active site substrate analogue 5-fluoro-indole-propanol-phosphate to identify the structural basis for the changed kinetic properties of the mutant (Anderson, K. S., Kim, A. Y., Quillen, J. M., Sayers, E., Yang, X. J., and Miles, E. W. (1995) J. Biol. Chem. 270, 29936–29944). Comparison with the wild-type enzyme showed that the βTrp170 side chain occludes the tunnel connecting the α- and β-active sites, explaining the accumulation of the intermediate indole during a single enzyme turnover. To prevent a steric clash between βLeu169 and βGly125, located in the β-sheet of the COMM (communication) domain (βGly102-βGly185), the latter reorganizes. The changed COMM domain conformation results in a loss of the hydrogen bonding networks between the α- and β-active sites, explaining the poor activation of the α-reaction upon formation of the aminoacylase complex at the β-active site. The 100-fold reduced affinity for serine seems to result from a movement of βAsp98 away from the β-active site so that it cannot interact with the hydroxyl group of a pyridoxal phosphate; IPP, indole-propanol phosphate; F-IPP, 5-fluoro-indole-propanol phosphate; βA169L/βC170W, F-IPP complex of tryptophan synthase containing the mutations βA169L and βC170W; IGP, indoleglycerol-phosphate; PLP, pyridoxal phosphate; PDB, Protein Data Bank; r.m.s., root mean square.

Tryptophan synthase (TRPS) is a bifunctional tetrameric enzyme with a linear αββα architecture that catalyzes the last two steps in the biosynthesis of L-tryptophan. In the α-subunit indole-glycerol-phosphate (IGP) is cleaved to indole and glyceraldehyde-3-phosphate (α-reaction; for reviews see Refs. 1–3). At the β-active site, the second substrate L-serine binds to and is then activated by a pyridoxal phosphate (PLP) cofactor to form a highly reactive aminoacylate intermediate, which reacts with indole to form the product L-tryptophan (β-reaction). The failure to detect indole even in rapid chemical quench experiments (4) and the existence of a ~25 Å-long tunnel that connects the α- and β-active sites (5) strongly suggests that indole is transferred through this tunnel from the active site in the α-subunit to the one in the β-subunit. This process, the direct transfer of an intermediate or metabolite between two sequential enzymes without free diffusion through the solvent, is known as substrate channeling. Channeling is thought to play an important role in metabolite regulation and cellular modulation of enzymatic activities (1, 6, 7). In the case of tryptophan synthase, it prevents loss of the intermediate indole through the cell membranes.

An important requirement for enzymes exhibiting substrate channeling is the tight regulation of the two coupled reactions so that they remain in phase. In tryptophan synthase, synchronization is achieved first by the influence of the α-site ligand on the affinity for serine and the distribution of the intermediates formed at the β-active site upon binding and subsequent reaction of serine and the PLP cofactor (8) (for review see Ref. 2). Second, the IGP cleavage rate is increased ~150-fold upon formation of the aminoacylase at the β-active site (4). Indole does not accumulate in the tunnel or at the β-active site, as the transfer of indole through the tunnel is fast (>1000 s⁻¹) and the reaction with the aminoacylate is largely irreversible. This kinetic model for efficient channeling predicts that obstruction or occlusion of the tunnel should lead to an accumulation of indole such that it may be detected in single-turnover rapid quench experiments. To test this hypothesis, βCys170, which lines the tunnel, was mutated to phenylalanine or tryptophan (9, 10). The latter mutant also contained the substitution βA169L, which introduces a restriction enzyme site for convenient verification of the mutation. The low resolution crystal structure of the double mutant shows that the introduced tryptophan, βTrp170, obstructs the tunnel (11). In agreement with the kinetic model, indole can indeed be detected in the two βCys170 mutants by single-turnover chemical quench-flow experiments (10). The rate constant for channeling, which is ~1000 s⁻¹ for wild-type TRPS, is 100 s⁻¹ for βC170F and 0.2 s⁻¹ for βA169L/βC170W (Table I). It is intriguing that this difference is related to the size of the obstructing residue at position β170. In contrast, it was rather unexpected to find that the activation of the α-reaction by the aminoacylase is the
same in the wild-type and the βC170F mutant, whereas it is reduced 100-fold in the βA169L/βC170W mutant (Table I). This observation was interpreted as an impairment of the α ↔ β subunit communication. It was speculated that the activation of IGP cleavage at the α-site upon formation of the aminoacyl-enzyme complex at the β-active site is prevented by the presence of the indole moiety of βTrp(170), which may mimic an indole intermediate in the tunnel (10).

Recently, we deduced a structural model for the allosteric α ↔ β subunit communication (12). Briefly, the substrate (or substrate analog) binding to the α-active site induces an ordering of the catalytically important loops αL2 and αL6 and a concerted rearrangement of both gating residues βTrp(279) and βPhe(280) (9, 13, 14), resulting in tunnel blockage and thus preventing untimely passage of indole. The ordering of a rigid but movable domain (βGly(102)-βGly(189)) is concomitant. We named it the “COMM domain” because it is central to the α ↔ β-active site communication. Rhee et al. (15) introduced the domain βGly(97)-βGly(189) as the “mobile region.” This COMM domain interacts with residues of α-subunit loops αL2 and αL6, β-subunit residues including βAsp(305), which is involved in binding of the metal ion, and active site residues βGlu(109) and βGln(114). Changes on either active site disrupt the respective interactions with the COMM domain, which results in a rigid body movement of the COMM domain, thereby changing the interactions at the other active site and thus transmitting the signal.

We decided to revisit the βA169L/βC170W mutant to see whether our model provides the structural basis for the changed kinetic properties of the mutant. Our previous study of the βA169L/βC170W mutant was hampered by the low resolution (3.2 Å) of the structure determined using x-rays from a rotating anode. Therefore, we reetermined the structure of the mutant complexed with the noncleavable IGP analogue using synchrotron radiation. The comparison of the kinetic properties of the single mutant βC170F and the double mutant βA169L/βC170W, together with the comparison of the structures of the latter and the wild-type enzyme, suggests the reason for the impaired inter-subunit communication being the βA169L mutation, whereas the βC170W replacement seems to be responsible for the modified channeling characteristics.

### MATERIALS AND METHODS

The βA169L/βC170W mutant of TRPS was purified (9, 16) and crystallized (11) as described previously. Diffraction data were collected of a crystal mounted in a capillary and kept at 4 °C at beam-line X12C at the National Synchrotron Light Source using a MAR Research image plate detector. The data were integrated and scaled with the HKL suite of programs (17). Refinement to 2.5 Å resolution was started with CNS 0.9a (18) by performing rigid body and simulated-annealing steps.

The coordinates of the wild-type TRPS\textsubscript{IPP} complex (PDB code 1Q0P) were used as a starting model, omitting the coordinates of loops αL2 and αL6, IPP, the cofactor PLP, and all water molecules. The mutated residues were modeled as alamines to avoid model bias. The final model was built by cyclic rounds of manual model building with the program “O” (19) and Maximum Likelihood refinement with the program REFMAC (20) using all reflections from 20 to 2.25 Å. Water molecules were incorporated by ARP (21) using the automatic cut-off option. All waters were checked manually and removed if displaying unusual H-bonding geometry. Apart from residues at the C termini of both polypeptide chains, the only part of the βA169L/βC170W\textsubscript{IPP} complex that remains too disordered to be built into electron density is the C-terminal part of loop αL6 (αArg(188)-αPro(192)). As shown in Fig. 1A, two regions in the β-subunit show disproportionately high B-factors (2σ × ⟨B⟩ = 72 Å², mean value ⟨B⟩ = 36 Å²); the first residues (βAla(136)-βSer(145)) of helix βH5 and the residues (βHis(163)-βAla(164)) preceding helix βH6 (secondary structure definition according to Ref. 12). Because both amino acid stretches belong to the COMM domain and may have structural and/or communicative functions, we retained these coordinates in the final model, although the final Sigma A-weighted 2 mF\textsubscript{o} – DF\textsubscript{o} (20) and a composed-omit (18) maps show only very weak electron density. The reason for this high flexibility and/or bad model definition is discussed below.

The final model consists of 4960 protein atoms, the α-ligand F-IPP, the cofactor PLP, and 190 water molecules. The R-factors are R = 17.0% and R\textsubscript{free} = 22.4%. Data and refinement statistics are given in Table II. The coordinates and structure factor amplitudes have been deposited with the PDB (accession code 1FU5).

### RESULTS AND DISCUSSION

The structure of the double mutant βA169L/βC170W is of high quality as indicated by the low R-factors and the good stereochemistry (see Table II). The side chain conformations of the mutated amino acids are very well defined by the electron density (Fig. 2A). The mutant has the same secondary and tertiary structure as the wild-type enzyme, and the overall topology is similar to the one assigned by Schneider et al. (12) that is observed in all TRPS structures. F-IPP binds (not shown) in a depression on the surface of the α-subunit and is held in place by several hydrogen bonds to the phosphate at the one end and hydrophobic interactions with the indole moiety at the other end, similar to the wild-type enzyme (12). Following, we compare the structures of the βA169L/βC170W\textsubscript{IPP} complex and the wild-type IPP complex (TRPS\textsubscript{IPP}) Protein Data Bank (PDB) code 1Q0P (14). The reason is that the latter was determined to a much higher resolution than the TRPS\textsubscript{IPP} complex (PDB code 1A50) (12), and a comparison of both complexes shows high structural similarity; the superposition of

### Table I

| Reaction | Wild-type | βC170F | βA169L/βC170W |
|----------|-----------|--------|---------------|
| IGP → E* | 0.16      | 0.16   | 0.08 (0.5)    |
| IGP – E* → indole-G3P-E* | 24 | 24 | 0.12 (0.005) |
| Activation by Ser | 150-fold | 150-fold | 1.5-fold (0.001) |
| E + Ser → E-Ser [μM⁻¹ s⁻¹] | 0.135 | 0.03 (0.22) | 0.002 (0.015) |
| E-Ser → E + Ser [s⁻¹] | 20 | 31 (1.55) | 156 (7.8) |
| E-Ser → E- AA [s⁻¹] | 46 | 12 (3.75) | 12 (3.75) |
| Indole channeling [s⁻¹] | >1000 | 100 (0.1) | 0.2 (0.0002) |
| E- AA-indole → E+ -Trp [s⁻¹] | >1000 | 250 (0.25) | 500 (0.5) |
| E+ -Trp → E + Trp [s⁻¹] | 5 | 3 (0.375) | 1 (0.125) |
| K\textsubscript{m} for L-Ser [mM] | 0.5 | 0.5 | 50 (100) |
Several CONNEXON domains have significantly different conformations in the βA169L/βC170W-IPP complex compared with IPP has no influence on the TRPS structure. In particular, it does not change the CONNEXON domain conformation (12, 14).

The βA169L/βC170W-IPP and TRPS-IPP structures differ significantly in the conformations of the CONNEXON domains and the tunnel. Because they have been observed in positions that result in open (5, 12) or closed (13, 12) tunnel, respectively, they have been thought of as a molecular gate. As described previously (11), the βTrp170 side chain points into the tunnel, thereby blocking it. In addition, because the bulky side chain of βC170W would clash with the βPhe280 side chain, the latter rotates by about 110° in the wild type (Figs. 1B and 2B). The changes are caused primarily by the introduction of leucine βLeu169, because its side chain would be too close to the adjacent amide nitrogen of βGly135 (distance 1.6 Å) and the Cα of βMet134 (distance 2.5 Å) located at the end of strand βS4. Their movement prevents this backbone clash and results in a domino effect-like rearrangement of the CONNEXON domain; because strand βS4 is in the middle of the β-strands of the four-stranded parallel β-sheet and forms hydrogen bonds to residues of both neighboring strands βS3 and βS5, these strands and the N-terminal part of β-strand βS6 also move. The shift of all four CONNEXON domain β-strands is conveyed by the movement of helix βH5 and loops βL3 and βL5. The maximum Cα atom shift of ~3.5 Å is found at lysine βLyr137. It is noteworthy that the catalytically important glutamate βGlu109 (4, 22) moves away from the β-active site with its carboxylate carbon atom shifting by ~2.1 Å.

A further consequence of the changed CONNEXON domain conformation in the βA169L/βC170W-IPP complex is found in the hydrogen bonding network involving aspartate βAsp305, which is positioned near the sodium binding site (13) and involved in the β-elimination reaction yielding the external aldimine (15, 23). Although the side chain of aspartate βAsp305 points toward the β-active site ("swing in" position (13)) in both the TRPS-IPP (14) and the βA169L/βC170W-IPP complex, there are significant differences. In the wild type, the distance between the carboxylate oxygen Oα′ of βAsp305 and the amide nitrogen of βGly111 is 3.4 Å, and the βAsp305 carboxylate is linked via one or two water molecules to the CONNEXON domain residues βAsp138, βGlu142, and βThr165, respectively. This network is broken in the βA169L/βC170W-IPP complex because the respective CONNEXON domain residues are shifted from the core of the β-subunit due to the introduction of the leucine βLeu169 as described above (distance between Oα′ of βAsp305 and the amide of βGly111 is 6.1 Å). This results in a loss of all water molecules involved in the hydrogen bonding network between βAsp305

| Crystal parameters, data collection, and refinement statistics |
|---------------------------------------------------------------|
| **Unit cell (a x b x c) [Å]**: 184.0 x 60.7 x 67.4 |
| **Data statistics** |
| Resolution [Å]: 20.0–2.25 |
| No. of observations/unique reflections: 68906/32603 |
| Completeness (total/high%): 91.3/92.4 |
| R_screw (total/high%): 4.2/19.9 |
| (I/σI) (total/high%): 13.4/4.2 |
| Refinement statistics |
| Resolution range (Å): 20.0–2.25 |
| Included amino acids: αMet1–αAla187, αLeu193–αArg267, βThr2–βArg394 |
| Mutated amino acids: βA169L, βC170W |
| r.m.s. deviation bonds/angles (Å/deg): 0.017/2.5 |
| Mean coordinate error estimated from a Luzzatti plot (Å): 0.28 |

| Table II |
|---------------------------------------------------------------|
| **Crystal parameters, data collection, and refinement statistics** |
| **Unit cell (a x b x c) [Å]**: 184.0 x 60.7 x 67.4 |
| **Data statistics** |
| Resolution [Å]: 20.0–2.25 |
| No. of observations/unique reflections: 68906/32603 |
| Completeness (total/high%): 91.3/92.4 |
| R_screw (total/high%): 4.2/19.9 |
| (I/σI) (total/high%): 13.4/4.2 |
| **Refinement statistics** |
| Resolution range (Å): 20.0–2.25 |
| Included amino acids: αMet1–αAla187, αLeu193–αArg267, βThr2–βArg394 |
| Mutated amino acids: βA169L, βC170W |
| r.m.s. deviation bonds/angles (Å/deg): 0.017/2.5 |
| Mean coordinate error estimated from a Luzzatti plot (Å): 0.28 |

| **Note** |
|---------------------------------------------------------------|
| a Highest resolution shell 2.35–2.25 Å. |
| b R_screw = Σ[fobs − fcalc]/Σfobs. |
| c R_work = Σ[fobs − fcalc]/Σfobs. |
| d Mean coordinate error estimated from a Luzzatti plot (Å). |

654 Ca pairs, excluding the CONNEXON domain residues, gives a r.m.s. deviation of 0.36 Å for Ca atoms and 0.13 Å for the 17 common α-ligand atoms, respectively (Fig. 1B). The low r.m.s. deviation value for the common α-ligand atoms indicates a high similarity of the α-ligand binding site and also for the enzyme, excluding the CONNEXON domain; this shows that the additional fluorine atom in F-IPP compared with IPP has no influence on the TRPS structure. In particular, it does not change the CONNEXON domain conformation (12, 14).
and the COMM domain. Although we cannot exclude the possibility that the nonobservation of the bridging water molecules is due to the lower resolution of the diffraction data, careful analysis of the final $mF_o - DF_i$ electron density map at a very low cut-off shows no indication for bound water molecules in this region. In contrast, the final $2mF_o - DF_i$ map shows clear backbone and side chain conformations for Asp$^{305}$ with $\Phi/\Psi$ torsion angles of $-91.2^\circ/16.5^\circ$ compared with $-90.8^\circ/-6.2^\circ$ in the TRPS$_{IPP}$ structure, indicating a small but significant conformational change because of the missing water contacts. Despite the changed conformation of Asp$^{305}$, the $\alpha$A169L/ $\beta$C170WF$_{IPP}$ complex contains a sodium ion. This fact can be attributed to the altered side chain conformation of the gating residues, because the side chain conformation of Phe$^{280}$ stabilizes the metal binding loop through hydrophobic interactions with the side chain rings of Phe$^{280}$ and proline Pro$^{307}$.

Based on the structure of the $\alpha$A169L/$\beta$C170WF$_{IPP}$ complex presented here, one can discuss the kinetic properties of both $\beta$Cys$^{170}$ mutants (see Table I) (4, 10). Both should have a more or less blocked tunnel, because the side chain of residue 170 points into the tunnel. A manually built $\beta$C170F model (figure not shown) based on the TRPS$_{IPP}$ structure indicates that introduction of the phenylalanine side chain retains a larger conformational freedom of the tunnel residues Phe$^{170}$, Tyr$^{279}$, and Phe$^{280}$ compared with the double mutant. This result is in line with the single turnover experiments on the $\beta$C170F mutant that showed, in contrast to the $\alpha$A169L/ $\beta$C170W mutant, only little indole accumulation in the $\alpha$-$\beta$ reaction (10). Introduction of the bulky tryptophan residue at the $\beta$Cys$^{170}$ position effects only the side chain conformations of the “gating residues” Tyr$^{279}$ and Phe$^{280}$. The rest of the $\beta$-subunit, in particular the COMM domain conformation, seems not to be affected by the $\beta$C170W mutation. Therefore, we postulate an unchanged wild type-like COMM domain conformation in the $\beta$C170F$_{IPP}$ or IPP complex; this would be in line with the observed turnover number of the $\alpha$-reaction and the activation of the $\alpha$-reaction by L-serine, neither of which changes upon the $\beta$C170F mutation. Furthermore, the $\beta$C170F mutant has the same L-serine affinity as wild-type TRPS (Table I). The only (small) kinetic differences between the wild-type and the $\beta$C170F mutant exist in the $\beta$- and the combined $\alpha$/$\beta$-reactions. Both require indole transport to the $\beta$-active site, but this would be hindered (and is consistent with our x-ray structure presented here) by the $\beta$C170F or $\beta$C170W mutation.

There are, however, striking differences between the $\beta$C170F and the $\alpha$A169L/$\beta$C170WF$_{IPP}$ complex in the activation of the $\alpha$-reaction upon formation of the aminoacrylate complex at the $\beta$-active site and in their affinity for serine (Table I). These must be caused (given our assumption that the COMM domain conformation is largely unaffected by the $\beta$C170W mutation) by the introduction of a leucine at position 169. As described in detail above, introduction of Leu$^{169}$ sterically induces a major rearrangement of the COMM domain; this results in an opening of the $\beta$-subunit, a withdrawal of catalytically important residues such as Glu$^{159}$ from the active site of the $\beta$-subunit, and a disruption of hydrogen bonding networks that connect the $\alpha$- and $\beta$-active sites or stabilize conformations of catalytically important residues such as Asp$^{305}$ (15). The latter is stabilized in the wild-type TRPS$_{IPP}$ complex by a number of water molecules in a conformation in which its carboxylate can interact...
with the hydroxyl group of the PLP-bound serine (external aldimine). It is interesting to note that the backbone conformation of βAsp$^{305}$ is the same in the βA169L/βC170W$^{P-IPP}$ complex and the wild-type aminoacrylate TRPS$^{P-IPP}$ complex (12). In this conformation, the βAsp$^{305}$ carboxylate is further away from PLP and cannot stabilize the bound serine, which is most
likely the reason for the 100-fold lower L-serine affinity of the βA169L/βC170W mutant.

In conclusion, the impaired channeling of the βA169L/βC170W mutant is caused by a complete occlusion of the tunnel by the βTrp\(^{170}\) side chain (despite a stabilization of the open conformation of the gating residues βTyr\(^{279}\) and βPhe\(^{280}\)). We predict that the situation is similar in the βC170F mutant. The remarkable differences in the kinetic parameters of the αβ-reaction in both of the βCys\(^{170}\) mutants, in particular the lack of activation of the α-reaction upon formation of the aminoacyl-ylate intermediate at the β-active site in the double mutant and the reduced serine affinity, seems to be caused mainly by the βA169L substitution. The longer βLeu\(^{169}\) side chain induces a rearrangement of the COMM domain. This affects the α ↔ β subunit communication as it is mediated by rigid body displacements of the COMM domain. In particular, the prerequisite for activation of the α-reaction is the closing of the β-subunit by a COMM domain movement. In addition to sterically preventing the closing of the COMM domain, the introduction of βLeu\(^{169}\) changes the COMM domain conformation such that the water molecules that hold βAsp\(^{305}\) in a position where it can interact with the hydroxyl group of the PLP-bound serine are destabilized. In the βA169L/βC170W mutant, βD305 is shifted away from PLP, which explains the 100-fold reduction in serine affinity.

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