Identification of the Geometric Requirements for Allosteric Communication between the α- and β-Subunits of Tryptophan Synthase*

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The pyridoxal 5'-phosphate-dependent tryptophan synthase α β complex is a paradigmatic protein for substrate channeling and allosteric regulation. The enzymatic activity is modulated by a ligand-mediated equilibrium between open (inactive) and closed (active) conformations of the α- and β-subunit, predominantly involving the mobile α loop 6 and the β-COMM domain that contains β helix 6. The α ligand-triggered intersubunit communication seems to rely on a single hydrogen bond formed between the carbonyl oxygen of βSer-178 of β helix 6 and the NH group of αGly-181 of α loop 6. We investigated whether and to what extent mutations of αGly-181 and βSer-178 affect allosteric regulation by the replacement of βSer-178 with Pro or Ala and of αGly-181 with either Pro to remove the amide proton that forms the hydrogen bond or Ala, Val, and Phe to analyze the dependence on steric hindrance of the open-closed conformational transition. The α and β activity assays and the equilibrium distribution of β-subunit catalytic intermediates indicate that mutations do not significantly influence the intersubunit catalytic activation but completely abolish ligand-induced α- to β-subunit signaling, demonstrating distinct pathways for α-β-site communication. Limited proteolysis experiments indicate that the removal of the interaction between βSer-178 and αGly-181 strongly favors the more trypsin-accessible open conformation of the α-active site. When the hydrogen bond cannot be formed, the β-subunit is unable to attain the closed conformation, and consequently, the allosteric signal is absent at the subunit interface.

Regulation of enzyme activity by interaction with metabolites is a key feature for the maintenance of cell life. This goal is achieved through conformational changes that, depending on protein complexity, involve tertiary and/or quaternary states. A common event that accompanies ligand binding to proteins is the transition between open and closed states (Ref. 1 and the references therein). Usually, the closed state is a more active conformation, bringing substrates and catalytic residues in close proximity, conferring special features to the active site environment, and protecting the productive catalysis from side reactions, generally involving water molecules.

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The α β complex of tryptophan synthase (TS) represents a particularly interesting case (2) because the open to closed transition serves a dual role: the increase in catalytic activity and the allosteric communication between subunits (3–9). The enzyme catalyzes the biosynthesis of L-tryptophan in two steps: the α-subunit cleaves indole-3-glycerol phosphate to di-glycer-aldehyde-3-phosphate and indole, which, intramolecularly channeled to the β-subunit (10, 11), reacts with β-serine in a β replacement reaction. The β-subunit contains a pyridoxal 5'-phosphate bound to Lys-87 as an internal aldime (12). During catalysis, a series of chromophoric intermediates are formed, including an external aldime, an α-aminoacrylate and quinonoid species (Scheme 1). The catalytic activity of α- and β-subunits is kept in phase by a fine-tuning associated with intersubunit communication (3, 13, 14). This allosteric regulation between active sites that are 20 Å apart (4, 10) involves alternative open and closed states of both the α- and β-subunit (5, 9, 15–17). The open to closed transition not only leads to an increase in the catalytic activity of the subunit but also is involved in signaling the catalytic state of one subunit to the other (3, 13, 14).

Structural and functional investigations on the wild type and on a variety of mutants of both subunits have allowed the unveiling of several aspects of this coordinated series of events at a molecular level (6–9, 15, 17–31). Specifically, the comparison of the three-dimensional structure of TS in the absence (both α- and β-subunits in the open state) and presence of a ligand in the α-active site and the catalytic intermediate α-aminoacrylate in the β-active site (both α- and β-subunits in the closed state) (5, 15) evidenced several structural differences: (i) the formation of hydrogen bonds between α loop 2 and β helix 6, (ii) the stabilization of α loop 6, which is crystallographically undetectable in the open form due to high mobility (5, 10), in a closed form (Fig. 1), (iii) the presence of a single hydrogen bond between α loop 6 and β helix 6, and (iv) the movement of part of the β-subunit, including β helix 6. This mobile portion of the β-subunit was called the COMM domain because of its role in communicating to the β-active site conformational changes occurring at the α-active site. The relevance of the α loop 6-β helix 6 hydrogen bond between the carbonyl oxygen of βSer-178 and the amide NH of αGly-181 for allosteric signaling was tested by introducing the S178P mutation (7). Indeed, the enzyme was still active but allosterically “knocked out.” A structural study showed several alterations of the α-β interface and the inability of the α-subunit to achieve the closed state even in the presence of α-subunit ligands (17).

1 The abbreviations used are: TS, tryptophan synthase; GP, D,L-glycerol-3-phosphate; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.
Because all mutants of βSer-178 are potentially able to form a hydrogen bond with αGly-181, we prepared the βS178A mutant to verify whether the disruption of the βSer-178-αGly-181 hydrogen bond was due to proline geometric features. Furthermore, by taking into account that αGly-181, but not βSer-178, is a conserved residue among TS from several species (7, 32), we have investigated the geometric requirements for the achievement of the βSer-178-αGly-181 hydrogen bond by preparing αG181A, αG181V, and αG181F, mutants characterized by a progressively increasing side chain steric hindrance, and αG181P.

Activity assays, distribution of catalytic intermediates, and limited proteolysis kinetics in the absence and presence of substrates and ligands concordantly indicate that the βSer-
nm was recorded using 1-cm-path length cuvettes. The reaction mixture contained 50 mM bicine (sodium N,N-bis(2-hydroxyethyl)glycine), 100 mM L-serine, 500 mM NaCl, 0.2 mM NADH, pH 7.8, and 80 units of lactate dehydrogenase from bovine heart. When present in the assay solution, the concentration of GP was 80 mM.

Spectrophotometric Measurements—Absorption spectra were collected using a Cary400 spectrophotometer (Varian) for a solution containing 50 mM bicine, 500 mM NaCl, and 100 mM L-serine, pH 7.8, in the absence and presence of 80 mM GP at 22 °C.

Limited Proteolysis—Solutions containing TS (1.22 mg/ml), 50 mM bicine, and 1 mM EDTA, at pH 7.8, were treated with 1 μg/ml TPCK-trypsin in the presence and absence of 80 mM GP and 100 mM L-serine at 22 °C (37). Digestions were stopped by boiling solutions for 3 min in the presence of SDS-PAGE loading buffer. Proteolysis products were resolved by SDS-PAGE, visualized by Coomassie Blue staining, and quantified by densitometric analysis carried out on digitalized images with Quantity One 4.2.0 Software (Bio-Rad).

RESULTS

Reverse α and β Replacement and β Elimination Activities—The rate of the reverse α reaction between tisole and glyceraldehyde-3-phosphate was found to be almost 2–3-fold slower for the α mutants and βS178P with respect to the wild type enzyme (Table I). Not surprisingly, the βS178A mutant exhibited only a 20% decrease. These findings suggest that mutations might prevent the formation of the closed, active conformation of the α subunit. The β replacement activity for each mutant was measured in the absence and presence of GP (Table I). All mutants are as active as the wild type, with the exception of βS178P and αG181P, which show slightly reduced β activity. GP exhibits a significant inhibitory effect on βS178A and a much smaller one on βS178P, whereas no inhibition is observed for all other mutants. These data indicate that the ligand-mediated intersubunit communication has been interrupted, suggesting that mutations do not allow the formation of the hydrogen bond between positions β178 and α181. In addition, these data confirm that intersubunit activation exploits a different pathway. In fact, αG181A, αG181F, and αG181V mutants exhibit unaltered catalytic activity despite the completely abolished ligand-induced allosteric regulation. The 1.4- and 2-fold decrease in the β activity observed for αG181P and βS178P, respectively, might be ascribed to slightly altered intersubunit contacts due to different conformations imposed by this mutation to α loop 6 and/or β helix 6. This decrease in activity is very modest when compared with the 30- and 100-fold reduction observed for the isolated α- and β-subunits, respectively (12).

In addition to the specific β replacement reaction, tryptophan synthase catalyzes a β elimination side reaction, in which L-serine deamination yields pyruvic acid and ammonia (12). The deaminase activity of wild type TS and mutants in the absence and presence of GP (Table I) indicates that the mutation causes a decrease of activity only for βS178P. Furthermore, addition of GP significantly reduces the deaminase activity of the wild type and βS178A mutant, whereas it has no effect on α181 mutants. For βS178P, a 30% reduction in the specific activity was observed in the presence of the allosteric effector.

Equilibrium Distribution between External Aldimine and α-Aminoaacrylate in the Absence and Presence of GP—The UV-visible spectra of the internal aldime for wild type TS and mutants (Fig. 2) show, for some mutants, the presence of a band at 325 nm, which is likely to be attributed to a derivative of the coenzyme with active site residues. This band also appears with aging in pyridoxal 5'-phosphate-dependent enzymes (38). When L-serine reacts with TS, an equilibrium between the catalytic intermediates α-aminoaacrylate and external aldime is attained, with the former being favored by α-subunit ligands, cesium ions, low pH, and high temperature (39–41). These
Reverse α, β replacement and β elimination activity of wild type and mutant TS in the absence and presence of the α ligand GP

Determination of the reverse α activity was performed at 37 °C using 1-mm-path length quartz cuvettes in 100 mM BTP, 100 mM NaCl, 2 mM indole, and 2 mM glyceraldehyde-3-phosphate, pH 7.8 (17). The α activity assay was carried out as described previously (34) at 37 °C. The β elimination activity was measured in a coupled assay with lactate dehydrogenase, according to Crawford and Ito (35), in 50 mM bicine, 100 mM L-serine, pH 7.8, at 22 °C in the absence (solid line) and presence of 0.1 μmol product in 20 min. The percentage of activity, reported in parentheses, is in reference to the wild type in columns 1, 2, and 4 and to the enzyme activity in the absence of GP in columns 3 and 5.

| Reverse α activity | β Replacement activity | β Elimination activity |
|--------------------|------------------------|-----------------------|
|                    | −GP                     | +GP                   | −GP                     | +GP                   |
|                    | units/mg                | units/mg              | units/mg                | units/mg              |
| Wild type          | 1651 ± 62 (100)         | 1204 ± 24 (100)       | 378 ± 14 (31)           | 6.31 ± 0.01 (100)     |
| βS178A             | 1359 ± 80 (82)          | 1282 ± 23 (106)       | 712 ± 33 (55)           | 5.02 ± 0.11 (79)      |
| βS178P             | 510 ± 05 (31)           | 609 ± 08 (51)         | 522 ± 03 (86)           | 2.31 ± 0.01 (37)      |
| αG181A             | 599 ± 64 (36)           | 1174 ± 35 (97)        | 1156 ± 30 (98)          | 5.35 ± 0.11 (85)      |
| αG181F             | 610 ± 55 (37)           | 1216 ± 02 (101)       | 1158 ± 17 (95)          | 6.08 ± 0.26 (90)      |
| αG181P             | 780 ± 82 (47)           | 825 ± 17 (69)         | 815 ± 37 (99)           | 7.12 ± 0.01 (113)     |
| αG181V             | 636 ± 130 (33)          | 1234 ± 38 (102)       | 1171 ± 51 (95)          | 7.78 ± 0.01 (123)     |

![Absorption spectra of the wild type TS and mutants](image)

**FIG. 2.** Absorption spectra of the wild type TS and mutants in the absence and presence of either L-serine or L-serine plus GP. The top left panel shows spectra for a solution containing 1.22 mg/ml enzyme and 50 mM bicine, pH 7.8; wild type, bold solid line; βS178A, diamond line; βS178P, dotted line; αG181A, dash-dot line; αG181V, dashed line; αG181F, light solid line; and αG181P, dash-dot-dot line. The other panels show absorption spectra recorded for solutions containing 1.22 mg/ml enzyme, 50 mM bicine, 500 mM NaCl, and 100 mM L-serine, pH 7.8, at 22 °C in the absence (solid line) and presence (dash-dot-dot line) of 80 mM GP.

Species absorb at 350 and 422 nm, respectively. The spectra of the equilibrium distribution of intermediates formed in the reaction of L-serine with the wild type and mutant enzymes, in the absence and presence of GP, are reported in Fig. 2. In the absence of GP, spectra obtained for the wild type and mutants are similar, except for αG181P and βS178P. In the former, the mutation favors the accumulation of the external aldime, whereas in the latter, the mutation stabilizes the aminoaacrylate species. Furthermore, differences in the relative absorption intensities for the bands at 422, 350, and 325 nm also arise from distinct amounts of the 325 nm species. Addition of GP to the βS178A mutant shifts the equilibrium toward the aminoaacrylate species, whereas in βS178P and α181 mutants, the distribution is unaffected by GP binding. The same pattern of effects was observed by monitoring the change in the amount of the external aldime as a function of GP recording the fluorescence emission at 500 nm upon excitation at 420 nm (data not shown).

**Effect of α- and β-Subunit Ligands on the Rate of Limited Proteolysis for Wild Type TS and Mutants**—In order to detect changes in the open to closed transition of α loop 6 upon mutation, the rate of tryptic cleavage of the α-subunit in the αβ complexes of wild type and mutant TS was measured in the absence and presence of α- and β-subunit ligands. The time course of proteolysis was analyzed by SDS-PAGE (Fig. 3A) and quantified by densitometric analysis (Fig. 3B). Proteolysis of the wild type and βS178A mutant in the absence of ligands results in rapid and almost complete cleavage of the α-subunit at a single site, αArg-188, to produce α-1 and α-2 fragments (34, 42, 43). Binding of GP to the α-subunit dramatically decreases the rate of proteolysis, as observed previously (43). Addition of L-serine in combination with GP almost totally protects the α-subunit, whereas L-serine alone has no effect (37, 43). On the contrary, αG181A and αG181P mutants are less susceptible to proteolysis in the absence of ligands with respect to the wild type. However, a more rapid and extensive degradation occurs when α and β ligands are bound. The proteolysis site in αG181F and αG181V mutants remains highly accessible in both the absence and presence of bound substrates. The α-subunit proteolysis rate of the βS178P mutant in the absence of L-serine and GP is slower than that of the wild type and is substantially unchanged in the presence of GP alone, whereas the addition of both ligands slightly reduces it. For all α mutants, the addition of L-serine alone makes αArg-188 more susceptible to cleavage, suggesting that binding of the β ligand may stabilize a more flexible and mobile conformation of α loop 6.

**DISCUSSION**

The characterization of mutants at the α181 and β178 position of *S. typhimurium* TS allows detailed analysis of the mechanism of intersubunit communication. In the proposed mecha-
nism of allosteric regulation between $\alpha$- and $\beta$-subunits (5, 7), $\alpha$ ligands interact with the $\alpha$ loop 6 residue $\alpha$Gly-184, reducing the flexibility of the loop and favoring the formation of the hydrogen bond between $\alpha$Gly-181 and $\beta$Ser-178. In turn, this hydrogen bond further reduces $\alpha$ loop 6 mobility, stabilizing the closed conformation of the $\alpha$-subunit. The relocation of $\alpha$ loop 6 causes a shift in the $\beta$ helix 6 of the COMM domain (5). The movement of $\alpha$ loop 6 is also propagated to $\alpha$Asp-60 via the hydrogen bond with $\alpha$Thr-183 (5, 15). This displacement orients $\alpha$Asp-60 for the efficient catalytic cleavage of indole-3-glycerol phosphate and concomitantly allows the formation of new interactions between $\alpha$ loop 2 and the COMM domain (5).

The present data support the hypothesis that the inter-subunit hydrogen bond between the $\alpha$Gly-181 amide proton and the $\beta$Ser-178 carbonyl oxygen atom is fundamental for the $\alpha$-$\beta$ communication and that the loss of this contact is sufficient to

FIG. 3. Effect of $\alpha$- and $\beta$-subunit ligands on the time course of proteolysis by trypsin of the wild type and mutant TS, as determined by SDS-PAGE (A) and quantitative densitometry analysis (B). Solutions of the wild type and mutant forms of TS (1.22 mg/ml in bicine buffer, pH 7.8) were treated with trypsin (1 $\mu$g/ml) at 22 °C for various times. $A$, times of incubation in the absence and presence of $\alpha$ and $\beta$ ligands are indicated. $B$, the fraction of cleavage as a function of incubation time with trypsin is reported in the absence of ligands (●) and in the presence of 80 mM GP (△), 100 mM L-serine (■), and both 80 mM GP and 100 mM L-serine (●). Lines through data points are intended as a guide for the reader. The fraction of cleavage is defined as the ratio between the intensity of the $\alpha$-1 fragment and the sum of the intensity of the $\alpha$ and $\alpha$-1 bands. The calculation disregards the $\alpha$-2 fragment (35).
abolish intersubunit allosteric regulation. In fact, when the interaction cannot be formed due to either lack of the donor amide proton (αG181P) or steric hindrance (αG181A, αG181V, and αG181F), the α ligands lose the ability to modulate β activity and the equilibrium distribution of catalytic intermediates at the β-active site. The proteolysis experiments demonstrate that all the allosterically silent mutants fail to attain the closed conformation of α loop 6 even in the presence of α and β ligands. These findings agree with the previously determined crystallographic structures of the βS178P (17) and αT183V mutants (30), which established that the lack of α loop 6 closure is the basis for the missing intersubunit signaling. When the equilibrium between closed and open conformations of the active site is shifted in favor of the open state (5, 44), the α activity is low, αGly-181 cannot be correctly positioned for hydrogen bonding with βSer-178, the hydrogen bond cannot be formed, and the signal of α-active site occupancy cannot be transduced to the β-subunit. The loss of influence of α-subunit ligands on the β-subunit was previously reported for other mutants of α loop 6 (αT183A, αT183V, and αR179L) (21, 30, 45). The mutated residues are involved in the ordering and maintenance of the correct position of α loop 6. Thus, these amino acid substitutions disrupt the stabilizing hydrogen bond network fundamental for loop relocation and formation of the αGly-181-βSer-178 interaction.

When no ligands are present, α loop 6 in the wild type and βS178A mutant is flexible and mobile, and αArg-188 is highly susceptible to proteolysis. Binding of GP to the wild type α-active site alters the conformation and flexibility of the loop and reduces the accessibility of the site of proteolysis. The closure of the loop caused by binding of GP can be further stabilized by an L-serine-induced conformational change in the β-subunit. This highlights that communication between α loop 6 and the β-active site is reciprocal. Our data indicate that the βS178A mutant retains ligand-induced communication between the α- and the β-site. On the contrary, Phe and Val mutations at position α181 prevent α loop 6 from attaining the closed conformation in the presence of bound ligands. In αG181A and αG181P, α loop 6 in the absence of ligands likely adopts a different conformation, characterized by an impeded interaction with trypsin. Upon binding of either GP alone or GP and L-serine, the α loop 6 of these mutants is not in the closed state, and its conformation seems to resemble the open state. Furthermore, the results confirm that no conformational changes in the α loop 6 of βS178P occur after a ligand binding, as evidenced by the crystal structure of the βS178P mutant, in which α loop 6 has no definite electron density in the absence and presence of α ligands (17). In addition, these experiments suggest that, similarly to the αG181P and αG181A mutants, the conformation of α loop 6 in βS178P is endowed with different flexibility with respect to the open state.

It is important to notice that all mutants, which had lost ligand-induced allosteric regulation, were almost as active as wild type TS in the β reaction, with the unique exception of S178P and αG181P. In these mutants, the substitution with proline is likely to distort the α loop 6 and β helix 6 conformation and modify contacts at the interface, as shown crystallographically for the former (17). In contrast, replacement of residues in α loop 2 or residues contacting α loop 2 was reported to critically reduce β activity without perturbing ligand-induced communication (24, 27, 29). Consequently, α loop 2 was proposed to be predominantly involved in the intersubunit interaction that leads to a reciprocal modulation of the activity of the α- and β-subunits within the tetramer (7). These findings collectively indicate that different pathways for intersubunit signal transmission do exist, which specifically involve α loop 2 and α loop 6. This conclusion is also supported by the recently determined structure of the TS α-subunit from E. coli (31).

Multiple sequence alignment of TS α and β chains from different sources reveals that βSer-178 is not conserved among the analyzed species (7), indicating that the side chain of L-serine is not relevant for allosteric communication and that, at this position, amino acids with different steric hindrance can be accommodated without perturbing the subunit interface. On the contrary, αGly-181 is invariant (32). The invariance of αGly-181 suggests that although the interaction is formed between backbone atoms, precise steric constraints exist due to the limited space to accommodate the α181 side chain in the closed form of α loop 6. We verified that serine to alanine substitution at position β178, which is not likely to alter the position of the carbonyl oxygen, does not perturb hydrogen bond formation and does not interrupt signal transmission. On the contrary, the results on α181 mutants show that glycine at position α181 is necessary for the maintenance of signal transmission, suggesting that only this residue allows α loop 6 closure and the correct positioning of the αG181 amide proton for formation of the hydrogen bond. The geometric restrictions imposed at position α181 were simulated by “in silico” mutagenesis. From inspection of these virtual structures, it appears that the closed conformation of α loop 6 is incompatible with the presence of side chains bulkier than a hydrogen atom. A detailed molecular dynamics modeling of the mobility of α loop 6 in the wild type and α181 mutants in the absence and presence of α-subunit ligands is currently under way and will be reported elsewhere.2

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