Crystal Structures of a New Class of Allosteric Effectors Complexed to Tryptophan Synthase*

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Tryptophan synthase is a bifunctional αβ2 complex catalyzing the last two steps of L-tryptophan biosynthesis. The natural substrates of the α-subunit indole-3-glycerolphosphate and glyceraldehyde-3-phosphate, and the substrate analogs indole-3-propanolphosphate and DL-α-glycerol-3-phosphate are allosteric effectors of the β-subunit activity. It has been shown recently, that the indole-3-acetyl amino acids indole-3-acetyglycine and indole-3-acetyl-L-aspartic acid are both α-subunit inhibitors and β-subunit allosteric effectors, whereas indole-3-acetyl-L-valine is only an α-subunit inhibitor (Marabotti, A., Cozzini, P., and Mozzarelli, A. (2000) Biochim. Biophys. Acta 1476, 287–299). The crystal structures of tryptophan synthase complexed with indole-3-acetyglycine and indole-3-acetyl-L-aspartic acid show that both ligands bind to the active site such that the carboxylate moiety is positioned similarly as the phosphate group of the natural substrates. As a consequence, the residues of the α-active site that interact with the ligands are the same as observed in the indole-3-glycerolphosphate-enzyme complex. Ligand binding leads to closure of loop L6 of the α-subunit, a key structural element of intersubunit communication. This is in keeping with the allosteric role played by these compounds. The structure of the enzyme complex with indole-3-acetyl-L-valine is quite different. Due to the hydrophobic lateral chain, this molecule adopts a new orientation in the α-active site. In this case, closure of loop L6 is no longer observed, in agreement with its functioning only as an inhibitor of the α-subunit reaction.

Allosteric effectors are defined as compounds that influence protein function by binding to a site distant to the functionally affected site. The mechanism of action is based on the stabilization of alternative tertiary or quaternary structures, depending on protein subunit composition. A paradigm of allosteric effectors is 2,3-diphosphoglycerate that binds to the central cavity of hemoglobin and decreases oxygen affinity by stabilizing the T state (1). In monomeric proteins noncompetitive inhibitors can be regarded as allosteric effectors and their action takes place via stabilization of tertiary conformations. The discovery of new allosteric effectors and the elucidation of the molecular basis of their action is relevant for the understanding of the plasticity of the protein matrix and the influence of cellular components for the control of metabolic pathways.

Tryptophan synthase (TRPS) (EC 4.2.1.20) is a tetrameric enzyme, consisting of two α- and two β-subunits arranged in a linear αβ2β mode (2), that catalyzes the last two steps of the biosynthesis of L-tryptophan in a concerted way. It is known that ligand-induced intersubunit signals keep the catalytic activities of the α- and β-active sites in phase (3–8). For recent reviews of the TRPS allosteric regulation, see Refs. 9–11. In particular, the α-subunit substrates indole-3-glycerolphosphate (IGP) and glyceraldehyde phosphate and the substrate analogs indole-3-propanolphosphate (IPP) and glyceraldehyde 3-phosphate (GP) are able to influence the activity of the β-subunit (3–6).

Detailed crystallographic studies of the wild-type enzyme and its mutants, in the absence and presence of α-subunit ligands were carried out (2, 12–16). The indole moiety is located in a hydrophobic cleft with the nitrogen atom N1 of the indole ring interacting with Asp235, a residue participating in α-subunit catalysis (17). No ionizable residues are present in the subsite occupied by the phosphate moiety of the ligands. However, the negative charge of the phosphate group is partially neutralized by the positive dipole moment created by helix αH8. (The secondary structure elements are used as defined by Schneider et al. (14)). The phosphate oxygen atoms form hydrogen bonds with several amino acids. In particular, the nitrogen amide protons of αGly234 and αSer235 interact with the phosphate oxygen atoms. Serine, αSer235, is localized at the end of α-helix αH8’ and was proposed to be involved in intersubunit regulation (18). Another important amino acid involved in the interaction with the phosphate group is αGly184’, located in loop L6. This structural element plays a crucial role for the propagation of the conformational events triggered by α-subunit ligands toward the β-active site (14). Loop L6 is highly mobile, as

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1 The abbreviations used are: TRPS, tryptophan synthase; IGP, indole-3-glycerolphosphate; IPP, indole-3-propanolphosphate; GP, DL-α-glycerol-3-phosphate; IAAA, indole-3-acetyl-L-amino acid; IAD, indole-3-acetyl-L-aspartate; IAG, indole-3-acetylglutamic acid; IAV, indole-3-acetyl-L-valine; TRPSPP, tryptophan synthase IPP complex; TRPSIGP, tryptophan synthase IGP complex; TRPSAD, tryptophan synthase IAD complex; TRPSAAG, tryptophan synthase IAG complex; TRPSIAV, tryptophan synthase IAV complex; αL6, loop 6 of the α-subunit of tryptophan synthase (14); βH6, helix 6 of the β-subunit of tryptophan synthase (14); COMM domain, (βGly182–βGly186) for the communication between α- and β-subunit (14); Bicine, N,N-bis(2-hydroxyethyl)glycine; r.m.s., root mean square.
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TABLE I  Crystal parameters, data collection, and refinement statistics

| Complex   | IAG      | IAD      | IAV      |
|-----------|----------|----------|----------|
| Protein Database code | 1K7E | 1K3U | 1K7F |
| Crystal parameters |          |          |          |
| Space group | C2     | C2      | C2      |
| Unit cell (Å) |  Å     | Å      | Å      |
| a | 184.0  | 184.0  | 183.7  |
| b | 60.0   | 60.0   | 59.1   |
| c | 67.5   | 67.2   | 67.5   |
| α | 94.6   | 94.5   | 94.7   |
| Data statistics |          |          |          |
| Resolution (Å) | 35.2–2.3  | 31.1–1.7 | 31.1–1.9 |
| No. of observations | 95,120 | 236,589 | 148,762 |
| No. of unique reflections | 29,178 | 77,570 | 53,470 |
| Compl. (total/high) (Å²) | 190.0/88.7 | 97.794/2.9 | 90.979/7.7 |
| R₁ (total/high) | 5.8/18.3 | 4.9/12.5 | 6.7/24.0 |
| R_f (total/high) | 11.3/22.2 | 14.4/25.5 | 9.7/23.3 |
| Refinement statistics |          |          |          |
| Resolution range (Å) | 20.0–2.3 | 20.0–1.7 | 20.0–1.9 |
| Included amino acids | αPhe²¹² | αPhe²¹² | αPhe²¹² |
| No. of protein atoms | 4944 | 5034 | 4948 |
| No. of waters | 237 | 712 | 150 |
| No. of ligand atoms | 32 | 36 | 35 |
| No. of metal ions | 1 | 1 | 0 |
| R_work, R_free (%) | 16.6/24.4 | 15.3/18.8 | 19.5/25.1 |
| Alternative side chain conformations | αGlu⁴⁰ | αGlu⁴⁰ | αGlu⁴⁰ |

° Completeness, R_work, and (I/obs) are given for all data and for the highest resolution shell: IAG, 2.4–2.3 Ǻ; IAD, 1.8–1.7 Ǻ; and IAV, 2.0–1.9 Ǻ.

evidenced by the lack of electron density in native enzyme structures (2, 14–16) and by limited proteolysis and protein engineering studies (19–21), and switches between an open and closed conformation (14). In the TRPS complexes with IPP or GP αL6 is closed, and there is a hydrogen bond between αThr¹⁵² located in αL6 and the catalytic residue αAsp⁶⁰. The closed state of the α-subunit is communicated to the β-subunit via several interactions with the COMM domain, a rigid but moveable domain (βGly⁴¹⁸–βGly⁴³⁹) of the β-subunit (14). A critical element of the intersubunit interface is the hydrogen bond between αGly¹⁸¹ and βSer¹⁷⁸, as functionally demonstrated (22) and structurally characterized in the accompanying paper (23).

In the course of an investigation aimed at the development of new inhibitors of the α-subunit of tryptophan synthase (24), eventually useful as herbicides (15, 25), a new class of α-subunit ligands of tryptophan synthase was discovered, the indole-3-acetyl amino acids (IAAA). These molecules are also physiologically interesting, since several indole-3-acetyl amino acid conjugates are storage forms for the auxin indole-3-acetic acid (26), a very important plant hormone (27). In particular, it was found that some IAAA, as indole-3-acetylglucose (IAG) and indole-3-acetyl-L-aspartate (IAD), are potent allosteric effectors of tryptophan synthase, whereas indole-3-acetyl-L-valine (IAV) and indole-3-acetyl-L-alanine are inhibitors of the α-subunit activity (24). In the present work, the three-dimensional structures of the complexes between tryptophan synthase and IAG, IAD, and IAV, respectively, are presented. The investigation of the interaction of these derivatives with the α-subunit of tryptophan synthase is relevant both for the understanding of the residues that are involved in the intersubunit communication and the design of more potent allosteric effectors and herbicides.

MATERIALS AND METHODS

Crystallization and Substrate-Complex Preparation—IAG, IAD, and IAV were purchased from Sigma and used without further purification. TRPS was purified as described previously (28). The protein was stored at a concentration of 10 mg/ml in a solution containing 50 mM Na-Bicine, pH 7.8, 10 mM Na-EDTA, 1 mM dithioerythritol, and 20 μM pyridoxal phosphate. Crystals were grown in the dark at room temperature within 1 week using the hanging drop method by mixing equal volumes (2–4 μl) of protein and reservoir solutions. The latter contained 9–12% polyethylene glycol 8000, 1.5 mM spermine, 1 mM EDTA, 50 mM Na-Bicine buffer, pH 7.8. The IAD, IAG, and IAV complexes were generated by soaking TRPS crystals for 10–30 min in cryo-protectant solution (100 mM Na-Bicine buffer, pH 7.8, 15% (v/v) polyethylene glycol 8000, and 20% (v/v) glycerol) containing 10–25 mM IAD, IAG, or IAV, respectively before flash-freezing in liquid nitrogen. To increase the IAV solubility 15% (v/v) Me₂SO were added to the soak solution.

X-ray Data Collection and Refinement—Diffraction data of the TRPS/IAA⁺ complexes were collected at beamlines BW7B at EMBL c/o DESY, Hamburg (IAD, IAG) and at ID14–1 at the European Synchrotron Radiation Facility, Grenoble, France (IAY) using wavelengths of 0.842 or 0.934 Å, and a MAR 345 image plate or MAR CCD detector, respectively. The crystals were kept at 100 K during measurements. The diffraction data were processed with XDS and scaled with XSCALE (29). Data statistics are given in Table I. Refinement was started to 2.5-Å resolution with CNS 1.0 (30) by performing rigid-body and simulated-annealing steps. The coordinates of the wild-type TRPS structure (Protein Database accession code 1Q0P (16)) were used as a starting model, omitting the coordinates of loops αL2 and αL6, IPP, the cofactor pyridoxal phosphate, and all water molecules. In each case the final model was obtained by cyclic rounds of manual model building with the program O (31) and Maximum-Likelihood refinement with the program REFMAC (32) using all reflections to maximum resolution. Water molecules were incorporated by "ARP" (33) using the automatic
cut-off option. All waters were checked manually and removed if displaying unusual hydrogen-bonding geometry. Apart from residues at the C termini of both polypeptide chains, two parts of the TRPSIAV structure are too disordered to be built into the electron density: loop \( \text{H}9251 \text{L}6 \) (\( \text{H}9251 \text{Arg}179 \text{–} \text{H}9251 \text{Leu}192 \)) and a part of loop \( \text{H}9252 \text{L}8 \) (\( \text{H}9252 \text{Gly}261 \text{–} \text{H}9252 \text{Gly}274 \)). Only weak density was found for several residues of loop \( \text{H}9251 \text{L}2 \), but we decided

![Fig. 1. 2mFo – DFo electron density for the TRPSIAAA structures at the α-active site. The density is shown at 1σ-contouring for the IAAA molecule, αGlu\(^{49} \text{, } \alpha\text{Asp}^{60} \text{, } \alpha\text{Thr}^{183} \text{, and for water molecules, which form hydrogen bonds with the α-ligand. The hydrogen bonds of the enzymatic important amino acids αGlu}\(^{49} \text{ and αAsp}\(^{60} \text{ are shown as dashed lines (see Table II). The figure was prepared using BOBSCRIPT} \text{, MOLSCRIPT} \text{, and RASTER3D.} \)](image)

![Fig. 2. Stereo plot of the structure superposition of TRPSIPP, TRPSIAAD, and TRPSIAV. The C-atom trace is shown for TRPSIPP; the α-subunit is colored in gray, loops αL2 and αL6 in cyan, and the β-subunit in pink. The IPP, IAD, and IAV ligand C-atoms are colored in yellow, green, or orange, respectively. Nitrogen atoms are colored in blue, oxygen atoms are colored in red, and phosphate atoms are colored in magenta. The figure was prepared using MOLSCRIPT and RASTER3D.](image)
not to remove this part from the final model. Also residues αPhe⁶⁰, βLys¹⁷⁵, βArg¹⁷⁵, and βTyr⁷⁷⁷ are modeled as alanine. The higher flexibility of this region is reflected in significantly higher temperature factors. In case of the TRPS⁸⁹ structure a number of amino acids have two conformations. Data and refinement statistics are given in Table I. The coordinates and structure factor amplitudes have been deposited with the Protein Database (34) (accession codes 1K3U, 1K7E, 1K7F).

Structure Superposition—To investigate the influence of the different α-active site ligands on the allosteric communication, we superimposed the TRPS₁₆₆ structures with each other and also with the wild-type TRPS₁₆₆ (16) structure. The superposition was done with the program O (31) using all common Cα atoms coordinates of both structures except the Cα atoms belonging to the COMM domain (βGly¹⁰²–βGly¹⁰⁸). The resulting r.m.s. deviations were calculated with the program BRAGI (35).

RESULTS AND DISCUSSION

The IAAAs bind at the IGP substrate binding site (13, 16) in the TRPS α-subunit (Fig. 1, A–C). No indication for a different binding site is found in the electron density of any of the three IAA ligand complexes. Also, the overall TRPS topology is not effected by the binding of the indole-3-acetyl amino acids compounds. Fig. 1 shows the electron density at the α-active site for the IAD, IAG, and IAV complexes. All three ligands could be placed easily in the electron density. The distances of the hydrogen bonds between the IAA ligands and the protein are shown in Table II.

A superposition of the new TRPS₁₆₆₆ structures with the TRPS₁₆₆₆₆ structure was calculated, excluding the Cα-atoms of loop αL₂, αL₆, and the COMM domain (Fig. 2), and, in the case of the TRPS₁₆₆₆ structure, also the Cα-atoms of the missing loop βL₈ were not used in structure superposition calculation either. We chose the TRPS₁₆₆₆ structure for the comparison because the IPP ligand, which binds with high affinity to the TRPS α-subunit (36), served as a lead compound for the α-subunit effector/inhibitor mechanism (24). The result of this superposition is shown in Figs. 2 and 3. There are no larger structural differences between the IAG and IAD TRPS complexes (Fig. 2). In particular, both TRPS₁₆₆₆ structures adopt, apart from some surface loops, the same subunit conformations observed in the TRPS₁₆₆₆ structure, as shown by the superposition between the TRPS₁₆₆₆ and TRPS₁₆₆₆₆ structure (Fig. 3). This is also valid for the loop αL₆, which is in the closed conformation in all three structures.

A common feature for all three IAA ligands is the substitution of the IPP/IGP phosphate group on the N-terminal side of α-helix αH₈ with a carboxylate group. In each case the two carboxylate oxygen atoms replace the OP1 and OP2 phosphate oxygen atoms, in the IAG complex the OP2 and OP3 oxygen atoms, and in the IAD complex the OP1 and OP3 oxygen atoms. In the case of the IAD ligand the additional side chain carboxylate group also replaces one phosphate oxygen and a water molecule that is found near the phosphate group in the TRPS₁₆₆₆₆ structures. The different IAA ligand binding modes indicate less specific binding of the carboxylate compared with the IPP/IGP phosphate group. This is in line with the finding that the binding affinity of IAD and IAG is about 50-fold lower than of phosphate-containing ligands (24). Based on the common orientation of the negatively charged carboxylate group at the N-terminal side of α-helix αH₈, the indole ring of the IAG and IAD ligands adopts a similar orientation as in the TRPS₁₆₆₆₆ structures, although the acetyl side chain has a different orientation than the propyl side chain of IPP. As a consequence, both IAA compounds have a shorter hydrogen bond between the indole nitrogen atom and the carboxylate group of aspartate, αAsp⁶⁰, and the acetyl oxygen atom of the IAG and IAD ligands points in the same direction as the IGP OH₃’ hydroxyl group. Since IAG lacks the IAD aspartate side chain, the IAG ligand has less negative charges and is shifted by approximately 0.6 Å from the IPP/IGP phosphate binding loop toward the catalytically important glutamate αGlu⁴⁹ (8, 37, 38). Interestingly, in the TRPS₁₆₆₆₆ complex αGlu⁴⁹ has two conformations, an “inactive” one interacting with αTyr¹⁷³ (2.6 Å), and an “active” one that forms a 2.7-Å hydrogen bond with the acetyl oxygen of IAG (see Table II and Fig. 1). This nomenclature reflects the finding that an active site conformation of αGlu⁴⁹ was also observed in case of the wild-type TRPS₁₆₆₆ (16) and the mutant αD60NIGP (39), βS178PIGP (23), and βS178PIAG (23) complexes. In the case of the IGP and GP molecules the α-ligand serves as a proton donor, whereas the hydrogen bond between the IAG acetyl oxygen and the αGlu⁴⁹ carboxylate group implies that the αGlu⁴⁹ carboxylate group is protonated. This has a direct consequence for the mechanism of the α-reaction: the cleavage of the C3-C3’ carbon-carbon bond in IGP (17, 39) is activated by tautomerization of the indole ring to yield the indolene tautomer. This tautomerization is probably facilitated by two basic groups B₃H and B₄, by “push-pull” general acid-base catalysis. B₃H protonates the indole ring at C₃, while B₄ abstracts the proton of the N₁ nitrogen atom of the indole ring. The aldol cleavage is catalyzed by B₅, which abstracts a proton from the 3’-hydroxyl to yield indole and glyceraldehyde 3-phosphate. Since the TRPS₁₆₆₆ structure implies that the αGlu⁴⁹ carboxylate group is protonated, we have for
the first time experimental evidence that αGlu⁴⁹ may act as the putative residues B₁-H and B₃ of the α-reaction.

The binding mode of the IAV compound is very different from that of the IAG and IAD ligands, since the hydrophobic valine side chain is bound in a hydrophobic pocket near the α-active site, formed by the αPhe²², αIle⁶⁴, and αLeu¹⁰⁰ side chains. The re-orientation of the IAV molecule leads to a rotation of the indole ring by nearly 90° around the C₇–C₈ carbon bond. In this conformation the indole nitrogen forms only a weak hydrogen bond (3.1 Å) to aspartate, αAsp⁶⁰, and a new hydrogen bond to the carbonyl oxygen of alanine, αAla⁵⁹ (2.6 Å). As a consequence, the acetyl carbon atoms point toward the side chain of phenylalanine, αPhe²¹², which, therefore, is also shifted. As a direct consequence of this different binding mode and side chain movement, loop αL₆ is not closed in the TRPSIAV structure. Apart from the side chain shift of phenylalanine, αPhe²¹², the side chain of threonine, αThr¹⁸³, would clash with the re-oriented IAV indole ring in a closed loop αL₆ conformation. This missing loop closure is the reason for the, compared with the TRPSIAG and TRPSIAD, structures, different COMM domain conformation, as indicated by the large r.m.s. deviation values for several parts of the COMM domain (Fig. 3). This second COMM domain conformation is in good agreement (r.m.s. deviation plot not shown) with the observed open conformation in the wild-type TRPSIGP structure (16). Interestingly, in contrast to the TRPSIAG and TRPSIAD, but in agreement with the TRPSIGP structures, there is no electron density for a sodium ion, although the soaking conditions (especially the pH) were the same for all three IAAA complexes. This observation might support our belief that the enzyme loses the sodium ion upon

**Fig. 3.** Cᵦ-r.m.s. deviation plots of different wild-type TRPS structures for the α- (left panel) and β- (right panel) subunits. A, comparison of TRPSIAD and TRPSIAV; B, comparison of TRPSIAD and TRPSIPPF; and C, comparison of TRPSIPPF and TRPSIAV.
opening of the COMM domain (16). The reason for the reduced sodium affinity of the open COMM domain conformation might be the higher flexibility of the sodium binding loop (\(\beta_{305-307}\)) as indicated by higher temperature factors in both sodium-free TRPS structures (data not shown), which is a consequence of the loss of side chain interactions of these loop region with COMM domain residues (16).

CONCLUSIONS

The analysis of the structural features of the TRPSAAA complexes allows to better define the mechanism for intersubunit regulation mediated by \(\alpha\)-subunit ligands, and, in particular, the residues that participate in the communication of allosteric signals. IAG and IAD share the IPP/IPG binding mode at the \(\alpha\)-subunit, in which the terminal carboxylate moiety replaces the phosphate group, the acetyl oxygen atom points in the same direction as the IGP OH\(^{-}\) hydroxyl group, the indole ring interacts with \(\alpha\)Asp\(^{60}\), and loop \(\alpha\)Lo6 is in the closed state. The hydrogen bond between the acetyl oxygen atom of IAG and the carboxylate oxygen atom of \(\alpha\)Glu\(^{69}\) indicates that the latter is protonated. This has important implications for the mechanism of the \(\alpha\)-reaction as it suggests a double role for \(\alpha\)Glu\(^{69}\) as a push-pull acid-base catalyst. The binding mode of IAV is very different, due to rearrangement of the lateral chain of the amino acid in a hydrophobic cavity. This results in loss of the hydrogen bond between the indole nitrogen and \(\alpha\)Asp\(^{60}\). In this complex, the loop \(\alpha\)Lo6 of TRPS remains in the open conformation and the intersubunit communication is lost.

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