Cryo-crystallography of a True Substrate, Indole-3-glycerol Phosphate, Bound to a Mutant (αD60N) Tryptophan Synthase \( \alpha_2\beta_2 \) Complex Reveals the Correct Orientation of Active Site \( \alpha\text{Glu}49^* \)

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The reversible cleavage of indole-3-glycerol by the \( \alpha \)-subunit of tryptophan synthase has been proposed to be catalyzed by \( \alpha\text{Glu}49 \) and \( \alpha\text{Asp}60 \). Although previous x-ray crystallographic structures of the tryptophan synthase \( \alpha_2\beta_2 \) complex showed an interaction between the carboxylate of \( \alpha\text{Asp}60 \) and the bound inhibitor indole-3-propanol phosphate, the carboxylate of \( \alpha\text{Glu}49 \) was too distant to play its proposed role. To clarify the structural and functional roles of \( \alpha\text{Glu}49 \), we have determined crystal structures of a mutant (\( \alpha\text{D60N} \) \( \alpha_2\beta_2 \) complex in the presence and absence of the true substrate, indole-3-glycerol phosphate. The enzyme in the crystal cleaves indole-3-glycerol phosphate very slowly at room temperature but not under cryo-conditions of 95 K. The structure of the complex with the true substrate obtained by cryo-crystallography reveals that indole-3-glycerol phosphate and indole-3-propanol phosphate have similar binding modes but different torsion angles. Most importantly, the side chain of \( \alpha\text{Glu}49 \) interacts with 3-hydroxyl group of indole-3-glycerol phosphate as proposed. The movement of the side chain of \( \alpha\text{Glu}49 \) into an extended conformation upon binding the true substrate provides evidence for an induced fit mechanism. Our results demonstrate how cryo-crystallography and mutagenesis can provide insight into enzyme mechanism.

Various biochemical and physical methods, including site-directed mutagenesis, kinetic analysis, and x-ray crystallography, have revealed many mechanistic aspects of the bifunctional bacterial tryptophan synthase \( \alpha_2\beta_2 \) complex (EC 4.2.1.20) (for reviews see Refs. 1 and 2). These studies provide evidence that \( \alpha \)-subunit residues Asp60 and Glu49 catalyze the reversible cleavage of IGP\(^1\) to glyceralddehyde 3-phosphate and indole (\( \alpha \)-reaction; see Scheme 1) (3–6). Recent x-ray crystallographic studies, however, demonstrated that whereas the side chain of \( \alpha\text{Asp}60 \) interacts with the bound inhibitor IPP, the side chain of \( \alpha\text{Glu}49 \) is too distant (\( \sim6 \) Å) from IPP to play its proposed role (7). However, IPP lacks the hydroxyl groups of the true substrate IGP, and this could affect the orientation of \( \alpha\text{Glu}49 \). To investigate interactions between active site residues of the \( \alpha \)-subunit and IGP, we have formed the enzyme-IGP complex using the mutant (\( \alpha\text{D60N} \) \( \alpha_2\beta_2 \) complex, in which the other catalytic residue \( \alpha\text{Asp}60 \) is replaced with Asn. This enzyme has no measurable activity in the reaction catalyzed by the \( \alpha \)-subunit but retains substantial \( \beta \)-subunit activity (5). Here we present crystal structures obtained by cryo-crystallography of the mutant (\( \alpha\text{D60N} \) tryptophan synthase in the presence and absence of the bound true substrate IGP.

EXPERIMENTAL PROCEDURES

The expression and purification of the mutant (\( \alpha\text{D60N} \) tryptophan synthase \( \alpha_2\beta_2 \) complex from *Salmonella typhimurium* has been described (5). Although four different substitutions were made at position 60, only the \( \alpha\text{D60N} \) \( \alpha_2\beta_2 \) complex yielded crystals suitable for further study. Crystals of the \( \alpha\text{D60N} \) \( \alpha_2\beta_2 \) complex were grown under the conditions used previously for crystallization of the wild-type enzyme (50 mM \( \mathrm{N}_2\mathrm{H}_2\mathrm{bis}(2\text{-hydroxyethyl})\)glycine, 1 mM Na-EDTA, 0.8–1.5 mM spermine, and 12% polyethylene glycol 8000 adjusted to pH 7.8 with NaOH) (8) and belong to the space group C2. \( \alpha\text{D60N} \) crystals grown in the presence of \( \mathrm{Na}^+ \) were soaked for 1–2 days in a standard \( K^+ \)-soaking solution containing 100 mM \( \mathrm{N}_2\mathrm{H}_2\mathrm{bis}(2\text{-hydroxyethyl})\)glycine (pH 7.8 titrated with KOH), 1 mM EDTA, and 20% polyethylene glycol 8000 (9), and these \( K^+ \)-soaked crystals were used for further soaking experiments with ligands (see below).

Preliminary x-ray diffraction data collected at room temperature from IGP-soaked \( \alpha\text{D60N} \) crystals indicated that there was no electron density for IGP bound to the \( \alpha \)-active sites of the enzyme and suggested that the crystalline enzyme still catalyzes the slow cleavage of IGP. Therefore, to eliminate low enzymatic activity of crystalline tryptophan synthase, we have flash frozen substrate-bound \( \alpha\text{D60N} \) crystals and then collected diffraction data from 95 K.

Substrate IGP was prepared enzymatically with tryptophan synthase from indole and glyceraldehyde 3-phosphate (10). IGP was introduced into \( \alpha\text{D60N} \) crystals by soaking the crystals in a IGP soaking solution (0.4 mM IGP with the standard solution) for 1 day. The crystals were then transferred to a IGP soaking solution for 30 min into each of a series of solutions having 5, 10, 15, 20, and 25% glycerol as cryoprotectant and then were flash frozen for data collection. To evaluate the effects of the \( \alpha\text{D60N} \) mutation on the structure, the unliganded \( \alpha\text{D60N} \) crystals prepared as above were also flash frozen and subjected to data collection. Diffraction data were collected at 95 K on a Raxis IIC imaging plate system mounted on a Rigaku RU-200 rotating anode x-ray generator operating at 50 kV and 100 mA. All diffraction data were integrated with DENZO and scaled with SCALEPACK (11). Table I summarizes data statistics and refinement statistics. In refining these structures with X-PLOR (12), the 2.0 Å wild-type structure determined in the presence of \( K^+ \) at room temperature (Protein Data Bank entry 1TTQ) (9) served as a starting model. The starting model was divided into three substructures (corresponding to the \( \alpha \)-subunit and N- and C-terminal domains of the \( \beta \)-subunits) and subject to a rigid body refinement followed by simulated annealing refinement. Subsequent manual rebuilding was carried out using the program O (13). At this stage, \( F_o - F_c \) maps of \( \alpha\text{D60N}-\text{IGP} \) revealed the bound IGP in the \( \alpha \)-subunit. An idealized model of IGP was modeled using QUANTA and fitted to the density then refined again by simulated annealing refinement followed by positional and temperature factor refinements.

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‡ The abbreviations used are: IGP, indole-3-glycerol phosphate; IPP, indole-3-propanol phosphate.
RESULTS AND DISCUSSION

**Glu49 Interacts with the True Substrate IGP**—Structural comparisons indicate that αD60N and the starting model (the wild-type structure) are almost identical within root mean square deviation of 0.52 Å for the main chain atoms of all residues, suggesting that the mutation does not produce any significant structural perturbations. Subsequent comparisons between αD60N and αD60N-IGP also indicated that there are no noticeable conformational changes induced by the binding of IGP. The αD60N-IGP structure is similar to that of the wild type-IPP complex (14) in that loop 6 (residues 179–191) is invisible and loop 2 (residues 53–62 including residue 60) is highly disordered.

**TABLE I**

| Data statistics | αD60N | αD60N-IGP |
|-----------------|-------|-----------|
| Resolution (Å)  | 1.9   | 2.0       |
| No. of unique reflections | 49520 | 45501 |
| Average redundancy | 3.7   | 4.3       |
| Refinement Rmerge (%)
| 5.9 (25.0) | 5.7 (42.4) |
| Completeness (%) | 86.4 (62.6) | 92.8 (79.4) |

**Cell parameter**

| a (Å) | 183.5 | 182.6 |
| b (Å) | 59.4  | 59.7  |
| c (Å) | 67.3  | 67.4  |
| β (deg) | 94.6 | 94.6 |

**Refinement statistics**

| Resolution range (Å) | 8.0–1.9 | 8.0–2.0 |
| No. of reflections (>2σ) | 43791 | 44729 |
| Average B-factors (Å²) | 27.6 | 26.4 |
| No. of protein atoms | 4896 | 4848 |
| No. of solvent water | 393 | 343 |
| Cation | 1 (K⁺) | 1 (K⁺) |
| R factor/Rfree (%) | 23.8/29.8 | 22.0/28.3 |

**Root mean square deviations from ideals**

| Bond length (Å) | 0.007 | 0.008 |
| Bond angle (deg) | 1.61 | 1.96 |

* Rmerge = Σ[I(I) − <I>]/ΣI. The values in parentheses are those in a shell of 2.0–1.9 Å for αD60N and of 2.1–2.0 Å for the αD60N-IGP.

Due to the variations in the quality of the electron density, residues 177–189 and 177–195 of the α-subunit were not modeled in the structure of αD60N and the αD60N-IGP, respectively.

Fig. 1A shows the electron density map for IGP and nearby residues in the αD60N-IGP structure. The overall binding site is almost identical to that of other structures complexed with IPP that have been determined previously (7, 14). However, the αD60N-IGP structure reveals that the side chain of Glu49 adopts an extended conformation and is within 2.8 Å from the hydroxyl group of C39 of IGP (Fig. 1B). This interaction has been proposed in the mechanism of α-reaction (Scheme 1) but has not been seen in other structures with the bound substrate analog IPP (7, 14). The positions of Asn60 in the αD60N-IGP structure is almost identical to that of the IPP-complexed structure, but Asn60 is very mobile (refined temperature factors for the all atoms in Asn60 are about 80 Å²).

Despite the similarity in the overall binding site, there are differences in positions (0.9–1.4 Å) of the corresponding atoms.
including phosphate, C1, C2, and C3 but not in positions of indole ring (Fig. 1B). The torsion angle around the C2'−C3' bond is 169° for IGP but 28° for IPP. Because there is relatively weak electron density for the C1' atom of IGP, we initially modeled IGP according to the torsion angles of IPP. This fitting keeps the hydroxyl group at C2' away from its corresponding density, suggesting that the current C1' atom represents the reliable position in IGP. Interaction between αGlu49 and the C3' hydroxyl group is unambiguous based on clear density. The C2' hydroxyl group is in L-enantiomeric configuration but does not form any hydrogen bonds with active site residues.

Other Active Site Residues around Bound IGP—Fig. 1 also shows that the phenolic hydroxyl of αTyr175 interacts with the C3' hydroxyl group of IGP. However, the finding that the mutant enzyme in which αTyr175 is replaced by Phe (Y175F) has substantial activity indicates that αTyr175 is not essential for catalysis or substrate binding (5). Early studies showed that whereas the Y175C mutant was inactive, a second site revertant (Y175C/G211E) exhibited partial activity in the -subunit (5) led to the conclusion that the C3' hydroxyl group of IGP interacts with the active site. This conclusion supports the view that Tyr175 serves a structural role but is not an essential catalytic residue.

Catalytic Role of α-Subunit αGlu49—The structure of the inactive mutant (αD60N) with bound IGP reveals two new structural features that were not observed in structures in the presence of substrate analog IPP. These features are the interaction between αGlu49 and the hydroxyl group at C3' and the location of the hydroxyl groups at C2' and C3'.

There is substantial evidence from site-directed mutagenesis studies that αGlu49 (4, 6) and αAsp60 (5) are catalytic bases in the reaction catalyzed by the α-subunit. The mechanism of this reaction (Scheme 1), based on previous proposals (3–5), suggested that αAsp60 facilitates tautomerization of the indole ring of IGP (I) to form II and that αGlu49 abstracts a proton from the C3' hydroxyl group of the glycerolphosphate moiety to form III.

Although previous x-ray crystallographic structures of the tryptophan synthase αβ6 complex demonstrated interaction between the carboxylate of αAsp60 and the indole nitrogen of the bound inhibitor (IPP), the side chain of αGlu49 was too distant to play its proposed role (7). The carboxylate of αGlu49 was folded away from the IPP and was located approximately 6.1 Å from the modeled C3' hydroxyl group (Fig. 1B). In the new structure, the side chain of αGlu49 interacts with the C3' hydroxyl group of IGP as proposed. This interaction is made possible by the movement of the side chain of αGlu49 into an extended form in the presence of IGP. These results provide an example of an induced fit mechanism in which an active site residue adopts a catalytically correct orientation when a substrate is bound to the active site. This type of induced fit is much smaller and more localized than the larger changes observed in some other structures which involve domain movement, loop closure, or conversion from an open to a closed conformation.

REFERENCES
1. Miles, E. W. (1991) Adv. Enzymol. Relat. Areas Mol. Biol. 64, 93–172
2. Miles, E. W. (1995) in Subcellular Biochemistry: Proteins: Structure, Function, and Protein Engineering (Biswas, B. B. & Roy, S., eds) Vol. 24, pp. 207–254, Plenum Press, New York
3. Kirschnerr, K., Lanne, A. N. & Strasser, A. W. M. (1991) Biochemistry 30, 472–478
4. Miles, E. W., McPhie, P. & Yutani, K. (1988) J. Biol. Chem. 263, 8611–8614
5. Nagata, S., Hyde, C. C. & Miles, E. W. (1989) J. Biol. Chem. 264, 6268–6275
6. Yutani, K., Ogashara, K., Tsujita, T., Kanemoto, K., Matsumoto, M., Tanaka, S., Miyashita, A., Matsushiro, A., Sugino, Y. & Miles, E. W. (1987) J. Biol. Chem. 262, 13429–13433
7. Rhee, S., Parris, K. D., Hyde, C. C., Ahmed, S. A., Miles, E. W. & Davies, D. R. (1997) Biochemistry 36, 7664–7680
8. Ahmed, S. A., Miles, E. W. & Davies, D. R. (1985) J. Biol. Chem. 260, 3716–3718
9. Rhee, S., Parris, K. D., Ahmed, S. A., Miles, E. W. & Davies, D. R. (1996) Biochemistry 35, 4211–4221
10. Kawasaki, H., Bauerle, R., Zen, G., Ahmed, S. A. & Miles, E. W. (1993) J. Biol. Chem. 268, 10678–10683
11. Otwinowski, Z. (1993) Data Collection and Processing (Sawyer, L., Isaacs, N. & Bailey, S., eds) pp. 56–62, Science and Engineering Research Council, Warrington, UK
12. Brugger, A. T. (1992) X-PLOR, version 3.1, a system for X-ray crystallography and NMR, Yale University Press, New Haven, CT
13. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991) Acta Cryst. Sec. A 47, 110–119
14. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W. & Davies, D. R. (1988) J. Biol. Chem. 263, 17857–17871
15. Heiniksi, D. R. & Yanofsky, C. (1963) J. Biol. Chem. 238, 1043–1048