Molecular insights into substrate recognition and catalysis by tryptophan 2,3-dioxygenase

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Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) catalyze the oxidative cleavage of the L-tryptophan (L-Trp) pyrrole ring, the first and rate-limiting step in L-Trp catabolism through the kynurenine pathway (1–3). In addition, IDO has been implicated in a diverse range of physiological and pathological conditions, including suppression of T cell proliferation, maternal tolerance to allogenic fetus, and immune escape of cancers (4–8), and is an attractive target for drug discovery against cancer and autoimmune and other diseases (2, 9–12).

Despite catalyzing identical biochemical reactions (Fig. 1a), the sequence similarity between TDO and IDO is extremely low. An alignment of their sequences is only possible based on their structures, which suggests a sequence identity of 10% between them (Fig. 1b). In comparison, Xanthomonas campestris TDO shares 34% sequence identity with human TDO (Fig. 1b), demonstrating the remarkable evolutionary conservation of this enzyme. TDO is a homotetrameric enzyme and is highly specific for L-Trp and related derivatives such as 6-fluoro-Trp as the substrate. In comparison, IDO is monomeric, and shows activity toward a larger collection of substrates, including L-Trp, d-trypotphan (d-Trp), serotonin, and tryptamine (3), although the Km for d-Trp is ~100-fold higher than that for L-Trp (13). The structure of human IDO in the catalytically inactive, ferric [Fe(III)]-heme state in complex with the 4-phenylimidazole inhibitor has recently been reported (14). Although this structure gave information about important active site residues, the inhibitor is coordinated to the heme iron and does not provide any information regarding Trp or oxygen binding.

To provide direct insight into substrate recognition and catalysis by these enzymes, we report here the crystal structures at up to 1.6-Å resolution of the active, reduced (Fe(II)) TDO from X. campestris in a binary complex with the substrate L-Trp or 6-fluoro-Trp. Our structures reveal for the first time the structural basis for the stereospecificity of this important enzyme. Our structural information is confirmed by biochemical studies and offers significant molecular insight into tryptophan dioxygenation by TDO and IDO.

Results

Structure Determination. Crystals of the reduced (Fe(II)) TDO from X. campestris in a binary complex with the substrate L-Trp or 6-fluoro-Trp were obtained after extensive efforts and by using anaerobic conditions, because the oxidized (Fe(III)) enzyme has much lower affinity for L-Trp (see below). The structures at up to 1.6-Å resolution of these binary complexes [Table 1; and see supporting information (SI) Table 3] as well as that of the free enzyme were determined by molecular replacement based on the structure of the apo enzyme, in the absence of heme, which we had determined by the selenomethionyl single-wavelength anomalous diffraction method (PDB entry 1YW0) (15).

The structure of the SO4414 protein from Shewanella oneidensis (16) was determined at 2.4-Å resolution by molecular replacement based on our structure of the apo enzyme (PDB entry 1ZEE).

Structure of TDO. The structure of X. campestris TDO monomer contains 12 helices (named aA through aL) and no b-strands (Figs. 1b and 2a). TDO is an intimately associated tetramer (Fig. 2b), and ~5,400 Å2 of the surface area of each monomer is buried in the tetramer. Helices bB and aC are located in the extensive, mostly hydrophobic interface between two of the monomers. The N-terminal segments (residues 21–40, including helix aA) of the two monomers are swapped in this dimer (Fig.


The authors declare no conflict of interest.

Abbreviations: D-Trp, d-tryptophan; IDO, indoleamine 2,3-dioxygenase; L-Trp, L-tryptophan; TDO, tryptophan 2,3-dioxygenase.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 1YW0, 2NW7, 2NW8, 2NW9, 1ZEE, and 2NWB). 3To whom correspondence should be addressed. E-mail: ltong@columbia.edu.

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which is important for the catalysis by TDO because several residues in this segment are part of the binding site for the Trp residue in the active site (see below).

**Binding Mode of the L-Trp Substrate to TDO.** Our structure of the binary complex defines the molecular mechanism for the recognition of the L-Trp substrate by TDO. Clear electron density was observed for heme and L-Trp in the active site based on the crystallographic analysis at 1.6-Å resolution (Fig. 3). The L-Trp substrate is located in a pocket over the distal face of the heme, having interactions with residues in helices /H9251B and /H9251D, and the /H9251D–/H9251E and /H9251J–/H9251K loops (Figs. 1 and 3). The carboxylate group of Trp is recognized by bidentate ion-pair interactions with the side chain of Arg 117 (in helix /H9251D). The carboxylate group is also hydrogen-bonded to the side chain hydroxyl of Tyr113 (helix /H9251D) and the main chain amide of Thr 254 (/H9251J–/H9251K loop). The ammonium ion of L-Trp is recognized by the 7-propionate side chain of the heme group (Fig. 3). In addition, the N1 nitrogen of the indole ring is hydrogen-bonded to the side chain of His 55 (helix /H9251B) (Fig. 3). A water molecule is present in the active site of this binary complex (Fig. 3), hydrogen-bonded to the ammonium ion of L-Trp and the main-chain amide of residue Gly 125 (Fig. 3). The water is 3.5 Å from the ferrous atom in the heme, too far for ligating interactions. The iron atom is still 0.3 Å out of the plane of the heme, on the side of the proximal His 240 ligand (SI Fig. 5).

The crystal was exposed to a solution saturated with nitric oxide (NO) before being flash-frozen, but we did not observe the binding of this dioxygen analog in the structure. This is confirmed by our structure of the binary complex with 6-fluoro-Trp, which was not exposed to NO but contained the same density for the water molecule (SI Fig. 6). The structure of the 6-fluoro-Trp binary complex is essentially identical to that of the L-Trp binary complex (SI Fig. 6). NO probably dissociated from the heme during the cryofreezing manipulations in the anaerobic box.

**Induced-Fit Behavior of TDO.** Our structural information suggests that TDO is an induced-fit enzyme. Although the active site (Fig. 3b). In addition, the N1 nitrogen of the indole ring is hydrogen-bonded to the side chain of His 55 (helix /H9251B) (Fig. 3b). A water molecule is present in the active site of this binary complex (Fig. 3), hydrogen-bonded to the ammonium ion of L-Trp and the main-chain amide of residue Gly 125 (Fig. 3b). The water is 3.5 Å from the ferrous atom in the heme, too far for ligating interactions. The iron atom is still 0.3 Å out of the plane of the heme, on the side of the proximal His 240 ligand (SI Fig. 5).

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**Induced-Fit Behavior of TDO.** Our structural information suggests that TDO is an induced-fit enzyme. Although the active site
this finding, the another TDO tetramer in the crystal. The main chain atoms of 7-propionate of heme is ion-paired with an Arg residue from may also be stabilized by crystal packing interactions, as the active site of the other monomer (Fig. 3). This complex may have the same binding mode to the active site of IDO (SI Fig. 9), because Tyr 113 of TDO is replaced by Phe 226 in IDO (SI Fig. 9), and the Thr 254 residue may not have an equivalent in IDO because the αJ–αK loop, disordered in the IDO structure (SI Fig. 9) (14), has highly divergent sequences in IDO compared with TDO (Fig. 1b). Therefore, IDO may have weaker interactions with tryptophan, which may be the reason why it cannot completely distinguish among the indoleamine substrates.

The structure comparison explains why 1-methyltryptophan is a micromolar inhibitor of IDO (18) but is essentially inactive against TDO (10). The N1 atom is directly hydrogen-bonded to the His 55 side chain in TDO (Fig. 3b), and its methylation will cause steric clash with this residue. In comparison, His 55 is replaced by Ser 167 in IDO (SI Table 4), which creates a small pocket that can accommodate the 1-methyl group (SI Fig. 9).

Table 1. Summary of crystallographic information

<table>
<thead>
<tr>
<th>Protein</th>
<th>TDO (holoenzyme)</th>
<th>TDO (holoenzyme)</th>
<th>TDO (holoenzyme)</th>
<th>TDO (apoenzyme)</th>
<th>TDO (apoenzyme)</th>
<th>SO4414 (holoenzyme)</th>
<th>SO4414 (apoenzyme)</th>
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</thead>
<tbody>
<tr>
<td>Ligand</td>
<td>L-Trp</td>
<td>6-fluoro-Trp</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Maximum resolution, Å</td>
<td>1.6</td>
<td>1.8</td>
<td>2.7</td>
<td>2.7</td>
<td>2.4</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Rmerge, %</td>
<td>6.9 (52.7)</td>
<td>8.6 (60.9)</td>
<td>13.0 (32.3)</td>
<td>16.6 (74.0)</td>
<td>10.4 (63.0)</td>
<td>6.6 (30.6)</td>
<td>2.3</td>
</tr>
<tr>
<td>Beam line</td>
<td>ESRF BM14</td>
<td>ESRF BM14</td>
<td>NSLS X4A</td>
<td>APS 21BM</td>
<td>NSLS X4A</td>
<td>NSLS X4A</td>
<td>NSLS X4A</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>87 (65)</td>
<td>87 (61)</td>
<td>72 (54)</td>
<td>82 (68)</td>
<td>83 (60)</td>
<td>87 (71)</td>
<td></td>
</tr>
<tr>
<td>R factor, %</td>
<td>17.1 (18.9)</td>
<td>16.6 (17.9)</td>
<td>25.7 (25.7)</td>
<td>25.0 (32.1)</td>
<td>21.7 (23.5)</td>
<td>23.4 (26.7)</td>
<td></td>
</tr>
<tr>
<td>Free R factor, %</td>
<td>18.9 (22.0)</td>
<td>18.4 (21.1)</td>
<td>26.3 (26.2)</td>
<td>29.4 (35.1)</td>
<td>22.5 (24.1)</td>
<td>27.5 (31.5)</td>
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<tr>
<td>rms deviation in bond lengths, Å</td>
<td>0.005</td>
<td>0.005</td>
<td>0.008</td>
<td>0.011</td>
<td>0.007</td>
<td>0.006</td>
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<tr>
<td>rms deviation in bond angles, °</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
<td>1.6</td>
<td>1.1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Most-favored region, %</td>
<td>92</td>
<td>91</td>
<td>89</td>
<td>86</td>
<td>92</td>
<td>91</td>
<td></td>
</tr>
</tbody>
</table>

* \( R_{	ext{merge}} = \sum_{i<j}^{N} f_{i} - f_{j}/\sum_{i<j}^{N} f_{i}^{2} \). The numbers in parentheses are for the highest-resolution shell.

\( R = \sum_{i<j}^{N} f_{i} - f_{j}/\sum_{i<j}^{N} f_{i}^{2} \).

Additional evidence for the induced-fit behavior is observed in the active site of the second TDO monomer in the asymmetric unit. The binding mode of the L-Trp substrate is very different in this monomer (Fig. 3d; and see SI Fig. 7). The side chain of TDO is not positioned as deeply into the pocket, and the hydrogen bond between the ring nitrogen and the side chain of His 55 is lost (distance of 3.8 Å). Both nitrogen atoms are instead hydrogen-bonded to a water molecule, located 3.5 Å from the heme iron but at a position distinct from that of the water in the active site of the other monomer (Fig. 3d). This conformation may also be stabilized by crystal packing interactions, as the 7-propionate of heme is ion-paired with an Arg residue from another TDO tetramer in the crystal. The main chain atoms of the Trp substrate appear to be disordered, because no clear electron density was observed for them (Fig. 3e). Consistent with this finding, the αJ–αK loop is disordered in this molecule, similar to that in the free enzyme (Fig. 3d). This complex may represent an initial stage in the formation of the Michaelis complex of TDO. Proper positioning of the L-Trp substrate for catalysis would lead to the recognition of its main chain atoms and the ordering of the αJ–αK loop.

Implications for Substrate Binding by IDO. Our structure of the binary complex of TDO also has significant implications for substrate recognition by the IDOs. The rms distance for 201 structurally equivalent Ca atoms between TDO and the large domain of human IDO (PDB entry 2D0T) (14) is 3.1 Å (SI Fig. 8), calculated with the program Dali (17). Despite sharing only 10% overall sequence identity, key active-site residues are similar in TDO and IDO (Fig. 1b; and see SI Table 4), and L-Trp may have the same binding mode to the active site of IDO (SI Fig. 9). Ionic interactions that are important for recognizing the L-Trp ammonium ion and carboxylate group are conserved in IDO (Arg 231 and 7-propionate), explaining why L-Trp is a much better substrate than D-Trp for IDO. At the same time, hydrogen-bonding interactions to these atoms may be absent in IDO, because Tyr 113 of TDO is replaced by Phe 226 in IDO (SI Fig. 9), and the Thr 254 residue may not have an equivalent in IDO because the αJ–αK loop, disordered in the IDO structure (SI Fig. 9) (14), has highly divergent sequences in IDO compared with TDO (Fig. 1b). Therefore, IDO may have weaker interactions with tryptophan, which may be the reason why it cannot completely distinguish among the indoleamine substrates.

A Model for the Michaelis Complex. To help provide further insight into the catalysis by these enzymes, we built a model for the Michaelis complex, by placing one oxygen atom (O1) of the dioxygen substrate directly over the heme iron, at a distance of 2.1 Å (Fig. 4a). The distal oxygen atom (O2) was placed such that the O1–O2 bond is parallel to the C2–C3 bond of indole ring, giving a Fe–O1–O2 angle of 135°. This conformation places the O2 atom within 0.5 Å of the water molecule observed in our structure (Fig. 4a), suggesting that this water should be ejected from the active site upon dioxygen binding. The active site is therefore completely devoid of solvent molecules in this Michaelis complex.

The modeled dioxygen-binding mode reveals the activation mechanism of this substrate for the reaction. It has been established that TDO has an ordered catalytic cycle in which the protein first binds L-Trp to the ferrous form, and then binding of dioxygen is facilitated, and nucleophile attack from the substrate C3 is initiated (3, 13, 19). In the model, the distal oxygen atom interacts with the L-Trp ammonium moiety and the backbone amide nitrogen of Gly 125 (Fig. 4a). The Lewis acidity...
of the hydrogen-bonding donors, coupled with the electron-withdrawing nature of the heme, would increase the electrophilicity of the bound dioxygen and render it more susceptible to nucleophilic attack by the substrate C3 atom. The increased hydrophobicity of the active site upon the exclusion of water would also aid the stabilization of an oxyferrous species. Studies with heme oxygenase suggest that the hydrogen-bonding interactions to the dioxygen substrate may also help to prevent its withdrawing nature of the heme, would increase the electrophilicity of the dioxygen substrate. After the initial actions to the dioxygen substrate may also help to prevent its becoming a hydrogen-bond competitor to the dioxygen. After the initial attack by the C3 atom, the reaction may proceed via a Criegee rearrangement pathway (SI Fig. 11). The Criegee rearrangement or a dioxetane intermediate (SI Fig. 11) has been reported for the enzyme at high concentrations (Table 2). Our binding data show that D-Trp has much lower affinity for the enzyme than L-Trp (Table 2), consistent with our structural information and explaining why D-Trp cannot be oxygenated by TDO.

Biochemical Studies Confirm the Structural Observations. X. campes-tris TDO has robust catalytic activity toward L-Trp and 6-fluoro-Trp but is inactive toward D-Trp, tryptamine or indolepropionic acid (Table 2), confirming its designation as a TDO. In fact, D-Trp is a weak, competitive inhibitor of the enzyme at high concentrations (Table 2). Our binding data show that D-Trp has much lower affinity for the enzyme than L-Trp (Table 2), consistent with our structural information and explaining why D-Trp cannot be oxygenated by TDO.

The biochemical studies also provide direct evidence for the induced-fit behavior of TDO. There is a large increase in the affinity of the enzyme for L-Trp when the heme iron is reduced (Kd [ferrous Fe(II) heme] = 3.8 mM, whereas Kd [ferri-c Fe(III) heme] = 4.1 μM) (Table 2). The electrochemistry data show a large, positive shift in reduction potential (+136 mV) in the presence of 15 mM L-Trp (Fig. 4c). In fact, the shift in reduction potential almost perfectly correlates with the increase in affinity for L-Trp on reduction, both giving an estimated ΔΔG of 15 kJ/mol. These data show that there is a significant stabilization of the ferrous form when substrate is bound. This stabilization could also play a physiological role to keep the protein reduced, and therefore active, when L-Trp is present.

Our structural studies have defined the binding mode of the substrate L-Trp to TDO, revealing the structural basis for the stereospecificity of this important enzyme. The induced-fit behavior of TDO, confirmed by our biophysical studies, appears crucial for the exclusion of water from the active site and for stabilizing the enzyme in the presence of the substrates. Finally, structural comparisons among these enzymes reveal the striking evolutionary conservation of the heme-dependent dioxygenases.

Materials and Methods

The experimental protocols are summarized here. More detailed information can be found in SI Materials and Methods.

Protein Expression and Purification. Full-length X. campes-tris TDO (NESG ID XcR13) and S. oneidensis SO4414 (NESG ID SoRS2) were cloned into a pET-21d (Novagen, San Diego, CA) derivative, with a C-terminal hexahistidine tag, and overexpressed at 17°C in Escherichia coli BL21(DE3) pMGK cells. Hemin (7 μM

An Allosteric Binding Site in the Tetramer Interface. We also observed the binding of four L-Trp residues to an allosteric site in the interface of the tetramer (Fig. 2b), with well defined electron density (SI Fig. 12). The L-Trp residue appears to be recognized specifically by the enzyme in this pocket (SI Fig. 12). There have been reports of allosteric activation by the substrate L-Trp (21, 22), and our observations offer a possibility for this effector site. Unfortunately, our kinetic studies so far have not shown any allosteric effects with X. campes-tris TDO. This site is not occupied in the 6-fluoro-Trp complex, possibly because of the lower concentration of this compound in the crystallization solution.
final concentration) was included in the media for preparation of the holoenzyme samples (23). The protein was purified by using nickel-affinity and gel-filtration chromatography.

Point mutations were created with the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations and their associated ORFs were verified by DNA sequence analysis.

**Protein Crystallization.** To obtain the structure of TDO in the ferrous state, the protein was reduced by the addition of sodium dithionite, and all steps were performed in an anaerobic glove box (Belle Technology, Dorset, U.K.), with the O₂ concentration maintained ≤2 ppm. Excess sodium dithionite was removed by gel filtration (Sephadex G25 column) before crystallization. Crystals of TDO were grown by the sitting-drop vapor diffusion method with a well solution comprising 100 mM Mes (pH 6.3), 10–12% (wt/vol) PEG 4000, 60 mM MnCl₂, 10 mM sodium dithionite, and 2 mM L-Trp. Before mounting in nylon loops and flash-freezing in liquid nitrogen, crystals were immersed in a cryoprotectant solution composed of mother liquor (with L-Trp concentration increased to 50 mM) supplemented with 23% (vol/vol) glycerol and bubbled with nitric oxide for 15 min before use.

**Data Collection and Processing.** X-ray diffraction data were collected at the X4A beam line of National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY), the 21BM beam line at Advanced Photon Source (Argonne National Laboratory, Argonne, IL), and the BM14 beam line at the ESRF synchrotron in Grenoble, France. The data were processed and integrated with XDS (26) and processed with CrysAlisPro (27).

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**Table 2. Summary of kinetic data on X. campestris TDO**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>( k_{\text{cat}} ) (s⁻¹)</th>
<th>( K_m ) (μM)</th>
<th>( K_d ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>L-Trp</td>
<td>19.5 ± 1.2</td>
<td>114 ± 1</td>
<td>3.84 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>D-Trp</td>
<td>0</td>
<td>16.5 mM ± 3.3*</td>
<td>&gt;50</td>
</tr>
<tr>
<td></td>
<td>5-F-D/L-Trp</td>
<td>37.3 ± 0.6</td>
<td>186 ± 12</td>
<td>2.45 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>6-F-D/L-Trp</td>
<td>2.40 ± 0.10</td>
<td>100 ± 6</td>
<td>1.51 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Indolepropionic acid</td>
<td>0</td>
<td>0</td>
<td>&gt;10*</td>
</tr>
<tr>
<td></td>
<td>O₂§</td>
<td>35.4 ± 0.9</td>
<td>119 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>H55A mutant</td>
<td>L-Trp</td>
<td>2.86 ± 0.10</td>
<td>133 ± 7</td>
<td>ND</td>
</tr>
</tbody>
</table>

NA, \( K_d \) for O₂ cannot be measured in the absence of substrate because of oxidation or in the presence of substrate because of turnover; NC, No spectral change detected; ND, not done.

*Inhibitory constant, \( K_i \).

†Although a spectral change was evident, substrate solubility prevented accurate measurement of \( K_d \). Values were estimated based on the maximum substrate concentration attainable.

‡Binding was too tight to be measured. Values quoted represent the minimum \( K_d \) that can be measured under standard assay conditions.

§The peak positions of the oxyferrous complex (O₂-TDO) are at 420 nm, 548 nm, and 578 nm.
European Synchrotron Radiation Facility (Grenoble, France). The diffraction images were processed and scaled with the HKL package (24). The data-processing statistics are summarized in Table 1, and more complete information can be found in SI Table 3.

Structure Determination and Refinement. The structures of the apo enzymes of TDO and SO4414 were determined by the selenomethionyl single-wavelength anomalous diffraction method (15). The selenium sites were located with SnB (25), and the reflection phases were calculated with Solve/Resolve (26). The structures of the holoenzymes and the ternary complex were determined by the molecular-replacement method, with the programs COMO (27) and AMoRe (28). The atomic models were built with the program XtalView (29) and TURBO-FRODO (30), and the structure refinement was carried out with CNS (31).

Electronic Spectroscopy, Steady-State Assays, and Dissociation Constant Measurements. Electronic absorption spectra were recorded by using a Cary 50-Probe UV-Visible spectrophotometer at 25°C. Assays for the steady-state turnover (at pH 7.5) of L-Trp and derivatives were performed as described (32, 33), except that substrate concentrations of 0–15 mM were used. The kinetic data were fitted to the Michaelis–Menten equation. The pH dependence of the steady-state kinetics was determined in the same manner, by using phosphate (pH 6.0–8.0) and Tris (pH 8.0–9.0) buffers. The electronic absorption spectra of the steady state were recorded by using a stopped-flow spectrophotometer (SX.17MV; Applied-Photophysics, Surrey, U.K.) in conjunction with a diode array detector, housed in an anaerobic glove box (O₂, <5 ppm; Belle Technology).

OTTLE Electrochemistry. Anodic potentiometric titrations were carried out as described (34) at 25°C by using a modified quartz EPR OTTLE cell. Titrations were performed in both the absence and presence of L-Trp (15 mM), and the heme reduction potentials were determined by fitting the data to the Nernst equation for a single-electron process by using Origin software (MicroCal, Northampton, MA). Reduction potentials are quoted versus the standard hydrogen electrode.

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