High-resolution structures of inhibitor complexes of human indoleamine 2,3-dioxygenase 1 in a new crystal form

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Human indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-dependent enzyme with important roles in many cellular processes and is a potential target for drug discovery against cancer and other diseases. Crystal structures of IDO1 in complex with various inhibitors have been reported. Many of these crystals belong to the same crystal form and most of the reported structures have resolutions in the range 3.2–2.3 Å. Here, three new crystal forms of human IDO1 obtained by introducing a surface mutation, K116A/K117A, distant from the active site are reported. One of these crystal forms diffracted to 1.5 Å resolution and can be readily used for soaking experiments to determine high-resolution structures of IDO1 in complex with the substrate tryptophan or inhibitors that coordinate the heme. In addition, this mutant was used to produce crystals of a complex with an inhibitor that targets the apo form of the enzyme under the same conditions; the structure of this complex was determined at 1.7 Å resolution. Overall, this mutant represents a robust platform for determining the structures of inhibitor and substrate complexes of IDO1 at high resolution.

1. Introduction

Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-dependent enzyme that catabolizes tryptophan and related compounds, enabling their degradation through the kynurenine pathway (Hayaishi, 1993; Khan *et al.*, 2007; Badawy, 2017; Raven, 2017). IDO1 is expressed in many tissues and is up-regulated during inflammatory responses to interferon γ or lipopolysaccharides. The depletion of local tryptophan and the production of active compounds from the kynurenine pathway give IDO1 an important role in many cellular processes.

Significant recent interest in IDO1 stems from the discovery that it mediates the immune tolerance of tumor cells (Munn & Mellor, 2016; Mellor *et al.*, 2017; Prendergast *et al.*, 2018). As a result, a large collection of IDO1 inhibitors have been reported (Prendergast *et al.*, 2017; Cheong *et al.*, 2018). Some of these have shown promise in phase II human clinical trials, although most recently a phase III clinical trial combining epacadostat (INCB024360) with a PD-1 antibody failed to demonstrate the benefit of IDO1 inhibition in patients with unresectable or metastatic melanoma.

The first structure of human IDO1 was reported in 2006 at 2.3 Å resolution in a complex with the inhibitor 4-phenyl-imidazole (PI; Sugimoto *et al.*, 2006). A large number of structures have been reported since then in complex with various compounds [Tojo *et al.*, 2014; Peng *et al.*, 2016; Wu *et al.*, 2017; Lewis-Ballester *et al.*, 2017; Crosignani *et al.*, 2017;

Alexandre *et al.*, 2018; Nelp *et al.*, 2018; PDB entry 6e35 (Northeast Structural Genomics Consortium, unpublished work)]. Most of these structures are in the same crystal form as the PI complex, although the resolution is often lower, with the lowest resolution of a published structure being 3.2 Å.

To produce crystals that can routinely be used to collect X-ray diffraction data to high resolution, we introduced mutations on the surface of human IDO1 with the hope of generating new crystal forms by reducing the surface entropy (Derewenda & Vekilov, 2006). We used the *SERP* server (Goldschmidt *et al.*, 2007) as well as manual inspection of the structure to identify mutation sites. We found that the K116A/K117A double mutant, that was designed based on manual inspection, can be crystallized in three different crystal forms, all of which are distinct from the other reported crystal forms. More importantly, various inhibitors that coordinate the heme, as well as the substrate tryptophan, could be soaked into these crystals, allowing the rapid structure determination of their complexes at high resolution. In addition, we were able to co-crystallize this mutant with a compound that targets the apo form using the same conditions as the holoenzyme and determine the structure of this complex at 1.7 Å resolution. We will focus on the new features observed in these structures and comparisons with previous structures in the descriptions below.

2. Materials and methods

2.1. Protein expression and purification

The human IDO1 gene (encoding residues 12–403) was cloned into the pET-28a vector using NdeI and XhoI restriction sites. Surface mutations were introduced using site-directed mutagenesis and were confirmed by sequencing. The protein was expressed in the *Escherichia coli* BL21 Star (DE3) strain. Cells were grown in LB medium with shaking at 37°C and were induced by adding 0.4 mM isopropyl β-D-1-thiogalactopyranoside and 0.75 mM 5-aminolevulinic acid to the cell culture when the OD₆₀₀ reached 0.6. The cell culture was then incubated for 18 h at 18°C and harvested by centrifugation. The cell was resuspended in lysis buffer consisting of 50 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol and lysed by sonication. The protein was bound to Ni-NTA beads and eluted using lysis buffer supplemented with 500 mM imidazole. The protein was further purified by gel-filtration chromatography in buffer consisting of 5 mM HEPES pH 7.6, 200 mM NaCl, 5 mM DTT. Peak fractions were collected, concentrated to around 50 mg ml⁻¹, flash-frozen in liquid nitrogen and stored at -80°C. The purified protein has a typical heme-incorporation rate of 0.6 and the iron is in the oxidized form.

2.2. Protein crystallization

All crystals were grown using the sitting-drop vapor-diffusion method at 20°C by mixing 20 mg ml⁻¹ protein solution with crystallization buffer consisting of 0.1 M phosphate pH 6.2, 15% (w/v) PEG 3350. Crystals of the free IDO1

enzyme typically appeared in 2 d and were flash-cooled in mother liquor supplemented with 20–30% (v/v) glycerol using liquid nitrogen.

To obtain an IDO1 structure with the heme iron in the reduced state, crystals were soaked in mother liquor supplemented with 20% (v/v) glycerol and 20 mM sodium dithionite for 2 min before being flash-cooled in liquid nitrogen.

To obtain structures in complex with inhibitors that bind heme, crystals were generally soaked in mother liquor supplemented with 25 mM compound for 5 h and cooled in the same way as free-enzyme crystals. For 4-chlorophenyl imidazole, crystals were soaked in mother liquor with 20 mM compound for 5 h before transferring to cryo-buffer for flash-cooling.

To obtain a structure in complex with the BMS-978587 analog, which binds to the apo form of IDO1, the protein was treated following a published protocol before crystallization (Nelp *et al.*, 2018). Briefly, the protein at 100 μM concentration was mixed with the compound at 500 μM concentration in gel-filtration buffer supplemented with 1% (v/v) DMSO and incubated at 42°C for 4 h. The sample was centrifuged to remove the precipitated heme. The supernatant was then concentrated to 20 mg ml⁻¹ for crystallization using the same condition as for the free enzyme. Crystals appeared in about 3 d and were cooled in mother liquor supplemented with 100 mM NaCl and 15% (v/v) glycerol.

2.3. Data collection and structure determination

The data set for IDO1 with reduced heme iron was collected on beamline FMX at The National Synchrotron Light Source II (NSLS-II), while all other data sets were collected on NE-CAT beamline 24-ID-C at the Advanced Photon Source (APS). Diffraction to 1.5 Å resolution was observed for some crystals at the beginning of data collection, but there was significant decay during data collection, especially for crystals that contained heme. Low-dose exposure was used to reduce radiation damage to the crystals. All diffraction images were processed using *XDS* (Kabsch, 2010) and the highest resolution was set to that at which CC_{1/2} is about 0.7 (Karplus & Diederichs, 2012). Structures were solved by molecular replacement using a monomer of PDB entry 4u72 (Sugimoto *et al.*, 2006) as the model. Manual model rebuilding was performed using *Coot* (Emsley & Cowtan, 2004) and structure refinement was carried out using *PHENIX* (Adams *et al.*, 2002).

3. Results and discussion

3.1. Surface engineering to produce new crystal forms

Protein surface engineering has often been successful in enabling and/or improving crystallization (Derewenda & Vekilov, 2006). To identify mutations that could produce new crystal-packing interactions in human IDO1, we used the *SERP* server (Goldschmidt *et al.*, 2007) and examined the structure of the protein manually. Three mutations, 135-KKK-137 to AAA, 363-KENK-366 to AANA and 13-KE-14 to AA, were

Table 1

Data-collection and refinement statistics for IDO1-inhibitor complexes.

Values in parentheses are for the highest resolution shell.

Structure	Epacadostat complex	Epacadostat sulfur analog complex	4-Chlorophenyl imidazole complex	BMS-978587 analog complex
Data collection				
Beamline	24-ID-C, APS	24-ID-C, APS	24-ID-C, APS	24-ID-C, APS
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$
a, b, c (Å)	80.4, 200.1, 114.7	80.9, 201.7, 114.9	79.7, 197.9, 113.0	80.5, 195.4, 118.2
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	46.7–2.3 (2.39–2.30)	55.3–2.3 (2.37–2.30)	49.1–2.1 (2.18–2.10)	47.6–1.7 (1.77–1.70)
R_{merge}	0.064 (0.81)	0.095 (0.75)	0.063 (0.73)	0.062 (0.83)
$CC_{1/2}$	0.99 (0.76)	0.99 (0.81)	0.99 (0.73)	0.99 (0.72)
$\langle I/\sigma(I) \rangle$	12.0 (1.6)	9.68 (1.7)	12.2 (1.6)	11.5 (1.3)
Completeness (%)	98.5 (95.9)	97.5 (89.5)	98.3 (96.6)	99.2 (98.0)
Multiplicity	3.8 (3.9)	4.9 (5.0)	3.7 (3.5)	4.4 (4.4)
Refinement				
Resolution (Å)	46.7–2.3 (2.39–2.30)	55.3–2.3 (2.37–2.30)	49.1–2.1 (2.18–2.10)	47.6–1.7 (1.77–1.70)
No. of reflections	81121 (7768)	83151 (7533)	102766 (9960)	201129 (19654)
R_{work}	0.208 (0.300)	0.203 (0.298)	0.203 (0.298)	0.194 (0.309)
R_{free}	0.254 (0.351)	0.251 (0.346)	0.240 (0.337)	0.230 (0.317)
No. of atoms				
Protein	11735	11912	11506	11820
Ligand/ion	247	322	369	172
Water	173	286	295	1253
B factors (Å ²)				
Protein	55.9	48.5	44.1	32.9
Ligand/ion	60.7	50.5	40.8	28.3
Water	51.7	46.9	45.3	40.2
R.m.s. deviations				
Bond lengths (Å)	0.012	0.016	0.008	0.007
Bond angles (°)	1.16	1.40	0.98	0.87
Ramachandran plot				
Favored (%)	96.46	96.31	97.09	98.17
Allowed (%)	3.54	3.69	2.91	1.83
Outliers (%)	0.00	0.00	0.00	0.00
PDB code	6e40	6e41	6e42	6e43

suggested by the server, and our manual examination suggested the K116A/K117A mutation and the internal deletion of residues 363–374. All of these mutants were generated and sequenced to confirm their identity. The 135-KKK-137 to AAA mutant failed to produce soluble protein in *E. coli*, while the other four mutants were expressed and purified. Three mutants, 363-KENK-366 to AANA, 13-KE-14 to AA and the internal deletion of residues 363–374, produced crystals that were similar to those of the wild-type enzyme.

Crystallization screening indicated that the K116A/K117A double mutant designed by our manual examination of the structure (Fig. 1a) produced crystals in a new buffer condition. These crystals were further improved by removing the N-terminal 14 residues of IDO1 and reducing the length of the linker between the His tag and the protein (to SSGSAA). Three different crystal forms have been observed for this mutant from essentially the same crystallization conditions. Crystal form I belongs to space group $P2_12_12$, with unit-cell parameters $a \simeq 90$, $b \simeq 198$, $c \simeq 116$ Å. Crystal form II also belongs to space group $P2_12_12$, but with unit-cell parameters $a \simeq 80$, $b \simeq 116$, $c \simeq 218$ Å. Crystal form III belongs to space group $C2$, with unit-cell parameters $a \simeq 180$, $b \simeq 125$, $c \simeq 110$ Å, $\beta \simeq 117^\circ$.

The diffraction quality of crystal form I is the best as judged by the resolution limit. These crystals routinely diffracted to 2.3 Å resolution or better, and diffraction can extend to 1.5 Å

resolution; they were used for all of the structures reported here. Diffraction data sets were collected to 2.2 and 2.5 Å resolution for crystal forms II and III, respectively, and their structures were readily solved by the molecular-replacement method. These two crystal forms were also successfully used to determine the structures of inhibitor complexes.

The asymmetric unit of crystal form I contains four molecules of IDO1, arranged as two dimers related by a pseudo-translation of (0.16, 0.0, 0.5), with peaks roughly 45% of the height of the origin in a Patterson map. The presence of this pseudo-translation does not affect the structure determination or refinement. The same dimer exists in crystal forms II and III and has essentially the same overall structure, with r.m.s.d.s of less than 0.5 Å to equivalent C $^\alpha$ atoms in crystal form I.

This IDO1 dimer is formed as a result of the mutations introduced into the protein (Fig. 1a), as the K116A and K117A mutations are located in the center of the dimer and the lysine side chains of the wild-type protein cannot be accommodated in this interface (Fig. 1b). The establishment of these interactions is likely to be the molecular basis for the new crystal forms that are observed for this mutant. This dimer is only formed in the crystal, as the mutant protein migrated as a monomer on a gel-filtration column. The buried surface area is 500 Å² at the dimer interface, suggesting that the dimer is stabilized by the high protein concentration in the crystal.

Both mutation sites are located on the surface of the IDO1 monomer, and the overall structure of the mutant protein is similar to that of the wild-type enzyme (Fig. 1*a*). The r.m.s.d. between equivalent C α atoms of the mutant structure and the PI complex of wild-type IDO1 (Sugimoto *et al.*, 2006) is 0.4 Å.

3.2. Structures of inhibitor complexes from soaking

We soaked crystals of free IDO1 enzyme in crystal form I (as well as crystal forms II and III) with various inhibitors that are known to bind to the heme and were able to determine the structures of many complexes, typically at resolutions of 2.3 Å or better. Three of these structures are reported here (Table 1), including the complexes with epacadostat (2.3 Å resolution), a

Table 2
Data-collection and refinement statistics for the free IDO1 enzyme and the tryptophan complex.

Values in parentheses are for the highest resolution shell.

Structure	Free enzyme (ferric)	Free enzyme (ferrous)	Tryptophan complex (ferrous)
Data collection			
Beamline	24-ID-C, APS	FMX, NSLS-II	24-ID-C, APS
Space group	<i>P</i> ₂ ₁ ₂ ₁ ₂	<i>P</i> ₂ ₁ ₂ ₁ ₂	<i>P</i> ₂ ₁ ₂ ₁ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	80.1, 197.0, 116.2	80.0, 196.5, 116.2	79.9, 197.2, 115.0
α , β , γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	47–1.9 (1.97–1.90)	65.9–2.0 (2.07–2.00)	46.7–2.1 (2.16–2.10)
<i>R</i> _{merge}	0.060 (0.72)	0.090 (0.88)	0.091 (0.86)
<i>CC</i> _{1/2}	0.99 (0.77)	0.99 (0.71)	0.99 (0.77)
$\langle I/\sigma(I) \rangle$	12.3 (1.6)	10.55 (1.7)	9.2 (1.4)
Completeness (%)	99.3 (98.0)	99.4 (94.9)	99.5 (97.6)
Multiplicity	3.7 (3.8)	4.9 (4.6)	5.0 (5.1)
Refinement			
Resolution (Å)	47–1.9 (1.97–1.90)	65.9–2.0 (2.07–2.00)	46.7–2.1 (2.16–2.10)
No. of reflections	143535 (13977)	123958 (11670)	107965 (10474)
<i>R</i> _{work}	0.194 (0.312)	0.189 (0.285)	0.208 (0.302)
<i>R</i> _{free}	0.235 (0.356)	0.226 (0.286)	0.247 (0.342)
No. of atoms			
Protein	11722	11690	11840
Ligand/ion	187	217	182
Water	938	1047	417
<i>B</i> factors (Å ²)			
Protein	34.8	33.0	44.8
Ligand/ion	37.3	34.5	41.1
Water	41.2	39.4	44.9
R.m.s. deviations			
Bond lengths (Å)	0.010	0.010	0.009
Bond angles (°)	0.97	1.03	1.03
Ramachandran plot			
Favored (%)	97.67	98.02	97.63
Allowed (%)	2.33	1.98	2.37
Outliers (%)	0.00	0.00	0.00
PDB code	6e44	6e45	6e46

Figure 1
Introducing surface mutations to obtain new crystal forms of human IDO1. (*a*) Overlay of the structure of the human IDO1 K116A/K117A mutant in complex with epacadostat (colored) with that of wild-type IDO1 in complex with PI (gray; Sugimoto *et al.*, 2006). The side chains of Lys116 and Lys117 (the mutation site) are shown in red and are distant from the active site. (*b*) Overall structure of a dimer of the human IDO1 K116A/K117A mutant. One monomer is in cyan and the other is in green. The mutation sites are shown in red. Several other residues in the dimer interface are also shown as stick models. All structural figures were produced with PyMOL (<http://www.pymol.org>).

sulfur analog of epacadostat (2.3 Å resolution) and 4-chloro-phenyl imidazole (Cl-PI; 2.1 Å resolution).

Clear electron density is observed for epacadostat in two of the four complexes in the asymmetric unit (Fig. 2*a*), while the electron density in the other two complexes is weaker. The electron density for the sulfur analog is of better quality (Fig. 2*a*) and is observed in all four complexes. The binding modes of the two inhibitors are essentially the same (Fig. 2*b*), and the overall structures of IDO1 in the two complexes are highly similar as well, with an r.m.s.d. of 0.2 Å for their equivalent C α atoms. The hydroxyl group of the hydroxyl-amidine is directly coordinated to the heme iron, and it is also hydrogen-bonded to the main-chain amide of Ala264 (Fig. 2*b*). It is most likely that this hydroxyl is in the deprotonated form, and the crucial role of this group in the interaction with IDO1 is consistent with the reported structure–activity relationship (Yue *et al.*, 2009) and quantum-chemical calculations (Röhrig *et al.*, 2017). The phenyl ring is positioned in a pocket that is lined with hydrophobic and aromatic side chains. Compared with the structure of the epacadostat complex reported previously (Lewis-Ballester *et al.*, 2017), the electron density for the phenyl ring is much stronger in the current structure and there is a difference of ~0.5 Å in the overall position of the compound in the binding site (Fig. 2*b*).

In contrast to PI (Sugimoto *et al.*, 2006), we found that two molecules of Cl-PI were bound near the heme in each IDO1 molecule (Fig. 2*c*). One is coordinated to the heme iron and its phenyl ring is located at a position close to that of the phenyl ring of epacadostat. This position of Cl-PI is essentially the same as that reported previously for PI (Fig. 2*c*; Sugimoto *et al.*, 2006). The other Cl-PI is located on the proximal side of the heme (Fig. 2*c*) and is equivalent to the binding site for indole ethanol (Si site; Lewis-Ballester *et al.*, 2017). A conformational change of Phe270 and neighboring residues is required for the compound to bind (Fig. 2*d*). The chlorophenyl ring is sandwiched between the side chains of Phe270 (in its new position) and Leu342, and the imidazole ring interacts with a side-chain propionate of the heme (Fig. 2*d*). The Cl atom makes interactions with several hydrophobic side chains, including Val170, Ala174 and Ala210 (Fig. 2*d*), which may

explain why PI cannot exploit this binding site. Three additional molecules of Cl-PI are bound in the dimer interface, π -stacked against each other and located next to the K116A mutations of the two monomers. This binding site is likely to be an artifact owing to the mutations.

3.3. Structure of an inhibitor complex from co-crystallization

Two classes of IDO1 inhibitors have recently been shown to target the apo form of the enzyme and compete with heme for binding (Nelp *et al.*, 2018). To obtain the structure of IDO1 in complex with these compounds, we first incubated the enzyme (K116A/K117A mutant) with an analog of BMS-978587 (Nelp *et al.*, 2018; Markwalder *et al.*, 2017; Williams *et al.*, 2018) at 42°C to remove the heme. A brownish precipitate developed and the resulting supernatant was colorless, confirming the

Figure 2

Structures of inhibitor complexes of human IDO1. (*a*) The OMIT $F_o - F_c$ electron density for epacadostat at 2.3 Å resolution, a sulfur analog of epacadostat at 2.3 Å resolution and Cl-PI in the distal and proximal sites at 2.1 Å resolution contoured at 2.5σ . (*b*) Overlay of the binding site for epacadostat (green), its sulfur analog (pink) and that reported previously (gray; Lewis-Ballester *et al.*, 2017). (*c*) Overlay of the binding mode of 4-chlorophenyl imidazole (Cl-PI; light brown) with those of epacadostat (green) and PI (gray; Sugimoto *et al.*, 2006). (*d*) Detailed interactions of Cl-PI in the proximal site with IDO1 (wheat). The conformation of residues in the epacadostat (green) and PI (gray) complexes are also shown.

removal of heme from the protein. Remarkably, this inhibitor complex could be crystallized using the same crystallization buffer as the free holo enzyme, and the crystals also had essentially the same unit-cell parameters (Table 1). This led to a structure of the complex at 1.7 Å resolution, owing to the lower radiation damage to these crystals, representing a major improvement over the earlier structure at 2.76 Å resolution (Nelp *et al.*, 2018). Well defined electron density is observed for the BMS-978587 analog (Fig. 3*a*), and the structure at high resolution also allowed the placement of many water molecules in the binding-site region.

Interestingly, we also observed electron density for another compound near the inhibitor (Fig. 3*a*), bound in essentially the same position as the second CI-PI molecule (Figs. 3*b*). The electron density and the chemical environment indicate that the compound is most likely to be benzoate or a close analog. This compound was not introduced during purification or crystallization, and it was probably bound to the enzyme during bacterial expression and co-purified. The heme incorporation of the purified enzyme was about 60%, and it might

be possible that the compound preferred to bind to the apo form of IDO1.

As observed in the CI-PI complex, the binding of benzoate necessitates a change in the conformation of Phe270, although the conformation observed in the CI-PI complex is not possible for this complex as the side chain of Phe270 would clash with the cyclohexyl group of the inhibitor (Fig. 3*c*). Instead, Phe270 assumes another conformation, including a shift of nearly 2 Å for its C α atom, although smaller changes are seen for the neighboring residues 269 and 271. The benzoate phenyl ring is sandwiched between Phe270 in this new position and the side chain of Leu342. Residues 336–350 in helix α J move by up to 0.9 Å to accommodate the benzoate molecule compared with the earlier structure of the complex (Nelp *et al.*, 2018), and as a result there is also a recognizable difference in the positions of the inhibitors in the two structures (Fig. 3*b*). Interestingly, the conformation of helix α J in the benzoate complex is similar to that in the CI-PI complex (Fig. 3*c*). The benzoate carboxylate group interacts with the side chains of Arg343 and His346 (Fig. 3*c*), with the latter

Figure 3

Structure of the human IDO1 apoenzyme in complex with an inhibitor. (a) OMIT $F_o - F_c$ electron density at 1.7 Å resolution for the BMS-978587 analog and for the putative benzoate contoured at 3σ . (b) Overlay of the binding mode of the BMS-978587 analog observed here (colored) with that reported previously (gray; Nelp *et al.*, 2018) and CI-PI. The heme in the CI-PI complex is not shown for clarity. (c) Detailed interactions of the putative benzoate molecule in the proximal site of IDO1 (green). The conformations of the residues in the epacadostat (green) and PI (gray) complexes are also shown.

Figure 4

Structures of free human IDO1 and the complex with tryptophan. (a) OMIT $F_o - F_c$ electron density at 2.1 Å resolution for tryptophan contoured at 2.5σ . (b) Overlay of the active-site region of the free IDO1 enzyme in the ferric (gray) and ferrous (cyan) states and the complex with tryptophan (green) in one molecule. The conformational changes for the DE and JK loops and for the Arg231 side chain are indicated by red arrows. (c) Overlay of the active-site region of the free IDO1 enzyme in the ferric (gray) and ferrous (cyan) states and the complex with tryptophan (green) in another molecule. The coordination bond between the carbonyl O atom of Ala264 and the heme iron is shown as a dashed line (gray).

being the proximal ligand of heme. Overall, the benzoate molecule appears to make extensive interactions with apo IDO1, which could explain why it co-purified with the enzyme.

3.4. Structures of free IDO1 and the tryptophan complex

We also determined the structure of the free IDO1 enzyme with the heme iron in the ferric state at 1.9 Å resolution (Table 2). To determine the structure of the free IDO1 enzyme in the ferrous state, we incubated the crystals with sodium dithionite, which caused a clear change in their color. The crystals were cooled before re-oxidation of the iron, and the structure was determined at 2.0 Å resolution (Table 2).

We soaked IDO1 crystals in the ferric state with tryptophan at 24 mM concentration, but did not observe any binding. In contrast, when IDO1 crystals were soaked with tryptophan and treated with sodium dithionite, clear electron density for tryptophan was observed in the resulting structure at 2.1 Å resolution (Table 2). The density was best for two of the four complexes in the asymmetric unit (Fig. 4a), while the density was weaker for the phenyl portion of the tryptophan side chain in the other two molecules.

In the free IDO1 structures, residues 261–270 assume a different conformation in the ferric and ferrous states such that Ala264 is positioned directly above the heme iron, which would clash with the binding of tryptophan and O_2 (Fig. 4b). Moreover, the carbonyl O atom of Ala264 (in the DE loop) is a direct ligand of the heme iron in two of the molecules in the ferric state (Fig. 4c). Upon reduction, the heme molecule as

well as His346 and helix αJ move away from the DE loop, which undergoes a conformational change such that the carbonyl O atom of Ala264 no longer coordinates the heme iron (Fig. 4c). On the binding of tryptophan residues 261–270 assume a different conformation, with residues 263–264 flanking one face of the indole ring of the tryptophan (Fig. 4b). The side chain of Arg231 becomes ion-paired with the carboxylate of the tryptophan. Residues 374–383 (the JK loop) change from a helical conformation to a loop, covering the tryptophan binding site. These observations indicate that the DE and JK loops are highly flexible and assume different conformations in response to the oxidation state of the heme iron and the presence of the substrate tryptophan.

4. Conclusions

Our success with the K116A/K117A double mutant is another indication of the impact of surface mutations on crystallization and crystal diffraction quality. The studies with the new IDO1 crystals indicate that they are a useful tool for determining the structures of inhibitor complexes to high resolution both by soaking with inhibitors that coordinate the heme as well as by co-crystallization with inhibitors that target the apo form of the enzyme.

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References

- Adams, P. D., Grosse-Kunstleve, R. W., Hung, L.-W., Ioerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K. & Terwilliger, T. C. (2002). *Acta Cryst.* **D58**, 1948–1954.
- Alexandre, J. A. C., Swan, M. K., Latchem, M. J., Boyall, D., Pollard, J. R., Hughes, S. W. & Westcott, J. (2018). *Chembiochem*, **19**, 552–561.
- Badawy, A. A.-B. (2017). *Int. J. Tryptophan Res.* **10**, <https://doi.org/10.1177/1178646917691938>.
- Cheong, J. E., Ekkati, A. & Sun, L. (2018). *Expert Opin. Ther. Pat.* **28**, 317–330.
- Crosgnani, S. *et al.* (2017). *J. Med. Chem.* **60**, 9617–9629.
- Derewenda, Z. S. & Vekilov, P. G. (2006). *Acta Cryst.* **D62**, 116–124.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* **D60**, 2126–2132.
- Goldschmidt, L., Cooper, D. R., Derewenda, Z. S. & Eisenberg, D. (2007). *Protein Sci.* **16**, 1569–1576.
- Hayaishi, O. (1993). *Protein Sci.* **2**, 472–475.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 133–144.
- Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
- Khan, J. A., Forouhar, F., Tao, X. & Tong, L. (2007). *Expert Opin. Ther. Targets*, **11**, 695–705.
- Lewis-Ballester, A., Pham, K. N., Batabyal, D., Karkashon, S., Bonanno, J. B., Poulos, T. L. & Yeh, S.-R. (2017). *Nature Commun.* **8**, 1693.
- Markwalder, J. A., Seitz, S. P., Blat, Y., Elkin, L., Hunt, J. T., Pabalan, J. G., Jure-Kunkel, M. N., Vite, G. D. & Covello, K. (2017). *Bioorg. Med. Chem. Lett.* **27**, 582–585.
- Mellor, A. L., Lemos, H. & Huang, L. (2017). *Front. Immunol.* **8**, 1360.
- Munn, D. H. & Mellor, A. L. (2016). *Trends Immunol.* **37**, 193–207.
- Nelp, M. T., Kates, P. A., Hunt, J. T., Newitt, J. A., Balog, A., Maley, D., Zhu, X., Abell, L., Allentoff, A., Borzilleri, R., Lewis, H. A., Lin, Z., Seitz, S. P., Yan, C. & Groves, J. T. (2018). *Proc. Natl Acad. Sci. USA*, **115**, 3249–3254.
- Peng, Y.-H. *et al.* (2016). *J. Med. Chem.* **59**, 282–293.
- Prendergast, G. C., Malachowski, W. P., DuHadaway, J. B. & Muller, A. J. (2017). *Cancer Res.* **77**, 6795–6811.
- Prendergast, G. C., Malachowski, W. J., Mondal, A., Scherle, P. A. & Muller, A. J. (2018). *Int. Rev. Cell. Mol. Biol.* **336**, 175–203.
- Raven, E. L. (2017). *J. Biol. Inorg. Chem.* **22**, 175–183.
- Röhrig, U. F., Zoete, V. & Michielin, O. (2017). *Biochemistry*, **56**, 4323–4325.
- Sugimoto, H., Oda, S., Otsuki, T., Hino, T., Yoshida, T. & Shiro, Y. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 2611–2616.
- Tojo, S., Kohno, T., Tanaka, T., Kamioka, S., Ota, Y., Ishii, T., Kamimoto, K., Asano, S. & Isobe, Y. (2014). *ACS Med. Chem. Lett.* **5**, 1119–1123.
- Williams, D. K. *et al.* (2018). *Bioorg. Med. Chem. Lett.* **28**, 732–736.
- Wu, Y., Xu, T., Liu, J., Ding, K. & Xu, J. (2017). *Biochem. Biophys. Res. Commun.* **487**, 339–343.
- Yue, E. W. *et al.* (2009). *J. Med. Chem.* **52**, 7364–7367.