Supporting Information

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SI Materials and Methods

IDO1 Cloning, Expression, Purification, and Crystallization. To generate human IDO1 protein for assays, an *Escherichia coli* expression vector was constructed by PCR amplification of IDO1 from a human cDNA library and insertion of the resulting the Nde–XhoI fragment encoding the full IDO1 protein into a modified pET28 vector, thereby introducing a thrombin-cleavable His tag at the N terminus of the resultant protein. A similar strategy was used to generate the expression construct for human IDO1 protein used for crystallography and biophysics assays, except that sequence encoding the first four amino acids of IDO was removed, such that, after His-tag cleavage, the resultant protein was composed of three residual, vector-encoded linker residues (–GSH–) followed by human IDO1 residues 5–403.

IDO1 constructs were transformed into *E. coli* strain BL21(DE3) and plated on LB/kanamycin agar plates. A single colony was inoculated into LB/kanamycin for overnight starter cultures that were then used to inoculate shaker flasks containing a defined medium containing 1× M9 salts plus 0.5% (wt/vol) D-glucose, 0.5% (wt/vol) casamino acids, trace minerals, and 30 μg/mL kanamycin sulfate. Flasks (2.5 L Thomson Ultra Yield) containing 1-L cultures were shaken at 240 rpm in a rotary incubator at 37 °C for ∼2 h until the OD at 600 nm reached 0.5. To boost heme biosynthesis, 5-aminolevulinic acid hydrochloride was added from a 250 mM stock to each culture to a final concentration of 1.0 mM, and the cultures were incubated at 37 °C for another 20–30 min until the OD at 600 nm reached 0.9–1.0. The flasks were removed from the incubator, placed in an ice bath, and chilled for 30 min. Isopropyl β-D-1-thiogalactopyranoside (0.5 mM) was added to each culture, and the flasks were returned to the shaker and incubated at 20 °C for 24 h. The cells were harvested by sedimentation ($9{,}000 \times g_{max}$) for 20 m at 4 °C, frozen, and stored at –80 °C. Typical yields were 11–13 g of wet cell paste per liter of culture.

To purify the full-length His-Tb-IDO1 protein, the frozen cell paste was thawed by resuspending in 10 vol Buffer A (50 mM potassium phosphate, 0.3 M potassium chloride, 25 mM imidazole, 5% glycerol, 0.1 mM Tris(2-carboxyethyl)phosphine, pH 7.1) supplemented with 1 tablet of cOmplete EDTA-free protease inhibitor mixture (Roche) per 50 mL. The chilled, suspended cells were lysed by passage through an APV 1000 homogenizer (SPX) pressurized to 800 bar, and the lysate was collected, chilled on ice, and incubated with 25 U/mL Benzonase Nuclease (Sigma) on ice for 15 min. The lysate was clarified by sedimentation ($16{,}000 \times g_{max}$) for 45 m at 4 °C, and the supernatant was loaded onto a HisTrap FF column (GE Healthcare Life Sciences) equilibrated with Buffer A. The HisTrap column was washed with Buffer A containing 60 mM imidazole and then eluted with Buffer A containing 350 mM imidazole. The red His-Tb-IDO1 protein in the elution fraction was supplemented with 2 mM EDTA, concentrated by ultrafiltration using 10-kDa cutoff Amicon Ultra centrifugal concentrators (Millipore), and immediately loaded onto a Superdex 200pg SEC column (GE Healthcare Life Sciences) equilibrated with 25 mM potassium phosphate, 100 mM KCl, 10% glycerol, and 1 mM EDTA (pH 6.8). The His-Tb-IDO1 protein was eluted from the SEC column in a volume consistent with monomeric protein, and the final pool had a 404- to 280-nm absorption ratio of 2.02, indicating high heme occupancy. The IDO1 protein concentration of 3.0 mg/mL was determined using the 404-nm Soret band extinction coefficient of 172 mM^{-1} cm^{-1}. The identity of the final purified His-Tb-IDO1 was confirmed using an LTQ mass spectrometer (Thermo) and was consistent with calculated mass for the *des*-Met protein (47,359 Da). The purified His-Tb-IDO1 protein was divided into aliquots, flash frozen in liquid nitrogen, and stored at −80 °C. A typical final yield was 25 mg of purified His-Tb-IDO1 per liter of expression.

The IDO1(5-403) protein was purified in a similar fashion, except that, after HisTrap purification, it was dialyzed into 25 mM potassium phosphate, 150 mM KCl, 1 mM EDTA, and 5% glycerol (pH 6.5); divided into large (∼100-mg) aliquots; flash frozen in liquid nitrogen; and stored at −80 °C. Aliquots were thawed rapidly in a 25 °C water bath; 5 U of human alpha-thrombin enzyme (3,000 NIH U/mg; Enzyme Research Labs) was added per 1 mg of His-Tb-IDO1(5-403), and the mixture was incubated at 25 °C for about 60 min. Complete thrombin cleavage of the His tag in the mixture was confirmed by MS. The mixture was passed through a Benzamidine-Sepharose 6B column (GE Healthcare Life Sciences) equilibrated with 50 mM Tris·HCl and 500 mM NaCl (pH 7.5), and the flow through was collected, combined, and washed (4 bed vol) using the same buffer. The sample was chilled on ice, concentrated by centrifugal ultrafiltration, and loaded onto a Superdex 200-pg SEC column equilibrated with 25 mM MES (pH 6), 5% glycerol, 105 mM NaCl, and 0.1 mM EDTA. The IDO1(5-403) protein was pooled from the SEC, concentrated by ultrafiltration to 8 mg/mL, and frozen. To purify for crystallization, a 24-mg portion of the SEC pool was diluted 1:3 with 25 mM MES (pH 6), 5% glycerol, and 0.1 mM EDTA (Mono S Buffer) and loaded onto a Mono S HR10/10 column (GE Healthcare Life Sciences) equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl in Mono S buffer; the main peak containing purified IDO1(5-403) eluted around 110 mM NaCl. This final pool had a 404- to 280-nm absorption ratio of 2.08, indicating high heme occupancy. The protein was confirmed using MS by observation of a species with the expected calculated mass of 45,197 Da. The final Mono S-purified sample was concentrated to 4–5 mg/mL and frozen in aliquots. A typical yield for this protein was 26 mg/L culture after SEC purification and 14 mg/L culture after Mono S purification.

Diffraction quality crystals of the IDO1 holoenzyme without bound inhibitor were obtained as a by-product from an unsuccessful complexing experiment with a compound related to compound **1**. Briefly, Mono S-purified IDO1(5-403) protein at 350 μM was mixed with 700 μM compound in buffer with 6.6% (vol/vol) DMSO and incubated for 15 h at 4 °C. Crystals were obtained from sitting drop trays using 0.4 μL of protein solution and 0.4 μL of reservoir solution incubated at room temperature (23 °C). Reddish crystals were harvested after about 1 wk from wells containing 0.1 M Bis·Tris (pH 6.5), 20% PEG 3000, and 0.2 M calcium acetate as the reservoir solution. Harvested crystals were briefly incubated with reservoir solution containing 25% ethylene glycol and flash frozen in liquid nitrogen. For cocrystallization, compound **1** was dissolved in DMSO to a concentration of 50 mM and added to Mono S-purified IDO1(5-403) to produce final complex conditions of 90 μM protein, 180 μM compound **1**, and 3% (vol/vol) DMSO in Mono S buffer at 110 mM NaCl. The complex was incubated in a water bath at 42 °C for 4 h, after which time a heavy reddish brown precipitate was observed. The solution was clarified by centrifugation at $14{,}000 \times g$ for 5 min, and the A404/A280 ratio of the protein in the supernatant was determined to be 0.38. The protein in the supernatant was concentrated by ultrafiltration with a 0.5 mL 10-kDa cutoff Amicon Ultra centrifugal concentrator (Millipore) to 12 mg/mL as determined by the Bio-Rad Bradford assay using a BSA standard curve. Crystallization was performed in sitting-drop plates using 0.3 μL of concentrated complex and

0.3 μL of reservoir solution, and crystal trays were incubated at room temperature (23 °C). Single crystals lacking the deep reddish color typical for IDO1 were observed after 1 wk in wells containing 22% (wt/vol) PEG 3350 and 0.2 M citrate tribasic (pH 7) in the reservoir. Crystals were frozen by plunging in liquid nitrogen after a brief incubation in cryoprotectant composed of 75% reservoir solution and 25% ethylene glycol. Synchrotron X-ray diffraction data for the IDO–compound **1** cocrystal were collected to 2.8 Å. Protein and cocrystals of IDO with compound **3** were prepared, and X-ray diffraction data were collected by contract with Proteros Biostructures GmbH. Purified IDO1 protein at 25 mg/mL was combined with 20 mM compound **3** with 10% DMSO final concentration and was crystallized at room temperature using a precipitant solution containing 16% PEG 8000, 0.2 ammonium acetate, and 0.1 M *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) (pH 8.75).

Myoglobin Protein Expression and Purification. Sperm whale myoglobin modified with H64Y and V68F mutations to have a unique absorbance spectrum as described by Hargrove et al. (1) was expressed with a C-terminal His_{6x} tag from the corresponding gene obtained from Genscript inserted into pET22b(+) between the NdeI and XhoI cloning sites. This was expressed in BL21(DE3) grown in 2.8-L Fernbach flasks containing LB with 50 μg/mL ampicillin at 37 °C. After these cultures reached OD at 600 nm of ~0.5, the temperature was lowered to 22 °C, and expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to 100 μM and ferrous iron chloride to 50 μM. These were further incubated for 12 h, after which cells were collected by centrifugation at 5,000 × *g*. The resultant cell paste was resuspended in 0.2 L of 50 mM potassium phosphate (pH 7.0), 100 mM sodium chloride, and 10 mM imidazole. To this was added 2 mg lysozyme (Sigma-Aldrich), 1 mg DNase (Sigma-Aldrich), and 1 mM PMSF (Research Products International). After 1 h stirring on ice, this was sonicated for 20 min, and insoluble cell debris was removed by centrifugation at 25,000 × *g* for 30 min at 4 °C. Protein was loaded onto a 5-mL HisTrap FF column (GE Healthcare) preequilibrated in the same buffer. After washing with 5 column vol, protein was eluted with buffer containing 500 mM imidazole. Imidazole was removed by buffer exchange into 50 mM potassium phosphate (pH 7.0) using a Bio-Rad 10-DG column. The apo-version was made from this stock using the method as described by Oohora and Hayashi (2) and modified to remove 2-butanone under vacuum and on ice before dialysis to minimize precipitation. The concentration of the apo-version was determined using the theoretical extinction coefficient at 280 nm of 16,980 M^{-1} cm^{-1} using the ExPASy protparam tool.

Cell-Free Activity Assays. IDO1 was incubated with **1**, **2**, and equine apo-myoglobin (Sigma-Aldrich) with various concentrations, times, and temperatures from 1× to 16× IDO1, from 0 to 20 min, and from 15 °C to 40 °C, respectively. The concentration of IDO1 was held at 5.2 μM and determined using the previously reported extinction coefficient at 404 nm of 172 mM^{-1} cm^{-1} (3). These reactions contained 50 mM potassium phosphate (pH 7.0) and 7% (vol/vol) DMSO, from which **1** and **2** were added when present.

Activity was tested after these incubations by addition of the total incubation volume (30 μL) to a final volume of 1 mL with the reactions conditions as previously described, which included 50 mM potassium phosphate (pH 6.5), 10 mM D-tryptophan, 10 μM methylene blue, 10 mM sodium ascorbate, and 10 μg/mL catalase (Sigma-Aldrich) (4). This was modified slightly to include the chelator diethylenetriaminepentaacetic acid (Sigma-Aldrich) at 50 μM to maintain ascorbate concentration. Kynurenine production was monitored using its absorbance at 321 nm (ε = 3.75 mM^{-1} cm^{-1}) (5).

Incubation reactions that tested the effects of turnover conditions on inhibition included 10 μM methylene blue, 10 mM sodium ascorbate, 10 μg/mL catalase, and 500 μM L-tryptophan. Activity was tested as with the other incubation reactions using D-tryptophan as substrate.

Cell-Free Heme Dissociation Assays. Heme dissociation from IDO1 was measured by addition of IDO1 (final concentration of 5.8 μM) to a solution of apo-H64YV68F myoglobin (final concentration of 95 μM) in 50 mM potassium phosphate buffer at various temperatures. The accumulation of holo-H64YV68F myoglobin was followed using its unique absorbance at 600 nm (ε = 9.54 mM^{-1} cm^{-1}) (1) and corrected for absorbance at 800 nm to control for scattering that results from protein precipitation.

The redox state dependence of heme loss from IDO1 was measured at 37 °C in 50 mM potassium phosphate (pH 7.0) and 0.1% (vol/vol) DMSO in the presence of 10 μM compound **2** and 1.1 μM IDO1. This was followed using the loss in absorbance of the IDO1 heme Soret peaks at 404 nm for ferric IDO1 and 425 nm for ferrous IDO1. Ferrous IDO1 was made by the addition of sodium dithionite to a final concentration of 5 mM. The absorbance of the aggregated heme product of this dissociation was tested under these conditions using free heme (Sigma-Aldrich) dissolved in 0.1 M sodium hydroxide and diluted into the same buffers to the same final concentration: 1.8 μM ($\varepsilon_{385\ nm}$ = 5.84 × 10^4 M^{-1} cm^{-1}) (6).

Cell-Free Cold Chase Assays. Measurements were made using 1 μM IDO1 and 2 μM ^{14}C-labeled **1** and **2**. Labeled compound and IDO1 were allowed to incubate for 2–5 h at 37 °C, after which 100 μM cold compound or DMSO was added and incubation was continued at 37 °C. At various time points, aliquots were removed and desalted using a Zeba spin column, and the eluant was counted in a scintillation counter. Data were fit to a single-phase exponential decay equation $Y = (Y_0 - NS) \times \exp(-k \times X) + NS$ in Graphpad prism, where Y is cpm measured as an indication of binding, NS is nonspecific binding, X is time, Y_0 is the initial binding signal, and k is the dissociation rate constant. The half-life is equal to $0.69/k$.

SKOV-3 Cell Activity Assay. SKOV3 cells (ATCC) cultured at 5 × 10^4 per well in 200 μL RPMI [with 10% FCS and 1 mM tryptophan (Sigma-Aldrich)] were activated with 25 ng/mL hIFN-γ (Peprotech) for 24 h and supplemented with vehicle (0.2% DMSO) or heme (40 μM). Heme (Frontier Scientific) was dissolved in DMSO as a 20 mM stock. After 24 h, the media were removed, and the cells washed for 1 h at 37 °C with fresh media containing 5 mg/mL cycloheximide (Calbiochem). After 1 h, the wash media were removed, and media containing vehicle (0.2% DMSO) or heme (40 μM) plus 1 mM tryptophan and 5 mg/mL cycloheximide were added to both the control-activated cells and the cells activated in the presence of 40 μM hemin. After another 24 h, the media were collected and tested for the formation of kynurenine using the Ehrlich's colorimetric assay. Briefly, 10 μL 30% trichloroacetic acid was added to 150 μL of sample media, and the solution was heated for 30 min at 50 °C to precipitate proteins. After clearing the samples by centrifugation at 1,000 × *g* for 10 min, equal volumes of cleared supernatants and 20 mg/mL *p*-dimethylamino-benzaldehyde (Affymetrix) dissolved in glacial acetic acid were added together. The yellow color change was read on a plate reader at 490 nm. Kynurenine concentrations were determined from kynurenine standard curves.

HeLa Cell Activity Assay. For assays to determine if heme concentration affects the inhibitory concentration of **1** and **2**, human HeLa cells (ATCC) suspended in RPMI phenol-red free:Hepes (10 mM) medium (containing 10% FBS) were added to individual wells of a 384-well tissue culture plate (20,000 cells per well) and allowed to incubate for 3–4 h (37 °C, 5% CO_2). Compound or DMSO was subsequently dispensed (50 nL per well) into individual wells containing cells and maintained at 37 °C and 5% CO_2.

After 1 h, RPMI phenol-red free:Hepes (10% FBS) medium supplemented with IFN-γ (10 ng/mL) and tryptophan (125 or 1,000 μM) with or without heme (40 μM) was added, and the treated cells were incubated for 20 h. Reactions were terminated by the addition of trichloroacetic acid (3% for 0.5 h at 50 °C), and precipitate was removed by centrifugation (1,000 × *g* for 5 min at ambient temperature). Supernatants were subsequently transferred to individual wells of a new 384-well plate, and an equal volume of Ehrlich colorimetric reagent was added to each well. Kynurenine production was measured using an Envision plate reader (490 nm) 30 min post-Ehrlich reagent addition. Compound concentration–response curves were calculated using proprietary Bristol-Myers Squibb Co. data analyses software. The percent inhibition equation was applied as follows: [1 − (sample value − average no stimulation-only control)/(average IFN-γ stimulation-only control − average no stimulation only control)] × 100. IC_{50} determinations were based on 50% inhibition on the fold induction between IFN-γ stimulation and unstimulated treatments.

1. Hargrove MS, et al. (1994) His64(E7) → Tyr apomyoglobin as a reagent for measuring rates of hemin dissociation. *J Biol Chem* 269:4207–4214.
2. Oohora K, Hayashi T (2016) Reconstitution of heme enzymes with artificial metalloporphyrinoids. *Methods Enzymol* 580:439–454.
3. Papadopoulou ND, et al. (2005) Redox and spectroscopic properties of human indoleamine 2,3-dioxygenase and a His303Ala variant: Implications for catalysis. *Biochemistry* 44: 14318–14328.
4. Sono M, Taniguchi T, Watanabe Y, Hayaishi O (1980) Indoleamine 2,3-dioxygenase. Equilibrium studies of the tryptophan binding to the ferric, ferrous, and CO-bound enzymes. *J Biol Chem* 255:1339–1345.
5. Lu C, Yeh SR (2011) Ferryl derivatives of human indoleamine 2,3-dioxygenase. *J Biol Chem* 286:21220–21230.
6. Yang F, Xia X, Lei HY, Wang ED (2010) Hemin binds to human cytoplasmic arginyl-tRNA synthetase and inhibits its catalytic activity. *J Biol Chem* 285:39437–39446.

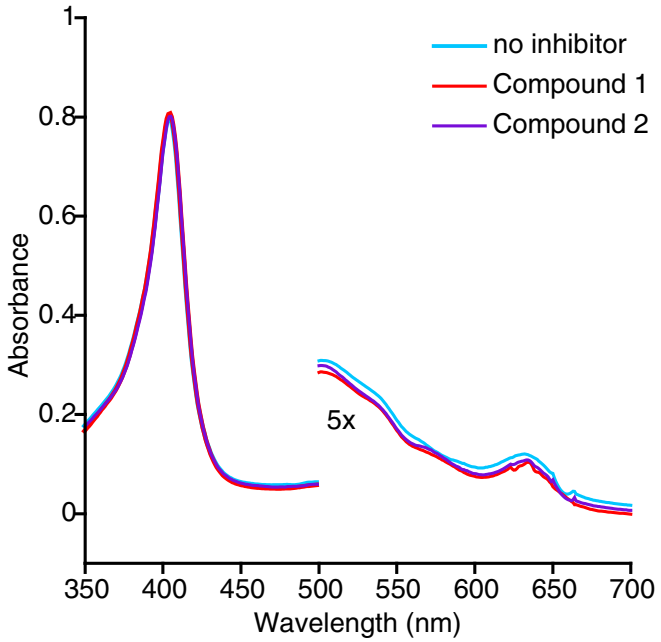

Fig. S1. UV-visible spectra of IDO1 (5 μM) in the presence of **1** and **2** (10 μM). Spectra were recorded at room temperature ~1 min after mixing inhibitor and IDO1 in 50 mM potassium phosphate (pH 7.0) with 7% (vol/vol) DMSO.

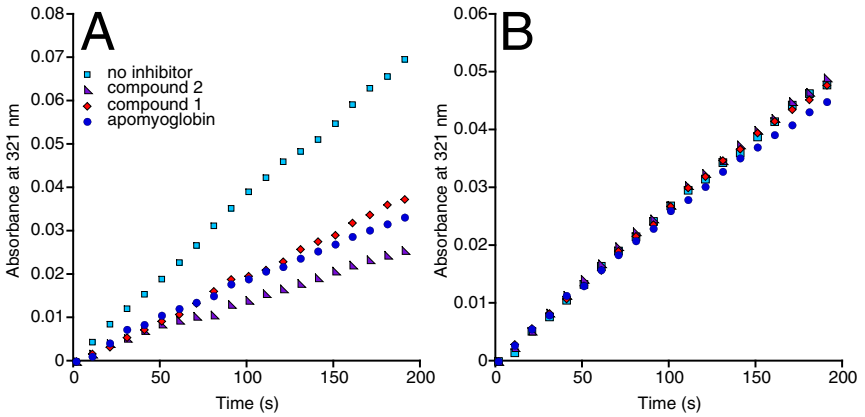

Fig. S2. Activity of IDO1 as monitored by the increase in absorbance at 321 nm corresponding to the production of *N*-formylkynurenine ($\varepsilon = 3.75\ mM^{-1}\ cm^{-1}$) (1). Reaction conditions were IDO1 (~70 nM), 10 mM D-tryptophan, 50 mM potassium phosphate (pH 7.0), 10 μg/mL catalase, and 10 mM sodium ascorbate at room temperature. Inhibitors and apo-myoglobin were at 0.6 μM carried over from incubations. *A* depicts typical traces after incubation of IDO1 with inhibitors for 15 min at 37 °C, and *B* shows typical traces after incubation with inhibitors for 15 min at 15 °C.

1. Lu C, Yeh SR (2011) Ferryl derivatives of human indoleamine 2,3-dioxygenase. *J Biol Chem* 286:21220–21230.

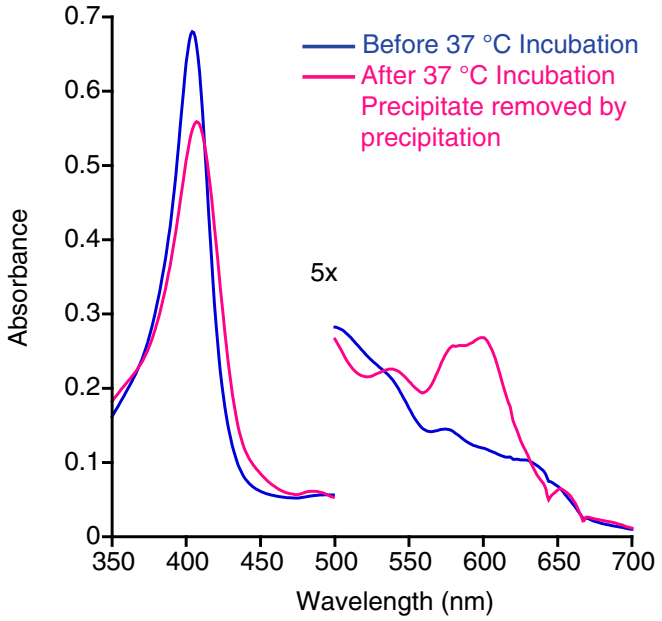

Fig. S3. UV-visible spectra of IDO1 with apo-myoglobin H64YV68F before incubation for 40 min at 37 °C (blue) and after incubation and centrifugation to remove precipitated protein (pink). Incubation conditions were IDO1 (5 μM) and apo-myoglobin H64YV68F (95 μM) in 50 mM potassium phosphate (pH 7.0) with 7% (vol/vol) DMSO.

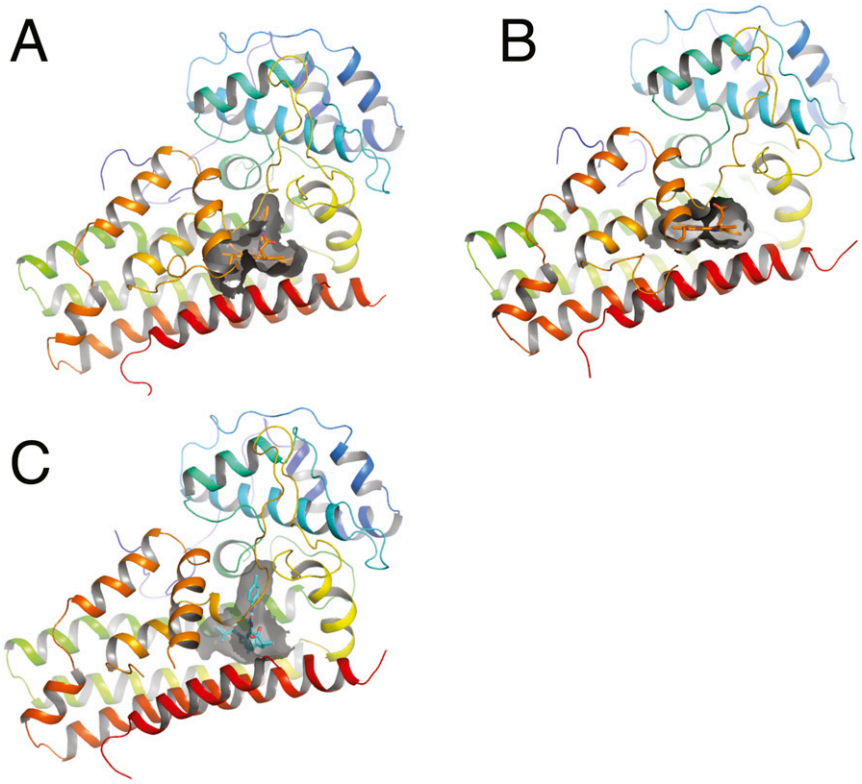

Fig. S4. Pocket rendering of the IDO1 holoenzyme (*A*), phenyl imidazole bound to IDO1 (*B*), and compound **1** bound to apo-IDO1. In the holoenzyme, access to the lateral binding pocket is restricted by the heme-coordinating loop. (*C*) Ligand-induced changes in loop geometry reveal a substrate binding channel that is exploited by the phenyl urea moiety of compound **1** and the phenyl carboxamide of compound **3** (the structure of IDO1 bound to **1** is shown).

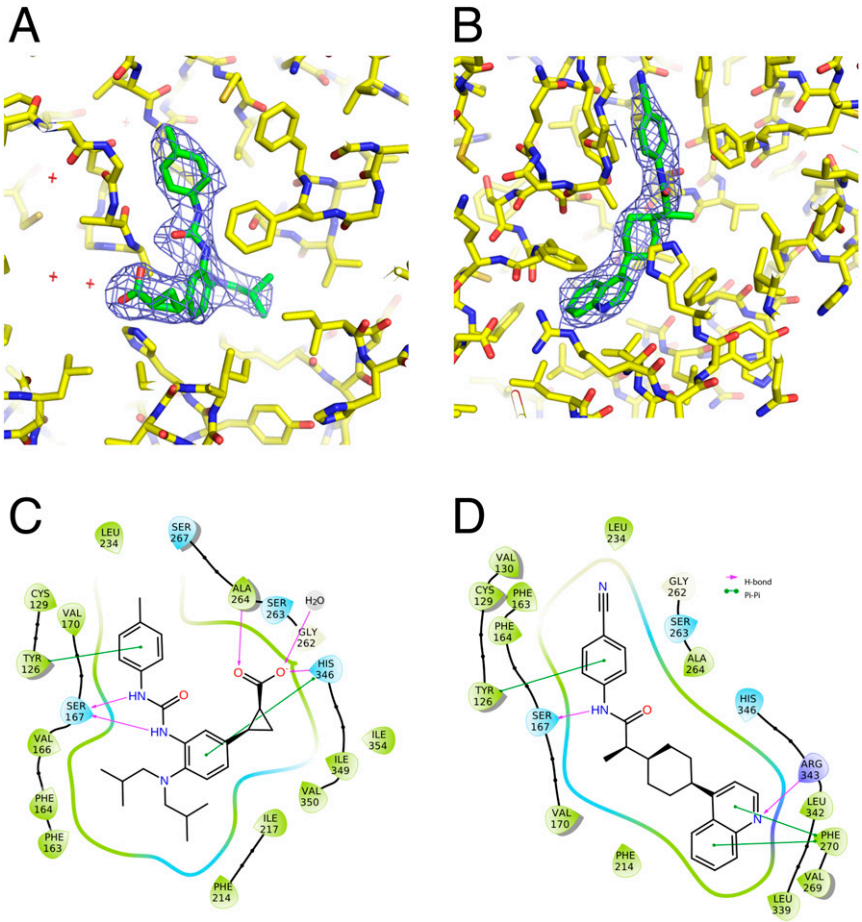

Fig. S5. Electron density for ligands bound to IDO1 with 2Fo − Fc map contour at rmsd 1.0 shown in blue for **1** (*A*) and **3** (*B*). Interactions formed with IDO1 on binding are shown in the 2D ligand interaction diagrams for **1** (*C*) and **3** (*D*).

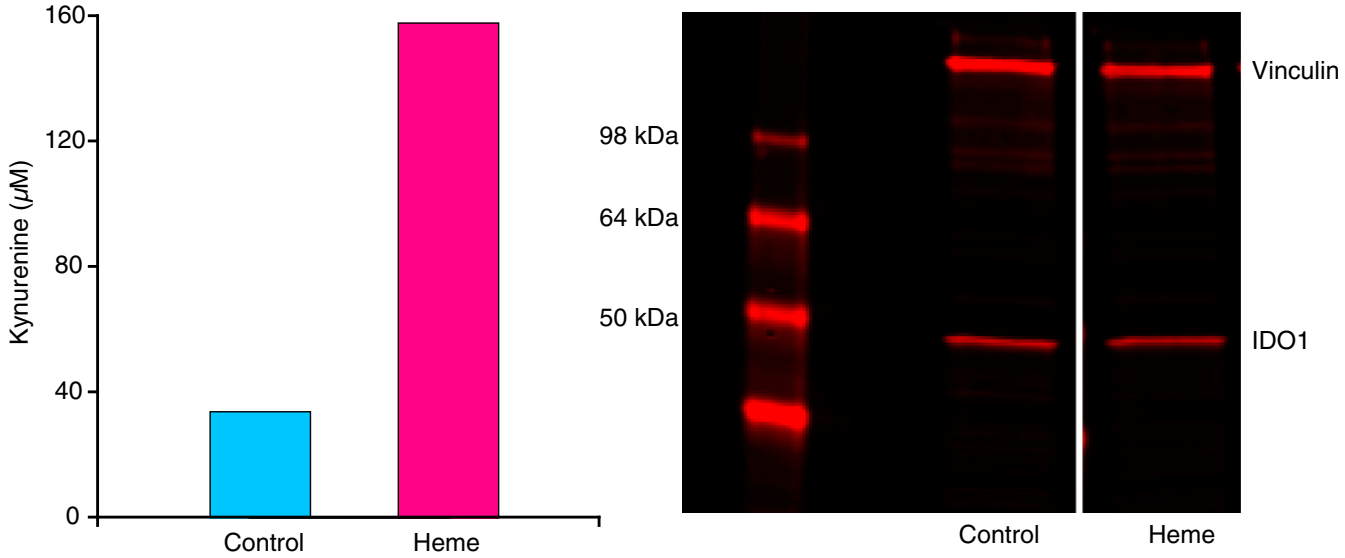

Fig. S6. Heme increases kynurenine production but not IDO1 concentration in SKOV3 human ovarian tumor cells. The graphs in *Left* depict kynurenine production from cells stimulated for 24 h with IFN-γ in the absence (blue) or presence (pink) of added 20 μM heme (separate experiment from data in Fig. 6). The Western blot in *Right* shows corresponding IDO1 protein levels from those cultures. The ratio of IDO1 to vinculin was 0.33 for the vehicle and 0.37 for heme.

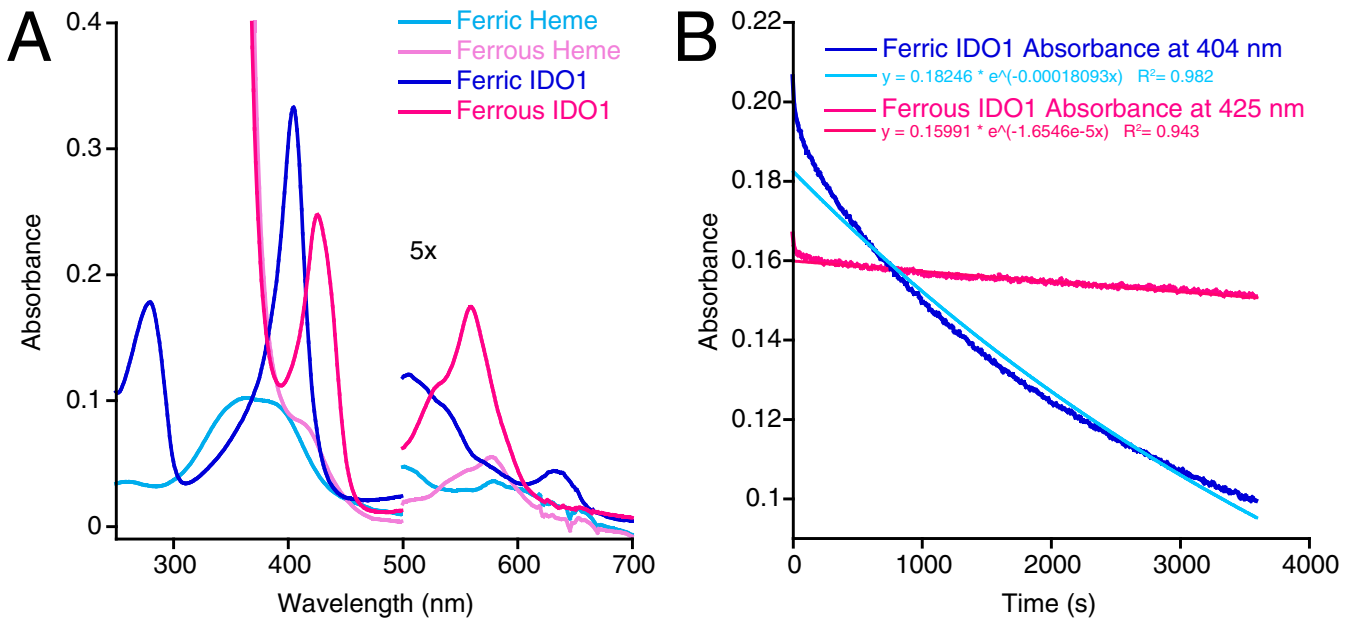

Fig. S7. (*A*) Comparison of ferric and ferrous IDO1 (1.8 μM) and free heme (1.8 μM) in 50 mM potassium phosphate (pH 7.0) and 0.1% (vol/vol) DMSO. (*B*) Traces of ferric and ferrous IDO1 at the respective Soret λ_{max} as in Fig. 7. This includes traces fitted using a single exponential that were then used to obtain half-lives to compare the heme off rates (not taking into account the absorbance of the free heme, which is very similar for ferric and ferrous heme). This shows that heme off rates differ by at least an order of magnitude.

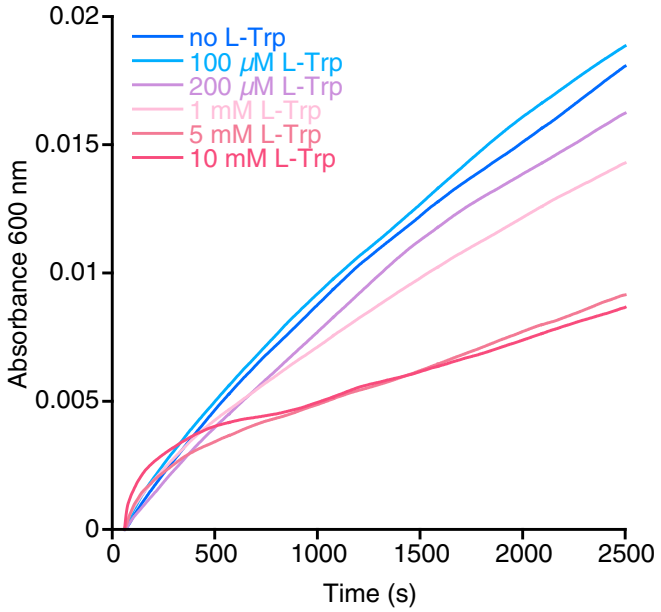

Fig. S8. Heme transfer from IDO1 to apomyoglobin H64YV68F with various concentrations of L-tryptophan. The heme transfer is monitored at 600 nm, where myoglobin H64YV68F possesses a unique absorbance ($\varepsilon = 9.54\ mM^{-1}\ cm^{-1}$) (1). Reactions took place at 37 °C with IDO1 (5 μM) and apo-myoglobin H64YV68F (95 μM) in 50 mM potassium phosphate (pH 7.0).

1. Hargrove MS, et al. (1994) His64(E7) → Tyr apomyoglobin as a reagent for measuring rates of hemin dissociation. *J Biol Chem* 269:4207–4214.

Table S1. X-ray statistics

Parameters	Holo-IDO1	IDO1 + compound **1**	IDO1 + compound **3**
Data collection			
Wavelength, Å	1.000	1.000	1.000
Space group	$P2_12_12_1$	C2	$P2_12_12_1$
Resolution, Å	2.82 (2.97–2.82)	2.75 (2.90–2.75)	2.78 (3.03–2.78)
Cell dimensions			
a, b, c, Å	119.11, 177.51, 101.25	203.39, 120.87, 101.53	85.21, 92.56, 128.70
α, β, γ, °	90, 90, 90	90, 119.11, 90	90, 90, 90
Rsym, %	15.0 (70.2)	9.1 (42.7)	4.6 (43.5)
I/σ(I)	9.3 (2.2)	10.5 (2.6)	18.7 (3.2)
Completeness, %	99.9 (100.0)	98.5 (99.7)	96.0 (96.4)
Redundancy	6.6 (6.8)	3.3 (3.4)	3.7 (3.8)
No. of unique reflections	52,390	54,682	25,234
Refinement			
Resolution, Å	2.82–34.02	2.76–45.11	2.78–46.28
No. of reflections	52,263	54,642	25,189
R_{work}/R_{free}, %	0.211/0.272	0.195/0.272	0.218/0.308
No. of waters	0	106	0
Mean *B* value, $Å^2$	49.91	54.95	70.28
Molprobity clash score	6.22	3.72	2.78
rmsd Bond length, Å	0.010	0.016	0.022
rmsd Bond angles, °	1.34	1.37	1.88
PDB ID code	6AZU	6AZV	6AZW

Parentheses show values in the highest-resolution shell. Rsym = $\sum$hkl$\sum$j|Ihkl,j−⟨Ihkl⟩| ÷ $\sum$hkl$\sum$jIhkl,j, where I is the measured intensity over the h, k, l Miller indices and j allows integration over the full set of measured intensities. PDB, Protein Data Bank.