Tryptophan Oxidation by the Heme-Containing Dioxygenases

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract

In biology, the kynurenine pathway is the major degradation pathway of tryptophan (L-Trp). The first and rate-limiting step is the oxidation of L-Trp to N-formylkynurenine (NFK). The mechanism of this oxygen-dependent reaction has not been established, but is catalysed by two heme-containing dioxygenase enzymes: indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). Early proposals suggested a base-catalysed abstraction mechanism, but most of the recent studies argue against that. Instead, formation of a ferryl (Compound II intermediate) seems more likely. In this thesis, aspects of the reaction mechanism have been investigated.

A Compound II intermediate was detected during the oxidation of L-Trp by hIDO using stopped flow photodiode array spectroscopy. A Compound II intermediate was also detected during the oxidation of a number of different tryptophan analogues. The results suggest a common mechanism of oxidation between L-Trp and other substrates of hIDO. The difference in reactivity between the tryptophan analogues 5-hydroxy-tryptophan and 5-methoxy-tryptophan with hIDO have been interpreted to indicate that initial oxygen atom insertion occurs by radical rather than electrophilic addition.

An intermediate was detected during the oxidation of L-Trp by hTDO and XcTDO. The spectrum of this intermediate did not appear to be characteristic of a Compound II based on comparison with the spectrum of Compound II from hIDO. Weaker binding of L-Trp to both hTDO and XcTDO has been used to interpret these results. It is suggested that Compound II in TDO has a different spectrum or that the rate-limiting step is altered and an alternative catalytic intermediate accumulates.

Crystal trials have been conducted for hIDO and hTDO, with some conditions producing micro-crystals. The structure of XcTDO in complex with potassium cyanide and L-Trp was solved. The binding mode of L-Trp within the distal pocket does not correlate with the L-Trp binding mode in the published ferrous XcTDO-L-Trp binary complex structure.

Oxidation of L-Trp by ferric TDO without the addition of reducing agent was investigated. The results suggest the recruitment of hydrogen peroxide from solution to activate ferric heme.

A summary of the mechanistic information gathered from all of the above experiments is presented.
Acknowledgements

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Publications
Abbreviations

**Enzymes**

IDO  indoleamine 2,3-dioxygenase
TDO  tryptophan 2,3-dioxygenase
hIDO  human indoleamine 2,3-dioxygenase
hTDO  human tryptophan 2,3-dioxygenase
XcTDO  *Xanthomonas campestris* tryptophan 2,3-dioxygenase
DmTDO  *Drosophila melanogaster* tryptophan 2,3-dioxygenase
CmTDO  *Cupriavidus metallidurans* tryptophan 2,3-dioxygenase
PaTDO  *Pseudomonas aeruginosa* tryptophan 2,3-dioxygenase
SIDO  *Shewanella oneidensis* indoleamine 2,3-dioxygenase
DNase  deoxyribonuclease

**Chemicals**

L-Trp  L-tryptophan
1-Me- L-Trp  1-Methyl- L-tryptophan
D-Trp  D-tryptophan
5-OH-Trp  5-hydroxy-tryptophan
5-MeO-Trp  5-methoxy-tryptophan
IPA  indole 3-propionic acid
5-F-Trp  5-fluoro-tryptophan
5-Me-Trp  5-methyl-tryptophan
S-Trp  beta-[3-benzo(b)thienyl]-alanine
O-Trp  beta-(3-benzofuranyl)-alanine
Amp  Ampicillin
Kan  Kanamycin
IPTG  isopropyl-β-D-thiogalactopyranoside
SDS  sodium dodecyl sulphate
DTT  dithiothreitol
NTA  nitrilotriacetic acid
EDTA  ethylenediaminetetraacetic acid
4-PI  4-phenylimidazole
Tris  tris(hydroxymethyl)aminomethane
TAE  tris-acetate-EDTA
TEMED  tetramethylethylenediamine
APS  ammonium persulphate
MPD  2-methyl-2,4-pentanediol
PEG  polyethylene glycol

**Units and symbols**

ε  extinction coefficient
λ  Wavelength
OD  optical density
A  Absorption
c  concentration
nm  Nanometre
M  Molar
mM  millimolar
µM  micromolar
min  minutes
s  seconds
ms  milliseconds
kg  kilograms
g  Grams
mg  milligrams
μg  microgram
l  Litres
ml  millilitres
nl  nanolitres
μl  microlitre
°C  degrees Celsius
rpm  revolutions per minute
v/v  volume to volume
w/v  weight to volume
mV  millivolts
kDa  kilodaltons
Da  daltons
g mol⁻¹  grams per mole
CV  column volume
ppm  parts per million
V  Volt
Å  Angström
kb  kilobases
Techniques

HPLC  high performance liquid chromatography
FPLC  fast performance liquid chromatography
LC-MS liquid chromatography mass spectrometry
UV-vis ultraviolet-visible spectroscopy
NMR nuclear magnetic resonance
PCR polymerase chain reaction
PAGE polyacrylamide gel electrophoresis
$R_z$ Reinheitzahl
ENDOR electron nuclear double resonance
EPR electron paramagnetic resonance

Amino acids

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<tr>
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**Miscellaneous**

- $R_z$: Reinheitzahl
- PDB: Protein Data Bank
- *E.coli*: Escherichia coli
- DNA: deoxyribonucleic acid
- IFN-Y: interferon gamma
- m/z: mass to charge ratio
- NADH: Nicotinamide adenine dinucleotide
Chapter 1: Introduction
1.1 Oxygenases

In the early part of the 20th century there was a general consensus that enzymatic incorporation of oxygen into organic substrates did not occur in biological systems. Wieland’s theory (1) was that oxygen accepts hydrogen atoms and is reduced to water or hydrogen peroxide, but it is never incorporated directly into organic substrates. In 1955 Hayaishi (2) and Mason (3) demonstrated that one or both atoms of oxygen could be directly incorporated into organic substrates by metalloenzymes using oxygen-18 labelling studies. This new class of metalloenzyme was termed the ‘oxygenases’. The oxygenases can be classified into two sub-categories: monooxygenases and dioxygenases. Monooxygenase enzymes catalyse the incorporation of only one atom of oxygen into a substrate (Equation 1.1) and dioxygenase enzymes catalyse the incorporation of both oxygen atoms into a substrate (Equation 1.2).

**Equation 1.1:** Monooxygenase reaction.

\[
\text{RH} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O}
\]

**Equation 1.2:** Dioxygenase reaction.

\[
\text{RH} + \text{O}_2 \rightarrow \text{RO}_2\text{H}
\]

1.2 Monooxygenases

Non-heme iron dependent monooxygenases include methane monooxygenase, toluene monooxygenase (4), tryptophan hydroxylase and phenylalanine hydroxylase (5). The heme-containing monooxygenases include the secondary amine monooxygenases, heme oxygenases (6) and the cytochrome P450s (7).
1.3 Dioxygenases

1.3.1 Non-heme dioxygenases

The non-heme iron-dependent catechol dioxygenases discovered by Hayaishi (2,8,9), catalyse the oxidative cleavage of catechol substrates, which is part of the bacterial aromatic compound degradation pathway (10). The intradiol dioxygenases (e.g. catechol 1,2-dioxygenase) require a mononuclear ferric iron (III) cofactor, ligated by two tyrosine and two histidine ligands. They cleave the carbon-carbon bond between the hydroxyl groups of catechol to produce muconic acid (Figure 1.1) (2). The extradiol dioxygenases (e.g. catechol 2,3-dioxygenase) require a mononuclear ferrous iron (II) cofactor, ligated by two histidine ligands and one glutamic acid ligand. They cleave the carbon-carbon bond adjacent to the hydroxyl groups of catechol to yield 2-hydroxymuconaldehyde (Figure 1.1) (8). Examples of non-heme dioxygenases include cysteine dioxygenase (11), beta-carotene dioxygenase (12) and acetylacetone dioxygenase (13).

Figure 1.1: The reactions catalysed by the intradiol and extradiol dioxygenases, reproduced from reference (14).
1.3.2 Heme-containing dioxygenases

The general family of the heme-containing dioxygenases includes the enzymes linoleate diol synthase (15,16), prostaglandin H synthase (17,18), fatty acid α-dioxygenase (19,20) as well as indoleamine 2,3-dioxygenase (IDO) (21,22) and tryptophan 2,3-dioxygenase (TDO) (23). IDO and TDO are the focus of this work.

IDO and TDO catalyse the initial and rate-limiting step of the kynurenine pathway (Figure 1.3). The reaction involves the oxidation of tryptophan (L-Trp) to N-formylkynurenine (NFK) (Figure 1.2).

![Figure 1.2](image_url): The reaction catalysed by heme-containing dioxygenases involved in tryptophan oxidation.

1.4 Tryptophan metabolism and the kynurenine pathway

L-Trp is the least abundant of the essential amino acids required by mammals. It is used for the synthesis of proteins as well as for the synthesis of the neurotransmitter serotonin, which is further converted to melatonin in the brain. Only ~1% of dietary L-Trp is converted to serotonin and the majority of L-Trp (~95%) is metabolised by the kynurenine pathway. The first step of the kynurenine pathway is the reaction catalysed by IDO and TDO (Figure 1.2) (24). The kynurenine pathway leads ultimately to the biosynthesis of the key biological cofactor nicotinamide adenine dinucleotide (Figure 1.3).
Figure 1.3: Tryptophan metabolism in mammalian cells, the kynurenine pathway.
1.5 Discovery of IDO and TDO

In 1937, Kotake and Masayama (23) discovered the existence of an enzyme that catalysed the oxidation of L-Trp to NFK, the first step of the kynurenine pathway (Figure 1.3). The enzyme, initially named tryptophan pyrrolase, was purified and identified as a heme-containing dioxygenase (25). This enzyme was found to be highly specific for L-Trp (26) and was later renamed tryptophan 2,3-dioxygenase (TDO). TDO is expressed in the liver, but not in other tissues. In 1937, Kotake and Ito (27) discovered that rabbits fed on D-Trp excreted D-kynurenine in the urine. This result suggested that an enzyme present in non-hepatic tissue had the ability to oxidise the ring cleavage of D-Trp. In 1967, an enzyme capable of catalysing the conversion of D-Trp to N-formyl-D-kynurenine was discovered (22,28). In 1978, this new enzyme was purified from rabbit intestine and identified as a heme-containing dioxygenase (21). This enzyme was named indoleamine 2,3-dioxygenase (IDO). Due to the broader spectrum of substrate it was found to oxidise (e.g. L-Trp, D-Trp, serotonin, tryptamine, 5-hydroxy-tryptophan) as compared with TDO. Unlike TDO, this enzyme was found to be expressed ubiquitously in non-hepatic tissues such as the lung, brain, spleen, kidney and stomach (29).

1.6 Comparison between IDO and TDO

TDO is a homotetrameric enzyme found in mammals, mosquitos, fruit flies and bacteria. Its expression is limited to the hepatic tissue, brain and skin in mammals (30) and induced by L-Trp, kynurenine and hydrocortisone. The sequence similarity between human tryptophan 2,3-dioxygenase (hTDO) and Xanthomonas campestris (XcTDO) is ~34% and the similarity between human indoleamine 2,3-dioxygenase (hIDO) and hTDO is only ~10% (31). IDO is a monomeric enzyme found mainly in mammals and is expressed in all tissues except hepatic tissue. Expression of IDO is induced by multiple immunological signals including interferon-γ, lipopolysaccharides and inflammatory cytokines (32).
1.7 Biological role of IDO and TDO

1.7.1 Quinolinic acid and neuro-degenerative diseases

Some of the metabolites produced during the kynurenine pathway (Figure 1.3) are neuroactive (33). The intermediate metabolite quinolinic acid has received considerable attention. It was found to cause neural death by direct intracerebral injection (34) or when applied to neurons in vitro (35). Quinolinic acid toxicity is due to selective activation of the neuronal N-methyl-D-aspartic acid (NMDA) subtype of glutamate receptors (36). High levels of quinolinic acid in the cerebrospinal fluid have been found to occur in a number of disease states such as; cerebral malaria (37), ischemic brain disease (38), poliovirus brain infection (39), Alzheimer's, Parkinson's and Huntingdon's (40).

1.7.2 Age-related cataracts

Kynurenine pathway metabolites have been implicated in cataract formation (41). The UV filter compounds found in the human lens are metabolites from the kynurenine pathway and include kynurenine, 3-hydroxy-kynurenine, 3-hydroxy-kynurenine glucoside and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid glucoside (42). These UV filters are synthesised from L-Trp in the lens epithelial cells (42); with age these UV filters tend to accumulate in the lens and ‘kynurenilation’ occurs causing yellowing of the lens (43).

1.7.3 Defence against pathogens

Infection with viruses or parasites causes induction of IDO within the infected tissues (44). The local induction of IDO suppresses the growth of pathogens through IDO-mediated L-Trp deprivation from the infected area. IDO is induced by IFN-ϒ which is a cytokine produced by natural killer cells as part of the innate immune response (32,45). The degradation of L-Trp by IDO thus plays a role in the defence mechanism against bacterial and viral infections.
1.7.4 The role of IDO and TDO in immunosuppression

Immune escape is a critical property of cancer progression. It was discovered that most human malignant tumour cells express IDO. Those tumours expressing high concentrations of IDO escape immune surveillance of the host by degrading local L-Trp which inhibits the cells immune responses (46). It was also found that placental IDO prevents rejection of the foetus based upon the finding that pharmacological inhibition of the placental IDO caused rejection of the foetus in mice (47).

The expression of TDO in cancer cells has also been reported (48). TDO also causes suppression of the immune system by depletion of local L-Trp in a similar manner to IDO.

1.8 Inhibitors of dioxygenase activity

Reports of IDO and TDO expression in cancer cells have identified these enzymes as potential targets for pharmacological intervention. Initial studies involving β-carboline derivatives (49) were found to inhibit the oxidation of L-Trp by both IDO and TDO. The most potent was a compound called norharman, which competed with oxygen to bind directly to ferrous heme iron thereby inhibiting the enzymes (50). A study by Sono et al in 1991, found that three analogues of L-Trp, 1-methyl-tryptophan (1-Me-Trp), β-(3-benzofuranyl)-alanine (O-Trp) and β-(3-benzo(b)thienyl)-alanine (S-Trp) were competitive inhibitors of IDO (51). The most potent inhibitor was 1-Me-Trp, which is currently in clinical trials, with a reported $K_i$ of 6.6 μM at pH 8 (51). Screening studies were undertaken against libraries of natural products to find inhibitors with an increased potency than those already reported, which tend to be active at concentrations > 10 μM. From the natural products screened; exiguamine A ($K_i = 210$ nM), annulin B ($K_i = 120$ nM) and annulin C ($K_i = 140$ nM) were found to be the most potent inhibitors of IDO (52–54). There have been numerous studies to identify potent inhibitors of both IDO (55–57) and TDO (58,59) (Figure 1.4). These studies have highlighted the importance of understanding
the mechanism of L-Trp oxidation by IDO and TDO in order to facilitate the rational design of inhibitors.

Figure 1.4: Structures of some IDO inhibitors reproduced from reference (56).

1.9 Substrate specificity

TDO is highly specific for L-Trp. Only bacterial XcTDO appears to tolerate minor substitutions on the indole ring, for example 5- or 6- L-fluoro-tryptophan (60,61). In comparison, IDO has the ability to oxidise a wider range of L-Trp analogues such as D-Trp, 5-hydroxy-L-tryptophan, 5-methyl-L-tryptophan and 1-methyl- L-tryptophan (60,62). The structures of these tryptophan analogues are shown in Figure 1.5.
1.10 Heme reduction potentials of IDO and TDO

The reported reduction potentials vary considerably for the heme-containing dioxygenases and are summarised in Table 1.1. These reduction potentials are low for oxygen binding proteins with the exception of XcTDO. The reduction potentials are lower than those typically found for the globins (≈ 150 mV haemoglobin and ≈ 50 mV myoglobin (63)) and are closer to the reduction potential of the peroxidase enzymes (≈ -200 mV) (64). The reduction potentials in Table 1.1 highlight how hIDO and XcTDO favour binding of L-Trp to the ferrous form and discriminate against binding to the ferric form of these enzymes. Binding of L-Trp increases the reduction potential thereby facilitating reduction of the heme. In comparison, hTDO does not discriminate against L-Trp binding to either the ferrous or ferric forms of the enzyme, as there is no
change in reduction potential upon L-Trp binding. The functional implications of this at present are unknown.

Table 1.1: Reported reduction potentials of IDO and TDO.

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<tr>
<th>Protein</th>
<th>Summary of reduction potentials</th>
<th>References</th>
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<tr>
<td></td>
<td>Fe^{2+}/Fe^{3+} (mV)</td>
<td>Fe^{2+}/Fe^{3+}</td>
</tr>
<tr>
<td>hIDO</td>
<td>-63</td>
<td>-16</td>
</tr>
<tr>
<td>hTDO</td>
<td>-92</td>
<td>-76</td>
</tr>
<tr>
<td>XcTDO</td>
<td>+8</td>
<td>+144</td>
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</table>

1.11 The source of reducing equivalents in IDO and TDO

In order for L-Trp oxidation to occur, reduction of the heme iron is required prior to oxygen binding. The physiological reductase for IDO and TDO has not yet been clarified, however, it has been suggested for IDO that cytochrome b₅ and cytochrome b₅ reductase keep the heme reduced in vivo (67,68). Majority of in vitro assays involve the use of L-ascorbate and methylene blue (60,65,66,69–71) in order to provide the reducing equivalents required for L-Trp oxidation. The use of L-ascorbate alone (72–75) or a combination of NADPH-cytochrome P450 reductase and cytochrome b₅ (76) has also been used to reduce TDO during in vitro assays. A recent study has demonstrated that NADH has the capability to reduce ferric IDO directly (77).
1.12 Crystallographic studies

The X-ray crystal structures of *Drosophila melanogaster* (*DmTDO*) (78), *Cupriavidus metallidurans* (*CmTDO*) (79), hTDO (80) and *Shewanella oneidensis* (sIDO) (31) have been published. However, the structural information provided by these structures, to facilitate the understanding of L-Trp binding and the mechanism of L-Trp oxidation, is limited in comparison to the structures of hIDO (81) and XcTDO (31).

1.12.1 The crystal structure of hIDO

In 2006, Sugimoto *et al* solved the X-ray crystal structure of ferric hIDO (81) in complex with a known inhibitor of hIDO, 4-phenylimidazole (82) (Figure 1.6) as well as in complex with cyanide. The structure of hIDO consists of two distinct domains (large and small) connected by a loop of 17 residues (250 - 267), which lies above the distal face of the heme. The small domain comprises of six α-helices, two short β-sheets and three 3_10 helices. The large domain is an all α-helical domain composed of thirteen α-helices and two 3_10 helices as well as harbouring the active site of the enzyme. In the large domain four long helices (G, I, Q, and S) run parallel to the heme plane. Helix Q provides an endogenous ligand (His346) for the heme iron at the fifth coordination position on the proximal side.
**Figure 1.6:** Ribbon representation of the overall structure of hIDO in complex with 4-phenylimidazole (PDB code 2D0T) (81). The small domain is depicted in red; the large domain in green and the small loop connecting both domains in blue. The inhibitor 4-phenylimidazole and heme are shown in magenta as a ball and stick representation.

The distal heme pocket (Figure 1.7) is made up of residues from the small and large domains as well as the small loop. The distal heme pocket contains a significant number of hydrophobic residues in order to accommodate binding of the hydrophobic substrate, L-Trp. The only relatively polar residue in the active site is Ser167, which is also one of the closest residues to the heme iron. In the 4-phenylimidazole bound hIDO crystal structure, Phe163 interacts with the phenyl group of 4-phenylimidazole through π-π stacking.
Figure 1.7: The active site of hIDO in complex with the inhibitor 4-phenylimidazole shown in magenta. The active site residues and heme are shown in green.

1.12.2 The crystal structure of XcTDO

In 2007, Forouhar et al solved the X-ray crystal structure of XcTDO (31). The enzyme was crystallised in the catalytically active ferrous form in complex with L-Trp and 6-fluoro-tryptophan. The overall structure of XcTDO is tetrameric with each monomer (Figure 1.8) consisting of twelve α-helices.
The active site of XcTDO (Figure 1.9) contains mainly hydrophobic residues to accommodate binding of the hydrophobic substrate L-Trp. Residue His240 occupies the proximal fifth coordination site of the heme iron. An electrostatic interaction with the side chain Arg117, as well as hydrogen bonding to the hydroxyl side chain of Tyr113 and main chain amide of Thr254 is formed between the carboxylate group of L-Trp. The amino group of L-Trp is recognised by the propionate side chain of the heme group, as well as hydrogen bonding to the side chain hydroxyl group of Thr254. The indole ring is held in place above the heme group by Van der Waals interactions with the side chain of Phe51 and several other hydrophobic residues. The nitrogen of the indole ring is hydrogen-bonded to the side chain of His55 (Figure 1.9). The Arg117 residue alters its orientation in the distal pocket to the closed conformation in the presence of L-Trp and to the open conformation in the absence of L-Trp.
**Figure 1.9**: Active site of XcTDO showing L-Trp (magenta) bound to the ferrous heme.

### 1.12.3 Comparison between IDO and TDO

Many of the active site residues are equivalent in both the hIDO and XcTDO structures (Table 1.2).

### Table 1.2: Comparison of hIDO and XcTDO active sites. Residues that align in each active site are presented on the same line.

<table>
<thead>
<tr>
<th>hIDO active site residues</th>
<th>XcTDO active site residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser167</td>
<td>His55</td>
</tr>
<tr>
<td>Phe226</td>
<td>Tyr113</td>
</tr>
<tr>
<td>Phe163</td>
<td>Phe51</td>
</tr>
<tr>
<td>Arg231</td>
<td>Arg117</td>
</tr>
<tr>
<td>Phe164</td>
<td>Ile52</td>
</tr>
<tr>
<td>Phe227</td>
<td>Met114</td>
</tr>
</tbody>
</table>
The most significant difference between the active sites of hIDO and XcTDO is the presence of a histidine (His55) residue in XcTDO and absence in hIDO (equivalent residue is Ser167). An active site base (a histidine residue) has been implicated as critical for the mechanism of L-Trp oxidation by the heme-containing dioxygenases (6,83).

1.13 Substrate inhibition of IDO

Since the inhibition of hIDO at elevated levels of L-Trp was observed over 40 years ago (84,85), a number of differing proposals have been put forward in the literature. Initially inhibition was understood to be the result of unproductive L-Trp binding to the ferric form of hIDO (86). This interpretation is inconsistent with the reported reduction potentials of hIDO, where an increase in the reduction potential is observed upon binding of L-Trp (65) thereby discriminating against the binding of L-Trp to the catalytically inactive ferric form of the enzyme.

A secondary, weaker L-Trp binding site was proposed from steady state measurements (71). It was understood that substrate inhibition could be a result of L-Trp binding to this secondary site, other sites in hIDO or even in multiple conformations within the distal cavity itself (71,87–89). It is important to note the physiological concentrations of L-Trp, which are in the ranges of 40 - 100 μM (90,91). Therefore, the question arises as to whether binding to any external sites would occur at these physiological concentrations.

Substrate inhibition was proposed to occur due to the sequential, binding of L-Trp and oxygen (92). At low concentrations of L-Trp, oxygen binds to hIDO prior to L-Trp; at higher concentrations of L-Trp the order of binding is reversed. It was demonstrated that the heme reduction potential has a regulatory role in controlling the overall rate of catalysis through the correlation between the reduction potential and the rate constant for oxygen binding (92).

The authors of this study have also suggested this explanation to account for the lack of substrate inhibition observed in hTDO. Binding of L-Trp to ferrous hTDO is much weaker in comparison to hIDO (60,74). Increases in $K_D$ will
correspondingly increase the effective inhibition constant so that substrate inhibition is not observed (92).

1.14 Reactions of IDO and TDO with hydrogen peroxide

1.14.1 Reactions of hydrogen peroxide and ferric IDO

When exposed to low levels of hydrogen peroxide ($\text{H}_2\text{O}_2$) L-Trp oxidation by IDO was inhibited and structural changes were observed (93). It was recently reported that exposure of IDO to hydrogen peroxide inhibits dioxygenase activity via Compound I initiated oxidative damage to both the heme centre and protein structure (94). The presence of L-Trp prevented inactivation of dioxygenase activity by reacting with Compound I to yield a Compound II species, thereby protecting IDO from further oxidative damage (94).

It was found that IDO has the ability to catalyse the oxidation of indole by hydrogen peroxide to generate two major products; 2-oxindole and 3-oxindole (95). The results demonstrate that IDO has the ability to oxidise indole (not L-Trp) by a previously unreported peroxygenase activity (95) in a manner that is analogous to the ‘peroxide shunt’ pathway of cytochrome P450.

1.14.2 Reactivation of ferric TDO by hydrogen peroxide

The activation of ferric TDO by hydrogen peroxide in the presence of L-Trp was reported in the literature 65 years ago (96) however, the mechanism has remained unknown. Liu et al demonstrated, using spectroscopy, that the addition of hydrogen peroxide to ferric CmTDO resulted in the formation of ferrous CmTDO (97). The catalytic competence of the ferrous CmTDO to oxidise L-Trp to produce NFK was confirmed using the inhibitor carbon monoxide.

Activation of ferric TDO occurs through oxidation using hydrogen peroxide to form a Compound ES intermediate ($\text{Fe}^{IV}=\text{O}$ and a protein based radical).
Compound ES is then reduced by L-Trp to produce ferrous TDO, which oxidises the remaining L-Trp to NFK (Figure 1.10).

The physiological relevance of this reaction stems from the fact that TDO is found in the liver, which is known to have an oxidising environment. To prevent inactivation of TDO, through oxidation of the heme iron, TDO could react with physiological levels of hydrogen peroxide. This reaction could lead to the formation of ferrous TDO, which binds oxygen and oxidises L-Trp to form NFK.

**Figure 1.10:** The proposed reactivation pathway of ferric TDO using hydrogen peroxide leading to formation of ferrous TDO and subsequently NFK formation. Reproduced from reference (97).
1.15 Mechanism of tryptophan oxidation by IDO and TDO

Since their discovery (22,23,84), the heme-containing dioxygenases involved in tryptophan oxidation have had a complicated history. It might be perceived from the literature that the mechanism of L-Trp oxidation by IDO and TDO has been fully established. However, this is not the case and significantly more work is required to be undertaken in order to completely understand the subtle differences between IDO and TDO and establish the precise mechanism of L-Trp oxidation, reviewed in Millett et al (98).

1.15.1 First step of the mechanism

The mechanism of L-Trp oxidation was initially proposed to involve base-catalysed abstraction (6,83) of the indole proton of L-Trp by an active site base (a histidine residue), Figure 1.11. This process initiates the nucleophilic attack of the electron-rich indole carbon (position C3) on the distal oxygen atom of the heme iron bound oxygen and results in the formation of an intermediate 3-indolenylperoxy-ferrous complex. This intermediate can then be converted to NFK via one of two pathways either a Criegee (83,99) or dioxetane (100) rearrangement.

Figure 1.11: Original mechanistic proposal for the oxidation of L-Trp by the heme-containing dioxygenases (Criegee rearrangement shown in blue and dioxetane rearrangement shown in red) (6,83)
The presumed presence of an active site base (a histidine residue) in the heme-containing dioxygenases provided the foundation for the mechanistic proposal in Figure 1.11. Sequence homology (~35%) was found between gastropod mollusc IDO-like myoglobins and hIDO (101,102). It was revealed that two histidine residues (His335 and His380) were conserved in all sequences and that His380 was understood to be the heme-binding fifth proximal ligand and His335 the sixth distal histidine ligand. It was proposed from the amino acid sequence of hIDO that the corresponding residues would be His303 (distal histidine ligand) and His346 (proximal histidine ligand). From EPR spectroscopy (84,103) it was suggested for both IDO and TDO, that the fifth proximal heme ligand was a nitrogen atom; possibly from a histidine residue. It was also suggested that the sixth distal heme ligand was also a nitrogen atom from a histidine residue.

The mechanism in Figure 1.11 was also based upon the idea that only substrates containing a proton on the indole nitrogen were active. Sono et al observed that tryptophan analogues, such as 1-methyl-tryptophan (1-Me-Trp) which contains a methyl group at the indole nitrogen were inhibitors of dioxygenase activity (51). This observation was interpreted to mean that deprotonation of the indole nitrogen was essential for catalysis.

The absence of an active site histidine in hIDO (81) meant that the mechanism required revision. It was proposed for hIDO that the heme bound dioxygen interacts with L-Trp through a hydrogen bond between the proximal oxygen atom and the NH group of the indole ring of L-Trp (Figure 1.12) (70,81). This interaction then facilitates the electrophilic addition of dioxygen to L-Trp.
Figure 1.12: Proposed mechanisms for the activation of oxygen by the heme-containing dioxygenases (A) The base-catalysed abstraction mechanism (6,83) (B) An alternative proposal to the base-catalysed abstraction mechanism involving abstraction of the indole proton by the heme bound oxygen (70,81).

More recently, difficulties with both mechanisms of oxygen activation in Figure 1.12 have become apparent. Spectroscopic work using $^1$H ENDOR (104) has shown that the heme bound dioxygen is not hydrogen bonded to L-Trp thereby ruling out the mechanism proposed in Figure 1.12B. Density functional theory calculations (105) do not support base-catalysed abstraction and alternative mechanisms were suggested including electrophilic addition or radical addition of the heme bound oxygen. The indole NH group of L-Trp has a very high theoretical $pK_a \approx 17$ (106) making it very difficult for weak bases such as a distal histidine or the heme bound oxygen to abstract a proton. The mechanistic proposal in Figure 1.12 was inconsistent with the chemistry of indoles (107), which do not react by base-catalysed abstraction. It was found that the replacement of the distal histidine in TDO, through mutagenesis studies, does not shut down enzymatic activity completely (61,74,108,109). This suggests that the distal histidine is not essential for L-Trp oxidation by TDO. The most conflicting experimental evidence with the base-catalysed abstraction mechanism (Figure 1.11) is that 1-Me-Trp was found to be a slow substrate of hIDO (62), CmTDO (110) as well as variants of hTDO and XcTDO (62).
1.15.2 Last step of the mechanism

A Criegee (83,99) or dioxetane (100) rearrangement was proposed as the final step of the original mechanistic proposal (Figure 1.11) leading to the formation of NFK. The Criegee rearrangement mechanism is well known in the non-heme iron literature (111,112).

Yeh et al detected a stretching frequency characteristic of a ferryl heme species assigned as Compound II (FeIV=O) in hIDO using resonance Raman spectroscopy during the oxidation of L-Trp (113). Interestingly a ferryl species was not detected in hTDO under the same experimental conditions. In a separate study also involving resonance Raman, Ogura et al observed a ferryl heme species during L-Trp oxidation by hIDO (114,115). Detection of a ferryl heme lead to the suggestion that dioxygen addition to L-Trp occurs sequentially (113,116) rather than simultaneously.

The formation of an epoxide was suggested to accompany formation of a ferryl heme (113). Computational studies support formation of an epoxide (116,117) and indirect evidence for an epoxide intermediate has been detected using mass spectrometry (118).

1.15.3 Alternative mechanistic proposal

An alternative mechanism has been proposed based upon the latest experimental and computational studies, Figure 1.13 (113–115,117,118). This alternative mechanism involves insertion of the first oxygen atom by either electrophilic addition or radical addition. This leads to the formation of a Compound II heme intermediate and a 2,3-epoxide intermediate. The second oxygen atom is inserted into the 2,3-epoxide, which is rearranged to form NFK. A recent heme modification study using *Pseudomonas aeruginosa* (PaTDO) suggested that the insertion of the initial oxygen atom occurs by electrophilic addition rather than radical addition (119).
Figure 1.13: An alternative mechanistic proposal for the oxidation of L-Trp by the heme-containing dioxygenases based on current experimental and computational evidence (113–115,117,118). Step 1a involves electrophilic addition of the first oxygen atom and step 1b involves radical addition of the first oxygen atom.

1.16 Future Direction

Although there has been a significant surge in experimental and computational information surrounding the heme-containing dioxygenases over recent years, (113–115,117,118) there is still much to learn with regard to IDO and TDO:

- It has not been determined whether L-Trp oxidation by IDO and TDO proceeds by either electrophilic addition (step (i) in Figure 1.13) or by radical addition of the initial oxygen atom (step (ii) in Figure 1.13).
- A Compound II (Fe$^{IV}$=O) intermediate has only been observed during the oxidation of L-Trp by IDO. Therefore, further investigation is required to determine whether other substrates (e.g. 1-Me-Trp) are oxidised by the same mechanism as L-Trp.
- A Compound II intermediate has not been observed during the oxidation of L-Trp by TDO. The assumption in the literature is that both IDO and TDO oxidise L-Trp by a common mechanism.
There is limited evidence to support formation of a 2,3-epoxide in the literature; this intermediate has only been detected indirectly by mass spectrometry.

The aim of the work undertaken in this study was to investigate these issues in order to gain further information in relation to IDO and TDO.
1.17 References


15. Hamberg, M., Zhang, LY., Brodowsky, ID., Oliw, EH (1994) Sequential oxygenation of linoleic acid in the fungus Gaeumannomyces graminis:


51. Cady, SG., Sono, M (1991) 1-methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan) and beta-[3-benzo(b)thienyl]-alanine (the sulfur analog of tryptophan) are competitive Inhibitors for indoleamine 2,3-dioxygenase. *Archives of Biochemistry and Biophysics* 291(2):326–33.


Chapter 2: Materials and Methods
This Chapter describes the experimental methods and techniques used throughout this thesis.

2.1 Chemicals and reagents

All chemicals of analytical and reagent grade were obtained from commercial sources (Sigma-Aldrich, Apollo Scientific, Fischer, Acros Organics and Melford) and used without further purification unless otherwise stated. All media, buffers and solutions were prepared using ultra-pure, doubly deionised water from an Elga PureLab water purifier. All molecular biology kits and enzymes were used according to the manufacturers protocols.

2.2 Media

Media used for *Escherichia coli* (*E.coli*) cell growth and protein expression are given in Table 2.1. All media was autoclaved at 121 °C for 20 minutes prior to use.

<table>
<thead>
<tr>
<th>Table 2.1: Media used for <em>E.coli</em> cell growth and protein expression.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LB</strong></td>
</tr>
<tr>
<td>1% (w/v) tryptone</td>
</tr>
<tr>
<td>1% (w/v) NaCl</td>
</tr>
<tr>
<td>0.5% (w/v) yeast extract</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.3 Expression vectors

The expression vectors used in this study are summarised in Table 2.2 and vector maps shown in Appendix A.

Table 2.2: Expression vectors.

<table>
<thead>
<tr>
<th>Vector</th>
<th>His-tag</th>
<th>Supplier</th>
<th>Antibiotic Resistance</th>
<th>Recombinant DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQE30</td>
<td>N-terminal</td>
<td>Qiagen</td>
<td>100 μg/ml ampicillin</td>
<td>hIDO</td>
</tr>
<tr>
<td>pET151d</td>
<td>N-terminal</td>
<td>Invitrogen</td>
<td>100 μg/ml ampicillin</td>
<td>hIDO</td>
</tr>
<tr>
<td>pET28a</td>
<td>C-terminal</td>
<td>Novagen</td>
<td>30 μg/ml kanamycin</td>
<td>hTDO</td>
</tr>
<tr>
<td>pET21d</td>
<td>C-terminal</td>
<td>Novagen</td>
<td>100 μg/ml ampicillin</td>
<td>XcTDO</td>
</tr>
<tr>
<td>pLeics05</td>
<td>C-terminal</td>
<td>Protex</td>
<td>100 μg/ml ampicillin</td>
<td>hTDO</td>
</tr>
</tbody>
</table>

2.4 *E. coli* cell strains

The *E. coli* cell strains used in this study are listed in Table 2.3.

Table 2.3: *E. coli* cell strains.

<table>
<thead>
<tr>
<th><em>E. coli</em> cell strain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>DNA isolation</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Expression of hTDO and XcTDO</td>
</tr>
<tr>
<td>SG1300</td>
<td>Expression of hIDO</td>
</tr>
</tbody>
</table>
2.5 Molecular biology

2.5.1 Primer Design

A number of primers were designed for the cloning and truncation of hTDO, Table 2.4. Forward and reverse primer pairs were designed to have similar melting temperatures \(T_m\) that were calculated using the formula in Equation 2.1.

Table 2.4: The forward and reverse primers used to generate alternative constructs of hTDO.

<table>
<thead>
<tr>
<th>Variant</th>
<th>(T_m/\degree C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Full length hTDO pLeics05:</strong></td>
<td></td>
</tr>
<tr>
<td>Forward: 5' AGGAGATATACATATGAGTGGGTGCCCATTTTTTA 3'</td>
<td>50.5</td>
</tr>
<tr>
<td>Reverse: 5' GAAGTACAGGGTTCTCTTAATCTGATTTCATTCGCTGAA 3'</td>
<td>50.6</td>
</tr>
<tr>
<td><strong>Truncated hTDO pLeics05 deleted residues 1 - 17 and 389 - 406:</strong></td>
<td></td>
</tr>
<tr>
<td>Forward: 5' AGGAGATATACATATGCTCCCCGTAGAAGGCAGCGA 3'</td>
<td>57.9</td>
</tr>
<tr>
<td>Reverse: 5' GAAGTACAGGGTTCTCAAATTTGTGAATGGTTGGGTTCATCT 3'</td>
<td>53.2</td>
</tr>
<tr>
<td><strong>Truncated hTDO pLeics05 deleted residues 1 - 19 and 388 - 406:</strong></td>
<td></td>
</tr>
<tr>
<td>Forward: 5' AGGAGATATACATATGGTAGAACAGCGAAGAAGA 3'</td>
<td>51.8</td>
</tr>
<tr>
<td>Reverse: 5' GAAGTACAGGGTTCTTTTGTGAATGGTTGGTGCATCTT 3'</td>
<td>52.3</td>
</tr>
</tbody>
</table>

Equation 2.1:

\[
T_m (\degree C) = 64.9 + 41(G+C-16.4) / (A+T+G+C)
\]

Where A, T, G and C are the number of each respective DNA base.
Once the required primers had been designed, they were checked using the website OligoCalc (http://www.basic.northwestern.edu/biotools/OligoCalc.html), which analyses the potential for self-complementary sequences. Primers were purchased from Invitrogen and were dissolved in sterile water to give a stock concentration of 100 μM and stored at -20 °C.

2.5.2 Polymerase chain reaction (PCR)

Cloning of hTDO was performed by Dr Xiaowen Yang at the PROTEX facility, University of Leicester.

2.5.3 Transformation of competent *E.coli* cells with plasmid DNA

Recombinant hTDO, hIDO or XcTDO DNA 1 μl (50-100 nM) was added to 50 μl of competent *E.coli* cells and incubated on ice for 20 minutes. The cells were heat shocked at 42 °C for 30 seconds, chilled on ice for 2 minutes and 80 μl of LB-media added. The cells were incubated at 37 °C for 60 minutes with shaking (200 rpm) before being plated onto LB-agar containing the appropriate antibiotic(s) and incubated (inverted) overnight at 37 °C.

2.5.4 DNA isolation

A single colony was selected from an LB-agar plate and used to inoculate 5 ml of LB-media supplemented with the appropriate antibiotic(s). The cell culture was incubated (shaking at 200 rpm) overnight at 37 °C. DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen) by following the manufacturers protocol. A small sample (1 ml) of the overnight cell culture was harvested by centrifugation at 13,000 rpm for 5 minutes (using a microcentrifuge). The supernatant was discarded and the cell pellet resuspended in 250 μl buffer P1 (containing RNase). Buffer P2 (250 μl) was added and the tube gently inverted 4-6 times prior to centrifugation at 13,000 rpm for 10 minutes. The supernatant was added to a QIAprep spin column and centrifuged at 13,000 rpm for 1 minute.
and the flow-through discarded. The column was washed with 500 μl of PB buffer, centrifuged at 13,000 rpm for 1 minute and the flow-through discarded. The column was washed with 750 μl PE buffer, centrifuged at 13,000 rpm for 1 minute and the flow-through discarded. The column was centrifuged at 13,000 rpm for a further 1 minute. The column was placed in a clean 1.5 ml eppendorf tube, 50 μl of EB buffer (10 mM Tris-HCl buffer, pH 8.5) was added to the column and left to stand for 1 minute before centrifuging at 13,000 rpm for 1 minute to recover the DNA. The DNA concentration was determined using a NanoDrop spectrophotometer and stored at -20 °C.

2.5.5 DNA sequencing

All DNA sequencing was performed by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) based at the University of Leicester using an Applied Biosystems 3730 automated fluorescent sequencer. The sequencing primers are listed in Table 2.5.

Table 2.5: The forward and reverse primers used for sequencing.

<table>
<thead>
<tr>
<th>Variant and sequencing primer</th>
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<tbody>
<tr>
<td><strong>Full length hTDO pLeics05:</strong></td>
</tr>
<tr>
<td>Forward: 5’ TAATACGACTCACTATAGGG 3’</td>
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<tr>
<td>Reverse: 5’ GCTAGTTATTTGCTCAGCGG 3’</td>
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<tr>
<td><strong>Truncated hTDO pLeics05 deleted residues 1 - 17 and 389 - 406:</strong></td>
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<tr>
<td>Forward: 5’ TAATACGACTCACTATAGGG 3’</td>
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<td>Reverse: 5’ GCTAGTTATTTGCTCAGCGG 3’</td>
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<td><strong>Truncated hTDO pLeics05 deleted residues 1 - 19 and 388 - 406:</strong></td>
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<tr>
<td>Forward: 5’ TAATACGACTCACTATAGGG 3’</td>
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<tr>
<td>Reverse: 5’ GCTAGTTATTTGCTCAGCGG 3’</td>
</tr>
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</table>
2.6 Electrophoresis

2.6.1 Agarose gel electrophoresis

Agarose gels 0.7-1.0% (w/v) containing 1 μg/ml ethidium bromide, were cast on a horizontal bed. Electrophoresis was performed in 0.5 x TAE buffer (40 mM Tris-HCl, 2 mM acetic acid, 1 mM EDTA, pH 8.5) at 100 V. DNA samples (10 μl) were mixed with 2 μl 6 x sample loading buffer prior to loading onto the gel. DNA ladder 4 μl (1 kb DNA ladder, New England BioLabs) was also loaded onto the gel to aid location of the desired bands. Nucleic acids were visualised by exposing the gel to long wavelength UV radiation on a transilluminator.

2.6.2 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels consisted of a 12% (w/v) polyacrylamide running gel and 4% (w/v) polyacrylamide stacking gel.

Protein samples (10 μl) were prepared by mixing with an equal volume of sample buffer and boiling for 5 minutes. The samples were loaded onto the gel along with a pre-stained molecular marker (New England BioLabs). The gels were run at 180 V in running buffer until the dye-front reached the end of the gel. The gels were stained with Coomassie Blue staining solution for 60 minutes before being de-stained by soaking in de-staining solution overnight. All solutions used for SDS-PAGE analysis are listed in Table 2.6.
Table 2.6: Solutions used for SDS-PAGE.

<table>
<thead>
<tr>
<th>Running gel</th>
<th>Stacking gel</th>
<th>Running buffer</th>
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<tbody>
<tr>
<td>12% (w/v) acrylamide</td>
<td>4% (w/v) acrylamide</td>
<td>25 mM Tris</td>
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<tr>
<td>375 mM Tris-HCl pH 8.8</td>
<td>125 mM Tris-HCl pH 6.8</td>
<td>250 mM glycine</td>
</tr>
<tr>
<td>1% (v/v) SDS</td>
<td>1% SDS</td>
<td>0.1% SDS</td>
</tr>
<tr>
<td>0.5% (v/v) APS</td>
<td>0.5% APS</td>
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</tr>
<tr>
<td>0.1% (v/v) TEMED</td>
<td>0.1% (v/v) TEMED</td>
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</table>

<table>
<thead>
<tr>
<th>Sample loading buffer</th>
<th>Staining solution</th>
<th>De-staining solution</th>
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<tbody>
<tr>
<td>50 mM Tris-HCl pH 8.8</td>
<td>0.125% (v/v) Coomassie blue R250</td>
<td>30% (v/v) methanol</td>
</tr>
<tr>
<td>1% SDS</td>
<td></td>
<td>10% (v/v) acetic acid</td>
</tr>
<tr>
<td>0.5% (v/v) bromophenol blue</td>
<td>30% (v/v) methanol</td>
<td></td>
</tr>
<tr>
<td>10% (v/v) glycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM DTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.7 Protein expression

Recombinant proteins were over-expressed in *E.coli* cells. A 200 ml starter culture of 2xYT-media supplemented with the appropriate antibiotic(s) was inoculated from either, a colonised LB-agar plate or glycerol stock and incubated overnight at 37 °C with shaking at 200 rpm. The starter culture was used to inoculate 12 x 600 ml of 2xYT-media supplemented with the same quantity of antibiotic(s) as the starter culture. This larger culture was incubated at 37 °C with shaking at 200 rpm until the absorbance at 600 nm (OD$_{600}$) reached ~0.8. Protein expression was induced by the addition of 0.2 mM IPTG. The cells were then incubated for 24 hours with shaking at 200 rpm and the temperature reduced (Table 2.7). Cells were harvested by centrifugation at 4,000 g for 15 minutes at 4 °C and pellets stored at -80 °C.

Table 2.7: Protein expression temperatures.

<table>
<thead>
<tr>
<th>Protein (construct)</th>
<th>Expression temperature / °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>hIDO (pQE30)</td>
<td>27</td>
</tr>
<tr>
<td>hIDO (pET151d)</td>
<td>25</td>
</tr>
<tr>
<td>hTDO (pET28a, pLeics05)</td>
<td>25</td>
</tr>
<tr>
<td>XcTDO (pET21d)</td>
<td>18</td>
</tr>
</tbody>
</table>
2.8 Protein purification

The buffers used during the purification of hIDO, hTDO and XcTDO are listed in Table 2.8.

2.8.1 Bacterial cell lysis

Cell pellets were re-suspended in 50 ml lysis buffer (Table 2.8) supplemented with two complete protease inhibitor tablets (Roche) and lysed by the addition of 5 mg of lysozyme and by sonication (on ice, 6 x 30 seconds pulses with 1 minute intervals using an MSE Soniprep 150 sonicator). After sonication, 5 mg of DNase I and 20 mM MgCl₂ were added and the suspension stirred for 30 minutes at 4 °C. The lysate was then centrifuged at 38,000 g for 50 minutes.

2.8.2 Ni-NTA metal-affinity chromatography

A 20 ml column of Ni-NTA superflow resin (Qiagen) was equilibrated with lysis buffer (Table 2.8) and the cell free extract loaded onto the column. The resin was washed with 15 column volumes (CV) of wash buffer (Table 2.8) and protein eluted using either a linear gradient ranging from 20-250 mM imidazole (hTDO and XcTDO, Table 2.8) or 100 mM EDTA (hIDO, Table 2.8).
Table 2.8: Buffers used for the purification of hIDO, hTDO and XcTDO.

<table>
<thead>
<tr>
<th>Protein</th>
<th>hIDO</th>
<th>hTDO</th>
<th>XcTDO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysis buffer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>50 mM NaH$_2$PO$_4$</td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>300 mM NaCl,</td>
<td>300 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>pH 8.0</td>
<td>10 mM imidazole,</td>
<td>pH 8.0</td>
<td>pH 8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wash buffer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash buffer</td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>50 mM NaH$_2$PO$_4$</td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>300 mM NaCl</td>
<td>300 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>pH 6.0</td>
<td>20 mM imidazole</td>
<td>20 mM imidazole</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 8.0</td>
<td>pH 8.0</td>
<td></td>
</tr>
<tr>
<td><strong>Elution buffer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elution buffer</td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>50 mM NaH$_2$PO$_4$</td>
<td>50 mM NaH$_2$PO$_4$</td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>300 mM NaCl</td>
<td>300 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>100 mM EDTA</td>
<td>20-250 mM imidazole</td>
<td>20-250 mM imidazole</td>
<td></td>
</tr>
<tr>
<td>pH 8.0</td>
<td>pH 8.0</td>
<td>pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>
2.8.3 Size exclusion chromatography

A pre-packed column (HiLoad 16/60 Superdex 75 (hIDO) or 200 (hTDO and XcTDO), GE Healthcare) was equilibrated with 50 mM Tris, 100 mM NaCl, pH 8.0 using an AKTA FPLC (GE Healthcare). The protein sample (2 ml) was injected onto the column and subsequently eluted using a flow rate of 1 ml/minute collected as 1 ml fractions (absorbance measured at 280 nm). The fractions were analysed using SDS-PAGE and those fractions primarily containing the protein of interest (hIDO, hTDO or XcTDO) were collected together and dialysed against 50 mM Tris, pH 8.0.

2.8.4 Heme reconstitution

Each protein was reconstituted with hemin prior to storage at -80 °C. Hemin (1.5 equivalents) was added to the protein stock and then incubated on ice in the dark for 60-120 minutes. Free hemin was removed from the protein by passage through a small gel filtration column (Bio-Rad 10 DG) before being concentrated using a Millipore Amicon Ultra 30 kDa filtration unit.

2.8.5 Protein concentration determination

Purified protein was concentrated using a Millipore Amicon Ultra centrifugal filter with a molecular weight cut-off of 30 kDa.

Protein concentration was determined by measuring the absorbance at the Soret band and the concentration calculated using the Beer Lambert law (Equation 2.2) using the following extinction coefficients for each protein hIDO $\varepsilon_{404} = 172 \text{ mM}^{-1}\text{cm}^{-1}$ (1), hTDO $\varepsilon_{408} = 196 \text{ mM}^{-1}\text{cm}^{-1}$ (2), XcTDO $\varepsilon_{404} = 180.5 \text{ mM}^{-1}\text{cm}^{-1}$ (3).
Equation 2.2:

\[ A = \varepsilon c l \]

Where \( A \) is the absorbance of the stock solution, \( c \) is the concentration of the protein, \( \varepsilon \) is the molar absorption coefficient and \( l \) is the pathlength of the cuvette.

2.9 UV-visible spectroscopy

All absorbance spectra and equilibrium ligand binding experiments were measured in 50 mM Tris-HCl buffer, pH 8.0 at 25 °C using a Perkin Elmer lambda 25, 35 or 40 UV–visible spectrophotometer. A JASCO spectrophotometer housed in an anaerobic glovebox (Belle Technology, \([O_2] < 5 \text{ ppm}\)) was used for oxygen sensitive measurements.

Typically a 200-700 nm scan was obtained using a scan speed of 960 nm/minute, acquiring at 0.5 nm intervals, using either a 0.5 ml, 1 ml or 3 ml (10 mm pathlength) quartz cuvette. Baseline corrections were made against all buffers and solvents.

2.10 Anaerobic glovebox solution preparation

Buffers were made anaerobic by bubbling oxygen-free nitrogen gas through them for ~60-120 minutes. Buffers were then placed in the glovebox overnight in order to remove any residual traces of oxygen. Solutions of sodium dithionite and substrates were prepared by adding the appropriate solid to anaerobic buffer inside the glovebox.

Buffers (~5 ml) were saturated with oxygen by bubbling with oxygen gas for ~15 minutes in a septum sealed flask. Final oxygen concentrations were calculated
on the basis of a saturating concentration of oxygen of ~1.2 mM at 25 °C and 1 atm.

2.11 Heme reduction

The ferrous forms of hIDO, hTDO and XcTDO were generated by the addition of microlitre volumes (1-5 μl) of sodium dithionite (prepared in anaerobic 50 mM Tris-HCl buffer, pH 8.0) to ferric hIDO, hTDO and XcTDO. The concentration of the sodium dithionite stock was not determined directly. Reduction was monitored spectrophotometrically using a JASCO spectrophotometer housed in an anaerobic glovebox (Belle Technology, [O₂] < 5 ppm) until no further spectral changes occurred.

2.12 Ligand bound derivative spectra

Ligands were dissolved in 50 mM Tris-HCl buffer pH 8.0 and microlitre volumes (5-10 μl) of excess ligand were added to ferric hIDO, hTDO or XcTDO (1-5 μM). Ligand binding was monitored spectrophotometrically until no further spectral changes occurred.

2.13 Ligand binding equilibria

Equilibrium binding constants (K_D) were determined by the addition of microlitre volumes (0.5-2.0 μl) of ligand from an appropriate stock solution (50 mM Tris-HCl buffer, pH 8.0) to ferric hIDO, hTDO or XcTDO (1-5 μM) until no further spectral changes occurred. Binding constants were determined spectrophotometrically by monitoring the change in absorbance at the appropriate wavelength and fitting to Equation 2.3. Data was fitted using the GraphPad Prism software package (version 6).
Equation 2.3

\[ \Delta A = \Delta A_\infty \left[ \text{Free} \right] \frac{K_D}{K_D + [\text{Free}]} \]

Where \( \Delta A \) and \( \Delta A_\infty \) are the absorbance changes corresponding to the intermediate and saturating ligand concentrations, \([\text{Free}]\) is the total concentration of unbound ligand and \( K_D \) is the equilibrium dissociation constant.

Ligand binding to ferrous XcTDO was carried out in an anaerobic glove box (Belle Technology, \([O_2] < 5 \text{ ppm}\)). Ferric XcTDO was reduced by stoichiometric titration with sodium dithionite (described in section 2.11, Chapter 2) prior to ligand binding as described above.

2.14 Steady state kinetics

Enzyme activity was measured by monitoring the formation of NFK at 321 nm. Reactions were performed at 25 °C in 50 mM Tris-HCl buffer, pH 8.0 containing: 10 \( \mu \text{M} \) methylene blue, 100 \( \mu \text{g} \) of catalase, 20 mM \( \text{L-ascorbate} \) and a fixed concentration of hIDO, hTDO or XcTDO (typically 100-300 nM). The reaction was initiated by the addition of tryptophan (\( \text{L-Trp} \)) or tryptophan analogue and initial rates calculated from the absorbance increase at 321 nm (\( \varepsilon_{321} = 3750 \text{ M}^{-1}\text{cm}^{-1} \)). The \( k_{\text{cat}} \) and \( K_M \) values were determined by varying the concentration of each substrate and by fitting the data to the Michaelis–Menten equation (Equation 2.4). Data was fitted using the GraphPad Prism software package (version 6).

Equation 2.4

\[ V = V_{\text{max}} \frac{[S]}{[S] + K_M} \]
Where, \( V_{\text{max}} \) \((k_{\text{cat}})\) is the maximal rate, the Michaelis constant \( (K_M)\) is the concentration at which the reaction rate is half its maximal value and \([S]\) is the substrate concentration.

In the steady state assay, L-ascorbate reduces the heme by transferring an electron via methylene blue to ferric enzyme (hIDO, hTDO or XcTDO). Catalase is required to remove peroxide formed during the reaction, which could lead to bleaching of the heme thereby negating enzymatic turnover.

### 2.15 Stopped flow experiments

Stopped flow experiments were carried out using an Applied Photophysics SX.18MV stopped flow spectrometer housed in an anaerobic glove box (Belle Technology Ltd., \([O_2] < 5 \text{ ppm}\)) and fitted with a Neslab RTE-200 circulating water bath (25 ± 0.1 °C). Multiple-wavelength absorption studies were carried out using a photodiode array detector and Pro SX software (Applied Photophysics Ltd.).

Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics Ltd.). The data was fitted to a 2-step model to extract spectra of intermediate species.

#### 2.15.1 Single mix stopped flow experiment

The single mix stopped flow experiment was initiated by mixing either ferrous or ferric hIDO, hTDO and XcTDO (2-5 μM) with substrate(s) (e.g. oxygen, hydrogen peroxide and tryptophan) and the resulting spectral changes were monitored.

#### 2.15.2 Sequential mix stopped flow experiment

The sequential mix stopped flow experiment (pseudo-first order conditions) was initiated by mixing ferrous hIDO, hTDO or XcTDO with oxygen-saturated buffer
(50 mM Tris-HCl buffer, pH 8.0, \([O_2] = 1.2 \text{ mM}\)). This solution is then aged for 50 milliseconds (to ensure complete formation of ferrous-oxy species \([\text{Fe}^{II}-\text{O}_2]\)) before a second mix with either \(\text{L-Trp}\) or tryptophan analogues ([substrate] \(\geq 10 \times K_M\) in cases where \(K_M\) is known). The resulting spectral changes were monitored.

2.16 Mass spectrometry

Samples were prepared in a glove box (Belle Technology, \([O_2] < 5 \text{ ppm}\)) through incubation of ferrous enzyme (1-2 \(\mu\)M) with either \(\text{L-Trp}\) or tryptophan analogue ([substrate] \(\geq 10 \times K_M\) in cases where \(K_M\) is known) prior to addition of aerobic solutions of buffer (50 mM Tris-HCl buffer, pH 8.0, \([O_2] = 258 \mu\text{M}\)). Mass spectrometry samples were also prepared using the steady state (\(\text{L-ascorbate, methylene blue, catalase}\)) assay. Samples were allowed to react for varying amounts of time (10-60 minutes) before being centrifuged at 13,000 rpm for 3 minutes (using a microcentrifuge) and the supernatant frozen directly on dry ice. Samples were stored at -80 °C until required for mass spectrometry analysis.

Samples were analysed by liquid chromatography mass spectrometry by Mr. Michael Lee (Department of Chemistry at the University of Leicester). Compounds were analysed by LC-MS using a Xevo QTof mass spectrometer (Waters) coupled to an Acquity LC system (Waters) using an Acquity UPLC BEH C18 column (2.1 x 50 mm, Waters). A linear gradient (95%-0% solvent A (0.1% formic acid in water) and 5%-100% solvent B (0.1% formic acid in acetonitrile)) was run over 3 minutes at a flow rate of 0.6 ml per minute. The ESI capillary voltage was 3 kV, cone voltage 30 V and collision energy 4 eV. The acquisition rate was 10 spectra per second and m/z data ranging from 50 to 2000 Da was collected. Mass accuracy was achieved using a reference lock mass scan, once every 10 seconds.
2.17 Liquid chromatography

Commercial 1-methyl-tryptophan (1-Me-Trp, Sigma-Aldrich, 95% purity) is contaminated with L-Trp and was purified further, prior to use in biophysical assays.

Analytical and semi-preparative RP-HPLC was performed on an ULTIMAT 3000 (DIONEX) coupled to a photodiode array detector set to monitor at 280 nm. A Phenomenex Gemini-NX C18 column (150 x 4.60 mm) was used for analytical HPLC and a Phenomenex Gemini-NX C18 (250 x 21.20 mm) used for semi-preparative work. A linear gradient of 10-60% buffer B was run over 45 minutes (Buffer A: water, Buffer B: acetonitrile). Fractions containing only 1-Me-Trp were combined and acetonitrile was evaporated before freeze-drying from water to recover 1-Me-Trp as a white powder.

2.18 Protein crystallisation

2.18.1 Crystal trials

Both hIDO and hTDO were initially screened against commercial crystallography screens: JCSG+ (Molecular Dimensions), PACT premier (Molecular Dimensions), Crystal screen I & II (Hampton Research) and Wizard classic crystal screen I & II (Rigaku).

Each commercial crystallography screen was manually dispensed (80 μl x 96 different conditions) using a multi-channel pipette into the deep wells of an MRC 96-well sitting drop vapour diffusion plate (Molecular Dimensions). The MRC 96-well plate was transferred to either a Cartesian or Mosquito robotic system where 100 nl of protein and 100 nl of well solution were mixed to form a 200 nl drop. Plates were covered with clear plastic film, stored at 18 °C and monitored under a light microscope for successful crystallisation conditions.

Anaerobic crystal trials of ferrous hIDO and hTDO, using the commercial screens listed above, were undertaken using an Oryx 4 (Douglas Instruments) robotic system housed in an anaerobic glovebox (Belle Technology, [O₂] < 5
ppm). Protein stock was reduced using sodium dithionite (see section 2.11) prior to mixing with the crystal screen. Plates were covered with clear plastic film, stored under anaerobic conditions (room temperature) and monitored under a light microscope (attached to the anaerobic glovebox) for successful crystallisation conditions.

Larger scale crystallisation trials using published crystallisation conditions (5–7) were carried out for hIDO and XcTDO using 24-well sitting (Molecular Dimensions) or hanging drop (Hampton Research) plates with drop sizes ranging from 1 to 4 μl. Protein was mixed with crystal screen (1:1 ratio), covered with clear plastic film and stored at 18 °C. Similar experiments were also conducted in an anaerobic glovebox (Belle Technology, [O₂] < 5 ppm) using ferrous XcTDO.

2.18.2 Seeding experiments

Seeding was undertaken to improve the crystal diffraction quality of XcTDO crystals. Drops containing a reduced concentration of XcTDO (< 50% of the initial concentration used to crystallise XcTDO) were equilibrated overnight; ensuring the drops remained clear after equilibration. Micro-seeding was undertaken by touching a previously grown crystal of XcTDO with a fine needle to collect microscopic crystal fragments, before it was then run through an equilibrated drop.

2.18.3 Crystal soaking

Crystals of ferric XcTDO were soaked with different ligands (30 mM L-Trp, 10 mM KCN and 100 μM H₂O₂ supplemented into the cryoprotectant) in order to obtain ligand bound crystal structures. Crystals of XcTDO were soaked with ligand for varying times (30 seconds - 20 minutes) before being flash frozen (see section 2.18.5).
Crystals of ferric XcTDO were grown in an anaerobic glovebox (Belle Technology, \([\text{O}_2] < 5 \text{ ppm}\)) and were soaked in sodium dithionite (5 - 10 seconds) to produce ferrous XcTDO crystals. In an attempt to generate turnover inside an XcTDO crystal (to trap the ternary complex \([\text{Fe}^{II}\text{-O}_2, \text{L-Trp}]\) or catalytic intermediates) cryoprotectant was supplemented with 258 \(\mu\text{M}\) or 130 \(\mu\text{M}\) oxygen and 1 mM \text{L-Trp}, which was soaked into crystals of ferrous XcTDO for varying times (10 - 60 seconds) before being flash frozen (see section 2.18.5). Crystals were soaked by Dr. Hanna Kwon (Department of Biochemistry at the University of Leicester)

### 2.18.4 Co-crystallisation

Ferric XcTDO was co-crystallised with L-Trp (30 mM) and KCN (10 mM), by adding the ligand to ferric XcTDO prior to mixing with crystal screen.

### 2.18.5 Freezing XcTDO crystals

A published cryoprotectant was used to avoid the formation of ice crystals during the freezing of XcTDO crystals (100 mM Mes pH 6.3, 12\% PEG 4000 and 60 mM MnCl\(_2\) supplemented with 25\% glycerol (5)).

Crystals of XcTDO were soaked in cryoprotectant prior to being flash frozen in nylon loops (0.2 - 0.3 mm, Hampton Research) by either immersion into liquid nitrogen or being placed into a cyrostream. Crystals were frozen by Dr. Hanna Kwon (Department of Biochemistry at the University of Leicester).

### 2.18.6 X-ray diffraction data collection

Diffraction data was collected at the Diamond Light Source in Oxford on the I04 - 1 beam line, via remote access from the University of Leicester.
2.19 X-ray data collection and structure determination

X-ray data collected from XcTDO crystals was processed by Dr Hanna Kwon using the CCP4 suite of programs (8). Recorded intensities were indexed and measured using MOSFILM then scaled and merged using AIMLESS.

The program Phaser (9) was used to solve the structure by molecular replacement adopting as a starting model the structure with PDB code 2NW7. Model refinement was done by REFMAC5 (10) and all model modifications were done by the modeling program COOT (11).
2.20 References


Chapter 3: Investigating the mechanism of tryptophan oxidation by hIDO
3.1 Introduction

The initial and rate-limiting step of the kynurenine pathway involves the oxidation of L-tryptophan (L-Trp) to N-formylkynurenine (NFK), Scheme 3.1. The reaction is oxygen-dependent and catalysed by two heme enzymes: tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), both enzymes are part of the heme-containing dioxygenase family.

Since the discovery of IDO and TDO (1,2), the exact mechanism of L-Trp oxidation has proven difficult to establish. The mechanism of L-Trp oxidation was proposed to involve base-catalysed abstraction of the indole proton (3,4), followed by either a Criegee (4,5) or dioxetane (6) rearrangement to produce NFK (Scheme 3.2). This proposal became widely reproduced in the literature due to the presumed presence of an active site base (histidine residue), but also the assignment of the tryptophan analogue, 1-methyl-L-tryptophan (1-Me-Trp) as an inhibitor. Deprotonation cannot occur at the methylated N¹ position (7).
Difficulties began to arise with the mechanistic proposal in Scheme 3.2 when the crystal structure of human indoleamine 2,3-dioxygenase (hIDO) (8) revealed the absence of a residue (histidine) that could act as an active site base. The mechanistic proposal in Scheme 3.2 was also inconsistent with the chemistry of indoles (9). It was also determined that the indole NH group of L-Trp has a very high pKₐ ≈ 17 (10) making it very difficult for weak bases such as a distal histidine or the heme bound oxygen to abstract a proton. There is no formal change in oxidation state of the heme iron, unlike other oxygen-dependent heme enzymes (e.g. cytochrome P450s) where the formation of a high oxidation state intermediate (Compound I) is always observed (11,12). The most conflicting experimental evidence with the base-catalysed abstraction mechanism (Scheme 3.2) is that 1-Me-Trp was found to be a slow substrate of hIDO (13). Since base-catalysed abstraction of the methyl group (N¹ position of L-Trp) is chemically impossible; this result was interpreted to imply that L-Trp oxidation must occur by a different mechanism. However, the assumption that L-Trp oxidation occurs by the same mechanism as 1-Me-Trp has not been established experimentally. Overall there has been little mechanistic information on the reaction of hIDO with other tryptophan analogues. The current mechanistic proposal for L-Trp oxidation is shown in Scheme 3.3.
Scheme 3.3: An alternative mechanistic proposal for the oxidation of L-Trp by the heme-containing dioxygenases based on current experimental and computational evidence (14–18). Step 1a involves electrophilic addition of the first oxygen atom and step 1b involves radical addition of the first oxygen atom.

The emergence of hIDO as a therapeutic target for the treatment of diseases such as cancer and the growing commercial interest in the search for hIDO inhibitors (rational design and high-throughput screening) has meant that establishing the precise mechanism of L-Trp oxidation has become increasingly important (19–21).

In this Chapter, the oxidation of L-Trp by hIDO has been analysed using an anaerobic stopped flow experiment to observe and characterise reaction intermediates, as proposed in Scheme 3.3. The activity of hIDO with a range of tryptophan analogues (particularly 1-Me-Trp) has been assessed with the aim of establishing whether or not oxidation occurs through a common mechanism across all substrates.
3.2 Methods

General methods for stopped flow and mass spectrometry are presented in Chapter 2. Here, detailed methodologies relating to this particular Chapter are given.

3.2.1 Preparation of hIDO

Samples of hIDO were prepared as described in Chapter 2 and concentrations were determined using the reported absorption coefficient $\varepsilon_{404} = 172 \text{ mM}^{-1}\text{cm}^{-1}$ (22).

3.2.2 Sequential mix stopped flow experiment: detection of intermediates

Stopped flow experiments were carried out using an Applied Photophysics SX.18MV stopped flow spectrometer housed in an anaerobic glove box (Belle Technology Ltd., $[O_2] < 5$ ppm) and fitted with a Neslab RTE-200 circulating water bath (25.0 ± 0.1 °C). Multiple-wavelength absorption studies were carried out using a photodiode array detector and Pro SX software (Applied Photophysics Ltd.).

The sequential mix stopped flow experiment was initiated by mixing ferrous hIDO (10 μM, generated by stoichiometric titration of ferric hIDO with sodium dithionite) with oxygen-saturated buffer (50 mM Tris-HCl buffer, pH 8.0, $[O_2] = 1.2$ mM). The solution was then aged for 50 milliseconds (ms) (pre-determined in separate experiments), to ensure complete formation of ferrous-oxy [Fe$^{II}$-O$_2$], before a second mix with either L-Trp or tryptophan analogues ([substrate] $\geq 10 \times K_M$ in cases where $K_M$ is known, Table 3.2). Formation and decay of ferryl heme (Compound II, [Fe$^{IV}$=O]) or formation of NFK was followed at 593 nm or 321 nm respectively (except 5-methoxy-DL-tryptophan (5-MeO-Trp) where the wavelength maxima for product formation was at 354 nm). In cases where the concentration of L-Trp or tryptophan analogue was in excess of oxygen, decay of Compound II led to the formation of ferrous [Fe$^{II}$] heme at the end of the experiment. Where the concentration of L-Trp or tryptophan analogue was low
compared to oxygen, the decay of Compound II led to formation of ferric [Fe\textsuperscript{III}] heme instead. This is consistent with a reported increase in reduction potential in the presence of substrate (23,24).

Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics Ltd.). The data was fitted to a two-step model (A > B > C) in order to extract spectra of intermediate species.

Detection of Compound II was observed in sequential mixing mode, monitoring absorbance changes at 593 nm, which report on the formation and decay of the ferryl species without complication from any other absorbing species. The stated hIDO and reagent concentrations throughout the rest of this Chapter relate to final concentrations after mixing.

### 3.2.3 Mass spectrometry analysis

Mass spectrometry experiments to detect NFK and the intermediate 2,3-epoxide species were carried out. Samples were prepared in an anaerobic glove box (Belle Technology Ltd., [O\textsubscript{2}] < 5 ppm) through incubation of ferrous hIDO (0.5 - 1 μM, generated by stoichiometric titration of ferric hIDO with sodium dithionite) with either L-Trp or a tryptophan analogue ([substrate] ≥ 10 x \(K_M\) in cases where \(K_M\) is known, Table 3.2) prior to the addition of aerobic solutions ([O\textsubscript{2}] = 258 μM) of 50 mM Tris-HCl buffer, pH 8.0. Mass spectrometry samples were also prepared using the steady state assay (utilises L-ascorbate, methylene blue and catalase; described in Chapter 2). Samples were allowed to react for varying amounts of time (10 - 60 minutes) before being centrifuged (13,000 rpm, 3 minutes) and the supernatant frozen directly on dry ice (25). Samples were stored at -80 °C until required for mass spectrometry analysis.
3.2.4 Tryptophan analogues

All chemicals used in this work were purchased from either Sigma-Aldrich, Apollo Scientific or Acros Organics and were of the highest purity (≥98%), except for 1-Me-Trp (95% purity) which is contaminated with L-Trp and required further purification via semi-preparatory liquid chromatography prior to use (Chapter 2) (13). The structures of the tryptophan analogues used in this study are shown in Figure 3.1.

Figure 3.1: Structures of the tryptophan analogues used in this study.
3.3 Results

3.3.1 Identification of reaction intermediates

The spectral identification of reaction intermediates during oxidation of L-Trp by the heme-containing dioxygenases has proven difficult. The ferrous-oxy species are often unstable and spectra of pure ferrous-oxy challenging to obtain. It is particularly difficult to distinguish between a ferrous-oxy species, a ternary complex [enzyme-O₂, substrate] and a Compound II intermediate [Fe⁴=O].

To overcome these challenges, all relevant wavelength maxima relating to hIDO have been collated in Table 3.1 and a pure hIDO ferrous-oxy spectrum collected for reference, Figure 3.2. In the following stopped flow experiments, ferrous-oxy has been allowed to form in high yield (>95%) prior to the addition of substrate to initiate turnover.

<p>| Table 3.1: Wavelength maxima for the ferric and ferrous derivatives of hIDO. |
|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Derivative</th>
<th>λ_max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric [Fe³⁺]</td>
<td>404, 500, 533, 635</td>
</tr>
<tr>
<td>Ferric-L-Trp [Fe³⁺-L-Trp]</td>
<td>410, 540, 576_sh</td>
</tr>
<tr>
<td>Ferrous [Fe²⁺]</td>
<td>425, 527_sh, 558</td>
</tr>
<tr>
<td>Ferrous- L-Trp [Fe²⁺-L-Trp]</td>
<td>425, 527_sh, 558_a</td>
</tr>
<tr>
<td>Ferrous-oxy [Fe²⁺-O₂]</td>
<td>416, 543, 577 nm</td>
</tr>
</tbody>
</table>

*No change in absorbance detected on binding of L-Trp (saturating concentrations) to ferrous hIDO.*

sh = shoulder
Figure 3.2: Stopped flow spectrum showing formation of ferrous-oxy in hIDO. Ferrous hIDO (2.5 µM) was mixed with O$_2$ (600 µM). The solid line is ferrous hIDO and the dashed line is ferrous-oxy hIDO. Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
3.3.2 Detection of a Compound II intermediate during L-Trp oxidation by hIDO

Under anaerobic conditions, ferrous hIDO (2.5 μM) was incubated for 50 ms with aerobic buffer ([O₂] = 300 μM), to allow for complete formation of the ferrous-oxy complex, before a second mix with L-Trp (50 μM). The first spectrum (collected 4 ms after mixing with L-Trp) is consistent with a ferrous-oxy species (λₘₐₓ = 413, 543, 577 nm, Figure 3.3) however; the α/β ratio (<1) and the Soret band (at 413 nm) are slightly different in comparison to a ‘pure’ ferrous-oxy (λₘₐₓ = 416, 543, 577 nm, α/β ratio = 1.06, Figure 3.2). The differences are assigned as being due to the binding of L-Trp to the ferrous-oxy species to form a ternary complex [Fe^{II}-O₂, L-Trp]. After a lag phase of 0.5 seconds (s), NFK formation was observed at 321 nm (Figure 3.3, inset). During NFK formation, an intermediate accumulated (λₘₐₓ = 413, 545, 574, 593 nm) evidenced by an increase in absorbance at 593 nm (Figure 3.3, inset). The intermediate existed during NFK production over 5 s and then decayed back to ferric hIDO when NFK production ceased due to L-Trp depletion. The intermediate observed in this experiment was assigned as a Compound II. Formation and decay of Compound II correlated exactly with NFK production which is evidence that Compound II is an intermediate in the mechanism and that its decay is rate limiting.
Figure 3.3: Stopped flow diode-array spectra showing the oxidation of L-Trp by ferrous hIDO. The solid line is the first spectrum recorded after mixing and represents the ternary [Fe\textsuperscript{II}-O\textsubscript{2}, L-Trp] complex; the dashed line is Compound II; the dotted line represents the final spectrum. Ferrous hIDO (2.5 μM) was premixed with O\textsubscript{2} (300 μM) for 50 ms followed by mixing with L-Trp (50 μM) and monitored over 100 s. Inset shows absorbance changes that report on NFK formation (321 nm) and Compound II formation and decay (593 nm, absorbance changes multiplied by a factor of 20). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
3.3.3 Independent Compound II formation using hydrogen peroxide

To provide further verification for the identity of the intermediate (Compound II) detected during L-Trp oxidation; experiments were carried out to prepare Compound II using hydrogen peroxide (H$_2$O$_2$) (26).

3.3.3.1 Reaction of ferrous hIDO with hydrogen peroxide

In a single mixed stopped flow experiment ferrous hIDO (2.5 μM) was rapidly mixed with H$_2$O$_2$ (5 equivalents). Figure 3.4A shows conversion of ferrous hIDO ($\lambda_{\text{max}} = 425, 527^{\text{sh}}, 558$ nm) to a second species with wavelength maxima ($\lambda_{\text{max}} = 416, 525^{\text{sh}}, 551, 591^{\text{sh}}$ nm). Reaction of ferrous hIDO with hydrogen peroxide leads to formation of Compound II (Equation 3.1) however the spectral form in Figure 3.4A is not similar to Compound II observed during L-Trp oxidation Figure 3.3.

Equation 3.1:

$$\text{Fe}^{\text{II}} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{\text{IV}=\text{O}} + \text{H}_2\text{O}$$

3.3.3.2 Reaction of ferrous hIDO with hydrogen peroxide and L-Trp

Reaction of ferrous hIDO (2.5 μM) with hydrogen peroxide (5 equivalents) was repeated in the presence of L-Trp (50 μM). Figure 3.4B shows the conversion of ferrous hIDO ($\lambda_{\text{max}} = 425, 527^{\text{sh}}, 558$ nm) to a second species with wavelength maxima ($\lambda_{\text{max}} = 413, 547, 579, 593^{\text{sh}}$ nm) identical to the Compound II intermediate observed during L-Trp oxidation ($\lambda_{\text{max}} 413, 547, 577, 593^{\text{sh}}$ nm, Figure 3.3).

It has been interpreted that the presence of L-Trp provides additional electronic contributions to the spectrum of Compound II; resulting in the spectrum observed in Figure 3.4B and Figure 3.3.
Figure 3.4: Stopped flow photodiode array spectra showing the formation of Compound II in hIDO using H₂O₂ in the absence and presence of L-Trp. (A) Ferrous hIDO (2.5 μM, solid line) reacted with H₂O₂ (5 equivalents). The dashed line is Compound II without the presence of L-Trp. (B) Ferrous hIDO (2.5 μM, solid line) was premixed with H₂O₂ (5 equivalents) for 50 ms followed by mixing with L-Trp (50 μM). The dashed line is Compound II. Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
3.3.4 Detection of a Compound II intermediate during the oxidation of different tryptophan analogues by hIDO

The activity of a range of different tryptophan analogues, listed in Table 3.2, with hIDO were examined with the aim of detecting Compound II (observed during L-Trp oxidation) in order to determine if oxidation of all substrates occurs via a common mechanism.

Table 3.2: Literature summary of kinetic data (steady state experiments) for hIDO with various tryptophan analogues used in this study.

<table>
<thead>
<tr>
<th>tryptophan analogue</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Trp</td>
<td>1.4 ± 0.05</td>
<td>7 ± 0.8</td>
</tr>
<tr>
<td>1-Me-Trp</td>
<td>0.027 ± 0.001</td>
<td>150 ± 11</td>
</tr>
<tr>
<td>5-F-Trp</td>
<td>0.76 ± 0.01</td>
<td>6 ± 0.8</td>
</tr>
<tr>
<td>5-Me-Trp</td>
<td>3.78 ± 0.16</td>
<td>98 ± 0.8</td>
</tr>
<tr>
<td>d-Trp</td>
<td>3.93 ± 0.07</td>
<td>1570 ± 100</td>
</tr>
<tr>
<td>5-OH-Trp</td>
<td>0.025 ± 0.0004</td>
<td>17 ± 1.1</td>
</tr>
<tr>
<td>5-MeO-Trp</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>IPA</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>S-Trp</td>
<td>**</td>
<td></td>
</tr>
</tbody>
</table>

*Activity not determined.
**No activity reported in the literature.
3.3.4.1 Oxidation of 1-Me-Trp by hIDO

The tryptophan analogue 1-Me-Trp (structure shown in Figure 3.1) was demonstrated to be a slow substrate of hIDO suggesting reassessment of the mechanism of L-Trp oxidation. The interpretation assumes that 1-Me-Trp and L-Trp are oxidised by a common mechanism (13), which is not established.

On reaction of ferrous hIDO (2.5 μM) with oxygen (300 μM) followed by 1-Me-Trp (1.5 mM), formation of a ternary complex [Fe^{II}-O_2, 1-Me-Trp] was observed ($\lambda_{\text{max}}$ = 413, 545, 577 nm, Figure 3.5A). After a lag phase of 4 s, NFK production was observed and an intermediate accumulated ($\lambda_{\text{max}}$ =413, 543, 577, 593$^{\text{th}}$ nm) as evidenced by increases in absorbance at 593 nm (Figure 3.5A and inset). The intermediate persisted during NFK production over 150 s before decaying to ferrous hIDO. The intermediate observed in this experiment was assigned as a Compound II and is identical to the Compound II intermediate detected during L-Trp oxidation ($\lambda_{\text{max}}$ = 413, 547, 577, 593$^{\text{th}}$ nm, Chapter 3: section 3.3.2). Formation and decay of Compound II was correlated with NFK production (Figure 3.5A, inset) confirming the role of Compound II as an intermediate in the oxidation of 1-Me-Trp in line with L-Trp oxidation by hIDO.

Indirect evidence for the intermediate 2,3-epoxide, proposed to form simultaneously with Compound II (Scheme 3.3), was detected by mass spectrometry (m/z = 235) and the data shown in Figure 3.5B. The intermediate 2,3-epoxide has previously been detected and characterised by mass spectrometry for the reaction of L-Trp with hIDO (25). Detection of both Compound II and the intermediate 2,3-epoxide are in support of a common mechanism for the oxidation of L-Trp and 1-Me-Trp by hIDO.
Figure 3.5: (A) Stopped flow diode-array spectra showing the oxidation of 1-Me-Trp by ferrous hIDO. Ferrous hIDO (2.5 μM) was premixed with O₂ (300 μM) for 50 ms followed by mixing with 1-Me-Trp (1.5 mM) and monitored over 500 s. The solid line is the first spectrum recorded after mixing and represents the ternary [Fe\textsuperscript{II}-O₂, 1-Me-Trp] complex; the dashed line is Compound II and the dotted line represents the final spectrum. Inset shows absorbance changes that report on NFK (at 321 nm) and Compound II (at 593 nm, absorbance changes multiplied by a factor of 20) formation and decay. Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5. (B) Detection of the intermediate 2,3-epoxide by mass spectrometry (m/z = 235) during the oxidation of 1-Me-Trp by hIDO.
3.3.4.2 Oxidation of S-Trp by hIDO

The tryptophan analogue S-Trp (structure shown in Figure 3.1) was originally reported to be one of the first inhibitors of hIDO along with 1-Me-Trp and O-Trp (7). This was based on the premise that reactive substrates were those containing a hydrogen atom on the N¹ position of L-Trp, in line with the base-catalysed abstraction proposal, Scheme 3.2. With the reclassification of 1-Me-Trp as a substrate of hIDO (13) and evidence for a common mechanism with L-Trp (Chapter 3: section 3.3.2), the activity of S-Trp was re-examined.

On reaction of ferrous hIDO (2.5 μM) with oxygen (300 μM) followed by S-Trp (200 μM), formation of a ternary complex [Fe²⁺O₂, S-Trp] was observed (λ max = 412, 543, 577 nm, Figure 3.6A) prior to the formation of an intermediate species assigned as Compound II (λ max = 413, 546, 577, 593 nm, Figure 3.6A) which decays to ferric hIDO. The Compound II intermediate detected during oxidation of S-Trp is identical to that observed for the reaction of L-Trp and 1-Me-Trp with hIDO.

A trace of the intermediate 2,3-epoxide (m/z = 238) was detected by mass spectrometry, Figure 3.6B. Product formation was observed neither by mass spectrometry (expected m/z = 254), steady state assay (no increase at 321 nm indicating NFK production) or during Compound II detection using stopped flow. An explanation for a lack of NFK could be due to alignment of the lone pairs on sulphur does not favour the ring-opening step of epoxide to allow for nucleophilic attack of the oxygen atom from Compound II.
Figure 3.6: (A) Stopped-flow diode-array spectra showing the oxidation of S-Trp by ferrous hIDO. Ferrous hIDO (2.5 μM) premixed with O₂ (300 μM) for 50 ms followed by mixing with S-Trp (200 μM) monitored over 200 s. The solid line is the proposed ternary [hIDO-O₂, S-Trp] complex, the dashed line is assigned as Compound II and the dotted line is the final spectrum (ferric hIDO). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5. (B) Detection of the intermediate 2,3-epoxide by mass spectrometry (m/z = 238) during the oxidation of S-Trp by hIDO.
3.3.4.3 Oxidation of d-Trp, 5-F-Trp and 5-Me-Trp by hIDO

Identical experiments were carried out with the tryptophan analogues: d-Trp, 5-F-Trp and 5-Me-Trp (structures shown in Figure 3.1). In these experiments, a Compound II intermediate was identified as observed during the reaction of L-Trp, 1-Me-Trp and S-Trp with hIDO. All Compound II wavelength maxima and mass spectrometry results are summarised in Table 3.3 and spectra shown in Figure 3.7.

<table>
<thead>
<tr>
<th>tryptophan analogue</th>
<th>Compound II λ_{max} (nm)</th>
<th>Mass spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2,3-epoxide</td>
</tr>
<tr>
<td>L-Trp</td>
<td>413, 547, 577, 593^{sh}</td>
<td>\bullet^a</td>
</tr>
<tr>
<td>1-Me-Trp</td>
<td>413, 543, 577, 593^{sh}</td>
<td>\bullet^b</td>
</tr>
<tr>
<td>5-F-Trp</td>
<td>414, 546, 576, 593^{sh}</td>
<td>\bullet</td>
</tr>
<tr>
<td>5-Me-Trp</td>
<td>413, 546, 576, 593^{sh}</td>
<td>\bullet</td>
</tr>
<tr>
<td>d-Trp</td>
<td>413, 546, 578, 593^{sh}</td>
<td>\bullet</td>
</tr>
<tr>
<td>S-Trp</td>
<td>413, 546, 577, 593^{sh}</td>
<td>\bullet</td>
</tr>
</tbody>
</table>

\(^a\)NFK and intermediate 2,3-epoxide were detected and characterised in (25) by mass spectrometry.
\(^b\)NFK and intermediate 2,3-epoxide were detected and characterised in (13,18) by mass spectrometry.
\(^c\)NFK formation not detected through either an increase in absorbance at 321 nm (stopped flow or steady state assays) or by mass spectrometry.
Figure 3.7: Stopped flow photodiode array spectra showing Compound II formation during the oxidation of (A) D-Trp (B) 5-Me-Trp and (C) 5-F-Trp by ferrous hIDO. Ferrous hIDO (2.5 μM) was premixed with O₂ (300 μM) for 50 ms followed by mixing with D-Trp (7 mM), 5-Me-Trp (1 mM) or 5-F-Trp (50 μM). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
3.3.5 Probing the reactivity of other tryptophan analogues with hIDO

In identical experiments with the tryptophan analogues: 5-OH-Trp and IPA (structures shown in Figure 3.1) it was recognised that both of these tryptophan analogues do not allow for accumulation of Compound II. The data collected was scrutinised in order to provide further mechanistic insights.

3.3.5.1 Oxidation of 5-OH-Trp and 5-MeO-Trp by hIDO

On reaction of hIDO (2.5 μM) with oxygen (300 μM) followed by 5-OH-Trp (200 μM), formation of a ternary complex [Fe^{II}-O_2, 5-OH-Trp] was observed ($\lambda_{\text{max}} = 414, 543, 577$ nm, Figure 3.8A). There was no evidence for a Compound II intermediate, instead decay of the ternary complex lead to the formation of an unknown intermediate ($\lambda_{\text{max}} = 409, 543, 577, 615^{\text{sh}}$ nm, Figure 3.8A), which persisted for 1 s before forming ferric hIDO. There were no significant absorbance increases at 321 nm that could be attributed to NFK formation. It is important to note that 5-OH-Trp is an active substrate, confirmed by steady state assays (described in Chapter 2) and mass spectrometry (NFK formation, m/z = 253), 5-OH-Trp is the slowest substrate of those reported for hIDO, $k_{\text{cat}} = 0.025$ s$^{-1}$ (Table 3.2). The mass spectrometry results provide no evidence for the intermediate 2,3-epoxide (m/z = 237).

If it is assumed that oxidation of 5-OH-Trp occurs by a common mechanism as L-Trp to form NFK, there are two possible pathways for the addition of the initial oxygen atom: electrophilic addition or radical addition, Scheme 3.3. From those two initial pathways, radical addition could account for the lack of accumulation of Compound II. If radical addition from ferric-superoxide occurs at the $C^3$ atom of 5-OH-Trp (Scheme 3.3), hydrogen atom abstraction from the 5-OH substituent group could occur. This could lead to a change in the rate-limiting step allowing for an accumulation of another intermediate species prior to Compound II. The hypothesis was tested using 5-MeO-Trp (structure shown in Figure 3.1), hydrogen atom abstraction cannot occur with a 5-MeO substituent group.
In contrast to 5-OH-Trp, 5-MeO-Trp is a good substrate of hIDO with a calculated turnover rate of $k_{cat} = 0.78 \text{ s}^{-1} (K_M = 40 \mu\text{M})$, determined during this study using the steady state assay method (Chapter 2). The data in Figure 3.9B shows that during oxidation of 5-MeO-Trp, Compound II accumulates ($\lambda_{max} = 413, 545, 577, 593^{sh}$). Mass spectrometry analysis (Figure 3.9) of the reaction of 5-MeO-Trp confirmed product formation ($m/z = 267$) and provided evidence for the intermediate 2,3-epoxide ($m/z = 251$). The data for 5-MeO-Trp is in line with the data collected during oxidation of L-Trp and 1-Me-Trp but also S-Trp, D-Trp, 5-Me-Trp and 5-F-Trp.

The data for 5-MeO-Trp supports the hypothesis outlined above. Full activity was restored on replacement of 5-OH with the 5-MeO substituent group, providing substantial support for the radical addition pathway in Scheme 3.3.

It is interesting to note that although Compound II does not accumulate during oxidation of 5-OH-Trp by hIDO, formation of this intermediate was observed on reaction of ferrous hIDO (2.5 μM) with 5 equivalents of hydrogen peroxide and 5-OH-Trp (50 μM), $\lambda_{max} = 413, 546, 593^{sh}$ nm.
Figure 3.8: Stopped-flow diode-array spectra show the oxidation of 5-OH-Trp and 5-MeO-Trp by ferrous hIDO. Ferrous hIDO (2.5 μM) premixed with O$_2$ (300 μM) for 50 ms followed by mixing with (A) 5-OH-Trp (200 μM) monitored over 100 s or (B) 5-MeO-Trp (250 μM) monitored over 100 s. The solid line is the proposed ternary [hIDO-O$_2$, substrate] complex. The dashed line in (A) is an unassigned intermediate and in (B) Compound II. The dotted line is the final spectrum. Inset: shows absorbance changes that report on NFK (354 nm) and Compound II (593 nm, absorbance changes at 593 nm have been multiplied by a factor of 20). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
Figure 3.9: (A) Detection of the intermediate 2,3-epoxide by mass spectrometry (m/z = 251) during the oxidation of 5-MeO-Trp by hIDO and (B) Detection of the NFK by mass spectrometry (m/z = 267) during the oxidation of 5-MeO-Trp by hIDO.
3.3.5.2 Oxidation of IPA by hIDO

The oxidation of IPA (structure shown in Figure 3.1) by hIDO has never been reported in the literature, the data presented here supports oxidation of this tryptophan analogue.

On reaction of ferrous hIDO (2.5 μM) with O\textsubscript{2} (300 μM) followed by IPA (1 mM), formation of a ternary complex [Fe\textsuperscript{II}-O\textsubscript{2}, IPA] was observed (λ\textsubscript{max} = 414, 542, 576 nm, Figure 3.10A) after which there was a long lag phase of 50 s. No product formation (at 321 nm) occurred during this lag phase (Figure 3.10A, inset). An intermediate was observed (λ\textsubscript{max} = 404, 503, 536, 576, 635 nm, with ferric/ferrous-oxy character, Figure 3.10A) and its decay coincided with product formation observed as an increase at 321 nm. An accumulation of a Compound II intermediate did not occur during oxidation of IPA by hIDO. Mass spectrometry analysis (Figure 3.10B) confirmed product formation (m/z = 222) and provided evidence for the intermediate 2,3-epoxide (m/z = 206).

The oxidation rate of IPA could not be determined under steady state conditions because the higher concentrations of hIDO and IPA required to observe turnover in the steady state assay resulted in significant changes in absorbance at 321 nm in control experiments (i.e. assays performed in the absence of enzyme or substrate).

The binding of IPA to ferrous XcTDO was monitored (binding of L-Trp or tryptophan analogues to ferrous hIDO or hTDO does not give a measurable signal change). A K\textsubscript{D} of 341 ± 37 μM (Figure 3.11A) was measured and this is 10-fold higher than L-Trp binding to ferrous XcTDO (33 ± 1.6 μM. Figure 3.11B). This experiment was undertaken to confirm that activity is a result of IPA binding.
Figure 3.10: (A) Stopped-flow diode-array spectra showing the oxidation of IPA by ferrous hIDO. Ferrous hIDO (2.5 μM) premixed with O₂ (300 μM) for 50 ms followed by mixing with IPA (1 mM) monitored over 500 s. The solid line is the proposed ternary [hIDO-O₂, IPA] complex. The dashed line is an unassigned intermediate. The dotted line is the final spectrum. Inset: shows absorbance changes that report on NFK formation (321 nm, highlighting the 50 s lag phase prior to product formation). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5. (B) Detection of NFK (m/z = 222) and the intermediate 2,3-epoxide by mass spectrometry (m/z = 206) during the oxidation of IPA by hIDO
Figure 3.11: Determination of the $K_D$ for the binding of (A) IPA and (B) L-Trp to ferrous XcTDO. Absorbance changes were monitored at 433 nm during titration with either IPA or L-Trp to ferrous XcTDO and the data fitted to Equation 2.3, Chapter 2.
3.4 Discussion

**Oxidation of L-Trp: detection of Compound II intermediate.**

The data shown in Figure 3.3 provides clear spectroscopic evidence for the accumulation of a Compound II intermediate during the oxidation of L-Trp by hIDO. Formation of a ferryl intermediate (Compound II) has been suggested from resonance Raman experiments (14–16). A spectrum identical to the Compound II spectrum shown in Figure 3.3 has been observed however, its identity was not confirmed and it was referred to as a ‘593 nm species’ (15).

Verification of the identity of the spectrum assigned as Compound II comes from experiments using ferrous hIDO, hydrogen peroxide and L-Trp (Figure 3.4B). In this experiment, a similar spectral form was observed with identical wavelength maxima ($\lambda_{\text{max}} = 413, 547, 579, 593^{\text{th}} \text{ nm}$) to Compound II detected during L-Trp oxidation ($\lambda_{\text{max}} = 413, 547, 577, 593^{\text{th}} \text{ nm}$). Wavelength maxima and spectra are not too dissimilar to Compound II observed in other heme proteins such as myoglobin and ascorbate peroxidase (28,29).

The role of Compound II as a catalytic intermediate can be confirmed through the correlation of NFK production with the accumulation and decay of Compound II during L-Trp oxidation. An accumulation of this intermediate, which allows for its detection, indicates that its decay is rate limiting in the mechanism. Nucleophilic attack of the ferryl oxygen on to the C₂ atom of the intermediate 2,3-epoxide must be the rate-limiting step of the current mechanistic proposal, scheme 3.3.

**Original base-catalysed abstraction mechanism versus the current mechanistic proposal.**

The tryptophan analogues 1-Me-Trp and S-Trp were considered to be inhibitors of dioxygenase activity (7), which was formulated on the basis that reactive substrates were those containing a hydrogen atom on the N¹ position of L-Trp, in line with the base-catalysed abstraction proposal (Scheme 3.2). It was later confirmed that 1-Me-Trp is a slow substrate of hIDO ($L$-Trp $k_{\text{cat}} = 1.4 \text{ s}^{-1}$ and 1-
Me-Trp $k_{\text{cat}} = 0.027 \text{ s}^{-1}$) and this was interpreted as being inconsistent with the literature proposal for L-Trp oxidation (13). The assumption behind this interpretation was that L-Trp and 1-Me-Trp are oxidised by a common mechanism which, until now, there was no evidence to support.

During the oxidation of 1-Me-Trp by hIDO, a Compound II (Figure 3.5A) and the intermediate 2,3-epoxide (Figure 3.5B) were detected, in line with the data collected during L-Trp oxidation. Compound II formation and decay (593 nm) was correlated with NFK production (321 nm), confirming the role of Compound II as a catalytic intermediate. The similarity between the data collected for both L-Trp and 1-Me-Trp is highly suggestive that both substrates are oxidised by a common mechanism, supporting a move away from the original base-catalysed abstraction proposal.

With regard to the oxidation of S-Trp, the data provide evidence for the formation of a Compound II species and a trace of the intermediate 2,3-epoxide detected by mass spectrometry (Figure 3.6), in line with L-Trp and 1-Me-Trp. Dioxygenated S-Trp was not observed by mass spectrometry, stopped flow or in steady state assays, possibly because the alignment of the lone pairs on sulphur do not favour ring-opening of the intermediate 2,3-epoxide. The data collected for S-Trp provides support for the current mechanistic proposal (Scheme 3.3) and is inconsistent with the original base-catalysed abstraction mechanism.
The oxidation of an N₁ fluoroalkylated tryptophan analogue (structure shown in Figure 3.12) by hIDO has recently been reported (30). Product formation (NFK) was confirmed by mass spectrometry analysis. The rate of oxidation could not be quantified although the reaction was observed to be slower than that of L-Trp, 1-Me-Trp and 5-OH-Trp (slowest reported substrate of hIDO). In order for NFK formation to occur, base-catalysed abstraction could not have taken place due to the presence of the fluoroalkylated group at the N₁ position. This data is in line with data reported for both 1-Me-Trp and S-Trp.

**Oxidation of 5-OH-Trp, 5-MeO-Trp: further mechanistic insights.**

The oxidation of 5-OH-Trp does not lead to the accumulation of a Compound II intermediate during catalytic turnover. An explanation for this result comes from the radical addition pathway of the current mechanistic proposal (Scheme 3.3), assuming oxidation of 5-OH-Trp occurs by a common mechanism with L-Trp. Radical formation at the C³ position of the indole ring in L-Trp could lead to hydrogen atom abstraction from the 5-OH substituent group. It is this process that prevents the accumulation of Compound II, altering the rate-limiting step of the mechanism, allowing for accumulation of another species and slowing the turnover rate even further (L-Trp $k_{cat} = 1.4 \text{ s}^{-1}$ and 5-OH-Trp $k_{cat} = 0.025 \text{ s}^{-1}$). This hypothesis was tested using 5-MeO-Trp because hydrogen atom abstraction cannot occur with a 5-MeO substituent group. The data provides evidence for a Compound II and intermediate 2,3-epoxide. Utilising the substituent group 5-MeO in place of 5-OH restores wild type hIDO activity confirming the hypothesis above. The data for 5-OH-Trp and 5-MeO-Trp align

![Figure 3.12: Oxidation of N₁ fluoroalkylated tryptophan by hIDO.](image)
with the data for L-Trp and 1-Me-Trp and are consistent with a radical addition mechanism.

A similar trend in the relative rates of oxidation for 5-OH-Trp and 5-MeO-Trp has been reported in IDO2. There is no reported activity of 5-OH-Trp though oxidation of 5-MeO-Trp ($k_{\text{cat}} = 0.161 \text{ s}^{-1}$) is similar to L-Trp ($k_{\text{cat}} = 0.103 \text{ s}^{-1}$) (31).

A recent study analysed the oxygen insertion reaction catalysed by Pseudomonas aeruginosa TDO (PaTDO) through the use of heme-modification (32). An increase in the electron-withdrawing power of the heme substituent increased the redox potential of PaTDO, decreased electron density on the heme iron, which in turn destabilised the ferrous-oxy intermediate. As a consequence of this, rates of L-Trp oxidation were increased. It was concluded that initial oxygen insertion must occur by direct electrophilic addition in PaTDO (Scheme 3.3). Although this publication is in opposition to the conclusion derived from the work presented here, the authors only assess this effect in PaTDO and comment that radical addition could occur in hIDO. The L-Trp oxidation rates reported after heme-modification do not show a significant increase in turnover rate compared with wild type PaTDO, the most electron-withdrawing modified heme increases the rate of L-Trp oxidation by a factor of 2.5 (wild type PaTDO + L-Trp $k_{\text{cat}} = 10 \text{ s}^{-1}$ compared with the most electron-withdrawing heme PaTDO + L-Trp $k_{\text{cat}} = 25 \text{ s}^{-1}$). The data presented in this Chapter provides compelling evidence in support for radical addition of the initial oxygen atom during L-Trp oxidation by hIDO.

**Oxidation of IPA: further mechanistic insights.**

Oxidation of the tryptophan analogue IPA has not been reported in the literature. In fact, it was positively asserted that IPA oxidation could not occur. The basis for this assertion was that the amino group in L-Trp (absent in IPA) is critical in facilitating ferric superoxide formation (33). This is the initial species required for the radical addition pathway, Scheme 3.3, achieved through hydrogen bonding to the iron bound oxygen as well as through electrostatic interactions. The
amino group is also understood to facilitate ring opening of the 2,3-epoxide intermediate through proton transfer (34).

In this study, the oxidation of IPA has been evidenced through an increase in absorbance at 321 nm (stopped flow experiment) and from the mass spectrometry data m/z = 222, both confirming NFK formation. During oxidation of IPA, Compound II is not observed suggesting an alteration in the rate-limiting step (formation of ferric superoxide and subsequent insertion of the initial oxygen atom) compared with L-Trp, preventing its accumulation. The intermediate 2,3-epoxide has been indirectly detected by mass spectrometry in line with L-Trp data. A 50 s lag phase prior to NFK formation is evidence of a significantly slower oxidation process than in L-Trp. The 50 s lag phase for IPA compared to 0.5 s lag phase for L-Trp oxidation suggests an unknown process (perhaps a rearrangement step or recruitment of another species within the active site) is required to facilitate NFK formation in the absence of the ammonium group.

It is interpreted that the altered kinetics of oxidation (long lag phase) as being consistent with a role of the ammonium group in stabilising the ferric superoxide complex via the radical addition pathway.

**Common mechanism of oxidation by hIDO.**

The overall conclusions of this work are that L-Trp and other substrate analogues are all oxidised by a common mechanism via a Compound II intermediate. Comparison of the Compound II spectra detected during the oxidation of L-Trp and other substrates is shown in Figure 3.13 along with the hIDO ternary [Fe^{II}-O_{2}, substrate] complex spectrum for reference. Initial oxygen addition occurs by radical addition rather than by electrophilic addition. The role of the ammonium group in L-Trp is necessary for efficient oxidation of L-Trp to form NFK, through its role in facilitating the formation of ferric-superoxide.
Figure 3.13: Comparison of the Compound II spectrum detected during the oxidation of L-Trp, 1-Me-Trp, S-Trp, 5-MeO-Trp, D-Trp, 5-Me-Trp, 5-F-Trp by hIDO. The spectrum also includes Compound II formed from the reaction between Fe^{II} hIDO with hydrogen peroxide as well as the hIDO ternary [Fe^{II}-O_2, substrate] complex.

Oxidation of L-Trp by hIDO via a Compound II intermediate is in line with other oxygen-dependent heme enzymes. Both cytochrome P450 and nitric oxide synthase form a Compound I intermediate (Fe^{IV}=O with a porphyrin radical cation). Formation of a Compound II during L-Trp oxidation also correlates with the peroxidase enzymes, which form both high oxidation state intermediates: Compound I and Compound II. The non-heme iron dependent dioxygenases also form a ferryl (IV) oxo intermediate during oxidative cleavage of catechol substrates.
3.5 References


7. Cady. SG, Sono. M (1991) 1-methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan) and beta-[3-benzo(b)thienyl]-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. *Archives of Biochemistry and Biophysics* 291(2):326–33.


Chapter 4: Investigation into the mechanism of tryptophan oxidation by hTDO and XcTDO
4.1 Introduction

Oxidation of L-tryptophan (L-Trp) to N-formylkynurenine (NFK) is catalysed by two heme enzymes: tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) known as the heme-containing dioxygenases. Since their discovery (1,2) the precise mechanism of L-Trp oxidation by both IDO and TDO has proven difficult to establish, particularly with regard to TDO. In comparison with IDO; TDO has been found to be more challenging to express and purify as well as being highly specific for L-Trp.

The first proposal for the mechanism of L-Trp oxidation involved base-catalysed abstraction of the indole proton (3,4), followed by either a Criegee (3) or dioxetane (5) rearrangement to form NFK (Scheme 4.1). Support for the proposal in Scheme 4.1 was based upon the supposed presence of an active site base (a histidine residue). A distal histidine (His55) in close proximity to the bound L-Trp was observed from the crystal structure of ferrous Xanthomonas campestris (XcTDO) in complex with L-Trp (6). The proposal in Scheme 4.1 was also based upon the idea that only substrates containing a proton on the indole nitrogen could be oxidised. The assignment of 1-methyl-L-tryptophan (1-Me-Trp) as an inhibitor of hIDO is consistent with this idea (7).

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**Scheme 4.1:** Original mechanistic proposal for the mechanism of L-Trp oxidation by the heme-containing dioxygenases (Criegee rearrangement shown in blue and dioxetane rearrangement shown in red) (3–5).
Further experimental studies to determine the precise mechanism of the heme-containing dioxygenases have cast serious doubts upon the validity of the base-catalysed abstraction proposal. The mechanistic proposal in Scheme 4.1 was found to be inconsistent with the chemistry of indoles, which do not react by base-catalysed abstraction (8). It was determined that the indole NH group of L-Trp has a very high pK$_a \approx 17$ (9) making it difficult for weak bases such as a distal histidine to abstract a proton. The mechanistic proposal in Scheme 4.1 does not show a formal change in oxidation state, unlike other oxygen-dependent heme enzymes (e.g. cytochrome P450 (10,11)) where formation of a high oxidation state intermediate (Compound I) is always observed. The most significant evidence against the mechanistic proposal in Scheme 4.1 is the fact that 1-Me-Trp was demonstrated to be a substrate of hIDO (12), *Cupriavidus metallidurans* (*CmTDO*) (13) and histidine variants of hTDO and XcTDO (12). Mutagenesis studies showed that replacement of the distal histidine within both XcTDO and hTDO did not inhibit L-Trp oxidation (14). An alternative mechanistic proposal for L-Trp oxidation is shown in Scheme 4.2 based on recent experimental and computational work.

**Scheme 4.2:** An alternative mechanistic proposal for the oxidation of L-Trp by the heme-containing dioxygenases based on current experimental evidence and computational evidence (15–20). Step 1a involves electrophilic addition of the first oxygen atom and step 1b involves radical addition of the first oxygen atom.
The role of hIDO as an emerging therapeutic target was discussed in Chapter 3, however, more recently it has been discovered that TDO is expressed in tumour cells (21). TDO has been shown to play an immune-regulatory role in many cancer systems by preventing tumour rejection (22,23); highlighting the importance of understanding L-Trp oxidation by IDO and TDO in order to facilitate rational inhibitor design.

In this Chapter, the oxidation of L-Trp by XcTDO and hTDO has been analysed using an anaerobic stopped flow experiment to observe and characterise catalytic intermediates proposed in Scheme 4.2. The aim of this work is to further the understanding of the mechanism of L-Trp oxidation by the TDO enzymes and to establish if there is a common mechanism between the TDOs and hIDO.
4.2 Methods

General methods for stopped flow and mass spectrometry are presented in Chapter 2. Here, detailed methodologies relating to this particular Chapter are given.

4.2.1 Preparation of hTDO and XcTDO

Samples of hTDO and XcTDO were prepared as described in Chapter 2 and concentrations determined using the reported absorption coefficients hTDO $\varepsilon_{408} = 196 \text{ mM}^{-1}\text{cm}^{-1}$ (24); XcTDO $\varepsilon_{404} = 180.5 \text{ mM}^{-1}\text{cm}^{-1}$ (14).

4.2.2 Sequential mix stopped flow experiment: detection of transient intermediates

Stopped flow experiments were carried out using an Applied Photophysics SX.18MV stopped flow spectrometer housed in an anaerobic glove box (Belle Technology Ltd., $[O_2] < 5 \text{ ppm}$) and fitted with a Neslab RTE-200 circulating water bath (25.0 ± 0.1 °C). Multiple-wavelength absorption studies were carried out using a photodiode array detector and Pro SX software (Applied Photophysics Ltd.).

The sequential mix stopped flow experiment was initiated by mixing ferrous hTDO or XcTDO (10 $\mu$M, generated by stoichiometric titration of ferric hTDO or XcTDO with sodium dithionite) with oxygen-saturated buffer (50 mM Tris-HCl buffer, pH 8.0, $[O_2] = 1.2 \text{ mM}$) and then aging the solution for 50 milliseconds (ms), to ensure complete formation of ferrous-oxy [Fe$^{ll}$-O$_2$], before a second mix with either L-Trp or tryptophan analogues ([substrate] ≥ 10 x $K_M$ in cases where $K_M$ is known, Table 4.2). Formation of NFK was followed at 321 nm and the decay of an unknown intermediate at 577 nm. In all cases L-Trp or tryptophan analogue was present in excess compared with the oxygen concentration and decay of unknown intermediate led to the formation of ferrous [Fe$^{ll}$] heme at the end of the experiment.
Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics Ltd.). The data was fitted to a two-step model (A > B > C) in order to extract spectra of intermediate species. The stated $XcTDO$, $hTDO$ and reagent concentrations throughout the rest of this Chapter relate to final concentrations after mixing.

4.2.3 Mass spectrometry analysis

Mass spectrometry experiments to detect NFK and the intermediate 2,3-epoxide species were carried out. Samples were prepared in an anaerobic glove box (Belle Technology Ltd., $[O_2] < 5$ ppm). Ferrous $XcTDO$ or $hTDO$ (0.5 - 1 $\mu$M, generated by stoichiometric titration of ferric $hTDO$ or $XcTDO$ with sodium dithionite) was incubated with either $L$-Trp or tryptophan analogue ([$\text{substrate}$] $\geq 10 \times K_M$ in cases where $K_M$ is known, Table 4.2) prior to the addition of aerobic solutions ($[O_2] = 258$ $\mu$M) of 50 mM Tris-HCl buffer, pH 8.0. Mass spectrometry samples were also prepared using the steady state assay (utilises $L$-ascorbate, methylene blue and catalase; described in Chapter 2). Samples were allowed to react for varying amounts of time (10 - 60 minutes) before being centrifuged (13,000 rpm, 3 minutes) and the supernatant frozen directly on dry ice (25). Samples were stored at - 80 °C until required for mass spectrometry analysis.
4.2.4 Tryptophan analogues

All chemicals used in this work were purchased from Sigma-Aldrich, Apollo Scientific and Acros Organics and were of the highest purity. The structures of the tryptophan analogues used in this study are shown in Figure 4.1.

![Figure 4.1: Structures of the tryptophan analogues used in this study.](image)
4.3 Results

4.3.1 Ferrous-oxy characterisation to support identification of reaction intermediates

The identification of reaction intermediates using absorbance spectroscopy during the oxidation of L-Trp by IDO and TDO has been challenging. Ferrous XcTDO and hTDO do not form a stable ferrous-oxy species in the absence of L-Trp and are oxidised back to ferric TDO (26). An experiment designed to try to form a ferrous-oxy species in both XcTDO and hTDO is shown in Figure 4.2A and Figure 4.2B. A ferrous-oxy species did not form at all in XcTDO (Figure 4.2A). A ferrous-oxy species formed in hTDO only at high concentrations of oxygen ([O₂] = 600 μM) and rapidly decayed to ferric TDO (Figure 4.2B). The spectrum of ferrous-oxy in hIDO is included as reference from Section 3.3.1, Chapter 3 Figure 4.2C. A ferrous-oxy species did not form in XcTDO even at high concentrations of oxygen. Distinguishing between transient catalytic intermediates and species such as the ternary complex [Fe^II-O₂, L-Trp] or ferrous-oxy species are complicated.
Figure 4.2: Reaction of ferrous XcTDO, hTDO or hIDO with O₂ (600 μM) monitored by stopped flow photodiode array spectroscopy. (A) Shows the time dependent spectral changes on mixing XcTDO with O₂ over a 1 s time base, no ferrous-oxy species was formed (B) hTDO ferrous-oxy species and (C) hIDO ferrous-oxy species (added for comparison Chapter 3). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
To facilitate the identification of catalytic intermediates during L-Trp oxidation by TDO, relevant wavelength maxima have also been collated in Table 4.1.

**Table 4.1**: Wavelength maxima for ferric and ferrous derivatives of *Xc*TDO and *h*TDO.

<table>
<thead>
<tr>
<th>Derivative</th>
<th><em>Xc</em>TDO</th>
<th><em>h</em>TDO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>$\lambda_{\text{max}}$ (nm)</td>
</tr>
<tr>
<td>Ferric [Fe$^{\text{III}}$]</td>
<td>404, 500, 550, 635</td>
<td>408, 533, 621</td>
</tr>
<tr>
<td>Ferric-L-Trp [Fe$^{\text{III}}$-L-Trp]</td>
<td>408, 536, 578, 633</td>
<td>410, 536, 565$^\text{sh}$</td>
</tr>
<tr>
<td>Ferrous [Fe$^{\text{II}}$]</td>
<td>432, 554, 588</td>
<td>428, 533, 560</td>
</tr>
<tr>
<td>Ferrous- L-Trp [Fe$^{\text{II}}$-L-Trp]</td>
<td>433, 556, 587</td>
<td>426, 533, 560</td>
</tr>
<tr>
<td>Ferrous-oxy [Fe$^{\text{II}}$-O$_2$]</td>
<td>$^a$</td>
<td>$^a$</td>
</tr>
</tbody>
</table>

$^a$No change in absorbance detected on binding of L-Trp (saturating concentrations) to ferrous *hIDO*.  
$^\text{sh}$ = shoulder
4.3.2 Detection of an intermediate during the oxidation of L-Trp by XcTDO

The oxidation of L-Trp by XcTDO was monitored in order to detect catalytic intermediates, particularly a Compound II intermediate as observed during the oxidation of L-Trp by hIDO in Chapter 3. Under anaerobic stopped flow conditions, ferrous XcTDO (2.5 μM) was incubated for 50 ms with oxygen (300 μM), prior to a second mix with L-Trp (1 mM). The first spectrum (collected at 4 ms after mixing with L-Trp) had spectral characteristics of ferrous XcTDO (\(\lambda_{\text{max}} = 432, 552, 580\) nm, Figure 4.3A). This initial ferrous species rapidly decayed to form an unknown intermediate (\(\lambda_{\text{max}} = 418, 546, 578\) nm, figure 4.3A). The intermediate accumulated during the 0.4 s lag phase prior to NFK formation. During NFK production, the intermediate decayed to ferrous XcTDO. Decay of the unknown intermediate correlates with NFK formation (Figure 4.3A, inset) suggesting its role as a catalytic intermediate in the mechanism of L-Trp oxidation.

4.3.3 Detection of an intermediate during the oxidation of L-Trp by hTDO

Under anaerobic stopped flow conditions, on reaction of ferrous hTDO (2.5 μM) with oxygen (300 μM) followed by L-Trp (2 mM), a tentatively assigned ternary complex [Fe\(\text{II}\)-O\(_2\), L-Trp] was observed (\(\lambda_{\text{max}} = 417, 543, 577\) nm). A second species (\(\lambda_{\text{max}} = 416, 543, 577\) nm) was extracted from the spectra after spectral deconvolution was performed by global analysis and numerical integration using PROKIN software (Applied Photophysics Ltd.). Although this species is very similar to the ternary complex it was assigned as an unknown intermediate. This intermediate accumulated during the lag phase of 0.8 s prior to NFK formation. During NFK production, the intermediate decayed to ferrous hTDO. There is a correlation between NFK formation and decay of the unknown intermediate (Figure 4.3A, inset) that is indicative of this species as a catalytic intermediate.

Indirect evidence for the intermediate 2,3-epoxide using mass spectrometry during the reaction of L-Trp with hIDO, XcTDO and hTDO has been reported in the literature (25).
Figure 4.3: Stopped flow diode-array spectra showing the oxidation of L-Trp by ferrous (A) XcTDO and (B) hTDO. The solid line is the first spectrum recorded after mixing and is assigned as a ternary complex; the dashed line is an unknown intermediate species; the dotted line represents the final spectrum. (A) Ferrous XcTDO or hTDO (2.5 μM) was premixed with O₂ (300 μM) for 50 ms followed by mixing with L-Trp (for XcTDO 1 mM, monitored over 10 s; for hTDO 2 mM, monitored over 500 s). Insets show absorbance changes that report on NFK formation (at 321 nm) and an intermediate species (at 577 nm, absorbance changes have been multiplied by a factor of 20). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
4.3.4 Detection of an intermediate during the oxidation of different tryptophan analogues by XcTDO and hTDO

A summary of the kinetic parameters ($k_{\text{cat}}$ and $K_M$ from steady state experiments) reported in the literature for hTDO and XcTDO with various tryptophan analogues are summarised in Table 4.2. The variety of tryptophan analogues available to probe the mechanism of hTDO and XcTDO is very limited in comparison to hIDO due to the high specificity of the active site of both enzymes for L-Trp, Table 4.2.

Table 4.2: Literature summary of kinetic data (steady state experiments) for XcTDO and hTDO with various tryptophan analogues.

<table>
<thead>
<tr>
<th>tryptophan analogue</th>
<th>XcTDO (6,14)</th>
<th>hTDO (24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat}}$ (s$^{-1}$)</td>
<td>$K_M$ (µM)</td>
</tr>
<tr>
<td>L-Trp</td>
<td>19.5 ± 1.2</td>
<td>114 ± 1</td>
</tr>
<tr>
<td>1-Me-Trp</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>5-F-Trp</td>
<td>2.4 ± 0.10</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>5-Me-Trp</td>
<td>3.59 ± 0.05</td>
<td>357 ± 12</td>
</tr>
<tr>
<td>D-Trp</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5-OH-Trp</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>5-MeO-Trp</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>IPA</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>S-Trp</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

*Activity not determined.
**No activity reported in the literature.
***Detectable activity (steady state parameters not determined due to substrate concentration limitations).
4.3.4.1 Oxidation of 5-F-Trp by XcTDO

The tryptophan analogue 5-F-Trp (structure shown in Figure 4.1) has previously been reported as a substrate of XcTDO. This substrate has a similar affinity as L-Trp to XcTDO \((K_M = 100 \ \mu\text{M})\) however the \(k_{\text{cat}}\) is about 10-fold lower \((k_{\text{cat}} = 2.4 \ \text{s}^{-1} \text{ compared to 20 s}^{-1} \text{ for the wild type XcTDO})\) (6,14). The reactivity of 5-F-Trp with XcTDO was analysed further in order to draw comparisons with L-Trp oxidation.

On addition of ferrous XcTDO (2.5 μM) with oxygen (300 μM) followed by 5-F-Trp (1 mM), the first species had spectral characteristics of ferrous XcTDO, \(\lambda_{\text{max}} = 432, 551, 583 \ \text{nm} \) (Figure 4.4A). This initial ferrous species rapidly decayed to form an unknown intermediate \((\lambda_{\text{max}} = 417, 548, 577 \ \text{nm}, \text{ Figure 4.4A})\). The intermediate accumulated during the 1 s lag phase prior to NFK formation. During NFK production, the intermediate decayed to ferrous XcTDO. The decay of this intermediate correlates with NFK formation (Figure 4.4A inset), which indicates its requirement for NFK production. The spectra collected during the oxidation of 5-F-Trp by XcTDO are identical to those collected during the oxidation of L-Trp by XcTDO. Indirect evidence for the intermediate 2,3-epoxide was detected using mass spectrometry \((m/z = 239)\) and the data shown in Figure 4.4B.
Figure 4.4: (A) Stopped flow diode-array spectra showing the oxidation of 5-F-Trp by ferrous XcTDO. The solid line is the first spectrum recorded after mixing and is assigned as a ternary complex; the dashed line is an unknown intermediate species; the dotted line represents the final spectrum. Ferrous XcTDO (2.5 μM) was premixed with O₂ (300 μM) for 50 ms followed by mixing with 5-F-Trp (1 mM, reaction monitored over 500 s). Inset shows absorbance changes that report on NFK formation (at 321 nm) and an intermediate species (at 577 nm, absorbance changes have been multiplied by a factor of 20). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5. (B) Mass spectrometry analysis of oxidation of 5-F-Trp by XcTDO, detection of intermediate 2,3-epoxide (m/z = 239) and NFK (m/z = 255)
4.3.4.2 Oxidation of S-Trp by XcTDO and hTDO

The tryptophan analogue S-Trp (structure shown in Figure 4.1) was originally reported to be one of the first inhibitors of hIDO along with 1-Me-Trp and O-Trp (7). This was based on the premise that reactive substrates were those containing a proton on the indole nitrogen of L-Trp, in line with the base-catalysed abstraction proposal Scheme 4.1. With the reclassification of 1-Me-Trp as a substrate of hIDO (12) and demonstration in Chapter 3 that S-Trp was partially oxidised via a Compound II intermediate by hIDO. The activity of S-Trp with XcTDO and hTDO was examined.

On reaction of ferrous hTDO (2.5 μM) with oxygen (300 μM) and S-Trp (1 mM), a tentatively assigned ternary complex [Fe\textsuperscript{II}-O\textsubscript{2}, S-Trp] was observed for hTDO ($\lambda_{\text{max}} = 416, 542, 577$ nm), which decayed to ferric hTDO. Spectral deconvolution was performed by global analysis and numerical integration using PROKIN software (Applied Photophysics Ltd.), however no other intermediate species were extracted from the data.

On reaction of ferrous XcTDO (2.5 μM) with oxygen (300 μM) and S-Trp (1 mM), the first spectrum had the spectral characteristics of ferrous XcTDO which rapidly decayed to ferric XcTDO. An intermediate species was not observed.

Production of NFK was not observed through an increase in 321 nm during steady state assays (described in Chapter 2, section 2.14 using the methylene blue, L-ascorbate reducing system) or stopped flow photo diode array spectroscopy. Neither NFK (m/z = 254) nor the intermediate 2,3-epoxide (m/z = 238) were detected using mass spectrometry.
4.3.4.3 Oxidation of IPA by XcTDO and hTDO

The oxidation of IPA (structure shown in Figure 4.1) by the heme-containing dioxygenases has not been reported in the literature. It was assumed that the amino group in L-Trp (absent in IPA) is critical in facilitating ferric superoxide formation (27) required for the radical addition pathway (Scheme 4.2). This was understood to occur through hydrogen bonding to the iron bound oxygen as well as through electrostatic interactions (27). The amino group in L-Trp is also understood to facilitate ring opening of the intermediate 2,3-epoxide through proton transfer (20). In Section 3.3.5.2, Chapter 3 it was demonstrated that IPA is a slow substrate of hIDO, therefore the activity of IPA with XcTDO and hTDO has been assessed.

On reaction of ferrous XcTDO or hTDO (2.5 μM) with oxygen (300 μM) and IPA (2 mM for XcTDO, 5 mM for hTDO), formation of a tentatively assigned ternary complex [FeII-O2, IPA] was observed for both XcTDO (λmax = 432, 550, 587sh nm, Figure 4.5A) and hTDO (λmax = 414, 545, 576 nm, Figure 4.5B). A lag phase of 50 s was recorded, during which the ternary complexes for both XcTDO and hTDO decayed to an intermediate species with ferric-like character (XcTDO λmax = 405, 500, 535sh, 635 nm Figure 4.5A, hTDO λmax = 405, 500, 545, 635 nm Figure 4.5B). After the lag phase, the intermediate decayed to a mixture of ferric/ferrous XcTDO and ferric hTDO. Production of NFK was observed through an increase at 321 nm after the 50 s lag phase and formation of the intermediate species Figure 4.5.

The binding of IPA to ferrous XcTDO was monitored (binding of L-Trp or tryptophan analogues to ferrous hIDO or hTDO does not give a measurable signal change). A KD of 341 ± 37 μM was measured and this is 10-fold higher than L-Trp binding to ferrous XcTDO (33 ± 1.6 μM). This experiment was undertaken (described in Chapter 2, Section 2.13) to confirm that activity is a result of IPA binding.

Analysis of the reaction of IPA with XcTDO or hTDO by mass spectrometry confirmed the formation of NFK (m/z = 222) and provided indirect evidence for the intermediate 2,3-epoxide (m/z = 206), Figure 4.6 and Figure 4.7.
The oxidation rate of IPA could not be determined under steady state conditions because the higher concentrations of XcTDO and hTDO as well as IPA required to observe turnover in the steady state resulted in significant changes in absorbance at 321 nm in control experiments (i.e. assays performed in the absence of enzyme or substrate).

In Chapter 3, oxidation of IPA by hIDO was demonstrated by an increase in absorbance at 321 nm and mass spectrometry. The data presented in this Chapter provide evidence for the oxidation of IPA by both XcTDO and hTDO.
Figure 4.5: Stopped flow diode-array spectra showing the oxidation of IPA by ferrous (A) XcTDO and (B) hTDO. Ferrous XcTDO or hTDO (2.5 μM) premixed with O₂ (300 μM) for 50 ms followed by mixing with IPA (2 mM for XcTDO, 5 mM for hTDO, both reactions were monitored over 500 s). The solid line is the proposed ternary [Fe^{II}-O₂, IPA] complex. The dashed line is an unassigned ferric like intermediate. The dotted line is the final spectrum. Inset: shows absorbance changes that report on NFK formation (321 nm, highlighting the 50 s lag phase prior to product formation). Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5.
Figure 4.6: Mass spectrometry evidence for the detection of the intermediate 2,3-epoxide (m/z = 206) (A) XcTDO (B) hTDO.
Figure 4.7: Mass spectrometry evidence for the detection of NFK (m/z = 222) by mass spectrometry (A) XcTDO (B) hTDO during the oxidation of IPA.
4.4 Discussion

Oxidation of L-Trp by XcTDO and hTDO

Distinguishing between different species of TDO (i.e. the ternary complex [Fe$^{II}$-O$_2$, L-Trp], the ferrous-oxy, ferrous and ferric species) and transient catalytic intermediates accumulated during L-Trp oxidation has been challenging. It has limited development of ideas on the mechanism of L-Trp oxidation by TDO. Modelling the data collected in Sections 4.3.2 - 4.3.4 has helped to aid somewhat in the distinction of these catalytic intermediates and has allowed for comparisons with the data collected for hIDO in Chapter 3.

The data collected in this Chapter provides support for the accumulation of an intermediate during the oxidation of L-Trp by XcTDO and hTDO. Correlation between the decay of the intermediate (decrease at 577 nm) and NFK formation (increase at 321 nm) observed for both enzymes suggest the role of this intermediate in the catalytic production of NFK. A similar intermediate was detected using stopped flow photodiode array spectroscopy during the oxidation of 5-F-Trp by XcTDO (Figure 4.4A), which can also be correlated with NFK formation. An intermediate 2,3-epoxide, suggested by the mechanistic proposal in Scheme 4.2 was shown to form during the oxidation of L-Trp by hIDO, XcTDO and hTDO through indirect detection by mass spectrometry (25). The same intermediate 2,3-epoxide was detected using mass spectrometry during the oxidation of 5-F-Trp by XcTDO (Figure 4.4B).

The mechanistic proposal in Scheme 4.2 suggests simultaneous formation of the intermediate 2,3-epoxide and Compound II. Does this indicate that the intermediate observed spectrally during oxidation of L-Trp by XcTDO and hTDO could be Compound II? The spectral intermediates detected during the oxidation of L-Trp by hTDO and XcTDO with the Compound II intermediates or the ternary complex observed from the oxidation of L-Trp and 1-Me-Trp by hIDO have been plotted together in Figure 4.8 and Figure 4.9.
Figure 4.8: Comparison of the Compound II spectrum from the oxidation of (A) L-Trp and (B) 1-Me-Trp by hIDO with the intermediate spectrum generated during the oxidation of L-Trp by (C) hTDO and (D) XcTDO.

Figure 4.9: Comparison of the ternary spectrum from the oxidation of (A) L-Trp and (B) 1-Me-Trp by hIDO with the intermediate spectrum generated during the oxidation of L-Trp by (C) hTDO and (D) XcTDO.
It is clear from Figure 4.8 and Figure 4.9 that the spectra of the unknown intermediates of hTDO and XcTDO do not have a shoulder at 593 nm, a characteristic feature of Compound II in hIDO (Figure 4.8) and appear to be more in line with the hIDO ternary complex (Figure 4.9). The spectra presented in Figure 4.8 suggest that the identity of the unknown intermediate in hTDO and XcTDO is not Compound II. This interpretation of the spectra assumes that Compound II would present the same spectral features in hIDO, hTDO and XcTDO. Alternatively, Compound II has a different spectrum in TDO than hIDO; both interpretations of the data are explained below.

The concentration of L-Trp required for maximal turnover in XcTDO (hTDO ($K_M = 222 \mu M$ (24) and XcTDO $K_M = 114 \mu M$ (14))) is higher than in hIDO ($K_M = 7 \mu M$ (28)). This shows that binding of L-Trp during oxidation is significantly weaker than for hIDO. Weaker binding of L-Trp to XcTDO and hTDO may also be reflected in weaker binding of NFK to both enzymes. This would result in more rapid dissociation of NFK from XcTDO and hTDO in comparison to hIDO. The consequence of this could be an alteration in the rate-limiting step of the mechanism. During the oxidation of L-Trp by hIDO, Compound II accumulates suggesting that insertion of the second oxygen atom and subsequent decay of Compound II are rate limiting (Scheme 4.3, red box). Oxidation of L-Trp by XcTDO and hTDO leads to a spectral form similar to the spectrum assigned as the ternary complex in hIDO (Chapter 3) Figure 4.9. This suggests that insertion of the first oxygen atom could be rate limiting in both hTDO and XcTDO (Scheme 4.3, blue box) and that Compound II forms and decays rapidly.

It is possible that tighter binding of L-Trp to hIDO ($K_M = 7 \mu M$ (28)) perturbs the spectrum of Compound II slightly; leading to a shoulder in the spectrum at 593 nm. If binding of L-Trp to TDO is weaker (hTDO ($K_M = 222 \mu M$ (24) and XcTDO $K_M = 114 \mu M$ (14)) this could account for the absence of a shoulder in the spectrum.
Scheme 4.3: An alternative mechanistic proposal for the oxidation of L-Trp by the heme-containing dioxygenases based on current experimental and computational evidence (15–19). Step 1a involves electrophilic addition of the first oxygen atom and step 1b involves radical addition of the first oxygen atom. The red box is the suggested rate-limiting step for hIDO and the blue box is the suggested rate-limiting step for XcTDO and hTDO.

The accumulation of a ferryl species (Compound II) was suggested from resonance Raman experiments for the oxidation of L-Trp by hIDO. Attempts to observe a Compound II during the oxidation of hTDO by resonance Raman failed (17). Explanation for this is Compound II did not accumulate to a sufficient population for detection (17).

Oxidation of L-Trp occurs by a common mechanism in hIDO and TDO?

The overall conclusions of this work are that XcTDO and hTDO oxidise L-Trp to form NFK via an intermediate species. The identity of the intermediate species identified in both hTDO and XcTDO during L-Trp oxidation is unknown. Comparisons of the spectra of the intermediates in hTDO and XcTDO with the spectrum of Compound II in hIDO lead to two interpretations of the data. Weaker substrate binding and consequently weaker product binding in both hTDO and XcTDO could result in a change in the rate-limiting step in the mechanism of L-Trp oxidation. The result of this is that Compound II does not accumulate. Alternatively tighter binding of L-Trp to hIDO perturbs the spectrum
of Compound II leading to a shoulder. Weaker binding of L-Trp in the TDOs could account for the absence of a shoulder and the fact that the intermediate spectra resemble the ternary complex in hIDO.

Whether a common mechanism between hIDO and hTDO exists still requires further investigation. However, the evidence appears to be pointing in that direction and away from the original base-catalysed abstraction mechanistic proposal.
4.5 References


Chapter 5: Crystallographic studies of
XcTDO, hTDO and hIDO
5.1 Introduction

It has become increasingly apparent that structural work could potentially be a powerful tool to support investigations into the mechanism of L-Trp oxidation by the heme-containing dioxygenases. Unfortunately, these enzymes have proved to be challenging to crystallise, which has resulted in a slow advance in gaining structural information in this area.

The first structure published, in 2006, was of hIDO in complex with the inhibitor 4-phenylimidazole (4-PI) (1). The structure revealed two distinct domains (large and small), connected by a small loop of 17 residues. The structure of hIDO provided the first glimpse into the active site of these enzymes. The coordination of a proximal histidine (His346) to the heme iron was confirmed as that predicted by spectroscopy (2,3). The structure contained a large number of hydrophobic residues, including those within the distal heme pocket required to accommodate the hydrophobic L-Trp molecule. Unexpectedly, the active site did not contain a distal histidine residue, which had been predicted from spectroscopic work and sequence alignments with a group of related globins (4–7). No further structure was put forward in the literature and no further structures of hIDO were published until 2014 (8), when hIDO had been crystallised in complex with two novel imidazothiazole derivative inhibitors using different crystallisation conditions than had been used for the earlier hIDO structure.

The most revealing structure identified was that of ferrous XcTDO in complex with L-Trp (9). This structure revealed for the first time the binding interactions used to hold L-Trp inside the distal heme pocket. In contrast with hIDO, XcTDO contains a histidine residue (His55) in the distal heme pocket, equivalent to the serine residue (Ser167) in hIDO and forms hydrogen bonds to the indole proton on N\textsuperscript{1} position of L-Trp. The active sites of hIDO and XcTDO are shown in Scheme 5.1 for comparison.
The most challenging enzyme to crystallise appears to be hTDO (10). In 2014, Liu et al published the apo structure of hTDO. However, without the heme co-factor bound key details of the active site are difficult to extract. The structures of Drosophila melanogaster (DmTDO) and Cupriavidus metallidurans (CmTDO) have also been solved with heme co-factor bound (10,11).

The data described above has allowed some clarification in the literature. However, there is still a significant quantity of information lacking in relation to substrate binding interactions, substrate activation and how the structure of the active site is related to its function. The first part of this Chapter describes the crystallisation trials of XcTDO with different ligands (e.g. L-Trp, hydrogen peroxide and potassium cyanide) as well as the crystal structure of XcTDO in complex with potassium cyanide and L-Trp [XcTDO-CN, L-Trp]. The second part of the Chapter describes the initial crystallisation trials undertaken in order to obtain crystals of both hIDO and hTDO.
5.2 Results: crystallisation of XcTDO

5.2.1 Expression and purification of XcTDO

The Mowat Group (Department of Chemistry, University of Edinburgh) provided the XcTDO construct (pET21 d with a C-terminal histidine tag). XcTDO was expressed and purified as described in Sections 2.7 and 2.8 in Chapter 2. The purity of the purified protein was assessed using SDS-PAGE, Figure 5.1.

![Figure 5.1: 12% SDS-PAGE gel of purified XcTDO. Lane A: molecular markers (masses shown next to lane A) and Lane B: XcTDO.](image)

5.2.2 Initial crystallisation of ferric XcTDO

In 2007, Forouhar et al solved the structures of ferric XcTDO and ferrous XcTDO in complex with L-Trp (12). The crystal screen used by Forouhar et al was identified as a starting point for the crystallisation of ferric XcTDO.

Crystals were grown using the sitting drop vapour diffusion technique. A reservoir solution comprising of 100 mM MES pH 6.3, 12% PEG 4000 and 60 mM MnCl₂ (12) was mixed with ferric XcTDO in 50 mM Tris-HCl at a concentration of 20 mg/ml in the crystal drop. The reservoir solution was mixed with ferric XcTDO in equal volumes (2 μl: 2 μl) and crystals typically grew within
2 days at 18 °C appearing in the form of thin red/orange square plates (1 mm x 1 mm), Figure 5.2A. The crystals of ferric XcTDO were very thin and fragile with some of the crystals growing as overlapping multi-crystals. Further crystals were grown as described above at a reduced stock concentration of ferric XcTDO (10 mg/ml) in order to improve the crystal quality. The crystals at a lower protein concentration took ~ 5 days to grow and appeared in the form of red/orange square plates (<200 μm x <200 μm). The plates were thicker and no overlapping multi-crystals were observed however, these crystals were significantly smaller Figure 5.2B.

Figure 5.2: (A) Crystals of XcTDO, crystal screen: 100 mM MES pH 6.3, 12% PEG 4000 and 60 mM MnCl₂, final protein concentration in the crystal drop is 20 mg/ml (B) Crystals of XcTDO, crystal screen: 100 mM MES pH 6.3, 12% PEG 4000 and 60 mM MnCl₂, final protein concentration in the crystal drop is 5 mg/ml.
5.2.3 Micro-Seeding experiments

Micro-seeding experiments were undertaken to improve the crystal quality using the standard seeding protocol (Section 2.18.2, Chapter 2). Drops were set up with a final protein concentration of 8 mg/ml and equilibrated overnight. Micro-fragments (seeds) of ferric XcTDO crystals were transferred to the pre-equilibrated drops using a needle. Crystals took ~2 days to grow however crystal quality did not appear to be improved, Figure 5.3.

Figure 5.3: Crystals of XcTDO grown after micro-seeding.

The crystal quality was not improved after seeding therefore the first crystallisation conditions: 100 mM MES pH 6.3, 12% PEG 4000 and 60 mM MnCl₂, final protein concentration in the drop of 20 mg/ml as described in Section 5.2.2 were used to grow further crystals of XcTDO.

A crystal of ferric XcTDO (Figure 5.2A) was tested to check for diffraction. The cryoprotectant (100 mM MES pH 6.3, 12% PEG 4000, 60 mM MnCl₂ and 25% glycerol (12)) was prepared and used to protect crystals of XcTDO from forming ice-crystals when frozen in liquid nitrogen. The ferric XcTDO crystal was found to diffract to 2.3 Å.
5.2.4 Co-crystallisation of XcTDO with L-Trp

Ferric XcTDO (and ferric hTDO (13,14)) has the ability to oxidise L-Trp to form NFK in the presence of oxygen and without the addition of a reducing agent. The mechanism for this unusual phenomenon is unknown and is investigated in Chapter 6. The structure of XcTDO in complex with the product of L-Trp oxidation, NFK has not been solved. Co-crystallisation or soaking with NFK cannot be undertaken because NFK is not available commercially, therefore this phenomenon was utilised as a method of obtaining crystals of XcTDO in complex with NFK. Ferric XcTDO was mixed with L-Trp (30 mM to ferric XcTDO + L-Trp $K_D = 3.8$ mM (15)), prior to crystallisation as outlined in Section 5.2.2.

5.2.5 Soaking of XcTDO with different ligands

The structures of ferric XcTDO in complex with potassium cyanide and L-Trp to form a ternary complex mimic [XcTDO-CN, L-Trp] and the structure of XcTDO Compound I using hydrogen peroxide have not been solved. Ferric XcTDO was crystallised using the method outlined in Section 5.2.2. Crystals were soaked in cryoprotectant supplemented with either hydrogen peroxide (100 $\mu$M, to obtain Compound I) or soaked in a solution of potassium cyanide (10 mM) followed by a solution containing both potassium cyanide (10 mM) and L-Trp (30 mM) in order to obtain the [XcTDO-CN, L-Trp] complex. Sequential soaking to obtain the [XcTDO-CN, L-Trp] ternary complex mimic was undertaken to overcome the phenomenon described above by blocking the ferric heme with the cyanide ligand. Crystals were soaked for a range of times (5 - 60 seconds for H$_2$O$_2$ experiments and 30 seconds - 15 minutes for CN-L-Trp experiments) before being immediately frozen in liquid nitrogen.

5.2.6 Crystals of XcTDO under anaerobic conditions

Crystals of ferric XcTDO were grown in an anaerobic glove box using the method described in Section 5.2.2. Crystals were soaked with a solution of sodium dithionite (unknown concentration) to produce ferrous XcTDO crystals
(a colour change was observed from orange/red ferric XcTDO crystal to yellow ferrous XcTDO crystal). Ferrous XcTDO crystals were then soaked for varying times (30 seconds to 5 minutes) with cryoprotectant containing L-Trp (1.5 mM) in order to obtain crystals of ferrous XcTDO in complex with L-Trp.

Ferric XcTDO crystals in the glove box were also soaked for varying times (30 seconds to 5 minutes) with a cryoprotectant solution containing potassium cyanide (10 mM) and L-Trp (30 mM) to obtain crystals of [XcTDO-CN, L-Trp]. Crystals were frozen directly into liquid nitrogen within the glove box to avoid exposure to oxygen.

### 5.2.7 Capturing intermediates within an XcTDO crystal

Soaking experiments described above in Section 5.2.6 were repeated with L-Trp and oxygen in an attempt to capture any catalytic intermediates during L-Trp oxidation within the crystal. Lipscomb et al carried out a similar experiment where a crystal of Fe^{II}-containing homoprotocatechuate 2,3-dioxygenase was soaked in the slow substrate 4-nirocatechol in a low oxygen atmosphere; three catalytic intermediates were structurally characterised (16).

Crystals of ferric XcTDO in the anaerobic glove box were initially soaked with a solution of sodium dithionite (unknown concentration) to produce ferrous XcTDO crystals. The ferrous XcTDO crystals were soaked with cryoprotectant containing L-Trp (1.5 mM) and oxygen (258 μM or 130 μM) for varying times (5 - 60 seconds). The crystals were immediately frozen in liquid nitrogen within the anaerobic glovebox.

### 5.2.8 Data collection and structure refinement

Diffraction data was collected at the Diamond Light Source in Oxford on the I04 - 1 beam line by myself, Dr Hanna Kwon and Professor Peter Moody (Department of Biochemistry, University of Leicester), via remote access from the University of Leicester. Seven data sets were collected, however after taking an initial assessment of the data and due to time constraints only one
data set was refined. Some of the crystals subjected to the soaking experiments appeared to have melted around the edges and did not diffract at all.

Dr. Hanna Kwon (Department of Biochemistry, University of Leicester) refined the data set (ferric XcTDO in complex with potassium cyanide and L-Trp) collected at the Diamond Light Source. The structure contained electron density for the ligands potassium cyanide and L-Trp. The data collection and refinement statistics are shown in Table 5.1.

**Table 5.1:** Data collection and refinement statistics for the structure of XcTDO in complex with potassium cyanide and L-Trp.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P 2 2 ı 2 ı</th>
</tr>
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<tbody>
<tr>
<td>Space group</td>
<td>P 2 2 ı 2 ı</td>
</tr>
<tr>
<td>Unit cell</td>
<td>109, 87, 62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Data collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
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<tr>
<td>I/σ</td>
</tr>
<tr>
<td>Completeness (%)</td>
</tr>
<tr>
<td>Rmerge</td>
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</table>

<table>
<thead>
<tr>
<th>Refinement statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rvalue %</td>
</tr>
<tr>
<td>Rfree %</td>
</tr>
<tr>
<td>Bond length (Å)</td>
</tr>
<tr>
<td>Angles (°)</td>
</tr>
</tbody>
</table>
5.2.9 Overall structure of XcTDO in complex with cyanide and L-Trp

The overall structure of XcTDO (Figure 5.4) in complex with potassium cyanide and L-Trp [XcTDO-CN, L-Trp] is identical to the published structure of ferrous XcTDO in a binary complex with L-Trp (12). There was clear electron density for the binding of L-Trp to an allosteric binding site at the interface between the tetramer. Each monomer unit binds one L-Trp molecule at this allosteric binding site. The same has also been reported for published structures of ferrous XcTDO (12), H55A and H55S mutants of XcTDO (15) all in complex with L-Trp.

Figure 5.4: Monomer of XcTDO, the heme is shown in magenta and L-Trp bound to the allosteric site highlighted in red.
5.2.10 Active site of XcTDO in complex with cyanide and L-Trp

The active site of XcTDO (Figure 5.5) shows ligation of the cyanide ligand to the heme iron at the sixth distal coordination site, mimicking oxygen binding. The L-Trp molecule is bound within the active site with the carboxylate and ammonium moieties of L-Trp facing towards the His55 residue. The indole ring is orientated so that the indole nitrogen is almost pointing away from the potassium cyanide ligand and the heme iron. There appear to be no significant electrostatic or hydrogen bonding interactions between L-Trp and any of the active site residues. The binding mode of L-Trp within this complex ternary complex is completely different to the L-Trp binding mode reported in the published ferrous XcTDO structure in a binary complex with L-Trp (12), see discussion (Figure 5.11) for further comparison.

Figure 5.5: Active site of XcTDO in complex with potassium cyanide and L-Trp (shown in cyan).
5.3 Results: hIDO crystallography trials

5.3.1 Preparation of hIDO for crystallography trials

5.3.1.1 Construct preparation

There were two constructs of hIDO available in the laboratory. The first was a pQE30 construct containing a non-cleavable N-terminal hexa-histidine tag\(^1\) (17). The second was a pET151/d construct containing a cleavable N-terminal hexa-histidine tag\(^2\). The pET151/d construct was prepared to replicate the Sugimoto et al construct (pET15b with a cleavable N-terminal hexa-histidine tag), which was successfully crystallised in 2006 (1). It was presumed that the flexible hexa-histidine tag of the pQE30 construct prevented crystallisation.

In 2014, hIDO was crystallised for the first time since 2006 using an alternative crystallisation screen and construct of hIDO (pET28a with cleavable N-terminal hexa-histidine tag). The hexa-histidine tag was not removed for crystallisation (8). It has become apparent that the crystallisation of hIDO is not solely dependent upon the construct design. Factors such as protein purity, protein concentration, crystal screen and ligand binding also appear to play an important role in hIDO crystallisation.

Both the pQE30 and pET151/d constructs of hIDO were taken forward for crystallisation trials and were expressed and purified as described in Chapter 2.

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\(^1\) Dr. Papadopoulou prepared the pQE30 construct of hIDO in 2005.

\(^2\) Dr. Rafice prepared the pET151/d construct of hIDO in 2009.
5.3.1.2 Construct expression and purification

Both hIDO constructs described in Section 5.3.1.1 were expressed and purified as described in Sections 2.7 and 2.8 in Chapter 2. The purity of the purified protein was assessed using SDS-PAGE (Figure 5.6).

![Image of SDS-PAGE gel](A) pQE30 hIDO construct and (B) pET151/d hIDO construct. Lane A: molecular markers (masses shown next to lane A) and lane B: hIDO.

Figure 5.6: 12% SDS-PAGE gel of the purified hIDO constructs. (A) pQE30 hIDO construct and (B) pET151/d hIDO construct. Lane A: molecular markers (masses shown next to lane A) and lane B: hIDO.

5.3.2 Crystallisation trials of hIDO: published screens

The conditions used to crystallise hIDO by both Sugimoto et al (screen 1, (1)) and Isobe et al (screen 2, (8)) have been reproduced in this work. Details of both crystallisation conditions are described in Table 5.2. Crystal trials were set up using both sitting and hanging drop vapour diffusion techniques. Protein was mixed with crystallisation screen in a 1:1 ratio.
Table 5.2: Published crystallisation conditions used to crystallise hIDO.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Crystallisation Screen</th>
<th>Protein buffer</th>
<th>[hIDO] mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% PEG 8000</td>
<td>10 mM MES</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>200 mM Ammonium acetate</td>
<td>25 mM NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM CHES</td>
<td>pH 9.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10% PEG 20,000</td>
<td>10 mM MES</td>
<td>10 - 20</td>
</tr>
<tr>
<td></td>
<td>2% Dioxane</td>
<td>25 mM NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM Bicine</td>
<td>pH 9.0</td>
<td></td>
</tr>
</tbody>
</table>

Crystallisation plates replicating the conditions highlighted in Table 5.2 mainly showed an orange/red amorphous precipitation and <10% produced gels. Screening was undertaken around these two sets of published crystallisation conditions using minor variations such as, varying the protein concentration between 5 - 20 mg/ml and the % PEG. Variations are described in Table 5.3.

Table 5.3: Variations around the two published hIDO conditions.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Variations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[hIDO] mg/ml</td>
</tr>
<tr>
<td>1</td>
<td>5 - 20</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td>1</td>
<td>5 - 20</td>
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<td>2</td>
<td>5 - 20</td>
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<td></td>
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<tr>
<td>2</td>
<td>5 - 20</td>
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<td></td>
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</tbody>
</table>
Screening around the published conditions produced orange/red amorphous precipitate. Reducing the protein concentration to 5 mg/ml produced a non-amorphous precipitate, which may lead to crystals if further investigations were to be undertaken and is in contrast to the hIDO concentrations used in Table 5.2. Neither crystals nor micro-crystals were observed during these experiments.

The effect of L-Trp (3 mM) or 4-PI (1mM) binding to hIDO during crystallisation was investigated (Table 5.4). A structure of hIDO in complex with L-Trp has not yet been solved. However, if it were to be solved in the future then it would provide direct insight into substrate recognition and catalysis in hIDO that could be compared to the XcTDO binary L-Trp complex (9). The inhibitor 4-PI was utilised due to the fact that the first structure hIDO published with this inhibitor bound to the active site. Binding of either ligand to the active site of hIDO should induce changes in the active site leading to conformational changes throughout the overall structure of hIDO, which could stabilise the structure for crystallisation.

**Table 5.4:** Variations involving ligand binding around the two published hIDO conditions.

<table>
<thead>
<tr>
<th>Screen</th>
<th>[hIDO] mg/ml</th>
<th>Protein buffer</th>
<th>Ligand</th>
<th>[Ligand] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 - 20</td>
<td>10 mM MES 25 mM NaCl pH 6.5</td>
<td>4-PI</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>5 - 20</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>4-PI</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5 - 20</td>
<td>10 mM MES 25 mM NaCl pH 6.5</td>
<td>4-PI</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5 - 20</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>4-PI</td>
<td>1</td>
</tr>
</tbody>
</table>
Screening around the two binary ligand complexes (L-Trp and 4-PI) of hIDO produced orange/red amorphous precipitate with <10% producing gels. There was no clear difference between the two different ligands and their effect on the crystallisation of hIDO.

5.3.3 Crystallisation trials of hIDO: crystallisation screens

Crystal trials were set up between hIDO and the following commercial crystallisation screens: JCSG+, PACT premier, crystal screen I & II and Wizard classic crystal screen I & II using a Mosquito and Cartesian robotic system. The crystallisation plates were stored at 18 ºC and the final hIDO concentrations were 10 mg/ml and 5 mg/ml. Trials were also conducted with hIDO in complex with 3 mM L-Trp.

Crystallisation plates showed >50% heavy or amorphous precipitation with ~10% of conditions producing phase separation or gels. The remaining conditions failed to produce any kind of precipitation. There was no clear improvement on the crystallisation of hIDO in complex with L-Trp compared to hIDO alone. Two conditions during the initial screening grew micro-crystals from the light precipitate shown in Figure 5.7 and the conditions are described in Table 5.5.

The two conditions producing micro-crystals of hIDO could be assessed further through the use of finer screening techniques (e.g. altering precipitant concentrations) in order to obtain diffraction quality crystals. The few screens producing gels could also be investigated. The gel could be used to streak seed into new protein-screen drops to encourage crystal nucleation.
Figure 5.7: Micro-crystals of hIDO observed in wells (A) E4, (B) E4 (hIDO + 3mM L-Trp) and (C) D10 during initial crystallisation trials.

Table 5.5: Details Conditions where micro-crystals of hIDO were observed during initial crystallisation trials.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein buffer</th>
<th>[hIDO] mg/ml</th>
<th>Buffer</th>
<th>Salt</th>
<th>Precipitant</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM imidazole-HCl</td>
<td>200 mM magnesium chloride</td>
<td>35% (v/v) 2-methyl-2,4-pentanediol (MPD)</td>
<td>8.0</td>
</tr>
<tr>
<td>D10</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM imidazole-HCl</td>
<td>200 mM sodium chloride</td>
<td>1000 mM ammonium phosphate dibasic</td>
<td>8.0</td>
</tr>
</tbody>
</table>
5.3.4 Crystallisation trials after trypsin digestion of hIDO

Prior to crystallisation trials trypsin was mixed with hIDO in two different ratios; 1:100 and 1:1000. Trypsin was employed at very dilute concentrations to fragment hIDO by removing flexible loops or ends, which could inhibit crystallisation without fragmenting the protein completely (18).

Crystallisation plates showed; >40% heavy or amorphous precipitation with ~10% of conditions producing phase separation or gels. The remaining conditions failed to produce any kind of precipitation. Two conditions during the initial screening grew micro-crystals from the light precipitate shown in Figure 5.8 and the conditions are described in Table 5.6. Note that one of the screens (E4) produced micro-crystals during these experiments as well during crystallisation experiments in Section 5.3.3.
Figure 5.8: Micro-crystals of hIDO observed in wells (A) E4 (hIDO + trypsin 1/1000), (B) E4 (hIDO + trypsin 1/100), (C) E12 (hIDO + trypsin 1/1000) (D) E12 (hIDO + trypsin 1/100) during initial crystallisation trials.

Table 5.6: Details of the conditions where micro-crystals of hIDO + trypsin were observed during initial crystallisation trials.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein buffer</th>
<th>[hIDO] mg/ml</th>
<th>Buffer</th>
<th>Salt</th>
<th>Precipitant</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM imidazole-HCl</td>
<td>200 mM magnesium chloride</td>
<td>35% (v/v) MPD</td>
<td>8.0</td>
</tr>
<tr>
<td>E12</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM imidazole-HCl</td>
<td>200 mM calcium chloride</td>
<td>20% (w/v) PEG 1000</td>
<td>8.0</td>
</tr>
</tbody>
</table>
The two conditions producing micro-crystals could be assessed further. The fragmentation of hIDO could also be investigated by MALDI-TOF and SDS-PAGE in order to produce a new construct of hIDO that could be put through further rounds of crystallisation trials. The few screens producing gels could also be investigated. The gel could be used to streak seed into new protein-screen drops to encourage crystal nucleation.
5.4 Results: Crystallography trials hTDO

5.4.1 Preparation of hTDO for crystallography trials

5.4.1.1 Construct preparation

Full-length hTDO had previously proven difficult to express and purify due to the formation of inclusion bodies when expressed in *E.coli* as well as a tendency to aggregate in free solution. A truncated version of hTDO was prepared by Yeh *et al* using the sequences of XcTDO and *Pseudomonas aeruginosa* (PaTDO) as a guide, deleting fragments 1 - 17 and 389 - 406 as they were not present in the bacterial TDOs. The new construct (pET14b expression vector) was shown to be soluble and functional, indicating that these fragments might not be critical for the structure and function of hTDO (14,19).

At the same time Raven *et al* developed a system for the expression of full-length hTDO (pET28a expression vector), where it was shown that the protein could be expressed as a mixture of the apo and holo forms and its activity introduced by reconstituting with hemin (13).

The structure of apo hTDO published by Liu *et al* was crystallised from a truncated construct of hTDO with deleted fragments 1 - 18 and 389 - 406 (pMCSG7 expression vector) (10).

Constructs of hTDO were prepared based on the constructs described above for crystallisation trials (Table 5.7) although pET28a full-length construct was already available in the lab.³

³ Dr. Basran prepared the pET28a construct of hTDO in 2007.
Table 5.7: Summary of the hTDO constructs used for crystallography trials.

<table>
<thead>
<tr>
<th>Vector</th>
<th>His-tag</th>
<th>Recombinant DNA</th>
<th>Protein size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28a</td>
<td>C-terminal</td>
<td>hTDO</td>
<td>Full-length 1-406</td>
</tr>
<tr>
<td>pLeics05</td>
<td>C-terminal</td>
<td>hTDO</td>
<td>Full-length 1-406</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>truncated 17-389</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>truncated 19-388</td>
</tr>
</tbody>
</table>

5.4.1.2 Construct expression and purification

The hTDO constructs described in Section 5.4.1.1 were expressed on a small scale (5 ml) to check for efficient expression in BL21 (DE3) E.coli cells. Expression was subsequently scaled up and the resulting protein purified as described in Section 2.7 and 2.8 in Chapter 2. The purity of the purified protein was assessed using SDS-PAGE (Figure 5.9).
Figure 5.9: 12% SDS-PAGE gel of the purified hTDO constructs. (A) pET28a full length hTDO, (B) pLeics05 full length hTDO, (C) pLeics05 truncated hTDO residues 17 - 389 and (D) pLeics05 truncated hTDO residues 19 - 388. Lane A: molecular markers (masses shown next to lane A) and lane B: hTDO.
5.4.2 Crystallisation trials of hTDO

Crystal trials were set up between different constructs of hTDO (Table 5.7) and the commercial crystallisation screens: JCSG+, PACT premier, crystal screen I & II and Wizard classic crystal screen I & II using a Mosquito robotic system. The crystallisation plates were stored at 18 ºC and the final protein concentrations were 5 mg/ml and 2.5 mg/ml.

Crystallisation plates showed >50% heavy or amorphous precipitation with ~10% of conditions producing phase separation or gels. The remaining conditions failed to produce any kind of precipitation. Three conditions during the initial screening grew micro-crystals from the light precipitate shown in Figure 5.10 and the conditions are described in Table 5.8.
Figure 5.10: Micro-crystals of hTDO observed in wells (A) A7 JCSG+ screen (pLeics05 full length hTDO), (B) E5 JCSG+ screen (pET28a full length hTDO) (C) F6 crystal screen I & II (pLeics05 truncated hTDO).

Table 5.8: Conditions where micro-crystals of hTDO were observed during initial crystallisation trials.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein buffer</th>
<th>[hIDO] mg/ml</th>
<th>Buffer</th>
<th>Salt</th>
<th>Precipitant</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM CHES</td>
<td>-</td>
<td>20% (w/v) PEG 8000</td>
<td>9.5</td>
</tr>
<tr>
<td>E5</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM CAPS</td>
<td>-</td>
<td>40% (v/v) MPD</td>
<td>10.5</td>
</tr>
<tr>
<td>F6</td>
<td>50 mM Tris-HCl pH 8.0</td>
<td>5</td>
<td>100 mM Tris.HCl 200 mM calcium acetate</td>
<td>20% (w/v) PEG 3000</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>
The three conditions producing micro-crystals of hTDO could be assessed further using finer screening techniques in order to obtain diffraction quality crystals. The few screens producing gels could also be investigated. The gel could be used to streak seed into new protein-screen drops to potentially produce crystals. From the initial crystallisation trials it is not clear whether using a truncated construct of hTDO has improved the chances of crystallising hTDO.
5.5 Discussion

The principal aim of the experiments reported in this Chapter was to generate protein crystals suitable for X-ray crystallography studies. Although crystals were only obtained for XcTDO, some screening conditions trialed with both hIDO and hTDO produced micro-crystals. These conditions are a step towards finding suitable crystallisation conditions for both these proteins through finer crystallisation screening techniques.

Crystals of XcTDO and ligand binding experiments

Crystals of XcTDO were grown using the published screen (12); they appeared in the form of very thin orange/red plates and diffracted. The ferric XcTDO test crystal diffracted to 2.3 Å and the crystal soaked with both potassium cyanide and L-Trp diffracted to 2.7 Å at the Diamond Light Source. It was noticed that the older the crystal the lower the diffraction quality therefore fresh crystals were prepared prior to crystal harvesting or any soaking experiments.

To obtain different ligand complexes of XcTDO, co-crystallisation and soaking experiments were set up. It was difficult to determine: the concentrations of ligand to which the crystal should be exposed and the length of time the crystals should be soaked in order to avoid decreasing the overall diffraction quality of the crystal. It was clear that when the crystals were sent to the beam line at the Diamond Light Source that some of the crystals had melted around the edges during the soaking experiments and were therefore no longer viable.

Structure of XcTDO in complex with potassium cyanide and L-Trp

The structure of ferric XcTDO in complex with potassium cyanide and L-Trp was solved with a resolution of up to 2.7 Å. The overall structure of the protein was identical to the published structure of ferrous XcTDO in complex with L-Trp (12). The binding of L-Trp to residues to an allosteric binding site was also observed in the ternary structure [XcTDO-CN, L-Trp] and in the published structure (12). Binding of L-Trp to this site could potentially induce a conformational change in
the overall structure of the protein that could facilitate substrate binding and catalysis at the active site. At the moment the role of L-Trp as an allosteric regulator is unknown.

After comparison of the active site of the ternary complex mimic [XcTDO-CN, L-Trp] and the published binary ferrous [XcTDO-L-Trp] complex, the immediate difference is the binding mode of L-Trp. In the active site of the binary complex (Figure 5.11A), the carboxylate and ammonium moieties of L-Trp are recognised by electrostatic and hydrogen-bonding interactions with the enzyme and a propionate group of the heme. The indole group is positioned as expected for oxidation of the C\(_2\)-C\(_3\) bond, the nitrogen atom (N\(_1\)) of the indole ring hydrogen bonds directly with the active site His55 residue. In the ternary mimic complex (Figure 5.11B) the L-Trp molecule is bound in completely the opposite orientation with the carboxylate and ammonium moieties of L-Trp facing towards His55 residue. The indole ring is orientated so that the indole nitrogen is almost pointing away from the potassium cyanide ligand and the heme iron. There appears to be no electrostatic or hydrogen bonding interactions between L-Trp and any active site residues. It is interesting to note that Arg117 is in the closed conformation in Figure 5.11A and in the open conformation in Figure 5.11B, clearly a result of the different binding modes of L-Trp within each complex.

It is unclear why the L-Trp molecule in the ternary complex mimic is bound in a different conformation within the active site. Perhaps it is a result of the presence of potassium cyanide or due to the ferric heme iron centre. It is unknown if this conformation of L-Trp in the active site is catalytically viable if it were in the presence of reduced heme and oxygen although first impressions would deem that it is not.
Figure 5.11: Comparison of the active site of (A) [XcTDO-CN, L-Trp] and (B) published binary ferrous [XcTDO-L-Trp] complex.

Constructs of hIDO and hTDO

During the crystallisation trials with both commercial and published screens there did not appear to be an observable difference between either of the hIDO constructs (pQE30 and pET151/d). As discussed previously, the literature suggests that it is the type and mode of ligand bound to the active site of hIDO that dictates whether the enzyme will crystallise. The only structures of hIDO are in complex with inhibitors (1,8).

Truncated constructs of hTDO were prepared and purified based on the constructs of hTDO in the literature. There did not appear at this stage to be a significant advantage of the truncated forms of hTDO over the full-length constructs in finding suitable crystallisation conditions. The advantage of the truncated constructs may become apparent during further screening for crystallisation conditions.
Crystallisation trials

The crystallisation conditions for hIDO from the literature were replicated in this work. However, crystals were not produced and attempts at modifying the conditions (e.g. altering the protein concentration) did not appear to improve the conditions significantly enough that crystal formation was observed. Addition of the ligands L-Trp and 4-PI were understood to induce conformational changes to the structure of hIDO through binding to the active site that could stabilise the structure for crystallisation. Crystals did not result from these hIDO binary complexes. Investigations using isothermal calorimetry (ITC) could determine which ligands have the greatest overall conformational effect on hIDO in order to support crystallisation attempts. Binding of L-Trp to hTDO was not utilised during these crystal trials due to the phenomenon that ferric hTDO can oxidise L-Trp in the presence of oxygen and without the addition of a reductant (discussed further in Chapter 6).

The commercial screens resulting in micro-crystals for both hIDO and hTDO could be taken forward for further analysis and finer screening. This will involve scaling up from 200 nl drops (100 nl hTDO mixed with 100 nl crystal screen) to 2 - 4 μl drop sizes in order to improve the size of the crystals. Screening around the condition could also be undertaken by altering, for example: the pH, protein concentration and precipitant concentration. Some conditions screened with hIDO and hTDO produced gels that could be used in streak seeding experiments to facilitate crystal growth. It is important to undertake more crystal trials in order to screen for more potential crystallisation conditions. Further crystallisation trials would need to be undertaken for both hIDO and hTDO using other commercial screens such as Morpheus (Molecular Dimensions), MDL structure screen (Molecular Dimensions) and Midas (Molecular Dimensions).
5.6 References


Chapter 6: Oxidation of tryptophan by ferric TDO
6.1 Introduction

The heme-containing dioxygenases only require a single, initiating reduction of the heme iron for oxidation of tryptophan (L-Trp) to proceed. Interestingly, incubation of L-Trp with ferric human tryptophan 2,3-dioxygenase (hTDO) under atmospheric oxygen concentrations, in the absence of a reducing agent resulted in N-formylkynurenine (NFK) formation (Scheme 6.1) (1,2). The exact mechanism of this reaction has not been established, although it was reported to be oxygen dependent (1). It was proposed that oxidation of L-Trp by ferric hTDO was likely to result from a trace amount of hydrogen peroxide and/or superoxide from solution (1). This was based on kinetic measurements where the addition of catalase or superoxide dismutase significantly reduced the rate of NFK production by up to 90% (1).

A truncated form of ferric hTDO has also been reported to oxidise L-Trp to produce NFK without the requirement of a reducing agent (3). Activation of ferric hTDO for catalysis was proposed to result from a one-electron reduction of the ferric hTDO (Fe^{III}) to ferrous (Fe^{II}) hTDO by either L-Trp itself or an active site amino acid situated in the proximity of the heme iron (3).

In contrast, human indoleamine 2,3-dioxygenase (hIDO) is only reported to be capable of oxidising L-Trp in the reduced, ferrous (Fe^{II}) form (1), the same was also reported for bacterial Xanthomonas Campestris TDO (XcTDO) (4).
The purpose of the work in this Chapter was to attempt to understand the mechanism of L-Trp oxidation by ferric TDO, particularly the mechanism of ferric TDO activation.
6.2 Results

6.2.1 Oxidation of L-Trp by ferric hTDO, XcTDO and hIDO: NFK formation

UV-visible spectroscopy was used to monitor the oxidation of L-Trp by ferric hTDO, XcTDO and hIDO (1 - 3 μM) in the absence of a reducing agent (e.g. sodium dithionite). Each enzyme was separately incubated with L-Trp (3 mM) and oxygen (258 μM). Increases in absorbance at 321 nm resulting from NFK formation were observed only for hTDO and XcTDO (Figure 6.1 and Figure 6.2).

![Figure 6.1](image)

**Figure 6.1**: Oxidation of L-Trp by ferric hTDO and XcTDO. (A) Spectral changes observed upon reaction of ferric hTDO with L-Trp under aerobic conditions, (B) Spectral changes observed upon reaction of ferric XcTDO with L-Trp under aerobic conditions for 60 minutes; arrows indicate the direction of the absorbance change during the course of the reaction. Reaction conditions: 50 mM Tris-HCl, pH 8.0, 25.0 °C.
Figure 6.2: Spectral changes observed upon reaction of ferric hIDO with L-Trp under aerobic conditions for 60 minutes. Reaction conditions: 50 mM Tris-HCl, pH 8.0, 25.0 °C.

Oxidation of L-Trp by XcTDO has only been reported for the ferrous form of the enzyme [4] however, the spectra shown in Figure 6.1B provide evidence to the contrary. Ferric XcTDO also possesses the ability to oxidise L-Trp, along with hTDO, and neither require addition of a reducing agent.

6.2.2 Oxidation of L-Trp by ferric hTDO and ferric XcTDO: oxygen and L-Trp dependence

To confirm the oxygen dependence of this reaction, both ferric hTDO and ferric XcTDO were separately incubated with L-Trp (3 mM) in the absence of oxygen. All buffers and solutions were degassed using nitrogen and the experiment carried out in an anaerobic glove box ([O₂] < 5 ppm). UV-visible spectroscopy was used to monitor absorbance changes. There were no increases in absorbance at 321 nm resulting NFK production for either hTDO or XcTDO (Figure 6.3). This experiment confirms that oxidation of L-Trp by both ferric hTDO and ferric XcTDO utilises oxygen.
Figure 6.3: Oxidation of L-Trp by ferric XcTDO and hTDO. (A) Spectral changes observed upon reaction of ferric XcTDO with L-Trp under anaerobic conditions, (B) Spectral changes observed upon reaction of ferric hTDO with L-Trp under anaerobic conditions. All reactions were monitored over 60 minutes. Reaction conditions: 50 mM Tris-HCl, pH 8.0, 25.0 °C.
The oxidation of L-Trp by ferric TDO is also dependent on the concentration of L-Trp. The turnover rate for the oxidation of L-Trp by ferric XcTDO in the absence of reducing agent is $k_{cat} = 1 \text{ s}^{-1}$ and $K_M = 116 \text{ μM}$. In comparison, the reported turnover rate for the oxidation of L-Trp by ferrous XcTDO (typical dioxygenase L-Trp oxidation assay) was reported to have a $k_{cat} = 19.5 \text{ s}^{-1}$ and $K_M = 114 \text{ μM}$ (4). There is a significant decrease in the rate of L-Trp oxidation between ferric XcTDO and ferrous XcTDO although the binding of L-Trp is very similar for both.

### 6.2.3 Oxidation of L-Trp by ferric hTDO and ferric XcTDO: addition of potassium cyanide

Potassium cyanide was utilised in the following experiments due to the fact that this small cyanide ligand (CN) can bind to ferric heme at the sixth distal coordination site. The purpose of using potassium cyanide in this experiment was to confirm that ferric heme is the initiating species for the oxidation of L-Trp, in the presence oxygen and without addition of a reducing agent.

Ferric hTDO and XcTDO (1 μM) were separately incubated with oxygen (258 μM) and potassium cyanide (500 μM). The reaction was initiated by the addition of L-Trp (3 mM). UV-visible spectroscopy was used to monitor NFK production through a change in absorbance at 321 nm over time. There was no overall increase in absorbance over 15 minutes. Interpretation of this result is that the cyanide ligand has saturated the ferric heme distal coordination sites, preventing NFK formation. This indicates that ferric heme is the initiating species for this reaction.

This experiment was repeated with potassium cyanide (500 μM) being added during the course of L-Trp oxidation by ferric hTDO and XcTDO. The purpose of this experiment was to observe if the overall rate of NFK formation was reduced or inhibited due to binding of the cyanide ligand to ferric hTDO and ferric XcTDO. A reduction in the rate of NFK formation was not observed for either hTDO or XcTDO. This result suggests that ferric TDO is only required for initial protein activation and is not the catalytic centre for the production of NFK.
6.2.4 Activation of ferric TDO by hydrogen peroxide and/or superoxide

It was investigated whether ferric hTDO and ferric XcTDO can recruit hydrogen peroxide and/or superoxide from solution to activate ferric heme for L-Trp oxidation. Both ferric hTDO and ferric XcTDO (1 μM) were separately incubated with L-Trp (3 mM), oxygen (258 μM) and either catalase (100 μg) or superoxide dismutase (100 μg). Catalase removes traces of hydrogen peroxide from solution through disproportionation to form oxygen and water. Superoxide dismutase removes superoxide from solution through the formation of either oxygen or hydrogen peroxide.

An 80% reduction in the rate of L-Trp oxidation by ferric hTDO and a 90% reduction in the rate of L-Trp oxidation by ferric XcTDO were observed in the presence of catalase. There was no reduction in the rate of L-Trp oxidation by either ferric hTDO or XcTDO in the presence of superoxide dismutase, contrary to that reported in the literature for hTDO (1). These results indicate involvement of hydrogen peroxide from solution in the activation of ferric hTDO and XcTDO prior to L-Trp oxidation.

6.2.5 Analysis of the oxidation of L-Trp by ferric hTDO and XcTDO using stopped flow photodiode array spectroscopy

During the initial experiments shown in Figure 6.1 there appeared to be no significant changes in the absorbance spectra of ferric hTDO or ferric XcTDO during NFK production. The reaction of both enzymes was monitored using single mixing stopped flow photodiode array spectroscopy (the stopped flow spectrometer is housed in an anaerobic glove box ([O₂] < 5 ppm)). The purpose of these experiments were to try to identify any transient protein species populated during L-Trp oxidation, which could aid in the understanding of this reaction.
6.2.5.1 Oxidation of L-Trp by ferric hTDO and ferric XcTDO monitored using stopped flow photodiode array spectroscopy

After mixing either ferric hTDO or ferric XcTDO (1 - 3 μM) with L-Trp (2 mM) and oxygen (600 μM) data was collected over a period of 200 s and the resulting spectra shown in Figure 6.4. Formation of NFK was evident through increases in absorbance at 321 nm over 50 - 100 s. A lag phase (~10 s) was observed before NFK production, which suggested ferric enzyme activation prior to L-Trp oxidation. A similar lag phase was not observed during ferrous hTDO and ferrous XcTDO steady state turnover experiments (described in Chapter 2 - L-ascorbate/methylene blue reducing system). An accumulation of ferrous hTDO and ferrous XcTDO was observed after NFK production appeared to cease (figure 6.4 and inset).
Figure 6.4: Stopped flow diode-array spectra showing oxidation of L-Trp by ferric hTDO and ferric XcTDO. (A) Spectral changes observed after mixing ferric hTDO (3 μM) with L-Trp (3 mM) and O$_2$ (600 μM) (B) Spectral changes observed after mixing ferric XcTDO (1.5 μM) with L-Trp (2 mM) and O$_2$ (600 μM). Reactions were monitored over 200 s. Absorbance values within the 480-700 nm region of the spectrum have been multiplied by a factor of 5 and arrows indicate the direction of the absorbance change during the course of the reaction. Insets show NFK formation at 321 nm, ferrous TDO accumulation (XcTDO 432 nm and hTDO 425 nm) and decay of ferric TDO (XcTDO 404 nm and hTDO 406 nm.)
To determine if ferrous TDO generated during the experiments described above (Figure 6.4) is catalytically competent, carbon monoxide (CO) was bubbled into ferric XcTDO prior to being mixed with L-Trp and oxygen (Figure 6.5). The presence of carbon monoxide did not appear to inhibit NFK formation by binding to ferrous XcTDO to form an XcTDO-CO complex (wavelength maxima of 420 nm). This is assuming that carbon monoxide can out-compete oxygen binding to ferrous XcTDO in the presence of L-Trp.

![Stopped flow diode-array spectra showing oxidation of L-Trp by ferric XcTDO in the presence of CO. Spectral changes observed after mixing ferric XcTDO (5 μM, pre-mixed with CO) with L-Trp (2 mM) and O₂ (600 μM). Reaction was monitored over 200 s. Absorbance values within the 480 - 700 nm region of the spectrum have been multiplied by a factor of 5 and arrows indicate the direction of the absorbance change during the course of the reaction. Inset shows NFK formation at 321 nm, accumulation of ferrous XcTDO with CO bound at 420 nm and decay of ferric XcTDO at 404 nm.](image)

The experiment above was repeated using a bench top spectrophotometer in order to observe the effect of adding CO (binds to ferrous heme) part way through L-Trp oxidation by ferric hTDO and XcTDO. The experiment was undertaken to determine if addition of CO would reduce the rate of NFK
formation or inhibit activity completely. It was also intended to determine if ferrous XcTDO is the catalytic centre for the formation of NFK. Ferric hTDO and ferric XcTDO were separately incubated with L-Trp in the presence of oxygen. The formation of NFK was monitored for ~5 minutes prior to CO addition. A decrease in the rate of NFK formation was not observed which can be interpreted as ferrous heme is not the catalytic centre for NFK production. However, this is again assuming that carbon monoxide has the ability to out-compete oxygen binding in the presence of L-Trp.

6.2.6 Reduction of ferric hTDO and ferric XcTDO by L-Trp

Using UV-visible spectroscopy, ferric hTDO and ferric XcTDO were incubated separately with L-Trp in the absence of oxygen (anaerobic glove box). After monitoring each protein for up to 5 hours ferrous hTDO or ferrous XcTDO were not produced. This result indicates that L-Trp does not directly reduce the heme iron.
6.3 Discussion

It was demonstrated in the literature that incubation of ferric hTDO (or a truncated version of ferric hTDO) with L-Trp and oxygen in the absence of a reducing agent could lead to the formation of NFK (1,3). The same has been demonstrated during this work for ferric hTDO as well as ferric XcTDO, which has only been reported to oxidise L-Trp in the reduced, ferrous form (4).

The ability of ferric hTDO and XcTDO to catalyse the oxidation of L-Trp is not one that hIDO possesses. This difference was accounted for in the literature as most likely relating to the fact that hIDO discriminates against substrate binding to the ferric enzyme ($K_D$ for Fe$^{III} +$ L-Trp binding is 285 μM and the $K_M$ for ferrous oxidation of L-Trp is 7 μM (5)) unlike hTDO ($K_D$ for Fe$^{III} +$ L-Trp binding is 200 μM and the $K_M$ for ferrous oxidation of L-Trp is 222 μM (1)). This explanation does not account for XcTDO, which also discriminates against substrate binding to the ferric enzyme ($K_D$ for Fe$^{III} +$ L-Trp binding is 3.8 mM and for Fe$^{II} +$ L-Trp binding is 4 μM and $K_M$ for ferrous oxidation of L-Trp is 114 μM (4,6)).

Product formation does not occur during incubation of L-Trp with ferric hTDO and XcTDO in the absence of oxygen subsequently confirming the role of oxygen in this reaction. This suggests parallels between ferric and ferrous oxidation of L-Trp. The mechanism of ferric oxidation is unknown particularly the activation of ferric TDO towards both oxygen and L-Trp.

Experiments with potassium cyanide confirmed that ferric TDO is the starting point thereby ruling out catalytic activity from any small populations of ferrous TDO in solution. The lack of inhibition after addition of potassium cyanide during NFK production indicates that another form of TDO is the primary catalytic centre, most likely ferrous TDO.

Ferric TDO activation by hydrogen peroxide and/or superoxide was investigated through the addition of catalase or superoxide dismutase during the incubation of L-Trp with oxygen and ferric hTDO and XcTDO. A reduction in the rate of NFK formation was only observed in the presence of catalase suggesting that only hydrogen peroxide plays a role in activating TDO for oxidation of L-Trp. The
activation of TDO by H₂O₂ has been examined at great length by Liu et al (7). The proposed pathway involves hydrogen peroxide binding to form a Compound I intermediate (Fe⁴=O with a porphyrin radical) that rapidly decays to form Compound ES (Fe⁴=O with a protein radical). The protein radical and ferryl heme (Fe⁴=O) react with L-Trp resulting in either a monooxygenated L-Trp species (Trp-O) or L-Trp dimer (Trp-Trp) and reduced TDO. Once reduced TDO is produced, oxygen binding can occur and formation of NFK ensues (Scheme 6.2)

Scheme 6.2: The proposed activation pathway ferric TDO by hydrogen peroxide (7).

Scheme 6.3: Current mechanistic proposal for the oxidation L-Trp by the heme containing dioxygenases (8). Step 1a involves electrophilic addition of the first oxygen atom and step 1b involves radical addition of the first oxygen atom.

In summary, the results presented in this Chapter suggest an overall mechanism that starts with the activation of ferric TDO by traces of hydrogen
peroxide in solution through the pathway proposed by Liu et al (Scheme 6.2). Once ferrous TDO has accumulated the mechanism of oxidation occurs via the current literature proposal (Scheme 6.3). The physiological relevance for the oxidation of L-Trp by ferric TDO comes from the fact TDO is found in more oxidising environments such as the liver. In comparison, it is proposed that IDO is maintained in a reduced state in vivo by cytochrome b5 and cytochrome b5 reductase (9). The tolerance for hIDO with hydrogen peroxide is low before inactivation of the enzyme occurs through the formation of Compound I mediated protein radicals (10,11). A similar system to maintain TDO in a reduced state in vivo has not yet been established. It is understood that TDO can overcome inactivation of the heme iron by recruiting hydrogen peroxide, which is present under physiological conditions at low levels in cells. Overall, the data in this Chapter provides a platform for further studies into the mechanism of L-Trp oxidation by ferric TDO.
6.4 References


Chapter 7: Conclusions and Future Work
7.1 Conclusions

Over the years, the heme-containing dioxygenases involved in tryptophan (L-Trp) oxidation have periodically been the subject of various studies and consequently received sporadic attention in the literature. Recently there has been a surge in experimental and computational investigations into the mechanism of L-Trp oxidation (1–5). This sudden interest in the mechanism is mainly due to the important physiological roles (reviewed in Section 1.7, Chapter 1) of IDO and TDO. The role of both enzymes in supporting the evasion of cancer cells from the immune response (6,7) has set precedence for understanding the mechanism of L-Trp oxidation in order to facilitate the rational design of inhibitors.

Since the initial mechanistic proposal of base-catalysed abstraction of the indole NH group of L-Trp by an active site base (8,9) was found to be flawed, an alternative mechanism has been proposed based upon the current experimental and computational evidence (1–5). Some of this work was reviewed during the early part of this PhD work (10). It could be wrongly perceived that there is little left to learn regarding the mechanism of these enzymes. However, there are still significant questions surrounding the mode of action of these enzymes and consequently a significant amount more to be investigated. The work presented in this thesis has aimed to answer some of the questions in the literature regarding the mechanism of L-Trp oxidation by both IDO and TDO.

Involvement of a Compound II intermediate (Fe\textsuperscript{IV}=O) in the mechanism of L-Trp oxidation had been first suggested by computational studies (4,11,12). The vibrational frequency resulting from Compound II was also detected using resonance Raman by two different studies (1–3). However, full characterisation of the absorbance spectrum of Compound II resulting from the heme-containing dioxygenases has never fully been undertaken, probably because of the difficulty in differentiating incompletely formed ferrous-oxy species from a genuine Compound II species. The spectrum with identical features to the Compound II spectra shown in Chapter 3 had previously been published although it was called a ‘593 nm species’ and its identity as Compound II never
fully confirmed (2,3). In Chapter 3, a Compound II intermediate was observed using absorption spectroscopy during the oxidation of L-Trp by hIDO. Other substrates (e.g. 5-fluoro-tryptophan, 5-methyl-tryptophan and 5-hydroxy-tryptophan) reportedly oxidised by hIDO were also examined to determine if they were oxidised by a common mechanism with L-Trp through a Compound II intermediate. The most significant result was that the tryptophan analogue 1-methyl-tryptophan (1-Me-Trp) was oxidised by a common mechanism with L-Trp through a Compound II intermediate. A common mechanism between 1-Me-Trp and L-Trp had previously been assumed but never confirmed since it was observed that 1-Me-Trp was a slow substrate of hIDO (13). It was found in this work that majority of the substrates tested are oxidised by hIDO through a common mechanism involving a Compound II intermediate. During this study the differences in reactivity between the 5-hydroxy-tryptophan and 5-methoxy-tryptophan with hIDO lead to the interpretation that the addition of the initial oxygen atom occurs by radical addition. Although, a recent heme-modification study suggested that the addition of the initial oxygen atom in PaTDO occurs by electrophilic addition (14).

In Chapter 4, the mechanism of L-Trp oxidation by hTDO and XcTDO has been studied in order to provide evidence for the accumulation of a Compound II intermediate and support for a common mechanism between TDO and hIDO. An intermediate was detected in the case of both hTDO and XcTDO although the spectrum was more comparable to that of the ternary complex of hIDO rather than Compound II. It has been interpreted that due to the weaker binding of L-Trp to hTDO and XcTDO (15,16) in comparison to hIDO, that the dissociation rate of L-Trp and therefore possibly NFK would be faster. This could result in either the spectrum of Compound II appearing differently in the TDOs compared to hIDO or an alteration of the rate-limiting step in the TDOs therefore Compound II does not accumulate. This second interpretation of an alteration in the rate limiting step could explain why a Compound II was not detected using resonance Raman spectroscopy (1). From the data collected during this study it is difficult to confirm a common mechanism between TDO and hIDO although it appears that this is likely and further study is required.
The heme-containing dioxygenases, in particular hIDO and hTDO, have proven reluctant participants in structural studies (reviewed in Chapter 5) therefore crystallisation trials have been undertaken to find suitable crystallisation conditions. Although crystals of hIDO and hTDO have not been obtained, for the purposes of this study, a couple of conditions have been found that produce micro-crystals that could be carried forward for further investigation. The structure of ferrous XcTDO has already been published in a binary complex with L-Trp (17) and is currently the most revealing structure with regard to substrate binding. Initial attempts to soak ligands into crystals of XcTDO and initiate turnover within a crystal of ferrous XcTDO soaked with L-Trp and oxygen were unsuccessful. The structure of ferric XcTDO in complex with both potassium cyanide and L-Trp was solved. The binding of L-Trp in the presence of potassium cyanide is completely different to the published structure of ferrous XcTDO in complex with L-Trp. The mechanistic implications of this alternate binding mode of L-Trp are presently unknown and further study is required.

In Chapter 6, the activity of ferric hTDO and XcTDO in the absence of a reducing agent has been investigated. The current data points towards the recruitment of hydrogen peroxide from solution to activate ferric heme for oxidation of L-Trp. The physiological implications of this are that TDO is often found within oxidising environments such as hepatic tissue, skin and hair where the protein is prone to deactivation through oxidation of the heme. Ferric TDO could recruit hydrogen peroxide found at low levels in cells to activate the ferric heme for oxidation of L-Trp to form NFK.

### 7.2 Future work

Since TDO has become of interest from a pharmaceutical perspective further investigations into the mechanism of L-Trp oxidation by TDO are required in order to understand if there is a common mechanism with hIDO. Currently within the literature there appears to be an assumption that these enzymes have a common mechanism. Understanding the differences and similarities between IDO and TDO will help to support the rational design of inhibitors.
It is still unclear whether the initial oxygen atom is inserted by radical addition or electrophilic addition. If the mechanism of L-Trp oxidation by both IDO and TDO occurs through radical addition then freeze quench coupled with EPR spectroscopy could be utilised. The reaction could be quenched at varying time points by freezing in an isopentane bath. The frozen samples can then be analysed using EPR for the presence of an L-Trp radical.

It has become increasingly apparent that structural work has the potential to be a powerful tool in order to investigate the mechanism of L-Trp oxidation by heme-containing dioxygenases. The drawback is the difficulty in obtaining crystals of these enzymes. The structures of three catalytic intermediates have been solved in the non-heme dioxygenase literature (18). If the structures of catalytic intermediates could be solved for IDO and TDO this would herald a significant advance in the field of study and support the direction of future studies using different techniques.

Overall the understanding of the mechanism of L-Trp oxidation has moved forward since the initial mechanistic proposal however there is much more to be learnt about these particular enzymes and their potential to form the basis of advances in the field of pharmacology.
7.3 References


Appendix A
Vector – pQE30

Vector - pET151d
Vector – pET28a

Vector – pET21d
Vector - pLeics05
Publications
Substrate Oxidation by Indoleamine 2,3-Dioxygenase
EVIDENCE FOR A COMMON REACTION MECHANISM

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The kynurenine pathway is the major route of l-tryptophan (l-Trp) catabolism in biology, leading ultimately to the formation of NAD+1. The initial and rate-limiting step of the kynurenine pathway is oxidation of l-Trp to N-formylnkynurenine. This is an O2-dependent process and catalyzed by indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase. More than 60 years after these dioxygenase enzymes were first isolated (Kotake, Y., and Masayama, I. (1936) Z. Physiol. Chem. 243, 237–244), the mechanism of the reaction is not established. We examined the mechanism of substrate oxidation for a series of substituted tryptophan analogues by indoleamine 2,3-dioxygenase. We observed formation of a transient intermediate, assigned as a Compound II (ferryl) species, during oxidation of l-Trp, 1-methyl-l-Trp, and a number of other substrate analogues. The data are consistent with a common reaction mechanism for indoleamine 2,3-dioxygenase-catalyzed oxidation of tryptophan and other tryptophan analogues.

Although support for the early mechanistic proposals (5) has recently waned, what happens instead is not known across the family of IDO and tryptophan 2,3-dioxygenase enzymes. Later steps of the mechanism are only partly clarified. Formation of a transient Compound II (ferryl) intermediate is implicated from resonance Raman (8, 9) data, and computational work supports this (10, 11). However, the evidence for ferri heme formation during turnover has so far been limited to IDO (with no evidence as yet for tryptophan 2,3-dioxygenase) and for only one substrate (l-Trp). There is no mechanistic information on the reactivity of IDO with any substrates other than l-Trp and therefore no indication of whether other substrates react by the same mechanism.

These questions lie at the heart of the debate on heme dioxygenase reactivity. It is important in the context of drug discovery programs because IDO has a wide substrate specificity and has attracted considerable interest as a therapeutic target in neurological disease and cancer (12–14), and there is commercial interest in the search for IDO inhibitors (with 1-Me-Trp already in clinical trials). The aim of this work was therefore to examine the mechanism of IDO-catalyzed oxidation across a range of Trp substrates and to establish whether all react using a common mechanism.

Experimental Procedures

Materials—All chemicals used in this work were purchased from Sigma-Aldrich and were of the highest purity (≥99% purity), except for 1-methyl-l-tryptophan (1-Me-l-Trp; 95% purity) which is contaminated with l-Trp and requires further purification by HPLC as noted previously (6). l-Trp, 3,5-dioxygenase (3,5-DIO), 1-Me-l-Trp, indole-3-propionic acid (IPA), l-hydroxy-l-tryptophan (5-OH-l-Trp), l-fluorol-l-tryptophan (5-F-l-Trp), and 5-methoxy-l-tryptophan (5-MeO-Trp) were purchased from Sigma-Aldrich; 5-methyl-l-tryptophan (5-Me-5-Trp) was purchased from Acros Organics; and 3-[3-benzofuran-2-yl]-alanine (3-Trp) was purchased from Sigma-Aldrich.

Preparation of IDO—hIDO was purified as described previously (15, 16), and the protein concentration was determined from the reported absorption coefficient (hIDO εmax = 172 mm−1 cm−1).

Kinetics—Pre-steady state stopped-flow experiments were carried out using an Applied Photophysics SX.18MV stopped-flow spectrometer housed in an anaerobic glove box (Belle Technology Ltd.; [O2] < 5 ppm) and fitted with a Neslab RTE-
Substrate Oxidation by Indoleamine 2,3-Dioxygenase

Scheme 1. Possible mechanisms for tryptophan oxidation in the heme tryptophan dioxygenases. Route 1 shows an electrophilic mechanism. Route 2 shows a radical addition mechanism.

Scheme 2. Structures of the tryptophan analogues used in this study. A, l-Trp; B, 2-Trp; C, 1-Me-l-Trp; D, general structure for the series of 2-substituted tryptophan analogues (R = Me (5-Me-Trp), R = OH (5-OH-l-Trp), R = OMe (5-MeO-Trp), R = F (5-F-l-Trp); E, IPA; F, 5-Trp.

200 circulating water bath (25.0 ± 0.1 °C). In stopped-flow experiments, stated concentrations of protein and reagents relate to final concentrations in the flow (after mixing). Detection of Compound II under turnover conditions (i.e., in the presence of O₂ and substrate) was observed in sequential mixing mode, monitoring absorbance changes at 593 nm that report on the formation and decay of the ferryl species without complication from any other absorbing species. Spectral deconvolution was performed by global analysis and numerical integration methods using Pro-Kinetis software (Applied Photophysics Ltd.). The experiment was initiated by mixing ferrous enzyme (10 μM) generated by stoichiometric titration of ferric enzyme with sodium dithionite) with oxygen-saturated buffer (50 mM Tris-HCl, pH 8.0, [O₂] = 1.2 mM) and then aging the solution for 50 ms to ensure complete formation of Fe(II)-O₂ before a second mix with l-Trp or tryptophan analogues ([Trp] = 10 × Kₘ in cases where Kₘ is known; Table 1). Formation and decay of ferryl heme or NFK was followed at 593 or 321 nm, respectively (except for the case of 5-methoxy-l-tryp isophorone wherein the wavelength maximum for product formation was at 354 nm). In cases where substrate was present in excess, decay of Compound II led to formation of ferrous heme at the end of the experiment except where [substrate] was low in which case decay to ferric heme was observed instead (consistent with the reported increase in reduction potential in the presence of substrate (17, 18)). In other, non-turnover reactions, ferrous hIDO (2.5 μM) was mixed with H₂O₂ (5 eq) in either single mixing mode or with H₂O₂/substrate in sequential mixing mode. Steady-state assays (50 mM Tris-HCl buffer, pH 8.0, 25.0 °C) measuring formation of NFk at 321 nm were performed in solutions containing 20 mM l-ascorbate, 10 μM methylene blue, 100 μg of catalase, and a fixed concentration of enzyme (~100 nM or less) according to published protocols (16).

Mass Spectrometry—For mass spectrometry experiments, formation of product or the intermediate 2,3-epoxide species was carried out in a glove box ([O₂] < 5 ppm) by incubation of ferrous enzyme (0.5–1 μM; generated by stoichiometric titration of ferric enzyme with ≤2 eq of dithionite) with either l-Trp or a tryptophan analogue ([Trp] = 10 × Kₘ in cases where Kₘ is known; Table 1) prior to addition of aerobic solutions ([O₂] = 258 μM) of buffer (50 mM Tris-HCl, pH 8.0) (19). Samples were allowed to react for varying amounts of time (15–60 min) before being centrifuged (13,000 rpm, 3 min), and the supernatant was frozen directly on dry ice. Samples were stored at −80 °C until required for LC-MS analysis.

Results

Detection of a Compound II Intermediate during Steady-state Oxidation of l-Trp by IDO

Identification of the reaction intermediates in IDO has been difficult (see "Discussion"). We designed an anaerobic stopped-flow experiment to cleanly differentiate between ferrous-oxo species and the transient intermediates formed later in the catalytic cycle by forming the ferrous-oxo species in high (>95%) yield prior to addition of substrate to initiate turnover.

Reaction with l-Trp

Under anaerobic stopped-flow conditions, ferrous hIDO was incubated for 50 μs with O₂-saturated buffer, to allow for complete formation of the Fe(II)-O₂ complex, prior to a second mix with l-Trp. The first spectrum (at 4 ms after mixing with l-Trp) is consistent with formation of a ternary Fe(II)-O₂ (l-Trp) complex (λmax = 413, 543, and 577 nm; Fig. 1A), although the α/β ratio (<1) and the Soret band (at 413 nm) are very slightly different from a "pure" sample of ferrous-oxo prepared in the same way but without the second mix (α/β ratio = 1.06, λmax = 416, 543, and 577 nm; Fig. 2A). An overlay of the spectra of the oxy and ternary complexes is shown in Fig. 2B; these alignments of the oxy and ternary spectra allow identification of the intermediate ferryl species below.
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After a lag phase of 0.5 s, NFK formation was observed at 321 nm (Fig. 1A, inset). During NFK formation, an intermediate accumulated (λmax = 416, 547, 577, and 593 nm; Table 1) as evidenced by the appearance of a shoulder in the visible region (absorbance increases at 593 nm; Fig. 1A and inset). This intermediate was assigned as arising from a Compound II species. Formation of a ferryl (Compound II) species has been suggested previously from resonance Raman work (8), and there is agreement on this point (9) (a similar species to that detected in Fig. 1 has been observed previously under similar conditions (9), but its identity was not confirmed). In our experiments, the Compound II species existed during NFK production over 5 s and then decayed when i-Trp was depleted and NFK production ceased. Compound II formation (measured at 593 nm; Fig. 1A, inset) correlates exactly with NFK production and decay (measured at 321 nm), which is clear evidence that Compound II is an intermediate in the mechanism and that its decay is rate-limiting.

To verify the identity of the intermediate species identified in the above reactions, we carried out experiments in which Compound II was prepared directly, under similar anaerobic conditions, but by reaction of ferrous hemin with peroxide (a method previously used for preparation of Compound II in cytochrome c peroxidase (20)). Reaction of ferrous hIDO with H2O2 (5 eq) in the presence of i-Trp (Fig. 3) shows conversion of the ferrous species (λmax = 425, 527, 552, and 558 nm) to a second species that has wavelength maxima (λmax = 413, 547, 579, and 593 nm) that are essentially identical to the species observed under turnover conditions above (Fig. 1).

Detection of a Compound II Intermediate during Turnover of hIDO with Other Substrates

S-Trp—Along with 1-Me-i-Trp (examined below) and O-Trp, S-Trp was originally reported (21) as an inhibitor of IOD. This was rationalized by assuming a base-catalyzed abstraction mechanism (which is not possible with S-Trp, hence the inhibition). But 1-Me-i-Trp is now reclassified as a
TABLE 1
Summary of kinetic and turnover data for hIDO with various substrates from steady-state (kcat and Km) and pre-steady-state (Compound II maxima) experiments
NFR formation was observed (by LC-MS and by increases in absorbance at 321 nm) for all substrates except for S-Trp; ND, not detected.

<table>
<thead>
<tr>
<th>Substrate/analogue</th>
<th>kcat</th>
<th>Km</th>
<th>Compound II λmax</th>
<th>Epoxide</th>
<th>LC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Trp</td>
<td>1.4 ± 0.1†</td>
<td>7.0 ± 0.8†</td>
<td>416, 547, 577, 593†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1-Me-1-Trp</td>
<td>0.27 ± 0.01*</td>
<td>150 ± 11*</td>
<td>413, 543, 577, 593*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-F-Trp</td>
<td>0.76 ± 0.04*</td>
<td>6.0 ± 0.8*</td>
<td>414, 546, 576, 593*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-Me-Trp</td>
<td>3.8 ± 0.2*</td>
<td>98 ± 14*</td>
<td>413, 547, 576, 593*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>n-Trp</td>
<td>3.9 ± 0.1*</td>
<td>1600 ± 100*</td>
<td>413, 546, 578, 593*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-OH-Trp</td>
<td>0.025 ± 0.001†</td>
<td>17 ± 1†</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-MeO-Trp</td>
<td>0.78 ± 0.06</td>
<td>40 ± 10</td>
<td>413, 545, 577, 593*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IPA</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S-Trp</td>
<td>-</td>
<td>-</td>
<td>413, 546, 577, 593*</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Kinetic constants taken from Ref. 28.
† Kinetic constants taken from Ref. 6.
‡ Epoxide formation reported in Ref. 19 and 28.
§ There are no reports of steady-state rate constants for IPA in the literature likely because the increased enzyme and substrate concentration needed to observe turnover lead to higher background absorbances.
¶ Kinetic parameters for S-Trp have not been reported previously and could not be determined from steady-state assays in this work because there are no changes in absorbance at 321 nm with this substrate.

FIGURE 3. Formation of Compound II from reaction with peroxide.
Stopped-flow spectra show the formation of Compound II monitored over 5 s. Ferric HIDO (2.5 μM; solid line) was premixed with H2O2 (5 eq) for 50 ms followed by mixing with 1-Trp (50 μM). The dashed line is Compound II. Absorbance values in the visible region have been multiplied by a factor of 5.

slow substrate (6), and this is so far the main experimental evidence used to rule out the base-catalyzed abstraction mechanism (assuming that all substrates react by a common mechanism). Because of these ambiguities in the assignments of reactivities for IDO substrates, the activity of S-Trp was reassessed. For S-Trp, formation of a ternary [Fe(II)-O2, S-Trp] species was observed (Fig. 4A, solid line) prior to formation of an intermediate species which, by analogy with the data for 1-Trp above, was assigned as Compound II (λmax = 413, 546, 577, and 593 nm; Fig. 4A). These kinetic data provide convincing evidence against base-catalyzed abstraction because formation of Compound II is not possible if the indole NH of the substrate is replaced with a sulfur atom; it is instead consistent with either of the two mechanisms shown in Scheme 1.

We have previously used mass spectrometry to identify products formed during IDO-catalyzed oxidations (19). In separate experiments on S-Trp using mass spectrometry, we observed evidence for formation of the corresponding 2,3-epoxide (m/z = 238), but product formation (m/z = 254) was not detected by mass spectrometry or in steady-state assays (at 32
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nm) probably because the alignment of the lone pairs on sulfur do not favor the ring-opening step leading to product formation (Scheme 1). Identification of an epoxide is consistent with the formation of a ferryl intermediate during the mechanism (Scheme 1).

1-Me-\(\text{i-Trp}\) — In an identical stopped-flow experiment as that carried out for \(\text{i-Trp}\) above, we also observed formation of a ternary [Fe(II)-O\(_2\), 1-Me-\(\text{i-Trp}\)] complex (\(\lambda_{\text{max}} = 413, 543, 577, \text{and } 593 \text{ nm}\); Fig. 1B). After a lag phase of 4 s, NFK formation was observed (Fig. 1B, inset), and an intermediate accumulated (\(\lambda_{\text{max}} = 413, 543, 577, \text{and } 593 \text{ nm}\) as evidenced by the appearance of the shoulder at 593 nm and increases in absorbance at this wavelength (Fig. 1, B and inset) that persisted over 150 s. We assigned this as a Compound II. As in the case of \(\text{i-Trp}\), formation of the Compound II (monitored at 593 nm) is correlated to NFK production (at 321 nm), which confirms a role for Compound II as an intermediate in the oxidation of 1-Me-\(\text{i-Trp}\).

We conclude that oxidation of \(\text{i-Trp}\) and 1-Me-\(\text{i-Trp}\) by ferrous hIDO proceeds via the same intermediate. This is the first evidence that other substrates react via the same oxidative mechanism as that used for \(\text{i-Trp}\) and that a common reaction intermediate (Compound II) is involved.

5-F-Trp, 5-Me-Trp, and \(\text{i-Trp}\) — We extended the scope of our study to examine the reactivity of hIDO with other substrates. To date, there is no mechanistic information available for these substrates. Data for all substrates, including steady-state activities (determined separately), are summarized in Table 1.

Parallel experiments were carried out with 5-F-Trp, 5-Me-Trp, and \(\text{i-Trp}\) (Scheme 2), and these experiments identified the same intermediate as observed for \(\text{i-Trp}\) and 1-Me-\(\text{i-Trp}\) (Table 1). This is the first observation of a Compound II intermediate for these numerous substrates, and the data are consistent with all substrates reacting by the same mechanism.

Probing the Reactivity of Other Substrates by \(\text{IDO}\) — Further information was extracted from reaction of hIDO with three other substrates, 5-OH-\(\text{i-Trp}\), 5-MeO-Trp, and \(\text{IPA}\) (Scheme 2).

5-OH-i-Trp and 5-MeO-Trp — On reaction of ferrous hIDO with \(\text{O}_2\) and then 5-OH-i-Trp, formation of a ternary complex ([Fe(II)-O\(_2\), 5-OH-i-Trp]) was initially observed (\(\lambda_{\text{max}} = 431, 543, \text{and } 577 \text{ nm}\); data not shown). No evidence for formation of a Compound II intermediate was observed, and there were no significant increases in absorbance at 321 nm that could be attributed to NFK formation. In steady-state assays, however, product formation at 321 nm was observed, although 5-OHTrp is a very slow substrate (\(k_{\text{cat}} = 0.025 \text{ s}^{-1}\); Table 1). \(^1\) LC-MS analysis of the steady-state reaction products identified a product (\(m/z = 253\)); however, there was no evidence for the formation of a 2,3-epoxide as has been observed (19) for \(\text{i-Trp}\). If the mechanism of oxidation of 5-OH-i-Trp proceeds by radical addition (Scheme 1, Route 2), then in the case of 5-OH-i-Trp radical formation at C\(^3\) could lead to hydrogen atom abstraction from the 5-OH group and a failure of Compound II to accumulate as in the case of the other substrates. We tested this hypothesis using 5-MeO-Trp in which hydrogen atom abstraction from the 5-OH group is not possible. In contrast to 5-OH-\(\text{i-Trp}\), the 5-MeO-Trp is a good substrate (\(k_{\text{cat}} = 0.8 \text{ s}^{-1}\)). \(^4\) Furthermore, oxidation of 5-MeO-Trp by ferrous hIDO clearly showed formation of a Compound II intermediate (Fig. 4B and Table 1), and mass spectrometry confirmed product formation (\(m/z = 267\) and evidence for a 2,3-epoxide (\(m/z = 251\)). These different characteristics of the 5-OH-\(\text{Trp}\) and 5-MeO-Trp substrates align with the data for \(\text{i-Trp}\) and 1-Me-\(\text{i-Trp}\) as above and are consistent with a radical mechanism being used (presumably for all substrates; Scheme 1).

Indole-3-propionic Acid — Oxidation ofIPA has never been reported for a heme dioxygenase, but our data support oxidation of this substrate. On reaction of ferrous hIDO with \(\text{O}_2\) and IPA, a ternary [Fe(II)-O\(_2\), IPA] species forms normally (\(\lambda_{\text{max}} = 415, 543, \text{and } 577 \text{ nm}\); data not shown) after which there is a long lag phase of 50 s during which no product formation occurred (at 321 nm). An intermediate was observed (data not shown), and its decay coincided with product formation (the latter was confirmed by LC-MS, which detected \(m/z = 222\) as well as \(m/z = 206\) for the corresponding epoxide). We interpret this to mean that the rate-limiting steps are different from the other substrates examined above so that Compound II does not accumulate, but product formation is still possible. The ammonium ion is presumed (23) to facilitate formation of a ferric superoxide complex (Scheme 1) through hydrogen bonding to the bound oxygen in the ternary complex. We interpret the altered kinetics for IPA (very long lag phase) as being consistent with a role for the ammonium group in stabilizing the ferric superoxide complex (via the radical pathway).

Discussion

The mechanism of tryptophan oxidation by the heme-containing dioxygenases is a subject of topical recent debate. A base-catalyzed proton abstraction mechanism (5) was widely reproduced in the literature despite the fact that there was barely any experimental evidence for it. Aside from the fact that the aromatic chemistry of indoles dictates that they cannot react in this way (24), the base-catalyzed mechanism is additionally problematic because there is no change in oxidation state of the heme iron during catalysis, which is out of line with all other O\(_2\)-dependent heme enzymes (e.g. P450s and NO synthases) that are known to use ferryl intermediates (Compound I).

There is now general agreement that base-catalyzed proton abstraction does not occur (3, 8–11, 23, 25, 26), although the experimental evidence against it is still rather limited. Our data for S-Trp also provide convincing evidence against base-catalyzed abstraction because we observed Compound II formation (Fig. 4A), and this is not possible if (as in the case of S-Trp) there is no proton on the indole NH that can be abstracted.

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3 Although Compound II does not accumulate during oxidation of 5-OH-i-Trp by hIDO, formation of this intermediate (\(\lambda_{\text{max}} = 413, 543, 577, \text{and } 593 \text{ nm}\)) was observed on reaction of ferrous hIDO (2.3 \(\mu\)M) with 5 eq of \(\text{H}_2\text{O}_2\) and 5-OH-i-Trp (50 \(\mu\)M).

4 It is probably the case that these relative rates of activity of 5-OH-i-Trp and 5-MeO-Trp apply for other heme dioxygenases, although there are few reports. For example, in the case of IDO2 (22), there is no reported oxidation of 5-OH-Trp, but oxidation of 5-MeO-Trp (\(k_{\text{cat}} = 0.161 \text{ s}^{-1}\)) is similar to \(\text{i-Trp}\) (\(k_{\text{cat}} = 0.103 \text{ s}^{-1}\)).
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of an incompletely formed (impure) ferrous-ox-y species from that of a ternary complex or an intermediate Compound II species is not straightforward.

Our kinetic experiments allow us to differentiate all three species. We demonstrate clean formation of ferrous-ox-y IDO (λ\text{max} = 416, 543, and 577 nm) under non-turnover conditions (Fig. 2A); these spectra are in exact agreement with previously reported spectra for ferrous-ox-y IDO prepared using different conditions (27). We show that the spectrum of this ferrous-ox-y species is subtly different from that of the ternary [Fe(II)-O\text{y} Trp] complex (λ\text{max} = 413, 543, and 577 nm) isolated during turnover conditions (Fig. 2B); we assign these minor differences as being due to binding of l-Trp to the Fe(II)-O\text{y} species. Furthermore, during IDO-catalyzed oxidation of l-Trp, we identified a Compound II intermediate (λ\text{max} = 416, 547, 577, and 593 nm) (Fig. 1A) that is spectroscopically distinct from either the ferrous-ox-y species or the ternary [Fe-O\text{y}-Trp] complex.

This same Compound II intermediate was also observed during turnover of l-Me-Trp, S-Trp, and S-MeO-Trp by ferrous hIdO (Figs. 1, A and B, and 4, A and B). Fig. 5A demonstrates the consistency of these Compound II spectra identified in each of these turnover experiments with the four different substrates with the feature at 593 nm present in all cases. The spectra of these Compound II species observed under turnover co\text{nditions are similar to those observed for a "genuine" ferro-enzyme species formed in IDO directly but under different conditions (by reaction of ferrous enzyme with peroxide) (Fig. 5B) and are subtly different from spectra of the ternary complexes observed in the same turnover experiments (as shown in Fig. 5C).

In summary, we detected a transient intermediate, assigned as a Compound II, in the reactivity of IDO with a number of different substrates. The evidence for the range of compounds examined herein strongly suggests that Compound II is the intermediate for all IDO-catalyzed reactions and that oxidation of all substrates by IDO occurs by a common mechanism. IDO has a very wide substrate specificity, much wider than that of tryptophan 2,3-dioxygenase, so this information will provide a basis for development of heme dioxygenases as therapeutic targets as an understanding of mechanism underpins structure-based inhibitor design.

Author Contributions—E. L. R. and S. H. conceived and coordinated the work. E. S. B. and J. B. collected the kinetic data. E. S. B. and J. B. analyzed the kinetic data with assistance from E. L. R. and S. H. M. L. was responsible for collecting and analyzing the mass spectrometry data. E. L. R. coordinated writing of the paper with assistance from all authors.

References
Substrate Oxidation by Indoleamine 2,3-Dioxygenase


Heme-containing dioxygenases involved in tryptophan oxidation
Elizabeth S Millett¹, Igor Efimov¹, Jaswir Basran², Sandeep Handa¹, Christopher G Mowat³ and Emma Lloyd Raven¹

Heme iron is often used in biology for activation of oxygen. The mechanisms of oxygen activation by heme-containing mono-oxygenases (the cytochrome P450s) are well known, and involve formation of a Compound I species, but information on the heme-containing dioxygenase enzymes involved in tryptophan oxidation lags far behind. In this review, we gather together information emerging recently from structural, mechanistic, spectroscopic, and computational approaches on the heme dioxygenase enzymes involved in tryptophan oxidation. We explore the subtleties that differentiate various heme enzymes from each other, and use this to piece together a developing picture for oxygen activation in this particular class of heme-containing dioxygenases.

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Introduction
The heme-containing dioxygenases involved in tryptophan oxidation have a somewhat unusual provenance. The activity of these enzymes was first noted in extracts of liver in the 1950s [1], but it was not until the 1990s that the enzymes were first purified [2-5] and that oxygen was identified as the source of oxygen atoms incorporated into the substrate [4]. They have attracted attention on an irregular basis ever since because the literature shows considerable activity, for example during the later 1970s and into the 1990s, and periods of relative obscurity. They are currently in vogue again. The ease and simplicity of recombinant expression systems is undoubtedly one of reasons for that, and the more recent realization that secondary metabolites of tryptophan are implicated in a range of disease states has provided new motivations in a therapeutic context (see for example [6,7]). But the widely cited review by Sono and Dawson [8] may well have been another reason, as buried within that magisterial work was a short but comprehensive review of the heme-containing dioxygenase literature to 1996, and between the lines a glimpse of questions as yet unresolved.

Nomenclature
The broader family of heme-containing dioxygenases includes the enzymes prostaglandin H synthase, illoleucine diol synthase, and the plant fatty acid α-dioxygenases, as well as the enzymes involved in tryptophan oxidation. The dioxygenase activity being considered in this review is the oxidation of 1,2-dihydroxyanthracene (1,2-phenanthrene-β-ketone) to 1,2-formylanthracene (NFK, Scheme 1a), which is the first and rate-limiting step in tryptophan catabolism. There are two kinds of heme-containing dioxygenase involved in tryptophan oxidation, both catalyzing the same reaction. The early literature is most confusing at first glance because different enzymes are inconsistently named, with ‘tryptophan pyrolylase’ [1,5,9], ‘tryptophan oxygenase’ (as noted in reference [8]), and even ‘tryptophan peroxidase-oxidase’ [2,5] all appearing in print. Only later on did the nomenclature converge on either tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO).

The monomeric IDOs have a much wider substrate specificity that can accommodate various substituted indoles (hence their name). The TDOs are more specific for 1,2-tryptophan; they are typically tetrameric, although recent spectroscopic work [10] indicates that the heme sites are not equivalent. As yet, there are no functional implications associated with the tetrameric versus monomeric structure.

Structural work
A landmark contribution was the publication of the structure for human IDO [11**]. The active site is shown in Figure 1. When the structure appeared, it was dichotomous in the sense that it answered a number of fundamental questions, but raised several others at the same time. For instance, there is a proximal histidine ligated to the heme group – which was in agreement with most of the early spectroscopic data (reviewed in [8]) and with recent mutagenesis work [12] – and a predominantly hydrophobic distal pocket, which was consistent with the substrate binding requirements (1-1'rp being similarly hydrophobic). But most unexpectedly, the active site contained no distal histidine, which had long been
predicted from both spectroscopic work (reviewed in [8]) and from sequence alignments with a group of related globins [13]. For IDO at least, the idea of a distal histidine looks now to have been a red herring, one of a number that have populated the literature (the suggestion that copper was essential for activity was another, reviewed in [14]).

A further advance was made when the structure of a bacterial (Xanthomonas campestris) TDO was published [15], with the Trp substrate bound at the active site. In this enzyme, there is a histidine in the distal pocket, Figure 1b, and it plays a role in substrate binding as there is a hydrogen bond from His55 to the substrate. We discuss this further below. Like IDO, the active site of xTDO is hydrophobic, but the structure nicely shows the few ionic interactions that are needed to tie the substrate to the active site.

Additional structural information has been very slow to arrive, and is a particularly challenging aspect of the field. There is a crystal structure of the Ralstonia metallidurans TDO enzyme [16], but other related enzymes have not yet yielded structural information.

**Heme reduction potentials and substrate binding**

The redox properties of the heme group are a key factor in determining reactivity but the early reports on redox data are a little misleading (we have briefly addressed this previously [17]).

Turning to more recent data, reported reduction potentials for human IDO (−63 mV [18]), human TDO (−92 mV [17]), and X. campestris TDO (+8 mV [15]) vary quite considerably (the value for human IDO was adjusted slightly from a preliminary report of −30 ± 4 mV [20]). With the exception of xTDO, these reduction potentials are rather low for an obligate O2-binding protein: lower than those typically found in the globins (+50 mV for myoglobin [21] and +150 mV for hemoglobin [22], but there are exceptions in the globin family where potentials are lower [23–25]) and closer to what one might expect for the peroxidase enzymes (usually ≈−200 mV, reviewed in [26]). It is not yet known how the positioning of the reduction potential affects tryptophan oxidation, but it is not unknown for a family of related proteins to exhibit a range of potentials. It is worth remembering that IDO and TDO operate through the ferrous form by binding O2: this most probably occurs with at least partial oxidation to ferric superoxide (see below), which would require re-reduction to the resting

![Scheme 1](image-url)
ferrous state upon completion of catalytic cycle. Consequently, one might imagine that the heme reduction potential should neither be too high, which would otherwise slow down the conversion to ferric superoxide upon O₂ binding, nor too low, which would slow down reduction back to ferrous. A somewhat intermediate positioning of the potential, between globins and peroxidases, may sensibly satisfy both requirements.

Some enzymes heavily discriminate against substrate binding to the ferric enzyme and this is reflected in the reduction potentials. Human IDO is an example: binding to the ferrous enzyme is more favorable than to the ferric enzyme, and the reduction potential correspondingly increases upon binding of substrate [20]. xTDO behaves similarly [15**,19], and so does the *Pseudomonas* enzyme studied in the 1980s [27]; but human TDO does not discriminate against substrate binding to the ferric enzyme in this way and the reduction potentials for the substrate-free and substrate-bound proteins are correspondingly similar [17]. It is not yet known whether these differences have functional or catalytic implications. One suggestion for hTDO [17] is that it might be linked to the reported activity of the ferric enzyme.

The source of the reducing equivalents

The mechanism of oxygen activation inevitably involves binding of O₂ to ferrous heme, and reduction of the heme iron is thus required. So far as has been established so far, only a single, initiating reduction of the heme is required. This distinguishes IDO and TDO from other catalytic heme enzymes in which continuous re-reduction of the heme (from Compound I) is required for turnover. However, the dioxygenases are more highly prone to autoxidation of the ferrous-oxy species than their globin counterparts, which may mean that further reduction in *vivo* is required. The physiological reductase is not yet fully clarified. The best information is for IDO, where it has been suggested that a cytochrome b₅ [28,29*] fulfills this role in *vivo*, which keeps the heme reduced in the same way as in the hemoglobins. Alongside cytochrome b₅, cytochrome b₃ reductase might also be involved in keeping IDO reduced [29*]. *In vitro*, most of the recent work [11**,15**,17,18,20,30,31*,32] uses a methylene blue/ascorbate cocktail to provide the reducing equivalents (a method that is based on an original enzyme assay [9,33,34]) although other work for TDO has used ascorbate only [16*35–38] or even a combination of NADPH–cytochrome P450 reductase/cytochrome b₅ [39].

It is interesting that NADH has also been recently shown to reduce ferric IDO directly [40**, through a mechanism involving ferrous-oxy formation. This could have physiological implications, since ferric human TDO (but not IDO) is known to be active [17,36], but the mechanism of the reaction is not yet known.

The mechanism of NFK formation

Proposals for the reaction mechanism appeared in the very early literature [41]. The suggestions were not based on experimental data, but this did not prevent them from being widely reproduced. Even now, the mechanism of the reaction is not definitively established, and much work needs to be done before it is.

The first step of the mechanism

The early literature converged on a base-catalyzed proton abstraction mechanism [8], Scheme 2a, and there were several good reasons for doing so. The supposed presence of an active site base (histidine), as described above, was one of them. Another, perhaps more compelling, was the assignment [42] of Me-·Trp (which cannot

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**Scheme 2**

(a) The previously proposed [8,41] mechanism for dioxygenase catalysis, showing a base-catalyzed abstraction mechanism followed by either Orkogee (blue) or directo (red) rearrangements. (b) A more recent proposal [11**,15**,31*], which does not involve an active site base.

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undergo base-catalyzed abstraction, Scheme 1) as an enzyme inhibitor. But the absence of an appropriately located histidine residue in human IDO \([11^{**}]\) has cast a long shadow on the plausibility of this mechanism.

An alternative mechanism therefore was discussed \([11^{**},15^{**},31^{*}]\), Scheme 2b, which did not depend on an obligate active site base. In fact, it has been pointed out \([45^{**}]\) that indoles do not typically react by loss of the indole proton \([43]\), and the \(pK_a\) of the indole proton is very high \((\approx 17)\) \([44]\), which makes both mechanisms (Scheme 2a and b) problematic \([45^{**}]\). Computational work has made a real contribution here: it was Chung and co-workers \([46^{**}]\) who first noted, based on DFT calculations, that base-catalyzed deprotonation of the indole proton may not be energetically feasible (a conclusion later confirmed in other computational work \([47,48^{**}]\)).

An additional difficulty with the base-catalyzed abstraction idea is that it has been demonstrated \([45^{**}]\) that Me-Trp is actually a slow substrate, which has been interpreted \([45^{**}]\) to mean that if Me-Trp reacts by the same mechanism as L-Trp then neither Scheme 2a nor Scheme 2b are possible. ENDOR data \([49]\) support this, because they show that the indole proton is unlikely to be hydrogen bonded to \(O_2\) in the ternary [enzyme-\(O_2\)-Trp] complex, arguing against the proposal suggested in Scheme 2b.

The involvement of an active site base, or not, has yet to be fully resolved, but the pendulum of experimental \([45^{**}]\) and computational \([46^{**},47,48^{**},52]\) evidence is currently swinging away from the idea. Scheme 3 shows alternative possibilities, as discussed below.

The involvement of ferroly heme

After formation of the ternary complex, the literature \([8]\) describes either a Criegee rearrangement or a dioxygen intermediate, Scheme 2a. The suggestions originate from proposals put forward in 1969 \([41]\) but experimental evidence for either mechanism has never been published.

The proposed mechanism, Scheme 2a, dictates that both oxygen atoms are incorporated into the substrate in a concerted fashion, but in a further twist it has now been suggested that this may too be incorrect. In a thoroughly intriguing paper \([48^{**}]\), Yeh and co-workers have used resonance Raman to observe transient formation of ferroly heme (FeO\(_{2}^{\Delta}\)) assigned as a Compound II species, during the reaction of human IDO with \(O_2\), an observation that was later confirmed \([30,51]\). This was interpreted to mean \([48^{**}]\) that the O-O bond is cleaved (activated) in a separate step and that oxygen atom insertion occurs sequentially. Computational data \([48^{**}]\) have again proved helpful and seem to support these conclusions. Curiously, ferroly heme was not observed for human TDO \([48^{**}]\), and its formation under turnover conditions has yet to be confirmed in any other enzyme.

The last step of the mechanism

Formation of an epoxide has been suggested \([48^{**}]\) to be concomitant with formation of ferroly heme, but the evidence for it, so far, is limited. Two independent computational studies have been published that examine

![Scheme 3](image_url)
epoxide formation [46,52], and there is indirect evidence for its formation from mass spectrometry [53]. If epoxide formation occurs, it has yet to be established how the final conversion to NFK is achieved. It has been pointed out [53] that epoxides can undergo facile ring opening, through cleavage of the C2-O bond [54], and this has been proposed as an initiating step in the final conversion to NFK [52,53]. A suggested heterolytic mechanism, which also includes most of the recent suggestions on the first step of the mechanism, is shown in Scheme 3. Mechanistic similarities have been noted [53,55] with the O2-dependent heme enzyme PmB, the second of a group of four enzymes involved in pyrroline synthase synthesis, which binds its substrate in a similar way [56]. Figure 1c. In this case, a similar abreast of 7-Chl-Trp has been proposed [56] (although in PmB, oxygen atom insertion into the substrate does not occur, Scheme 1b).

Peroxide reactivity

IDO and TDO also react with peroxide; this is not altogether unexpected as many heme proteins do that, to give Compound I as the initial product. This was first noted in quite early work [2]. In the P450s, peroxide can substitute for O2 (the peroxide shut mechanism, through Compound I [57]). But peroxide is an awkward substrate: even the specialist heme peroxidases have difficulty with fairly modest concentrations of peroxide in the absence of a reducing substrate, so that enzyme inactivation by complex mechanisms (after initial Compound I formation) can rapidly occur. A secondary consideration is that the peroxide reaction leads to non-specific radical formation on the protein (and at more than one site). This can occur particularly for proteins that are not designed to work with peroxide, as painstaking experiments on myoglobin have proved (see for instance [58]), but is even also possible in the peroxidases that otherwise react quite cleanly to give Compound I (see for example [59]).

In IDO and TDO, the reactivity with peroxide is as yet not completely clarified. In the absence of Trp, the reaction of ferric heme with peroxide has recently been examined and assigned as Compound II in IDO [60] and as a Compound ES (i.e., as cytochrome c peroxidase Compound I [61], with a ferryl heme with a protein radical) in TDO [62**]. The nature and reactivity of this species has yet to be fully examined. Liu and co-workers have noted the possible complications from heme degradation on reaction with peroxide [62**], but have convincingly shown [62**) that reaction of ferric TDO with peroxide in the presence of Trp leads to formation of the catalytic ferrous form (through reduction of the protein by Trp), in a process that they refer to as 'reactivation'. It has been suggested that an alternative route for generation of ferrous heme might have physiological implications for TDO [62**] if the pool of reduced, ferrous heme is otherwise depleted (for example in an oxidizing biological environment).

Relationship to other oxidative heme enzymes

Oxygen activation in heme enzymes typically occurs via formation of an initial Compound I intermediate (e.g., in the P450s, NO synthases, and peroxidases), and it is instructive to continue to bear these comparisons in mind when considering the reactivity of the heme-containing dioxygenase group. Compound I formation is something that IDO and TDO are apparently capable of [60,62**] but may wish to avoid under turnover conditions because, so far as we know, there is no sacrificial reductase to supply the electrons needed to re-reduce Compound I. It has been suggested [49] that limiting the supply of protons might offer one level of regulatory control, by disfavoring Compound I formation during turnover conditions [55]. For the enzymes involved in tryptophan oxidation, the responsibility for the oxygen activation process instead lies within the realm of the ferrous-oxo (or ferric superoxide [48**]) derivative. But if Compound I does form (perhaps in the presence of trace amounts of peroxide), the reactivation mechanism suggested recently [62**], in which ferrous heme is recovered by reaction with substrate, might be an 'escape route' through which the enzyme can recover itself in extremis.

Outlook

The potted history of IDO and TDO is full of discrepancies and contradictions, and more are surely yet to come. They have floated in and out of fashion over the last decades, and in some senses (not least, mechanically) have been peripheral to the broader heme protein family. But while they catalyze a reaction that is unique in heme chemistry, recent work has shown that the underlying mechanisms may not be so very different from those used in other well-known catalytic heme enzymes and that subtle discrimination of reactivity is used to differentiate their behavior from other catalytic heme enzymes.

In 2012, as a result of some intensive experimental scrutiny over recent years, IDO and TDO find themselves more closely aligned with the heme protein family than ever they once were. And yet, they continue to raise questions that are relevant to the whole heme family. Compound I may not, after all, have a complete monopoly on oxygen activation.

Acknowledgements

ER thanks Grant Mask for clarification on early literature and nomenclature, and Alimin Liu, Syun-Ru Yeh, and Oscar Chung for helpful discussions on their work during preparation of this article.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


16. Following on from the original Sugimoto paper [11], the crystal structure of a bacterial TDO in complex with α-tryptophan, showing the nature of the substrate-binding interactions.


18. The third of the three crystal structures so far published for IDO or TDO.


32. The first paper to address the possible physiological reductant for IDO. It suggests potential roles for cytochrome b5, and cytochrome b5 reducse b5 as a reductant of IDO (see also [26,38]).


35. One of the first detailed studies to appear on recombinant human IDO.


This paper shows that NAD oxidizes NADH under aerobic conditions in the absence of other enzymes or reagents, a previously unreported observation that could be very useful in terms of assay and formation of the ferrous-oxo species of IDO at ambient temperature.


42. Cadar SG, Sonc M. γ-Methyl-α-tryptophan, beta-[3- benzofuranoyl]-α-alamine (the oxygen analog of tryptophan), and beta-[3-benzoyl]-benzyl]-α-alamine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. Arch Biochem Biophys 1981; 211:326-333.


44. Yagi G. The proton dissociation constant of pyrrole, indole and related compounds. Totalhishon 1961; 25:2835-2861.


This paper presented evidence, from resonance Raman data, for a ferriyl intermediate (compound b) in hIDO, thus undermining long-established proposals for the mechanism of oxygen insertion. On the basis of this evidence, it was proposed that oxygen atom insertion occurs in a step-wise fashion, not concurrently as previously assumed [8]. Yang et al. later published similar observations using the same methodology [90,29].


This recent review of IDO and TDO.


The structure of this related PmB enzyme provides a useful framework for comparison with the dioxygenases.


Detection of a Compound ES in TDO and resolution of a long-standing question of enzyme reactivation in dioxygenases, showing that ferrous heme is formed on reaction of ferric TDO with peroxide and Trim.
How is the distal pocket of a heme protein optimized for binding of tryptophan?

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Keywords
dioxygenase; heme; iron; N-formylkynurenine; tryptophan

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Introduction

The first and rate-limiting step in the kynurenine pathway is the O2-dependent oxidation of L-tryptophan to N-formylkynurenine (Scheme 1). The enzymes that catalyze this reaction are referred to in the literature as ‘tryptophan pyrrolase’ [1–3] (and even as ‘tryptophan peroxidase-oxidase’) [4]; only later did the nomenclature evolve more systematically to describe either one of the two types of enzyme that catalyze this same reaction: tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO). Although both

\[
\begin{align*}
\text{Tryptophan} & \xrightarrow{O_2} \text{N-formylkynurenine} \\
\text{TDO/IDO} & \quad \text{Scheme 1. The reaction catalyzed by indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase. In 1-Me-Trp, the proton on N1 is replaced with a Me group; in tryptamine, the carboxylate group of the side chain is replaced with a proton.}
\end{align*}
\]

Abbreviations
hIDO, human indoleamine 2,3-dioxygenase; hTDO, human tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase; rTDO, \textit{Ralstonia metallidurans} TDO; TDO, tryptophan 2,3-dioxygenase; xTDO, Xanthomonas campestris TDO.
enzymes catalyze the same reaction, there are a number of differences between them: TDO is typically tetrameric and, in mammals, it is located primarily in the liver, whereas IDO is monomeric and located ubiquitously around the body, apart from in the liver. The substrate specificities are also different, as reflected in the nomenclature, with the IDOs having generally a broader substrate specificity for numerous indole-derived substrates compared to the more substratespecific TDOs.

A considerable volume of spectroscopic and functional studies was carried out on both IDO and TDO from the 1970s to the 1990s [5], after which time interest in these enzymes waned. The more recent realization that tryptophan oxidation is implicated in a wide range of disease states means that IDO and TDO find themselves once again in the limelight, and they are currently the subject of a great deal of interest [6,7]. An important breakthrough was the structure for recombinant human IDO (hIDO) [8], which was crystallized with the inhibitor 4-phenylazozole bound to the heme iron. The active site structure not only confirmed some of the earlier predictions from spectroscopic work, but also contained some unexpected revelations. Predictably perhaps, considering the nature of the substrate, the IDO active site contains a large number of hydrophobic aromatic residues, including in the distal pocket (Fig. 1A). Most unexpectedly, no histidine residue was found in the distal pocket, which was in direct contradiction to the predictions obtained from low-temperature spectroscopic work [9-11]. Indeed, the entire IDO active site is almost completely devoid of polar residues: Ser167 is the only polar residue (Fig. 1A), although two mutagenesis studies suggest that this is not essential for catalysis [8,12]. The later crystal structure of a bacterial TDO from Xanthomonas campestris (xTDO) [13] to a large extent not only confirmed the generally hydrophobic nature of the dioxigenase active site (Fig. 1B), but also included information on the interactions that tie the substrate to the enzyme in the substrate-bound complex.

A structure has also been published for Ralstonia metallidurans TDO (xTDO) [14], which has an active site structure similar to that of the hIDO and xTDO enzymes (Fig. 1C). There is as yet no full analysis of the individual contributions of various active site residues to the overall process of tryptophan oxidation. This is in contrast to other heme proteins (e.g. the globins and the peroxidases) [15,16], for which very extensive studies have been carried out with the aim of dissecting the contributions of individual residues. In this sense, although all of the IDO and TDO enzymes that have been studied so far have active sites that are broadly recognizable as heme enzymes, in some ways are structurally analogous to other heme proteins (such as the globins and the heme peroxidases), we still do not understand how the active site pocket is optimized for its specialized role in the oxidation of exogenous tryptophan bound in the active site (a reaction that, so far as is known, lies only within the gift of IDO and TDO). To begin to address this, we have examined the functional consequences of individual mutations in two human dioxigenases: hIDO and human tryptophan 2,3-dioxygenase (xTDO). The results obtained reveal some of the subtle influences that are imposed upon the molecule by different amino acids and, by comparison with known bacterial enzymes, we use the information to build a more comprehensive picture of the individual roles of key residues on the catalytic process.

Results

Comparison of active sites

The R. metallidurans active site structure [14] is similar to that of hIDO [8] and xTDO [13] (Fig. 1). Because the active site structures of these enzymes appear to be similar, mutational analysis of hIDO
and hTDO, as reported in the present study, is presumed to be representative of the family as a whole. Active site residues targeted for mutagenesis in hIDO, based on the published structure, are shown in Table 1. For hTDO, no crystal structure is available. Hence, a model of hTDO, created using hidden Markov modelling and sequence homology, was calculated using the mfold server [17] and the template structures were found to correspond to Protein Data Bank entries 2NOX (rTDO), 2NW8 (xTDO) and 2DOT (hIDO) (which are the same structures shown in Fig. 1). Superposition of these published structures and the model for hTDO suggest the spatial correspondence of the active site amino acid residues, and provide a rationalization for the mutations in hTDO shown in Table 1.

### Ligand binding

Wavelength maxima for the various ferric and ferrous derivatives of the hIDO and hTDO variants are presented in Table 1. These are broadly similar to those for the corresponding wild-type proteins and are not discussed in further detail.

Equilibrium binding constants for binding L-Trp ($K_{D,Trp}$) are presented in Table 2. For comparison, binding constants for binding of cyanide ($K_{D,CN}$) are also presented and do not deviate markedly from wild-type values.

### hIDO

For the hIDO variants, most values of $K_{D,Trp}$ for the ferric protein were broadly similar to those for the wild-type, and the most substantial changes are confined to the F226A and R231K variants ($K_{D,Trp}$ increased by approximately 10-fold), which suggests a role for both of these residues in L-Trp binding. This is explored further in the steady-state analyses below.

### hTDO

These effects for hIDO are largely duplicated in hTDO because none of the mutations had large effects on either L-Trp or cyanide binding (Table 2), with the exception of the R144A mutation, which resulted in an approximately 10-fold increase in $K_{D,Trp}$.
Table 3. Steady-state kinetic data for oxidation of L-Trp by hIDO, hTDO and variants (50 mM Tris-HCl, pH 8.0, 25.0 °C).

<table>
<thead>
<tr>
<th></th>
<th>kcat (s⁻¹)</th>
<th>KM (μM)</th>
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<th>kcat (s⁻¹)</th>
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<td>1.4 ± 0.01</td>
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<tr>
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<td>H76A</td>
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<td>S167H</td>
<td>0.00006 ± 3 × 10⁻⁸</td>
<td>26 ± 1</td>
<td>H76S</td>
<td>0.10 ± 0.01</td>
<td>600 ± 70</td>
</tr>
<tr>
<td>F226A</td>
<td>0.39 ± 0.01</td>
<td>640 ± 70</td>
<td>F140A</td>
<td>0.604 ± 0.001</td>
<td>520 ± 60</td>
</tr>
<tr>
<td>F226Y</td>
<td>0.6 ± 0.2</td>
<td>26 ± 2</td>
<td>F140Y</td>
<td>0.60 ± 0.02</td>
<td>460 ± 40</td>
</tr>
<tr>
<td>F163A</td>
<td>0.03 ± 0.001</td>
<td>47 ± 5</td>
<td>F72A</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>R231K</td>
<td>0.31 ± 0.01</td>
<td>3300 ± 400</td>
<td>R144K</td>
<td>0.16 ± 0.01</td>
<td>830 ± 60</td>
</tr>
<tr>
<td>F164A</td>
<td>0.74 ± 0.01</td>
<td>210 ± 10</td>
<td>F144A</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>F227A</td>
<td>0.43 ± 0.01</td>
<td>50 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From Chauhan et al. (12). † Measureable activity was not observed under the conditions measured. The F22A variant was particularly unstable and denatured under normal steady-state assay conditions (pH 8.0); at pH 9, increases at KM could be observed, although reproducibility at each L-Trp concentration was very poor and a full steady-state profile could not be extracted. Instead, we measured Vmax at a single concentration of substrate (2 mM) to extract an apparent kcat for both F22A and hIDO at pH 9.0 and found that the F22A variant had < 3% of the activity of hTDO (apparent KMhIDO = 1.9 s⁻¹; apparent KMhTDO = 0.06 s⁻¹), which is comparable to the corresponding F163A variant in hIDO (2% of wild-type activity).

Fig. 2. Steady-state oxidation of L-Trp by (A) hIDO and (B) the F163A variant of hIDO. Solid lines show a fit of the data to the Michaelis-Menten equation conditions: 50 mM Tris-HCl, pH 8.0, 25.0 °C.

Steady-state oxidation of L-Trp

Steady-state parameters for L-Trp oxidation are reported in Table 3; representative data sets are shown in Fig. 2 for hIDO and the F163A variant.

hIDO

For hIDO, the effects on L-Trp binding in the ferrous enzyme (K_D,Trp, as above) are also seen in the steady-state (which reports on L-Trp binding to the ferrous enzyme), although the effects are more pronounced because the F226A variant (an approximately 130-fold increase in KM) and the R231K variant (an approximately 470-fold increase) show severely decreased substrate binding affinities (note, however, that the F226Y variant retains its ability to bind substrate; Tables 2 and 3). More modest effects on substrate binding are observed for the F163A and F164A variants (approximately 10-fold and 30-fold increases in KM, respectively). Regardless of the effects on KM, values of kcat for most variants are largely unchanged compared to the wild-type protein, with the exception of the F163A variant where kcat is considerably reduced (approximately 30-fold slower). Overall, we interpret these effects as indicating that, although binding affinity is lowered in some cases, the rate-limiting steady-state turnover (i.e. the conversion of Trp to N-formylkynurenine) is less seriously affected by the mutations and remains close to wild-type levels in most cases.

hTDO

These observations for hIDO are broadly mirrored in hTDO, although the effects are less pronounced. By comparison to hIDO, both the F140A (assumed to be equivalent to F226A in hIDO) and the R144K (assumed to be equivalent to R231K) variants show...
simply but more marginally (approximately five-fold) weakened affinity for L-Trp binding: the F72A (equivalent to F163 in hIDO) and R144A variants were found to be completely inactive and the data for R144A suggest that this may arise from a weakened affinity of the ferrous enzyme for substrate under steady-state conditions (we were unable to measure a $K_M$ for the F72A variant). The H76A variant (equivalent to S167 in hIDO and H55 in xTDO) has activity that is essentially unchanged from the wild-type enzyme (Table 2). The H76S variant shows a more marked effect on $k_{cat}$ (an approximately 10-fold decrease) but neither variant shows a substantial effect on $K_M$. We interpret this as indicating that His76 does not play an essential role. This is discussed in more detail below.

Redox potentiometry

We have noted previously [9] that reduction potentials in the presence and absence of substrate are correlated with the binding affinity of the ferric and ferrous forms, which means that the measured reduction potentials report directly on the relative binding affinity of the substrate. In hTDO, the reduction potential does not shift upwards on the binding of substrate [18], which differentiates it from both hIDO and xTDO (both of which show upwards shifts in potential on binding of L-Trp) [9,13]. The reduction potential data for the hTDO variants are in accordance with these conclusions because the measured Fe$^{2+}$/Fe$^{3+}$ reduction potentials for the hTDO variants in the presence and absence of substrate (Table 4; with a representative data set shown in Fig. 3) shows only minor increases in reduction potential (with the exception of H76A which shows a larger difference). We have interpreted this information [18] as indicating that hIDO needs to specifically favour binding to the reduced form, whereas hTDO does not discriminate in this way, possibly reflecting a requirement imposed upon TDO in the cellular environment (oxidizing versus reduced). Indeed, rather than exhibiting preferential binding of substrate to the reduced enzyme (as does hIDO), the binding of L-Trp to hTDO is typically much weaker (Table 2) and was previously suggested [19] to account for the fact that substrate inhibition is not observed in hTDO. The reduction potential data for the variants support these suggestions.

Discussion

In cases where structural information is available for IDO or TDO enzymes (Fig. 1), the active sites reveal a handful of residues that can potentially regulate substrate binding and catalysis. The data available in the literature on the effects of active site mutations on tryptophan dioxygenase activity are neither extensive nor conclusive. The first study [20] looked at the role of His346, which is the proximal ligand in hIDO. Along with their landmark structure, Sugimoto et al. [8] provided very preliminary activity data for variants at a number of positions (C129A, F163A, S167A, F226A, F227A, R231A and S263A), although only $K_D$ values for substrate binding were reported. Two studies have looked at the role of the distal histidine [21,22], although they differ in their conclusions. Additionally, the roles of F68, R134 and Y130 (Fig. 1c) have been examined briefly in rTDO [14], and the single T342A variant in hTDO has also been studied [23]. However, taken together, these investigations have not yet led to a clear view on the role of individual residues in the active site.

In the present study, we examined the likely roles of these active site residues in hIDO and hTDO and the main effects observed are summarized below.

<table>
<thead>
<tr>
<th></th>
<th>hIDO</th>
<th>+Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>H76A</td>
<td>-92 ± 3</td>
<td>-76 ± 3</td>
</tr>
<tr>
<td>H76S</td>
<td>-92 ± 2</td>
<td>-76 ± 3</td>
</tr>
<tr>
<td>H76G</td>
<td>-129 ± 5</td>
<td>-116 ± 3</td>
</tr>
<tr>
<td>F140A</td>
<td>-129 ± 5</td>
<td>-116 ± 3</td>
</tr>
<tr>
<td>F140Y</td>
<td>-129 ± 5</td>
<td>-116 ± 3</td>
</tr>
<tr>
<td>F140A</td>
<td>-129 ± 5</td>
<td>-116 ± 3</td>
</tr>
<tr>
<td>F140Y</td>
<td>-129 ± 5</td>
<td>-116 ± 3</td>
</tr>
<tr>
<td>R144K</td>
<td>-143 ± 3</td>
<td>-116 ± 3</td>
</tr>
<tr>
<td>R144A</td>
<td>-153 ± 3</td>
<td>-112 ± 3</td>
</tr>
</tbody>
</table>

Fig. 3. Redox potentiometry of the F72A variant of hTDO showing the Nernst plot in the absence of L-Trp (solid circles) and the presence of 3 mM L-Trp (open circles). Reaction conditions: 100 mM potassium phosphate (pH 7.0) at 25.0 °C.

Table 4. Fe$^{2+}$/Fe$^{3+}$ reduction potentials (mV) obtained for hTDO and variants in the absence (−Trp) and presence (−Trp) of L-Trp. Reaction conditions are described in the Experimental procedures. Reduction potentials for hIDO variants have been reported previously [19].
The role of the distal histidine

The question of whether there is a role for an active site histidine in the mechanism has been muddled in the literature: early data supported its involvement [24], although the subsequent ground swell of opinion does not. The only experimental evidence against the proposal so far is that 1-Me-Trp (Scheme 1) is a substrate for hIDO, albeit a slow one [25]. Assuming that Me-Trp and Trp react by the same mechanism (which is not conclusively established), we have interpreted [25] this observation as indicating that base-catalyzed abstraction of the indole proton by histidine cannot be involved in the mechanism (because it is not possible to remove a Me group via the same mechanism). Recent computational data [26–29] and electron-nuclear double resonance spectroscopy [30] all support our suggestion.

The data reported in the present study help to build a more extensive and persuasive body of experimental evidence backing up our original proposals [25]. The data for hTDO show only minor effects on both $k_{cat}$ (approximately 5- and 10-fold decreases) and $k_M$ (< 4-fold increases) for the H76 variants. Previously published data on xTDO (H55A, H55S) [22] are in agreement with these findings (<10-fold changes in $k_{cat}$ and negligible changes in $k_M$ on the removal of H55). Our interpretation of all of the data is that histidine is not involved in base-catalyzed abstraction and this would be consistent with our suggestions for the mechanism [25], which do not require abstraction of the indole proton. The fact that there is no histidine in hIDO appears to be in agreement with the general conclusions outlined above.

The distal arginine

The structure of xTDO in complex with substrate shows ionic interactions between the carboxylate group on the substrate and Arg117 (Fig. 1B). This residue is also present in hIDO (Arg231) and xTDO (Arg134) and, from our model, presumably also present in hTDO (Arg144).

Replacement of the active site Arg with Lys in both hIDO and hTDO has fairly minor (< 10-fold reduction) effects on $k_{cat}$ (Table 3), although substantially different consequences on substrate binding in the two enzymes. In hIDO, $K_M$ increases by approximately 500-fold in R231K and these changes are mirrored in the $K_M$ for Trp binding to the ferric form (an increase of approximately 10-fold in R231K). In hTDO, $K_M$ increases only by approximately five-fold in R144K, and this is mirrored in the $K_M$ for Trp binding to the ferric form; the R144A variant is found to be inactive, although the binding data for the ferric enzyme indicate that substrate binding is again affected by the mutation [$K_{D,Fe}^3$] is increased by approximately 10-fold]. The corresponding variant in R. metallidurans TDO (R134A) is also reported as inactive, although no detailed kinetic analyses have been provided [14].

Our summary of all of the data is that the interaction with Arg affects substrate binding in all of the enzymes examined so far; it is critical for substrate binding in hIDO but is less critical in hTDO. For hIDO at least, there is only one (critical) hydrogen-bonding interaction (to Arg) that fits with this interpretation because the residues equivalent to Y113 and His55 in xTDO (which provide the additional stabilization) are not present (replaced with Phe and Ser, respectively; Table 1). There are consistent reports in the literature that the binding of Trp is sensitive to substitution of the carboxylate group on the substrate because tryptamine (Scheme 1) is reported as being inactive in three different TDOs [13,14,18] and in hIDO [18]. These previous observations for the TDOs are thus in agreement with the conclusions that we draw regarding the role of the distal Arg. For IDO, the picture is less clear cut. Early work [19] reports very low (< 0.5%) activity for rabbit IDO against tryptamine, although we have not been able to reproduce this activity for the hIDO isolated from the expression vector reported in the present study or with the same hIDO in another expression vector with a cleavable His-tag. In our hands, we found hIDO to be inactive towards tryptamine, which is in accordance with the conclusions that we draw from the data on TDO above. A word of caution may be required. These early reports [19] have been widely cited [5,8,13] as providing evidence that IDO turns over substrates other than the native substrate (L-Trp); for tryptamine at least, this may need to be reassessed.

Hydrophobic residues

Hydrophobic residues are in abundance in the active site (Fig. 1). The most useful information on their role comes from mutagenesis data on the hIDO variants.

Phe163

Phe163 is critical for activity in hIDO, as indicated by a dramatic decrease in $k_{cat}$, and the effect of this residue is duplicated in hTDO because replacement of the equivalent residue (Phe72) inactivates the enzyme. Because the equivalent residue in R. metallidurans TDO (F68A) is also inactive (although no kinetic
parameters were reported) [1-4], we conclude that Phe163 in hIDO and its equivalent residues in hTDO and *R. metallidurans* TDO are key determinants of activity. This is also supported by steady-state data for the F51A variant of xTDO (\(k_{cat,\text{F51A}}\) approximately 0.05 \(k_{cat,\text{WT}}\); data not shown).

**Phe227**

Although Phe227 forms part of the substrate binding pocket in hIDO, removal of Phe227 has a very marginal effect (Table 3). In our hands, the F227A variant is catalytically competent, and we conclude that this residue has a non-essential role, presumably because it is too far from the substrate binding site (Fig. 1A) to affect the hydrophobic interactions to the substrate.

**Phe164 and Phe226**

Reducing the hydrophobic nature of the distal pocket would be expected to affect binding of the largely hydrophobic substrate. The data for hIDO support this: the largest effects are seen for F226A (approximately 130-fold decrease in \(K_d\)), with similar but smaller effects for F164A (an approximately 30-fold decrease). This is consistent with a role for these residues in providing \(
\pi-\pi\) stacking interactions with \(\text{L-Trp}\). Consistent with these conclusions, a conservative substitution at Phe266 (F226V) is tolerated and has no substantial effect (Table 3). Neither F226A, nor F164A show a substantial drop in \(k_{cat}\) and we conclude that the turnover activity of these enzymes is not affected by the mutation.

However, when the analysis is expanded more widely and other proteins are considered, it is found that the effects of Phe226 are not, overall, duplicated across other TDOs and an inconsistent pattern emerges. The F140Y substitution in hTDO is tolerated but the F140A substitution is not, and the corresponding Y130F variant of *R. metallidurans* TDO shows a modest increase in \(k_{cat}/K_m\). Further data are needed before clarification of the complex role of hydrophobic residues can be fully understood.

**Concluding remarks**

In summary, the conclusions based on the data obtained in the present study, as well as those from previous studies, are: (a) the distal histidine, where present, plays a non-essential role; (b) the active site arginine has a role to play in substrate binding but is more influential in some cases (hIDO) than in others (hTDO); and (c) hydrophobic interactions can affect substrate binding, with the most substantial effects being observed in hIDO Phe226 and Phe164. Clearly, further mutagenesis work on other proteins will be required before a full picture emerges.

**Experimental procedures**

**Materials**

\(\Pi\)-ascorbate, bovine liver catalase, DNase I, \(\alpha\)-glucose, glucose oxidase, methylene blue, xanthine, xanthine oxidase, all reductant potential dyes, \(\text{Tryptophan}\) and substrate analogues were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). *Escherichia coli* strains BL21 (DE3) and Rosetta (DE3) pLysS were obtained from Novagen (Madison, WI, USA). Hemin was obtained from Fluka (Buchs, Switzerland).

**Mutagenesis and protein purification**

Site-directed mutagenesis was performed in accordance with the Quikchange™ protocol (Stratagene Ltd, Cambridge, UK). All variants were expressed and purified in accordance with previously published procedures for the corresponding enzyme [12,18]. Absorption coefficients for all variants were determined using the pyridine hemochrome method [31].

**Steady-state kinetic assays**

Activities were measured spectrophotometrically by monitoring the formation of 9-formylkynurenine at 321 nm. Steady-state kinetic measurements were carried out using either a Perkin Elmer Lambda 35 UV-visible spectrophotometer (Perkin Elmer, Boston, MA, USA) or a Cary 50-Probe UV-visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). Reactions were performed at 25.0 °C in 50 mM Tris-HCl buffer (pH 8.0). Assays contained 10 \(\mu\text{M}\) methylene blue, 100 \(\mu\text{g}\) of catalase, 20 \(\mu\text{M}\) \(\Pi\)-ascorbate and the appropriate amount of enzyme. The reaction was initiated by the addition of \(\text{L-Trp}\) and initial rates were calculated from the increase in A\(_{321}\) (\(A_{321} = 3750 \text{ M}^{-1} \text{cm}^{-1}\)) [32]. Apparent \(K_m\) and \(k_{cat}\) values were determined by varying the concentration of each substrate and fitting the data to the Michaelis-Menten equation.

**Ligand-bound derivatives**

All ligand binding data were measured in 50 mM Tris-HCl buffer (pH 8.0) at 25.0 °C. Ligand bound derivatives (cytochrome, \(\text{L-Trp}\)) were obtained by the addition (typically 2-10 \(\text{mM}\)) of a concentrated stock solution to the enzyme. Ferrous proteins were generated by stoichiometric titration of the ferric enzyme with sodium dithionite. Equilibrium
binding constants, $K_D$, were determined in accordance with previously reported procedures [33].

**Redox potentiometry**

Redox potentiometry was carried out in accordance with previously reported procedures [18]. Reduction potentials (Fe$^{3+}$/Fe$^{2+}$) were determined at 25.0°C by the reduction of the protein with a dye of known potential [34]. The assay solution contained potassium phosphate buffer (0.1 M, pH 7.0), glucose (5 mM), xanthine (16 mM), xanthine oxidase (5 µM), glucose oxidase (50 µg·mL$^{-1}$), catalase (5 µg·mL$^{-1}$) (Sigma-Aldrich Co.) and enzyme (2 µM). Heme reduction potentials were determined by fitting the data to a Nernst equation for a single-electron process [34] using Origin software (Microcal, Inc., Northampton, MA, USA).

**Acknowledgements**

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**References**


Supporting information
Additional supporting information may be found in the online version of this article at the publisher's web site:
Table S1. Wavelength maxima for hIDO, hTDO and the corresponding variants examined in the present study.