Oxidation of l-tryptophan in biology: a comparison between tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase

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Abstract

The family of haem dioxygenases catalyse the initial oxidative cleavage of l-tryptophan to N-formylkynurenine, which is the first, rate-limiting, step in the kynurenine pathway. In the present paper, we discuss and compare structure and function across the family of haem dioxygenases by focusing on TDO (trypotphan 2,3-dioxygenase) and IDO (indoleamine 2,3-dioxygenase), including a review of recent structural information for both enzymes. The present paper describes how the recent development of recombinant expression systems has informed our more detailed understanding of the substrate binding, catalytic activity and mechanistic properties of these haem dioxygenases.

Introduction

The kynurenine pathway converts l-tryptophan into NAD, which is responsible for the metabolism of the majority of L-tryptophan in biology (Figure 1) [1]. The first, rate-limiting, step in this pathway is the formation of N-formylkynurenine by incorporation of dioxygen across the C2–C3 bond of l-tryptophan (Figure 2). TDO (trypotphan 2,3-dioxygenase) and IDO (indoleamine 2,3-dioxygenase) are haem-containing enzymes that both catalyse this reaction. TDO was initially discovered in mammalian liver in 1936 [2] and it was first characterized in rat liver in 1955 [3]. IDO was discovered approx. 30 years later in rabbit small intestine by Hayaishi and colleagues [4,5] and was observed to be similar to TDO because it had the capability of catalysing D-tryptophan ring cleavage.

Recently, expression systems for both IDO and TDO have appeared which has meant that more direct comparisons can be made across the entire dioxygenase family. In the present paper, we provide recent data for hTDO (human TDO) and hIDO (human IDO) and we summarize what is currently known about these enzymes. We use these comparisons between enzymes, together with structural information, to draw general conclusions about the substrate-binding and catalytic properties of the haem dioxygenase family.

Location and isolation of TDO and IDO

TDO has been characterized in many prokaryotes and eukaryotes, including rabbits [5], mice [6], insects such as Drosophila melanogaster [7], bacteria including Xanthomonas campestris [8] and Ralstonia metallidurans [9], and yeast [10]. TDO was originally thought to be primarily localized to the liver; however, the enzyme has also been identified in skin [11]. IDO is ubiquitously expressed throughout the body, apart from in the liver.

Bacterial expression

There are now a number of expression systems reported for various TDO and IDO enzymes. hTDOs [12,13] and all hIDOs [14–16], apart from one (which has been expressed in yeast [17]), have been expressed in Escherichia coli. The bacterial TDOs from X. campestris [8] and R. metallidurans [9] were also expressed in E. coli. We have reported expression for full-length hTDO, the gene being cloned into E. coli with a C-terminal hexahistidine tag that is cleavable by thrombin. This recombinant expression system yields ~15 mg/l purified protein [12]. A truncated form of the same hTDO has also been expressed in E. coli [13].

Overall structure

TDO has four subunits forming a tetramer of ~190 kDa in eukaryotes and ~120 kDa in prokaryotes. One of the TDO subunits is approximately the same size as the monomeric mammalian IDO enzyme, at ~45 kDa. Both dioxygenases contain a protohaem IX prosthetic group and use dioxygen for catalysis. The two enzymes have relatively low sequence identity [8], but X. campestris TDO shares 34% sequence identity with hTDO, suggesting a closer conservation between these enzymes [8]. In contrast, prokaryotic IDOs are less structurally homologous with the eukaryotic enzymes, with ~14% sequence identity [9].

Crystallography

A crystal structure for hIDO was reported in 2006 (Figure 3A). The overall monomeric structure of hIDO is folded into two domains: a large domain, which has an all α-helical
structure, and a small domain, connected via a long loop (Figure 3A) [16]. Subsequently, crystal structures for TDOs from X. campestris [8] (Figure 3B) and R. metallidurans [9] have been reported. The structure was a major step forward and, somewhat unexpectedly, showed an active site that, with the exception of Ser167, was devoid of polar residues (Figure 4). Hence, although the preponderance of hydrophobic residues in the active site was consistent with the idea that the enzyme needs to bind a relatively large hydrophobic substrate, the absence of hydrogen residues close to the iron active site was not expected at all and was not consistent with previously proposed mechanisms [18] in which the presence of an active-site base had been assumed. Despite the low sequence identity between TDO and IDO, a comparison of the published crystal structures for X. campestris TDO [8] and hIDO [16] showed that the active-site region is also relatively conserved (Figure 4), suggesting that they have a similar substrate-binding modes [8,9,16,19].
There is no crystal structure for hTDO yet reported. Highly conserved residues include the proximal histidine (His592 and His364 in *X. campestris* TDO and hIDO respectively), several hydrophobic residues (Phe45 and Tyr113 in *X. campestris* TDO; Phe663 and Phe26 in hIDO) and an arginine residue (Arg37 and Arg231 in *X. campestris* TDO and hIDO respectively) which has been proposed to be involved in substrate binding [8]. The only polar residue in the distal pocket of hIDO (Ser675) is replaced with a histidine residue in *X. campestris* TDO (His65), but, despite nicely fitting the bill as the long-sought active base, this residue is not essential for activity [19].

**Binding of L- and D-tryptophan**

EPR spectroscopy has shown that ferric hTDO is a mixture of high-spin iron (g values of 5.71, 2.01) and low-spin iron (g values of 2.89, 2.30, 1.62), which correlated with observations for ferric hIDO high-spin iron (g values of 5.82, 1.99) and low-spin iron (g values of 2.85, 2.27, 1.62) [12,20]. The spectroscopic features of the low-spin species are consistent with those expected for a bis-histidine species [21,22], but this assignment is problematic for hIDO in which there is no active-site histidine residue (see above). Upon addition of L-tryptophan to ferric hTDO, the formation of a new low-spin species (g values of 2.63, 2.20, 1.84 [12]) was observed that was also observed for ferric hIDO in the presence of the same substrate (g values of 2.53, 2.19, 1.86 [14]). The new low-spin species in both cases has been assigned as arising from histidine/hydroxide-ligated haem in the substrate-bound form. The formation of hydroxide-bound haem is not observed as a function of pH in these two human enzymes, which differentiate them from other haem proteins (e.g. the globins) in which conversion into hydroxide-bound haem is facile. For the dioxygenases, it might suggest conformational rearrangements of the protein structure on substrate binding which lead to alteration in haem-co-ordination geometry.

The substrate-binding affinity can be accessed by two measurable parameters. For the ferrous enzyme, the *Kₘ* can be used; for the ferric enzyme, the *Kₐ*. For hTDO, we have reported that the *Kₘ* for the ferrous form (222 μM) and the *Kₐ* for the ferric form (170 μM) are similar to each other and also to ferric IDO (see below) and other (ferrous) bacterial TDOs such as *X. campestris* TDO (*Kₘ* = 114 μM) [8]), showing that hTDO does not discriminate against binding of substrate through changes in oxidation state at the metal [12,13]. This has been confirmed by redox experiments, which provide a correlation with the binding data. Hence, reduction potentials for the Fe³⁺/Fe²⁺ couple of hTDO in the absence (~92 mV) and presence (~76 mV) of substrate are not very different, which is also consistent with the idea that there is no preferential binding of the substrate in one or the other oxidation state. In contrast, the substrate-binding affinity of ferric hIDO (*Kₐ* ≈ 200–300 μM) is ~20-fold lower than for the ferrous form (*Kₐ* ≈ 10 μM), so that ferrous hIDO has a much higher affinity for l-tryptophan [14,20]. These binding affinities for hIDO are similarly correlated in the redox data since there is an ~80 mV positive shift in the reduction potential on binding of substrate, demonstrating stabilization of the ferrous form of the enzyme upon binding of substrate. It is not yet known why hIDO discriminates in this way through oxidation state, but hTDO does not. It might be related to their physiological locations. IDO is located in a reducing environment in the cytoplasm, therefore the ferric form of the enzyme would presumably be at a low concentration, hence binding by the ferric form is not used catalytically. In contrast, TDO is primarily located in the more oxygen-rich environment of the liver, resulting in a significant amount of the enzyme being in the ferric form compared with the ferrous form, so that binding to the ferric protein would be required to be more efficient.

**Catalytic activity**

Steady-state rates of substrate oxidation for both hTDO and hIDO are comparable (*kₐ* = 1.4 s⁻¹ in each case) [12,20]. However, these are lower than for the bacterial forms of the enzyme which have higher values (*kₐ* = 19.5 s⁻¹ [8], 18.0 s⁻¹ [21], 17.5–19 s⁻¹ [24]). The reasons for this difference are not clear.

Activity has also been observed for the ferric form of hTDO and the truncated form of hTDO [12,13]. Hence, under aerobic conditions, ferric hTDO produces detectable amounts of product through oxidation of l-tryptophan. Under anaerobic conditions, no product formation was detected. The mechanism of this reaction is not yet known.

**Analogue of l-tryptophan**

TDO is a rather fussier enzyme than IDO and does not oxidize as wide a range of tryptophan analogues, only L-tryptophan, 5-fluorotryptophan and 6-fluorotryptophan (Figure 5) [8,12]. This is in contrast with IDO which utilizes a broader range of substrates, including L-tryptophan, D-tryptophan, 5-hydroxytryptophan, 5-methyltryptophan, tryptamine and 5-hydroxytryptamine (serotonin), in addition to those substrates utilized by TDO (Figure 5) [12,18,25,26]. Steady-state experiments with both hTDO and hIDO show that substrate analogues with the α-carboxylate and/or the α-ammonium group modified are not tolerated [12], which is consistent with an important hydrogen-bonding stabilization of the bound substrate.

**O₂ binding**

Formation of the catalytic ferrous-oxy complex is readily observed in hIDO on the stopped-flow timescale [20]. However, no formation of a stable ferrous-oxy complex has so far been observed for hTDO [12]. This is not because of a lack of reactivity of the ferrous haem group, since other diatomic ligands such as cyanide and carbon monoxide can bind to the ferrous haem [12]. It is interesting to consider that the ferric/ferrous reduction potential for both hTDO (~92 mV) and hIDO (~63 mV) enzymes are quite similar, which indicates that the intrinsic stability of the ferrous iron is not very different in both cases. This makes it all the more surprising that hTDO fails to form a stable ferrous-oxy complex. The reasons for this instability are not yet clear.
Figure 5 | Structures of tryptophan analogues

The main difference between the active sites of TDO and hIDO is that TDO has a histidine residue, whereas hIDO has serine (Ser^{167}) as the equivalent residue (Figure 4). Site-directed mutagenesis has shown that Ser^{167} in hIDO is not involved in dioxygen binding because the ferrous–oxy complex for the S167A variant forms normally [20]. Hence, whereas Ser^{167} is the only polar residue in the active site, it is unlikely to provide hydrogen-bonding stabilization of the bound dioxygen ligand, as observed in the globins (stabilization through the distal histidine). Therefore, for IDO hydrogen-bonding stabilization of the oxy complex, if it is needed, is more likely to occur through active-site water molecules.

The conversion of Ser^{167} into a histidine residue, to mimic TDO (S167H variant of hIDO) is also useful. In this case, formation of a ferrous–oxy complex is observed, but it is now more unstable than wild-type hIDO. Hence, both S167H variants of hIDO and hTDO form an unstable ferrous–oxy complex, which is in contrast with wild-type hIDO, and suggests that the histidine residue is particularly significant in this regard [12]. The very low reduction potential for S167H (−203 mV) is certainly likely to be influential in destabilizing the ferrous–oxy complex, but does not provide a complete explanation because the reduction potential of recombinant hTDO (−92 mV) is higher, and in this enzyme, the ferrous–oxy species is not detected at all.

Catalytic mechanism

Since the mid-1990s, all proposed mechanisms have been proposed to start with the ferrous form of the dioxygenase enzyme and it was suggested that a deprotonation of the indole NH catalysed by an active site base was the initial step (Figure 6A). This mechanism was proposed before structural information was available, and for hIDO, we now know that there is no active-site histidine residue and that Ser^{167} is not essential for catalysis [20]. When the structure of X. campestris TDO appeared [8], it showed that an active-site base (His^{55}) was present, but removal of His^{55} does not eliminate activity. The idea that the bound dioxygen acts as a base has also been suggested [16,27] (Figure 6B), but, at present, this has not been confirmed, so the precise mechanism remains to be clarified.

Summary

There are still a large number of unanswered questions for these dioxygenase enzymes. We do not know, for example, precisely how substrate binding and O₂-binding is controlled, and we do not understand the functional significance of the subtle differences in properties between the various...
dioxygenase enzymes. We do not know whether substrate oxidation of the ferric enzyme is physiologically relevant or not. The precise mechanism of substrate oxidation, and the role of the protein in controlling this, also remains to be fully elucidated. These questions provide the focus for further studies in this area.

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References