Archives of Biochemistry and Biophysics 544 (2014) 18–26

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Review

Heme-dependent dioxygenases in tryptophan oxidation

Jiafeng Geng, Aimin Liu *

Department of Chemistry, Georgia State University, 50 Decatur Street SE, Atlanta, GA 30303, United States

ARTICLE INFO

Article history:
Available online 1 December 2013

Keywords:
Metalloprotein
Oxygen activation
Peroxide reaction
Reactive oxygen species
High-valence iron
Free radical

ABSTRACT

L-Tryptophan is an essential amino acid for mammals. It is utilized not only for protein synthesis but also for the biosynthesis of serotonin and melatonin by the serotonin pathway as well as nicotinamide adenine dinucleotide by the kynurenine pathway. Although the kynurenine pathway is responsible for the catabolism of over 90% of L-tryptophan in the mammalian intracellular and extracellular pools, the scientific field was dominated in the last century by studies of the serotonin pathway, due to the physiological significance of the latter's catabolic intermediates and products. However, in the past decade, the focus gradually reversed as the link between the kynurenine pathway and various neurodegenerative disorders and immune diseases is increasingly highlighted. Notably, the first step of this pathway, which is catalyzed by heme-dependent dioxygenases, has been proven to be a potential target for immune regulation and cancer treatment. A thorough understanding of the intriguing chemistry of the heme-dependent dioxygenases may yield insight for the drug discovery of these prevalent illnesses. In this review, we survey enzymatic and mechanistic studies, initially started by Kotake and Masayama over 70 years ago, at the molecular level on the heme-dependent tryptophan dioxygenation reactions.

Introduction

Hemoproteins perform a wide range of biochemical functions including oxygen transport and storage, gas sensing, electron transfer, and chemical catalysis. The utilization of heme iron for dioxygen activation and oxygen insertion into organic substrates is prevalent in nature, with the most well-known examples being the heme-dependent monooxygenation reactions catalyzed by cytochrome P450s. Notably, hemoproteins rarely express dioxygenase activity as the native biological function. Thus far, only a few examples have been identified in lipid metabolism (fatty acid α-dioxygenase, prostaglandin H synthase, and linoleate diol synthase), tryptophan oxidation (tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase), and natural rubber degradation (rubber oxygenase). Heme-dependent dioxygenases are distinctive members of the dioxygenase family in that they utilize a histidine-coordinated heme rather than a non-heme iron or manganese to facilitate dioxygen activation and oxygen insertion reactions.

Tryptophan 2,3-dioxygenase (TDO)[1] is the first functionally defined heme-dependent dioxygenase [1–3]. It was initially referred to by several different names: tryptophan peroxidase-oxidase, tryptophan pyrrolase, and tryptophan oxygenase. TDO employs a *b*-type ferrous heme to catalyze the oxidative cleavage of the indole ring of L-tryptophan (L-Trp), converting it to *N*-formylkynurenine (NFK) (Scheme 1). In mammals, TDO is mainly a hepatic enzyme that participates in the initial and rate-limiting step of the kynurenine pathway, which is the primary route of L-Trp degradation [4–9]. The kynurenine pathway constitutes the major part of the *de novo* biosynthesis of nicotinamide adenine dinucleotide (NAD), an essential life-sustaining redox cofactor, in eukaryotic organisms and in some bacterial species [10,11]. In addition to mammals, TDO is also present in other sources such as insects and bacteria [3,10,12–14].

Hayaishi et al. discovered an isozyme of TDO in 1967 [15,16]. This enzyme is named indoleamine 2,3-dioxygenase (IDO) because it exhibits a much broader substrate-specificity than TDO. While TDO is highly specific for L-Trp, IDO can tolerate a collection of indoleamine derivatives, including D-Trp, tryptamine, and serotonin [17–21]. IDO participates only in the kynurenine pathway of mammals and is ubiquitously distributed in all tissues except the liver [20–22]. Although TDO and IDO were identified decades ago, their crystal structures were not solved until recently [14,23–25]. IDO is crystallized as a dimer with a disulfide bond connecting the two monomeric units [23], whereas TDO consists of four subunits arranged in a dimer of dimer quaternary structure [14,24,25] (Fig. 1). The two enzymes share only ~10% sequence identity but exhibit similar active-site architectures [14,23–25]. Recently, a potential TDO/IDO superfamily has been proposed upon incorporating another heme-dependent tryptophan-utilizing enzyme, PrnB, which possesses a common structural core as TDO and IDO [26].

* Corresponding author. Address: Department of Chemistry, Georgia State University, P.O. Box 3965, Atlanta, GA 30302, United States. Fax: +1 404 413 5505.
E-mail address: Feradical@gsu.edu (A. Liu).

[1] *Abbreviations used:* TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase; L-Trp, L-tryptophan; 1-Me-L-Trp, 1-methyl-L-tryptophan; NFK, *N*-formylkynurenine; NAD, nicotinamide adenine dinucleotide; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); cmTDO, *Cupriavidus metallidurans* TDO; xcTDO, *Xanthomonas campestris* TDO; dmTDO, *Drosophila melanogaster* TDO; hTDO, human TDO; H-bonding, hydrogen-bonding; DFT, density functional theory; MD, molecular dynamics; QM/MM, quantum mechanics/molecular mechanics; •NO, nitric oxide; WT, wild-type; 2MI, 2-methylimidazole; IPNS, isopenicilin *N* synthase.

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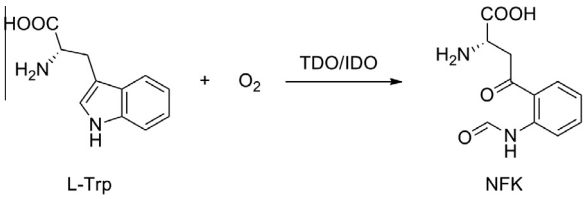
http://dx.doi.org/10.1016/j.abb.2013.11.009



Scheme 1. The chemical reaction catalyzed by TDO and IDO.

During the past decade, TDO and IDO have attracted enormous attention because of their physiological importance. IDO is inducible by interferon-γ as a result of inflammation and thus is highly related to immune functions [27–29]. It is proposed that local depletion of cellular L-Trp by IDO inhibits the growth of certain pathogens, contributing to innate host immune response [27–30]. However, in contrast, a growing body of evidence demonstrates that increased expression of IDO is frequently linked to host immunosuppression. It can promote immune tolerance under various physiological and pathophysiological conditions, which causes serious problems including maternal fetal tolerance and immune escape of cancer [20,27,29–36]. In recent years, TDO has been found to be expressed in tumor cells, and the expression of TDO has been shown to play an immune-regulatory role in many cancer systems via preventing tumor rejection, much like the reports for IDO [35,37]. These findings make it extremely beneficial to characterize the biochemical properties and elucidate the catalytic mechanism of TDO and IDO for inhibitor design and drug discovery.

The catalytic mechanism of oxygen activation and insertion for P450-type monooxygenases is well studied. It features a compound I intermediate, which is a ferryl species (Fe(IV)=O) coupled with a cation porphyrin radical [21,38]. The compound I intermediate is a catalytically competent oxidant and is able to insert the ferryl oxygen into organic substrates [39]. It should be noted, however, heme-dependent monooxygenation consumes electrons (from NADH/NADPH) and protons with one of the atoms of O_2 being reduced to water. In contrast, the TDO/IDO reaction does not consume any electrons or protons from external sources. Thus, the dioxygenation reaction is fundamentally distinct from those monooxygenation reactions in terms of oxygen reduction. This review seeks to synthesize recent findings on the mechanistic studies of TDO and IDO and share our perspectives on several critical aspects of the catalytic properties of these two isozymes, including their reactivity towards hydrogen peroxide, the involvement of high-valence ferryl species in the reaction cycle, and the catalytic roles of a distal histidine residue in TDO.

Reactivities towards hydrogen peroxide and physiological relevance

P450-type monooxygenases exhibit a "peroxide shunt" pathway in which the ferric form of enzymes can interact with single-oxygen donors such as peroxides, leading to direct formation of the compound I intermediate [21,38] (Scheme 2A). Compared to the native Fe(II)- and O_2-dependent reaction pathway, this alternative pathway allows the catalytic cycle to be completed without the participation of electron donors and associated electron transfer proteins.

The ferrous heme of TDO and IDO is the catalytic center that binds and activates dioxygen. Like many other Fe(II)-dependent enzymes, TDO and IDO become auto-oxidized in aerobic environments when the substrate L-Trp is absent. For quite a long time, the reactions involving hydrogen peroxide and the resting ferric state of TDO and IDO received little attention, despite several very interesting phenomena reported from discrete studies. For

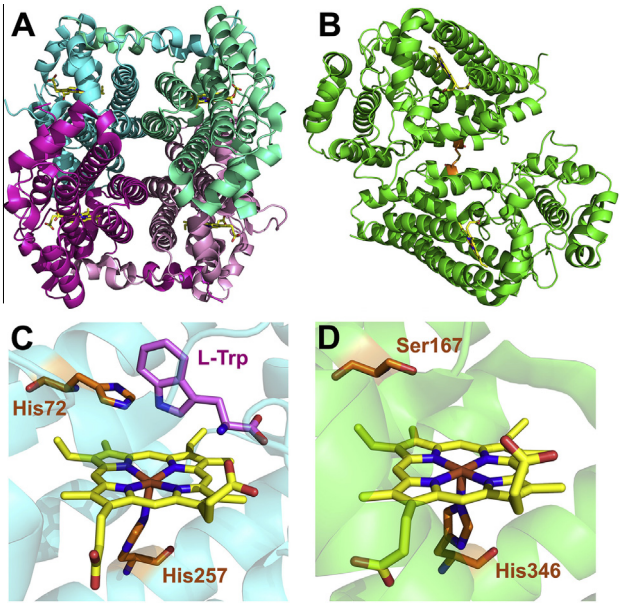
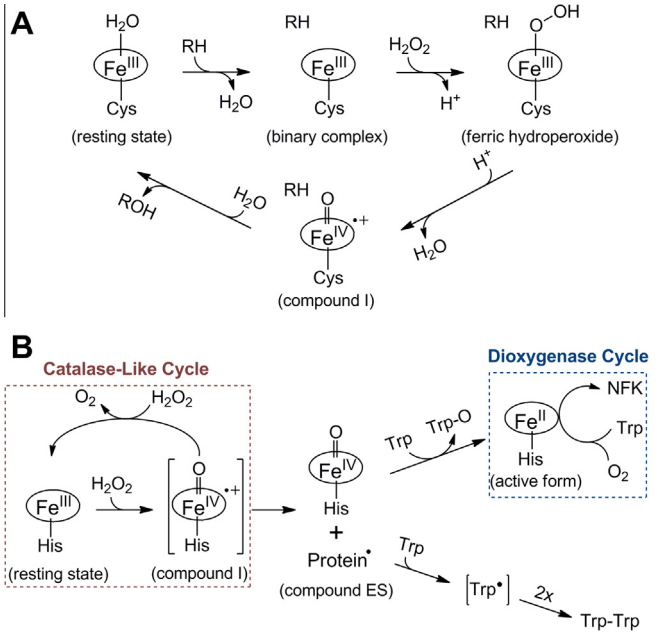


Fig. 1. Crystal structures of TDO and IDO. (A) The quaternary structure of TDO is tetrameric, arranged in a dimer of dimer pattern (PDB entry: 2NOX). Each subunit contains a *b*-type heme that is labeled in yellow. (B) IDO is crystallized as a dimer with a disulfide bond connecting the two monomeric units (PDB entry: 2DOT). The hemes are labeled in yellow and the two cysteine residues of the disulfide bond are labeled in orange. (C) The active-site architecture of substrate-bound TDO (PDB entry: 2NW8). The heme center is coordinated to a proximal histidine residue (His257 in *Cupriavidus metallidurans* TDO (cmTDO) amino acid numbering). The enzyme-bound L-Trp (labeled in pink) is H-bonded to a distal histidine residue (His72 in cmTDO amino acid numbering). (D) The active-site architecture of substrate-free IDO (PDB entry: 2DOT). The distal histidine residue present in TDO is replaced by a serine residue (Ser167) in IDO.

example, it was reported over 60 years ago that hydrogen peroxide is able to activate the resting ferric state of TDO in the presence of L-Trp [9]. This observation was later confirmed by independent studies from different laboratories and further proven by the observation that the activation effect is inhibited by catalase [3,40,41]. More than 30 years ago, IDO was found to possess peroxidase activity [18] and H_2O_2-dependent monooxygenase activity [42], but no studies regarding whether these activities occur in a physiologically meaningful context ensued. It was not until recently, when considerable attention was attracted to these research directions, that significant progress was made. As detailed below, a H_2O_2-mediated enzyme reactivation mechanism has been proposed in TDO based on intensive biochemical and spectroscopic investigations [43]. Several consecutive mechanistic studies have highlighted the versatile activities of IDO towards H_2O_2, which have revealed the fundamental differences between TDO and IDO in their peroxide reactions [44–47].

Hydrogen peroxide-mediated enzyme reactivation pathway in TDO

Although the activation of ferric TDO by H_2O_2 in the presence of L-Trp was discovered in 1950 [9], the mechanism of this phenomenon remained a mystery. We have recently demonstrated via unequivocal spectroscopic (optical and Mössbauer) evidence that ferrous TDO can be produced upon addition of H_2O_2 to ferric TDO in the presence of L-Trp [43]. Through an enzymatic assay with carbon monoxide (CO) as an inhibitor, the freshly generated ferrous enzyme is proven to be the catalytically competent species that gives rise to the observed dioxygenase activity [43]. As shown in Scheme 2B, a two-phase enzyme reactivation mechanism is proposed to illustrate how ferric TDO is reductively reactivated by



Scheme 2. Hydrogen peroxide-mediated alternative enzymatic pathways in heme-dependent oxygenases. (A) The "peroxide shunt" pathway in P450-type monooxygenases. "RH" represents the substrate and "ROH" represents the product. (B) The enzyme reactivation pathway in TDO. The enzyme reactivation occurs when the protein radical and the ferryl heme in the compound ES-type intermediate are each reduced by L-Trp. Intermediates shown in parentheses are predicted but not detected experimentally.

an oxidant, H_2O_2, based on identification of nearly all of the intermediates and products in the reaction system [43]. In the first phase, the ferric enzyme is oxidized by H_2O_2 to yield a metastable compound ES intermediate, which is a ferryl species plus a radical of a nearby aromatic amino acid residue (Scheme 2B). In the second phase, the protein-based radical and the ferryl heme in the compound ES intermediate are reduced by L-Trp in separate events. Reduction of the protein-based radical leads to radical-mediated dimerization of L-Trp; reduction of the ferryl heme leads to monooxygenation of L-Trp and production of the ferrous enzyme (Scheme 2B). In the absence of an exogenous reducing agent, the reducing power for the peroxide-mediated reactivation of ferric TDO is ultimately derived from L-Trp. Overall, the observed dioxygenase activity is generated through the native enzymatic cycle catalyzed by ferrous TDO with dioxygen as the co-substrate, rather than another version of the "peroxide shunt" described for cytochrome P450 enzymes.

Along with the discovery of the reactivation mechanism, a previously unknown catalase-like activity of TDO is identified and linked with the reactivation pathway [43] (Scheme 2B). Even though TDO is not as efficient as native catalase enzymes, its catalase-like activity provides a solid foundation for the H_2O_2-utilization capability. In the catalytic mechanism of catalase, the ferric heme reacts with the first H_2O_2 molecule to generate a compound I intermediate, which subsequently reacts with a second H_2O_2 molecule to produce dioxygen [48,49]. In the reactivation pathway of TDO, the compound I intermediate in the catalase-like reaction cycle is believed to be the precursor of the compound ES species, which in turn is the precursor of ferrous TDO (Scheme 2B). Moreover, during enzyme reactivation under anaerobic conditions, the catalase-like activity of TDO provides the only source of dioxygen to sustain the dioxygenase reaction of the ferrous enzyme generated by the reactivation pathway. Indeed, the amount of the dioxygenase reaction product, NFK, is quantitated to be roughly half the amount of H_2O_2 in an anaerobic reaction system [43].

Reactions involving ferric IDO and hydrogen peroxide

Unlike TDO, IDO does not possess a H_2O_2-mediated enzyme reactivation pathway. Rather, the catalytic activity of IDO is inhibited by H_2O_2 [50]. Recently, Freewan et al. reported that H_2O_2 inhibits cellular IDO dioxygenase activity and that the inactivation is achieved via compound I-initiated oxidative damage to the heme as well as the protein structure [47]. The inhibition effect can be significantly suppressed by L-Trp. The presence of L-Trp leads to either two-electron or one electron reduction on the IDO compound I species, which consequently regenerates ferric IDO or yields an IDO compound II species, respectively [47]. Moreover, excess L-Trp can form a lethargic complex with the newly generated IDO compound II species [44,47], thereby further protecting the protein from oxidative damage.

Despite lacking a catalase-like activity [18], IDO displays other types of activity towards H_2O_2. In addition to common peroxidase substrates, including 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and guaiacol [18,44], IDO can catalyze peroxidase reactions with physiological peroxidase substrates, such as ascorbate, tyrosine, and NADH [46,47]. The metabolisms of nitric oxide ($^{\bullet}NO$) and its oxidation product, nitrite NO_2^-, are recognized as key reactions catalyzed by heme-dependent peroxidases in inflammatory tissues. High-valence ferryl species of heme-dependent peroxidases can consume $^{\bullet}NO$ and convert NO_2^- into a nitrating species, nitrogen dioxide radical $NO_2^{\bullet}$, which is capable of performing nitration reactions on tyrosine residues in proteins [51–54]. In the presence of H_2O_2, IDO was demonstrated to catalytically consume $^{\bullet}NO$ and utilize NO_2^- to promote formation of 3-nitrotyrosine as a self-modification [47]. In another report, it was found that IDO can catalyze oxidation reactions of indole, but not L-Trp, by H_2O_2 with 2- and 3-oxoindole as the major products [45]. The reactions were proposed to proceed via a peroxygenase mechanism in which the reactive compound I species of IDO generated by peroxide oxidation of the ferric enzyme transfers its ferryl oxygen to indole to yield monooxygenated products, a process resembling the "peroxide shunt" in P450 enzymes [45]. Therefore, the IDO compound I intermediate is a catalytically competent species responsible for both the peroxidase and peroxygenase activities of the enzyme, and it is also the primary source of oxidative damage to the protein in the absence of any small-molecule substrates.

Physiological relevance

The different behaviors between TDO and IDO in their peroxide reactions are most likely rooted in the different reactivities of their high-valence ferryl species generated upon peroxide oxidation. The slightly different designs of the heme environment between these two enzymes may account for the observed differences. Specifically, a highly conserved distal histidine residue (His72 in *Cupriavidus metallidurans* TDO (cmTDO) amino acid numbering, Figure 1C), which is present in TDO but absent in IDO, is likely the major factor that differentiates the reactivities of the ferryl species via providing a hydrogen-bonding (H-bonding) interaction to the ferryl oxo group in TDO [55].

The differences in peroxide reactions between these two enzymes might be physiologically important. TDO is a hepatic enzyme, and hepatocytes are known to be in an oxidizing environment that may cause inactivation of TDO by oxidation of the heme iron. While IDO is proposed to be maintained in the reduced state *in vivo* by the action of cytochrome b_5 and cytochrome b_5 reductase [56,57], no biological reagents have been identified to perform the same function for TDO. Under normal physiological conditions, H_2O_2 is present at low levels in cells. However, a small amount of H_2O_2 is sufficient to cause enzyme reactivation in TDO

under aerobic conditions when the substrate L-Trp is present. Amino acids are neither stored in the human body nor excreted. Rather, they are utilized for protein synthesis or degraded with the highest priority relative to glucose and other energy sources. As a key enzyme responsible for L-Trp degradation, TDO may adopt the reactivation pathway to ensure sustainable catalytic efficiency. In general, the discovery of the enzyme reactivation mechanism is important for understanding how a ferrous enzyme maintains its catalytic activity in an oxidizing environment. Unlike TDO, IDO primarily utilizes its dioxygenase activity to ubiquitously regulate local cellular levels of L-Trp and L-Trp catabolites for immune regulatory functions. It should be noted that L-Trp can efficiently retard all of the aforementioned peroxidase/peroxygenase-type reactions of IDO by occupying the active site [44,45,47]. This observation suggests that the H_2O_2-mediated reactions of IDO are inhibited in normal tissues where L-Trp is present at a certain level. However, in inflammatory tissues where the levels of H_2O_2 and •NO are elevated, but the level of L-Trp is diminished, the biological actions of IDO might be modulated by its peroxidases/peroxygenase activities. This may cause dioxygenase inactivation, •NO consumption, and protein nitration.

Dioxygenase mechanism of TDO and IDO

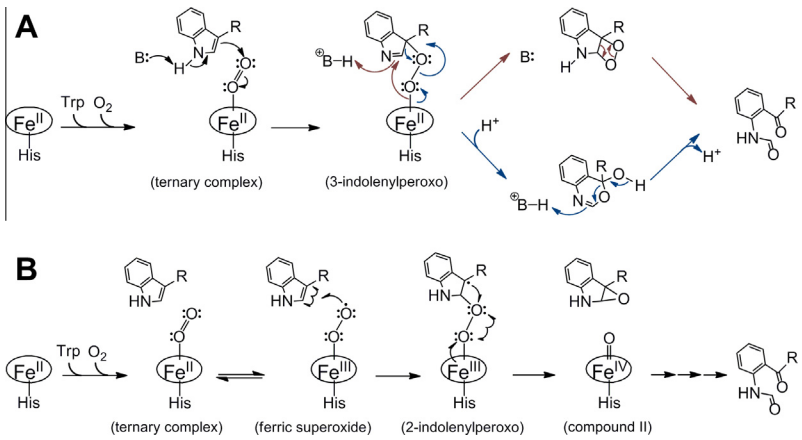
Despite accelerated efforts to investigate the mechanisms of TDO and IDO, the mechanism by which oxygen is activated and inserted to L-Trp is not yet definitively established. This conundrum is mainly due to a lack of direct and solid experimental evidence for the chemical identities of key catalytic intermediates. Nonetheless, recent crystallographic, spectroscopic, and computational studies have provided researchers with a diverse collection of information to draw a general picture of the reaction pathway and comment on several long-debated issues regarding the dioxygenase mechanism. These issues are related to the following topics: (a) the formation of the catalytic ternary complex, (b) the involvement of an acid–base catalyst for reaction initiation, (c) the existence of high-valence ferryl species in the reaction cycle, and (d) the catalytic roles of the distal histidine residue in TDO.

Catalytic ternary complex of TDO and IDO

A ternary complex of Fe(II)-O_2-Trp is regarded as the starting point of the dioxygenase reactions of TDO and IDO (Scheme 3). In this complex, the ferrous heme is coordinated by O_2 with the substrate L-Trp binding nearby. The direct coordination of O_2 to the heme iron plays an essential role for activation of the dioxygen molecule in a previously proposed base-dependent mechanism [21,23,25,58–61] (Scheme 3A) and a recently proposed ferryl-dependent mechanism [60–66] (Scheme 3B). In the former mechanism, the direct coordination significantly increases the electron-deficiency of the distal oxygen atom with the heme iron withdrawing a great portion of electron density from the dioxygen molecule. This facilitates an effective nucleophilic attack by the electron-rich C3 of the substrate after deprotonation of the indole NH group (Scheme 3A). In the latter mechanism, the direct coordination enhances the radical feature of the distal oxygen atom with the heme-bound O_2 possessing superoxide characteristics, enabling a direct radical addition of the distal oxygen to the C2 position of the substrate without deprotonation of the indole moiety (Scheme 3B).

Spectroscopic and computational studies have suggested that the conformation of the ternary Michaelis complex of TDO is properly tuned by the protein matrix via intricate hydrophobic and H-bonding interactions for efficient catalysis [24,62,64,67–69]. Structural comparisons show that most of the hydrophobic interactions between L-Trp and the substrate-binding pocket of TDO are conserved in IDO [14,23–25,69]. However, due to substitutions of active-site residues, several important H-bonding interactions within the TDO ternary complex are inevitably altered or missing in IDO. They are believed to be the key factors that differentiate the reactivities and substrate selectivity between these two isozymes. For example, an H-bonding interaction between the amine group of L-Trp and the side chain hydroxyl group of a distal threonine residue is suggested to be responsible for the strict substrate L-stereoselectivity of TDO [70,71]. Compared to the TDO ternary complex, the IDO ternary complex displays a higher degree of conformational freedom [64,67,68]. This is consistent with the relaxed substrate specificity of IDO. The elevated structural flexibility also destabilizes the IDO ternary complex, making it susceptible to autoxidation [21].

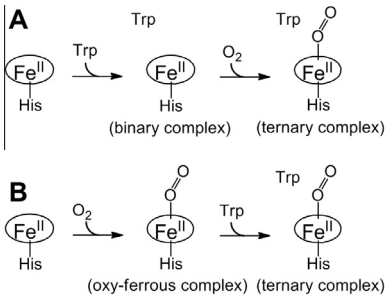
Interestingly, an emerging body of evidence indicates that the assembly processes of the ternary complex in TDO and IDO differ (Scheme 4). In TDO, the binding of the primary-substrate (L-Trp) is believed to precede the binding of the secondary-substrate (O_2) [21] (Scheme 4A). This notion was initially evidenced from a pioneer rapid-kinetic study by Hayaishi and coworkers [72]. They have shown that accumulation of the oxy-ferrous complex of



Scheme 3. Representative catalytic mechanisms of TDO and IDO. (A) A previously proposed base-dependent mechanism [21,23,25,58–61]. This mechanism is branched at the 3-indolenylperoxo intermediate, which is proposed to be decomposed via either a dioxetane pathway (red, upper branch) or a Criegee rearrangement pathway (blue, bottom branch). (B) A recently proposed ferryl-dependent mechanism, in which direct radical addition of the ferric superoxide to the indole C2 position is proposed [60–66]. The catalytic steps connecting the compound II intermediate to the final product is still under development.

TDO can be observed only in the presence of L-Trp, and that in the absence of L-Trp, the ferrous heme does not readily bind O_2 although it is eventually oxidized to the ferric state by O_2 [72]. An induced-fit behavior occurs in TDO upon L-Trp binding, as revealed from the crystal structures of substrate-free and substrate-bound *Xanthomonas campestris* TDO (xcTDO) [24] as well as from a recent modeling study based on the crystal structure of *Drosophila melanogaster* TDO (dmTDO) [14]. L-Trp recognition can establish a complex and extensive network of substrate-enzyme interactions, which stabilizes the active-site region and completely shields it from the solvent by switching from an open conformation to a closed conformation through loop movements [24]. Thus, the Trp-TDO binary complex represents an intermediate stage in the formation process of the ternary Michaelis complex of TDO. On the contrary, IDO is generally believed to bind O_2 prior to L-Trp [73,74] (Scheme 4B), despite the existence of different opinions in early literature [21]. This assembly mode of the ternary Michaelis complex is proposed based on a collection of interesting and consistent observations. Unlike TDO, IDO can form its oxy-ferrous adduct regardless of L-Trp [75]. The rate constants for O_2 and CO binding to the heme center of ferrous IDO are not significantly perturbed by L-Trp [75]. In addition, Yeh et al. have shown that conversion of ferric IDO to the ferryl form via peroxide oxidation significantly facilitates L-Trp binding [44]. Combining this phenomenon with a previous observation that cyanide-bound ferric IDO has a much higher affinity towards L-Trp than the ligand-free ferric enzyme [73,76], it is suggested that regardless of the heme redox state, ligand binding to the heme iron of IDO can introduce conformational changes that are in favor of L-Trp binding [44]. Moreover, since the early studies of IDO, inhibition of the dioxygenase activity at high L-Trp concentrations is noted [15,77]. A recent mechanistic study by Raven and colleagues on the substrate-inhibition effect has revealed that this phenomenon can be accounted for by the sequential, ordered binding of O_2 and L-Trp [74]. At low concentrations of L-Trp, O_2 binds first followed by the binding of L-Trp; at higher concentrations of L-Trp, the order of binding events is reversed, and L-Trp binding disfavors the subsequent O_2 binding step, diminishing the catalytic activity [74]. Overall, the proposed mechanisms of Michaelis complex assembly for TDO and IDO are in accordance with the results of steady-state kinetic studies. In TDO, the K_m value of L-Trp is larger than the K_d value of L-Trp for the ligand-free ferrous enzyme [78]. However, in IDO the K_m value of L-Trp is much smaller than the K_d value of L-Trp for the ligand-free ferrous enzyme, while the K_m value of O_2 is similar to the K_d value of O_2 for the ligand-free ferrous enzyme [73,76,79].

The preferential binding of the primary substrate prior to O_2 binding is prevalent in iron-dependent oxygenases and has been shown to be beneficial in many cases, including cytochrome P450s and α-KG dependent non-heme oxygenases [21,80]. Recognition of the primary substrate usually triggers alterations in the microenvironment and coordination status of the metal center, thereby facilitating the subsequent O_2 binding by expelling solvent molecules from the active site to increase O_2 affinity or by removing solvent-derived ligands from the metal center to generate a coordination vacancy [21,80]. This substrate-binding strategy is also considered an *in vivo* protection mechanism, as retarding O_2 binding in the absence of the primary substrate can prevent oxidative damages to the metal center and avoid the release of reactive oxygen species [81]. In addition to the aforementioned benefits, in P450s, binding of the primary substrate increases the heme redox potential, which facilitates heme reduction by associated electron donors [21]. Similarly, it has been shown in xcTDO that L-Trp binding causes a significant positive shift in the redox potential of the heme center [24]. Thus, the preferential binding of L-Trp in TDO may play an additional physiological role to keep the heme iron from being oxidized. In contrast, the sequential binding of O_2 and L-Trp in IDO is unique, and little is known about the physiological significance of this seemingly irrational design. Plausibly, the distinctive assembly mode of the IDO ternary complex is important for ensuring enzyme turnover under substrate-deficient conditions [44].



Scheme 4. Assembly modes of the ternary Michaelis complex in TDO (A) and IDO (B).

Involvement of an active-site base in the first step of catalysis

Since the initial proposal by Hamilton [58], it has been generally believed that TDO and IDO begin the catalytic cycle with proton abstraction from the indole NH group of L-Trp by an active-site base [21,23,25,59,67,79,82,83] (Scheme 3A). Deprotonation of the indole group can generate an electron-rich environment at the indole C3 position and thus stimulate a nucleophilic attack at the distal oxygen of the heme-bound O_2. An intermediate, 3-indolenylperoxo, is generated as a result of the nucleophilic attack (Scheme 3A). This proposal is supported by a few pieces of experimental evidence, including a solvent kinetic isotope effect study on TDO, which reveals that a proton-transfer step is partially rate-limiting in the catalytic cycle [59]. In line with this proposal, a 3-indolenylhydroperoxo intermediate is identified during the oxidation of L-Trp by a singlet O_2 from an enzyme-free reaction system [84,85]. Despite a much lower specificity, decomposition of this intermediate is shown to yield NFK with both oxygen atoms from the singlet O_2 conserved in the product [84,85].

In TDO, the distal His72 residue is regarded as the acid–base catalyst, as suggested by the crystal structure of the binary complex of xcTDO and L-Trp, which shows that the corresponding histidine residue is H-bonded to the indole NH group of L-Trp [24] (Fig. 1C). In IDO, there is no apparent active-site residue candidate that plays such a role. Instead, it is proposed that the heme-bound O_2 functions as the acid–base catalyst [23,67,79,86]. By using CO as an O_2 surrogate, resonance Raman studies by Yeh and coworkers support the aforementioned assignments of the acid–base catalyst, with the distal histidine for TDO [67,69] and the heme-bound O_2 for IDO [86].

In the recent years, more and more studies have cast serious doubt on the base-dependent catalytic mechanism. Firstly, the indole NH group has a very high pK_a value at *ca.* 17 [87], so it would be extremely difficult for weak bases such as the imidazole moiety of His72 and the heme-bound O_2 to abstract a proton from it. Recent computational studies, including density functional theory (DFT), molecular dynamics (MD), quantum mechanics/molecular mechanics (QM/MM) and ONIOM calculations, also disfavor the involvement of an acid–base catalyst by demonstrating that the base-catalyzed deprotonation of the indole group is not energetically favorable, compared to the direct addition of the heme-bound O_2 [62–66]. Moreover, replacement of the distal histidine residue in TDO failed to shut down the enzyme, and the distal histidine mutants from different sources all exhibited a detectable activity [55,67,78,83,88], suggesting that deprotonation of the indole group is not critical for catalysis in TDO. In a spectroscopic study on the

cryoreduced ternary complex of xcTDO, Davydov et al. show that mutation of the distal histidine or methylation of the indole nitrogen of L-Trp has no observable effect on the spectroscopic properties of the oxy heme moiety or its annealing behavior, thus arguing against the hypothesis that proton abstraction of the indole group is the initial catalytic step in TDO [68].

The most solid evidence against the base-dependent mechanism is the observation that 1-methyl-L-tryptophan (1-Me-L-Trp) is a substrate for IDO, cmTDO, and the distal histidine variants of human TDO (hTDO) and xcTDO [55,89]. Notably, cmTDO displays a significant activity towards 1-Me-L-Trp with a k_{cat} value comparable to that of cmTDO with L-Trp as the substrate [55]. Thus, substrate deprotonation is not an inevitable course; instead, direct addition of the heme-bound O_2 is more likely to occur in the first step of TDO and IDO.

Ferryl species of TDO and IDO

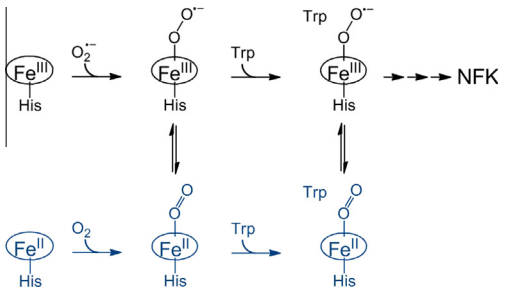
Regardless of whether deprotonation of the indole NH group occurs or not, an indolenylperoxo-type intermediate, in which the heme iron and L-Trp are linked by dioxygen, is generated upon addition of the distal oxygen to L-Trp (Scheme 3). However, the protonation state of the indole moiety can affect the addition position of the distal oxygen on the indole ring and further influence the manner of O—O bond cleavage. In the base-dependent mechanism, nucleophilic attack by the indole C3 at the distal oxygen yields a 3-indolenylperoxo intermediate (Scheme 3A). Subsequently, the O—O bond is cleaved heterolytically and the two oxygen atoms are inserted into L-Trp via either a Criegee rearrangement pathway or a dioxetane pathway (Scheme 3A). Notably, there is no high-valence heme species involved in this mechanism. As the base-catalyzed proton abstraction process is disproved by a large body of experimental and computational results, an alternative catalytic mechanism has received general recognition in the field [55,60–66,74] (Scheme 3B). In this mechanism, the oxy-ferrous heme adduct possesses ferric superoxide characteristics, and direct radical attack at the indole C2 position by the distal oxygen occurs (Scheme 3B). As a result, a 2-indolenylperoxo intermediate is generated. This intermediate is decomposed through homolytic O—O bond cleavage, generating a compound II-type ferryl species plus a monooxygenated form of L-Trp as an epoxide intermediate, which subsequently recombine to generate NFK (Scheme 3B).

The ferryl-dependent dioxygenase mechanism is supported by recent computational calculations [62,64–66] and experimental results [62,90–92], as well as some indirect evidence from the early studies of IDO. Notably, a unique "superoxide shunt" pathway has been previously identified in IDO, in which ferric IDO can utilize superoxide $O_2^{\cdot-}$ as a co-substrate to catalyze the dioxygenase reaction of L-Trp both *in vitro* and *in vivo* [21,93–96] (Scheme 5). Spectroscopic studies have revealed that ferric IDO can bind to and react with $O_2^{\cdot-}$ to yield the oxy-ferrous complex [46,75,93,97], which can be also generated by addition of O_2 to the ferrous enzyme as shown in Scheme 4 and 5. Moreover, it has been reported that the oxy-ferrous complex of IDO readily oxidizes itself to the ferric state by releasing O_2 as $O_2^{\cdot-}$, regardless of whether L-Trp is present or not [21,62,75,77]. Taken together, one can conclude that regardless of L-Trp, the oxy-ferrous adduct of IDO is in equilibrium with the corresponding ferric superoxide complex, which is the reactive species responsible for direct radical addition to L-Trp (Scheme 5). In fact, resonance Raman characterization of the ternary Michaelis complex of IDO proves that the heme-bound O_2 exhibits superoxide characteristics [62]. Significant progress in the mechanistic studies of TDO and IDO came in 2009 when the proposed compound II-type ferryl intermediate was trapped and characterized in IDO by Yeh et al. [62]. This observation was later confirmed by independent work from others [90,91]. Soon afterwards, the existence of the epoxide intermediate of L-Trp in the reaction cycle was inferred by a mass spectrometry study from the Raven laboratory, in which a side product of monooxygenated L-Trp was detected from the dioxygenase reaction of IDO, xcTDO, and hTDO [92].

Despite intensive efforts from different groups, the proposed ferryl intermediate has not yet been captured in the dioxygenase reaction of TDO. The ferryl intermediate observed in IDO is reported in a low occupancy [62,91] and lacks additional support and further characterization from different spectroscopic methods. To obtain detailed information on the chemical properties of the ferryl species in TDO and IDO, we and others have adopted an alternative pathway to produce the ferryl species in high yields via reactions with H_2O_2 [43,44,47,55]. In TDO, a compound ES-type ferryl species is generated by reacting ferric TDO with H_2O_2 and characterized by optical, EPR, and Mössbauer spectroscopy [43,55]. The Mössbauer characterization reveals a unique quadrupole splitting (ΔE_Q) value (1.755 mm/s determined at pH 7.4) for the ferryl moiety of this species [43], which lies between the ranges for protonated Fe(IV)—OH species (2.0–2.5 mm/s) and unprotonated Fe(IV)═O species (1.0–1.6 mm/s) [98–102]. Subsequent DFT calculations indicate that the unusual ΔE_Q value originates from H-bonding interaction to the ferryl-oxo group provided by the protein matrix [43]. In IDO, Lu and Yeh have described a compound II-type ferryl species which is produced upon addition of H_2O_2 to ferric IDO [44]. This high-valence species is capable of oxidizing peroxidases substrates such as ABTS, but not L-Trp [44], which is consistent with a previous report that by using H_2O_2 as a co-substrate, IDO converts some L-Trp catabolites including melatonin, serotonin, and tryptamine, but not L-Trp, to oxygenated products [103]. The poor reactivity of the ferryl species towards L-Trp also explains why the H_2O_2-mediated reactivation pathway is not present in IDO.

Catalytic roles of the distal histidine in TDO

One of the major differences between the active site architectures of TDO and IDO is a distal histidine residue, which is present in TDO (His72 in cmTDO amino acid numbering) but substituted by a serine (Ser167) in IDO (Fig. 1C and 1D). The distal histidine is highly conserved and is replaced by threonine in only three putative TDO sequences [25]. It has been demonstrated by various studies that His72 possesses multiple functions prior to and during the assembly of the Michaelis complex to ensure efficient catalysis of TDO. In the crystal structure of the binary complex of xcTDO and L-Trp, the corresponding distal histidine is H-bonded to the indole NH group of the substrate, suggesting its involvement in substrate binding [24]. Indeed, it has been shown in cmTDO that complete elimination of this H-bonding interaction by introducing 1-Me-L-Trp as a substrate results in a significant increase in the substrate K_m value but only moderately affected the k_{cat} value of



Scheme 5. The "superoxide shunt" pathway in IDO. The bottom branch in blue shows the native O_2-dependent pathway. The oxy-ferrous adducts are in equilibrium with the corresponding ferric superoxide complexes regardless of the presence of L-Trp, connecting the two pathways.

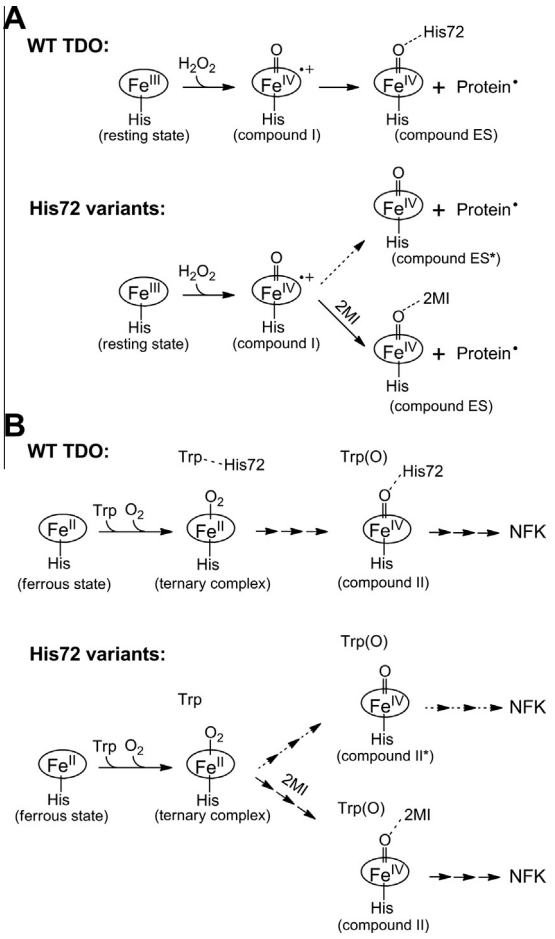
the dioxygenase reaction [55]. EPR studies on the binding events of small-molecule ligands, *i.e.* imidazole and its analogs, show that these molecules are capable of directly coordinating to the heme iron of the His72 mutants of cmTDO but not the wild-type (WT) enzyme [55]. This observation indicates that possibly via steric hindrance, His72 efficiently shields the heme center from nonproductive binding of exogenous small-molecule ligands. Consistent with this proposed function, it has been demonstrated in xcTDO that the distal histidine can retard L-Trp binding to the ferric enzyme and thus prevent formation of non-productive ferric enzyme-substrate complexes, as revealed by a sharp increase in the affinity of L-Trp to the ferric enzyme upon replacement of the distal histidine [78].

The interactions between the substrate and the His72 residue are important in maintaining the proper binding conformation of L-Trp as well as the tight contacts in the ternary Michaelis complex. The crystal structures of the binary substrate-enzyme complexes of the distal histidine mutants of xcTDO show observable displacements of the substrate away from the putative O_2-binding site, as compared to the WT protein structure [78]. EPR studies on the substrate-induced spin-transition phenomenon in cmTDO reveal that mutation of His72 alters the binding conformation of the substrate, which subsequently affects the pK_a value of the active-site water [55]. Resonance Raman studies on hTDO demonstrate that the tight interactions in the Michaelis complex of the WT enzyme becomes relaxed in the distal histidine mutants [67].

Although His72 is generally recognized to make considerable contributions prior to and during the assembly of the Michaelis complex, no consensus has been reached regarding whether and how it plays a catalytic role in later steps. As previously mentioned, His72 was initially proposed to function as an acid–base catalyst, deprotonating the indole NH group of L-Trp to initiate the reaction. Since the base-dependent catalytic mechanism receives more and more objections, the involvement of His72 in the chemical catalysis steps of the reaction cycle remains elusive. Nonetheless, distal histidine variants of TDOs from different sources all present a notable decline in the enzymatic activity, i.e. a decrease in the k_{cat} value plus an increase in the K_m value, as compared to WT enzymes [55,67,78,83,88].

Our recent chemical-rescue study on cmTDO has shown that the diminished catalytic activity due to the replacement of His72 can be recovered to an appreciable degree by an exogenous histidine analog, 2-methylimidazole (2MI) [55]. While 2MI is able to cause an significant increase in the k_{cat} values of the His72 mutants, it only minutely altered the substrate K_m values [55]. Thus, the chemical-rescue effect mainly acts on the chemical catalysis steps rather than the substrate binding steps. This suggests that the enzyme-bound 2MI molecule is able to participate in the chemical catalysis steps in a way that mimics His72.

As aforementioned, a compound ES species of TDO can be generated by addition of H_2O_2 to the ferric protein [43]. In the His72 mutants, accumulation of the compound ES species cannot be observed unless a substantially higher concentration of H_2O_2 (i.e. above 100 mM) is used. The chemical rescue agent, 2MI, is shown to rescue the disappearing compound ES species from the reactions between the ferric His72 mutants and H_2O_2 [55]. Notably, the optical spectroscopic features of the rescued mutant compound ES species are the same as those of the WT compound ES species. However, they are distinctive from those of the mutant compound ES species that are forced to accumulate by a large excess of H_2O_2 [55]. The differences of the spectral features between the former two groups of ferryl species and the latter one are believed to result from the loss of a H-bonding interaction to the Fe(IV)-oxo moiety. This interaction is contributed by either His72 in the case of the ferryl species from WT TDO or 2MI in the case of the rescued ferryl species from the mutants [55] (Scheme 6A). Similar to



Scheme 6. Proposed roles of the distal histidine (His72) in TDO during the peroxide reaction (A) and the dioxygenase reaction (B). Notably, "compound ES" differs from "compound ES*" by an H-bonding interaction with either His72 or 2MI. The same difference is also present between "compound II" and "compound II*. The accumulation of "compound ES*" can be observed only in the presence of highly concentrated H_2O_2 (i.e. over 100 mM).

Scheme 6A, Scheme 6B illustrates the proposed role of His72 in the dioxygenase reaction after assembly of the ternary complex. His72 is anticipated to facilitate chemical catalysis via H-bonding interactions to the oxygen-bound heme intermediates [55]. The key catalytic intermediate is the proposed compound II-type ferryl species, which is similar to but not the same as the compound ES species observed in the peroxide reactions. In both scenarios of Scheme 6, the physical properties and reactivities of the ferryl species are dependent on the presence of His72. In its absence, 2MI can effectively participate in the part played by His72, thus rescuing the enzymatic activities.

Concluding remarks

Unexpectedly, TDO and IDO appear to employ a catalytic strategy that is highly reminiscent of those in some non-heme iron-containing oxygenases and oxidases, such as α-KG-dependent oxygenases, pterin-dependent oxygenases, isopenicilin *N* synthase (IPNS), and CloR. These enzymes utilize a ferric superoxide species, to claim the electrons needed for hemolytic cleavage of the O—O bond of O_2 from the substrates or co-substrates [80,104]. This course of action provides the catalytic driving force for substrate activation and ensures that all the oxidizing equivalents from O_2 can be properly transferred into the substrates and co-substrates,

avoiding the incorporation of electron donors and associated mediators to the reaction cycle. The ferric superoxide species and subsequent ferryl intermediates allow the enzymes to sequentially target two positions of the substrates, making possible a collection of difficult reactions. In TDO and IDO, the utilization of ferric superoxide as an oxidant for substrate activation is unprecedented in heme-containing oxygenases. A recent computational study by Lai and Shaik has revealed the origin of the different activities of the superoxo species between P450s and TDO/IDO, and the axial heme ligand has been suggested to be the major determinant [105]. Moreover, direct oxygen insertion performed by a compound II-type ferryl species is also exceptional in heme chemistry. Compound II complexes are usually considered languid oxidants compared to Compound I complexes. This demanding task is achieved by the TDO/IDO compound II species, mostly because the other reactant, i.e. the putative epoxide intermediate of L-Trp, is already activated and fairly reactive. Currently, a detailed mechanism of the second oxygen insertion step mediated by the TDO/IDO compound II intermediate is under development, and the hypotheses from computational studies await confirmation by experimental results [65,66]. Nonetheless, the tryptophan oxidation reactions catalyzed by TDO and IDO have proven to be a distinctive prototype of heme-dependent reactions. The valuable information obtained by a collective effort from the field of enzymology has again demonstrated the remarkable catalytic versatility of hemoproteins and is inspiring to a broad science community.

Acknowledgement

This work was supported by NSF Grant MCB-0843537 (A.L.). J.G. acknowledges fellowship support from the Molecular Basis of Disease program of Georgia State University.

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