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# Reassessment of the Reaction Mechanism in the Heme Dioxygenases

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Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) are heme enzymes that catalyze oxidation of L-tryptophan to *N*-formyl-kynurenine (Scheme 1),[1−3] in a mechanism that involves binding of dioxygen to reduced iron. The mechanistic details of this oxidation are not yet known, but early studies[3] suggested base-catalyzed deprotonation of the indole NH group (Scheme 2A). This was based largely on the observation that 1-methyl-L-tryptophan (1-Me-L-Trp) is an inhibitor of dioxygenase activity and was plausible since base-catalyzed abstraction is not possible with the methylated compound. There are problems with this mechanism, however. To begin with, it is inconsistent with the chemistry of indoles,[4] which typically react by electrophilic addition across the $C_3$ position and subsequent formation of a cation at $N_1$. In addition, the structure of human IDO (hIDO)[5] reveals that there is no active-site base close enough for proton abstraction at $N_1$. The only polar active-site residue is Ser167, but this is not essential for activity.[5,6] Although *X. campestris* TDO (*Xc*TDO) does contain an active-site histidine (His55, equivalent to Ser167 in hIDO)[7] which hydrogen bonds to the indole $N_1$, it is not essential for activity.[7,8] Together, this led to the hypothesis[5,7] that the bound dioxygen might act as the active-site base (Scheme 2B), with no involvement from active-site residues.

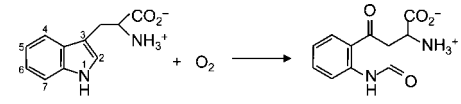
Here, we examine the activity of three heme dioxygenases (hIDO, human TDO (hTDO) and *Xc*TDO[9]) with 1-Me-L-Trp, including a number of site-directed variants focused on the His55/Ser167 location. In contrast to previous work,[10] we find that 1-Me-L-Trp is a slow substrate. These observations are inconsistent with current proposals for the mechanism of substrate oxidation, and we propose an alternative.

To determine if the purified enzymes could utilize 1-Me-L-Trp as a substrate, identification of the methylated product was essential.[11,12] Formation of *N*-formyl-kynurenine and *N*-formyl-methylkynurenine was confirmed at 321 nm (Figure S2, solid and dashed line, respectively). The reaction with 1-Me-L-Trp was repeated in the absence of enzyme (Figure S2, dotted line) and no absorbance change at 321 nm was observed, confirming that product formation is from enzymatic oxidation. LC−MS analysis using selected ion monitoring (shown for hIDO, Figure 1A) was then carried out. For the reaction with L-Trp, this gave an ion with $m/z = 237$ (Figure 1B, *top*) which corresponds to the mass of *N*-formyl-kynurenine ($m/z = 236$); for the reaction with 1-Me-L-Trp, an equivalent ion is detected at $m/z = 251$ (Figure 1B, *bottom*) as expected for *N*-formyl-methylkynurenine ($m/z = 250$).

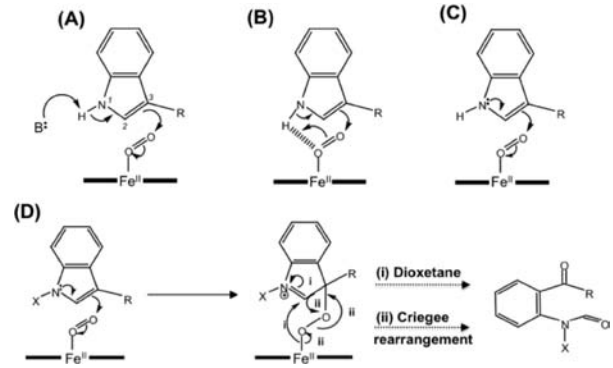
Steady-state kinetic parameters for the oxidation of 1-Me-L-Trp were also determined[6,7] (Figure S3 and Table 1).[13] Clear increases in absorbance, corresponding to product formation, are observed for hIDO (Figure S3), as well as for the S167A variant of hIDO and variants of both hTDO and *Xc*TDO (Table 1).[13]

These data clearly indicate that formation of *N*-formyl-methylkynurenine occurs upon reaction of 1-Me-L-Trp with hIDO and variants of hIDO, hTDO, and *Xc*TDO. However, no activity was detected for hTDO and *Xc*TDO with 1-Me-L-Trp in either steady-

***Scheme 1.*** Reaction Catalyzed by IDO and TDO




***Scheme 2.*** Possible Mechanisms for Oxidation of Trp ($C_2$, $C_3$, and $N_1$ of the Substrate Are Labeled)[a]




[a] (A) Abstraction of the indole proton by an active-site base.[3,15] (B) Abstraction of the indole proton by the heme-bound dioxygen.[5,16] (C) Direct electrophilic addition. (D) A revised mechanism for product formation in heme dioxygenases, where X = H or Me. The conversion to product is proposed to occur by either a Criegee or dioxetane mechanism.[3]

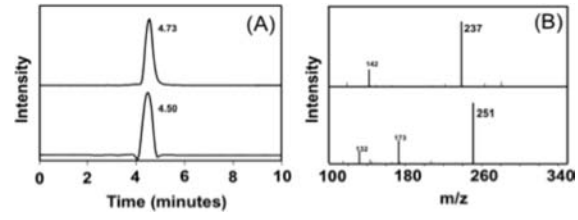



***Figure 1.*** LC−MS analyses of products obtained on reaction of hIDO with *N*-formyl-kynurenine (top) and *N*-formyl-methylkynurenine (bottom). Panel A: Elution profiles for selected ion chromatograms with *m/z* of (top) 237 and (bottom) 251, corresponding to *N*-formyl-kynurenine and *N*-formyl-methylkynurenine, respectively. Panel B: Corresponding positive ESI mass spectra for the products eluted at 4.73 and 4.50 min (top and bottom, respectively).

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***Table 1.*** Steady-State Parameters ($k_{cat}$, $K_M$) for Oxidation of 1-Me-L-Trp

| | variant | $k_{cat}$ ($s^{-1}$) | $K_M$ ($\mu$M) |
|---|---|---|---|
| hIDO | native | $0.027 \pm 0.001$ | $150 \pm 11$ |
| | S167A | $0.032 \pm 0.002$ | $31 \pm 5.0$ |
| hTDO | native | – | – |
| | H76S | $0.023 \pm 0.001$ | $2300 \pm 230$ |
| *Xc*TDO | native | – | – |
| | H55A | $0.048 \pm 0.011$ | $59 \pm 16$ |
| | H55S | $0.052 \pm 0.009$ | $70 \pm 11$ |

state or LC−MS analyses. The structure of *Xc*TDO[7] reveals a hydrogen bond (2.6 Å) between the indole NH of L-Trp and His55, which would be expected to lead to a steric clash between His55 and the methyl group of 1-Me-L-Trp. Indeed, a model of 1-Me-L-Trp binding to *Xc*TDO (Figure S4) shows a nonbonding distance of ~1.5 Å between the $N^{\epsilon}$ of His55 and the carbon atom of the Me group on 1-Me-L-Trp. We propose that binding of 1-Me-L-Trp to *Xc*TDO is weak as a consequence. Sequence alignments[7] indicate that hTDO also contains a histidine (His76) in the same position (in fact this histidine residue is conserved in all TDOs), so that the same steric restrictions apply and thus explain the observed inactivity. Substitution of histidine in *Xc*TDO (H55A, H55S) and hTDO (H76S) allows accommodation of the additional methyl group, and turnover of 1-Me-L-Trp occurs (Table 1). This hypothesis is further supported by the fact that hIDO and S167A, which are both able to accommodate the bulky Me group in their active sites, are also able to oxidize 1-Me-L-Trp (Table 1).

Overall, turnover numbers for 1-Me-L-Trp are lower than those for L-Trp.[6,8,14] When X = Me (Scheme 2D), the inductive effect of the Me group would presumably stabilize the cation intermediate. This would not be expected to slow down the electrophilic attack but might affect other steps in the mechanism that our experiments do not address. A completely different binding location/orientation for 1-Me-L-Trp (and therefore a different mechanism) is also possible, but this is not supported by inhibition data (Table S1 and ref 10) which show 1-Me-L-Trp as a competitive inhibitor for L-Trp.

Since hIDO and variants of both hTDO and *Xc*TDO can oxidize 1-Me-L-Trp, deprotonation of the indole NH cannot be essential for catalysis. Mechanisms involving abstraction of the indole proton, using either an active-site base[3,15] or dioxygen[5,16] (Scheme 2A, B), are therefore unlikely as they cannot proceed with a methyl group on $N_1$.

The chemistry of indoles is very well documented[4] and does not occur by loss of the indole proton. Instead, when an indole reacts with an electrophile (e.g., $O_2$), the lone pair on $N_1$ initiates the process (Scheme 2C) and the electrophile becomes attached preferentially at $C_3$ (Scheme 2D).[17] We propose this as a more likely mechanism for tryptophan oxidation in the heme dioxygenases, allowing both L-Trp and 1-Me-L-Trp to react (Scheme 2D), with the role of the iron merely as a donor of the required oxygen molecule. This mechanism is compatible with the absence of an active-site base in hIDO and avoids the need to deprotonate an $N_1$ atom with a $pK_a$ (≈17[18]) that is out of range.[18] In addition, a recent density functional theory study[19] found that direct electrophilic addition has a lower activation energy than a base-catalyzed deprotonation mechanism, consistent with our observations.

In conclusion, we report that 1-Me-L-Trp is a substrate for hIDO and variants of hTDO and *Xc*TDO in which the active-site histidine has been replaced. This shows that deprotonation of the indole $N_1$ is not essential for catalysis, and we propose that direct electrophilic addition to dioxygen, facilitated by the lone pair on the indole $N_1$, occurs instead.

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**Supporting Information Available:** HPLC/NMR of 1-Me-L-Trp, spectra of *N*-formyl-kynurenine and *N*-formyl-methylkynurenine, steady-state data, and a model of the *Xc*TDO/Me-Trp complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) All proteins were isolated as described previously.[6,7,14]
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(11) Commercially available 1-Me-L-Trp (95% purity) was purified by HPLC to remove contaminating species which could act as substrate (Figure S1A), and the purity was confirmed by further HPLC and $^1$H NMR (Figure S1B, C).
(12) Steady-state assays contained sodium ascorbate (20 mM), methylene blue (10 $\mu$M), catalase (10 $\mu$g), 1-Me-L-Trp (variable), and enzyme (1−5 $\mu$M),[6,7] in either 50 mM Tris/HCl buffer, pH 8.0 (hIDO and hTDO and respective variants), or 100 mM phosphate buffer, pH 7.5 (*Xc*TDO and variants). Reactions were allowed to proceed for at least 1 h and then quenched by addition of 30% (v/v) trichloroacetic acid, followed by centrifugation to remove the enzyme. Values for $K_i$ were measured in the same way by varying [L-Trp] as above through differing concentrations of 1-Me-L-Trp. Data were analyzed by plotting $1/v$ (the observed rate) against 1/[L-Trp].
(13) The corresponding LC-MS data for these proteins also showed ions at 251, as for hIDO (data not shown).
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(17) Attack at $C_3$ generates a positive charge on $C_2$ which is resonance stabilized by the lone pair on $N_1$ (Scheme 2D). Attack at $C_2$, as proposed recently,[19] is less favourable and not observed in indole chemistry because this generates a positive charge on $C_3$, which is less well stabilized (through the benzene ring). This preference for attack at $C_3$ would also apply for radical addition.[19]
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