Hydrogen Movements in the Oxidative Half-Reaction of Kynurenine 3-Monooxygenase from *Pseudomonas fluorescens* Reveal the Mechanism of Hydroxylation

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This research was supported by National Science Foundation Grant 1904480 to G.R.M. and a UWM Research Growth Initiative Grant to G.R.M.

RUNNING TITLE: Hydrogen Movements in the Oxidative Half-Reaction of KMO

Abbreviations:

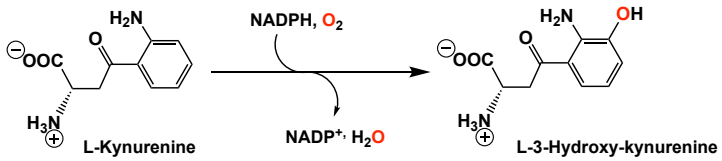
KMO; Kynurenine 3-monoxygenase, L-Kyn; L-kynurenine, 3-OHKyn; 3-hydroxykynurenine, BA; benzoyl-L-alanine, mNBA; meta- nitrobenzoyl-L-alanine.

Abstract

Kynurenine 3-monoxygenase (KMO) catalyzes the conversion of L-kynurenine (L-Kyn) to 3-hydroxykynurenine (3-OHKyn) in the pathway for tryptophan catabolism. We have investigated the effects of pH and deuterium substitution on the oxidative half-reaction of KMO from *P. fluorescens* (PfKMO). The three phases observed during the oxidative half reaction are formation of the hydroperoxyflavin, hydroxylation and product release. The measured rate constants for these phases proved largely unchanging with pH, suggesting that the KMO active site is insulated from exchange with solvent during catalysis. A solvent inventory study indicated that a solvent isotope effect of 2 - 3 is observed for the hydroxylation phase and that two or more protons are in flight during this step. An inverse isotope effect of 0.84 $\pm$ 0.01 on the rate constant for the hydroxylation step with ring perdeutero-L-Kyn as a substrate indicates a shift from sp^2 to sp^3 hybridization in the transition state leading to the formation of a non-aromatic intermediate. The pH dependence of transient state data collected for the substrate analog meta-nitrobenzoylalanine indicate that groups proximal to the hydroperoxyflavin are titrated in the range pH 5 - 8.5 and can be described by a pKa of 8.8. That the higher pH values do not slow the rate of hydroxylation precludes that the pKa measured pertains to the proton of the hydroperoxflavin. Together, these observations indicate that the C4a-hydroperoxyflavin has a pKa >> 8.5, that a non-aromatic species is the immediate product of hydroxylation and that at least two solvent derived protons are in-flight during oxygen insertion to the substrate aromatic ring. A unifying mechanistic proposal for these observations is proposed.

Keywords: kynurenine, monooxygenase, hydroxylase, aromatic, flavin, dioxygen, ischemia, stroke.

Kynurenine 3-monooxygenase (KMO) catalyzes the hydroxylation of L-kynurenine (L-Kyn) to 3-hydroxykynurenine (3-OHKyn) (Scheme 1). This activity is central to the tryptophan degradation pathway that in higher organisms leads to the production of nicotinamide and picolinic acid [1].



Scheme 1

KMO has been identified as a therapeutic target for limiting neuronal damage from ischemia and specific diseases [2-13]. The inhibition of KMO has been reported to have synergistic neuroprotective effects that include elevation of the concentration of kynurenate, that both slows glutamate release and antagonizes NMDA receptors, reducing aberrant excitation [14]. Inhibition of KMO also has the added benefit of halting the accumulation of specific neurotoxic and/or apoptotic metabolites, such as 3-OHKyn and quinolinic acid [4, 15].

KMO is a class A external flavoprotein aromatic hydroxylase (FAH)[16]. This class of enzymes use the isoalloxazine ring of FAD to mediate the delivery of electrons from singlet state NADPH to the molecular oxygen ground state triplet in order to promote subsequent hydroxylation of singlet state molecules. In these reactions, the flavin ring system is intimately involved in the ensuing chemistry and the spectrophotometric reporting power of the isoalloxazine has enabled the chemical mechanism of a number of FAH enzymes, including KMO, to be studied in considerable detail [17-19]. The mechanism of KMO is typical of class A FAH enzymes that exhibit ping-pong kinetics in which the aromatic substrate binds first, followed by NADPH and the reduction of FAD. The $NADP^+$ formed must then dissociate prior to the addition

of molecular oxygen and the commencement of the oxidative catalytic steps [15, 16]. The ping-pong mechanism thus facilitates the observation of the reductive and oxidative half-reactions in separate experiments by controlling the availability of the third substrate, dioxygen. For the paradigm FAH enzyme, para-hydroxybenzoate hydroxylase (PHBH), the association of substrate, stimulation of reduction, hydroxylation, and the dissociation of products have been found to be reliant on coordination of proton abstraction with dynamic movement of the flavin isoalloxazine [20, 21]. The movement of the flavin cofactor to the "out" position is required for reduction by NADPH in order to effectively couple aromatic substrate binding to hydride transfer [22]. Once reduced, the anionic flavin ring system returns to a more sheltered "in" position adjacent to the aromatic ring of the substrate. This proximity allows for reduction of molecular oxygen to be fully coupled to substrate hydroxylation via the intermediacy of a stabilized flavin-C4a-hydroperoxy species. For PHBH, it has been shown that a network of H-bonds both detects the native substrate and deprotonates it to activate the aromatic ring for the electrophilic substitution/hydroxylation to occur. It has also been suggested that this network is used to deprotonate the product resulting in a dianion that promotes flavin movement required for product release [20, 23, 24]. However, this proton network would appear to be unique to PHBH as a physiological imperative in order to discriminate between its substrate, p-hydroxybenzoate (pOHB) and p-aminobenzoate [23, 25, 26]. In most other FAH enzymes, including KMO, there is no evidence to indicate the existence of a similar H-bond network [27]. In truth, the aromatic substrate binding pocket is highly variable in this class of enzymes [17], and so each enzyme likely has unique strategies to execute the catalytic choreography required to complete the complex three substrate/three product redox reaction.

In this investigation, the movement of hydrogens in the oxidative half-reaction of KMO was studied. This includes the effect of pH on the observable steps of the oxidative half-reaction that revealed the active site is sheltered from solvent during this phase of catalysis. Kinetic isotope effects on the hydroxylation reaction using ring-perdeuterated L-Kyn as a substrate indicates that a non-aromatic intermediate form of the product is generated with hydroxylation. A proton inventory was used to estimate that two or more protons are in flight in the hydroxylation transition state. In addition, the first spectrophotometric estimation of a pKa associated with a FAH enzyme hydroperoxyflavin from comparison of multiple transient state datasets was made in the presence of a non-activated substrate analog. These data are discussed relative to the proposed mechanisms for the hydroxylation reaction of KMO.

Materials and Methods

Materials: Flavin adenine dinucleotide (FAD), L-kynurenine (L-Kyn), β-mercaptoethanol (βME), HEPES, Tris, Bis-Tris, MES and acetic acid buffers were purchased from ACROS. NADPH was purchased from Alexis. D_2O, NaOD, and D_2SO_4 were each purchased from Cambridge Isotopes. Dithiothreitol, xanthine, xanthine oxidase, meta-nitrobenzoylalanine and methyl viologen were purchased from Millipore-Sigma. Benzoylalanine (BA) was provided as a gift from Robert S Phillips at the University of Georgia, Athens, GA [28]. Ring perdeuterated L-Kyn ($^2H_4$-L-Kyn) was provided by Dr. Markey of the laboratory of Neurotoxicology at the National Institute of Mental Health, Bethesda, MD.

Preparation of Kynurenine 3-monooxygenase: PfKMO was expressed, and purified according to previously published methods [29]. All enzyme manipulations were undertaken at or below 4 ˚C. Enzyme samples were concentrated to ~200 μM in 20 mM HEPES, 2 mM βME, pH 7.5. For experiments carried out in buffered deuterium oxide solution, the enzyme was exchanged using repeated steps of centrifugal concentration with Amicon 10 kDa nominal molecular weight cutoff filters and subsequent dilution in the buffered deuterated solvent. Deuterium oxide buffer and substrate solutions were prepared by dissolving reagents in D_2O and making pH adjustments with concentrated NaOD and/or D_2SO_4 with correction for the response of the pH electrode for D_2O solvent [30]. For experiments that involved a wide range of pH values, a mixture of either 25 mM acetic acid, 25 mM Bis-Tris, and 50 mM Tris or 50 mM acetic acid, 50 mM MES, 100 mM Tris (all concentrations post mixing) was used to buffer for pHs from 5.2-8.5 [31].

Transient State Observation of the Oxidative Half-reaction of PfKMO: The influence of pH on the observable steps of the oxidative half-reaction were measured by pH-jump methods using a HiTech (TgK Scientific) DX2 stopped-flow spectrophotometer equipped with ceramic valves and a PEEK flow circuit. This involved mixing ~40 μM of reduced PfKMO, 2 mM DTT in the presence of saturating L-Kyn or inhibitor (BA or mNBA) in 5-20 mM HEPES pH 7.5 with defined concentrations of dissolved molecular oxygen in a dominant three component buffer titrated with nitric acid to a specific pH. Saturating ligand concentrations were based on measured dissociation constants for each ligand in the reduced enzyme complex [29]. The reduced enzyme•ligand complex was prepared anaerobically in a tonometer using published methods and reduced over 16 hours using xanthine (200 μM)/xanthine oxidase (1 U) with methyl viologen (5 μM) as a mediator [15, 32, 33]. The reduced anaerobic sample was then mounted onto the stopped-flow spectrophotometer for which residual oxygen had been eliminated by the introduction of a solution of anaerobic glucose (10 mM) and glucose oxidase (2 U/mL) for a minimum of 3 hrs. Solutions of dissolved dioxygen were prepared by sparging a buffer solution for 5 min with a defined ratio of dioxygen and dinitrogen gases blended with a Maxtec gas blender. The concentration was assessed by sparging the reaction vessel of a calibrated Hansatech Clark electrode until the signal recorded came to equilibrium.

The absorbance changes that occurred in the oxidative half-reaction were monitored using both single wavelength, photomultiplier tube (PMT) and multi-wavelength, charged couple device (CCD) detection. In PMT mode, absorption changes were observed at 415 nm, a wavelength that permitted delineated observation of each phase of the oxidative half-reaction. Each absorption trace was fit to a sum of three exponentials (Equation 1).

Equation 1 $$A_{415nm} = A_1(e^{-k_{1obs}t}) + A_2(e^{-k_2t}) + A_3(e^{-k_3t}) + C$$

From this equation, the absorbance amplitudes (A_n) and associated rate constants (k_n) were determined; C was the absorbance of the flavin cofactor at 415 nm in the resting enzyme. k_{1obs} is defined as such to denote a second-order reaction observed for one specific dioxygen concentration.

The intermediate spectra observed for the KMO_{RED}•mNBA oxidative half-reaction were obtained by deconvolution of CCD derived data sets using the Spectrafit module of KinTek Explorer software. The data were collected for two timeframes (1.6 and 168 seconds) and spliced together at 1.6 seconds to yield data sets with sufficient time resolution to describe the rapid and slow steps observed. The data were fit to a two-step irreversible model to yield the pure spectra of three species that comprise the reaction in the presence of this ligand. The effect of pH on rate constants or absorption changes was described by a single ionization expression (Equation 2) [34]. The Ka values determined from titratable phenomena (X) were determined by plotting proton concentration against the observed change; where X_{AH} and X_{A^-} represent the respective fully protonated and unprotonated arms of the titration.

Equation 2 $$Y = \frac{(X_{AH}[H^+] + K_a X_{A-})}{[H^+] + K_a}$$

Solvent Isotope Effects and Proton Inventories: The rate constants for observable steps of the oxidative half-reaction under varied fractions of D_2O were measured spectrophotometrically

using stopped-flow methods. For both the KMO_{red}•ligand complex and the dioxygen solution, two reaction mixtures were prepared, one in 100 % H_2O and one in 100 % D_2O. The enzyme was prepared in 5 mM HEPES, pL 7.5 while buffers to be equilibrated with dioxygen comprised a dominant three-component buffer (25 mM acetic acid, 25 mM Bis-Tris, 50 mM Tris) titrated to pL 6.0 and equilibrated with molecular oxygen (~1850 μM). Target D_2O fractions were then prepared by combining ratios of the two enzyme or two dioxygen solutions prior to preparing anaerobically in a tonometer or sparging with dioxygen respectively.

To evaluate the proton inventory resulting from medium effects or specific transition-states, first-order rate constants were fit to variations of the Kresge (Gross-Butler) equation, Equation 3 and 4, respectively [35, 36]. The ratio of rate constants (k_X/k_H), where H is 100% H_2O and X is the fraction of D_2O, were plotted against the atomic fraction of deuterium in solvent, *n*. The inverse of the transition state fractionation factor with deuterium, 1/θT, is equal to the kinetic isotope effect when there is a single proton contributing to the effect, or v = 1.

Equation 3 $$k_X = k_H(\frac{k_H}{k_D})^{-n}$$

Equation 4 $$k_X = k_H(1 - n + n\theta^T)^{\nu}$$

Kinetic Isotope Effects: Kinetic isotope effect experiments were carried out using a stopped-flow spectrophotometer and ring-per-deuterium labeled L-Kyn. Two KMO reaction mixtures (20 μM final) were made anaerobic and reduced as described above, each containing saturating concentrations of L-Kyn or $^2H_4$-L-Kyn in 20mM HEPES, 2 mM DTT, pH 7.5. Each reduced anaerobic sample was mixed with 20 mM HEPES pH 7.5 equilibrated to 1850 μM dioxygen concentration at

5˚C. Absorbance changes were monitored using PMT detection at 415 nm. Reaction traces were compared and twelve were combined into one average trace for each isotopomer. These data were then fit to the sum of three exponentials (Equation 1).

Results

Effects of pH on the Steps of the Oxidative Half-Reaction: The paradigm enzyme of the Class A FAHs, PHBH, exhibits influence of the solvent pH on steps of the oxidative half-reaction. This influence is imparted principally through a hydrogen-bond relay that links the protonation state of the substrate to a histidine at the surface of the enzyme [21]. It has been proposed that this structural feature has a role in ostensibly every sector of the catalytic cycle[16, 17, 19]. It is interesting that this specialized structural feature is largely absent in other FAH enzymes [17]. In order to assess the influence of pH on the observable steps of the oxidative half-reaction of KMO, a series of pH jump experiments were undertaken. In these experiments, the reduced enzyme substrate (or inhibitor) complex in a relative low concentration of HEPES buffer was mixed with solutions of dioxygen titrated to a range of pH values. The buffer combination used provided constant ionic strength and was of sufficient concentration to define the pH of the reaction when mixed. These studies were undertaken with reduced KMO in complex with L-Kyn and also in complex with the substrate-like inhibitor, BA, which has a non-activated aromatic ring that does not promote/permit hydroxylation.

Kinetic experiments reported here were completed using specific buffer mixtures and as a result the measured rate constants and intermediate spectra differ slightly from those previously reported at the same pH [15]. For the KMO_{RED}•L-Kyn complex, the reaction with dioxygen produces triphasic absorption trace at 415 nm (Figure 1A) indicating the formation of two intermediates. Previously reported assigned identities of the two intermediates were the C4a-hydroperoxyflavin and the product complex (KMO_{OX}•3-OHKyn)[15]. The decay of this latter species was observed to be the primary rate-limiting phase and involved spectrophotometric

changes consistent with product release [15]. Neither the rate of formation of the C4a-hydroperoxyflavin (phase I), hydroxylation reaction (phase II) or product release (phase III) was significantly influenced by pH (Figure 1B).

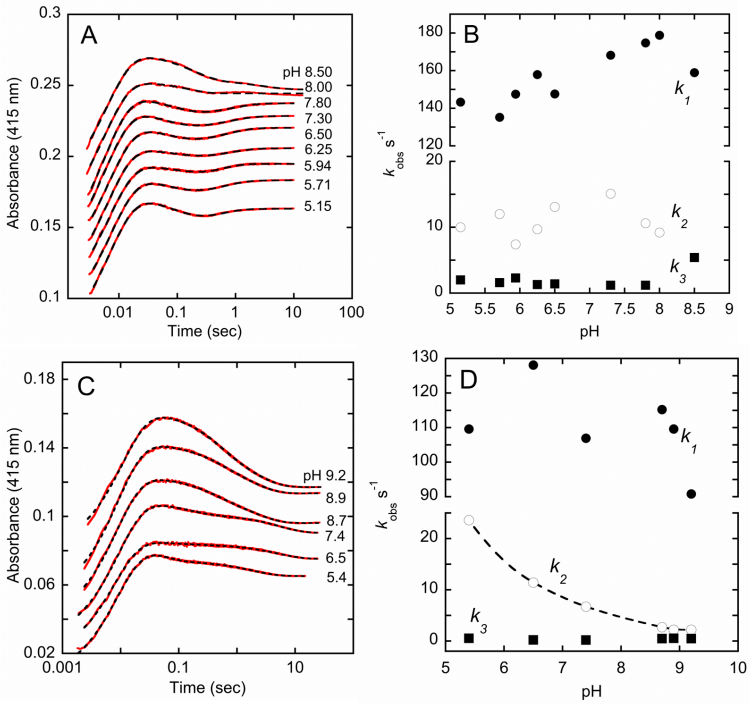


Figure 1: The dependence of pH upon the oxidative half-reaction for KMO_{RED}•L-Kyn and KMO_{RED}•BA. Anaerobic mixtures of 20 µM KMO_{RED}, in 5 mM HEPES, 1 mM DTT, pH 7.5 with saturating L-Kyn or BA were reacted with 1850 µM dioxygen concentration in 25 mM acetic acid, 25 mM Bis-Tris, 50 mM Tris (pH range 5–9) using a stopped-flow spectrophotometer at 5 ˚C. Kinetic traces were collected at 415 nm using photomultiplier tube (PMT) detection. The depicted traces are offset in absorbance to clearly show the apparent changes in amplitude and rate constants for each pH value. **A.** Absorption traces at 415 nm for the oxidative half-reaction of KMO_{RED}•L-Kyn complex. **B.** The rate constants measured from **A**. **C.** Absorption traces at 415 nm for the oxidative half-reaction of KMO_{RED}•BA complex. **D.** The rate constants measured **C.** The fit of the dependence for k_2 is an interpolated line.

The amplitudes associated with hydroxylation and product release did exhibit pH dependent changes and will be discussed more thoroughly below (Figure 1A & C).

The absorbance traces associated with the reaction of dioxygen with the KMO_{RED}•BA complex also have three phases and were fit to a sum of three exponentials (Equation 1, Figure 1B). Although hydroxylation of BA does not occur, it has been shown that dioxygen collision with reduced flavin in the presence of this ligand leads to a stabilized C4a-hydroperoxyflavin. This intermediate decays to hydrogen peroxide and, unexpectedly, the reaction concludes with a step that spectrophotometrically resembles what is observed for product release with the native product, 3-OHKyn. The rate constant observed of the formation of the hydroperoxyflavin and for the product release-like step exhibited no pH dependence (Figure 1C & D). Interestingly the

observed rate constant for the second phase decreased 11-fold with increasing pH. This phase represents decay of the hydroperoxyflavin to form hydrogen peroxide and so differs from the reaction observed with L-Kyn. Stabilization of the hydroperoxyflavin formed in the presence of BA at high pH isn't particularly mechanistically revealing as organic peroxides often show instability at higher pH values. Collectively, this and the lack of influence of pH on the rates observed for the oxidative half-reaction with L-Kyn suggests that the active site is largely insulated from the influence of the solvent.

***Solvent Isotope Effects and Proton Inventory*:** A proton inventory was carried out by observation of rate constant changes during the oxidative half-reaction using a three-buffer combination at pL 6.0. While no influence of pH was observed for rates of the oxidative steps, this pL value was selected as it corresponds to a condition well below the observed pKas determined from the absorption spectra of the hydroperoxyflavin and the oxidized enzyme (see below). For the KMO_{RED}•L-Kyn complex, complete assessment of the inventory was hampered in pre-steady state experiments by marked instability of KMO when incubated in high fractions of D_2O. As such, no reliable pre-steady state data was obtained for the D_2O fractions above 50%. The data for 0-50% D_2O clearly show that substrate hydroxylation (phase II) is slowed in deuterated solvent. An additional unassigned slower phase after product release was also unveiled with increasing deuterium oxide, no assignment of this phase has been made, and the rate constant for this event is below that of turnover (Figure 2).

The proton inventory data sets were fit to the two-proton in flight transition state and solvent medium effect equations (Equation 3 and 4 respectively), and neither returned a measurably better fit (Figure 2 inset) as judged objectively by the R-value.

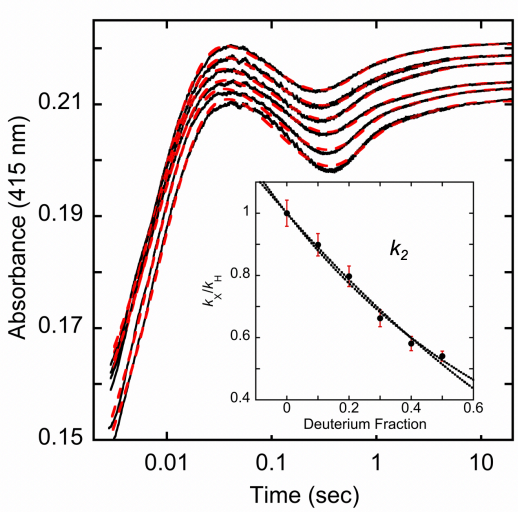


Figure 2. Solvent Isotope effects in the transient state. Absorbance traces for KMORED•L-Kyn complex reoxidation at various deuterium oxide fractions. Anaerobic mixtures of 20 μM KMO_{RED}, 1 mM DTT, and saturating L-Kyn in 5 mM HEPES, pH 7.5 were mixed against pseudo-first order dioxygen and varied deuterium oxide concentrations in a buffer mixture at pL 6.0, using a stopped-flow spectrophotometer at 5˚C. The changes in absorption were observed at 415 nm. Frpm top to bottom, the deuterium oxide percentages were 0%, 10%, 20%, 30%, 40%, and 50%. **Inset.** The dependence of deuterium oxide fraction on the ratios of the rate constants (k_X/k_H) for k_2. The data were fit to both two-proton transition state and medium effect equations.

Based on the fit to the medium effect, the solvent kinetic isotope effect (SKIE) was 3.6 ± 0.2. When fit to two-protons in flight an SKIE of 2.3 ± 0.1 was obtained. For either case, these data indicate that solvent derived protons are exchanged during substrate hydroxylation.

Oxidative Half-reaction in the Presence of Ring Perdeutero-L-Kyn: The oxidative half-reaction was also observed successively for the KMO_{RED}•L-Kyn and KMO_{RED}•$^2H_4$-L-Kyn (ring perdeutero) complexes. For native substrates that have a single activating substituent, insertion of the distal oxygen of the hydroperoxyflavin is believed to define the rate observed for hydroxylation. For this reason, it was expected that deuterium replacement on the ring at the C3 position (the site of hydroxylation) may result in a kinetic isotope effect arising from geometry changes at this site in the transition state for oxygen addition to the substrate (Scheme 2). Comparison of the

experiments undertaken in the presence and absence of the ring-deuterium yielded an inverse isotope effect of 0.84 ± 0.01 for the rate constant associated with hydroxylation (Figure 3).

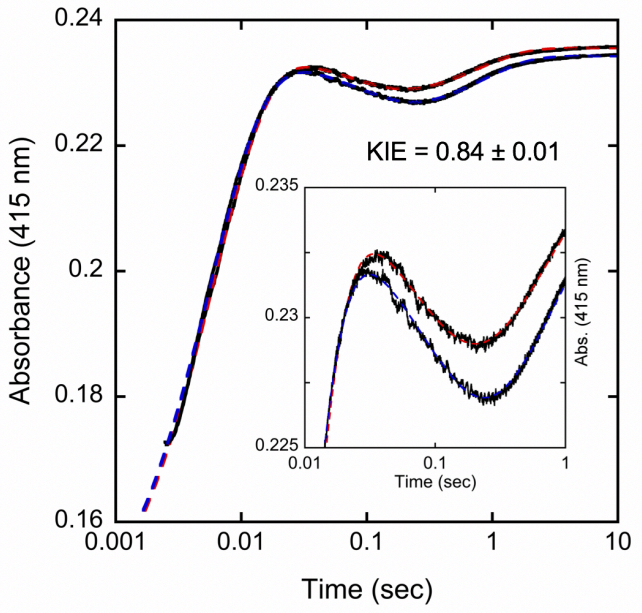


Figure 3. Absorbance traces for KMORED•L-Kyn and KMORED•$^2H_4$-L-Kyn complex reoxidation monitored at 415 nm. Anearobic mixtures of 20 µM KMO_{RED}, 1 mM DTT, and saturating ligand concentrations were mixed against pseudo-first order dioxygen (924 µM final) concentrations in 20 mM HEPES, pH 7.5 using a stopped-flow spectrophotometer at 5˚C. Rate constants for phase 1 and 3 remained constant. The KIE of 0.84±0.01 was derived from the average traces accumulated from 12 traces for k_H and 7 traces for k_D. Inset is an expansion of the data and fit for the isotope sensitive step.

No significant changes in rate constants for the first or third phases of the oxidative half-reaction with ring deuterium substitution were observed. An inverse effect at this stage in the reaction suggests that the second phase of the oxidative half-reaction is indeed the hydroxylation step and that the aromatic position C3 of L-Kyn experiences a sp^2 to sp^3 hybridization change for the transition state that occurs with electrophilic attack by the hydroperoxyflavin. This is consistent with the long-held consensus that the first species to occur with hydroxylation in FAH enzymes is a ring diene species that rearomatizes to form product. Despite data obtained for other FAH enzymes with substrate analogs that support this conclusion (see below), this is the first direct evidence for a non-aromatic intermediate arising during hydroxylation with a native substrate. We can therefore conclude that the non-aromatic (diene-imine) intermediate does form in the oxidative half-reaction, but in the net chemistry of hydroxylation the decay of this species is rapid.

Estimation of the pKa's Associated with the Oxidized flavin and Hydroperoxyflavin: An attempt to titrate the proton of the distal oxygen of the KMO hydroperoxyflavin was made by mixing anaerobic reduced KMO saturated with mNBA with dioxygen dissolved in a three-component dominant buffer. The mNBA analog was employed in place of L-Kyn principally to avoid the complication of the overlapping absorption contribution of the saturating substrate (L-Kyn λ_{max}= 360 nm, ε_{360} = 4000 $M^{-1}cm^{-1}$)[29]. In addition, the kinetics of the oxidative half-reaction with this analogue are relatively slow and simplify to two phases, providing greater certainty for the fit of the datasets. CCD data sets were collected for a range of pHs and the data were deconvoluted by fitting to a two-step model. The resultant spectra of the hydroperoxyflavin were then plotted and the pKa assessed at a wavelength where pH induced changes were greatest (480 nm) (Figure 4A).

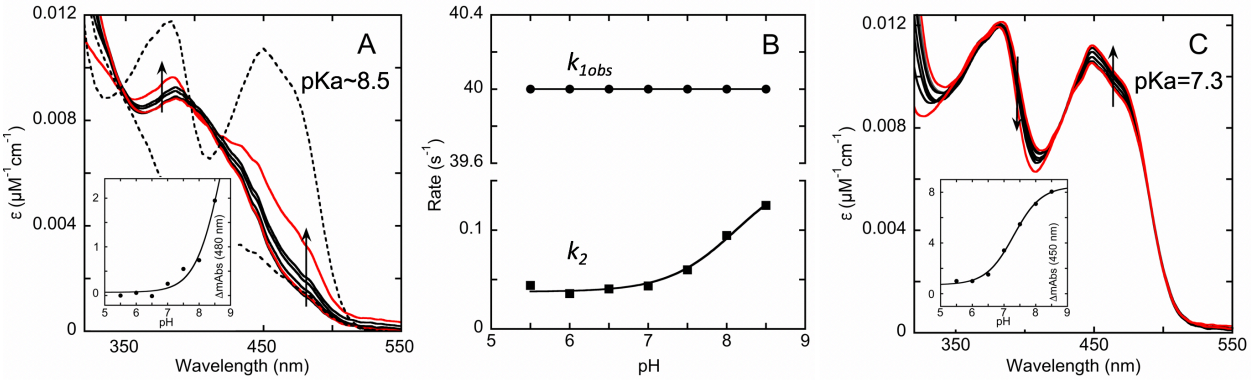


Figure 4. The pH dependence of the oxidative half reaction of KMO_{RED}•mNBA complex. KMO_{RED}•mNBA (20 μM KMO with 100 μM mNBA) in 20 mM HEPES pH 7.5 was reacted with 262 μM dioxygen prepared in 50 mM acetic acid, 50 mM MES, 100 mM Tris for buffering within pH range 5.5 – 8.5. The reaction was observed by CCD detection and individual datasets for analysis were constructed by splicing together data for short acquisition times (1.6 seconds) with data for long acquisition times (168 seconds). The resulting datasets were fit to a two step model using the Spectrafit singular value decomposition module of KinTek Explorer software to obtain the spectra of the hydroperoxyflavin. **A.** Deconvoluted spectra for the KMO flavin-hydroperoxyflavin recorded at pHs indicated. The arrow denotes the titration from low to high pH. Spectra for the oxidized (t_{end}) and reduced (t_{zero}) forms of KMO at pH 7.5 are shown for reference (dashed lines). **Inset.** The pH dependence of absorption changes at 480 nm for the data shown in **A.** fit to Equation 2. **B.** The pH dependence of rate constants derived from the fit of the data shown in **A.** The dependence of k_2 was fit to equation 2. **C.** Deconvoluted spectra for oxidized KMO flavin recorded at pHs indicated. The arrow denotes the titration from low to high pH. **Inset**. The pH dependence of absorption changes at 450 nm for the data shown in **C.**

Though incomplete, these data indicate a pKa of ~8.8 when fit to an equation that describes a single dissociable proton (Equation 4). The rate constants returned for the fit of each data set

confirmed the approximate value of the pKa by indicating that only the rate of decay of the hydroperoxyflavin is pH sensitive and that the rate dependence indicates a pKa of approximately 8.2 (Figure 4C). It is reasonable to expect that the measured pKa differs from what would occur with the native substrate, L-Kyn. However, an argument could be made that the extent of pKa shift in the presence of mNBA is modest as both L-Kyn and mBNA are expected to have a neutral charge during the events of the oxidative half-reaction. The reason for this is that the pKa of L-Kyn aniline amino group would be predicted to be ~4.5 [37] and maintenance of this protonation state would favor delocalization of electrons and attack by the relative weak hydroperoxyflavin electrophile.

Discussion

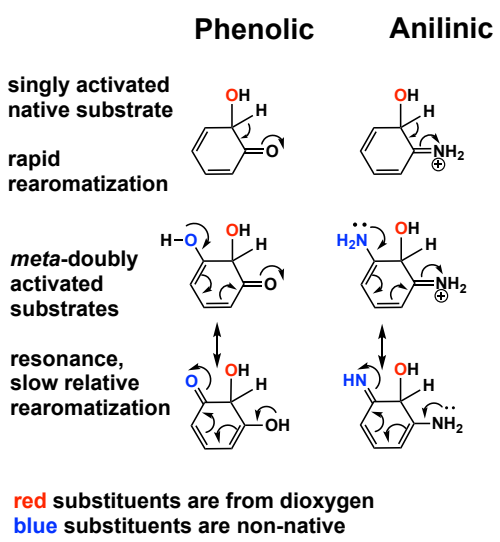
KMO is a class A flavoprotein aromatic hydroxylase (FAH) [16]. These enzymes are generally found in catabolic pathways and serve in the degradation of activated aromatic molecules; most commonly phenolic compounds. KMO is an anilinic hydroxylase that catalyzes an early step in L-tryptophan catabolism and has long been identified as a promising drug target to ameliorate the effects of deleterious neuron excitation that occurs with ischemia or specific diseases such as Alzheimer's and Huntington's [38, 39]. Though the structure of human KMO has recently been published [40], mammalian homologs have proven to be largely intractable to biophysical investigation [41-43]. In general, more resilient forms of the enzyme isolated from single-celled organisms have been used to elucidate the chemistry and/or the interaction of the enzyme with potential therapeutics [15, 27, 29]. Here we present an investigation of hydrogen movements the oxidative half reaction of KMO from *P. fluorescens*.

The consensus mechanism of the oxidative half-reaction of FAH enzymes is that formation of a C4a-hydroperoxyflavin results from a bimolecular reaction between reduced flavin and molecular oxygen [44, 45]. The activated substrate ring then undergoes electrophilic attack by this flavin intermediate delivering the distal hydroperoxy oxygen to the aromatic ring to form a non-aromatic species. This diene-one (or diene-imine) forms concomitantly with the C4a-hydroxyflavin, but because the non-aromatic species decays rapidly to form the aromatic product, only the hydroxyflavin is observed [26, 46-49]. The hydroxyflavin then decays releasing a molecule of water often concomitantly with the release of the hydroxylated product to regenerate the oxidized resting state of the enzyme. For KMO, the net chemistry of the oxidative half-reaction is the same as other FAH enzymes, but the kinetics of specific species differs.

Previous studies of this enzyme have established that there are three observable steps in the oxidative half-reaction that have been assigned successively as formation of a C4a-hydroperoxyflavin, substrate hydroxylation, and product release [15]. These three processes are therefore a variation of what is normally observed in the oxidative half-reaction of FAH enzymes in that the hydroxyflavin intermediate is not observed. This species decays rapidly, presumably by reaction with a water molecule [15] and so from an observational standpoint the decay of the C4a-hydroperoxyflavin results in the regeneration of the oxidized flavin. The final step of the oxidative half-reaction is then the release of 3-OHKyn from the active site which is observed as a perturbation of the oxidized absorption spectrum[15].

The data presented here are the first for a FAH enzyme that show the involvement a non-aromatic species during hydroxylation of the native aromatic substrate. Native substrates of FAH enzymes all carry a single activating substituent on the aromatic ring [17]. *para*-Hydroxybenzoate hydroxylase (PHBH) is the most completely studied of the FAHs. For PHBH an intermediate, designated as Intermediate II, was observed to accumulate with the decay of the hydroperoxyflavin in the presence of the substrate 2,4-dihydroxybenzoate [26, 46]. Though the chemical identity of this species remained unsettled for almost two-decades, it was deduced that the spectrum of Intermediate II was composite and comprised of both the C4a-hydroxyflavin and some intermediate form of the substrate/product [24, 50-52]. Intermediate II species have also been observed in phenol hydroxylase (PH) with the substrate resorcinol (1,3-dihydroxybenzene) [53]. In addition, kinetic isotope effect studies of PH using perdeuterated resorcinol yielded a primary kinetic isotope effect of 8.5 with the decay of Intermediate II. This effect is consistent with loss of the ring deuterium upon the aromatization of the proposed non-aromatic species.

This confirmed the hypothesis that the immediate product of hydroxylation is non-aromatic and the mechanism of hydroxylation by the FAH enzymes is now rather settled in the literature and generally follows the mechanism of Meada-Yorita and Massey that was proposed with observation of this kinetic isotope effect (see below).



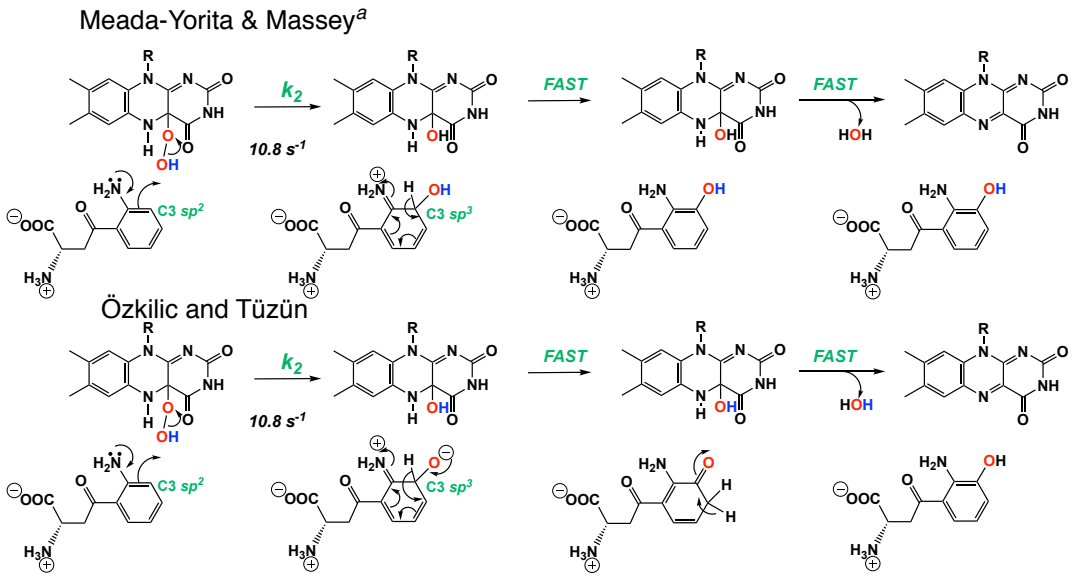
Scheme 2: The Substrate/Product Component of Intermediate II

Additional activating substituents *meta* to the native activating group are thought to stabilize the ring diene form by resonance (Scheme 2) and this explanation has become the basis for the currently accepted chemical mechanism of the hydroxylation reaction of FAH enzymes. However, in all cases for which it has been studied, the decay of the hydroperoxyflavin (Intermediate I) occurs with hydroxylation of the substrate and so this step can also be investigated using isotopic substitution at the site of hydroxylation with native substrates. The advantage of such approaches is that native substrates negate the possibility of substrate specific reaction coordinates.

The secondary isotope effect on the formation of the non-aromatic species with a native substrate, has hitherto not been observed for an FAH enzyme [54]. The kinetic isotope experiment conducted here using the native substrate L-Kyn resulted in identical rate constants for the formation of the C4a-hydroperoxyflavin, and for product release. A secondary isotope effect of 0.84 was observed for the hydroxylation rate, identifying a change from sp^2 to sp^3 hybridization at the substrate ring in the transition state for this step. More than any prior

observation, this verifies the presence of a non-aromatic cyclohexadiene species as the immediate product of hydroxylation in the FAH enzymes. The reason that this observation can be made for KMO, when it has not been made in similar experiments with other FAH enzymes [53] is that the C4a-hydroxyflavin is not observed to accumulate. This results in a more pronounced absorption change during hydroxylation and the added amplitude provides increased certainty for the rate constant measured with each isotopomer (Figure 3).

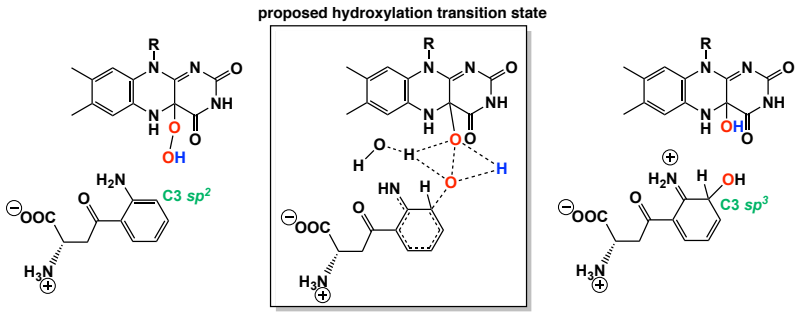
In addition to the secondary KIE observed during hydroxylation, a solvent isotope effect is also observed for this phase. The proton inventory data are similarly well described by two or many protons in flight during hydroxylation. While the mechanism for hydroxylation can be depicted in a variety of ways, it is a requirement that multiple protons be exchanged [50, 52, 55, 56]. The exchange of two or more protons during hydroxylation suggests a complex transition state for this step. In a recent computational investigation of the KMO hydroxylation mechanism by Özkilic and Tüzün it was proposed that the C4a-oxide that is generated from the C4a-hydroperoxide is protonated by the transferred hydroxyl. This return of the proton to the residual flavin adduct promotes the formation of an amino-dienone that must then acquire a proton to rearomatize (Scheme 3) [55]. Such a mechanism is inconsistent with our data as it does not account for two proton transfers occurring with the development of sp^3 character at the ring C3 position. Similarly, the consensus mechanism of Maeda-Yorita and Massey does not explicitly require the movement of two or more protons during the hydroxylation reaction step.



Scheme 3. Proposed Mechanisms of the Oxidative Half-Reaction of KMO.

a - adapted from observations made with phenol hydroxylase

As such we propose a hydroxylation transition state in which two protons are shared between the proximal and distal oxygens of the C4a-hydroperoxide as the O-O bond is broken and as sp^3 character is developed at the site of hydroxylation (Scheme 4). Such a mechanism has the C4a-oxide and the diene-imine protonated simultaneously with the requisition of one these two protons from a water molecule.



Scheme 4. The Proposed KMO Hydroxylation Transition State

Despite the fact that PHBH is the most completely studied and understood member of the class A FAH enzymes and regarded as the paradigm of this class, it has structural features that are unique to its function. The most striking of these is a proton relay that serves to activate the

phenol of p-hydroxybenzoate. This relay links the 4-hydroxyl of the substrate with two tyrosine hydroxyls, two water molecules and a histidine that lies at the protein's surface [21, 25, 57]. The protonation state of the phenol is influenced by a contour of positive potential that raises the dissociation constant of the hydroxyl proton of the phenol by two orders of magnitude (pka 9.1 vs 7.4) [58, 59]. As a consequence the latter steps of the oxidative half-reaction of PHBH are influenced by pH [24]. Interestingly, this proton relay is absent from ostensibly every other class A FAH enzyme including KMO [17]. It could be surmised that the relatively low pKa of aromatic amines, would negate the need for a means of deprotonating the substrate in anilinic flavoprotein hydroxylases. This was confirmed here by pH studies of the oxidative half-reaction of KMO in the presence of L-Kyn or BA that show the rates of the oxidative steps are largely immune to the concentration of protons in the solvent (Figure 1). This indicates that the active site of PfKMO is shielded from desultory proton exchange. The structure of PfKMO in complex with L-Kyn was published in 2019 [10]. This structure revealed that the aniline moiety of L-Kyn is stacked roughly perpendicular to the isoalloxazine of the FAD and that the C3 of the substrate is within 4.6 Å of the flavin C4a position. A prominent characteristic of the substrate binding pocket is the preponderance of hydrophobic residues that surround the substrate aniline moiety (Figure 5). No hydrogen bonding or direct charge-pair interactions are observed for this portion of the substrate. Moreover, the flavin isoalloxazine fills the outer portion of the active site, presumably

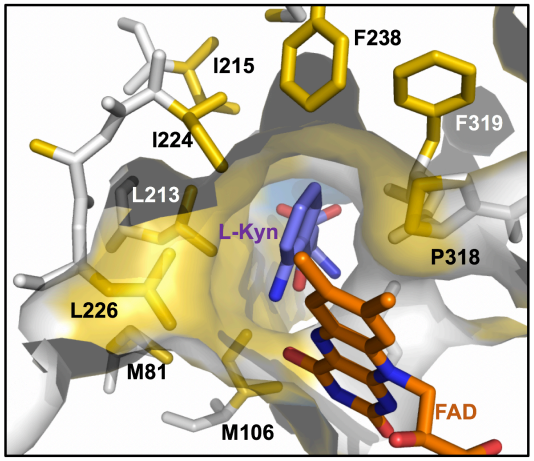


Figure 5: The binding cavity of L-Kyn in the active site of PfKMO (PDB ID: 6FOX). Yellow shading indicates relative hydrophobicity based on the script developed by Hagmans *et al.*, (Hagemans, D., et al. (2015). A script to highlight hydrophobicity and charge on protein surfaces. *Front Mol Biosci* **2**: 56).

restricting entrance or egress of both ligands and solvent. It is therefore reasonable to conclude that at pH values of 5.5 and above this cavity captures exclusively the base form of the L-Kyn aniline. The enzyme then maintains this state by secluding the aniline from solvent in a largely aprotic environment to ensure the ring remains activated.

Protonation of the peroxyflavin imparts electrophilic character. The source of the proton has not been established nor has the affinity of the proton for the peroxyflavin been measured. Here we deconvoluted multiple spectral datasets collected for a range of pHs to reveal pure spectra of the (hydro)peroxflavin formed in the presence of mNBA. This ligand was selected as it promotes a long-lived hydroperoxflavin and exhibits simplified kinetics compared to those observed with L-Kyn. These pH dependent changes in the intermediate spectra were analyzed in an attempt to define the pKa of the hydroperoxy group. The spectrum of the hydroperoxyflavin exhibited pH-dependent extinction coefficient increases at all wavelengths between 340 and 520 nm (Figure 4A). While the titration was not complete, the data fit to indicate a pKa of ~8.8. This perturbation of the hydroperoxyflavin spectrum cannot be ascribed to the deprotonation of the hydroperoxyflavin however, as the rate of decay of this species (k_2) increases only slightly with pH, suggesting that the proton of the hydroperoxyflavin remains intact and that the experiment captures instead the titration of some other species proximal to the active site that can perturb the peroxyflavin absorption spectrum (Figure 4B). This however does permit the conclusion that the pKa of the hydroperoxyflavin is substantially higher than the highest pH at which the oxidative steps were observed (pH 8.5).

These analyses also provided pH dependent absorption changes for the KMO_{OX}•mNBA complex spectrum that is observed at the end of the oxidative process. The changes observed

titrated to reveal a pKa of 7.3 and likely reflect the titration of a group or groups that influence the position of the flavin. Other FAH enzymes, most particularly PHBH,[60-62] have been shown to use flavin ring dynamics to gate access to the active site in order to bind the substrate, control reduction, and release the hydroxylated product [22, 62, 63]. It is thus reasonable to suggest that the pH induced flavin spectrum perturbations observed with KMO are evidence of flavin movement whose native function is to release 3-OHKyn from the active site. The most direct evidence in support of this assertion is that the position of the flavin of PfKMO in complex with L-Kyn occludes exchange of ligand with the active site (Figure 5), implicating that flavin movement is a requisite part of catalysis.

Conclusive Remarks

In terms of rate constant, the hydroxylation phase is sensitive to both solvent derived deuterons and deuteron substitutions on the L-Kyn aromatic ring. That both effects are observed on the same step in the oxidative half reaction narrows the mechanistic hypotheses for hydroxylation. In recent modelling studies the mechanism of hydroxylation proposed by Özkilic and Tüzün involves at least three steps (Scheme 3) none of which predict the simultaneous movement of two or more solvent protons [55]. Moreover, the mechanism first put forward by Maeda-Yorita & Massey based on observations made with phenol hydroxylase predict that one solvent derived proton is in flight during hydroxylation to protonate the flavin C4a-oxide [53]. We have proposed a combination of these hypotheses that are consistent with our data. We propose that one solvent derived proton is acquired to protonate the oxygen atom added to the substrate

and that the proton initially attached to the transferred oxygen is returned to protonate the C4a-oxide.

The lack of dependence of the observed rate constants of the oxidative half reaction are consistent with the available structural data that show the L-Kyn aniline is housed in a pocket of hydrophobic residues and isolated from proton exchange with the solvent. Sequestering the substrate in such a manner is proposed to maintain the activated state of the preponderate neutral aniline acquired from solvent. The kinetic data summarized here are consistent with the restricted enlisting of waters only to protonate anionic species generated with hydroxylation.

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