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Evidence for the Mechanism of Hydroxylation by 4-Hydroxyphenylpyruvate Dioxygenase and Hydroxymandelate Synthase from Intermediate Partitioning in Active Site Variants

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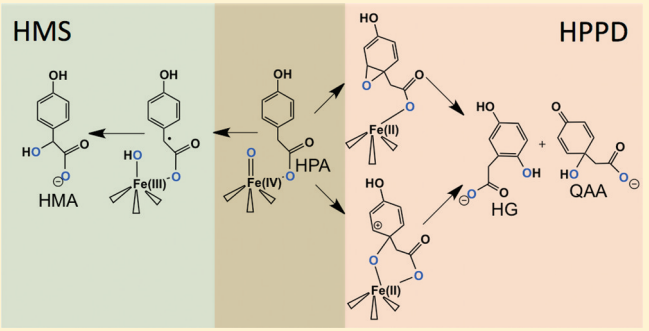
ABSTRACT: 4-Hydroxyphenylpyruvate dioxygenase (HPPD) and hydroxymandelate synthase (HMS) each catalyze similar complex dioxygenation reactions using the substrates 4-hydroxyphenylpyruvate (HPP) and dioxygen. The reactions differ in that HPPD hydroxylates at the ring C1 and HMS at the benzylic position. The HPPD reaction is more complex in that hydroxylation at C1 instigates a 1,2-shift of an aceto substituent. Despite that multiple intermediates have been observed to accumulate in single turnover reactions of both enzymes, neither enzyme exhibits significant accumulation of the hydroxylating intermediate. In this study we employ a product analysis method based on the extents of intermediate partitioning with HPP deuterium substitutions to measure the kinetic isotope effects for hydroxylation. These data suggest that, when forming the native product homogentisate, the wild-type form of HPPD produces a ring epoxide as the immediate product of hydroxylation but that the variant HPPDs tended to also show the intermediacy of a benzylic cation for this step. Similarly, the kinetic isotope effects for the other major product observed, quinolacetic acid, showed that either pathway is possible. HMS variants show small normal kinetic isotope effects that indicate displacement of the deuteron in the hydroxylation step. The relatively small magnitude of this value argues best for a hydrogen atom abstraction/rebound mechanism. These data are the first definitive evidence for the nature of the hydroxylation reactions of HPPD and HMS.



Both 4-hydroxyphenylpyruvate dioxygenase (HPPD) and hydroxymandelate synthase (HMS) catalyze very similar dioxygenation reactions, even to the extent that they require the same substrates, 4-hydroxyphenylpyruvate (HPP) and dioxygen (Scheme 1). With respect to the reactions catalyzed, HPPD and HMS principally differ in the placement of one of the atoms of dioxygen. For each, one oxygen atom is committed to an oxidative decarboxylation that converts the pyruvate side chain to an aceto substituent. The other oxygen atom is then added as a hydroxyl with HMS hydroxylating at the benzylic carbon forming hydroxymandelate (HMA) and HPPD at the adjacent C1 of the aromatic ring. Hydroxylation of the ring then induces a fascinating transformation involving a 1,2-shift of the aceto substituent forming 2,5-dihydroxyphenylacetate (homogentisate, HG).

HPPD is common to ostensibly all aerobes and is almost certainly the progenitor of HMS that is found in a relatively small number of bacteria. The HPPD reaction is the second step of tyrosine catabolism, a five-step pathway whose fundamental purpose is to produce the energy-yielding products, fumarate and acetoacetate.[1] However, pathway intermediates can be drawn into the biosynthesis of other molecules.[2−5] HMS participates in one example of this, as it functions to divert HPP from tyrosine catabolism to form HMA toward the biosynthesis of hydroxyphenylglycine that is incorporated into a variety of nonribosomal peptides such as vancomycin.[6,7] Another example occurs in plants where HG is the precursor to tocopherol and the critical photosynthetic cofactor, plastoquinone. It is this that has, for the past three decades, garnered considerable research interest in HPPD. Molecules that inhibit HPPD have potent herbicidal effects and a number of such molecules are now sold worldwide as broadleaf herbicides.[8−10] An unexpected additional use for one of the molecules originally developed as a herbicide has been as a therapeutic that can alleviate the symptoms of a number of inborn defects in tyrosine catabolism.[11−13] It could be argued that the study of the inhibition of HPPD has eclipsed the study of its reaction mechanism and key details of the reaction mechanism remain unsupported. Similarly, as it has only been described in the literature recently, relatively little is known of the chemistry catalyzed by HMS.[7,14]

HPPD and HMS are Fe(II)-dependent dioxygenases that, from a chemical standpoint, are grouped with the α-keto acid-dependent oxygenases (α-KAOs).[15] Structurally, however, both enzymes differ from the majority of α-KAOs that have a jellyroll fold. Instead, HPPD and HMS have a topology that is common

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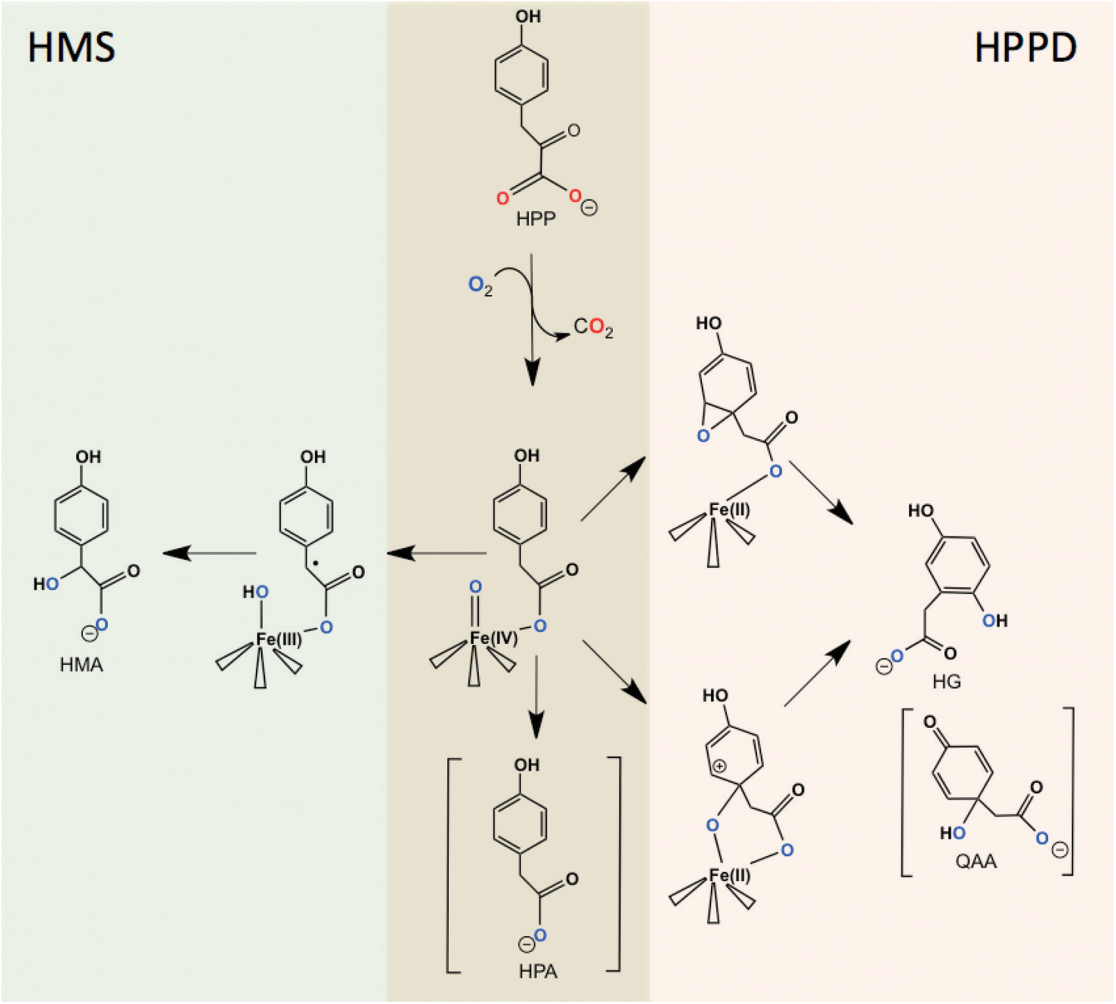
Scheme 1. [a]



[a]Brackets indicate non-native products.

to the vicinal oxygen chelate superfamily.[16] α-KAO enzymes all obtain electrons to activate molecular oxygen from oxidative decarboxylation of an α-keto acid. The most common ensuing reaction of this group of enzymes is hydroxylation. HPPD is somewhat unusual in that it hydroxylates an aromatic ring inducing the 1,2-shift of the aceto substituent (Scheme 1). While shifts of this type have been observed in a number of oxygenases, it is more common that only small substituents such as deuteriums, tritiums, halides, and methyl groups can be induced to shift in this manner and generally only in a fraction of total catalysis.[17−25] The identity of the intermediate that is the immediate product of hydroxylation in such reactions has been the source of conjecture for more than 40 years. The principal division is based around the intermediacy of an epoxide or a benzylic cation. Either species can be readily envisaged to resolve as the final aromatic product, and experiments that unambiguously show one mechanism over the other have proven exceedingly difficult to design. One definitive method to distinguish between these two mechanisms is to measure the secondary kinetic isotope effect on the hydroxylation step when a deuterium is substituted ortho to (or if applicable at) the point of hydroxylation.[26] However, in terms of direct observation or kinetic resolution, few if any of the systems that exhibit an NIH shift are amenable to this observation. That is to say that the hydroxylation rate constant cannot be measured with sufficient precision or else cannot be observed independently due to the balance of rate constants for steps before and after.[25,27−29] Moreover, this is a recurrent limitation in the study of many metal-dependent oxygenases, including for enzymes that carry out less complicated hydroxylation reactions such as hydroxylation at aliphatic carbon atoms, as is the case for HMS. These hydroxylation reactions exhibit primary kinetic isotope effects when deuterium atoms are substituted at the point of hydroxylation,[30] but these effects can also be completely shrouded.[31] The utility of intermediate partitioning and product analysis has emerged as a robust and accurate method that can be used to unveil otherwise hidden kinetic isotope effects.[26,32−35] In this report we describe the application of this methodology to variants of HPPD and HMS that exhibit bifurcation and trifurcation during the hydroxylation step and as such yield multiple distinct products in a defined ratio (Scheme 1).

MATERIALS AND METHODS

Materials. HPP and HG were purchased from Acros Chemicals. HMA and 4-hydroxyphenylacetate (HPA) were obtained from Sigma-Aldrich. Mutagenesis was performed using the materials and protocol of the Stratagene QuikChange

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Table 1. Steady-State Kinetic Values

HPPD variant	WT	N245I	N254Q	P243T	S230A
k_{cat} (s^{-1})	6.8 ± 0.6	0.64 ± 0.09	0.03 ± 0.01	2.1 ± 0.1	2.8 ± 0.4
K_{HPP} (μM)	27 ± 6	27 ± 5	15.0 ± 3.3	8.7 ± 2.8	47 ± 16
k_{cat}/K_{HPP} ($mM^{-1}s^{-1}$)	254 ± 70	24 ± 6	2.4 ± 0.4	241 ± 36	60 ± 15
HMS variant	WT	I216N		T214P	S201A
k_{cat} (s^{-1})	3.7 ± 0.1	1.4 ± 0.1		1.18 ± 0.04	3.0 ± 0.6
K_{HPP} (μM)	47.6 ± 5.6	14.3 ± 3.0		20.5 ± 2.8	28.0 ± 13.5
k_{cat}/K_{HPP} ($mM^{-1}s^{-1}$)	77 ± 5	98.5 ± 14.5		57.5 ± 6.5	108 ± 19

Lightning kit. Competent BL21(DE3) *Escherichia coli* cells were obtained from New England Biolabs. IPTG was from Gold Biochemicals. D_2O was from Cambridge Isotope Laboratories. Q-Sepharose was from Bio-Rad. Sephacryl S-200 was obtained from Amersham. Quinolacetate (QAA) was made using the P243T variant described in this article and purified as previously described.[13] Perdeutero-HPP and 3′,3′-dideutero-HPP were prepared according to our previously published methods.[27,31] All other chemicals, buffers, and biological media were obtained from Fisher Scientific or Sigma-Aldrich Chemicals and were of high purity.

Mutagenesis. pET17b-derived (Novagen) plasmids carrying the *Streptomyces avermitilis* HPPD gene (pSAHPPD[36]) and the *Amycolatopsis orientalis* HMS gene (pAOHMS[37]) were mutated using the Stratagene QuikChange Lightning kit. Mutations were identified in the transformants by either incorporating changes to the plasmid restriction pattern by the addition of silent mutations or by sequencing the plasmid from multiple transformants. In each case, sequencing the entire gene confirmed the desired mutations and the absence of additional spurious mutations (Sequetech, Mountainview, CA). The following oligonucleotides and their respective reverse complement oligonucleotides were used to mutate the HPPD gene(s):

HPPD Oligos. SAHPPDN245I: GGTCAAGTTCCCGATCATCGAGCCCGCCCTGGCCAAGAAGAA.

SAHPPDN245Q: CAAGTTCCCGATCCAGGAGCCCGCCCTGGCCAAGAAGAA.

SAHPPDP243T: CTCAAGGTCAAGTTCACGATCAACGAGCCCGCCTTGGCCAAGAAGAAGTCC.

SAHPPDS230A: GACATCGCGACCGAGTATTCGGCGCTGATGGCGAAGGTCGTGGC.

HMS Oligos. AOHMSI216N: CACCCTCACCCTGAACGAGCCCGACCG.

AOHMST214P: GCGGTCACCCTCCCGCTGATCGAGCCC.

AOHMSS201A: CAGGCGATGAACGCCACCGTCGTGCAG.

Expression and Purification. The apo-forms of WT and variants of HPPD and HMS were expressed and purified using ammonium sulfate fractionation, anion exchange chromatography, and size exclusion chromatography according to published methods.[36,37]

Standard Assay Conditions and Steady-State Analysis. HPPD/HMS activity was measured using a Hansatech Oxygraph dioxygen electrode. Standard activity assays included 1−2 μM of enzyme, 1 mM β-mercaptoethanol (βME), 20 μM ferrous sulfate, and 400 μM HPP in 20 mM HEPES pH 7.0 at 25 °C with atmospheric oxygen (250 μM). Reactions were initiated with HPP, and rates were measured between 20 and 50 s of initiation. Apparent kinetic parameters were measured by varying the concentration of HPP. Data were fit to the Michaelis−Menten equation (eq 1).

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

Kinetic Isotope Effects from HPLC Product Analysis. The ratio of products of each variant enzyme was determined using high-pressure liquid chromatography. Initially enzyme turnover reactions were undertaken at 25 °C and were comprised of 25 μM ferrous sulfate, 6−10 μM HPPD or HMS, 1 mM reductant (either βME or dithiothreitol), 250 μM O_2, and 100 μM HPP in 10 mM potassium phosphate or 20 mM HEPES buffer (pH 7.0). The initial background rate of dioxygen reduction due to Fenton chemistry was observed before the enzymatic reaction was initiated by either the addition of unlabeled or deutero-labeled HPP (ring-deutero for HPPD and 3′,3′-dideutero for HMS). After completion of the reaction (i.e, when the observed rate of dioxygen consumption approximated that due to background dioxygen reduction), 400 μL of the reaction mixture was withdrawn, added to a 0.5 mL, 10 kDa nominal molecular weight limit ultracentrifugal filter (Amicon), and centrifuged at 14000*g* for 7 min to remove the enzyme. The resulting filtrate was chromatographed using a Waters 600E HPLC system fitted with a 4.6 mm ×150 mm Waters 5 μm Xterra reverse phase C18 column. The column was run under isocratic conditions at 1 mL/min with a mobile phase consisting of 10 mM potassium phosphate (pH 2.0) and 0.5% acetonitrile. Elution was monitored at 220 and 276 nm using a Waters 2487 dual wavelength detector. Individual products were identified by coelution with standards for each. Quantitation was accomplished by preparing standard curves from 0−100 μM for each product under the same conditions.

Kinetic isotope effects were calculated using eq 2 that computes the factor of perturbation of the product ratio in the presence of the deuterated substrate for the case of a trifurcating mechanism.[35]

$$^{D}k_{obs} = \frac{(P_1/(P_1 + P_2 + U))^{H}}{(P_1/(P_1 + P_2 + U))^{D}} \quad (2)$$

In this equation, P_1 is the concentration of product for which the isotope effect is being determined and P_2 is the concentration of an alternate product formed in the same reaction. For both HPPD and HMS, U refers to uncoupling in the hydroxylation step that is isotopically insensitive and results in the production of HPA. For the case of bifurcating reactions where two products are evolved the P_2 term is omitted. To ensure that all of catalysis was considered, the total

consumption of molecular oxygen was compared to the total concentration of products formed.

The KIE values for HG would be skewed if QAA were to act as a shunt substrate for HPPD and converted to HG. In order to establish if HPPD can catalyze the conversion of QAA to HG, 60 μM QAA was incubated with 15 μM HPPD in the presence of 25 μM ferrous sulfate, 1 mM ascorbate in 10 mM potassium phosphate buffer pH 7.0. After incubation at 25 °C for 10 min (approximately twice as the length of time used for the KIE assays), 200 μL of the reaction was withdrawn, filtered, and chromatographed as above. The chromatogram obtained from this sample was then compared to the control incubated in an equivalent manner in the absence of HPPD.

RESULTS

Characterization of the Variant Enzymes. Each of the HPPD and HMS variants described could be expressed and purified using published methods for the wild-type enzymes.[31,36] Moreover, the yield of purified enzyme was generally comparable to the wild-type forms and each could be stored at −80 °C without appreciable loss of activity. Apparent steady-state turnover values for each variant are listed in Table 1. These values are not directly applicable to the conclusions made here but do indicate that each mutation yields an active and competent enzyme.

Product Analysis. The percentages of products formed by each variant of HPPD and HMS are shown in Table 2, and the position of each residue in the active site is shown in Figure 1. For each variant all major products were accounted for in that the percentages of all products summed to ∼100% based on the uptake of dioxygen. This indicates that for both HPPD and HMS hydroxylation is tightly coupled to dioxygen reduction and that all reducing equivalents committed to catalysis result in substrate decarboxylation. None of the variants presented made any fraction of the product of the other enzyme. In other words, HPPD variants were observed to make only HG, HPA, and QAA, while HMS variants made only HMA and HPA, indicating that the preference for hydroxylation regiospecificity was not altered by these mutations. Under the conditions used in this investigation wild-type HPPD is prone to uncoupling to form HPA. This suggests that the hydroxylation of the aromatic ring is less optimized than the hydroxylation of the benzylic position by HMS, which is fully coupled to the formation of HMA. For respective HPPD/HMS variants, alteration of the 245/216 position and the 230/201 position increases uncoupling in the hydroxylation reaction yielding more HPA than is observed in the wild type of either enzyme (Figure 1). Interestingly, mutating to counterpart residues at the 243/214 positions resulted in improved or equivalent hydroxylation efficiency. For HMS, this result is contrary to what would be predicted from structure as threonine 214 is observed to hydrogen bond to the newly added side chain hydroxyl in the HMS·HMA product complex, prompting the proposal that this threonine may participate in the delivery of the oxygen to the benzylic carbon.[37] P243 in HPPD clearly contributes an important steric component to the execution of the NIH shift. Mutating this residue to threonine results in the majority (∼80%) of the hydroxylation reaction terminating with the formation of QAA, where the hydroxyl has been delivered to the ring C1 without displacement of the aceto substituent bonded in this position. The N245 position is also critically important in the NIH shift. In previous work we have shown that mutation of this position to a serine residue results in a predominance of QAA.[13] Here, mutation to an isoleucine results in ∼90% uncoupling in the hydroxylation step and the formation of HPA. Curiously, the N245Q and N245I variants were able to complete the shift for most instances in which they hydroxylate the ring (∼50% and ∼10%, respectively) and uncouple to make HPA for the remaining fraction of total turnover. Together these results suggest that a hydrogen-bonding residue in the 245 position aids the initial ring hydroxylation reaction and that only in the presence of a large and/or amide side chain can the NIH shift occur.

Table 2. Percentage of Products Formed

HPPD variant	WT	N245I	N245Q	P243T	S230A
HG (%)	84.5	13	44.5	13	11.6
QAA (%)	0	0	4	78	57.8
HPA (%)	15.4	86.6	52	8.7	30.6
HMA (%)	0	0	0	0	0
total	99.9	99.6	100.5	99.7	100
HMS variant	**WT**	**I216N**		**T214P**	**S201A**
HG (%)	0	0		0	0
QAA (%)	0	0		0	0
HPA (%)	0	10		0	12
HMA (%)	100	90		100	88
total	100	100		100	100

The serine at position 230/201 is fully conserved in all HPPD and HMS primary structures, implying that it is vital in the mechanisms of both enzymes. This would appear to be correct for HPPD that either uncouples or predominantly fails to execute the shift when this residue is mutated to an alanine. However, S201A in HMS has a very modest effect on the product distribution, causing the enzyme to uncouple in only ∼10% of turnovers, prompting the question as to why this residue is fully conserved in HMS primary structure.

Kinetic Isotope Effects from Hydroxylation Partitioning. The kinetic isotope effects as measured from hydroxylation partitioning for both HPPD and HMS are listed in Table 3. In prior research we have shown, using the isotopic substitutions of HPP used here, that neither HPPD nor HMS accumulates the hydroxylating intermediate to any measurable extent. The decay of this species is not limiting for any of the observed catalytic events, and as such, simple isotopic substitution and kinetic investigation has not elucidated any part of the early hydroxylation mechanism for either enzyme. This has limited our ability to apply rapid mixing quench studies to characterize this step.[27,31] As such, it is assumed that the hydroxylating intermediate is an oxo-ferryl species as has been observed in a number of mononuclear Fe(II)-dependent hydroxylases.[38−42] In Figures 2 and 4, example HPLC chromatograms in the presence and absence of the isotopic label are included to illustrate how these otherwise shrouded kinetic isotope effects were measured.

The kinetic isotope effects for HPPD indicate that at least three hydroxylation reaction coordinate saddle points are possible and that a variant enzyme may bias one or use multiple of these pathways (Figure 2). As stated, one of the unexpected observations was that the native form of HPPD exhibits uncoupling to form HPA in a fraction of total turnover. The advantage of this is that it affords the measurement of the secondary isotope effect associated with hydroxylation to form

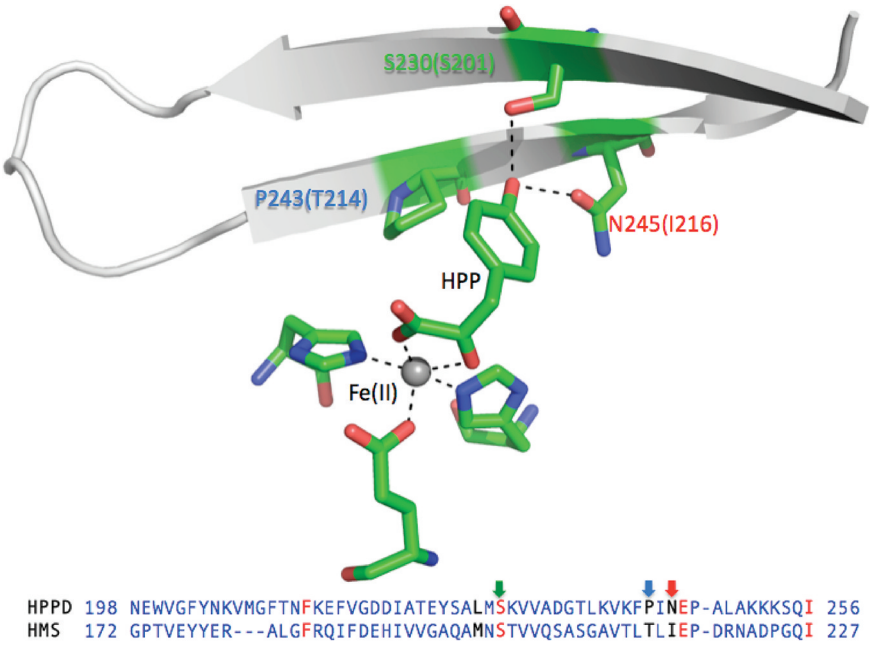

Figure 1. Positions of the residues targeted for mutagenesis. The positions are shown for HPPD with residues and numbers in parentheses indicating the corresponding residue in HMS. All three positions are clustered on the third and fourth β strands of the β-barrel that is common to the vicinal oxygen chelate superfamily of enzymes. The putative 4-hydroxyphenylpyuvate binding site was modeled in earlier work using molecular dynamics and was based the observed binding position for hydroxymandelate in hydroxymandelate synthase.[37] In this position the 4-hydroxyl of the substrate phenol is within hydrogen bonding distance to the conserved serine residue. The sections of amino acid sequence shown compare the primary sequences of HPPD from *Streptomyces avermitilis* and HMS from *Amycolatopsis orientalis* local to the residues altered by mutation.

Table 3. KIEs from Isotopic Perturbation of Hydroxylation Partitioning

HPPD variant	WT	N245I	N245Q	P243T	S230A
$^{D}k_{obs}$ HG	0.84 ± 0.01	1.02 ± 0.06	0.99 ± 0.01	1.05 ± 0.08	1.07 ± 0.10
replicates	3	2	2	3	2
$^{D}k_{obs}$ QAA	n.m.[a]	n.m.	1.09 ± 0.08	0.76 ± 0.04	1.02 ± 0.04
replicates	3	2	2	3	2
HMS variant	WT	I216N		T214P	S201A
$^{D}k_{obs}$ HMA	n.m.[a]	2.19 ± 0.01		n.m.	2.56 ± 0.24
replicates	1	3		1	3

[a]Not measurable.

HG for the native form of the enzyme. This value was 0.84 and strongly suggests that an sp^2 to sp^3 hybridization change is occurring with the hydroxylation event. Such a value is entirely consistent with the formation of a 1,2 ring epoxide during the hydroxylation step and is the first direct evidence for the native mechanism of hydroxylation by HPPD. Each of the variant enzymes investigated, however, show kinetic isotope effects of 1 within error for the formation of HG which indicates either no change in hybridization during hydroxylation, more consistent with the formation of a benzylic cation, or that multiple simultaneous pathways contribute to the effect. Similarly, the KIE for the formation of QAA by the variants can be either inverse or unity, indicating that it is possible to yield this product via either of the proposed branches in Figure 2.

The native form of HMS is fully coupled in hydroxylation and forms only HMA. As such the KIE for hydroxylation cannot be measured using partition analysis. This is also true for the T214P mutation. Therefore, we cannot observe the native function of the enzyme by these methods. However, the kinetic isotope effect for hydroxylation by HMS as it relates to mechanism is less disputed than is that for HPPD (Figure 4). The mechanism must include displacement of a hydrogen from the benzylic carbon and as such is expected to show a primary KIE when this atom is replaced with a deuterium atom. Two of the HMS variants studied do show uncoupling and the formation of HPA, and the measured KIEs for these are modest normal effects around 2.2−2.6. These values confirm the displacement of the hydrogen in the hydroxylation step but do not delineate between hydrogen atom abstraction or hydride transfer mechanisms. The former of these has been adopted by consensus, fundamentally due to the propensity of the iron cofactor to carry out one-electron chemistry, but either mechanism can account for the observed product, HMA. The labeled HPP substrate in this case is dideutero at the benzylic position. As such, the effect is multiplicative with the KIE on the concomitant formation of the benzylic radical (in the case of H atom abstraction) or cation (in the event of hydride transfer). The cation would be predominantly sp^2 in character

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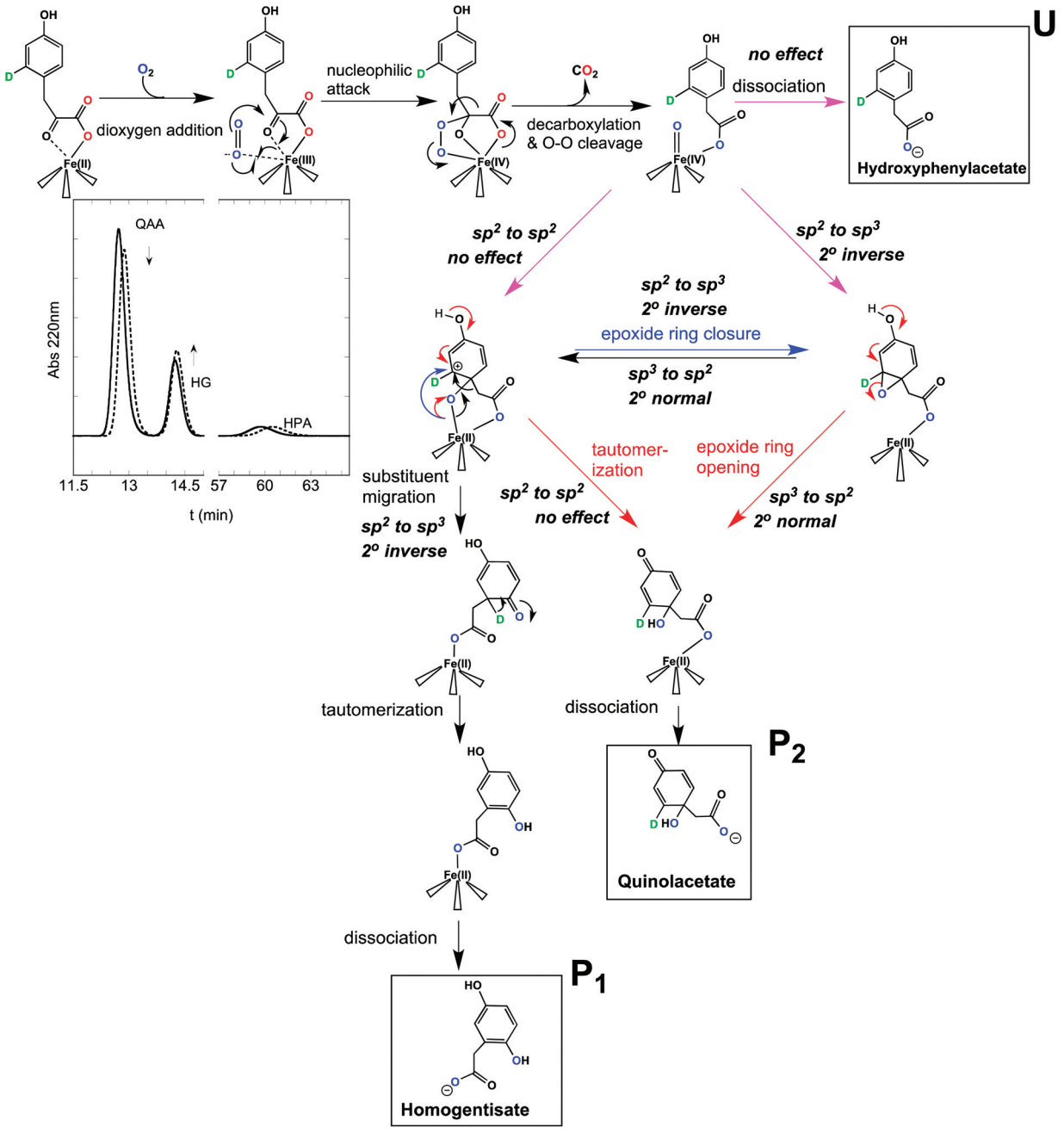

Figure 2. Hydroxylation mechanism of HPPD as deduced from intermediate partitioning. The proposed chemical mechanism of HPPD is shown. Molecules shown boxed are detected as products. The origin of the kinetic isotope effects is shown as italicized notations. Notations P_1, P_2, and U refer to the variables of eq 2. The inset depicts two chromatograms for P243T variant in the absence (solid line) and presence (dashed line) of the ring-deuterium label of HPP.

while the radical could be sp^2 or sp^3. Although the extent of these contributions cannot be established from the data presented, the relatively small KIE observed may argue for a single contribution altering the rate constant. This would occur with H atom abstraction that yields a largely sp^3 hybridized benzylic radical. This is the first evidence for the nature of the hydroxylation mechanism of HMS and indicates that the hydrogen at the point of substitution is displaced during the hydroxylation step. The effect is considerably smaller than that measured in other α-KAO enzymes that typically show KIEs for aliphatic hydroxylation as high as 40, suggesting greater hydrogen transfer optimization and tunneling in these enzymes.[30,39,40,42,43]

DISCUSSION

Understanding the role each residue plays in the reaction mechanism is the common objective of site-directed mutagenesis. The purpose of this study, however, is not to assign functions to the residues that were mutated. The principal objective was instead to determine the kinetic isotope effects for the hydroxylation step and thereby further define the chemical mechanism of the sister enzymes, HPPD and HMS. The mutations selected for this study target specific residues implicated to be important in the mechanisms of HMS and HPPD.[13,37,44−46] These form part of a broader effort to understand the basis of hydroxylation regiospecificity and the

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enzymatic machinery required to carryout the NIH shift. Each of the residues mutated is hypothesized to cluster about the phenol of HPP in both enzymes (Figure 1). Two of the residues are distinct in HPPD (N245, P243) and HMS (I216, T214), and these were mutated to their counterpart residues (N245I, P243T, I216N, and T214P). The conserved serine residue (S230/201) was shown to form a hydrogen bond with the HMA phenol hydroxyl in the product complex of HMS,[37] and this residue was mutated to an alanine in each enzyme. The HPPD variant N245Q was included also as it was one of the only variants to yield a large fraction of the native product, HG.

A number of qualitative mechanistic conclusions based on the ratio of products are possible. The first is that all enzymes investigated are fully coupled to formation of products; i.e., each molecule of dioxygen that is consumed results in the formation of a product that has, at a minimum, undergone decarboxylation. This is evidence that the oxidative decarboxylation reaction is tightly coupled to dioxygen reduction. Moreover, it also to some extent confirms the accepted order of events, in that the hydroxylation reaction is contingent on the decarboxylation reaction and thus must occur later in the catalytic cycle.

The variant forms of HPPD and HMS used in this investigation were selected for their capacity to form multiple products and most particularly HPA. HPA as an abortive singly oxygenated and decarboxylated product that is released when a form of either enzyme fails to hydroxylate the ligand (Figures 2 and 4). It is thus very likely that HPA is an intermediate in the catalytic cycle that is common to both enzymes. However, measurement of the fractional yield of HPA also allows us both to account for the total products formed and to have a necessary internal reference for a step in the bi- or trifurcation (depicted as magenta arrows in Figures 2 and 4) that, in terms of formation rate constant, is independent of the isotopic substitutions. However, for each instance in which HPA is made an equal number of oxygen atoms is unaccounted for. These atoms may result in self-hydroxylation of the enzyme as has been reported for a number of α-KAO enzymes.[47−49]

For cases that show kinetic isotope effects close to 1, the complexity of the hydroxylation mechanism of HPPD and the potential for simultaneous compensatory secondary isotope effects undermine a direct intuitive explanation. The predicted mechanistic possibilities depicted in Figure 2 are summarized (minus the substrate binding and product release steps) in Figure 3. The lower parts of Figure 3 show the unique linear pathways for the production of HG and QAA and in each case the competing branch point that forms HPA. Any one path has a unique predicted isotope effect that reflects only the first intermediate formed during ring oxygenation and this outcome is independent of isotope effects in subsequent steps including the interconversion of the epoxide and cationic species. If individual linear pathways were exclusively operative for any one product, the interpretation of the kinetic isotope effects would be simple and definitive. That is, an inverse effect would indicate the intermediacy of the epoxide and an isotope effect of 1 would indicate the benzylic cation intermediate. However, generally we cannot know which or how many or to what extent any of these possible pathways are biased for any form of the enzyme nor the value of the intrinsic kinetic isotope effect for any one step. Simulation of the interconnected mechanism depicted in Figure 2 including all possible steps shows that the result of multiple pathways occurring and interconverting for

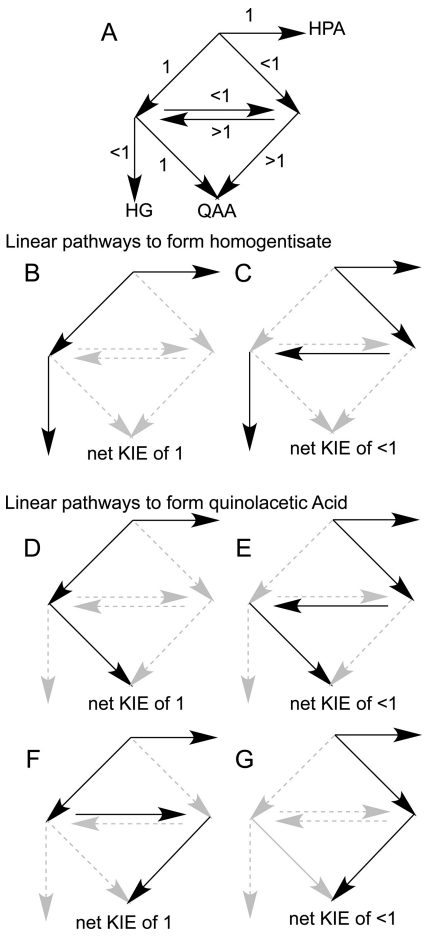

Figure 3. Summary of the unique linear pathways that yield HG and QAA based on the mechanism depicted in Figure 2. Part A shows the predicted kinetic isotope effects relative to 1 for each of the relevant isotopically sensitive steps in Figure 2. Parts B and C show the two unique pathways for the formation of HG, and parts D−G show the four unique pathways that yield QAA. Gray arrows are included for references only and are considered as nonoperative steps when depicting any one pathway.

any one product is that the observed net kinetic isotope effect will be ~1, and this is what we see for most observations (Table 3). However, we observe two cases that show substantially inverse kinetic isotope effects. These are for the production of HG for the wild-type enzyme and for the production of QAA in the P243T variant enzyme (Table 3). These values strongly suggest that for these two cases the epoxide pathway is dominant (pathways C, E, and G, Figure 3). This can be concluded for two reasons. First, these effects are observed for the major fractional product in both instances, negating the influence of competing pathways for the alternate product. Second, the effects are significantly inverse, and the contribution of other pathways would be to make the observed value less so. This suggests that for any one pathway the intrinsic oxygenation isotope effect arises only in this initial pathway selection (between HPA, the epoxide and the benzylic cation intermediates) and that it is only in the epoxide path that the possibility for significantly inverse isotope effects is established.

The conclusion is that once the hydroxylation pathway is selected from the three possibilities branching from the

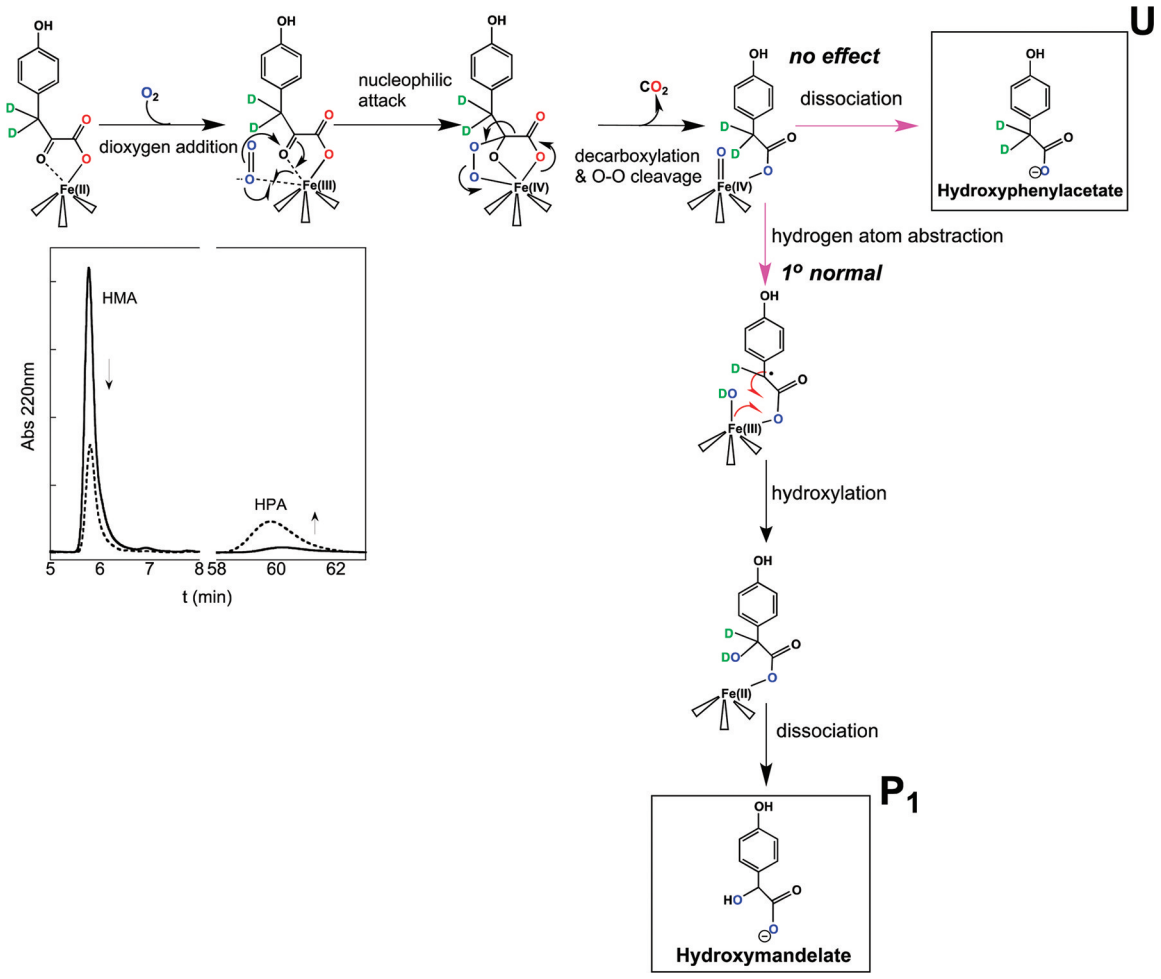

Figure 4. Hydroxylation mechanism of HMS as deduced from intermediate partitioning. The proposed chemical mechanism of HMS is shown. Molecules shown boxed are detected as products. The origin of the kinetic isotope effects is shown as italicized notations. Notations P_1 and U refer to the variables of eq 2. The inset depicts two chromatograms for I216N variant in the absence (solid line) and presence (dashed line) of the benzylic dideuterium label of HPP.

hydroxylating intermediate, the pathway to form either HG or QAA is linear, and as such, the isotope effect is defined only by the relative probability of one of the first three steps in the absence and presence of the deuteron. This conclusion is aided by the assumption that no species other than the epoxide and cationic forms can interconvert. This lack reversibility toward products is consistent generally as we expect the reduction of dioxygen to be significantly exothermic overall. However, it is also consistent with the mechanism presented in that we would predict high forward reactivity for the hydroxylating intermediate and the relative instability of the benzylic cation and epoxide species compared to QAA or the dienone intermediate that precedes rearomatization to HG.

The data obtained suggest that the native HPPD hydroxylation reaction results in the formation of a ring epoxide as the first intermediate. Inverse KIE values arising from deuterons placed ortho to the site of hydroxylation are reasonably explicit in this regard. The observation of a KIE of ~1 in the variant enzymes for the hydroxylation step that ultimately results in the liberation of HG differs from the wild-type observation and suggests either no change in hybridization at ring C2, in keeping with a benzylic cation, or that we are observing the net effect of multiple simultaneous pathways. Together these data suggest that the hydroxylation reaction can proceed through either intermediate and that changes in the active site promote to some extent the benzylic cation pathway for HG production. KIEs for the formation of QAA can only be measured for three of the variant enzymes. Again, two show KIEs close to unity and one is significantly inverse, bolstering the multiple similar pathway theory from above.

The concept of multiple pathways yielding a given product where the overall reaction traverses an energy landscape involving multiple saddle points has been suggested for a number of systems.[50,51] Such phenomena account for the multiple conformational states that an enzyme population adopts at any point in time and yet remains catalytic. It cannot be gleaned if the observations made here are a consequence of biasing one conformational state and/or a different dynamic population of states in the variant enzymes. However, it does seem to be a fascinating example of built in malleability in the reaction coordinate as a consequence of multiple energetically similar pathways being available.

The KIEs measured for variants of HMS each show small normal values consistent with abstraction of the hydrogen during hydroxylation (Figure 4). This narrows the mechanistic possibilities and shows that HMS, in all likelihood, functions much the same as other aliphatic hydroxylating α-KAO enzymes. The magnitude of the KIE (~2) suggests that the

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abstraction of the hydrogen is less optimized than in other αKAO enzymes and unlikely to be aided by tunneling effects in the hydrogen abstraction step.

Prior to this investigation, only two studies had offered evidence to suggest the hydroxylation reaction intermediate of HPPD. The first was the work of Gunsior et al., who observed the liberation of an oxepinone from the P243T variant, the conclusion was that the oxepinone was a rearrangement product derived from the abortive release of an epoxide intermediate following the second oxygenation.[46] Later, Raspail et al., perpetuated this notion without identifying the actual product formed from an S246A variant (equivalent to the S230A variant in this study).[52] Our data show that the P243T and S230A variants make predominantly QAA. The observations of Gunsior et al., who correctly identified the oxepinone, are accounted for by the conversion of QAA to the oxepinone under the conditions used to purify the product for identification. Specifically, the use of trifluoroacetic acid in the chromatography buffer, a molecule known to catalyze conversion of quinol derivatives to oxepinones.[53,54] We therefore respectfully propose that the earlier observation was an artifact. However, the irony of this assertion is that QAA formed by the P243T exhibits a KIE of 0.76, clearly suggesting the intermediacy of an epoxide (Table 3).

The remaining oddity is that QAA is known to convert quantitatively to HG in solution at pH 12.0,[55] suggesting that the NIH shift does not require elaborate enzymatic machinery. QAA however is not a substrate for HPPD as no amount of HG is observed when QAA is incubated with the enzyme (data not shown). With the delivery of the second oxygen atom, an accounting of electrons would indicate that the enzyme has returned to the ferrous state (Figure 2). As such, the resting enzyme should be in the correct form to carry out the NIH shift from QAA to yield HG. The tentative conclusion is that QAA is thus an off-pathway product and not an intermediate that occurs in normal catalysis.

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ABBREVIATIONS

HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPP, 4-hydroxyphenylpyruvate; HG, homogentisate; QAA, quinolacetic acid; HMS, hydroxymandelate synthase; HMA, hydroxymandelate; HPA, hydroxyphenylacetate; α-KAO, α-keto acid dependent oxygenase; βME, β-mercaptoethanol; TFA, trifluoroacetate; NIH, National Institutes of Health; KIE, kinetic isotope effect; HPLC, high pressure liquid chromatography; WT, wild type; NMR, nuclear magnetic resonance.

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