J|A|C|S COMMUNICATIONS

Published on Web 03/31/2010

Measurement of the Intramolecular Isotope Effect on Aliphatic Hydroxylation by *Chromobacterium violaceum* Phenylalanine Hydroxylase

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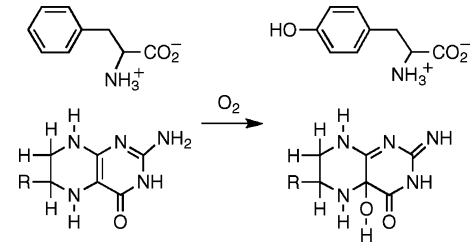
Phenylalanine hydroxylase (PheH) is a non-heme iron-dependent monooxygenase that catalyzes the hydroxylation of the amino acid phenylalanine to yield tyrosine (Scheme 1).[1] PheH is found in organisms ranging from bacteria to humans. In mammals, the enzyme is responsible for catabolism of dietary phenylalanine, and mutations in PheH are linked to the disorder phenylketonuria.[2] Among the bacterial enzymes, that from *Chromobacterium violaceum* (CvPheH) is the most studied.[3−6] PheH is a member of the family of aromatic amino acid hydroxylases, along with tyrosine hydroxylase (TyrH) and tryptophan hydroxylase.[1] Each of these enzymes catalyzes the hydroxylation of the corresponding aromatic amino acid using molecular oxygen and the electrons from a tetrahydropterin.[7] In addition to aromatic hydroxylation, the aromatic amino acid hydroxylases can catalyze the hydroxylation of benzylic and aliphatic substrates.[8−10] Previously, the isotope effect on benzylic hydroxylation was used to compare the reactivity of the eukaryotic enzymes with that of CvPheH.[6,10,11] These studies showed similar reactivities for all of the enzymes and suggested a common hydroxylating intermediate for all of the family members.

Crystal structures of the three eukaryotic enzymes and CvPheH have revealed a common fold for the catalytic domain.[12−14] The active sites are characterized by a ferrous iron coordinated on one face by two histidines and a glutamate. Three water molecules complete an octahedral geometry. When a tetrahydropterin and an amino acid substrate are present, the geometry around the iron changes from six- to five-coordinate, presumably opening a site for oxygen to directly coordinate to the iron.[15,16] The proposed hydroxylating intermediate is an Fe(IV)O species; direct evidence for such an intermediate has been obtained for TyrH.[17] Such a reactive intermediate could explain the rich chemistry displayed by these enzymes. Here we report the use of an intramolecular kinetic isotope effect as a probe of the chemical mechanism of aliphatic hydroxylation by CvPheH. The results shed light on the reactivity of the hydroxylating intermediate for the family of aromatic amino acid hydroxylases.

The hydroxylation of cyclohexylalanine by prokaryotic or eukaryotic PheH yields 4-HO-cyclohexylalanine as the only amino acid product, and amino acid hydroxylation is fully coupled to tetrahydropterin oxidation.[18] With 6-methyltetrahydropterin as the pterin substrate, we obtained k_{cat} and k_{cat}/K_m values of 2.8 ± 0.1 s^{-1} and 45 ± 6 $mM^{-1}s^{-1}$, respectively, for cyclohexylalanine as the substrate for CvPheH at 25 °C and pH 7, compared with values of 12 s^{-1} and 180 ± 20 mM^{-1} s^{-1}, respectively, with phenylalanine as the substrate.[6] In order to gain insight into the chemical mechanism of this reaction, we measured deuterium kinetic isotope effects using 3-[$^2H_{11}$-cyclohexyl]alanine.[18] The isotope effect on

Scheme 1



k_{cat} was 0.99 ± 0.1 with 6,7-dimethyltetrahydropterin and 1.03 ± 0.1 with 6-methyltetrahydropterin. These results suggest that a step that does not involve hydrogen atom abstraction is rate-limiting with this substrate.

As an alternative approach, the isotope effect on the hydroxylation of cyclohexylalanine was analyzed as an intramolecular isotope effect. Such an approach can avoid the problem of slow, nonchemical steps.[19] To do so, 3-[1,2,3,4,5,6-$^2H_6$-cyclohexyl]alanine,[18] in which the carbon of interest has one deuterium and one hydrogen, was used as the substrate. The amount of deuterium in the 4-HO-cyclohexylalanine formed from 3-[1,2,3,4,5,6-$^2H_6$-cyclohexyl]alanine was then determined by mass spectrometry of the isolated product.[11] When the 3-[1,2,3,4,5,6-$^2H_6$-cyclohexyl]alanine was synthesized by reduction of phenylalanine with D_2, the ratio of product that retained all the deuterium to that which had lost one deuterium was 2.5 ± 0.1. When similar experiments were carried out with 3-[2,3,4,5,6-$^2H_5$-cyclohexyl]alanine obtained by reduction of L-[ring-$^2H_5$]phenylalanine with H_2, the ratio of products was 3.03 ± 0.1.

The isotope effect for CH bond cleavage can be obtained from the partitioning between H and D abstraction if one makes two reasonable assumptions: (1) CH bond cleavage is irreversible, and (2) the cyclohexyl ring cannot flip in the active site more rapidly than CO bond formation occurs. The first assumption is reasonable for a hydroxylation reaction. The second is supported by the structure of human PheH with bound amino acid[15] and by the stoichiometric coupling of tetrahydropterin oxidation to amino acid hydroxylation with cyclohexylalanine as a substrate for CvPheH. The position of the hydroxyl group in the product is the result of the partitioning between hydroxylation of C4 at the axial (a) position and hydroxylation at the equatorial (e) position (Scheme 2). The ratio of the two products (P_a/P_e) is then described by eq 1. The monodeuterated substrate can be bound with deuterium in the axial or equatorial position. If deuterium is in the axial position, the rate constant for cleavage of the axial CH bond, k_a, will be subject to the primary kinetic isotope effect ^Dk, and that for cleavage of the equatorial CH bond, k_e, will be subject to the secondary isotope effect $_\alpha{}^Dk$. In this case, the ratio of the product that has lost

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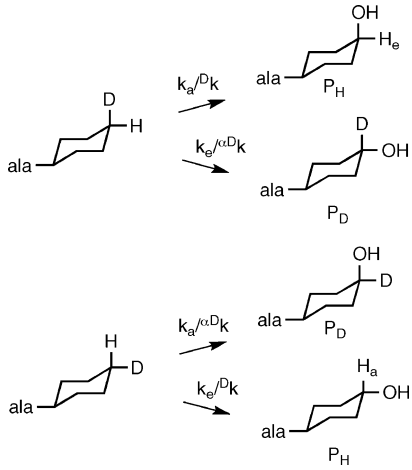
10.1021/ja101563t

Scheme 2. Intramolecular Isotope Effects on Cyclohexylalanine Hydroxylation



deuterium, P_H, to the one that has retained deuterium, P_D, is given by eq 2. If deuterium is in the equatorial position, the isotope effects are on the opposite steps (Scheme 2), and the ratio of products is given by eq 3. No preference is anticipated for binding of the substrate with deuterium in the equatorial versus the axial position. Thus, the isotopic content of the product is the average of the contents from the two binding orientations (eq 4). Previously, Carr et al.[18] reported that 90% of the hydroxylation occurs at the axial position with cyclohexylalanine, giving $k_a/k_e = 9$. Combining this value with the average value of 0.36 ± 0.02 for P_H/P_D reported here yields a value of 12.6 ± 1.0 for $^Dk/^{\alpha D}k$. If an upper limit of 1.2 is used for the secondary isotope effect, ^Dk has an upper limit of 15.1 ± 1.2.

$$P_a/P_e = k_a/k_e \tag{1}$$

$$(P_H/P_D)_a = (k_a/k_e)(^{\alpha D}k/^Dk) \tag{2}$$

$$(P_H/P_D)_e = (k_e/k_a)(^{\alpha D}k/^Dk) \tag{3}$$

$$(P_H/P_D)_{av} = 0.5(^{\alpha D}k/^Dk)[(k_a/k_e) + (k_e/k_a)] \tag{4}$$

The isotope effect on hydroxylation of cyclohexylalanine by CvPheH is larger than that on benzylic hydroxylation by the enzyme (10 ± 1).[6] In view of its magnitude, the intermolecular isotope effect of 12.6−15.1 is likely to be the intrinsic one. This value is comparable to the deuterium isotope effects on aliphatic hydroxylation by the α-ketoglutarate-dependent non-heme enzyme TauD ($^Dk = 16$),[20] which utilizes a Fe(IV)O center similar to that in PheH,[21] and by the heme-dependent cytochrome P450 ($^Dk = 12$).[22,23] The magnitude of the value with CvPheH is consistent with a mechanism involving hydrogen atom abstraction by the Fe(IV)O center followed by rebound of the hydroxyl radical, a mechanism also suggested for the other enzymes. The large isotope effect also suggests the involvement of tunneling in this reaction.[24,25] Evidence of tunneling has also been demonstrated for the hydroxylation of benzylic carbons by all of the aromatic amino acid hydroxylases.[6,10] The lack of an isotope effect on the k_{cat} value with 3-[$^2H_{11}$-cyclohexyl]alanine establishes that hydrogen atom abstraction is much faster than other steps in turnover, even though this enzyme is not evolved to carry out this reaction. Hydroxylation is similarly not the rate-limiting step in the hydroxylation of phenylalanine by CvPheH.[6] In the case of TyrH, product release has been shown to be substantially slower than hydroxylation of the normal substrate.[26]

The results presented here are consistent with the involvement of a highly reactive Fe(IV)O center as the hydrogen-atom-abstracting species for the aliphatic hydroxylation carried out by CvPheH. The comparable magnitudes of the isotope effects on aliphatic hydroxylation by the aromatic amino acid hydroxylases and by α-ketoglutarate and heme-dependent enzymes as well as the ability of all three systems to catalyze aliphatic hydroxylation suggest that their hydroxylating intermediates have comparable reactivities.

Acknowledgment. This work was supported in part by grants from the NIH (GM47291) and the Welch Foundation (AQ-1245).

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JA101563T