VOLUME 120, NUMBER 17
MAY 6, 1998

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Oxygen-18 Kinetic Isotope Effect Studies of the Tyrosine Hydroxylase Reaction: Evidence of Rate Limiting Oxygen Activation

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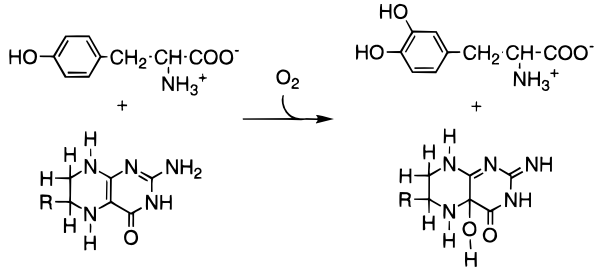
Received October 9, 1997

Abstract: Tyrosine hydroxylase converts tyrosine to dihydroxyphenylalanine utilizing a tetrahydropterin cofactor and molecular oxygen. Previous deuterium isotope effect studies have raised the possibility that the activation of oxygen might be the rate-limiting step for this reaction. To test the validity of this proposal, we have measured the ^{18}O kinetic isotope effects for the tyrosine hydroxylase reaction as a function of amino acid substrate, tetrahydropterin derivative, and pH. The measured ^{18}O isotope effects are nearly constant in every condition tested with an average value of 1.0175 ± 0.0019. These results are consistent with a change in the bond order to oxygen in the rate determining step. Furthermore, the isotope effects measured with the coupled substrate 4-methoxyphenylalanine and the completely uncoupled substrate 4-aminophenylalanine are identical, implying the same rate determining step independent of whether oxygen activation is coupled to substrate hydroxylation. The results of these studies provide strong support for a rate limiting reductive activation of molecular oxygen, most likely via a one-electron transfer from the tetrahydropterin to form superoxide anion as the first reactive intermediate.

Introduction

Tyrosine hydroxylase [EC 1.14.16.2] catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine (dopa) (Scheme 1), the first and rate-limiting step in the biosynthesis of catecholamine neurotransmitters.[1] Together with phenylalanine hydroxylase and tryptophan hydroxylase, they constitute a small family of non-heme iron enzymes which catalyzes the hydroxylation of aromatic amino acids, using molecular oxygen and tetrahydropterin as the additional substrates.

Scheme 1. Overall Reaction Catalyzed by Tyrosine Hydroxylase



The chemical mechanism of these enzymes is poorly understood. The kinetic mechanism of tyrosine hydroxylase has been extensively studied and is consistent with the ordered binding of tetrahydropterin first, followed by oxygen and then tyrosine;

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S0002-7863(97)03543-9 CCC: $15.00
Published on Web 04/17/1998

additionally, a dead-end enzyme tyrosine complex forms.[2] There is no primary deuterium[2] nor solvent[3] kinetic isotope effect on V_{max} or V/K_{Tyr}. These results rule out a rate-limiting hydrogen transfer step under steady-state conditions, suggesting instead the possibility of a rate limiting conformational change or a slow activation of molecular oxygen.

If the formation of the hydroxylating intermediate for the reaction catalyzed by tyrosine hydroxylase is the rate-limiting step, it is expected that the measurement of ^{18}O kinetic isotope effects can provide evidence for the validity of this proposal. A technique for the measurement of ^{18}O isotope effects has been presented by Tian and Klinman[4] and employed for the measurement of equilibrium isotope effects on oxygen binding to its biological carriers. Subsequent kinetic isotope effects measurements were carried out with the enzymes dopamine β-monooxygenase[5] and lipooxygenase.[6]

We now extend this methodology to tyrosine hydroxylase, providing direct evidence for a rate-limiting reductive activation of molecular oxygen. Among the mechanistic alternatives, a limiting one-electron transfer from tetrahydropterin to form superoxide anion appears most probable.

Materials and Methods

Materials. 4-Methoxyphenylalanine, 4-aminophenylalanine, sheep liver dihydropteridine reductase, and NADH were from Sigma Chemical Co., St. Louis, MO. 4-Methoxyphenylalanine was further purified by using a 19 × 300 mm Waters C18 μBondapak column in 6% CH_3CN. Tetrahydropterin (PH_4) was from Dr. B. Schircks (Jona, Switzerland). 6-Methyltetrahydropterin (MPH_4) was synthesized as described previously.[7]

Recombinant rat tyrosine hydroxylase was purified as described previously.[8] The enzyme concentration was determined with use of an $\epsilon_{280}^{1\%}$ value of 10.4 and a subunit molecular weight of 56000.[9] Catalase was from Boehringer Mannheim, Indianapolis, IN.

Methods: ^{18}O Isotope Effects. ^{18}O effects were measured by a competitive method described by Tian et al.[5] This method involves the isolation of O_2 remaining in the reaction mixture followed by determination of the extent of reaction and the isotope ratio in the isolated oxygen. First, samples of 125 mL of 50 mM MES (pH 6.5) or 50 mM Hepes (pH 8.0), 70−85 mg of NADH, and variable amounts of substrate (4-methoxyphenylalanine or 4-aminophenylalanine) were saturated with O_2 by bubbling pure O_2 through the buffer solution for ca. 20 min. The O_2-saturated buffer was then transferred to the vacuum apparatus. To the transferred solution, 100 μL of catalase (20 mg/mL), 500 μL of dihydropteridine reductase (1.4 mg/mL), and 1.5 mL of tyrosine hydroxylase (2.5−5.5 mg/mL) were added before the reaction vessel was sealed with a rubber plunger. The total volume was ca. 75 mL. The first sample removed was a blank. The O_2 was isolated and converted to CO_2. The total CO_2 concentration was measured by transferring the CO_2 sample to a pressure transducer. A solution of tetrahydropterin or 6-methyltetrahydropterin was injected into the reaction container to start the reaction. After a certain period of time, a sample was removed and the O_2 was isolated and converted to CO_2. Three time point samples were usually taken per experiment. The CO_2 samples were analyzed for ^{18}O content at Geochron Laboratories in Cambridge, MA, by isotope ratio mass spectrometry. The relative pressure values of CO_2 from blank and reaction samples were used to calculate the extent of reaction, f, and the isotope ratios were used to calculate R_f/R_0. The ^{18}O isotope effect was then calculated with eq 1.

$$^{18}(V/K) = 1/[1 + \ln(R_f/R_0)/\ln(1 - f)] \quad (1)$$

Table 1. $^{18}(V/K)$ Isotope Effects for Tyrosine Hydroxylase[a]

pH	amino acid substrate	pterin[b]	$^{18}(V/K)$
6.5	4-methoxyphenylalanine (0.6 mM)	$6MPH_4$	1.0175 ± 0.0010
6.5	4-methoxyphenylalanine (2.0 mM)	$6MPH_4$	1.0185 ± 0.0012
6.5	4-methoxyphenylalanine (3.8 mM)	$6MPH_4$	1.0175 ± 0.0012
6.5	4-methoxyphenylalanine (10 mM)	$6MPH_4$	1.0162
6.5	4-methoxyphenylalanine (2.0 mM)	PH_4	1.0186 ± 0.0002
8.0	4-methoxyphenylalanine (2.0 mM)	$6MPH_4$	1.0161 ± 0.0037
6.5	4-aminophenylalanine (100 μM)	$6MPH_4$	1.0171 ± 0.0017

[a] The conditions for the $^{18}(V/K)$ isotope effects measurements are described under Methods. [b] The concentration of $6MPH_4$ and PH_4 used was 25 to 50 μM.

Results and Discussion

The formation of the hydroxylating intermediate in the reaction catalyzed by tyrosine hydroxylase has been suggested to be rate limiting, based on steady-state kinetics and deuterium kinetic isotope effect experiments.[2,3] Therefore, it was anticipated that ^{18}O isotope effects could provide valuable insight into the validity of this proposal and possibly the nature of the hydroxylating intermediate. ^{18}O kinetic isotope effects have now been measured for the tyrosine hydroxylase reaction as a function of amino acid substrate, tetrahydropterin derivative, and pH. The results of the ^{18}O kinetic isotope effect experiments are summarized in Table 1. It can immediately be noted that the measured ^{18}O kinetic isotope effects are large under all experimental conditions studied. These results are consistent with a change in the bond order to oxygen in the rate determining step.

Tyrosine hydroxylase catalyzes the hydroxylation of a variety of aromatic amino acids and phenylalanine derivatives, the majority of which afford multiple products. 4-Methoxyphenylalanine is an excellent substrate for the study of the tyrosine hydroxylase for two main reasons: (1) its enzymatic oxidation affords 4-methoxy-3-hydroxyphenylalanine as the only product and (2) the oxidation of the amino acid is completely coupled to the oxidation of the tetrahydropterin cofactor.[10] For these reasons, 4-methoxyphenylalanine was chosen as the amino acid substrate for the initial ^{18}O kinetic isotope effect measurements.

The kinetic mechanism of tyrosine hydroxylase indicates that oxygen binds after tetrahydropterin and before tyrosine and that this binding is a rapid equilibrium process.[2] The magnitudes of ^{18}O kinetic isotope effects as a function of time and 4-methoxyphenylalanine concentration are shown in Figure 1 and summarized in Table 1. It is evident that these effects are independent of the amino acid concentration. This result is consistent with the substrate concentration dependence of the isotope effects in an equilibrium ordered mechanism, in which the V/K isotope effects are the same regardless of which substrate is varied, and regardless of the level of the second substrate.[11] If we consider the minimum kinetic mechanism shown in eq 2, where oxygen binds to the enzyme·tetrahydropterin complex before the amino acid, followed by an irreversible isotopic sensitive step,

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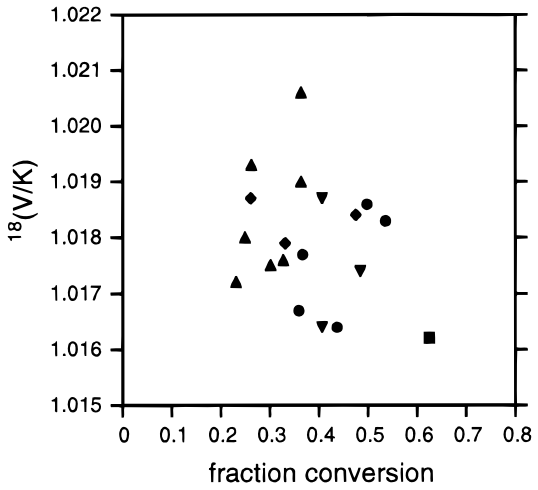

Figure 1. $^{18}(V/K)$ vs fraction conversion for the reaction of 4-methoxyphenylalanine (0.6 mM (●), 2.0 mM (▲), 3.8 mM (▼), and 10 mM (■) at pH 6.5 and 2.0 mM (◆) at pH 8.0) with tyrosine hydroxylase.

$$E{\cdot}PH_4 + O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}PH_4{\cdot}O_2 + aa \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}PH_4{\cdot}O_2{\cdot}aa \xrightarrow{k_5} \quad (2)$$

the expressions for the ^{18}O kinetic isotope effect on V/K can be easily derived as:

$$^{18}(V/K)_{O_2} = \frac{^{18}k_5 + \frac{k_5}{k_4}\left(1 + \frac{k_3[aa]}{k_2}\right)}{1 + \frac{k_5}{k_4}\left(1 + \frac{k_3[aa]}{k_2}\right)} \quad (3)$$

where $^{18}k_5$ is the ^{18}O intrinsic isotope effect. For an equilibrium ordered mechanism, k_2 is very fast with respect to k_3[aa]. Underthese conditions, the expression for $^{18}(V/K)_{O_2}$ reduces to:

$$^{18}(V/K)_{O_2} = (^{18}k_5 + k_5/k_4)/(1 + k_5/k_4) \quad (4)$$

which is independent of the concentration of amino acid and equal to $^{18}(V/K)_{aa}$. For such a mechanism, the full isotope effect on k_5 is seen when the amino acid substrate dissociates rapidly relative to catalysis, i.e., the commitment to catalysis is small ($k_4 > k_5$). Although this has not been established for tyrosine hydroxylase, we note that increase of pH from 6.5 to 8.0 has no effect on the measured isotope effects (Table 1), ruling out a pH-sensitive external commitment.

With nonphysiological substrates, the oxidation of tetrahydropterin can be uncoupled from the oxidation of the amino acid.[1] When 4-aminophenylalanine is used as the amino acid substrate for tyrosine hydroxylase, the oxidation of tetrahydropterin is completely uncoupled and no hydroxylated product is formed.[12] As shown in Table 1, the ^{18}O isotope effects obtained with 4-aminophenylalanine are virtually identical to those of the fully coupled substrate 4-methoxyphenylalanine. This implies the same rate determining step, independent of whether oxygen activation is linked to substrate hydroxylation.

^{18}O equilibrium isotope effects have been calculated for the reduction of molecular oxygen to the species $O_2^{\bullet-}$, $HO_2^{\bullet}$, O_2^{2-}, HO_2^-, H_2O_2, and H_2O.[4] These authors also measured the equilibrium isotope effects of the reversible oxygen carriers hemoglobin, myoglobin, hemerythrin, and hemocyanin and found excellent agreement with the expected equilibrium isotope effects inferred from the known structures of bound oxygen and the calculated ^{18}O equilibrium isotope effects. These calculated equilibrium isotope effects can thus be used as a frame of reference for the interpretation of the ^{18}O kinetic isotope effects, as has been done for dopamine β-monooxygenase[5] and lipooxygenase.[6] Although the calculated isotope effects could be slightly different when the hydrogen bonded to oxygen has been replaced by a carbon or a metal atom, there is indication that the changes, if any, are very small.[4,13]

The ^{18}O kinetic isotope effects measured for the tyrosine hydroxylase reaction in every condition tested are nearly constant, with an average value of 1.0175 ± 0.0019 (Table 1). The magnitude of this isotope effect rules out two very important cases as rate limiting. These are the reactions of dioxygen that involve a change in bonding without a concomitant change in bond order, namely, the formation of either protonated superoxide ($HO_2^{\bullet}$) or hydrogen peroxide, for which the equilibrium isotope effects have been calculated to be 1.0115 and 1.0089, respectively. On the other hand, the results indicate a change in the bond order to oxygen in the rate determining step. The three possibilities to consider are the one-electron reduction of oxygen to form superoxide, the two-electron reduction of oxygen to form O_2^{2-}, and the two-electron reduced, protonated product, HO_2^-. The equilibrium isotope effects calculated for these three species ($O_2^{\bullet-}$, O_2^{2-}, HO_2^-) are 1.0331, 1.0496, and 1.0340, respectively.[4]

The principal kinetic barrier to direct reaction of dioxygen with organic substrates is the spin-triplet ground state of dioxygen. Since organic and biological molecules have paired electrons and singlet ground states, their reactions with dioxygen are spin forbidden. This kinetic barrier can be overcome by exciting the O_2 molecule to the singlet state, by a free radical pathway, or by complexation with a paramagnetic metal ion. Tyrosine hydroxylase could utilize two of these three mechanisms for the activation of oxygen: a free radical mechanism involving the tetrahydropterin cofactor and the complexation of O_2 to the non-heme iron. These two possibilities are described below in detail.

Tyrosine hydroxylase contains an essential non-heme iron. The requirement of the iron has been demonstrated by the lack of activity in apoenzyme[14,15] or in protein variants in which ligands to the iron have been mutated.[16] No other transition metals have been found to be catalytically active, implying that the metal center is involved in catalysis and that it is not merely structural. The isolated enzyme contains a high-spin iron(III) with $S = {}^5/_2$, which is reduced before turnover to the active ferrous form by the tetrahydropterin cofactor.[17]

Due to the high affinity of Fe(II) for dioxygen,[18] it might be expected that oxygen would bind initially to the iron site. If this were the case, significant electron transfer from Fe(II) to O_2 would be anticipated to accompany binding, producing a species with extensive $Fe^{III}O_2^-$ character (Scheme 2). The formation of the iron−dioxygen complex would show an isotope effect of ≤ 1.01 upon its formation,[4] ruling out this step as rate determining. Subsequent one-electron transfer from the reduced

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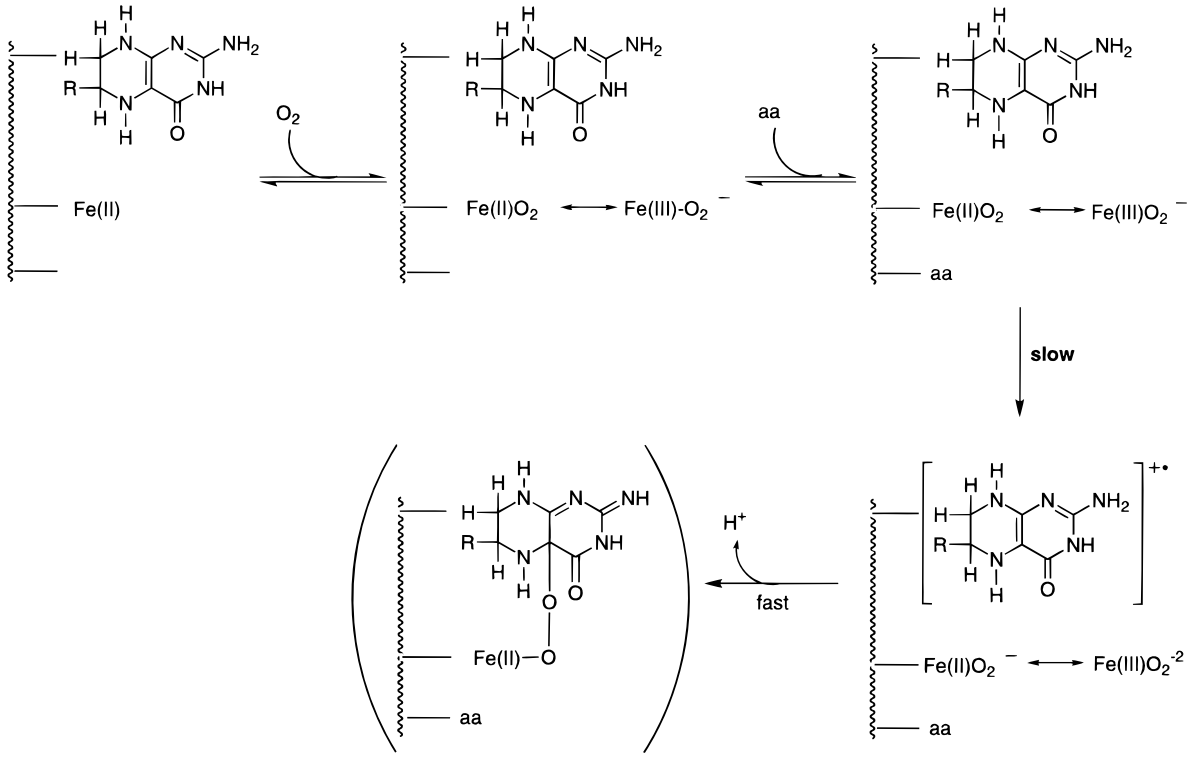
Scheme 2. Proposed Mechanism for the Tyrosine Hydroxylase Reaction Involving Initial Binding of Oxygen to the Fe(II) Center



pterin ring could, in principle, lead to a rate limiting decrease in bond order at the oxygen−oxygen bond by the formation of $Fe^{III}O_2^{2-}$ and a kinetic isotope effect of the magnitude observed in this study (1.0175).

The mechanism in Scheme 2 requires that the initial reaction of Fe(II) with O_2 be reversible and that O_2 dissociate rapidly from the $Fe^{III}O_2^-$ complex ($k_2 > k_3$[aa], as needed for an equilibrium ordered mechanism). An equilibrium ^{18}O isotope effect would occur on O_2 binding, followed by a kinetic isotope effect for the one-electron transfer from tetrahydropterin in the presence of bound substrate. The expression for the ^{18}O isotope effect on $^{18}(V/K)_{O_2}$ for the kinetic mechanism shown in eq 2 when O_2 binds to Fe(II) is:

$$^{18}(V/K)_{O_2} = \frac{^{18}K_1\left(^{18}k_5 + \frac{k_5}{k_4}\right) + \frac{^{18}k_1k_3k_5[\text{aa}]}{k_2k_4}}{1 + \frac{k_5}{k_4}\left(1 + \frac{k_3[\text{aa}]}{k_2}\right)} \qquad (5)$$

where k_1, k_2, and k_5 are isotope sensitive. This reduces to:

$$^{18}(V/K)_{O_2} = {}^{18}K_1(^{18}k_5 + k_5/k_4)/(1 + k_5/k_4) \qquad (6)$$

in the case of an equilibrium ordered mechanism. $^{18}K_1$ is the ^{18}O equilibrium isotope effect on oxygen binding ($^{18}K_1 = [(^{16}k_1/^{16}k_2)/(^{18}k_1/^{18}k_2)]$). The observed isotope effect in eq 6 will, therefore, range from $^{18}K_1$ (ca. 1.01)[4] to $^{18}K_1{}^{18}k_5$ depending on the ratio of k_5 to k_4.

One difficulty with the mechanism in Scheme 2 is that it requires that the overall rate determining step be the one-electron transfer from tetrahydropterin to an already activated form of oxygen ($Fe^{III}O_2^-$) resulting in the thermodynamically preferred two-electron reduction of dioxygen, a condition we consider unlikely. Although precise one-electron reduction potentials for $Fe^{III}O_2^-$ and $6MPH_4$ are not available, we estimate 0.74 V for the $Fe^{III}O_2^-/Fe^{III}O_2^{2-}$ couple (based on the reduction potential for $HOO^\bullet$ to HOO^- at pH 7.0[19]) and 0.26 V for $6MPH_4$ (based on the pH 7.0 reduction potential for the 6,7-blocked pterin, 5,6,6,7,7-pentamethyl-5,6,7,8-tetrahydropterin semiquinone[20]). This leads to an estimate for $\Delta G°$ of −11.1 kcal/mol for the one-electron reduction of $Fe^{III}O_2^-$ by tetrahydropterin.

Additionally, in the event that the mechanism depicted in Scheme 2 were operative, it seems likely that formation of $Fe^{III}O_2^-$ would be detectable by Mössbauer and/or Raman spectroscopy. Such species have been previously characterized in other systems, including Fe(II)-bleomycin[21] and cytochrome P450.[22,23] In the case of tyrosine hydroxylase, however, Mössbauer spectra of the Fe(II) form of tyrosine hydroxylase in the presence and absence of tetrahydropterin are not significantly different.[24] This argues against the formation of an $Fe^{III}O_2^-$ species, at least in the absence of amino acid substrate. Carbon monoxide and nitric oxide are known to bind reversibly to Fe(II) centers, often providing distinctive spectroscopic signatures for the formation of such complexes. Investigation of the spectroscopic properties of tyrosine hydroxylase with CO and NO may provide valuable information regarding a possible interaction of O_2 with iron when substrate is bound.

We further note that the mechanism in Scheme 2 requires that $Fe^{III}O_2^{2-}$ couple to the tetrahydropterin to form the postulated Fe(II) μ-peroxo 4a-tetrahydropterin intermediate (see

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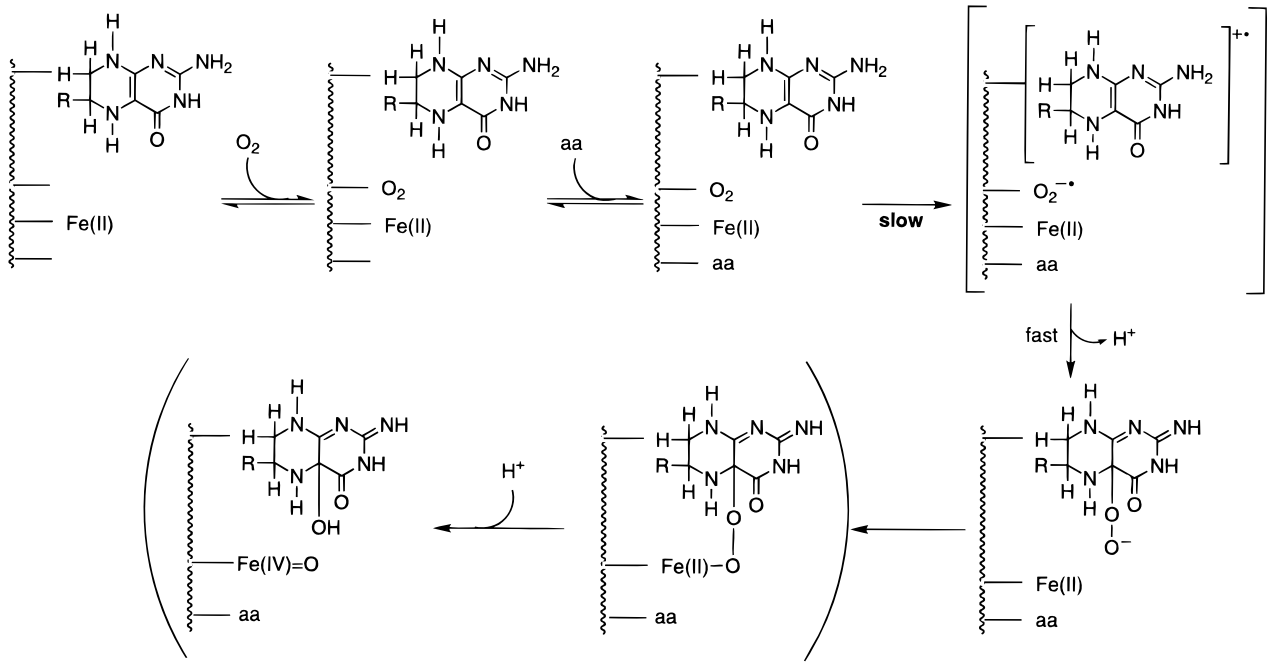
Scheme 3. Proposed Mechanism for the Tyrosine Hydroxylase Reaction Involving an Initial Rate Limiting One-Electron Transfer from Tetrahydropterin to Oxygen



below). As indicated in Scheme 2, this can only occur from a reduced iron species, $Fe^{II}O_2^-$, which is expected to be a minor contributor to the ground-state electronic configuration.

Another possibility for the reaction of O_2 with tyrosine hydroxylase is that oxygen binds weakly to a hydrophobic site on the protein, Scheme 3. Weak binding would be consistent with the rapid equilibrium interaction of O_2 to enzyme. Under this condition, the rate determining step becomes the one-electron transfer from tetrahydropterin to dioxygen to form $O_2^{\bullet-}$. For this step, a $\Delta G°$ of 9.7 kcal/mol can be calculated from the one-electron reduction potentials of oxygen[19] and 5,6,6,7,7-pentamethyl-5,6,7,8-tetrahydropterin semiquinone[20] at pH 7.0. The isotope effect expression for this mechanism is shown in eq 4 where it is assumed that k_1 and k_2 involve little or no change in bond order to O_2. If the commitment to catalysis is small ($k_4 > k_5$), the magnitude of the observed isotope effect reflects $^{18}k_5$, which could be as large as 1.0331 in the case of a late transition state.[4] We note that tetrahydropterins undergo autoxidization in neutral aqueous solutions to form quinonoid dihydropterin and hydrogen peroxide. Model studies with tetrahydropterin analogues have led to the conclusion that the reaction with dioxygen proceeds through an initial one-electron transfer from the tetrahydropterin to O_2. This one-electron transfer is slow and rate controlling.[20] It has been proposed that the transition state closely resembles a tetrahydropterin semiquinone-superoxide radical, which must couple to form a 4a-peroxytetrahydropterin intermediate.[20] This is analogous to the mechanism proposed for the reaction of reduced flavin analogues with oxygen[25] and for the oxygen activation by flavin-dependent phenol hydroxylases.[26]

In both of the mechanisms shown (Schemes 2 and 3), the rate-limiting step is the one-electron transfer from tetrahydropterin to either O_2 or $Fe^{III}O_2^-$. We therefore anticipated that altering the tetrahydropterin structure would affect the rate of the reaction between tetrahydropterin and either oxygen species, which could then have an effect on the measured isotope effects. Consequently, a number of alternate 6-substituted tetrahydropterins were examined as substrates. Among these (6,7-$(CH_3)_2$-PH_4, 6-phenyl-PH_4, 6-$HOCH_2$-PH_4, and tetrahydropterin), PH_4 had kinetic parameters most different from those of $6MPH_4$, with V/K values reduced 4-fold (results not shown). However, the ^{18}O isotope effects measured with PH_4 are identical to those measured with $6MPH_4$ (Table 1). We attribute this to the unchanged standard reduction potential of 600 mV[27] for PH_4 in relation to $6MPH_4$. Unless tetrahydropterins with sufficiently different rates and reduction potentials become available as substrates, it appears unlikely that it will be possible to observe altered isotope effects.[28] This comparison between PH_4 and $6MPH_4$ does suggest that differences in binding interactions between enzyme and cofactor are not affecting transition state structures significantly.

In deciding between Schemes 2 and 3, there are a number of factors that point toward Scheme 3: (1) There is no evidence for a direct interaction between O_2 with the Fe(II) center of tyrosine hydroxylase in the absence of substrate. (2) Thermodynamic arguments indicate that the first electron transfer to O_2 to form superoxide is a far less favorable process than reduction of $Fe^{III}O_2^-$ to $Fe^{III}O_2^{2-}$. (3) Coupling the reduced oxygen to the pterin radical cation is expected to be more facile with $O_2^{\bullet-}$ than with $Fe^{II}O_2^-$, since the latter is expected to have a large degree of $Fe^{III}O_2^{2-}$ character. Various experimental

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(28) The range of tetrahydropterins which could be used for the analyses was limited by the need to minimize the amount of nonenzymatic autoxidation. Consequently, low (25−50 μM) concentrations were used and the quininoid dihydropterin was recycled with use of dihydropteridine reductase and NADH. Tetrahydropterins which were poor substrates for the reductase or for which the autoxidation was too rapid were thus ruled out.

observations are consistent with the cofactor−oxygen intermediates shown in Scheme 3. The initial oxidized pterin product in the reaction of tyrosine hydroxylase, as well as in the reaction of phenylalanine hydroxylase, is the 4a-carbinolamine.[29,30] Experiments with $^{18}O_2$ have unambiguously demonstrated that the source of the oxygen atom in the tyrosine hydroxylase reaction is dioxygen.[31] One of the oxygen atoms of O_2 is found in the hydroxylated product and the other in the carbinolamine 4a-hydroxytetrahydropterin, which slowly dehydrates to quinonoid dihydropterin and water. In the phenylalanine hydroxylase from *Chromobacterium violaceum*, isotope effect studies have provided evidence for a rate determining step before the hydride shift, most likely the formation of an oxygen−pterin intermediate.[32]

In the context of Scheme 3, what role would the iron play in oxygen activation and substrate hydroxylation? One important reactivity difference between pterin- and flavin-dependent hydroxylases is the ability of the former to hydroxylate unactivated aromatic and aliphatic C−H substrates. In this respect, the chemistry of these non-heme iron pterin enzymes is more similar to the cytochrome P450. It is possible that the formation of an Fe(II) μ-peroxo 4a-tetrahydropterin intermediate serves to activate further the oxygen with the formation of a higher valent iron oxo species as the hydroxylating species, as has been previously proposed.[33] Since the formation of the pterin hydroperoxide is expected to be irreversible, any further activation would not appear in $^{18}(V/K)_{O_2}$, which measures steps up to and including the first irreversible step. NMR studies of the binding of the $6MPH_4$ to a cobalt-substituted tyrosine hydroxylase have shown that the metal center and the pterin are in very close proximity, approximately 5 Å apart.[34] This distance is appropriate for the formation of the postulated Fe(II) μ-peroxo 4a-tetrahydropterin. In the absence of direct spectroscopic evidence, however, the formation of such a species must remain speculative.

In conclusion, ^{18}O kinetic isotope effect measurements for the tyrosine hydroxylase have provided, for the first time, direct evidence of activation of molecular oxygen as rate limiting in the reaction catalyzed by tyrosine hydroxylase. While two possible mechanisms have been presented and described above, a slow one-electron transfer from the tetrahydropterin cofactor to oxygen to form superoxide anion (Scheme 3) is in better accordance with the available data. We note that earlier model studies of the oxidation of pterin[20] and flavin[25] analogues and studies of the related flavoprotein systems[26] have postulated a similar rate determining step.

Acknowledgment. This work was supported by National Institutes of Health Grants GM 25765 (J.P.K.) and GM 47291 (P.F.F.) and Robert A. Welch Foundation Grant A-1245 (P.F.F.). W.A.F. was supported by postdoctoral fellowships from the NIH (F32 GM 17026) and the Ford Foundation.

JA973543Q

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