

Dopamine β -Hydroxylase

COMPARATIVE SPECIFICITIES AND MECHANISMS OF THE OXYGENATION REACTIONS*

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Dopamine β -hydroxylase (EC 1.14.17.1), a copper-containing monooxygenase present in a variety of mammalian tissues, plays a key role in the biosynthetic interconversion of neurotransmitters and in the production of adrenalin. A systematic structure-activity analysis has now been carried out, establishing that this enzyme readily carries out the sulfoxidation and ketonization of a variety of substrates, and that these activities are kinetically comparable to the long-recognized methylene hydroxylation activity. Thus, dopamine β -hydroxylase exhibits wide specificity with respect to the functionality undergoing oxygenation, though it is considerably more selective than the broad specificity microsomal P-450 and flavin monooxygenases. A systematic analysis of the effects of phenyl substituents on both the sulfoxidation and hydroxylation reactions has been carried out, and the kinetic constants obtained have been correlated with σ ⁺ substituent constants in order to provide direct comparisons to peroxide and P-450 oxygenations. The striking difference in ρ values for sulfoxidation and hydroxylation, by comparison to those reported for P-450 and peroxide reactions, suggests that dopamine β -hydroxylase-catalyzed sulfoxidation proceeds via a nucleophilic mechanism, while hydroxylation involves much less charge development in the transition state. On the basis of these results, together with an analysis of the kinetic consequences of altering the electron donor for each of the dopamine β -hydroxylase-catalyzed oxygenation reactions, a detailed mechanism for dopamine β -hydroxylase-catalyzed oxygenation is proposed and correlated with other information in the literature. Taken together, the results reported provide both insight into the mechanism of dopamine β -hydroxylase action and basic structure-activity information with regard to both the chemical nature of the functionality undergoing oxygenation and the secondary structural features which critically affect substrate reactivity.

Dopamine β -hydroxylase (EC 1.14.17.1), a copper-containing monooxygenase present in a variety of mammalian tissues (1, 2), catalyzes methylene hydroxylation at the benzylic position in a variety of phenyl-substituted alkyl amines (1-4). Dopamine β -hydroxylase has attracted increasing interest

recently due to its key role in the biosynthetic conversion of neurotransmitters and in the production of adrenalin (5, 6). Accordingly, structure-activity data for dopamine β -hydroxylase must be critically considered in the design of novel neurotransmitter analogs that have potential pharmacological interest.

In preliminary reports from this laboratory, we have established two new oxygenase activities for dopamine β -hydroxylase: sulfoxidation of thioethers and ketonization of the enantiomers of hydroxylation products (7-10). Thus, it now becomes clear that a complete structure-activity analysis for dopamine β -hydroxylase must take into account both the chemical nature of the functionality undergoing oxygenation and those secondary structural features of substrates which have heretofore been presumed to critically affect substrate activity. In this paper, we report a systematic analysis of dopamine β -hydroxylase reactivity in hydroxylation, sulfoxidation, and ketonization reactions. Our results not only define the kinetic consequences of structural alterations on dopamine β -hydroxylase reactivity, but also provide insight into the mechanism of its action and a direct mechanistic comparison with P-450-catalyzed oxygenations and chemical systems.

MATERIALS AND METHODS

Dopamine β -hydroxylase was isolated and purified from bovine adrenals (9, 11) and exhibited a specific activity of 12-15 units/mg in the standard O₂ monitoring assay. Tyramine hydrochloride, ascorbic acid, sodium fumarate, *p*-fluorophenethylamine hydrochloride, *p*-chlorophenethylamine, *p*-bromophenethylamine, *p*-methoxyphenethylamine, and MES¹ were obtained from Sigma Chemical Co. 2-Phenylethylamine, *p*-tolylethylamine, 3-phenylpropylamine, and phenylethylenediamine were obtained from the Aldrich Chemical Co. Substrates which were obtained as free bases were converted to the hydrochlorides and crystallized before use in kinetic determinations. K₄Fe(CN)₆·3H₂O was a product of Fisher Scientific. (+)-Norpseudoephedrine was obtained from Knoll Fine Chemicals, and an authentic sample of 2-aminopropiophenone (cathinone) was kindly supplied by Dr. Sheldon Sparber of the University of Minnesota. Other chemicals and solvents were obtained from standard commercial sources, and were of the highest quality obtainable.

Determination of Kinetic Constants—Kinetic constants (k_{cat} , K_m , k_{cat}/K_m) were determined using either the polarographic O₂ monitor assay, which we have developed for ascorbate-supported reactions, or the spectrophotometric assay with ferrocyanide as reductant (12). The dopamine β -hydroxylase preparation used in kinetic determinations had a specific activity of 14.7 units/mg (1 unit is defined as 1 μ m/min of O₂ consumption) under standard O₂ monitor assay conditions. In determining k_{cat} values, the molecular weight of dopamine β -hydroxylase was taken to be 2.9×10^5 (6).

The O₂ monitor measurements were carried out in 0.1 M sodium acetate, pH 5.0, containing 10 mM sodium fumarate, 5 μ M CuSO₄, 10-12 mM ascorbic acid, 300 μ g/ml of catalase, atmospheric O₂ saturation, and various amounts of substrate, at 37.0 °C. Under these conditions, we find that air saturation corresponds to about 250 μ M O₂ (9). The kinetic measurements with K₄Fe(CN)₆ as reductant were carried out

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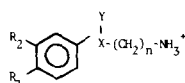
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¹ The abbreviation used is: MES, 2-(*N*-morpholino)ethanesulfonic acid.

in 0.1 M MES, pH 6.1, containing 10 mM sodium fumarate, 5 μ M CuSO₄, 1.5 mM K₄Fe(CN)₆, and atmospheric O₂ saturation, at 37.0 °C. Under these conditions we find that $\Delta\epsilon_{420} = 1100 \text{ M}^{-1} \text{ cm}^{-1}$. In both cases, reactions were initiated by addition of the variable substrate (Scheme 1), and the endogenous nonenzymatic rate was subtracted from the dopamine β -hydroxylase-dependent rate. Kinetic constants were calculated from the data using either the method of Eisenthal and Cornish-Bowden (13) or using the computer program of Cleland (14) to fit the hyperbolic form of the Michaelis-Menten equation.

Since the O₂ monitor assay has not been widely used (7, 9), the validity of using oxygen consumption as a measure of dopamine β -hydroxylase activity was confirmed by quantitative comparisons between the rates of oxygen consumption, product formation, and electron consumption. As shown in Table I, the amount of octopamine formed, as determined spectrophotometrically after periodate oxidation to *p*-hydroxybenzaldehyde (15), corresponds stoichiometrically to the total amount of oxygen consumed during ascorbate-supported tyramine hydroxylation. For comparison of electron and oxygen consumption rates, Fe(CN)₆⁴⁻ was used as the reducing agent instead of ascorbate to support dopamine β -hydroxylase hydroxylation of tyra-



- Ia : R₁ = R₂ = H, X = CH₃, Y = H, n = 1
 Ib : R₁ = CH₃, R₂ = H, X = CH₃, Y = H, n = 1
 Ic : R₁ = CH₃, R₂ = F, X = CH₃, Y = H, n = 1
 Id : R₁ = F, R₂ = H, X = CH₃, Y = H, n = 1
 Ie : R₁ = OCH₃, R₂ = H, X = CH₃, Y = H, n = 1
 If : R₁ = Cl, R₂ = H, X = CH₃, Y = H, n = 1
 Ig : R₁ = Br, R₂ = H, X = CH₃, Y = H, n = 1
 Ih : R₁ = CH₃, R₂ = H, X = CH₃, Y = H, n = 1
 IIa : R₁ = R₂ = H, X = CH₃, Y = H, n = 2
 IIb : R₁ = R₂ = H, X = Y = S, n = 2
 IIc : R₁ = F, R₂ = H, X = Y = S, n = 2
 IId : R₁ = Cl, R₂ = H, X = Y = S, n = 2
 IIe : R₁ = Br, R₂ = H, X = Y = S, n = 2
 IIf : R₁ = CH₃, R₂ = H, X = Y = S, n = 2
 IIg : R₁ = OCH₃, R₂ = H, X = Y = S, n = 2
 IIIa : R₁ = CH₃, R₂ = H, X = CH₃, Y = OH, n = 1 (S enantiomer)
 IIIf : R₁ = R₂ = H, X = CH₃, Y = OH, n = 1 (α -CH₃) (S,S diastereomer)

SCHEME 1

TABLE I

Comparison of oxygen uptake, product formed, and electron consumption

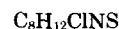
Run	Moles octopamine formed ^a mole oxygen consumed	Moles Fe(CN) ₆ ⁴⁻ consumed ^b mole oxygen consumed
1	1.06	2.1
2	0.97	1.8
3	0.82	

^a Oxygen consumption was followed with a YSI polarographic O₂ monitor at 37 °C for 15 min in 0.1 M potassium phosphate buffer, pH 6.48, containing 20 mM sodium fumarate, 10 mM ascorbic acid, 13,778 units/ml of catalase, and 10 mM tyramine in a total volume of 3.0 ml. The reaction was initiated by addition of 15 μ l of partially purified dopamine β -hydroxylase (through a DEAE-cellulose column). At the end of 15 min, 1 ml of the mixture was withdrawn and quenched by adding 2 ml of 4 M NH₄OH. To this solution, 0.1 ml of 6% NaIO₄ was added. The solution was blended on a Vortex mixer and allowed to stand for 4 min, followed by addition of 0.1 ml of 30% NaHSO₃ and blending on a Vortex mixer. The absorbance of the anion of *p*-hydroxybenzaldehyde thus formed was read at 333 nm and compared to a standard curve. Octopamine standards and blanks containing no enzyme were also run.

^b Fe(CN)₆⁴⁻ consumption was monitored spectrophotometrically as described under "Materials and Methods." The reaction was initiated by adding 6.8 μ g of dopamine β -hydroxylase to 2.0 ml of the reaction solution. Oxygen consumption was followed as described in Footnote a, except that the reaction was initiated by addition of 10.2 μ g of dopamine β -hydroxylase to 3 ml of the Fe(CN)₆⁴⁻ solution.

mine, since the amount of ferrocyanide oxidized is readily quantitated spectrophotometrically (12). Again, as shown in Table I, the expected stoichiometry of 2 electrons transferred per oxygen molecule consumed was obtained. We have previously demonstrated that the extremely rapid autooxidation of ascorbate under the assay conditions is controlled by appropriately adjusting catalase levels (9).

Synthesis of Phenyl-2-aminoethylsulfides—Phenyl-2-aminoethyl sulfide (Iib) was synthesized by the method of Wehrmeister (16). Thirty ml of ethanolamine (0.5 mol), 28.6 ml of glacial acetic acid (0.5 mol), and 51 ml of benzenethiol (0.5 mol) were combined in a 200-ml round bottom flask with 30 ml of benzene, and water was removed under reflux as the benzene azeotrope. After a total of 24 ml of H₂O was collected, the crude phenyl-2-aminoethylacetamide was crystallized from 95% ethanol to yield 96 g (98%) of white crystalline plates, m.p. after recrystallization from benzene/hexane, 79–81 °C. The NMR and mass spectra of this material were completely consistent with the structure. The crystalline amide (25.2 g) was hydrolyzed by refluxing with 40 ml of 12 N HCl overnight. The solution was then basified, extracted with ether, dried over anhydrous Na₂CO₃, the ether removed *in vacuo*, and the dark yellow oil was vacuum-distilled (b.p., 78–85 °C at 0.5 mm Hg (literature value, b.p., 90–95 °C at 0.2 mm Hg)) to yield 13.7 g of pale yellow oil. The amine was converted to the hydrochloride salt by addition of a mixture of 7.8 ml of 12 N HCl (1.05 eq) and 10 ml of absolute ethanol to the free base dissolved in 50 ml of anhydrous ether. An oil separated which crystallized upon standing to yield 9.46 g of snow-white needles. The crystalline hydrochloride softened to a gel at 109–112 °C, and melted at 162–163 °C (literature values, 109–116 °C and 162–163 °C (17)).



Calculated: C 50.65 H 6.38 N 7.38 S 16.90

Found: C 50.63 H 6.38 N 7.35 S 16.89

The substituted phenyl-2-aminoethylsulfides were synthesized in a similar manner, using the appropriate substituted benzenethiols (Aldrich Chemical Co.). 4'-Chlorophenyl-2-aminoethylsulfide (IId) gave a hydrochloride which softens to gel at 153–154 °C and melts at 231–232 °C (literature values, 147–152 and 231–232 °C (17)).



Calculated: C 42.87 H 4.95

Found: C 42.88 H 4.98

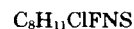
4'-Bromophenyl-2-aminoethyl sulfide (IIe) gave a hydrochloride which softens at 155–157 °C and melts at 195–197 °C.



Calculated: C 35.77 H 4.13

Found: C 35.78 H 4.13

4'-Fluorophenyl-2-aminoethyl sulfide (IIc) gave a hydrochloride which softens at 135–138 °C and melts at 203–204 °C.



Calculated: C 46.26 H 5.34

Found: C 46.27 H 5.38

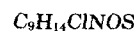
4'-Methylphenyl-2-aminoethylsulfide (IIf) gave a hydrochloride, which softens to gel at 130–132 °C and melts at 158–160 °C (literature value, m.p., 155–156 °C (18)).



Calculated: C 53.06 H 6.93

Found: C 53.03 H 6.93

4'-Methoxyphenyl-2-aminoethylsulfide (IIg) gave a hydrochloride, melting at 135–136 °C (literature value, m.p., 125 °C (18)).



Calculated: C 49.20 H 6.42

Found: C 49.19 H 6.38

Resolution of (*R,S*)-octopamine, synthesis and resolution of (*R,S*)- β -O-ethyloctopamine, and synthesis of 4-hydroxy- α -aminoacetophenone and phenyl-2-aminoethylsulfoxide were accomplished as previously described (7, 9). All synthetic materials were fully characterized by NMR, IR, mass spectral, and elemental analysis.

RESULTS

Comparison of Hydroxylation, Sulfoxidation, and Ketone Activities—The data in Table II clearly establish that dopamine β -hydroxylase readily carries out sulfoxidation and ketonization of appropriate substrates, and that these activities are kinetically comparable to the long recognized hydroxylation activity of this enzyme. Thus, with respect to the functionality undergoing oxygenation, dopamine β -hydroxylase begins to resemble the broad specificity microsomal P-450 and flavin monooxygenases, although these latter enzymes are much more tolerant of secondary structural alterations (10). We note here that we have already unequivocally established the identity of the ketonization and sulfoxidation products via NMR, IR, UV, and mass spectral analyses and by chromatographic comparisons with authentic standards (7, 9). In addition, we have demonstrated for both reactions the requirement for O_2 and electron donor, and the corresponding electron to O_2 to product stoichiometry of 2:1:1, diagnostic for monooxygenases. Finally, dopamine β -hydroxylase-catalyzed ketonization exhibits marked stimulation by Cu^{2+} and fumarate (Fig. 1), as has been well established for hydroxylation (21) and sulfoxidation (7), and the stereochemistry for all three reactions is consistent (7, 9).

It is immediately evident from Table II that, while the k_{cat} values for various substrates exhibit similar trends whether ascorbate or ferrocyanide is the reducing agent, in all cases, the actual values for ferrocyanide-supported oxygenations are greatly reduced. Reduced efficiency in turnover with ferrocyanide can likely be ascribed to the fact that the potential of ferrocyanide is about 300 mV higher than ascorbate, and, in fact, approximates or perhaps even exceeds that of dopamine β -hydroxylase itself (12, 22, 23). In all cases, apparent K_m values for ferrocyanide-supported oxygenations are reduced, and thus kinetic saturation is obtained at lower substrate concentrations. It is interesting to note that for all alternate substrates, regardless of the chemical nature of the functionality undergoing oxygenation, ferrocyanide k_{cat} values are about 10% of those obtained with ascorbate. In contrast, the reactivities of tyramine and phenylethylamine, with the "natural" alkyl chain configuration, are only reduced to 30% of the ascorbate values.

Turning to ascorbate-supported oxygenations, several striking facts emerge from the kinetic data in Table II.

1) Comparison of 3-phenylpropylamine and phenyl-2-aminoethylsulfide provides a measure of the relative ease of S versus C-H oxygenation. Since the K_m values for these sub-

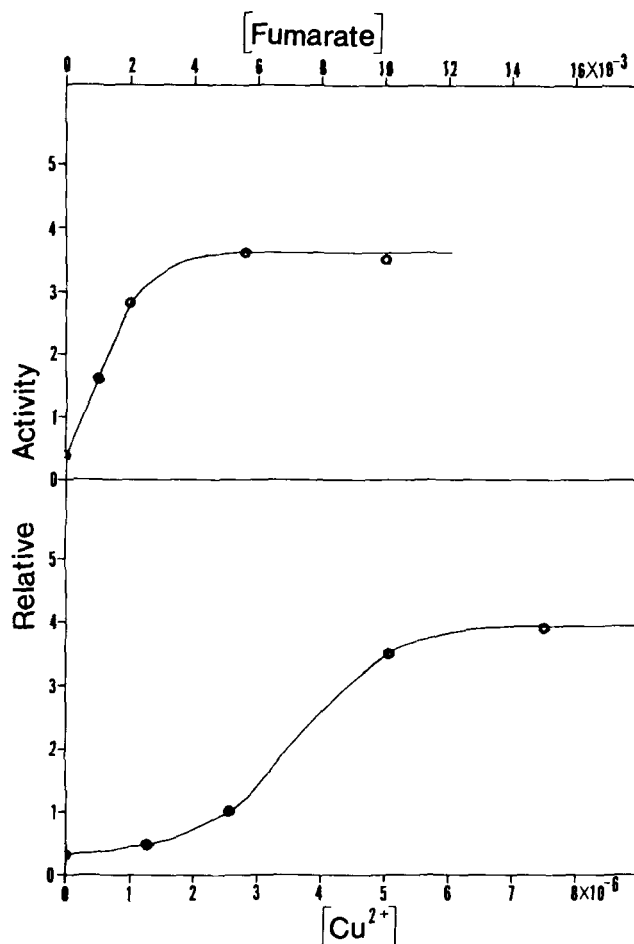


FIG. 1. Stimulation of dopamine β -hydroxylase-catalyzed ketonization by $CuSO_4$ and sodium fumarate. The reactions were carried out in 0.1 M MES, pH 6.1, containing 10 mM *S*-octopamine hydrochloride, 1.5 mM $K_4Fe(CN)_6$, and varying concentrations of Cu^{2+} and fumarate as indicated. Reactions were initiated by addition of 15 μ g of purified dopamine β -hydroxylase to 2.0-ml portions of the buffer, and the reaction was followed by measuring the change in absorbance at 420 nm. Top: effect of varying concentrations of fumarate on dopamine β -hydroxylase activity in the presence of constant 5 μ M Cu^{2+} . Bottom: effect of varying concentrations of Cu^{2+} on dopamine β -hydroxylase activity in the presence of constant 10 mM fumarate.

TABLE II
Kinetic constants for substrates of dopamine β -hydroxylase

Substrate	Reaction type ^a	Ascorbate ^b			$Fe(CN)_6^{4-}$ ^b		
		k_{cat} s^{-1}	K_m mM	k_{cat}/K_m	k_{cat} s^{-1}	K_m mM	k_{cat}/K_m
Tyramine(Ib)	H	80	2.0×10^{-4}	4.0×10^4	26	0.8	3.3×10^4
2-Phenylethylamine(Ia)	H	65	7.0×10^{-4}	0.9×10^4	19	1.0	1.9×10^4
3-Phenylpropylamine-(IIa)	H	12	20.4×10^{-4}	6.0×10^2	1	0.5	2.0×10^3
Phenyl-2-aminoethylsulfide(IIb)	S	68	26.5×10^{-4}	3.0×10^3	6	1.5	4.0×10^3
<i>S</i> -Octopamine(IIIa)	K	33	14.0×10^{-4}	2.4×10^3	2.8	4.4	6.4×10^2
(+)-Norpseudoephedrine(IIIb)	K	10	18.8×10^{-4}	5.4×10^2	0.9	5.4	1.7×10^2
Ascorbate ^c	H	80	1.3	6.1×10^4			
Ferrocyanide ^c	H				22	0.1	2.2×10^5

^a H, hydroxylation; S, sulfoxidation; K, ketonization.

^b Conditions are described in detail under "Materials and Methods."

^c Data obtained at a constant tyramine concentration of 10 mM.

strates are virtually identical, the 6-fold greater reactivity of the sulfide is almost completely reflected in its k_{cat} value. Since it seems clear that k_{cat} values for dopamine β -hydroxylase reflect to a large degree the oxygenation step (25, 26), the greater reactivity of sulfur is expected by analogy to chemical oxidizing agents. It is evident that for substrates of cognate secondary structure, sulfoxidation is now the process catalyzed most readily by dopamine β -hydroxylase, and designation of this enzyme as a "hydroxylase" is misleading.

2) Dopamine β -hydroxylase-catalyzed ketonization of *S*-octopamine and (1*S*,2*S*)-norpseudoephedrine is also obviously a kinetically facile process, and we have previously presented evidence that this process occurs through the normal oxygenation pathway (9) (presumably the *gem*-diol being the immediate product). Diminished reactivity in substrates with α -CH₃ substituents has been previously noted (4), and it is now clear from our data that for the two substrates examined, this is almost completely a k_{cat} effect.

3) Comparison of tyramine, 2-phenylethylamine, and 3-phenylpropylamine reveals that the latter compound is a

perfectly respectable substrate for dopamine β -hydroxylase. The lack of the *p*-OH substituent in 2-phenylethylamine has a minimal effect on k_{cat} , while extension of the alkyl chain to 3 carbons in 3-phenylpropylamine causes a more pronounced decrease in reactivity. On the basis of fixed-time, fixed-concentration assays, Creveling and co-workers (4, 24) reported that 3-phenylpropylamine exhibits about 2% of the activity of tyramine. It is obvious from our data that their observation of such low reactivity is accounted for by the increased K_m for this substrate, while in reality, the k_{cat} is about 20% that of 2-phenylethylamine. This underscores the value of a complete kinetic analysis, once a suitable continuous assay method is in hand.

4) The kinetic data for ascorbate and ferrocyanide, obtained under conditions of fixed tyramine concentration, show the expected agreement in k_{cat} values for the respective electron donors.

Substituent Effects on Hydroxylation and Sulfoxidation—In order to provide insight into the mechanism of dopamine β -hydroxylase action, and to allow for a direct mechanistic comparison with P-450-catalyzed oxygenations and chemical systems, we have carried out a systematic analysis of the effects of aryl substituents on both the sulfoxidation and hydroxylation reactions. Data of this type have very recently been reported for P-450-catalyzed sulfoxidations of thioanisole derivatives (27), and has provided evidence for a radical pathway for P-450-catalyzed sulfoxidations, differing sharply from the presumed nucleophilic mechanism for chemical sulfoxidation with H_2O_2 (28). Our kinetic data with the various substituted phenylethylamines and phenyl-2-aminoethylsulfides are presented in Tables III and IV and are correlated with σ^n substituent constants in Fig. 2. It is immediately evident from the figure that sulfoxidation and hydroxylation differ sharply in their sensitivities to substituent effects, with their respective ρ values being -3.6 and -0.4 . Since ρ values are a measure of the extent of charge development in the transition state (29, 30), these results suggest a nucleophilic mechanism for sulfoxidation, similar to that operative in

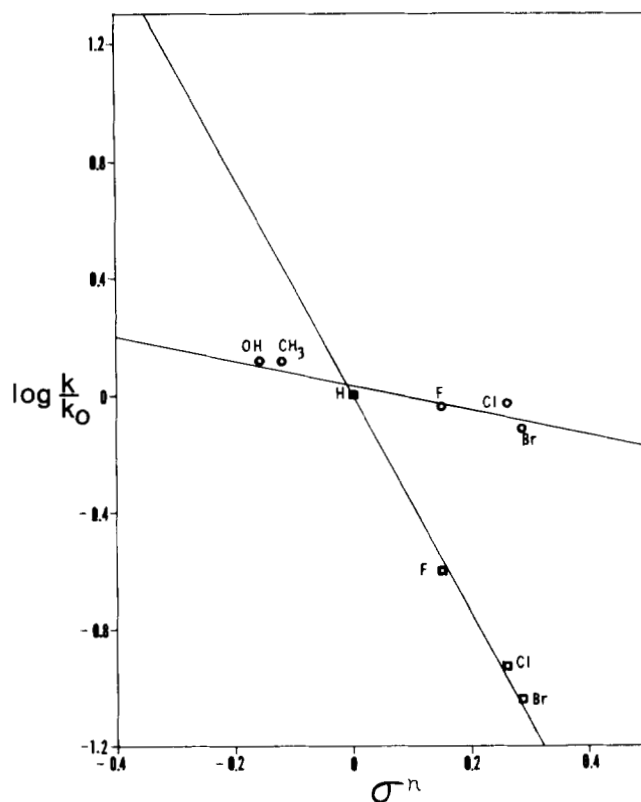


FIG. 2. Linear free energy plots for dopamine β -hydroxylase-catalyzed hydroxylation and sulfoxidation reactions. The data for the sulfoxidation series (\square) are plotted using the Hammett equation, $\log \frac{k}{k_0} = \rho\sigma^n$, where σ^n is the normal substituent constant and ρ is the slope (29, 30). In the case of the hydroxylation series (\circ), steric effects are apparently more significant, and an extended equation including a steric substituent constant was required, $\log \frac{k}{k_0} = \rho\sigma^n + nE_s$, where E_s is the Taft steric substituent constant (29, 30), and n is the corresponding slope. When plotted using the simple equation, points for Cl, Br, and CH_3 were found to be considerably less reactive than predicted, and from a secondary plot of the deviation versus E_s , the value of n was estimated to be -1.1 . The corrected values for these points were then calculated using n and the corresponding E_s values. The slopes, ρ , for sulfoxidation and hydroxylation were found to be -3.6 and -0.4 , respectively, using a linear regression analysis.

sulfoxidations with H_2O_2 where a large negative ρ value is observed (28). In contrast, dopamine β -hydroxylase-catalyzed hydroxylations resemble P-450-catalyzed oxygenations, where there is increasing evidence for a radical pathway (27, 33).

Turning to the data in Tables III and IV, it is apparent that *meta*-fluorotyramine exhibits kinetic parameters virtually identical with those obtained with tyramine. Thus, although the *meta*-fluoro substituent greatly perturbs the pK_a of the phenolic OH (31), this has little or no effect on reactivity with dopamine β -hydroxylase, in sharp contrast to what has been observed with dioxygenases, such as protocatechuate-3,4-dioxygenase (32). The *para*-methoxy substituent drastically decreases reactivity in hydroxylation; this compound is the least reactive of all those examined, and there is considerable uncertainty in the kinetic values obtained. We presume that the size of the *p*-methoxy substituent grossly interferes with reactivity at the active site, and the steric corrections in the Taft relationship are insufficient to account for this. Thus, although the *p*-methoxy sulfide exhibits reasonable activity in sulfoxidation (Table IV), where the nucleophilic mechanism

TABLE III

Comparison of kinetic constants for 4'-substituted 2-phenylethylamines

Kinetic data were obtained using the O_2 monitor with ascorbate as reductant as described under "Materials and Methods."

R		k_{cat} s^{-1}	K_m mM	k_{cat}/K_m
H	Ia	65	7.0	9.0×10^3
OH	Ib	80	2.0	4.0×10^4
<i>m</i> -F, <i>p</i> -OH	Ic	83	2.9	2.8×10^4
F	Id	59	11.0	5.5×10^3
OCH_3	Ie	1.8	9.4	2.0×10^2
Cl	If	28	11.3	2.5×10^3
Br	Ig	14	18.4	7.7×10^2
CH_3	Ih	24	11.0	2.2×10^3

TABLE IV

Comparison of kinetic constants for 4'-substituted phenyl-2-aminoethyl sulfides

Kinetic data were obtained using the O_2 monitor with ascorbate as reductant as described under "Materials and Methods."

R		k_{cat} s^{-1}	K_m mM	k_{cat}/K_m
H	IIb	68	26.5	3.0×10^3
OCH_3	IIg	15	32.7	4.7×10^2
F	IIc	17	26.0	6.2×10^2
Cl	IId	8	28.6	3.0×10^2
Br	IIf	6	21.7	2.9×10^2
CH_3	IIh	0		

makes electronic factors much more important, we chose not to apply an arbitrary steric correlation, and have eliminated the *p*-methoxy data from Fig. 2. Finally, the *p*-methyl sulfide is completely unreactive in sulfoxidation. This compound exhibits extremely poor binding to dopamine β -hydroxylase, and we have found it to be very weak competitive inhibitor, with K_i in the 0.5 M range. It seems likely that the hydrophobicity of the methyl group adversely affects binding to dopamine β -hydroxylase.

DISCUSSION

The data presented here clearly establish that dopamine β -hydroxylase readily carries out sulfoxidation and ketonization of a variety of substrates, and that these oxygenation activities are kinetically comparable to the long-recognized methylene hydroxylation activity of this enzyme. Thus, dopamine β -hydroxylase exhibits wide specificity with respect to the functionality undergoing oxygenation, though it is considerably more selective than the broad specificity microsomal P-450 and flavin monooxygenases (10). Analysis of the effects of phenyl substituents on substrate reactivity has allowed us now to focus on the oxygen transfer process, and to make a mechanistic comparison to other enzymatic and chemical oxygenation reactions. The striking difference in ρ values for sulfoxidation and hydroxylation, by comparison to those reported for P-450 and peroxide reactions, suggests that dopamine β -hydroxylase-catalyzed sulfoxidation proceeds via a nucleophilic mechanism. In contrast, our data indicate that dopamine β -hydroxylase-catalyzed hydroxylations involve much less charge development in the transition state.

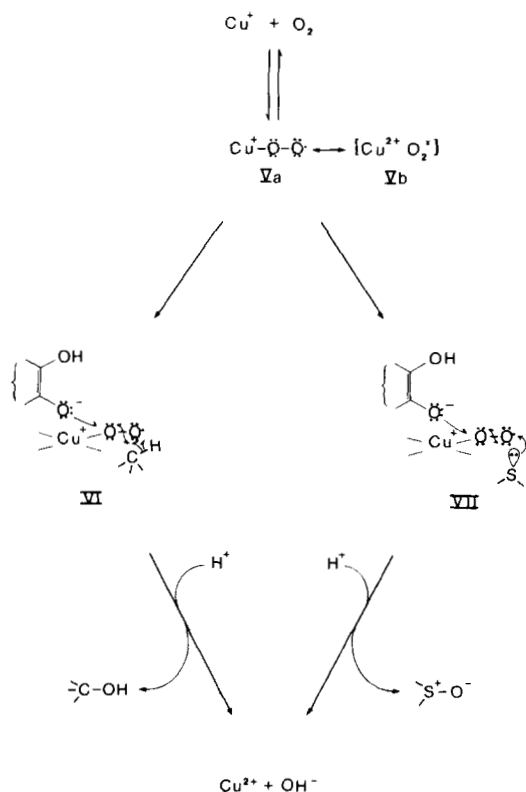
On the basis of these results and much other published information on dopamine β -hydroxylase reactions, we propose the mechanism illustrated in Scheme 2 for dopamine β -hydroxylase-catalyzed oxygenations. Subsequent to the first

electron transfer from ascorbate or ferrocyanide, the copper-oxygen species is best represented in the resonance form, Structure Va. The contribution of form Vb should be minimal, due to the high oxidation-reduction potential of dopamine β -hydroxylase-copper (12, 22, 23); this is in sharp contrast to ferrous-oxygen adducts in iron-containing oxygenases, which "leak" superoxide readily (33), and is consistent with our experience that superoxide dismutase does not inhibit dopamine β -hydroxylase oxygenations. In Scheme 2, the relative binding order of O_2 , organic substrate, and electron donor is not specified, since our data do not bear on this point. Structures VI and VII represent the catalytically competent ternary complexes immediately prior to the oxygenation step in hydroxylation and sulfoxidation, respectively. For Structure VI, a 1-electron transfer from ascorbate through copper initiates hydroxylation, involving at least partially concerted homolytic cleavages of the O-O and C-H bonds. The isotope effect studies have established that C-H bond cleavage must occur to a large extent in the rate-determining oxygen transfer step (25, 26). On the other hand, substantial involvement of the electron donor and a homolytic pathway, is indicated by the kinetic data presented in this paper. Turning to sulfoxidation, breakdown of Structure VII is facilitated via nucleophilic attack by the lone pair on sulfur, accounting for the greater k_{cat} values observed in the sulfoxidation reaction. Thus, the sulfoxidation pathway involves a much greater degree of charge development than does that for hydroxylation.

Several interesting points arise from consideration of the mechanism of Scheme 2. Clearly, the electron donor participates throughout the catalytic cycle; slower turnover with ferrocyanide has been observed by others (6), and the nature of the electron donor is shown to be reflected in k_{cat} values by our kinetic data (Table II). Moreover, the necessity for the presence of electron donor for completion of the catalytic cycle has been demonstrated (34), and the finding that semidehydroascorbate is released during dopamine β -hydroxylase catalysis (35-37) establishes that donation of the 2 electrons occurs separately. Thus, as has been repeatedly put forth by Skotland and Ljones (5) and co-workers, there is no necessity to postulate 2 coppers/active site. However, our data do not bear on this point, and the mechanism of Scheme 2 can just as readily accommodate the participation of 2 coppers in catalysis. It is not clear to us how the mechanism of Scheme 2 can be reconciled with the recent isotope effect treatment of Klinman *et al.* (38), since their kinetic analysis completely ignores the electron donor. In our view, the weight of evidence now demonstrates that the dopamine β -hydroxylase reaction cannot be viewed simply as a two-stage process, with electron donation separated from substrate and O_2 binding.

Although we establish in this paper that dopamine β -hydroxylase is relatively tolerant of substrate structure, we have observed no significant inhibition by any of a variety of product analogs we have tested. Among these are *R*-octopamine, (*R,S*)-phenyl-2-aminoethyl sulfoxide, and 4'-hydroxy- α -aminoacetophenone, the respective products of dopamine β -hydroxylase-catalyzed hydroxylation, sulfoxidation, and ketonization. In contrast, we have observed that *N*-phenylethylenediamine is a potent inhibitor of dopamine β -hydroxylase, most likely due to chelation of the Cu^{2+} , thus inactivating the enzyme. It is interesting to note that due to this inhibition we have been unable to observe nitrogen oxygenation with *N*-phenylethylenediamine, although with the broad specificity microsomal P-450 and flavin monooxygenases, nitrogen oxygenation occurs readily (10).

Taken together, the results reported here not only provide insight into the mechanism of dopamine β -hydroxylase action, but provide basic structure-activity information with regard



SCHEME 2. Proposed mechanism for dopamine β -hydroxylase oxygenation.

to both the chemical nature of the functionality undergoing oxygenation and the secondary structural features which affect substrate activity. From a pharmacological point of view, the development of potent selective inhibitors for dopamine β -hydroxylase would provide a valuable complement to the structure-activity results reported here. However, we note that product analogs may in general be poor inhibitors for dopamine β -hydroxylase, since this enzyme is located in catecholamine storage vesicles, where the catecholamine concentration has been reported to be very high, 0.54 M (39).

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