1-(2-Aminoethyl)-1,4-cyclohexadiene: A Probe To Examine the Chemistry and Energetics of the C−H Bond Cleavage in Dopamine β-Monooxygenase Catalysis[1]

Kandategе Wimalasena* and Kevin R. Alliston

Contribution from the Department of Chemistry, Wichita State University, Wichita, Kansas 67260-0051

Received May 16, 1994[⊗]

Abstract: The chemistry and energetics of the initial C−H bond cleavage step in dopamine β-monooxygenase (DβM, E.C. 1.14.17.1) catalysis has been explored using 1-(2-aminoethyl)-1,4-cyclohexadiene (CHDEA) as a probe. DβM has been previously shown (Wimalasena, K.; May, S. W. *J. Am. Chem. Soc.* **1989**, *111*, 2729) to catalyze the aromatization of CHDEA to phenylethylamine (PEA). We now report that the side chain hydroxylated product, 2-amino-1-(1,4-cyclohexadiene)ethanol (CHDEA-OH), is also a *direct* product of the DβM/CHDEA reaction. The PEA:CHDEA-OH product ratio is 2.7 at pH 5.2 and 37 °C for the DβM/CHDEA reaction. The side chain deuterated analog, 1-(2-amino-1,1-dideuterioethyl)-1,4-cylohexadiene, undergoes aromatization almost exclusively. The ring deuterated analog, 1-(2-aminoethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene, favors side chain hydroxylation 2.6 times over aromatization. The hexadeuterio derivative, 1-(2-amino-1,1-dideuterioethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene (CHDEA-d_6), prefers aromatization 3.9 times. The apparent kinetic isotope effects of CHDEA-d_6 are small, 1.8 for k_{cat} and 1.3 for k_{cat}/K_m, suggesting that the initial C−H bond cleavage is only partly rate limiting in the CHDEA/DβM reaction analogous to the normal DβM hydroxylation reaction. The intrinsic isotope effects for the exocyclic and ring methylene hydrogens are estimated to be 9.9 and 7.0, respectively. The alteration of the product ratio due to deuterium substitution suggests that activation energies of the initial C−H bond cleavage steps for the two pathways are similar. In contrast to the high thermodynamic driving force for the ring methylene hydrogen abstraction, the similar activation energies must be a consequence of the proximity and/or the relative orientation of the corresponding hydrogens of the enzyme-bound CHDEA with respect to the activated copper oxygen species.

Introduction

Dopamine β-monooxygenase (DβM;[2] E.C. 1.14.17.1), a copper-containing mammalian enzyme, catalyzes the *in vivo* conversion of dopamine to (*R*)-norepinephrine in the sympathetic nervous system.[3−4] In addition to the physiological reaction, DβM has also been shown to catalyze a wide variety of monooxygenations, including ketonization of phenylethanolamines[5] and β-halo phenylethylamines,[6] oxygenation of sulfides[7] and selenides,[8] epoxidation of olefins,[9] N-dealkylation of benzylic N-substituted analogs,[9,10] and allylic hydroxylation.[11] Although the generally believed *in vivo* reductant, ascorbic acid,[12] was reported to be the most efficient reductant for the enzyme, our recent work has shown that 6-*S*- and 6-*O*-phenylascorbic acid derivatives are much more efficient electron donors for the enzyme than ascorbic acid.[13] Other well-known electron donors such as $K_4Fe(CN)_6$, dopamine, hydroquinone, and dichlorophenolindophenol[12] are also reductants for the

[⊗] Abstract published in *Advance ACS Abstracts*, January 1, 1995.

(1) Preliminary accounts of this work were presented at the 36th West Central States Biochemistry Conference, Lawrence, KS, Oct. 29−30, 1993; 85th Annual Meeting of ASBMB, Washington, D. C., May 21−25, 1994.

(2) Abbreviations: 2-amino-1-(1,4-cyclohexadiene)ethanol, CHDEA-OH; 1-(2-amino-1,1-dideuterioethyl)-1,4-cyclohexadiene, CHDEA-d_2; 1-(2-amino-1,1-dideuterioethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene, CHDEA-d_6; 1-(2-aminoethyl)-1,4-cyclohexadiene, CHDEA; 1-(2-aminoethyl)-3,3,6,6,-tetradeuterio-1,4-cyclohexadiene, CHDEA-d_4; 2-amino-1-phenylethanol, PEA-OH; 2-(cyclohexa-1,4-dienyl)-2-(hydroxyethyl)-2-(*p*-nitrobezyl)acetamide, CHDEA-OH-*p*NPA; 2,2-dideuterio-2-phenylethylamine, PEA-d_2; dopamine β-monooxygenase, DβM; *p*-nitrophenylacetic acid *N*-hydroxysuccinimide ester, SNPA; *p*-nitrophenylacetic acid *N*-hydroxysuccinimide ester derivatized products, *p*NPA derivatives; 2-phenyl-2-(hydroxyethyl)-2-(*p*-nitrobezyl)acetamide, PEA-OH-*p*NPA.

(3) (a) Kaufman, S.; Bridges, W. F.; Eisenberg, F.; Friedman, S. *Biochem. Biophys. Res. Commun.* **1962**, *9*, 497−502. (b) Goldstein, M.; Lauber, E.; McKereghan, M. R. *J. Biol. Chem.* **1965**, *240*, 2066−2072. (c) Taylor, K. B. *J. Biol. Chem.* **1974**, *249*, 454−458.

(4) For recent reviews see: (a) Rosenberg, R. C.; Lovenberg, W. In *Essays in Neurochemistry and Neuropharmacology;* Youdim, M. B. H., Lovenberg, W., Sharman, D. F., Lagnado, J. R., Eds.; John Wiley & Sons: New York, 1980; Vol. 4, pp 163−209. (b) Stewart, L. C.; Klinman, J. P. *Annu. Rev. Biochem.* **1988**, *57*, 551−592. (e) Fitzpatrick, P. F.; Villafranca, J. J. *Arch. Biochem. Biophys.* **1987**, *257*, 231−250. (c) Kaufman, S. *J. Psychiatr. Res.* **1974**, *11*, 303−316. (d) Ljones, T.; Skotland, T. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raton, FL, 1986; Vol. II, pp 131−157.

(5) (a) May, S. W.; Phillips, R. S.; Herman, H. H.; Mueller, P. W. *Biochem. Biophys. Res. Commun* **1982**, *104*, 38−44. (b) May, S. W.; Phillips, R. S.; Mueller, P. W.; Herman, H. H. *J. Biol. Chem.* **1981**, *256*, 2258−2261.

(6) (a) Klinman, J. P.; Krueger, M. *Biochemistry* **1982**, *21*, 67−75. (b) Mangold, J. B.; Klinman, J. P. *J. Biol. Chem.* **1984**, *259*, 7772−7779. (d) Bossard, M. J.; Klinman, J. P. *J. Biol. Chem.* **1986**, *261*, 16421−16427.

(7) May, S. W.; Phillips, R. S. *J. Am. Chem. Soc.* **1980**, *102*, 5981−5983.

(8) May, S. W.; Herman, H. H.; Roberts, S. F.; Cicarello, M. C. *Biochemistry* **1987**, *26*, 8470−8475.

(9) (a) May, S. W.; Mueller, P. W.; Padgette, S. R.; Herman, H. H.; Phillips, R. S. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 161−168. (b) Padgette, S. R.; Wimalasena, K.; Herman, H. H.; Sirimanne, S. R.; May, S. W. *Biochemistry* **1985**, *24*, 5826−5839.

(10) Wimalasena, K.; May, S. W. *J. Am. Chem. Soc.* **1987**, *109*, 4036−4046.

(11) Sirimanne, S. R.; May, S. W. *J. Am. Chem. Soc.* **1988**, *110*, 7560−7561.

(12) (a) Skotland, T.; Peterssen, L.; Backstrom, D.; Ljones, T.; Flatmark, T.; Ehrenberg, A. *Eur. J. Biochem.* **1980**, *105*, 5−11. (b) Skotland, T.; Ljones, T. *Biochim. Biophys. Acta* **1980**, *630*, 30−35. (c) Rosenberg, R. C.; Gimble, J. M.; Lovenberg, W. *Biochim. Biophys. Acta* **1980**, *613*, 62−72. (d) Diliberto, E. J., Jr.; Allen, P. L. *J. Biol. Chem.* **1981**, *256*, 3385−3393. (e) Stewart, L. C.; Klinman, J. P. *Biochemistry* **1987**, *26*, 5302−5309.

(13) Wimalasena, K.; Dharmasena, S.; Wimalasena, D. S. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 113−119.

0002-7863/95/1517-1220$09.00/0

enzyme. In addition N-substituted phenylenediamines[14] and the ascorbate derivative, 2-aminoascorbic acid,[14,15] were shown to be efficient chromophoric reductants for the enzyme.

The chemistry and biochemistry of DβM have been a subject of current interest because of its central role in the catecholamine neurotransmitter biosynthesis. Although the exact nature of the activated copper-oxygen species involved in DβM catalysis is not known, a high energy copper−peroxo species, i.e. Cu^{II}−O−O−H(Cu) which is analogous to the initial ferrous−peroxo species proposed for heme and non-heme iron containing monooxygenases, has been proposed[4b,10,16] as a possible candidate (for an alternate novel mechanism, see ref 17). Detailed structure−activity studies of substrates and mechanism-based inhibitors[4b,9,10] and extensive deuterium isotope effect studies[16] are in agreement with the general conclusion that the DβM reaction is initiated by a hydrogen atom abstraction from the benzylic position of the substrate. Subsequent radical recombination of the resultant Cu^{II}−O• to the substrate radical results in the formation of a copper-bound alkoxide which is shown to hydrolyze and release from the active site in a rate-determining step.[16] In addition, thermodynamic arguments have been used to propose that homolysis of both the O−O bond of the activated copper oxygen species and the C−H bond of the substrate are concerted and nearly ergoneutral. However, the understanding of the detailed molecular mechanism of DβM catalysis is far from complete.

The use of molecular probes designed to partition between different catalytic pathways at a known chemical step should provide valuable information regarding the energetics of that step. The relative rates of product formation from these partitioning reaction pathways should primarily be dependent on the relative activation energies of the respective chemical step and must be independent from the energetics of the binding steps since in ordinary irreversible enzymatic reactions, substrate binding must occur prior to the initial chemical steps. These types of probes could be especially valuable in the study of kinetically complex enzymatic reactions. We now report the use of 1-(2-aminoethyl)-1,4-cyclohexadiene (CHDEA) as the first such probe to study the chemistry and energetics of the initial C−H bond cleavage step in DβM catalysis.

Results and Discussion

Previously DβM has been shown to catalyze the aromatization of CHDEA to phenylethylamine (PEA) with the characteristics of the normal DβM reductive monooxygenation pathway.[18] It was also reported that PEA is the only observable product of the DβM/CHDEA reaction.[18] However, careful re-examination of the reaction revealed that in addition to the aromatized product, a significant amount of a second product was also produced during the DβM/CHDEA turnover. The *p*-nitrophenylacetamide derivative (*p*-nitrophenylacetic acid *N*-hydroxysuccinimide ester (SNPA) derivatized product; *p*NPA) of the new product had a similar but resolvable retention time from 2-amino-1-phenylethanol (PEA-OH), which was identical to that of the *p*NPA derivative of synthetic (see Experimental Section and Figure 1B) 2-amino-1-(1,4-cyclohexadienyl)ethanol (CHDEA-OH-*p*NPA) in reversed-phase chromatography with several different solvent systems (Figure 1A). This observation establishes that the new product is the corresponding side-chain hydroxylated product (CHDEA-OH). The time courses of the formation of both products, PEA and CHDEA-OH, were linear

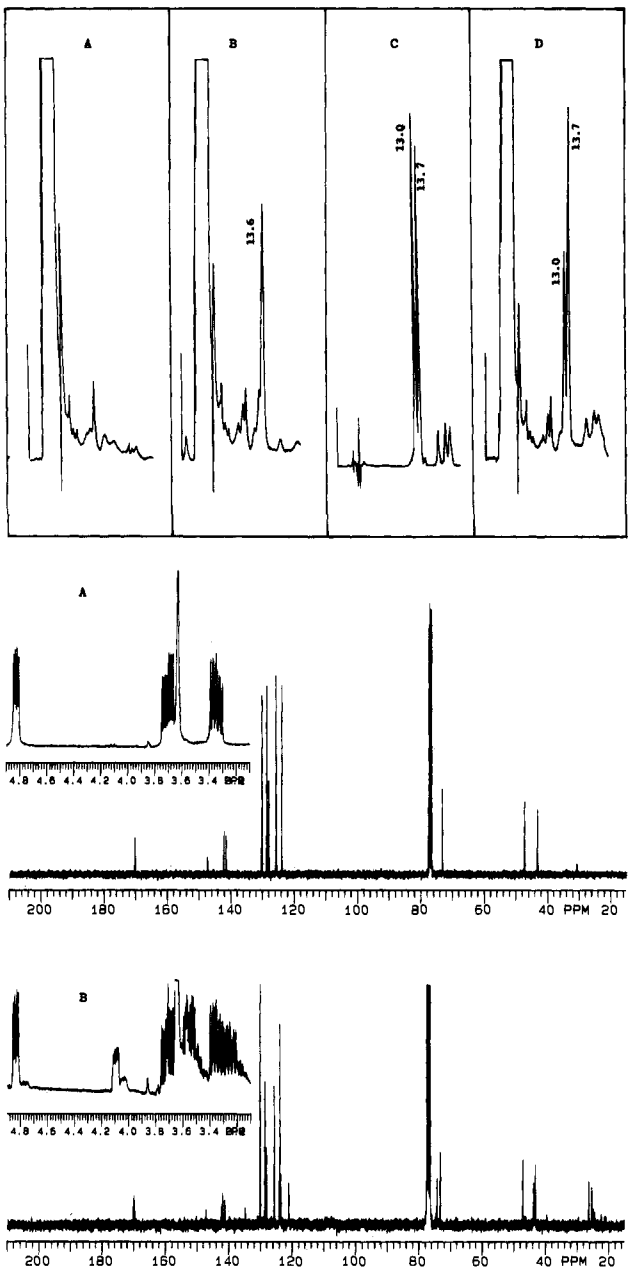

Figure 1. Identification of CHDEA-OH as a second product of DβM/CHDEA turnover. (A, top) The enzymatic reactions were carried out as detailed in the Experimental Section. The reaction mixtures were derivatized with SNPA and analyzed by C_{18}-reversed-phase HPLC using 35% acetonitrile in 50 mM potassium phosphate buffer (pH 6.7) as the mobile phase, observing the products at 280 nm. Panel A, control (same as the complete reaction except that the enzyme was absent); panel B, enzymatic reaction; panel C, authentic synthetic mixture of PEA-OH-*p*NP (retention time 13.0 min; identification by spiking with a synthetic authentic sample (data not shown)) and CHDEA-OH-*p*NP (retention time 13.6−13.7 min); panel D, B spiked with C. Note that the peaks with longer retention times were deleted from the chromatograms for clarity. (B, bottom) Panel A, ^{13}C- and partial ^{1}H-NMR spectra (insert) of *p*NPA derivatized synthetic 2-amino-1-phenylethanol in $CDCl_3$; panel B, ^{13}C- and partial ^{1}H-NMR spectra (insert) of partially separated synthetic mixture of CHDEA-OH-*p*NPA and PEA-OH-*p*NPA in $CDCl_3$. This mixture was used in the identification of CHDEA-OH (Figure 1A, panel C).

(14) Wimalasena, K.; Wimalasena, D. S. *Biochem. Biophys. Res. Commun.* **1991**, *175*, 920−927.
(15) Wimalasena, K.; Wimalasena, D. S. *Anal. Biochem.* **1991**, *197*, 353−361.
(16) (a) Miller, S.; Klinman, J. P. *Biochemistry* **1985**, *24*, 2114−2127. (b) Miller, S. M.; Klinman, J. P. *Biochemistry* **1983**, *22*, 3091−3096. (c) Ahn, N.; Klinman, J. P. *Biochemistry* **1983**, *22*, 3096−3106.
(17) Tian, G.; Berry, J. A.; Klinman, J. P. *Biochemistry* **1994**, *33*, 226.
(18) Wimalasena, K.; May, S. W. *J. Am. Chem. Soc.* **1989**, *111*, 2729.

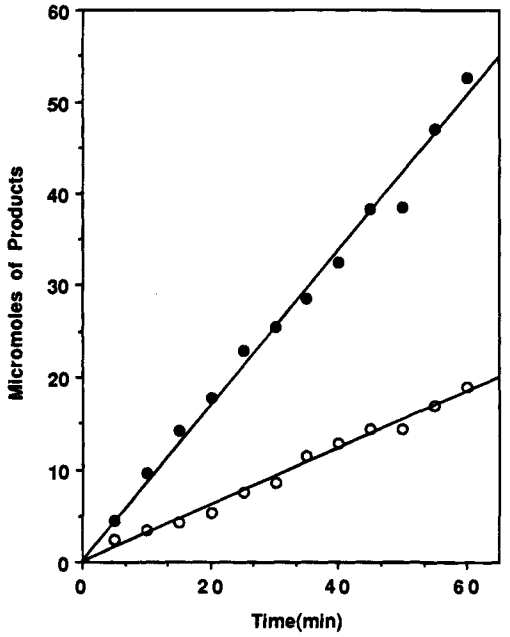

Figure 2. Time-courses of PEA and CHDEA-OH formation during CHDEA/DβM turnover. DβM/CHDEA reaction was carried out as detailed in the Experimental Section. Aliquots of 0.1 mL were withdrawn at 5 min intervals, derivatized with SNPA and analyzed by C_{18} reversed-phase HPLC at 280 nm using 45% acetonitrile, 55% 62.5 mM NaOAc buffer (pH 5.2) as the mobile phase. (●) PEA and (○) CHDEA-OH.

with no lag or burst periods (Figure 2), suggesting that PEA was not derived from the nonenzymatic dehydration of the initially generated CHDEA-OH, which is consistent with the results of previous[18] deuterium labeling experiments. These findings demonstrate that the DβM/CHDEA reaction *directly* produces both aromatic and side-chain hydroxylated products. The inability to detect the side-chain hydroxylated product, CHDEA-OH, in the previous study[18] may have been due to its sensitivity to the reaction and detection conditions and/or the low extinction and poor resolution of the underivatized CHDEA-OH under the HPLC−UV conditions used.

Previous quantitative deuterium labeling studies[18] have shown that the aromatization of CHDEA is initiated by the abstraction of a hydrogen from one of the ring methylenes by the activated copper−oxygen species in the DβM active site (Figure 3). Therefore, the product distribution of the DβM/CHDEA reaction must be solely determined by whether the corresponding exocyclic or ring methylene hydrogen is abstracted during the initial catalytic step, since the side chain hydroxylation must be initiated by the abstraction of one of the exocyclic methylene hydrogens analogous to the normal DβM monooxygenation pathway (Figure 3). Thus, assuming the C−H bond cleavage and the product release steps are irreversible in DβM catalyzed monooxygenation of CHDEA,[19] the relative rates of hydrogen abstraction from exocyclic methylene or from one of the ring methylenes could be determined by measuring the PEA:CHDEA-OH ratio.

A series of control experiments revealed that CHDEA-OH derived from the DβM/CHDEA reaction was stable under the present experimental conditions and its *p*NPA derivative could be accurately quantitated by reversed-phase HPLC. Quantitative analysis of the *p*NPA derivatives from the DβM/CHDEA reaction mixtures revealed that the PEA:CHDEA-OH ratio was

(19) The C−H bond cleavage and product release steps in the DβM monooxygenation of dopamine are shown to be irreversible (see ref 16).

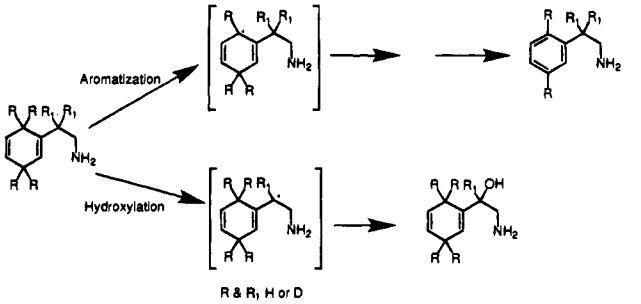

Figure 3. DβM-catalyzed monooxygenation of CHDEA. The side-chain hydroxylation is initiated by hydrogen abstraction from exocyclic methylene analogous to the normal monooxygenation pathway. The aromatization is initiated by the abstraction of hydrogen from one of the ring methylenes by the activated copper−oxygen species to generate the hexadienyl radical and Cu^{II}−O• species. The aromatization could be completed by direct transfer of a single electron to the Cu^{II}−O• species, followed by spontaneous ejection of a proton from the cyclohexadienyl cation or rebinding of oxygen to produce the ring hydroxylated product, followed by enzyme-assisted or spontaneous dehydration within or outside the active site. At present, we do not have sufficient experimental data to distinguish between these possibilities.

Table 1. Initial Rate DβM Kinetic Parameters of 1-(2-Aminoethyl)-1,4-cyclohexadiene Derivatives[a]

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	product ratio[b]
CHDEA	0.94 ± 0.05	19.6 ± 0.2	2.09 × 10^4	2.7
CHDEA-d_2	0.87 ± 0.06	16.9 ± 0.2	1.95 × 10^4	*c*
CHDEA-d_4	0.76 ± 0.06	14.6 ± 0.2	1.91 × 10^4	0.4
CHDEA-d_6	0.68 ± 0.04	10.7 ± 0.2	1.58 × 10^4	3.9
tyramine	0.92 ± 0.14	29.8 ± 0.7	4.25 × 10^4	*d*

[a] All the kinetic constants are apparent and determined at pH 5.2, 37 °C, and atmospheric oxygen saturation conditions (256 μM) with the same preparation of soluble bovine enzyme (sp act. 20 μmol mg^{-1} min^{-1}) using the spectrophotometric assay as previously described.[17] The K_m and k_{cat} values were obtained by computer-fitting of the data to the hyperbolic form of the Michaelis−Menten equation. [b] Product ratio is defined as aromatized/side-chain hydroxylated and were averages of five to seven separate determinations. [c] The product ratio was less exact due to the formation of relatively small amounts of the side-chain hydroxylated products and varies between 20 and 35. [d] Included for comparison purposes.

2.7[20] at pH 5.2 and 37 °C (Table 1) suggesting that the difference in activation energies for the hydrogen atom abstraction from the ring or exocyclic methylene must be less than 1 kcal/mol.[21] In contrast to the high thermodynamic driving force for the ring methylene hydrogen abstraction leading to aromatization (see below), the observed low partition ratio is somewhat unexpected. In order to further confirm this observation, a series of specifically deuterated CHDEA derivatives were synthesized, characterized, and examined with the enzyme. The steady-state apparent kinetic parameters of selectively deuterated CHDEA derivatives are presented in Table 1 together with those from the regular DβM substrate, tyramine, for comparison purposes. Analysis of the DβM products from the side-chain deuterated analog, 1-(2-amino-1,1-dideuterioethyl)-1,4-cylohexadiene (CHDEA-d_2), indicated that it undergoes DβM-catalyzed aromatization almost exclusively, producing 2-phenyl(1,1-dideuterio)ethylamine under these reaction conditions (Table 1). In contrast, the ring-deuterated analog, 1-(2-aminoethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene (CHDEA-d_4), favors side-chain

(20) In all experiments the substrate and enzyme concentrations and reaction times were adjusted to prevent the formation of the phenylethanolamine, an observed second-round product from the DβM/PEA reaction.

(21) This could be estimated from the Arrhenius equation, i.e. $\ln(PEA/CHDEA\text{-}OH) = -\Delta\Delta E_a/RT$.

hydroxylation over the aromatization by a factor of 2.6. The hexadeuterio derivative, 1-(2-amino-1,1-dideuterioethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene (CHDEA-d_6), favors aromatization by a factor of 3.9 (Table 1). These results confirm that the abstraction of the exocyclic methylene or one of the ring methylene hydrogens by the activated copper−oxygen species in the DβM active site is kinetically competitive and the activation energies for these two processes must be similar.

The kinetic data in Table 1 reveals that the apparent kinetic isotope effects on k_{cat} and k_{cat}/K_m for both CHDEA-d_2 and CHDEA-d_4 are very small. This is not surprising since the composite rates of aromatization and hydroxylation are calculated by measuring the consumption of the reductant (Table 1), and the major effect of deuterium substitution is on the partitioning between the two pathways. However, the apparent kinetic isotope effects of CHDEA-d_6 are also relatively small, 1.8 for k_{cat} and 1.3 for k_{cat}/K_m, suggesting that under the reaction conditions, the initial C−H bond cleavage steps are only partially rate-limiting in the CHDEA/DβM reaction (probably for both pathways), similar to that of the tyramine/DβM reaction.[16] An estimate of the intrinsic isotope effect for the exocyclic methylene C−H bond cleavage could be obtained from the product ratio, CHDEA/CHDEA-d_2 (see table footnote *c*), which is 9.9[22] and is in good agreement with the values reported for the tyramine hydroxylation reaction by Miller and Klinman.[16b] The comparison of the product ratios from CHDEA/CHDEA-d_4 gives an estimate for the intrinsic deuterium isotope effect for the ring methylene C−H bond cleavage step, which is 7.0.[22]

In light of the known chemistry of 1,4-cyclohexadiene the similar activation energies for the two pathways is intriguing and contrasts with the previous proposal[18] that the enzyme-mediated aromatization of CHDEA is a consequence of the thermodynamic favorability of the hydrogen abstraction from one of the ring methylenes over the exocyclic methylene. The experimentally determined bond dissociation energy of ring methylene hydrogens of 1,4-cyclohexadiene is 70−74 kcal/mol.[23] Although the bond dissociation energy of exocyclic methylene hydrogens of CHDEA is not available, it should be comparable with the allylic hydrogens of propene which is in the range of 85 kcal/mol.[23b] Thus, thermodynamically under normal conditions aromatization should be much more favorable than the side chain hydroxylation. Therefore, the similar activation energies for the C−H bond cleavage steps for these two pathways must be a consequence of the proximity and/or the relative orientation of the catalytically involved hydrogens of the enzyme-bound CHDEA with respect to that of the activated copper−oxygen species in the DβM active site in the transition state. The detailed study of the temperature dependence of the partition ratio of the DβM/CHDEA reaction using specifically deuterated CHDEA derivatives (currently in progress) should provide additional quantitative thermodynamic information on the initial C−H bond cleavage step in DβM catalysis. In addition, the discovery of a DβM substrate that can partition between two reaction pathways producing two different products may be useful both in understanding the very nature of the active site of the enzyme and in the future design of mechanism-based inhibitors for the *in vivo* modulation of adrenergic activity for therapeutic purposes.[24]

Experimental Section

Materials. Sodium fumarate, L-ascorbic acid, sodium hydride, lithium aluminum hydride, *N,N*-dimethyl-1,4-phenylenediamine dihydrochloride (DMPD), 2-amino-1-phenylethanol, benzyl cyanide, and phenylethylamine were purchased from Aldrich; dimethyl-d_6 sulfoxide was obtained from Cambridge Isotope Labs; tyramine and *p*-nitrophenylacetic acid *N*-hydroxysuccinimide ester (SNPA) were obtained from Sigma; and beef liver catalase was obtained from Boehringer Mannheim. All other chemicals and solvents were of the highest grade obtainable. Soluble dopamine β-monooxygenase was purified (sp act. 20 units/mg) using freshly prepared bovine adrenal chromaffin granules[25] according to the procedure of Ljones *et al.*[26] with minor modifications. The concentration of purified enzyme was estimated spectrophotometrically using ϵ_{280} = 1.24 mL mg^{-1} cm^{-1}. All the spectrophotometric measurements were carried out on a Hewlett-Packard 8452A spectrophotometer equipped with a temperature-regulated cell compartment. Kinetic constants (apparent k_{cat} and K_m at atmospheric oxygen saturation conditions, i.e. 256 mM) for various substrates were determined by the computer-fit of the data to the hyperbolic form of the Michaelis−Menten equation. All ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-300 in D_2O with 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionic acid (Aldrich) as an internal standard or in $CDCl_3$ with TMS. All the melting points were uncorrected.

Methods. 1. Spectrophotometric Assays of Dopamine β-Monooxygenase. The standard DβM assay solution contained 10 mM fumarate, 0.5 μM $CuSO_4$, and 100 μg/mL catalase in 125 mM NaOAc buffer at pH 5.2 in a total volume of 1.0 mL. All the enzymatic reactions were carried out at 37 °C unless otherwise stated. DβM concentrations were kept at a constant 0.92 μg of protein per assay. Concentrations of substrates were varied from 0.5 to 22.0 mM and the reactions were initiated with 12.5 mM DMPD. The rate of enzyme-mediated oxidation of DMPD to its cation radical was monitored at 514 nm as previously reported.[15] Extinction coefficient of 5450 M^{-1} cm^{-1} for the DMPD cation radical was used in rate calculations (see ref 15).

2. Product Identification from Various DβM/CHDEA Reactions. Standard product identification reactions were carried out in a total volume of 0.5 mL of 125 mM NaOAc buffer (pH 5.2) containing 10 mM ascorbate, 10 mM fumarate, 5 mM CHDEA analog, 100 μg/mL catalase, 0.5 μM $CuSO_4$, and 0.5 mM benzylamine (internal standard). The reactions were initiated with 9.1 μg of purified DβM. After the mixture incubated for 1 h at 37 °C, 0.5 mL of THF containing a 2-fold excess of SNPA was added along with 250 μL of ethanol. The pH was adjusted to 8.0 with a saturated Na_2CO_3 solution, and the resulting mixture was allowed to react for 30 min. The desired products were resolved and quantified by C_{18} reverse-phase HPLC with UV detection at 280 nm using 45−30% acetonitrile in 62.5 mM NaOAc buffer (pH 5.2) as the mobile phase. The products of the DβM/CHDEA turnover were identified by spiking with *p*NPA-derivatized authentic standards or the synthetic mixture of the two alcohols, CHDEA-OH and PEA-OH (see below). In quantification experiments the extinction coefficients of *p*NPA-derivatized enzymatic products of CHDEA-OH and PEA, at 280 nm, were assumed to be the same as the *p*NPA derivatized internal standard, benzylamine. When CHDEA-d_4 or CHDEA-d_6 were the substrates, corrections were made for the small contamination of PEA by carrying out parallel control experiments in which all conditions

(22) One reviewer suggested that both ring hydrogens might be accessible to the activated copper oxygen species, and this number should be divided by two. However, we do not have any experimental evidence for or against this proposal.

(23) (a) Huyser, E. S. In *Free Radicals;* McManus, S. P., Ed.; Academic: New York, 1973; Vol. 26, pp 1−59. (b) Benson, S. W. *J. Chem. Educ.* **1965**, *42*, 502.

(24) (a) Kruse, L. I.; Kaiser, C.; DeWolf, W. E., Jr.; Frazee, J. S.; Gravey, E.; Hilbert, E. L.; Faulkner, W. A.; Flaim, K. E.; Sawyer, J. L.; Berkowitz, B. A. *J. Med. Chem.* **1986**, *29*, 2465−2472. (b) Kruse, L. I.; Kaiser, C.; DeWolf, W. E., Jr.; Chambers, P. A.; Goodhart, P. J.; Ezkiel, M.; Ohlstein, E. H.; *J. Med. Chem.* **1988**, *31*, 704−706. (c) Bargar, T. M.; Broersma, R. J.; Creemer, L. C.; McCarthy, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 2975−2976. (c) May, S. W.; Wimalasena, K.; Herman, H. H.; Fowler, L. C.; Cicarello, M. C.; Pollock, S. H. *J. Med. Chem.* **1988**, *31*, 1066−1068.

(25) (a) Kirshner, N. *J. Biol. Chem.* **1962**, *237*, 2311−2317. (b) Njus, D.; Radda, G. K. *Biochem. J.* **1979**, *180*, 579−585.

(26) Ljones, T.; Skotland, T.; Flatmark, T. *Eur. J. Biochem.* **1976**, *6*, 525−533.

were identical except no DβM was present and by quantifying and subtracting the background contamination (see below).

3. Time Courses of PEA and CHDEA-OH Formation during CHDEA/DβM Turnover. Enzymatic reactions were carried out in a total volume of 2 mL of 125 mM NaOAc buffer (pH 5.2) containing 10 mM ascorbate, 10 mM fumarate, 10 mM CHDEA 100 μg/mL catalase, 0.5 μM $CuSO_4$, 0.5 mM benzylamine (internal standard), and 45.7 μg of purified DβM at 37 °C (under these reaction conditions the formation of PEA-OH due to the hydroxylation of enzymatically produced PEA was found to be insignificant). Aliquots of 0.1 mL were withdrawn at 5-min intervals, derivatized with SNPA, and analyzed by HPLC as described above.

Syntheses. 1,1-Dideuteriobenzyl Cyanide. This was synthesized as previously reported.[18] Briefly, 5.0 g of benzyl cyanide was added to a mixture of 10 g of D_2O and 5.0 g of K_2CO_3, and the mixture was stirred for 12 h at 50 °C. After the layers were allowed to separate, the D_2O layer was removed, an additional 10 g of D_2O and 5.0 g of K_2CO_3 were added, and the procedure was repeated. Extraction with diethyl ether, drying over anhydrous Na_2SO_4, and evaporation of ether *in vacuo* yielded 4.63 g (91%) of 1,1-dideuteriobenzyl cyanide with 100% deuterium incorporation by ^{1}H-NMR analysis. ^{1}H-NMR (D_2O): δ 7.32 (5H, m).

2. 2,2-Dideuterio-2-phenylethylamine (PEA-d_2). This was obtained by the $LiAlH_4/AlCl_3$ reduction of 1,1-dideuteriobenzyl cyanide according to the procedure of Nystrom *et al.*[27] A solution of 6.5 g (50 mmol) of $AlCl_3$ in 75 mL of ether was added dropwise to a suspension of 1.45 g (39.2 mmol) of $LiAlH_4$ in 100 mL of anhydrous diethyl ether (freshly distilled from Na). Then 4.63 g (39.2 mmol) of 1,1-dideuteriobenzyl cyanide in 100 mL of ether was added dropwise to the reaction mixture and stirred for 2 h at room temperature. Excess $LiAlH_4$ was destroyed by the cautious addition of water. The gelatinous white liquid was acidified with 6 M H_2SO_4 in an ice bath, washed with ether, and basified to pH 11 with NaOH pellets. The resultant mixture was extracted with (3 × 50 mL) ether, which were combined, washed with (2 × 20 mL) water and with (1 × 20 mL) brine, and dried over anhydrous Na_2SO_4. The crude product was precipitated by adding methanolic HCl and crystallized from ether/absolute EtOH to yield 2.8 g (45%) of 2,2-dideuterio-2-phenylethylamine hydrochloride, mp 217−218 °C, ^{1}H-NMR (D_2O): δ 3.28 (2H, s), 7.39 (5H, m), no observable proton signals for benzylic hydrogens.

3. 1-(2-Amino-1,1-dideuteriothyl)1,4-cyclohexadiene (CHDEA-d_2). A warm slurry of 2.7 g (16.9 mmol) of 2,2-dideuterio-2-phenylethylamine hydrochloride in 20 mL of *tert*-butyl alcohol was quickly added to 300 mL of distilled NH_3 in a three-necked flask equipped with a $CaCO_3$ drying tube and a Dewar condenser cooled with dry ice/acetone. Small portions of Li metal were added until the blue color persisted and then the reaction mixture was stirred for an additional 30 min. After the bulk of the NH_3 was allowed to evaporate in a fume hood, the reaction mixture was diluted with 50 mL of water and extracted with (3 × 50 mL) ether. The ether layers were combined, washed with (2 × 20 mL) water and with (1 × 20 mL) brine, and dried over anhydrous Na_2SO_4. The product was precipitated as the hydrochloride by adding methanolic HCl. The crude product was recrystallised from ether/absolute EtOH to yield 2.2 g (80%) of CHDEA-d_2, mp 153 °C (dec). ^{1}H-NMR (D_2O): δ 2.60−2.74 (4H, m), 3.12 (2H, s), 5.66 (1H, s broad), 5.79 (2H, s broad), no observable proton signals for benzylic hydrogens; ^{13}C-NMR (D_2O): δ 29.0, 30.4, 49.9, 125.5, 127.3, 132.7.

4. 1-(2-Aminoethyl)-1,4-cyclohexadiene (CHDEA). This compound was synthesized from phenylethylamine using a similar procedure as detailed above for CHDEA-d_2 with a 93% yield, mp 153 °C (dec). ^{1}H-NMR (D_2O): δ 2.37 (2H, t, J = 6.8 Hz), 2.62−2.70 (4H, m), 3.13-(2H, t, J=6.9 Hz), 5.65 (1H, s broad), 5.80 (2H, s broad); ^{13}C-NMR (D_2O): δ 28.9, 30.4, 37.1, 40.0, 125.5, 127.0, 127.3, 132.8.

5. 1-(2-Aminoethyl)-3,3,6,6,-tetradeuterio-1,4-cyclohexadiene (CHDEA-d_4). This compound was prepared from the base-catalyzed exchange of the diallylic protiums for deuterium with the dimsyl-d_5 anion in DMSO-d_6 according to the published procedure for 3,3,6,6-tetradeuterio-1,4-cyclohexadiene[28] with minor modifications. Briefly, a solution of CHDEA (0.5 g, 3.16 mmol), after exchange of amine protium for deuterium with D_2O, in 1 mL of DMSO-d_6 was injected under nitrogen into an 18 °C solution of 11.1 mmol of dimsyl-d_5 anion generated from 0.33 g of oil-free NaH and 4 mL of DMSO-d_6. The resulting red solution was stirred rapidly for 2 min and then quenched with 1 mL of D_2O. The reaction mixture was diluted with 50 mL of water and extracted with (3 × 50 mL) ether. The ether layers were combined and washed with (2 × 20 mL) water and with (1 × 20 mL) brine, and dried over anhydrous Na_2SO_4. The crude product was precipitated by adding methanolic HCl and recrystallized from ether/abssolute EtOH to yield CHDEA-d_4 with about 95% deuterium (by ^{1}H-NMR) in diallylic positions of the ring. After a repetition of the above procedure, CHDEA-d_4, with 98.2% deuterium incorporation, was obtained with an overall yield of 0.15 g (30%), mp 153 °C (dec). ^{1}H-NMR (D_2O): δ 2.37 (2H, t, J = 6.9 Hz), 3.13 (2H, t, J = 6.9 Hz), 5.65 (1H, s broad), 5.80 (2H, s broad). The presence of a trace of aromatic protons indicated a slight contamination by PEA.

6. 1-(2-Amino-1,1-dideuterioethyl)-3,3,6,6,-tetradeuterio-1,4-cyclohexadiene (CHDEA-d_6). This compound was synthesized from CHDEA-d_2 with 94% deuterium incorporation into the diallylic positions of the ring using a similar procedure as described for CHDEA-d_4, mp 153 °C (dec). ^{1}H-NMR (D_2O): δ 3.12 (2H, s), 5.65 (1H, s broad), 5.80 (2H, s broad); ^{13}C-NMR (D_2O): δ 39.8, 125.7, 127.3, 133.0. The presence of a trace of aromatic protons indicated a slight contamination by PEA.

7. 2-(Cyclohexa-1,4-dienyl)-2-(hydroxyethyl)-2-(*p*-nitrobenzyl)-acetamide (CHDEAOL-*p*NPA). Small portions of Li metal (0.063 g, 9.04 mmol) were added to a refluxing solution (16 °C) of 2-amino-1-phenylethanol (PEA-OH) (0.50 g, 3.64 mmol) in ethylamine (100 mL), and the mixture was stirred for 1 h. After the ethylamine was evaporated, the reaction products were diluted with water and extracted with (2 × 50 mL) ether and (2 × 50 mL) ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated to dryness. A portion of the above reaction mixture (100 mg) and 275 mg of SNPA were dissolved in 20 mL of 50% THF/H_2O, adjusted to pH 8 with saturated Na_2CO_3, stirred for 30 min, and evaporated to dryness *in vacuo*. The residue was partitioned between 50 mL of ether and 50 mL of water. The ether layer was washed with $NaHCO_3$ and 1 M HCl and then evaporated to dryness. The products were partially separated by silica gel column chromatography using $CHCl_3$ as the mobile phase. The HPLC analysis of the fraction (10 mg) containing the highest concentration of the desired product showed that it was a 1:1 mixture of CHDEA-OH-*p*NPA and PEA-OH-*p*NPA which was not further separable by conventional chromatography. ^{1}H-NMR ($CDCl_3$): δ 2.64−2.68 (4H, m), 3.25 (1H, ddd, J = 5.0, 7.6, 13.9 Hz), 3.55 (1H, ddd, J = 3.7, 6.5, 13.9 Hz), 3.65 (2H, s), 4.01 (1H, dd, J = 3.6, 7.3 Hz), 5.68 (2H, m), 5.87 (2H, m), 7.45 (2H, d, J = 8.8 Hz), 8.20 (2H, d, J = 3.3 Hz); ^{13}C-NMR-($CDCl_3$): δ 25.3, 26.3, 43.2, 43.9, 74.3, 121.1, 123.6, 123.9, 124.0, 130.2, 134.8, 142.2, 147.2, 169.7 (deduced by subtracting the corresponding signals of authentic PEA-OH-*p*NP from the ^{1}H- and ^{13}C-NMR spectra of the mixture). *Note*: The attempted complete reduction of 1-phenyl-2-aminoethanol under the above conditions or the use of traditional Birch conditions, i.e. NH_3/Li/^{t}BuOH, cleanly produced CHDEA.

Acknowledgment. We gratefully acknowledge the partial support of this work by the National Institutes of Health (R29 GM45026). We also thank the reviewers and the Associate Editor of JACS, Richard L. Schowen, for their valuable comments.

JA941496L

(27) Nystrom, R. F. *J. Am. Chem. Soc.* **1955**, *77*, 2544−2545.

(28) Lockhart, T. P.; Comita, P. B.; Bergman, R. G. *J. Am. Chem. Soc.* **1981**, *103*, 4082.