Mode of Substrate Interaction and Energetics of Carbon−Oxygen Bond Formation of the Dopamine β-Monooxygenase Reaction†

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ABSTRACT: Previous studies have shown that the dopamine β-monooxygenase (DβM; E.C. 1.14.17.1)/1-(2-aminoethyl)-1,4-cyclohexadiene (CHDEA) reaction partitions between side chain and ring H-abstraction to produce the side-chain-hydroxylated product, 2-amino-1-(1,4-cyclohexadienyl)ethanol, and the aromatized product, phenylethylamine, and that the two pathways do not crossover. [Wimalasena, K., and May, S. W. (1989) *J. Am. Chem. Soc. 111*, 2729−2731; Wimalasena, K., and Alliston, K. R. (1995) *J. Am. Chem. Soc. 117*, 1220−1224]. We now report that the ring H-abstraction pathway of the reaction further partitions to produce the ring hydroxylated product, CHDEA-6OH, and the aromatized product, PEA, at the carbon−oxygen bond formation step. The ring hydroxylation is shown to be stereospecific, exclusively producing the *(S)* product. The absolute stereospecificity of the ring and side-chain hydroxylations of the DβM/CHDEA reaction suggests that the side-chain *pro-R* hydrogen of the enzyme-bound substrate is close to perpendicular to the aromatic ring of the phenylethylamine substrate or cyclohexadiene ring of CHDEA. The relative activation energy parameters suggest that the partitioning of the ring H abstraction pathway between aromatized and ring hydroxylated products is due to the partitioning of the high-energy intermediates, the cyclohexadienyl radical and the Cu(II)−O• species, between carbon−oxygen bond formation and direct electron transfer. The relatively high activation enthalpic favorability and entropic unfavorability for the carbon−oxygen bond formation strongly suggest that the critical balancing of these two opposing forces is mandatory for the desired product formation.

Dopamine β-monooxygenase [DβM;[1] E.C. 1.14.17.1], a copper-containing mammalian enzyme, catalyzes the biosynthetic conversion of dopamine to *(R)*-norepinephrine in the sympathetic nervous system (for recent reviews see refs *1*−*4*). In addition to the physiological benzylic hydroxylation reaction, DβM has also been shown to catalyze a wide variety of monooxygenations including ketonization of phenylethanolamines (*5*, *6*) and β-halophenylethylamines (*7*, *9*), oxygenation of sulfides (*10*) and selenides (*11*), epoxidation of olefins (*12*, *13*), N-dealkylation of benzylic N-substituted analogues (*14*), allylic hydroxylation (*15*), and oxidative aromatization of cyclohexadienes (*16*, *17*). While ascorbic acid is presumed to be the physiological reductant (*18*−*22*), $K_4Fe(CN)_6$, catecholamines, (*20*−*23*), N-substituted phenylenediamines, and 2-amino- (*24*, *25*), 6-*O*-phenyl, 6-*O*-alkylphenyl- (*26*) substituted ascorbic acid derivatives are also good reductants for the enzyme.

The copper atoms in the DβM active site are responsible for binding, activation, and insertion of oxygen into the organic substrate. An optimum stoichiometry of two catalytically essential coppers per DβM active site has been determined (*27*, *28*). A high-energy Cu-peroxo species, i.e., Cu(II)−O−O−H which is analogous to the initial Fe(III)−O−O−H species proposed for cytochrome P_{450} monooxygenases (*50*), is presumed to be responsible for abstracting a H atom from the benzylic position of the substrate with the concomitant homolytic cleavage of the O−O bond[2] to generate Cu(II)−O•, a substrate-derived radical species, and water (*14*, *30*−*32*). Subsequent fast radical recombination of the Cu−O• to the substrate carbon radical results in the formation of a Cu-bound alkoxide, which was shown to be hydrolyzed in a rate-determining step and released from the active site (*30*−*32*). The absolute stereoselectivity of the DβM reaction for *(R)*-hydroxylation has been shown to be a consequence of the stereospecific abstraction of the *pro-R*

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[1] Abbreviations: CHDEA-1OH, 2-amino-1-(1,4-cyclohexadienyl)-ethanol; CHDEA-d2, 1-(2-amino-1,1-dideuterioethyl)-1,4-cyclohexadiene; 4Me-CHDEA-d2, 1-(2-amino-1,1-dideuterioethyl)-4-methyl-1,4-cyclohexadiene; CHDEA-d6, 1-(2-amino-1,1-dideuterioethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene; CHDEA, 1-(2-aminoethyl)-1,4-cyclohexadiene; CHDEA-d4, 1-(2-aminoethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene; PEA-OH, 2-amino-1-phenylethanol; ^{13}C NMR, carbon nuclear magnetic resonance spectroscopy; CHDEA-d2-6OH-pNPA, *N*-2-[2,2-dideuterio-2-(6-hydroxy-1,4-cyclohexadienyl)]ethyl-4-nitrobenzamide; PEA-d2, 1,1-dideuterio-2-phenylethylamine; DβM, dopamine β-monooxygenase; HPLC, high-performance liquid chromatography; CHDEA-d2-6OH, 2-(6-hydroxy-1,4-cyclohexadienyl)-2,2-dideuterioethylamine; CHDEA-6OH, 2-(6-hydroxy-1,4-cyclohexadienyl)ethylamine; CHDEA-d2-6OH-pNPA, *N*-2-[2,2-dideuterio-2-(6-hydroxy-1,4-cyclohexadienyl)]ethyl 4-nitrophenylacetamide; 5SRS and 5RSS, *N*-2-{*trans*-2-[(*S*)-2-methoxy-2-trifluoromethyl-2-phenylacetyloxy]cyclohexyl}-ethylbenzamide; SNPA, *p*-nitrophenylacetic acid *N*-hydroxysuccinimide ester.

[2] Tian et al. (*29*) have recently proposed a sophisticated description of the relative timing of the O−O bond cleavage based on the ^{18}O isotope effects of the O−O bond cleavage step. According to this mechanism, homolysis of the O−O bond must occur prior to the initial C−H bond cleavage. However, the interpretation of our results does not depend on the order of C−H and O−O bond cleavage.

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H from the benzylic position of the substrate followed by the binding of oxygen with the retention of configuration (*33*).

Our recent studies (*16*, *17*) have shown that 1-(2-aminoethyl)-1,4-cyclohexadiene (CHDEA) is an alternate substrate for DβM that partitions between side-chain hydroxylation and ring aromatization at the initial C−H bond cleavage step. Quantitative deuterium labeling studies have shown that the side-chain hydroxylation and aromatization are initiated by the side-chain methylene H and ring methylene H-abstractions, respectively, and that the two pathways do not cross over (*16*, *17*). In the present study we have shown that the ring H-abstraction pathway of the DβM/CHDEA reaction further partitions to produce ring-hydroxylated and aromatized products. The ring hydroxylation of CHDEA was shown to be highly stereospecific, producing only the *(S)* alcohol. The relative activation energy parameters derived from the temperature dependency of the partition ratios strongly suggest that aromatization and ring hydroxylation are a consequence of the partitioning of the cyclohexadienyl radical and the Cu(II)-O$^{\bullet}$ species between carbon−oxygen bond formation and direct electron transfer. Relevance of these findings to the molecular mechanism of DβM and other monooxygenases are discussed.

EXPERIMENTAL PROCEDURES

Materials

Sodium fumarate, L-ascorbic acid, *N*,*N*-dimethyl-1,4-phenylenediamine dihydrochloride (DMPD), 2-amino-1-phenylethanol, benzylcyanide, 3-phenylpropylamine, 4-methylbenzylcyanide, borane−methyl sulfide complex, and 2-phenylethylamine were purchased from Aldrich; dimethyl-d_6 sulfoxide was from Cambridge Isotope Labs; tyramine and *p*-nitrophenylacetic acid *N*-hydroxysuccinimide ester (SNPA) were from Sigma; and beef liver catalase was from Boehringer Mannheim. All other chemicals and solvents were of the highest grade obtainable. Soluble DβM was purified (specific activity 20−25 units/mg) by use of freshly prepared bovine adrenal chromaffin granules as previously reported (*34*). The concentration of purified enzyme was estimated spectrophotometrically using $\epsilon_{280} = 1.24$ mL mg^{-1} cm^{-1}. All spectrophotometric measurements were carried out on a Hewlett-Packard 8452A spectrophotometer equipped with a temperature-regulated cell compartment. All HPLC analyses were carried out on a LDC analytical HPLC system with variable UV detection. All ^{1}H- and ^{13}C NMR spectra were recorded on a Varian XL-300 or Varian INOVA-400 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

Product Identification and Quantification from Various DβM/CHDEA Reactions. DβM/CHDEA reactions were carried out in a total volume of 0.5 mL of 100 mM potassium phosphate buffer at pH 7.2 containing 10 mM fumarate, 5 mM CHDEA analogue, 100 μg/mL catalase, 0.5 μM $CuSO_4$, 0.5 μM benzylamine (internal standard), and 10−15 μg of purified bovine adrenal soluble DβM. The reactions were initiated by adding L-ascorbic acid to a final concentration of 10 mM after the reaction mixture was equilibrated for 10 min at the indicated temperature. The enzymatic reactions were terminated after 45 min and the mixture was derivatized with SNPA by adding a 2-fold excess of SNPA in 0.5 mL of acetonitrile and 0.5 mL of water and adjusting the pH to 8.5 with saturated Na_2CO_3. The desired SNPA-derivatized products were resolved and quantified by C_{18} reverse-phase HPLC with UV detection at 280 nm using 55−30% acetonitrile and 2−15% methanol in 50 mM potassium phosphate buffer (pH 8.0) as the isocratic mobile phase (depending on the CHDEA derivative). The products of the DβM/CHDEA turnover were identified by spiking with corresponding SNPA-derivatized, authentic synthetic products. Products from methylated and other structurally different analogues were identified by the relative retention times of their products compared to CHDEA. In product quantification experiments, the extinction coefficients of pNPA derivatives of enzymatic products at 280 nm were assumed to be the same as the pNPA derivative of the internal standard, benzylamine.

Time Courses of PEA, CHDEA-1OH, and CHDEA-6OH Formation during DβM/CHDEA Turnover. Enzymatic reactions were carried out in a total volume of 2 mL of 100 mM potassium phosphate buffer, pH 7.2, containing 10 mM L-ascorbate, 10 mM fumarate, 10 mM CHDEA, 100 μg/mL catalase, 0.5 μM $CuSO_4$, 0.5 μM benzylamine (internal standard), and 45.7 μg purified bovine adrenal soluble DβM at 32 °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.

Temperature-Dependent Studies. These experiments were typically carried out at the temperature range 8−44 °C under standard DβM assay conditions in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.2, containing 10 mM fumarate, 5 mM CHDEA or other analogue, 100 μg/mL catalase, 0.5 μM $CuSO_4$, 0.5 μM benzylamine (internal standard), and 10−15 μg of highly purified bovine adrenal soluble DβM. The reactions were initiated by adding L-ascorbic acid to a final concentration of 10 mM after preincubating for 10 min at the indicated temperature in order to ensure that the reaction mixture attained the correct temperature prior to initiation of the reaction. The products were derivatized with SNPA, resolved, and quantified by reversed-phase HPLC as mentioned above. In most cases 6−7 separate experiments were carried out for the full temperature range using the same enzyme preparation and identical experimental conditions (see individual figure captions). The averages of partition ratios and standard deviations were calculated from these sets of data.

Syntheses

1,1-Dideuteriobenzylcyanide, 1-(2-aminoethyl)-1,4-cyclohexadiene (CHDEA), 1,1-dideuterio-2-phenylethylamine (PEA-d2), 1-(2-amino-1,1-dideuterioethyl)-1,4-cyclohexadiene (CHDEA-d2); 1-(2-aminoethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene (CHDEA-d4); 1-(2-amino-1,1-dideuterioethyl)-3,3,6,6-tetradeuterio-1,4-cyclohexadiene (CHDEA-d6), and *N*-2-(*trans*-6-hydroxycyclohexyl)ethyl-4-nitrobenzamide were synthesized as previously described (*16*).

*1,1-Dideuterio-4-methylbenzylcyanide (***1***).* This compound was synthesized from 5 g of 4-methylbenzylcyanide by K_2CO_3-catalyzed deuterium exchange (*17*) with a 100% yield. ^{1}H NMR ($CDCl_3$) δ 2.34 (3 H, s), 7.2 (4 H, m).

*2,2-Dideuterio-2-(4-methylphenyl)ethylamine (***2***).* This compound was synthesized from **1** by $LiAlH_4/AlCl_3$ reduction (*35*) with an 84% yield. ^{1}H NMR (D_2O) δ 2.35 (3 H, s), 3.26 (2 H, s) 7.25 (4 H, m).

1-(2-Amino-1,1-dideuterioethyl)-4-methyl-1,4-cyclohexadiene. This compound was synthesized from **2** by traditional Birch reduction with Li/NH_3 (*16*, *36*), with absolute ethanol as a cosolvent, with a 25% yield. Mp 158−188 °C (dec); ^{1}H NMR (D_2O) δ 1.79 (3 H, s), 2.63 (4 H, br s), 3.11 (2 H, s), 5.51 (1 H, br s), 5.68 (1 H, br s).

*N-2-(Cyclohexenyl)ethylbenzamide (***3***).* This compound was obtained by stirring a mixture of 0.5 g (2.28 mmol) of benzoic acid *N*-hydroxysuccinimide ester, 0.5 g (4.0 mmol) of (cyclohexenyl)ethylamine, and 1 mL of saturated K_2CO_3 in a mixture of CH_3CN/H_2O (1:1 v/v) for 1.5 h (pH > 8). The reaction mixture was concentrated in vacuo to dryness, dissolved in 100 mL of H_2O and extracted with diethyl ether. The combined ether layers were washed with 1 M HCl, 1 M NaOH, H_2O, and brine, dried with Na_2SO_4, and concentrated in vacuo (yield 86%). ^{1}H NMR ($CDCl_3$) δ 1.53−1.67 (4 H, m), 1.95−2.02 (4 H, m), 2.24 (2 H, t, J = 6.8 Hz), 3.50 (2 H, quartet, J = 12.3 Hz), 5.25 (1 H, s), 6.3 (1 H, br s), 7.38−7.48 (3 H, m), 7.72−7.75 (2 H, m); ^{13}C NMR ($CDCl_3$) δ 22.3, 22.8, 25.2, 27.9, 33.9, 37.6, 123.6, 126.7, 126.8, 128.4, 131.2, 134.7, 134.9, 167.3.

*N-2-(trans-2-Hydroxycyclohexyl)ethylbenzamide (***4SR** *and* **4RS***).* Under N_2, 0.34 g (1.5 mmol) of **3** in 10 mL of CH_2Cl_2 was cooled to 0 °C and 49.4 mL of borane−methyl sulfide (0.5 mmol) was added. The solution was refluxed for 1.5 h and then 10 mL of ethanol, 99 mL of 5 M NaOH (0.5 mmol), and 174 mL of 30% H_2O_2 (1.53 mmol) were added. The reaction mixture was diluted with H_2O and the products were extracted with ether. The ether layers were combined and washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The reaction mixture was purified by silica gel column chromatography with 1:1 hexane/ethyl acetate to yield 47.5 mg of **4SR** and **4RS**. ^{1}H NMR ($CDCl_3$) δ 1.00−1.39 (5 H, m), 1.51−2.10 (6 H, m), 2.45 (1 H, br s), 3.22−3.45 (2 H, m), 3.58−3.70 (1 H, m), 7.0 (1 H, br s), 7.35−7.50 (3 H, m), 7.75−7.81 (2 H, m); ^{13}C NMR ($CDCl_3$) δ 24.9, 25.7, 31.8, 33.4, 36.3, 38.6, 43.4, 75.1, 126.9, 128.5, 131.2, 134.9.

*N-2-{trans-2-[(S)-2-Methoxy-2-trifluoromethyl-2-(phenylacetyloxy)]cyclohexyl}ethylbenzamide (***5SRS** *and* **5RSS***).* To 37 mg of the **4SR** and **4RS** mixture was added 5 mL of dry pyridine and 33.7 mL of *(S)*-2-methoxy-2-(trifluoromethyl)phenylacetyl chloride (Mosher's reagent). The reaction progress was monitored by HPLC and an additional aliquot of 25 μL of *(S)*-methoxy-2-(trifluoromethyl)phenylacetyl chloride was added until the starting material disappeared (monitored by HPLC). The resultant two diastereomers, **5SRS** and **5RSS**, were separated by preparative reversed-phase HPLC with 55:5:40 CH_3CN/CH_3OH/50 mM potassium phosphate buffer, pH 8.0. The structures of these two compounds were deduced by HPLC, mass spectrometry NMR, 2D NMR, and Mosher's model analysis (see Results). ^{1}H NMR of **5SRS** (the diastereomer corresponding to the enzymatic product) in $CDCl_3$, δ 1.09−1.40 (3 H, m), 1.46 (1 H, ddt, J = 7.58, 8.14, and 13.48 Hz), 1.62−1.79 (4 H, m), 1.94−2.02 (1 H, d, m, J = 12.36 Hz), 2.04−2.10 (1 H, d, m, J = 8.99 Hz), 3.39−3.47 (2 H, m), 3.50 (3 H, s), 4.76 (1 H, dt, J = 4.21 and 9.55 Hz), 5.97 (1 H, br s), 7.37−7.56 (8 H, m), 7.70−7.74 (2 H, m). Mass spectrometry [low resolution (CI)] M + 1 = 463 *m*/*z*.

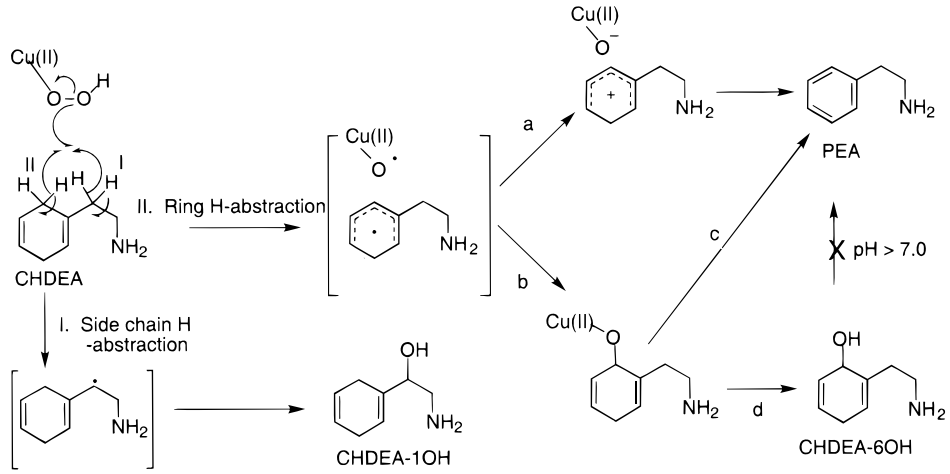
Isolation and Characterization of the Ring-Hydroxylated Product of the DβM/CHDEA-d2 Reaction. Large-scale enzymatic incubations were carried out in a total volume of 10 mL of 100 mM potassium phosphate buffer, pH 7.2, containing 10 mM fumarate, 5 mM CHDEA-d2, 100 μg/mL catalase, 0.5 μM $CuSO_4$, and about 1 mg of purified DβM (added in three portions over a 9 h incubation period). After preincubation for 10 min at 16−20 °C, the enzymatic reaction was initiated by adding L-ascorbic acid to a final concentration of 10 mM. The temperature was maintained at 16−20 °C throughout the incubation period. Aliquots of 20 μL were withdrawn at 1 h intervals, derivatized with SNPA, and analyzed by HPLC as described previously, to follow the progress of the reaction. After 9 h no further increase in the ring-hydroxylated product was observed and the reaction was terminated. The reaction mixture was derivatized with SNPA as described above and the products were extracted with ether, washed with brine, and dried over anhydrous Na_2SO_4. Then, 1 mL of pyridine-d_5 was added to the ether extract and the solution was concentrated in vacuo, to about 0.5 mL (CHDEA-6OH-pNPA was found to dehydrate to PEA-pNPA when removed from aqueous basic solutions unless pyridine was present). The product was isolated by preparative HPLC. Immediately after the isolation the sample was reconstituted with pyridine-d_5 and analyzed by ^{1}H NMR. ^{1}H NMR of the product was consistent with the expected product.

Assignment of the Stereochemistry of the Ring-Hydroxylated Product of the DβM/CHDEA-d2 Reaction. The benzamide derivative of the ring-hydroxylated product of the DβM/CHDEA-d2 reaction, {*N*-2-[2,2-dideuterio-2-(6-hydroxy-1,4-cyclohexadienyl)]ethylbenzamide}, was obtained from large-scale incubations in the same manner as CHDEA-6OH-pNPA except that *N*-hydroxysuccinimide ester of benzoic acid was used as the derivatization agent and the reaction was monitored by C_{18} HPLC at 254 nm. The reaction mixture was hydrogenated in benzene (15 mL) containing 20 mg of PtO_2, and 0.5 g of K_2CO_3 at 45 psi for 12 h. The product mixture was filtered and concentrated in vacuo to dryness. The residue was dissolved in 50 mL of H_2O and extracted with ether. The ether layers were combined and washed with 0.1 M HCl, 0.1 M NaOH, and H_2O, dried over Na_2SO_4, and concentrated *in vacuo*. The product mixture was dissolved in 0.5 mL of dry pyridine, and (*S*)-methoxy-α-(trifluoromethyl)phenylacetyl chloride was added in portions until the alcohol peak, as identified by HPLC, completely disappeared. The products were resolved and isolated by reversed-phase C_{18} HPLC and characterized by ^{1}H NMR and mass spectrometry. The ^{1}H NMR, mass spectra, and HPLC elution profile of this compound were found to correspond to those of the synthetic **5SRS** under identical conditions.

RESULTS

Identification of CHDEA-6OH as a Third Product from the DβM/CHDEA Reaction. Examination of the DβM/

Scheme 1: Partitioning Pathways of the DβM/CHDEA Reaction



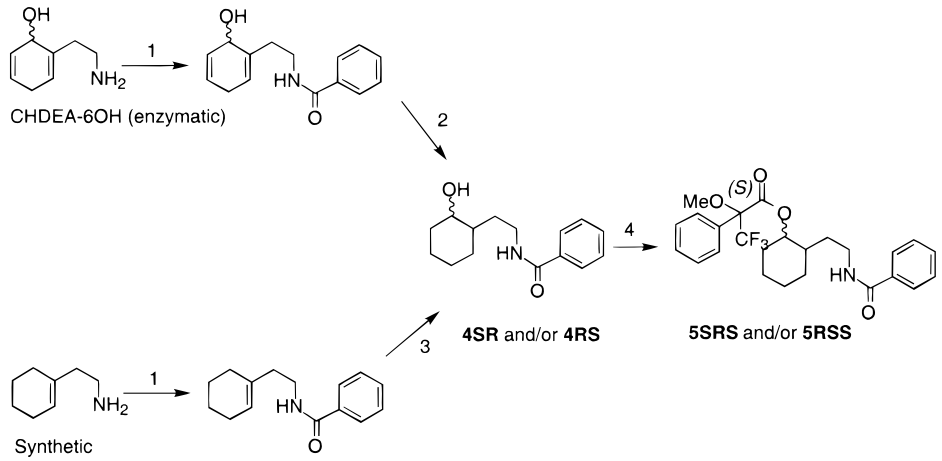
CHDEA reaction products at neutral pH (pH 7.2) revealed that in addition to previously characterized side-chain-hydroxylated and aromatized products, 2-amino-1-(1,4-cyclohexadienyl)ethanol (CHDEA-1OH) and phenylethylamine (PEA) (*16*, *17*), a third product was also produced in a time-, reductant-, and enzyme-dependent manner (Scheme 1). This new product had a retention time close to but resolvable from that of CHDEA-1OH under the standard HPLC conditions (see Experimental Procedures), suggesting it could be a second hydroxylated product of CHDEA. The new product was found to be stable at or above pH 7.0 under the standard assay conditions but readily and quantitatively decomposed to produce PEA, under mild acidic conditions (1 M acetic acid). Preliminary experiments also revealed that the side-chain-deuterated derivative of CHDEA, CHDEA-d2, produced a higher ratio of new product/side-chain-hydroxylated product than either CHDEA or CHDEA-d6 under similar experimental conditions.

To structurally characterize the novel product, large-scale DβM/CHDEA-d2 reactions were carried out under the optimum conditions (pH 7.2 and 18 °C) for the formation of the new product. The *p*-nitrophenylacetic acid derivative of the product was isolated by preparative HPLC and identified by ^{1}H NMR as *N*-2-[2,2-dideuterio-2-(6-hydroxy-1,4-cyclohexadienyl)]ethyl-4-nitrophenylacetamide (CHDEA-d2-6OH-pNPA), suggesting that the new product is the C-6 ring-hydroxylated product of CHDEA-d2, 2-(6-hydroxy-1,4-cyclohexadienyl)-2,2-dideuterioethylamine (CHDEA-d2-6OH; Scheme 1). The chemical properties of the new product, especially the acid-catalyzed dehydration to the corresponding aromatic product, are consistent with this structure, because 1,4-cyclohexadienols are known to undergo dehydration to produce the corresponding aromatic compounds under mildly acidic conditions (*37*). In addition, the observed higher ratio of new product/side-chain-hydroxylated product in the DβM/CHDEA-d2 reaction in comparison to that of either CHDEA or CHDEA-d6 under the same reaction conditions is also consistent with the ring-hydroxylated product, CHDEA-6OH, since the side-chain deuteration increases the favorability for the ring H abstraction relative to the side-chain H abstraction (unpublished results).

Stereochemistry of the DβM Ring Hydroxylation of CHDEA. To determine the stereochemistry of the ring-hydroxylated product of the DβM/CHDEA reaction, chiral derivatization of the ring hydroxyl group of CHDEA-d2-6OH-pNPA with Mosher's reagent [*(S)*-2-methoxy-2-(trifluoromethyl)phenylacetyl chloride; *38*, *39*] was attempted under a variety of reaction conditions. All these attempts failed, primarily due to the instability of CHDEA-d2-6OH-pNPA under the reaction conditions and led to the dehydration of the cyclohexadienol moiety to produce the corresponding aromatic derivative. Therefore, a different strategy in which the primary NH_2 group of the enzymatic reaction mixture was initially derivatized with *N*-hydroxysuccinimide ester of benzoic acid followed by catalytic hydrogenation of the mixture with PtO_2 as a catalyst in the presence of solid K_2CO_3 in benzene (*40*), was developed and successfully used in the stabilization and isolation of the enzymatic product (Scheme 2). Under the above hydrogenation conditions the cyclohexadienol ring was efficiently reduced to cyclohexanol without significant dehydration to the corresponding aromatic derivative. The reduced, ring-hydroxylated product was derivatized with Mosher's reagent under standard reaction conditions and was easily isolated by reversed-phase HPLC. An authentic diastereomeric mixture of *N*-2-[*trans*-2-hydroxycyclohexyl]ethylbenzamide (**4SR** and **4RS**) was synthesized from *N*-(2-cyclohexenyl)ethylbenzamide (**3**) by hydroboration with borane−methyl sulfide complex followed by H_2O_2 oxidation (Scheme 2; *49*). The resultant two trans products were derivatized with the same Mosher's reagent to produce an equimolar mixture of **RSS** and **SRS** *N*-2-{*trans*-2-[(*S*)-2-methoxy-2-trifluoromethyl-2-(phenylacetyloxy)]cyclohexyl}ethylbenzamide (**5SRS** and **5RSS**), which were used for spectroscopic and chromatographic comparison purposes.

The above two 2-OH-derivatized synthetic trans isomers, **5SRS** and **5RSS**, (which were separable by reversed-phase HPLC under the conditions given in Experimental Procedures), were compared with the catalytically reduced and similarly derivatized enzymatic products by reversed-phase HPLC. Analytical-scale HPLC comparisons revealed that the slow-eluting trans diastereomer of the authentic synthetic mixture corresponded to the enzymatic product and not even a trace of the fast-eluting trans diastereomer or any other similar products were present in the enzymatic reaction mixture (Figure 1). To further verify the above observations, a preparative-scale DβM/CHDEA-d2 reaction was carried out and the products were subjected to the above reaction

Scheme 2: Derivatization of Enzymatic and Synthetic Ring-Hydroxylated Products[a]



[a] (1) *N*-Hydroxysuccinimide ester of benzoic acid. (2) $H_2/PtO_2/K_2CO_3$/benzene. (3) $BH_3{\cdot}(CH_3)_2S/NaOH/H_2O_2$. (4) (*S*)-2-Methoxy-2-trifluoromethylbenzoyl chloride (Mosher's reagent).

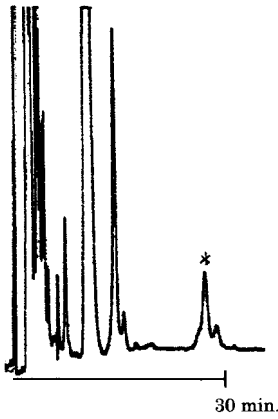


FIGURE 1: Characterization of the ring hydroxylation of the DβM/CHDEA reaction. The enzymatic products from analytical-scale DβM/CHDEA reactions were derivatized with the *N*-hydroxysuccinimide ester of benzoic acid and catalytically reduced as detailed under Experimental Procedures. The reduced products were derivatized with *(S)*-2-methoxy-2-(trifluoromethyl)phenylacetyl chloride and analyzed by C_{18} reversed-phase HPLC at 254 nm with 55% acetonitrile/45% 50 mM potassium phosphate buffer (pH 7.2) as the mobile phase. The peak eluting approximately between 26 and 28 min (*) corresponds to the enzymatic product (control experiments revealed that the small peak eluting soon after the enzymatic product is a breakdown product of one of the derivatizing reagents).

sequence and analyzed by reversed-phase HPLC. Again, only the slow-eluting trans product was detected; the faster eluting trans product was not detected (Figure 2). However, a second faster eluting product (faster than the faster eluting trans product) was detected in the large-scale reaction mixture that was not present in the analytical sample. Both these enzymatic products were isolated by preparative HPLC and subjected to ^{1}H NMR and mass spectroscopic analysis. The ^{1}H NMR and mass spectral analysis of the isolated reduced enzymatic product confirmed that it corresponds to the isolated slow-eluting synthetic trans product (Figures 2 and 3). The product eluting prior to the above two synthetic trans products, which was not present in the analytical enzymatic sample (Figures 1 and 2), was identified as the partially reduced *N*-2-{*trans*-6-[(*S*)-2-methoxy-2-trifluoromethyl-2-(phenylacetyloxy)]cyclohexenyl}-2,2-dideuterioethylbenzamide by ^{1}H NMR and mass spectral analysis (data not shown).

Absolute Stereochemistry of the Ring-Hydroxylated Product of the DβM/CHDEA Reaction. To establish the absolute stereochemistry of the ring-hydroxylated product by use of Mosher's empirical model (*38*, *39*), initially ^{1}H NMR spectra of the two isolated synthetic trans products were fully resolved by 2D-COSY experiments. Careful examination of the chemical shifts of the cyclohexane ring hydrogens of the derivatized products, **5SRS** and **5RSS**, revealed that while the hydrogens on carbons 2, 3, and 4 of the slow-eluting trans isomer displayed small but consistent downfield shifts, the hydrogens on carbons 5 and 6 shifted upfield relative to the corresponding protons of the fast-eluting trans isomer (Figure 4). This same trend was also observed for the exocyclic methylenes of the ethylamine side chain where the protons of the slow-eluting trans isomer displayed considerable downfield shifts relative to the fast-eluting trans isomer (Figure 4). On the basis of these observations and the empirical Mosher's rules, the absolute configuration of the 2-OH of the fast-eluting trans isomer was assigned to *(R)* (**5RSS**) and the slow-eluting isomer was assigned to *(S)* (**5SRS**) (Figure 4; *38*, *39*).

Stability of the CHDEA-6OH Product under Assay Conditions. The solution stability of the enzymatically generated CHDEA-6OH at elevated temperatures was determined by examining the time courses of the DβM-mediated production of PEA and CHDEA-6OH from CHDEA. In these experiments, the concentrations of all enzymatic products were determined by reversed-phase HPLC as a function of time up to 60 min at pH 7.2 and 32 °C. All resulting rates of synthesis were linear with no lag or burst periods (Figure 5), suggesting that both products are stable under the experimental conditions and accurately quantified by HPLC.

Relative Activation Energy Parameters of Partitioning of the Ring H-Abstraction Pathway of the DβM/CHDEA Reaction. The temperature dependency of the partition ratio, CHDEA-6OH/PEA, for the DβM/CHDEA reaction was examined in the temperature range 8−40 °C at pH 7.2 under standard experimental conditions. The enzymatic products, PEA and CHDEA-6OH, were derivatized with SNPA and carefully quantified by reversed-phase HPLC. These studies revealed that the above partition ratio sharply decreased with

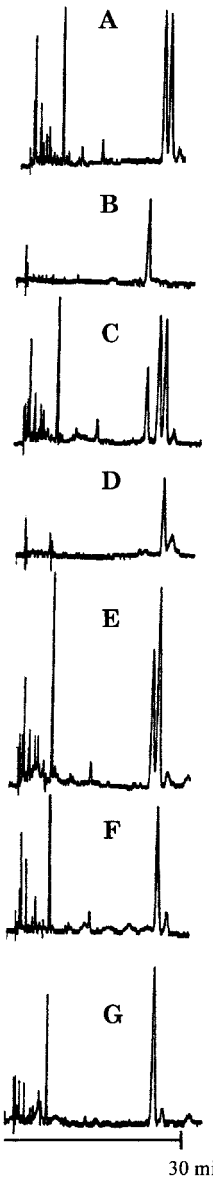


FIGURE 2: Isolation of reduced and derivatized CHDEA-6OH from large-scale Dβ M/CHDEA incubations. The enzymatic products were reduced and derivatized as described in the caption to Figure 1. All the products were separated by preparative HPLC and analyzed by analytical HPLC and mass spectrometry. (A) Synthetic trans mixture; (B) first fraction (from 20.0 to 25.5 min) of the preparative HPLC of the enzymatic reaction mixture; (C) fraction B spiked with mixture A. Note that the enzymatic product does not correspond to either of the synthetic trans products. This product has been identified as the partially reduced, derivatized enzymatic product, *N*-2-{*trans*-6-[(*S*)-2-methoxy-2-trifluoromethyl-2-(phenylacetyloxy)]cyclohexenyl}-2,2-dideuterioethylbenzamide, by mass spectroscopy [low resolution MS (CI) M + 1 = 463] and ^{1}H NMR analysis (data not shown). (D) Second fraction (from 25.5 to 28.8 min) of the preparative HPLC of the enzymatic reaction mixture; (E) fraction D spiked with mixture A. Note that the isolated enzymatic product corresponds to the slow-eluting synthetic trans product. Mass spectral characteristics (M + 1 = 465) and ^{1}H NMR (Figure 3) of the isolated enzymatic product is consistent with the proposed structure. (F) Preparative HPLC-isolated slow-eluting (from 25.5 to 28.8 min) diastereomer of the synthetic trans mixture. ^{1}H NMR of this compound corresponds to the enzymatic product (Figure 3). The molecular ion peak of this compound (M + 1 = 463) differs from the enzymatic product by 2 mass units due to the absence of benzylic deuterium in the synthetic product (data not shown). (G) Fraction D spiked with compound F. Note that the slow-eluting synthetic trans product corresponds to the enzymatic product.

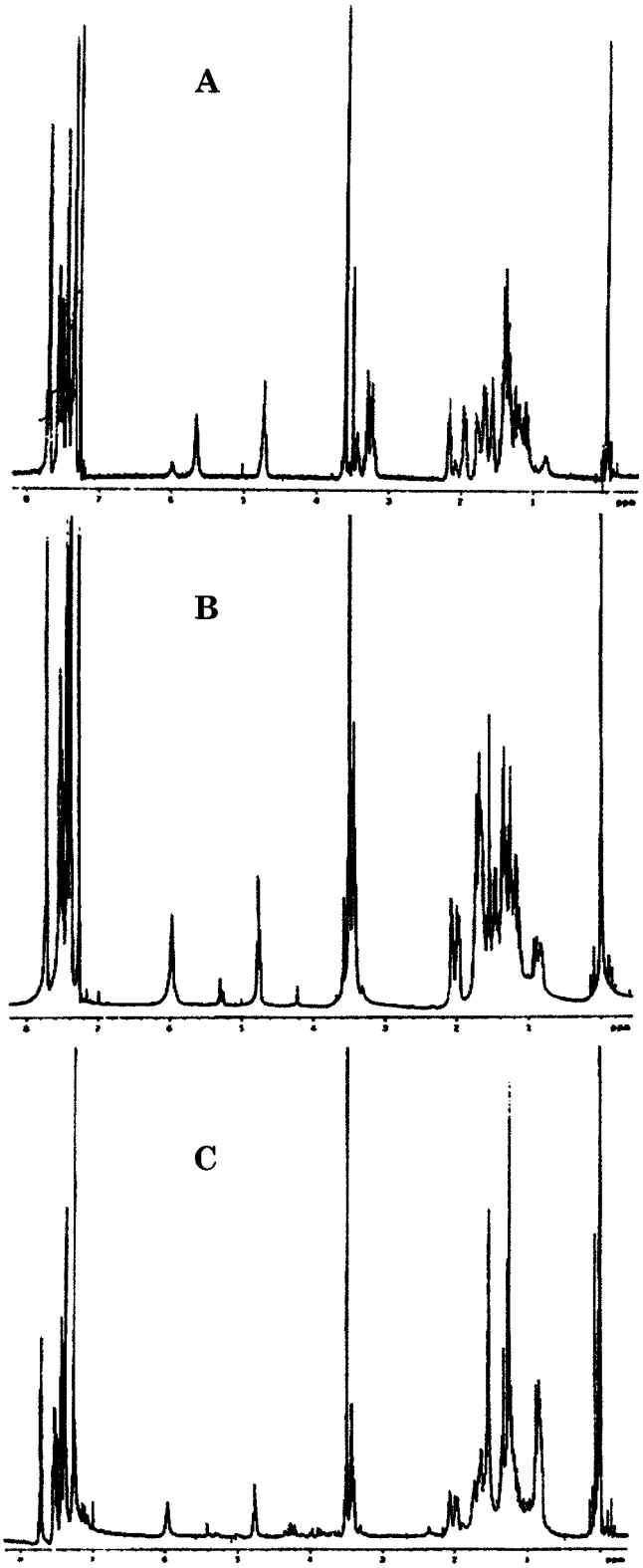
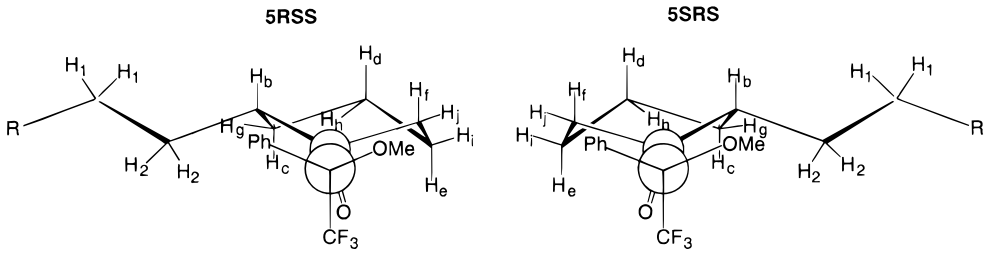


FIGURE 3: ^{1}H NMR spectra of synthetic and enzymatic products. (A) ^{1}H NMR spectrum of the fast eluting diastereomer of the synthetic trans mixture in Figure 2A; (B) ^{1}H NMR spectrum of the slow-eluting diastereomer of the synthetic trans mixture in Figure 2A,F; (C) ^{1}H NMR spectrum of the enzymatic product in Figure 2D.

increasing temperature for CHDEA and all the CHDEA derivatives tested, i.e., CHDEA-d2, CHDEA-d6, 4Me-CHDEA-d2, and CHDPA. In addition, the Eyring plots (*41*, *42*) for all these derivatives were linear and the results were



Proton	δ ppm for fast eluting (**5RSS**)	δ ppm for slow eluting (**5SRS**)	Δδ of **5SRS** relative to **5RSS** downfield (D) upfield (U)
1	3.35	3.4	0.05D
2	1.3	1.75, 1.25	0.45D, 0.05U
N-H	5.66	5.97	0.31D
a	4.75	4.8	0.05D
b	1.6	1.7	0.1D
c	1.15	1.2	0.05D
d	1.25	1.35	0.1D
e	1.35	1.25	0.10U
f	1.4	1.35	0.05U
g	1.95	2.0	0.05D
h	1.70	1.7	-
i	1.8	1.75	0.05U
j	2.2	2.1	0.1U

FIGURE 4: Assignment of absolute stereochemistry of the ring-hydroxylated product of the DβM/CHDEA reaction. All the ^{1}H NMR peaks were resolved and assigned by analyzing the COSY spectra of the two isolated synthetic trans products (**5RSS** and **5SRS**), and the difference in chemical shifts (δ) of the corresponding hydrogens were calculated and compared. By use of Mosher's empirical model, the slow-eluting isomer is assigned as the **5SRS** configuration on the basis of the small but consistent upfield shift of the hydrogens H_e, H_f, H_i, and H_j and downfield shifts of the hydrogens 1, N−H, H_b, H_c, H_d, H_g, and one of the H_2s in comparison to the fast-eluting diastereomer, **5RSS**.

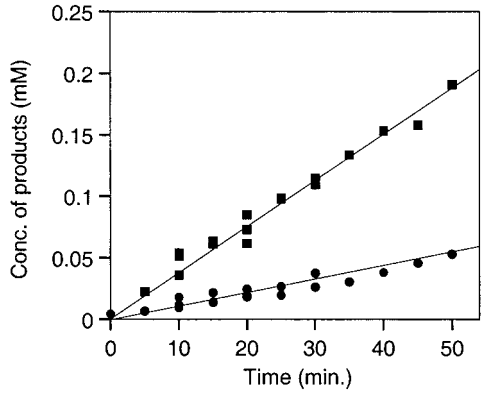


FIGURE 5: Time courses of the products of the DβM/CHDEA reaction. The DβM/CHDEA reaction was carried out as detailed under Experimental Procedures. Aliquots of 0.1 mL were withdrawn at 5 min intervals, derivatized with SNPA, and analyzed by C_{18} reversed-phase HPLC at 280 nm using 45% acetonitrile/55% 50 mM potassium phosphate buffer (pH 7.2) as the mobile phase. (●) PEA; (■) CHDEA-6OH (CHDEA-1OH is not shown for clarity). Note that the small deviation of the line for CHDEA-6OH from the origin is due to a small background in the chromatograms.

highly reproducible between experiments (Figure 6). The direct fit of the data to the logarithmic form of the Eyring equation (*41*, *42*) revealed that the difference in activation enthalpy ($\Delta\Delta H^{\#}$) and activation entropy ($\Delta\Delta S^{\#}$) between the two pathways was -15.44 ± 0.59 kcal/mol and -46.36 ± 2.08 eu, respectively (Table 1), for CHDEA. The activation Gibbs free energy difference ($\Delta\Delta G^{\#}$) for the two pathways was calculated to be -1.07 kcal/mol at 37 °C in favor of the ring hydroxylation (Table 1). The corresponding thermodynamic parameters, $\Delta\Delta H^{\#}$, $\Delta\Delta S^{\#}$, or $\Delta\Delta G^{\#}$ were not significantly changed for CHDEA-d6 or CHDEA-d2 (data not shown), suggesting that the deuterium substitution on either the ring or the side chain has no significant effect on the above partition ratio (Table 1). On the other hand substitution of a methyl group on the 4-position of the ring of CHDEA-d2 significantly decreased both $\Delta\Delta H^{\#}$ (from 15.44 ± 0.59 to -19.45 ± 0.20 kcal/mol) and $\Delta\Delta S^{\#}$ (from 46.36 ± 2.08 to -65.96 ± 0.71), resulting in the change of overall activation free energy, $\Delta\Delta G^{\#}$, from -1.07 to $+1.00$ kcal/mol at 37 °C, favoring the aromatization over ring hydroxylation. Furthermore, the extension of the side chain of CHDEA by one methyl unit [1-(3-aminopropyl)-1,4-cyclohexadiene (CHDPA)] had only a minor effect on the activation energy parameters, with the activation free energy for oxygen rebinding ($\Delta\Delta G^{\#}$) at 37 °C slightly favoring aromatization, similar to CHDEA and CHDEA-d6 (Table 1).

DISCUSSION

Stereochemistry of the DβM/CHDEA Ring Hydroxylation Reaction. The new enzymatic product of the DβM/CHDEA reaction was unequivocally identified as the ring-hydroxylated product of 1,4-CHDEA, CHDEA-6OH, by standard chemical and spectroscopic techniques (Figures 1−3). The stereospecificity of enzymatically produced CHDEA-6OH was determined by a multistep analysis procedure, involving the benzoylation of the NH_2 group and catalytic hydrogenation of the cyclohexadiene ring, followed by the derivatization of the OH group with excess Mosher's reagent (*38*, *39*). After the reaction sequence, the enzymatic product yielded

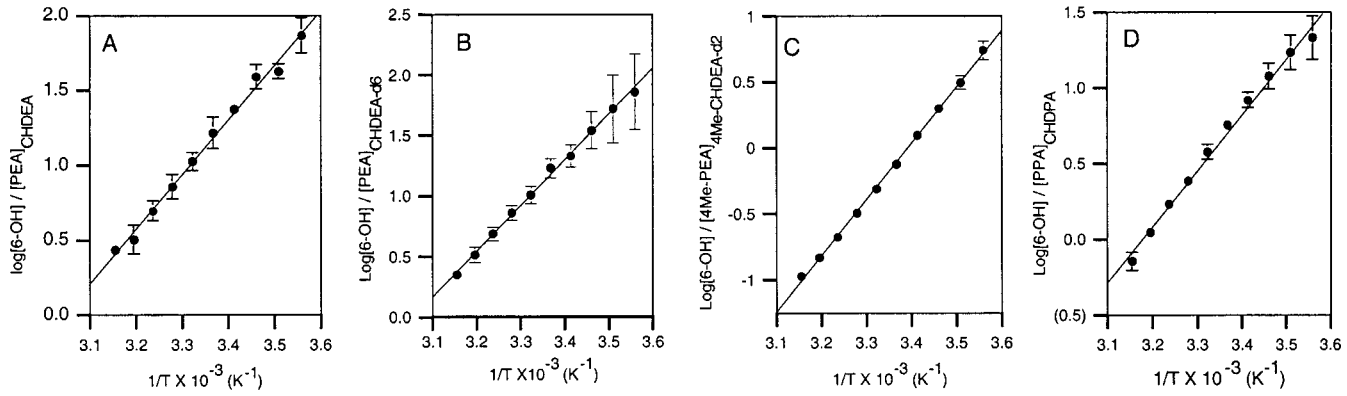


FIGURE 6: Eyring plots for the partition ratio, CHDEA-6OH/PEA, of the Dβ M/CHDEA reaction at pH 7.2. Each point is an average of 4−5 independent experiments that were carried out at the given temperature under identical conditions with the same enzyme preparation as described under Experimental Procedures. The errors were calculated from the standard deviation of the data. The average data sets were fit to straight lines. The activation energy parameters, $\Delta\Delta H^{\#}$ and $\Delta\Delta S^{\#}$, were calculated by direct fit of the data to the exponential form of the Eyring equation. (A) CHDEA; (B) CHDEA-d6; (C) 4Me-CHDEA-d2; (D) CHDPA.

Table 1: Relative Activation Energy Parameters of Oxygen Rebinding/Aromatization in the Dβ M/CHDEA Reaction at pH 7.2[a]

substrate	$\Delta\Delta H^{\#}$ (kcal/mol)	$\Delta\Delta S^{\#}$ (eu)	$\Delta\Delta G^{\#}$ at 37 °C[b]
CHDEA	−15.44 ± 0.59	−46.36 ± 2.08	−1.07 ± 0.09
CHDEA-d6	−15.90 ± 0.36	−48.06 ± 1.27	−1.00 ± 0.08
4Me-CHDEA-d2	−19.45 ± 0.20	−65.96 ± 0.71	+1.00 ± 0.03
CHDPA	−16.10 ± 0.37	−50.81 ± 1.27	−0.35 ± 0.04

[a] The activation energy parameters, $\Delta\Delta H^{\#}$ and $\Delta\Delta S^{\#}$, were calculated by direct fit of the partition ratio, CHDEA-6OH/PEA, to the exponential form of the Eyring equation (*41, 42*) at the temperature range 8−40 °C as described under Experimental Procedures. The errors of $\Delta\Delta H^{\#}$ and $\Delta\Delta S^{\#}$ were obtained from the fit of the average sets of data. [b] Directly calculated from partition ratios by use of the equation $\Delta\Delta G^{\#} = -RT \ln$ [CHDEA-6OH]/[PEA]; errors were calculated from the standard deviations.

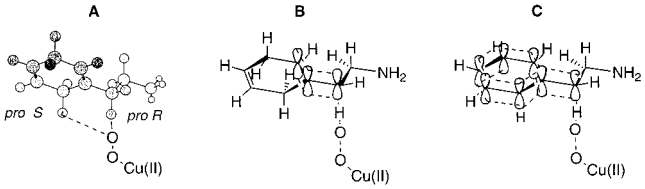


FIGURE 7: Proposed mode of interaction of amine substrates with Dβ M.

a single trans diastereomer that coeluted with the similarly derivatized slow-eluting authentic trans diastereomer in reversed-phase HPLC while not a trace of the faster eluting trans isomer was detectable (Figures 1 and 2). These results clearly show that, similar to the normal benzylic hydroxylation reaction, Dβ M-catalyzed ring hydroxylation of CHDEA is highly stereospecific. The corresponding cis isomers were absent in the enzymatic reaction mixture, suggesting that the catalytic hydrogenation of the cyclohexadiene ring of CHDEA-6OH exclusively produces the corresponding trans products. This finding is not unexpected since hydrogenation of cyclic alcohols is known to predominantly produce cis H addition products from the same face as the hydroxyl group due to the interaction of the OH group with the catalytic surface (*43*). In the case of CHDEA-6OH this effect together with the bulkiness of the benzoylated phenylethylamine side chain appears to exclusively direct hydrogenation toward the more stable trans isomer.

The ^{1}H NMR spectra of the isolated enzymatic product and slow-eluting authentic trans product were identical (except for deuterium at the benzylic position of the enzymatic product), suggesting that the absolute stereochemistry of the enzymatic product must be the same as that of the slow-eluting authentic trans diastereomer (Figure 3). The stereochemistry of the slow-eluting authentic trans product has been assigned by use of Mosher's empirical model (*38, 39*) as *(S, R)* with respect to the ring hydroxyl group and the side chain (Figure 4). Therefore, we conclude that Dβ M-catalyzed ring hydroxylation of CHDEA is highly stereospecific and produces only the corresponding *(S)* alcohol. Furthermore, on the basis of the known absolute stereospecificity of Dβ M toward benzylic (*10, 33*) and allylic (*15*) *(R)* hydroxylations, we are certain that the Dβ M/CHDEA side-chain hydroxylation reaction is also stereospecific and produces only the corresponding *(R)* alcohol.

A Model for the Mode of Interaction of the Substrate with Dβ M. The absolute stereospecificity of the Dβ M benzylic hydroxylation reaction has been shown to be due to the stereo- and regiospecific abstraction of the (*pro-R*) benzylic hydrogen followed by the carbon−oxygen bond formation with the retention of configuration (*33*). These results suggest that while the (*pro-R*) benzylic hydrogen of the enzyme-bound substrate must be accessible to the activated copper oxygen species, the (*pro-S*) hydrogen must be inaccessible and located away from the activated copper oxygen species (for further support for this proposal see ref *44*). Similarly, the observed *(S)* stereospecificity of the ring and *(R)* stereospecificity for side-chain hydroxylations of the Dβ M/CHDEA reaction suggests that while (*pro-S*) hydrogen of the C6 position of the ring and the (*pro-R*) hydrogen of the side chain are readily accessible to the activated copper oxygen species, the (*pro-R*) hydrogen of the ring and (*pro-S*) hydrogen of the side chain are not. Consequently, as shown in Figure 7A, the side-chain (*pro-R*) hydrogen and (*pro-S*) hydrogen of ring C6 must be located, along with the activated copper oxygen species, in the same face of the cyclohexadiene ring of enzyme-bound CHDEA. Such a binding mode will require the side-chain (*pro-R*) hydrogen to be approximately perpendicular to the plane of the cyclohexadiene ring (Figure 7A). The direct extrapolation of these findings to the regular Dβ M substrate, phenylethylamine, suggests that the plane of the aromatic ring of the

enzyme-bound substrate must also be perpendicular to the side-chain benzylic (*pro-R*) hydrogen.

The importance of the above binding mode for the catalytic efficiency of the enzyme can readily be seen by close examination of the possible transition-state structures of the C−H bond cleavage step in the DβM reaction. In the case of CHDEA and other allylic substrates, abstraction of the (*pro-R*) allylic hydrogen of the side chain could be facilitated by lowering of the transition-state energy through maximum delocalization of the radical character into the ring double bond, when the (*pro-R*) hydrogen is parallel to the π orbitals of the ring double bond (Figure 7B). Similarly, in the benzylic hydroxylation reaction, the abstraction of benzylic (*pro-R*) hydrogen could be facilitated by lowering of the transition-state energy through the delocalization of the benzylic radical character into the aromatic ring when the side-chain (*pro-R*) hydrogen is parallel to the aromatic ring π orbitals (Figure 7C). Therefore, the selective binding of this specific conformation of the substrate by the enzyme may be essential for the catalytic efficiency of the enzyme. Although a similar binding mode has also been proposed for the interaction of substituted benzene derivatives in P_{450} catalyzed benzylic hydroxylation reactions (for example see ref *45*), to our knowledge no direct experimental evidence is available to support this proposal.

Molecular Mechanism of the DβM/CHDEA Aromatization Reaction. Although it is conceivable that CHDEA-6OH is the terminal enzymatic product from the ring H abstraction pathway of the DβM/CHDEA reaction and PEA exclusively arises from the nonenzymatic dehydration of the initially generated CHDEA-6OH (Scheme 1, pathway c), the following experimental evidence strongly disfavors this possibility. First, stability tests of CHDEA-6OH under the experimental conditions clearly show that enzymatically generated CHDEA-6OH does not undergo significant solution dehydration to produce PEA under the experimental conditions (data not shown). Second, the time courses of the formation of all three enzymatic products, PEA, CHDEA-6OH, and CHDEA-1OH (data not shown), displayed no lag or burst periods for up to 1 h (Figure 5), demonstrating that all three products must have their own independent origins under the experimental conditions. These results, along with the observation that both PEA and CHDEA-6OH are produced in significant quantities in the enzyme reaction mixtures under appropriate conditions, strongly suggest that PEA and CHDEA-6OH were directly produced by the enzyme as terminal products and, especially, PEA is not derived from the solution decomposition of CHDEA-6OH under the experimental conditions.

The large negative relative activation enthalpy, $\Delta\Delta H^{\#}$, determined for CHDEA and its derivatives (Table 1) suggests that the ring hydroxylation pathway is highly favored by activation enthalpy compared to the aromatization pathway. However, the relative activation entropy, $\Delta\Delta S^{\#}$, for the ring hydroxylation pathway is very large and negative, suggesting that the transition state for the ring hydroxylation is much more ordered, organized, and entropically highly unfavorable in comparison to aromatization (Table 1). These results indicate that the transition states of these two partitioning pathways are significantly structurally different from each other (Scheme 1, pathways a and b). Therefore, a mechanism in which the aromatized product and the ring-hydroxylated

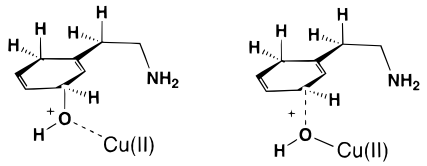


FIGURE 8: Possible transition states for the hydrolysis and dehydration of the enzyme-bound ring alkoxide of CHDEA.

products are derived from the partitioning of the copper alkoxide product species between the dehydration and the hydrolysis (Scheme 1, pathways c and d) could be eliminated. If such a mechanism was operative, $\Delta\Delta H^{\#}$ and $\Delta\Delta S^{\#}$ would be small due to the similarity of the respective transition-state structures (Figure 8).[3] In addition, abstraction or ejection of a H$^{\bullet}$ from the C3 position of the initially generated cyclohexadienyl radical, with or without any assistance from the enzyme, leading to the subsequent formation of the aromatized product would also be inconsistent with the observed lack of a significant deuterium isotope effect on the aromatization pathway (k_H/k_D for aromatization is 1.02 $\pm$ 0.20 at 37 °C).[4] Therefore, we propose that the partitioning of the ring H-abstraction pathway between hydroxylation and aromatization is a consequence of the partitioning of the cyclohexadienyl radical intermediate between carbon−oxygen bond formation from the Cu−O$^{\bullet}$ species or direct electron transfer from the Cu−O$^{\bullet}$ species. As shown in Scheme 1, most likely the aromatization is a consequence of the direct transfer of an electron from the cyclohexadienyl radical to the active-site Cu(II)−O$^{\bullet}$ species followed by a fast deprotonation of the highly unstable cyclohexadienyl cation to produce the aromatic product, PEA (Scheme 1, pathway a). Efficient and macroscopically irreversible electron transfer could be facilitated by the high thermodynamic driving force for aromatization of the cyclohexadienyl cation. In addition, the highly favorable $\Delta\Delta H^{\#}$ for the hydroxylation pathway would be a consequence of a high thermodynamic driving force for radical recombination to produce the ring-hydroxylated enzyme-bound alkoxide, similar to the normal DβM monooxygenation pathway, compared to direct electron transfer to produce the cyclohexadienyl cation and Cu−O^{-} species. Furthermore, the highly unfavorable $\Delta\Delta S^{\#}$ for the ring hydroxylation pathway is also consistent with the above proposal since radical recombination is expected to be

[3] As one reviewer suggested, the measured activation parameters include the entire system and the difference in activation energies could be due to the difference in energies of the enzyme conformations at the transition state. While this is a theoretical possibility, on the basis of the following arguments we strongly believe that the partitioning of the reaction pathways in the DβM/CHDEA reaction is due to the characteristics of CHDEA. DβM is a specific enzyme and known to interact exclusively with the benzylic positions of the substrate to produce the expected monooxygenated products from all known substrates, except CHDEA. Clearly, the unexpected ring H-abstraction pathway is a consequence of the structural and electronic properties of CHDEA. Therefore, it is very likely that the observed partitioning of the reaction pathways between the ring hydroxylation and aromatization is also a consequence of the unique characteristics of the cyclohexadienyl radical intermediate rather than the existence of different enzyme conformations at the transition state.

[4] A mechanism involving spontaneous ejection of a hydrogen atom from the cyclohexadienyl radical without assistance from the enzyme may not be possible simply due to the fact that the C−H bond energy of the cyclohexadienyl radical is about 20 kcal/mol (*46*). The isotope effect for aromatization is calculated from the partition ratios of CHDEA and CHDEA-d6.

entropically costly in comparison to the direct electron transfer, which does not involve carbon−oxygen bond formation.

Activation Entropic Cost of Carbon−Oxygen Bond Formation. Partitioning of the ring H abstraction pathway of the DβM/CHDEA reaction between the ring hydroxylation and aromatization is intriguing. The small activation free energy difference, $\Delta\Delta G^{\#}$, between the two pathways (Scheme 1; pathways a and b) clearly demonstrates that the high relative activation enthalpic favorability (Table 1) for the carbon−oxygen bond formation (Scheme 1, pathway b) is offset by the high relative activation entropic cost (Table 1) of a highly ordered transition state. On the other hand, the high relative enthalpic unfavorability for the direct electron transfer (Scheme 1, pathway a) is compensated by high entropic favorability of a loose transition state for direct electron transfer. Therefore, although the enzyme has been naturally optimized for efficient carbon−oxygen bond formation through the generation of highly reactive intermediates with an optimal arrangement under normal circumstances, the inherent high entropic burden associated with a highly ordered transition state could redirect the enzyme leading to the formation of undesirable products. Further support for this proposal is provided by the activation energy parameters presented in Table 1 for 4Me-CHDEA-d2. Both $\Delta\Delta H^{\#}$ and $\Delta\Delta S^{\#}$ for 4Me-CHDEA-d2 are noticeably lower than the corresponding values for CHDEA, suggesting that the transition state for the carbon−oxygen bond formation is more optimal and rigid for 4Me-CHDEA than for CHDEA. However, the overall relative activation free energy, $\Delta\Delta G^{\#}$, for 4Me-CHDEA-d2 at 37 °C is positive and disfavors the carbon−oxygen bond formation in contrast to CHDEA (Table 1). These results indicate that the enthalpic favorability gain by geometric and electronic optimization of the transition state for the carbon−oxygen bond formation is offset by the high entropic cost of such a highly ordered transition state to an extent that aromatization is more favored than with CHDEA. Therefore, the maximum efficiency and selectivity of the desirable reaction could only be achieved by optimization of the transition state to balance these two opposing forces.

Concluding Remarks. Taken together, we have shown that the ring H abstraction pathway of the DβM/CHDEA reaction partitions into two pathways after the initial C−H bond cleavage to produce the ring-hydroxylated product, CHDEA-6OH, and aromatized product, PEA. The ring hydroxylation is highly stereospecific, similar to the physiological benzylic and allylic hydroxylation reactions of DβM, and exclusively produces the *(S)* hydroxylated product. The absolute stereospecificities of the ring and side-chain hydroxylations of the DβM/CHDEA reaction led us to propose a model for the interaction of substrates with the enzyme. The salient feature of this model is that the enzyme effectively interacts with a conformation of the substrate in which the side-chain benzylic (*pro-R*) hydrogen is approximately perpendicular to the aromatic ring of the substrate. This specific interaction may provide an additional mechanism for lowering the activation energy of the initial hydrogen abstraction step through extended delocalization of the radical character into the aromatic ring of the substrates. On the basis of the magnitudes of the relative activation parameters for the two pathways (Scheme 1, pathways a and b), we propose that the formation of the aromatized and ring-hydroxylated products from CHDEA is a consequence of the partitioning of the high-energy intermediates, cyclohexadienyl radical and Cu(II)−O$^{\bullet}$ species, between the carbon−oxygen bond formation and direct electron transfer. Since the carbon−oxygen bond formation is highly favored by activation enthalpy and direct electron transfer is favored by activation entropy, critical balancing of these two opposing forces is mandatory for the desired specific product formation. Finally, the relative activation parameters of 4Me-CHDEA-d2 further suggest that the partition ratio is highly sensitive to small alterations of the structural parameters of the substrate and therefore the structure−activity studies of the carbon−oxygen bond formation step in enzymatic monooxygenations must be analyzed cautiously (for example see refs *47* and *48*).

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