Interaction of non-conjugated olefinic substrate analogues with dopamine β -monooxygenase: catalysis and mechanism-based inhibition

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The reaction of dopamine β -monooxygenase (DBM; EC 1.14.17.1) with the prototypical non-conjugated olefinic substrate, 2-(1-cyclohexenyl)ethylamine (CyHEA) [see Sirimanne and May (1988) *J. Am. Chem. Soc.* **110**, 7560–7561], was characterized. CyHEA undergoes facile DBM-catalysed allylic hydroxylation to form (*R*)-2-amino-1-(1-cyclohexenyl)ethanol (CyHEA-OH) without detectable epoxidation or allylic rearrangement, and with stereochemistry consistent with that of DBM-catalysed benzylic hydroxylation and sulfoxidation. The k_{cat} of 90 s⁻¹ for CyHEA oxygenation is about 75 % of the k_{cat} for tyramine, the substrate commonly used in assays of DBM activity. DBM-catalysed oxygenation of CyHEA also results in mechanism-based inactivation of DBM, with the inactivation reaction yielding $k_{\text{inact.}} = 0.3 \text{ min}^{-1}$ at pH 5.0 and 37 °C, and a partition ratio of 16000. Although both CyHEA turnover and inactivation exhibit normal kinetics, CyHEA processing also results in gradual depletion of copper from DBM; however, mechanism-based irreversible DBM inactivation occurs independent of this copper depletion when sufficient copper is present in the assay solution. A likely mechanism for turnover-dependent DBM inactivation by CyHEA involves initial abstraction of an

allylic hydrogen to form a resonance-stabilized allylic radical, which can then either partition to product or undergo attack by an active-site residue. Acyclic, non-conjugated olefinic analogues exhibit diminished substrate activity toward DBM. Thus, k_{cat} for oxygenation of *cis*-2-hexenylamine, which also produces only allylic alcohol product, is only 14 % of that for CyHEA. Similarly, $k_{\text{inact.}}/K_1$ for turnover-dependent inactivation by the acyclic olefin 2-aminomethyl-1-pentene is more than an order of magnitude smaller than that for benzylic olefins. Our results establish that DBM catalyses allylic oxygenation of a number of non-conjugated olefinic substrate analogues with neither epoxidation nor allylic rearrangement occurring. The absence of epoxide products from non-conjugated olefinic substrates implies an inability of the activated copper–oxygen species of DBM to effect radical cation formation from a non-conjugated olefinic moiety. The striking contrast between DBM and cytochrome *P*-450, which carries out both epoxidation and allylic oxidation with non-conjugated olefinic substrates, is probably a reflection of the differences in redox potential of the activated oxygen species operative for these two enzymes.

INTRODUCTION

Dopamine β -monooxygenase (DBM, EC 1.14.17.1) is a copper-containing monooxygenase which catalyses hydroxylation at the pro-*R* hydrogen of dopamine to form noradrenaline (nor-epinephrine) in a variety of mammalian tissues (Levin et al., 1960; Weinshilboum and Axelrod, 1971; Aberg et al., 1974; Goldstein and Cubeddu, 1976) and thus plays a key role in the biosynthesis of noradrenaline and in the production of adrenaline (epinephrine) (Winkler et al., 1970; Battersby et al., 1976). We and others have demonstrated that DBM exhibits broad specificity toward its organic substrate. Thus, we have shown that DBM is capable of catalysing stereoselective sulfoxidation (May and Phillips, 1980), oxygenative ketonization (May et al., 1981; Mangold and Klinman, 1984), olefin oxidation (May et al., 1983; Padgett et al., 1985), alkyne oxidation (Padgett et al., 1985; Colombo and Villafranca, 1984), selenoxidation (May et al., 1987) and oxygenative *N*-dealkylation (Padgett et al., 1985; Sirimanne et al., 1987; Wimalasena and May, 1987). We have also shown that DBM catalyses aromatization of 1-(2-aminoethyl)-1,4-cyclohexadiene (Wimalasena and May, 1989), which represents a redirection of the specificity of this enzyme. A number of substrate analogues have been shown to undergo

benzylic methylene oxygenation (Baldini and Villafranca, 1980; Colombo et al., 1984; Fitzpatrick et al., 1985; Fitzpatrick and Villafranca, 1985, 1986; Kruse et al., 1986; Bargar et al., 1986; Ross et al., 1987).

In a previous communication we reported DBM-catalysed stereoselective allylic hydroxylation of 2-(1-cyclohexenyl)ethylamine (CyHEA) (Sirimanne and May, 1988). We now extend and further characterize our recent findings on allylic hydroxylation, and we demonstrate that the prototypical non-conjugated olefinic substrate, CyHEA, is not only a highly active substrate but also a mechanism-based inhibitor for DBM. Moreover, we report here another unprecedented activity of DBM, that is hydroxylation of simple acyclic olefinic amines. The lack of DBM-catalysed epoxidation of these non-conjugated olefins, in contrast with the reactivity of cytochrome *P*-450, probably reflects the lower redox potential of the activated copper-oxy species operative in DBM catalysis.

EXPERIMENTAL

Materials

Tyramine hydrochloride, ascorbic acid, sodium fumarate and potassium phthalimide were obtained from Sigma Chemicals Co.

Abbreviations used: ACPP, 3-amino-2-cyclopentylpropene; *Cis*-HEA, *cis*-2-hexenylamine; CyHEA, 2-(1-cyclohexenyl)ethylamine; CyHEA-OH, 2-amino-1-(1-cyclohexenyl)ethanol; DCC, dicyclohexylcarbodiimide; DBM, dopamine β -monooxygenase; DMAP, 4-(dimethylamino)pyridine; NBP, 4-nitrobenzylpyridine; PEA, 2-phenylethylamine; SMPA, *N*-succinimidyl- α -methoxyphenylacetate; SNPA, *N*-succinimidyl(4-nitrophenyl)acetate; THF, tetrahydrofuran; TMEDA, tetramethylethylenediamine; Tr-HEA, *trans*-2-hexenylamine.

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CyHEA, 2-propylacrolein, *cis*- and *trans*-hex-2-enols, cyclopentanone, ethylcyanoacetate, phosphorus tribromide, (*S*)- α -methoxyphenylacetic acid and sodium borohydride were from Aldrich Chemicals. Beef liver catalase (specific activity 65000 units/mg of protein) was obtained from Boehringer–Mannheim. The h.p.l.c.-grade solvents were purchased from Fisher Scientific.

Isolation and assay of DBM

DBM was isolated from bovine chromaffin granules obtained from fresh adrenal medulla (Padgett et al., 1985; Colombo et al., 1987). The specific activity of DBM was 40–50 units/mg. The concentration of DBM was determined spectrophotometrically by measuring the absorbance at 280 nm ($E_{280}^{1\%} = 12.4 \text{ M}^{-1}\text{cm}^{-1}$). A polarographic oxygen monitor assay was used in the determination of kinetic constants of both substrates and inhibitors. Kinetic constants of substrates were calculated by computer fit of the data to the hyperbolic form of the Michaelis–Menten equation (Cleland, 1967). All k_{cat} and K_m values are apparent values obtained at an atmospheric oxygen saturation. Oxygen consumption was measured with a Clark electrode and YSI oxygen monitor. The standard oxygen monitor assay mixture contained 0.12 M sodium acetate buffer, pH 5.0, 10 mM sodium fumarate, 0.1 mg/ml of catalase, $0.5 \mu\text{M}$ CuSO_4 , 10 mM ascorbic acid and 1.2–2 μg of DBM/ml. In this assay mixture at atmospheric pressure oxygen saturation was found to be 250 μM . Initial rates were corrected for a small background due to ascorbic acid auto-oxidation.

4-Nitrobenzylpyridine (NBP) derivatization of epoxides

The procedure is essentially the same as that of Padgett et al. (1985). Standard DBM assay solutions were employed with 15 mM CyHEA and 10 μg of DBM in a total volume of 1 ml. Controls (without enzyme; without ascorbate; or without CyHEA) were run in parallel, substituting buffer for the omitted components. The reaction and controls were incubated at 37 °C with shaking for 15 min. To 0.4 ml of solution was added 6% (w/v) NBP in ethylene glycol (1 ml) and 200 mM Tris/HCl, pH 7.6, 27% ethanol (v/v) (0.40 ml), and the solutions were incubated at 60 °C with shaking for 1 h. These solutions were cooled in ice and 50% (v/v) triethylamine in acetone (2.0 ml) was added, and the spectrum was recorded with reference to distilled water treated similarly.

Detection, isolation and identification of the product of the CyHEA/DBM reaction

The standard assay mixture used in assay of DBM with 50 mM CyHEA was employed. Controls (without DBM; without ascorbate; or with boiled DBM) and the complete enzymic reaction mixtures were incubated overnight at 37 °C open to air with shaking in a New Brunswick Environmental Incubator Shaker. All the solutions were basified with 9 M NaOH and extracted with ethyl acetate (3 $\times$ 2 ml). The residues were dissolved in 2 ml of tetrahydrofuran (THF) and 20–25 mg of *N*-succinimidyl(4-nitrophenyl)acetate (SNPA) was added with swirling. The mixture was warmed in a steam bath and stirred for an additional 45 min. Excess SNPA was quenched with saturated aq. Na_2CO_3 . The reaction mixtures were first analysed by reverse-phase h.p.l.c. The resulting white residue obtained on the evaporation of the solvent was extracted with ethyl acetate (2 $\times$ 2 ml). The extracts were concentrated under vacuum and analysed by h.p.l.c. and t.l.c. H.p.l.c. analysis was carried out on a DYNAMAX silica-gel column (4.6 mm $\times$ 250 mm) with 50%

(v/v) hexane in ethyl acetate as the eluent; t.l.c. was performed using silica-gel t.l.c. plates (ANALTECH) and the same eluent. In preparative t.l.c., corresponding bands were visualized under u.v. light, scraped off and eluted with ethyl acetate. Evaporation of the solvent gave the product which was further compared with the SNPA derivative of the chemically synthesized 2-amino-1-(1-cyclohexenyl)ethanol (CyHEA-OH) by h.p.l.c. and characterized by 360 MHz Fourier transform (FT) n.m.r. and m.s.

O_2 /ascorbate and O_2 /product stoichiometry of the CyHEA/DBM reaction

Enzymic reactions were initiated by addition of DBM and O_2 uptake was followed using an oxygen monitor until 80% of O_2 was consumed. The enzymic reaction was then quenched with 500 μl of perchloric acid and filtered through a Centricon 10 filter. The amount of ascorbate consumed was determined from total ascorbate and residual ascorbate levels. The amount of ascorbate remaining was quantified using ion-paired reverse-phase h.p.l.c. with electrochemical detection (Herman et al., 1987). Deproteinized samples were applied to a 25 cm C_{18} reverse-phase h.p.l.c. column (Altech) equilibrated with a mobile phase composed of 0.15 M monochloroacetate (pH 3.0) containing 2 mM Na_2EDTA and 0.13 mM sodium octylsulphonate flowing at 1.0 ml/min. Ascorbate was oxidized at 650 mV (referenced to an Ag/AgCl electrode) using a thin-layer amperometric detector cell (TL5A) coupled to a potentiostat (Model LC4B, Bio-analytical systems). Parallel controls were run under identical conditions and worked up identically. The filtrate was then freeze-dried and extracted with ethyl acetate after basifying with 10 M NaOH.

Stereospecificity of the CyHEA/DBM reaction

The enzymic reaction product was prepared and isolated as before and derivatized with *N*-succinimidyl[α -(*S*)-methoxyphenyl]acetate (SMPA) (Husain et al., 1989). The derivatization procedure and the h.p.l.c. analysis of the SMPA-derivatized product were identical with that of SNPA derivatization and h.p.l.c. analysis of the enzymic product.

In this experiment, the derivatized product was dissolved in ether and oxidized by stirring with 20–25 mg of MnO_2 for 1 h. The oxidizing agent was filtered and the solution was concentrated in a rotary evaporator. Aminomethyl-(1-cyclohexenyl) ketone was reduced back to the racemic alcohol with NaBH_4 . The diastereomeric mixture thus obtained was analysed by silica-gel h.p.l.c. using identical conditions to those used for analysis of SNPA-derivatized product and compared with the enzymic product.

Determination of the absolute configuration of enzymically formed CyHEA-OH

The assignment of the absolute configuration was accomplished by the use of Mosher's model (Dale and Mosher, 1973) for mandelate esters of chiral alcohols. The racemic amino alcohol was acylated with (*S*)-*O*-methylmandelic acid as described in the synthesis section. Synthetic racemic amino alcohol derivatized with the chiral acid was analysed by silica-gel h.p.l.c. with u.v. detection at 254 nm using 40% hexane in ethyl acetate as the eluent at a flow rate of 1 ml/min. The two diastereomers were separated by preparative t.l.c. using 40% hexane in ethyl acetate as the solvent. FT n.m.r. spectra were obtained, and the signals for the vinyl protons were identified by n.m.r. decoupling experiments. The chemical shifts of vinyl protons in the cyclo-

hexenyl group were used in the prediction of the absolute configuration by Mosher's model.

Identification of the product from *cis*-2-hexenylamine (Cis-HEA)/DBM reaction

The standard assay mixture used in assay of DBM with 50 mM Cis-HEA was employed. Controls (without DBM; without ascorbate; or with boiled DBM) and the complete enzymic reaction mixtures were incubated at 37 °C overnight in an atmospheric oxygen concentration with shaking in the New Brunswick Environmental Incubator Shaker. All the solutions were basified with 9 M NaOH and extracted with ethyl acetate (3 × 2 ml). The residues were dissolved in 2 ml of THF and 20–25 mg of SMPA (Husain et al., 1989) was added with swirling. The mixture was warmed slightly in a steam bath and stirred for 1 h. Excess SMPA was quenched with saturated aq. Na₂CO₃ and the solvent was evaporated off under vacuum. The white residue was partitioned between water and ethyl acetate (2 × 2 ml). The extracts were concentrated under vacuum and analysed by t.l.c. The t.l.c. of the enzymic reaction product and controls was performed on t.l.c. plates (Analtech) using 50 % hexane in ethyl acetate as the eluent. The SMPA-derivatized product was isolated by preparative t.l.c. using the same eluent. The corresponding band was visualized under u.v. and scraped off and eluted with ethyl acetate. Evaporation of the solvent gave the product which was characterized by 360 MHz FT n.m.r. and m.s.

Time-dependent inactivation of DBM by mechanism-based inhibitors

The inactivation mixtures contained 0.1 M sodium acetate (pH 5.0), 10 mM fumarate, 0.5 μ M CuSO₄, 0.275 mg/ml catalase and 10 mM ascorbate. A sample (0.4 ml) of inactivation reaction mixture was incubated with the required amount of DBM and inactivator was added after removing an aliquot for the 100 % activity. Subsequently, 40 μ l aliquots were removed at required time intervals and assayed for residual activity by dilution into an assay solution.

From the slopes (k_{obs}) of the plots of $\ln(\%$ of residual activity) versus time, first-order inactivation kinetic constants were determined. k_{inact} and K_1 were calculated from the $1/y$ intercept and $1/x$ intercept of the linear fit of the $1/k_{\text{obs}}$ versus $1/[\text{inhibitor}]$ by linear regression analysis. All k_{inact} and K_1 values are apparent values obtained at atmospheric oxygen saturation.

DBM inactivation by mechanism-based inhibitors in the presence of preformed product

Normal DBM assay solution with saturating amounts of suicide substrate ascorbate was incubated at 37 °C and the enzymic reaction was initiated with the required amount of DBM. The O₂ consumption was recorded until there was no oxygen consumption. Another identical aliquot was added after the solution was re-saturated with air and the oxygen consumption was recorded again. Using the traces for oxygen uptake, time versus relative oxygen concentration was plotted.

Syntheses and characterization of compounds

CyHEA-OH

A three-step synthetic route was used to prepare this compound. First, cyclohexanonetoluenesulphonyl hydrazone was prepared according to the general method of Paquette et al. (1980). [Melting point 147–150 °C [literature value 145–150 °C (Paquette et al., 1980)]; ¹H n.m.r. (δ)(C²HCl₃): 7.1–8 (dd, 4H), 2.0–2.4 (m,

4H), 1.5–1.7 (m, 6H); mass spectrum (EI) m/e 266 (M^+).} Next, 1-cyclohexenealdehyde was prepared from this hydrazone using a modification of the method of Chamberlien et al. (1978). [Boiling point 85–90 °C/30 mmHg. ¹H n.m.r. (C²HCl₃)(δ): 9.37 (s, 1H), 6.74 (m, 1H), 2.0–2.6 (m, 4H), 1.4–2.0 (m, 4H).] Finally, CyHEA-OH was synthesized from 1-cyclohexenealdehyde according to the general method of Evans et al. (1974). The final product was purified as the hydrochloride by recrystallization from ethanol/ether (20 %). [Melting point 140 °C (dec.) [literature value 137–138 °C (Burger et al., 1961)]; ¹H n.m.r. (free amine)(C²HCl₃)(δ): 3.9 (t, 1H), 5.83 (s, broad, 1H), 1.0–3.06 (m, 12H); ¹H n.m.r. (SMPA-derivatized)(C²HCl₃): 7.1–7.4 (m, 4H), 5.64 (s, broad, 1H), 5.5 (s, 1H), 4.0 (m, 1H), 3.1–3.5 (m, 5H), 1.3–2.1 (m, 8H); (Found: C, 54.0; H, 9.1. Calc. for C₈H₁₆NOCl: C, 54.1; H, 9.0.)

Acylation of CyHEA-OH and (*R*),(*S*)-1-amino-2-propanols was carried out by dissolving the corresponding amino alcohol (0.45 g, 0.006 mol) in 50 ml of THF and treating with a catalytic amount of 4-(dimethylamino)pyridine (DMAP), two equivalents of dicyclohexylcarbodiimide (DCC) and (*S*)- α -methoxyphenylacetic acid (1 g, 0.006 mmol). The mixture was stirred at room temperature overnight. After cooling at –20 °C dicyclohexylurea was removed by filtration. THF was evaporated under reduced pressure and the residue was dissolved in methylene chloride and washed with dilute HCl, water and then saturated NaHCO₃ solution. The methylene chloride layer, dried over anhydrous sodium sulphate, was evaporated to dryness. The residue was chromatographed on silica gel using 50 % hexane in ethyl acetate and the diastereomers were isolated.

2-Aminomethyl-1-pentene hydrochloride

Synthesis of this compound was accomplished starting with NaBH₄ reduction of propylacrolein to 2-propyl-1-propen-3-ol, conversion of the alcohol into *N*-(2-propyl-2-propenyl)-phthalimide, and reaction with hydrazine monohydrate. The product was recrystallized as the hydrochloride salt from ethanol/ether. [m.p. 153–155 °C; ¹H n.m.r. (C²HCl₃)(δ): 5.14 (s, 1H), 5.03 (s, 1H), 3.59 (s, 2H), 2.10 (t, 2H), 1.45–1.53 (m, 2H), 0.9 (t, 3H.); mass spectrum (EI) m/e 99 (M^+); (Found: C, 53.0; H, 10.4. Calc. for C₆H₁₄NCl: C, 53.1; H, 10.3.)].

3-Amino-2-cyclopentyl-1-propene (ACPP)

This compound was prepared as follows. First, ethylcyclopentylidenecyanoacetate was prepared from cyclopentanone using the method of Cope et al. (1963), and then hydrogenated (Cormier et al., 1973) to give ethyl-3-amino-2-cyclopentylpropionate. Reaction with NaHCO₃ and methyl iodide in methanol (Cormier et al., 1973) produced ethyl-2-cyclopentyl-2-propenoate, which was converted into 2-cyclopentyl-3-hydroxy-1-propene by reaction with di-isobutyl aluminium hydride in hexane. This product was then converted into *N*-(2-cyclopentyl-2-propenyl)phthalimide, followed by hydrazinolysis to give the final product, ACPP, which was recrystallized as the amine hydrochloride from ethanol/ether. [m.p. 180–182 °C (dec); ¹H n.m.r. (C²HCl₃)(δ): 4.88 (m, 2H), 3.1–3.66 (s, broad, 2H), 2.33–2.73 (m, 1H), 1.2–2.2 (m, 8H); mass spectrum (EI) m/e 125 (M^+); (Found: C, 59.4; H, 10.1; N, 8.7. Calc. for C₈H₁₆NCl: C, 59.4; H, 10.0; N, 8.7.)].

Cis-HEA

This compound was prepared from *cis*-3-hexen-1-ol via the phthalimide and hydrazinolysis, and recrystallized from ethanol/ether (49 %). [m.p. 188–190 °C; ¹H n.m.r. (²H₂O)(δ):

5.33–6.33 (m, 2H), 3.76 (d, 2H), 1.9–2.5 (dd, 2H), 0.73–1.8 (m, 5H); mass spectrum (EI) m/e 99 (M^+); (Found: C, 53.1; H, 10.4. Calc. for $C_6H_{14}NCl$: C, 53.1; H, 10.3.).

trans-2-Hexenylamine (Tr-HEA)

This compound was prepared from *trans*-2-hexen-1-ol via the phthalimide and hydrazinolysis, and the hydrochloride salt of the product was recrystallized from ethanol/ether (3.72 g, 66%). [m.p. 198–200 °C; 1H n.m.r. (2H_2O)(δ): 5.26–6.4 (m, 2H), 3.6 (d, 2H), 2.06 (t, broad, 2H), 0.63–1.8 (m, 5H); mass spectrum (EI) m/e 99 (M^+); (Found: C, 53.1; H, 10.4. Calc. for $C_6H_{14}NCl$: C, 53.1; H, 10.3.).]

RESULTS

Reaction of DBM with CyHEA

The non-conjugated olefinic substrate analogue, CyHEA is a highly active substrate for DBM. This compound exhibits normal Michaelis–Menten kinetics (Figure 1), and its oxygenation is strictly dependent on the presence of the reductant, ascorbate, as expected for the normal reductive oxygenation pathway of DBM catalysis. The k_{cat} for CyHEA was found to be 90.1 s^{-1} which is about 75 % of the k_{cat} of tyramine, the substrate commonly used in assays of DBM activity (Padgette et al., 1985). As is evident

Figure 1 Double-reciprocal plot for the substrate kinetics of CyHEA with DBM

This experiment was carried out according to the method given in the Experimental section using $1\text{ }\mu\text{g/ml}$ of DBM. The kinetic parameters were calculated using the hyperbolic fit of the Michaelis–Menten equation.

from Table 1, the activity of CyHEA is comparable with those of other well-characterized substrates. The DBM activator, sodium fumarate, also stimulates oxygenation of CyHEA by DBM (Figure 2), approaching a maximal activation of about 230 % at 10 mM fumarate. Although this activation parallels that found for other known DBM substrates (May et al., 1981; Padgette et al., 1985), the degree of activation is much higher than the activation observed with the best substrates such as tyramine (30 %) (Figure 2).

By analogy to other monooxygenases such as cytochrome P-450, and to our previous findings with conjugated olefins (Padgette et al., 1985), it seemed likely that the product of CyHEA oxygenation by DBM would be the corresponding epoxide. Since we have successfully trapped epoxide products from styrene derivatives with NBP (Padgette et al., 1985), we decided to employ this trapping agent for the CyHEA/DBM reaction. However, no NBP-trappable enzymic products could be detected. This suggested to us that the corresponding epoxide, 1-aminoethyl-1,2-epoxycyclohexane, may, in fact, not be the product formed from DBM-catalysed oxygenation of CyHEA. Accordingly, we proceeded to carry out isolation and identification of the reaction products. Due to the absence of a u.v.-detectable group in CyHEA, SNPA was used to attach the u.v.-detectable group 4-nitrophenylacetyl to the amino functionality of the enzymic product. Derivatization with SNPA allowed both successful analysis of the enzymic reaction by reverse-phase h.p.l.c. (retention times: SNPA-CyHEA, 12.7 min; SNPA-product, 6.0 min) and isolation of the enzymic product from the CyHEA/DBM reaction by preparative silica-gel t.l.c. Product formation was strictly dependent upon the presence of both ascorbate and active DBM. The CyHEA/DBM reaction exhibits an ascorbate: O_2 :product ratio of 1.2:1:1.1 which is characteristic for the normal reductive oxygenation pathway of DBM catalysis. The enzymic product was identified as CyHEA-OH (Scheme 1) by n.m.r., m.s. and chromatographic comparison with the similarly derivatized authentic material. The SNPA-derivatized product exhibited 1H n.m.r. (C^2HCl_3)(δ): 7.8 (dd, 4H), 5.75–5.85 (m, 1H), 5.68 (s, broad, 1H), 4.02–4.09 (m, 1H), 3.67 (s, 2H), 3.5–3.6 (m, 1H), 3.2–3.3 (m, 1H), 1.4–2.1 (m, 8H); mass spectrum (EI) 304 (M^+).

Stereochemistry of the CyHEA/DBM reaction

The stereoselectivity of the CyHEA/DBM reaction was explored using a novel amino-directed chiral derivatizing reagent developed by May and co-workers (Husain et al., 1989). This reagent, SMPA, attaches the *S*-(α -methoxyphenyl)acetyl moiety, which is both u.v.-detectable and chiral, to the amino functionality of the enzymic product. Enzymically produced and chemically synthesized CyHEA-OH were derivatized with SMPA as described in the Experimental section and analysed by silica-gel h.p.l.c. As seen in Figure 3, the enzymic product yields one peak, while synthetic racemic CyHEA-OH yields two partially resolved peaks in h.p.l.c.. Enzymically produced CyHEA-OH co-elutes with the slow-eluting diastereomer, thus indicating the asymmetric nature of the allylic hydroxylation of CyHEA.

The absolute configuration of enzymically produced CyHEA-OH was assigned by making use of the elegant empirical model proposed by Dale and Mosher (Dale and Mosher, 1973). This model predicts absolute configuration of chiral alcohols by analysing the n.m.r. spectral data of *O*-methylmandelate esters of the alcohols. Trost and co-workers (Trost et al., 1986) expanded the applicability of this model using structurally diverse and complex chiral alcohols. In our experiments directed towards Mosher modelling, n.m.r. analysis established that *S*- α -methoxy-

Table 1 Substrate and inactivation kinetic parameters for non-conjugated olefinic and aromatic olefinic DBM substrates

Determined as described in the Experimental section. All values were determined in duplicate or triplicate at a single oxygen concentration (0.25 mM), using DBM of 30–50 units/mg specific activity. DBM tetramer of 290 000 Da was used in apparent k_{cat} calculations and all values were normalized to 21 units/mg for comparison. All k_{cat} , K_m , k_{inact} and K_i values are apparent values and obtained at atmospheric oxygen saturation (0.25 mM).

Compound	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	k_{inact} (min^{-1})	K_i (mM)	$k_{\text{cat}}/k_{\text{inact}}$
CyHEA	90.1	6.1	14.8	0.33	82.5	16380
Cis-HEA	13.0	6.5	2.0	—	—	—
Tr-HEA	+ *	—	—	—	—	—
AMP	15.0	13.3	1.13	0.05	19.0	18 000
2-PEA†	91	7.0	13.0	—	—	—
PAME†	14	6.7	2.1	0.23	4.7	3652
Tyramine	121	2.0	60.5	—	—	—

* Although a noticeable oxygen consumption was observed, the activity was too small for the evaluation of kinetic parameters.

† Values reported previously (Padgett et al., 1985), except that partition ratios listed here are based on the DBM tetramer of 290 000 Da. Partition ratios based on a monomer molecular mass of 73 000 Da are as follows: CyHEA, 4095 Da; 2-aminomethyl-1-pentene, 4500 Da; 3-amino-2-phenyl-1-propene, 913 Da.

Figure 2 Fumarate activation of CyHEA/DBM reaction

Standard DBM oxygen monitor assay conditions were used, with 0.1 mg/ml catalase, 10 mM ascorbate, 5 μg of copper sulphate and 2 μg /ml DBM. The reactions were initiated with CyHEA after incubating DBM with fumarate for 5 min. Fumarate activation for CyHEA ($\square$) and tyramine ($\triangle$).

	R_1	R_2	R_3	n_1	n_2
CyHEA	CH_2	H	CH_2	1	2
Cis-HEA	H	CH_3	H	2	0
Tr-HEA	CH_3	H	H	2	0
2-Aminomethyl-1-pentene	H	H	C_3H_7	0	0

Scheme 1

phenylacetic acid in the presence of DCC and DMAP resulted in acylation at both the amine and the alcohol functionalities of CyHEA-OH. Although amine derivatization was not required

Figure 3 Stereochemical course of the CyHEA/DBM reaction

H.p.l.c. chromatograms of (a) the SMPA-derivatized extract of DBM/CyHEA reaction carried out under standard enzymic conditions (see the Experimental section) using a mobile phase of 3:1 (v/v) hexane/ethyl acetate; (b) SMPA-derivatized, chemically synthesized (*R,S*) CyHEA-OH using the conditions of (a); and (c) a mixture of the sample of (b) with an equal aliquot of the sample of (a). The asterisk denotes the peak due to SMPA-derivatized CyHEA-OH.

for application of the Mosher model, it turned out to be beneficial for product separation by preparative t.l.c. H.p.l.c. analysis of the derivatives established that chemically synthesized CyHEA-OH produced two peaks whereas enzymically produced CyHEA-OH gave rise to only one peak in h.p.l.c. This indicates that acylation of CyHEA-OH occurred without racemization of either *S*- α -methoxyphenylacetic acid or CyHEA-OH. The derivatives of chemically synthesized and enzymically produced CyHEA-OH were separated by preparative t.l.c. and examined by n.m.r.; application of the Mosher model allowed us to assign the (*R*) configuration to enzymically produced CyHEA-OH (Sirimanee and May, 1988).

As a test of the Mosher modelling method, two model amino alcohols of known absolute configuration were subjected to the same treatment to predict the absolute configuration. In these experiments, (*R*)- and (*S*)-1-amino-2-propanols were separately derivatized under the same conditions with (*S*)- α -methoxy-

Figure 4 Kinetics of time-dependent inactivation of DBM by CyHEA

The standard dilution assay for inactivators was used with 17 $\mu\text{g}/\text{ml}$ of DBM and 10 mM ascorbate in the inactivation solutions. (a) Plot of percentage activity remaining versus time. CyHEA concentrations in the respective experiments were as follows: 0 mM, 5.96 mM, 15.97 mM, 20.02 mM and 23.83 mM. (b) Double-reciprocal plot of k_{obs} versus $[\text{CyHEA}]$ from which first-order rate constants were determined by linear regression analysis.

phenylacetic acid and the derivatives were isolated by preparative t.l.c. ^1H n.m.r. [*S*-isomer] (C^2HCl_3)(δ): 7.15–7.55 (m, 10H), 6.55–6.65 (t, 1H), 4.95–5.05 (m, 1H), 4.8 (s, 1H), 4.45 (s, 1H), 3.18–3.5 (m, 8H), 1.24 (d, 3H); ^1H n.m.r. [*R*-isomer] (C^2HCl_3)(δ): 7.27–7.5 (m, 10H), 6.9–7.0 (m, 1H), 5.0–5.14 (m, 1H), 4.75 (s, 1H), 4.6 (s, 1H), 3.25–3.57 (m, 8H), 1.06 (d, 3H). The chemical shift for the methyl group of the (*R*) isomer exhibited an up-field shift of 0.184 p.p.m. with respect to the (*S*) isomer, thus confirming that the prediction made by the Mosher model is in agreement with the known absolute configurations.

Acyclic olefinic amines

Among the other non-conjugated olefins examined in this study, (Table 1), *Cis*-HEA exhibited substrate activity with $K_m = 6.5$ mM and $k_{\text{cat}} = 13$ s^{-1} . The k_{cat} value is only about 14% of that of CyHEA while the K_m values are approximately the same. Product formation is dependent on the presence of ascorbate and active enzyme. Identification of the product from the *Cis*-HEA/DBM reaction was carried out by derivatizing the enzymic product with SMPA (Husain et al., 1989) and isolating the product by silica-gel preparative t.l.c. The t.l.c., n.m.r. and m.s. of the SMPA derivative confirmed that the only product formed is the allylic-hydroxylated derivative, 4-hydroxy-*cis*-2-hexenylamine. ^1H n.m.r. (C^2HCl_3)(δ): 7.2–7.4 (m, 5H), 7.0–7.1 (s, broad, 1H), 5.63–5.78 (m, 1H), 5.3–5.43 (m, 1H), 4.58 (s, 1H),

4.12–4.2 (m, 1H), 3.15–4.5 (m, 5H), 1.9–2.3 (m, 2H), 0.93 (t, 3H); mass spectrum (CI) m/e 264 ($M^+ + 1$).

The *trans* olefin, *Tr*-HEA was also examined as a DBM substrate. This compound exhibited only very weak substrate activity under standard oxygen monitor assay conditions; therefore, accurate kinetic parameters could not be obtained.

To examine how the olefinic geometry modulates the substrate activity of non-conjugated olefins, 2-aminomethyl-1-pentene, a structural isomer of *Cis*-HEA or *Tr*-HEA was also synthesized and examined. 2-Aminomethyl-1-pentene exhibited a substrate activity with DBM; $k_{\text{cat}} = 15.0$ s^{-1} , $K_m = 13.3$ mM. These values are comparable with those of *Cis*-HEA under standard conditions. In contrast, 2-aminomethyl-1-propene displayed no oxygen consumption when incubated in the presence of DBM and ascorbate under normal assay conditions.

It is known that addition of radicals to alkenes is favoured if the allylic position is highly substituted (Cadogan and Perkins, 1964). Therefore, we examined ACPP, in order to determine whether DBM would process this olefin to the corresponding epoxide. ACPP displayed neither substrate activity nor inhibitory activity when assayed under standard assay conditions, thus indicating the absence of any oxygenation by DBM. Similarly, *cis*-hex-2-ene, which lacks the terminal amino functionality, and hexylamine, which lacks double bonds, were both found to exhibit no substrate activity towards DBM under normal assay conditions.

Figure 5 Time course of oxygen uptake and inactivation rate in the absence and presence of DBM/CyHEA preformed products

The reaction mixture was exactly the same as the standard assay mixture described in the Experimental section. A 2.5 ml assay solution containing 40 mM CyHEA was incubated at 37 °C and oxygen consumption ($\square$) was measured using a standard dissolved oxygen electrode assay, after injecting 3 μ g of DBM. (a) After total inactivation of the enzyme, the system was resaturated with air, and an identical aliquot of DBM was added and the oxygen consumption ($\blacktriangle$) was monitored. (b) Conditions were exactly the same as that of above except copper was added before adding the second aliquot of DBM.

Inactivation by CyHEA

Inspection of the time course of enzymic turnover indicated that DBM undergoes turnover-dependent inactivation during the course of allylic hydroxylation of CyHEA. The kinetics of this inactivation reaction were examined using a dilution assay (see the Experimental section) and this reaction was found to follow pseudo-first-order kinetics (Figure 4). The kinetic constants obtained by linear regression analysis were $k_{\text{inact.}} = 0.33 \text{ min}^{-1}$ and $K_i = 82.5 \text{ mM}$. Although the $k_{\text{inact.}}$ of CyHEA is comparable with that of the prototypical olefinic mechanism-based DBM inactivator, 3-amino-2-phenyl-1-propene (Padgett et al., 1985), K_i is much larger than that of any of the mechanism-based inactivators known to date. Moreover, the partition ratio ($k_{\text{cat.}}/k_{\text{inact.}}$) is approximately 16000, a value 11 times greater than that for 3-amino-2-phenyl-1-propene.

To test whether the allylic-hydroxylated product of the CyHEA/DBM reaction can return to the active site and inactivate the enzyme independent of turnover, a 'double addition' experiment was performed, to allow examination of inactivation

in the presence of preformed enzymic product. For normal turnover-dependent irreversible inactivation, the time course of O_2 uptake and concomitant enzyme inactivation should be identical between a reaction mixture and the same reaction mixture when a fresh identical aliquot of DBM is added after 100 % enzyme inactivation and re-equilibration with air. However, it is evident that with CyHEA, the enzyme shows considerably diminished activity in the second cycle of such an experiment (Figure 5). This diminished activity was found to be a result of Cu^{2+} depletion, because, the addition of Cu^{2+} before the second cycle restores full enzyme activity and gives rise to an identical inactivation time course.

We examined the possibility that Cu^{2+} depletion in the previous experiment could be due to copper chelation by the enzymic product, CyHEA-OH. However, CyHEA-OH showed no DBM inhibitory activity whatsoever (reversible or irreversible). Cu^{2+} depletion might be expected from the reduction of the Cu^{2+} to $\text{Cu}(0)$ with concomitant formation of aminomethyl-(1-cyclohexenyl) ketone. However, no ketone was detected by h.p.l.c. analysis of the CyHEA/DBM enzymic reaction mixture with comparison with authentic ketone obtained by oxidizing CyHEA-OH with MnO_2 .

The acyclic olefinic amines, Cis-HEA or Tr-HEA, did not exhibit significant inactivation of DBM when assayed in the normal dilution assay for mechanism-based inactivators. In contrast, 2-aminomethyl-1-pentene was found to inactivate DBM in an ascorbate- and time-dependent manner. The kinetic constants for the inactivation are apparent $k_{\text{inact.}} = 0.05 \text{ min}^{-1}$ and $K_i = 19 \text{ mM}$ (Table 1). Interestingly, during the examination of the inactivation of DBM in the presence and absence of preformed products, the turnover of 2-aminomethyl-1-pentene was also found to be associated with depletion of Cu^{2+} similar to that observed with the turnover of CyHEA.

DISCUSSION

Previous work from this laboratory has established that the benzylic olefin substrate, 3-amino-2-phenyl-1-propene, and its derivatives undergo facile DBM-catalysed epoxidation, with the reaction proceeding via initial electron abstraction to give a substrate radical cation. We have further demonstrated that it is a species along this reaction pathway (and not the final epoxide product) which gives rise to turnover-dependent inactivation during processing of these benzylic olefin substrates (Padgett et al., 1985).

In examining the first non-conjugated olefinic DBM substrate, we wished to ascertain whether DBM is capable of effecting: (a) epoxidation of non-conjugated olefins; and (b) hydroxylation of the allylic carbon of non-conjugated olefins. In view of the physico-chemical similarity of allylic and benzylic systems, we expected that both allylic and benzylic hydroxylations should be equally facile. However, no prediction could be made regarding epoxidation of non-conjugated olefins since the redox properties of the activated copper-oxy species of DBM is unknown. The data presented in this paper establish (1) that the prototypical allylic substrate, CyHEA, indeed undergoes DBM-catalysed allylic hydroxylation with facility equal to benzylic hydroxylation (Table 1), (2) that the reaction proceeds via the normal reductive monooxygenation pathway of DBM catalysis, and (3) that no epoxide product is detectable. Moreover, DBM-catalysed oxygenation of CyHEA is stereospecific, producing CyHEA-OH product with absolute configuration (*R*). Thus, DBM-catalysed allylic oxygenation follows the same stereochemical course as the well-studied DBM-catalysed hydroxylation, sulphoxidation and ketonization of benzylic substrates (Winkler et al., 1970;

Battersby et al., 1976; May and Phillips, 1980; May et al., 1981; Mangold and Klinman, 1984).

Turning from CyHEA to acyclic, non-conjugated olefinic analogues, it is clear that such compounds exhibit diminished substrate activity toward DBM. Thus, the k_{cat} for Cis-HEA is only 14% of that for CyHEA. Similarly, k_{inact}/K_I for turnover-dependent inactivation by the acyclic olefin substrate 2-aminomethyl-1-pentene is also more than an order of magnitude smaller than that for benzylic olefins. As is the case for CyHEA, our results establish that DBM-catalysed oxygenation of Cis-HEA produces the corresponding allylic alcohol product rather than the corresponding epoxide. Since radical addition reactions to olefins are favoured if the allylic position is highly substituted (Cadogan and Perkins, 1964), we also examined ACP, which possesses a branched allylic carbon, in an attempt to observe DBM-catalysed epoxidation. However, we observed no enzymic activity toward this olefin.

While our results demonstrate that DBM catalyses allylic oxygenation of a number of non-conjugated olefinic substrate analogues, none of the compounds we examined undergoes DBM-catalysed epoxidation. We have previously presented evidence that DBM oxygenation of benzylic olefinic and heteroatom functionalities proceeds via initial electron abstraction to give a substrate radical cation (Padgett et al., 1985; Wimalasena and May, 1987). Thus, the absence of epoxide products from non-conjugated olefinic substrates implies an inability of the activated copper-oxygen species of DBM to effect radical cation formation from a non-conjugated olefinic moiety. This is consistent with the well-known difference in oxidation potential and reactivity between conjugated and non-conjugated double bonds. For example, styrene has an ionization potential of 8.47 eV and an E_2 of 1.68 V while propene has an ionization potential of 9.73 eV and E_2 of 2.84 V (Weast, 1983).

Groves and co-workers (Groves and Subramanian, 1984) found that cytochrome *P*-450 catalyses both epoxidation and allylic hydroxylation of cyclohexene, and they propose that epoxidation by *P*-450 proceeds via an olefinic cation radical while allylic oxygenation proceeds via an allylic radical. Moreover, they observed that allylic hydroxylation of cyclohexene is accompanied by allylic rearrangement, and that methylenecyclohexene gives rise to both 2-methylenecyclohexanol and 1-cyclohexenylmethanol. In contrast, we find that allylic hydroxylation of non-conjugated olefinic substrates by DBM proceeds without rearrangement. These results may be suggestive of a mechanism in which copper interacts with the olefin moiety during the catalysis, thus precluding rearrangement of the double bond. Such copper-olefin interactions have been proposed to account for the lack of allylic rearrangement in oxidations of olefins with peroxy compounds in the presence of Cu^{2+} ions (Denney et al., 1959; Kharasch and Fono, 1959; Cross and Whitham, 1961).

Results of the present study establish that CyHEA is a turnover-dependent irreversible inactivator of DBM. While the k_{inact} of 0.33 min^{-1} is comparable with that of the prototypical benzylic olefin inactivator, 3-amino-2-phenyl-1-propene, k_{inact}/K_I for CyHEA is an order of magnitude smaller and a high partition ratio is observed. Although both turnover and inactivation by CyHEA exhibit normal kinetics (i.e. pseudo-first-order inactivation in dilution assays), it is evident from the double-addition experiments that CyHEA turnover results in gradual depletion of copper from DBM. However, it should be noted that turnover-dependent, irreversible (i.e. not reversible by prolonged dialysis) DBM inactivation occurs independent of copper depletion provided that sufficient copper is present in the assay solution. A likely mechanism for this turnover-dependent

DBM inactivation by CyHEA, analogous to that proposed for benzylic inactivators (Goodhart et al., 1987), involves initial abstraction of an allylic hydrogen atom from CyHEA by the activated copper-oxy species to form a resonance-stabilized allylic radical. The resultant allylic radical can then either partition to product or undergo attack by an active-site residue during enzyme inactivation.

In an attempt to elucidate the mechanism of copper depletion, control experiments eliminated the possibility that copper depletion could be due to chelation by the enzymic product, CyHEA-OH. One possibility which we can visualize to account for copper depletion is that the copper alkoxy species produced after C-O bond formation at the allylic carbon partitions between hydrolysis to release the allyl alcohol product on the one hand, and breakdown to give (1-cyclohexenyl)aminomethyl ketone with concomitant formation of $\text{Cu}(0)$ on the other. Since formation of $\text{Cu}(0)$ and ketone by such partitioning are stoichiometric, only nanomolar amounts of ketone would be formed, thus accounting for our inability to detect ketone product by h.p.l.c. We note that there is chemical precedence for such a reaction, leading to the reduction of the metal ion with concomitant formation of ketone, in oxidations with manganese porphyrins (Fontecave and Mansuy, 1984), oxochromium(VI) and oxovanadium(V) (Sheldon and Kochi, 1981).

The striking contrast between cytochrome *P*-450 and DBM in catalysing oxygenation of non-conjugated olefinic substrates is probably a reflection of the differences in redox potential of the activated oxygen species operative for these two enzymes. The ability of *P*-450 to carry out both epoxidation and allylic oxidation indicates that the *P*-450-activated oxoiron(V) species is capable of both initial electron abstraction from the double bond and initial allylic hydrogen abstraction (Groves and Subramanian, 1984), whereas the absence of olefin cation radical formation and hence epoxide products in the DBM/non-conjugated olefin reaction may be ascribed to the lower redox potential of the DBM-activated 'copper-oxy' species. Direct chemical precedence for such a reactivity difference exists. It is well known that high-valent oxo-metal compounds of high oxidation potential [e.g. $\text{O} = \text{Cr}(\text{VI})$] react with olefins to give epoxidation products as well as allylic oxidation products, while oxidants of lower oxidation potential [e.g. $\text{O} = \text{Se}(\text{IV})$] give rise to only allylic oxidation products (Sheldon and Kochi, 1981).

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