Stereochemistry of Copper Amine Oxidase Reactions*

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The stereochemical course of the oxidation of stereospecifically deuterated dopamine and tyramine, catalyzed by porcine plasma amine oxidase, has been investigated using 'H NMR spectroscopy. The oxidation proceeds with loss of the pro-R hydrogen at C-1. This stereochemistry is in contrast to that observed with the analogous copper containing oxidases isolated from pea seedlings (pro-S) and bovine plasma (nonstereospecific). There is no precedent for these three distinct stereochemical reaction courses to be followed by enzymes in the same class. Mechanistic differences among the three enzymes are evident from the profiles of solvent exchange into reaction products; however, these differences cannot account for the overall differential stereochemical courses observed.

Two classes of enzymes catalyze the oxidative deamination of primary amines (Equation 1); the flavin-containing monoamine oxidases (EC 1.4.3.4) and the copper-containing amine oxidases (EC 1.4.3.6) (1).

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\text{RCHNH}_2 + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}
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The established physiological role of the monoamine oxidases in neurotransmitter metabolism has made these enzymes the subject of intensive investigation, notably as targets for antidepressant drugs. Both the biological function and role of copper in the latter enzymes is unclear, and they contain a different organic cofactor, believed to be pyrroloquinoline quinone in bovine and porcine plasma amine oxidase, porcine kidney diamine oxidase and l-lysyl oxidase (2-6). The two enzyme types are also mechanistically distinct as solvent exchange at C-2 of dopamine during catalysis has been observed only with the copper enzymes (7).

Previous stereochemical studies on copper amine oxidases have shown that the pro-S hydrogen is lost from C-1 of benzylamine and dopamine when oxidized by the enzyme isolated from pea seedlings (8, 9). Bovine plasma amine oxidase also catalyzes abstraction of the pro-S hydrogen from benzylamine and p-hydroxybenzylamine (10, 11). The bovine enzyme, however, exhibits mirror-image binding and processing of dopamine, resulting in nonstereospecific proton abstraction at C-1 and C-2 (12, 13). In this paper we apply 'H NMR spectroscopy to confirm and extend the stereochemistries deduced previously for the bovine and pea seedling enzymes using radiochemical labeling techniques. In addition, we establish that amine oxidation by a third copper enzyme, porcine plasma amine oxidase, occurs with removal of the pro-R hydrogen from C-1 of substrate. This represents the first instance where all possible stereochemical reaction courses are followed by enzymes in the same formal class.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemicals used were of reagent grade and were used without further purification. NADH, pyridoxal phosphate, tyrosine, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenethylamine, tyramine, and benzylamine were from Sigma. Cyclohexanol was obtained from American Chemicals Ltd. and 'H_2O (99.8% deuterium) from Aldrich. L-[U-'^14C]Tyrosine (50 mCi/nmol) was from ICN and ACS liquid scintillation mixture was from Amersham Corp. All solvents were reagent grade and were freshly distilled before use.

**Enzymes**—Porcine plasma amine oxidase was isolated from 15 liters of fresh citrated porcine blood as previously described (14). Isolation steps included (NH_4)_2SO_4 fractionation and sequential column chromatography on DE-52 cellulose, ConA-Sepharose, Bio-Gel A-1.5m, and hydroxypatite. The final specific activity of the isolated enzyme was 0.057 units/mg, when assayed spectrophotometrically at 250 nm, using 3.3 mM benzylamine as a substrate and an extinction coefficient of 11.3 mM^-1 cm^-1. Protein concentration was estimated using a Bio-Rad kit which is based on the Bradford method (15) with bovine serum albumin as a reference standard protein. pea seedling (Pisum sativum, cv Laxton’s Progress) amine oxidase was isolated from 500 g of 9-day-old etiolated seedlings. Isolation steps included protamine sulfate treatment, (NH_4)_2SO_4 fractionation, and chromatography on phosphocellulose (16). The final specific activity was 0.051 units/mg when assayed as above. Tyrosine decarboxylase apoenzyme, horse liver alcohol dehydrogenase, catalase, and bovine plasma amine oxidase were obtained from Sigma.

**Synthesis of DL-2-'H-Labeled Amino Acids—**3'-Methoxytyrosine was reacted with KOH in H_2O, in the presence of pyridoxal hydrochloride, using the general procedure described by Fujibara and Schoen (17) for 'H-deuteriation of amino acids. The product 3'-methoxy-dl-[2-'H]tyrosine was obtained as a solid after neutralization of the reaction mixture and was recrystallized from hot water by addition of ethanol. DL-[2-'H]Tyrosine and DL-[2-'H]phenylalanine were similarly prepared. The isolated yields were in the 20-30% range and 'H NMR spectroscopy showed over 95% deuterium incorporation into C-2 of all 3 amino acids. Treatment of 3'-methoxy-dl-[2-'H]tyrosine with HBr (18) gave DL-[2-'H]h dopa.

**Synthesis of Chiral Amines—**Tyrosine decarboxylase apoenzyme was extracted from 150 mg (90 units) of Streptococcus faecalis cells into 20 ml of 50 mM sodium acetate buffer, pH 5.7, containing 0.45 mM pyridoxal phosphate by grinding with alumina or pulsed sonication for 8 min. Following centrifugation, the supernatant was concentrated to 1 ml by ultrafiltration, resuspended in 80 ml of 50 mM sodium acetate buffer, pH 5.7, and concentrated to 1 ml to remove excess pyridoxal phosphate. This was reconstituted in 80 ml of buffer and reconcentrated to 5 ml. Extracted enzyme was assayed by measuring 14CO_2 evolution from radiolabeled tyrosine. Incubation mixtures contained 1.3 mM L-tyrosine, 0.16 mM L-[U-'^14C]tyrosine, 0.45 mM pyridoxal phosphate, and 5 uI of extract in 0.5 ml of 50 mM sodium acetate buffer, pH 5.7. Reactions were carried out in scintillation vials with a piece of filter paper impregnated with 20 uI of 1 M NaOH placed over the vial lids. Following incubation at 37 °C for 10 min, mixtures were quenched with the addition of 0.2 ml of 10% trichloroacetic acid, and shaken for 1 h to ensure that evolution of 14CO_2 was complete. The filter papers were removed and counted in 10 ml of ACS mixture. The enzyme was extracted at 20-30% yield and had a specific activity of 1-5 units/mg.

For (1S)-['H]amine synthesis, incubations contained 50 mg of the...
appropriate DL-[2-2H]dopamin acid and 5 units of tyrosine decarboxylase extract in 10 ml of 50 mM sodium acetate buffer, pH 5.7. After a 24-h reaction at ambient temperature, the mixture was applied to a column (2 x 5 cm) of Amberlite IRC 50 (H+ form) equilibrated with water. Unreacted amino acid was eluted with water, then amine eluted with 1 M acetic acid. Fractions were pooled and evaporated to dryness under reduced pressure, after the addition of 0.2 n HCl. (1R)-[2H]Amines were synthesized in an analogous fashion starting with unlabeled amino acids. In this case incubations were carried out in 10 ml of 50 mM sodium acetate buffer, pH = 6.0, where pH D is the incorrect pH meter reading.

**Stereochemistry of Amine Oxidase Reactions**—Incubations were carried out in a coupled fashion (7, 11) and contained in 1.0 ml: 15 µmol of steresspecifically deuterated amine, 0.3 units of amine oxidase, 11,000 units of catalase, 3 units of horse liver alcohol dehydrogenase, 1.1 µmol of NADH, 15 µmol of cyclohexanol, and 100 µmol of sodium or potassium phosphate buffer, pH 7.6. Following incubation for 16 h at ambient temperature, samples were diluted to 10 ml with water and loaded onto reverse-phase C8 Sep-Pak cartridges. The cartridges were rinsed with 5 ml of water, then the alcohol product was eluted with 10 ml of methanol. The methanol eluates were concentrated by evaporation under reduced pressure, then applied to preparative silica gel tic plates developed with ethyl acetate/ hexane (2:1) and 15 pmol of unlabeled dopamine or tyramine. All stereochemical studies were done at least in duplicate.

**Solvent Exchange into Product**—To monitor solvent exchange into alcohols, incubations were carried out as described for the stereochemical studies with chiral amines, except reactions were carried out in 100 mM deuterated sodium or potassium phosphate buffer, pH 6.9, containing 15 µmol of unlabeled dopamine or tyramine.

**Solvent Exchange into Dopamine**—Incubations were carried out as for the solvent exchange into alcohols, in 100 mM deuterated sodium or potassium phosphate buffer, pH 6.9, for 4 h. The extent of reaction was estimated by separation of starting dopamine from product 3,4-dihydroxyphenethyl alcohol on ion exchange Accell CM cartridges and quantitation by measuring absorbance at 280 nm.

**1H NMR Spectroscopy**—*H NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument operating at ambient temperature (22 ± 1°C). Spectra of amine hydrochloride salts were recorded in 1H09 with the residual 1H0 signal (4.81 ppm) used as an internal reference standard. Spectra of alcohols were recorded using 4:1 (H2O):C12H2O as solvent with the residual H2O signal (2.19 ppm) as internal reference standard. The absolute chemical shifts using these solvent peaks as reference standards were reproducible to better than 0.05 ppm. Spectra were accumulated into 16 K of computer memory using a 42.1 pulse with 7-s delays between pulses. Longer relaxation delays did not measurably affect the relative intensities of the signals as measured by integration.

**Results and Discussion**

Tyrosine decarboxylase, a pyridoxal phosphate containing enzyme, was used to synthesize stereospecifically deuterated amines. Reactions catalyzed by this enzyme have been well studied, and the enzyme has been shown to operate stereospecifically such that the replacement of the carboxyl group with a solvent proton occurs with retention of configuration (19). Since the enzyme decarboxylates only L-amino acids, unresolved mixtures of O-deuterated amino acids can be used as starting materials. In addition, tyrosine decarboxylase exhibits broad substrate specificity and can be employed for the synthesis of stereospecifically labeled dopamine and phenethylamine as well as tyramine.

Fig. 1 shows the *H NMR spectra of stereospecifically deuterated dopamines isolated after incubation of L-dopa in *H0 and DL-[2-2H]dopa in *H0, respectively, with tyrosine decarboxylase. The deuteration can be seen to be complete within the limits of sensitivity of the *H NMR spectra (≥98%) and the absolute configuration of the chiral dopamines are assigned based on the established stereospecificity of the tyrosine decarboxylase reaction. Caution must be exercised in prolonged incubations of aromatic amino acids with excess pyridoxal phosphate added as an enzyme cofactor, since non-enzymatic exchange of the protons by the cofactor alone can result in scrambling in the final amine products.

Incubations of the chiral amines with the three amine oxidases were carried out in a coupled fashion, such that the aldehyde products generated were converted directly into alcohols (Scheme 1). The *H NMR spectra of 3,4-dihydroxyphenethyl alcohol isolated from incubations with (1R)-[2H]dopamine with porcine, pea seedling, and bovine plasma amine oxidase are shown in Fig. 2. The alcohol obtained from incubation with porcine plasma amine oxidase (Fig. 2a) exhibits triplets at 3.73 ppm (C-1 proton signals) and at 2.74 ppm (C-2 proton signals), a pattern requiring full protonation at both positions. Loss of deuterium from (1R)-[2H]dopamine during reaction thus establishes that dopamine oxidation occurs with loss of the pro-R hydrogen (or deuterium in the (R)-labeled compound), at C-1 during oxidation. The alcohol sample isolated from an incubation containing pea seedling enzyme and (1R)-dopamine contained deuterium (Fig. 2b), confirming the pro-S hydrogen abstraction previously reported for this enzyme (7, 8). The clean doublet at 2.72 ppm attests to the complete retention of deuterium in this alcohol sample. We attribute the large downfield signal near 3.73 ppm to an impurity in the incubation mixture. The 3,4-dihydroxyphenethyl alcohol isolated from incubations with the bovine amine oxidase, yields a more complex spectrum (Fig. 2c). The complex pattern near 3.7 ppm is attributed to two overlapping triplets for C-1. The minor triplet at 3.71 ppm arises from 3,4-dihydroxyphenethyl alcohol, mono-
deuterated at C-1, which is approximately 0.02 ppm shifted to higher field due to a $\alpha$-deuterium isotope effect (20). The corresponding signals for the C-2 protons are seen at 2.74 ppm (triplet) for the minor diprotio-alcohol and an upfield $\beta$-deuterium isotope shifted doublet at 2.72 ppm for the major monodeutero product. The relative intensities of these NMR signals indicates that 85% of the original deuterium of $(R)$-dopamine is retained.

In complementary experiments with $(S)$-[2H]dopamine, deuterium was retained (>95%) in the 3,4-dihydroxyphenethyl alcohol isolated from incubations with bovine plasma amine oxidase, as predicted for a reaction with abstraction of the pro-$R$ hydrogen (Fig. 2d). Samples derived from reaction of pea seedling amine oxidase were devoid of deuterium (Fig. 2e), establishing the loss of the pro-$S$ hydrogen during catalysis. The alcohols obtained from incubations with bovine amine oxidase again show a complex spectrum (Fig. 2f), overlapping triplets near 3.7 ppm, and a doublet superimposed on a triplet near 2.7 ppm. These signals are assigned as for $(R)$-dopamine, above, and their relative intensities indicate that 94% of the deuterium was retained, again demonstrating a partially nonstereospecific reaction course. For a completely
Stereochemistry of Amine Oxidases

nonstereospecific reaction, one would predict a 1:1 mixture of monodeuterated and fully protonated alcohols. Bovine plasma amine oxidase, however, is known to exhibit a deuterium isotope effect of 6 for dopamine oxidation (18), resulting in the retention of a preponderance of deuterium in both alcohols derived from chiral substrates. Our results are consistent with the existence of dual catalytically competent binding modes for dopamine in the active site of bovine plasma amine oxidase as proposed in previous stereochemical studies (12, 13). We account for deviations from the =85:15 ratio predicted for a kinetic isotope effect of 6, to unequal flux through the two binding modes, with preferential processing of dopamine through the R mode, i.e. abstraction of the pro-R proton. This in turn is proposed to arise from different overall primary kinetic isotope effects in each mode, 6 for R substrates and 16 for S-substrates. Since the $K_{m}$ for dopamine is 40 μM (18), the reaction changes from saturating $V$ conditions to $V/K$ control as substrates become depleted in our stereochemical study. Any isotope effects estimated from our final product ratios will reflect isotope effects on both of these catalytic parameters. Our product ratios are, however, in general agreement with the predicted ratios of 75:25 for (R)-dopamine and 90:10 for (S)-dopamine extrapolated from primary tritium $V/K$ isotope effects (12, 13).

In order to further probe the unusual lack of overall stereospecificity in the bovine plasma amine oxidase reaction, the stereochemical course of the oxidation of tyramine was also investigated. Fig. 3a shows the spectrum for $p$-hydroxyphenethyl alcohol derived from (1R)-[3H]tyramine and bovine plasma amine oxidase. The retention of 82% deuterium (and loss of 18%), establishes a nonstereospecific reaction course for tyramine. This is confirmed in Fig. 3b, for the alcohols derived from the 1S isomer, which contained a mixture of 87% deuterated and 13% fully protonated molecules. Thus tyramine is also subject to mirror-image binding and processing by bovine plasma amine oxidase. The average measured primary deuterium isotope effect for tyramine oxidation of 6 (18), accounts for the net retention of deuterium in both samples. Tyramine has been shown to exhibit biphasic steady-state plots, yielding $K_{m}$ values of 1.3 and 52 mM. Our stereochemical experiments are under $V/K$ control and likely not as sensitive to differential isotope effects as the reaction progresses. As with dopamine, the increased flux through the R mode can be attributed to different isotope effects within each mode, however, the values of the isotope effects estimated from the present study are 4.6 for the R and 6.7 for the S mode. The chiral purity of the tyramine samples in this study was confirmed by incubation with porcine plasma and pea seedling amine oxidases. For both isomers, complete loss of the pro-R and pro-S hydrogens, respectively, was observed (data not shown), as with the labeled dopamines.

Temperature-dependent enantioselectivity in an alcohol dehydrogenase reaction has been recently demonstrated (21), suggesting that temperature can be a critical variable in asymmetric enzyme reactions. The effect of temperature on tyramine oxidation was examined by conducting a stereo-
chemical study with bovine plasma amine oxidase at 4 °C. The spectra of product p-hydroxyphenethyl alcohols obtained from parallel incubations with the bovine plasma enzyme are shown in Fig. 3. The alcohols derived from (1R)-tyramine were mixtures of 80% deuterated and 20% protonated species (Fig. 3c), while those from (1S)-tyramine were 93% deuterated (Fig. 3d). These results suggest isotope effects of 4 and 13 for the R and S modes, respectively, with a small increase in the flux through the R mode as the temperature decreases.

To our knowledge, there is no precedence for all possible stereochemical reaction courses to occur within a single class of enzyme acting on the same substrate. The closest example is that of maltase decarboxylase, acetolactate decarboxylase, and acetocacetate decarboxylase which have been shown to catalyze the decarboxylation of α-keto acids with net retention, inversion, and racemization, respectively (22). In this instance, while the chemical transformation is the same, the substrates, acetolactate, malate, and acetocacetate are different. The decarboxylases are further classified differentially by enzyme classification numbers.

Bovine plasma amine oxidase clearly exhibits two different binding modes, such that a catalytic base on the enzyme can abstract a proton from C-1 of substrate with absolute and opposite stereospecificity within each mode. While it is tempting to speculate that the porcine and pea seedling enzymes have evolved such that only one of the two binding modes has been conserved in each enzyme, this cannot be confirmed in the absence of genetic and structural information on the enzymes. It is possible, however, to use additional chemical probes to establish mechanistic inter-relationships for the three enzymes. Lovenberg and Beaven (7) showed that deamination of C-2 tritiated dopamine by bovine plasma amine oxidase occurred with release of label into solvent, while reaction with the flavin-containing monoamine oxidase resulted in retention of label in the deaminated product. This was ascribed to the reversible formation of an enamine off the main enzyme reaction pathway, Scheme 2. Thus, solvent exchange at C-2 provides a mechanistic distinction between the copper and flavin enzymes.

The solvent-exchange characteristics of the amine oxidase reactions catalyzed by the porcine, bovine, and pea seedling enzymes were compared by conducting the reactions using fully protonated dopamines in 2H2O. The 1H NMR spectra of the alcohols obtained from these reactions are presented in Fig. 4. Both the porcine and bovine enzymes catalyze incorporation of deuterium into C-2, but to different extents, 1.2 and 0.8 deuteriums, respectively (Fig. 4). The wash-in experiment was carried out in triplicate for the porcine enzyme and in each case 1.2 ± 0.05 atoms of deuterium were observed in the isolated products. Wash-in catalyzed by the bovine enzyme was more variable, duplicate experiments with dopamine showed 0.8 and 1.1 atoms of deuterium in product, while duplicates for tyramine both gave alcohols with 0.8 ± 0.05 atoms of deuterium. Deuterium incorporation at C-2 in the dopamine reaction catalyzed by the bovine enzyme has been shown to occur stereospecifically (13). Our general observation of the incorporation of less than one deuterium into product would be consistent with some nonstereospecificity during wash-in, and current studies in this laboratory are addressing the basis for this variability. No deuterium was detected in the alcohol obtained from the incubations with pea seedling amine oxidase and dopamine (Fig. 4b). The extent of reversibility of the wash-in step was further probed by reisolating amines from 2H2O incubations; in no case was deuterium found in the reisolated amines.

Incubations with unlabeled tyramine and pea seedling enzyme in 2H2O yielded alcohols which were 80% deuterated at C-1 (Fig. 4d). The degree of deuterium incorporation at C-1 was variable and ranged from 0 to 90%, for identical incubations with different enzyme preparations. This wash-in is attributed to solvent exchange into either the NADH or the cyclohexanol used in the coupling reaction with one batch of pea seedling enzyme which were used only in the solvent exchange experiments. The reasons for this variation in pea seedling enzyme preparations are unclear at present, however, in no instance was there any detectable deuterium incorporated at C-2.

From the 2H2O experiments, clear distinctions can be seen to exist between the three copper enzymes, with respect to substrate specificity, position, and extent of deuterium incorporation into products. The differential incorporation of label into 3,4-dihydroxyphenethyl alcohols seen for bovine and porcine amine oxidases arises from different ratios in the relative rates of hydrolysis and enamine formation. Although the pea seedling enzyme resembles the flavin-containing monoamine oxidase which lack the enamine exchange pathway, the spectral properties and presence of pyrroloquinoline quinone (23) preclude flavin as the cofactor in this enzyme.

Rapid-scanning stopped-flow studies are in progress to confirm the presence of pyrroloquinoline quinone in the porcine and pea seedling amine oxidases. It is also of interest to establish the stereochemical relationship between proton abstraction at C-1 and protonation at C-2 for the porcine enzyme by configurational analysis of the course of deuterium wash-in. Both processes are catalyzed by a single base in bovine plasma amine oxidase (13), and such work could support arguments for the conservation of a single catalytic group in the bovine and porcine plasma enzymes.

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