Kinetics and Stereospecificity of the Lysyl Oxidase Reaction*

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The structural specificity of amine oxidation by lysyl oxidase was investigated using kinetic and NMR spectroscopic analyses. Substrate efficiency increased with increasing molecular distance from the α-carbon of the aromatic moiety substituted on the aliphatic chains of a series of primary amines. The p-hydroxyl substituent of p-hydroxybenzylamine significantly increased kcat over that of benzylamine, whereas this was not the case when tyramine and phenethylamine were compared. Direct spectrophotometric measurement of p-hydroxybenzaldehyde formation yielded burst kinetics, the second, slower phase of which was eliminated under anaerobic conditions. Thus, enzyme reoxidation is the more rate-limiting of the two half-reactions catalyzed by this substrate by this ping-pong enzyme. 31P NMR spectroscopy of the alcohol reductively derived from the aldehyde product of the lysyl oxidase-catalyzed oxidation of deuterated tyramine indicated that the pro-S but not the pro-R α-deuteron was catalytically abstracted. Moreover, lysyl oxidase catalyzed solvent exchange of protons at the C-2 position. Such stereospecificity and proton exchange uniquely differentiates lysyl oxidase from all but an aortic semicarbazide-sensitive amine oxidase among the pro-S-specific copper-dependent amine oxidases analyzed thus far.

The copper-containing amine oxidases catalyze the oxidative deamination of mono-, di-, and polyamines forming stoichiometric amounts of the corresponding aldehydes, hydrogen peroxide, and ammonia in the presence of molecular oxygen as electron acceptor, as shown below.

\[ \text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{H}_2\text{O}_2 + \text{NH}_3 \]  

(Eq. 1)

The presence of copper(II) is essential to enzyme activity, as is that of a carbonyl cofactor (1). Although pyrroloquinoline quinone had been implicated as the cofactor of bovine plasma amine oxidase (2, 3), Janes et al. (4, 5) have shown that the quinone of 2,4,5-trihydroxyphenylalanine (topa quinone)\(^1\) is the carbonyl cofactor in several copper amine oxidases, including the bovine, porcine and sheep plasma, porcine kidney, and pea seedling enzymes. This residue could function as the carbonyl cofactor upon its oxidation to the para- or ortho-quinone tautomer. They also established a consensus sequence of Asn-topa-Asp/Glu. Uncertainties remain about the chemical identity of the carbonyl cofactor in lysyl oxidase.

Although the substrate specificities, inhibitor sensitivities, and immunological reactivities of the copper amine oxidases appear to differ, these enzymes do exhibit common mechanistic features (6). Thus, each catalyzes α-proton abstraction prior to substrate oxidation (7, 8). Evidence for the generation of a substrate-derived α-carbanion has been seen in the reductive half-reaction catalyzed by lysyl oxidase (9), and an active site histidine residue has been implicated as the general base which could function in α-proton removal in this enzyme (10). α-Proton abstraction has also been documented as a catalytic step in the reaction mechanism of plasma amine oxidase (11) as well as other amine oxidases (11, 12). Moreover, it has been shown that α-proton abstraction is stereospecific, occurring with abstraction of the pro-S hydrogen from tyramine by the amine oxidases of pea seedling, soybean, chick pea, and pig kidney (13, 14). The amine oxidases of bovine, sheep, and rabbit plasma appear to be nonstereospecific in this regard (11–13). Porcine plasma amine oxidase is the only enzyme among those studied which is pro-R-stereospecific (15). The enzymes with pro-R or with nonstereospecific behavior also catalyze solvent exchange of substrate protons at the C-2 position of tyramine or dopamine, contrary to the trend observed in the pro-S-specific enzymes (11, 13, 14). In the present report, we describe the structural specificity of lysyl oxidase for arylalkylamine substrates. The ability of this enzyme to utilize such chromophoric amines is exploited to assess the relative rates of the two half-reactions of this ping-pong enzyme. Using stereospecifically deuterated tyramine, lysyl oxidase exhibited pro-S stereospecificity for α-proton abstraction and catalyzed solvent exchange of protons at the C-2 position. These properties are discussed in relationship to those of other pro-S amine oxidases.

EXPERIMENTAL PROCEDURES

Materials—The hydrochloride salts of tyramine, benzylamine, phenethylamine, and phenylbutylamine were purchased from Aldrich. p-Hydroxybenzylamine was synthesized as described elsewhere (16). Homovanillic acid, n-hexylamine, horseradish peroxidase, liver alcohol dehydrogenase, glucose oxidase, catalase, and tyrosine decarboxylase (Streptococcus faecalis) were obtained from Sigma. All chemicals were of reagent grade unless otherwise specified, and all protonated solvents were distilled before use. Deuterated solvents (99.95% \(^5\)H) and (2.2,4,6,8-\(^2\)H)tyramine HCl (98 atom % \(^3\)H) were obtained from Merck Sharp and Dohme (99.35% \(^3\)H) or General Intermediate of Canada (99.9%). Reverse osmosis water that was passed through anion and cation exchangers, carbon, and 0.45-μm cartridges (Milli-Q) was used for all chemical solutions and buffers. Thin layer chromatography plates (Kieselgel 60 F254, 0.2 mm) were from E. Merck, Darmstadt, Germany.

Enzyme Purification and Assay—Lysyl oxidase was purified to apparent homogeneity from bovine aorta as described previously (17). Purity was indicated by the presence of a single band migrating at a...
position equivalent to 32,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). The purified preparations of lysyl oxidase consist of copurified mixtures of four ionic variants (17, 19), each of which has a molecular weight of 32,000 in sodium dodecyl sulfate. These variants exhibit a high degree of structural similarity and appear to use the same catalytic mechanism (12, 20).

Lysyl oxidase was assayed against the bovine endothelial elastin substrate prepared from chick embryo aorta which had been pulsed in organ culture with L-[4,5,3H]lysine, as described (21, 22). Assay enzymes included 125,000 cpm of the elastin substrate in 0.1 M sodium borate, 0.15 M sodium chloride at pH 8.0 in a total volume of 750 ml and were incubated for 2 h at 37°C. Trisodium water formed during the incubation was isolated by vacuum distillation and quantified by liquid scintillation spectrometry of the distillates. All activities were corrected for enzyme-free controls and were within the linear range of this assay (100-1200 cpm released per 2 h). One enzyme unit was defined as 1 cpm (30% counting efficiency) of 3H released by enzyme action in 2 h. Specific activities of the purified enzyme preparations used here varied from 0.4 to 1.13 x 106 units mg⁻¹. Functional active site content was quantified by comparing the specific activity of each enzyme preparation against the elastin substrate to the theoretical maximum value of 4 x 106 units mg⁻¹ previously estimated as the value for the fully functional enzyme (25). The enzymes were assayed against n-hexanolamine as a substrate by a peroxidase-coupled fluorescence method (23, 24). Assay mixtures contained 0.25 mg of sodium homovanillate, 40 μg of horseradish peroxidase, 1.2 M urea, 5.0 mM hexamethylenamine in 0.05 M sodium borate, pH 8.2, in a final volume of 2.0 ml. Lysyl oxidase (2-4 μg) was added to initiate the reaction and initial rates of product formation were monitored at a constant temperature of 50°C by spectrophotometry at excitation and emission wavelengths of 315 and 425 nm, respectively. The enzyme-dependent production of hydrogen peroxide was quantified by reference to standard plots relating nanomoles of hydrogen peroxide to fluorescence units. Error ranges for all assay data, including those used to express kinetic isotope effects, were determined according to Mendenhall (25).

Pre-steady state and steady state kinetics of p-hydroxybenzylamine oxidation by lysyl oxidase was assessed at 37°C using a Hewlett-Packard diode array spectrophotometer. Lysyl oxidase (32 μg, 1.13 x 106 units mg⁻¹ specific activity) was quickly added to an assay solution of 4.0 mM p-hydroxybenzylamine, 1.2 M urea, and 16 mM potassium phosphate buffer, pH 7.8, which had been preincubated at 37°C. The production of p-hydroxybenzylaldehyde was monitored by the change in absorbance at 332 nm, using 22,000 m⁻¹ cm⁻¹ as the molar extinction coefficient. This extinction coefficient was experimentally determined in a study with solutions of authentic p-hydroxybenzylaldehyde under the assay conditions described.

Anaerobic assays were carried out with the buffered substrate, 20 units of glucose oxidase, 25 units of catalase, and 10 mM glucose contained in the main chamber and the aliquot of enzyme in the side arm of the reaction cuvette. The cuvette was then sealed, evacuated, and successively evacuated and flushed with pure nitrogen for 20 min. further allowing the glucose oxidase system to consume residual oxygen. Catalysis was initiated by inverting the cuvette to mix the enzyme and substrate solutions and replacing the cuvettes in the light path of the instrument within 5 s.

'H NMR Spectroscopy—'H NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument at ambient temperature (22°C). The spectra of p-hydroxyphenethyl alcoholwere obtained in (CD₃)₂CO:2H₂O (4:1), with the residual HZH₂C signal set at 2.19 ppm as an internal reference standard. Spectra of p-hydroxyphenethyl alcohols isolated from these experiments were esterified with S-(+)-O-acetylmandelic acid to establish the position of proton or deuteron wash-in at C-5. Experiments to determine the stereoelectronic course of proton abstraction from C-1 of tyramine was established using (1R)-[2-'H] or (1S)-[3H]tyramine as substrates. Incubations to determine solvent exchange characteristics at C-2 of tyramine were carried out with [2,2-'H]tyramine in a protonated buffer and with [2-'H]tyramine in a deuterated buffer. In the latter case, lysyl oxidase was also exchanged into deuterated buffer. The corresponding (1R)-['H]- and (1S)-['H]-tyramine substrates—Two units of bovine plasma amine oxidase were incubated in 6 ml of 30 mM phosphate buffer, pH 7.6, containing 60 μmol of [2,2-'H]-tyramine or in deuterated buffer, pD 7.1, with 60 μmol of [2,2-'H]-tyramine for the production of (2S)-['H]- and (2R)-['H]-p-hydroxyphenethyl alcohol, respectively. Coupling reagents included 150 μmol of NADH, 5 units of alcohol dehydrogenase, and 10 units of catalase. Incubations were carried out at 37°C and allowed to proceed for 3 to 6 h or overnight. After completion of the reaction, p-hydroxyphenethyl alcohols were isolated and analyzed by 1H NMR spectroscopy as described above.

Coupled Lysyl Oxidase-Alcohol Dehydrogenase Incubations—Coupled enzyme oxidation reactions were carried out as described by Bartterby et al. (14) with modification. A typical incubation included 1600 units of (2R)-['H]- or (2S)-['H]-tyramine Substrates—Two units of bovine plasma amine oxidase were incubated in 6 ml of 30 mM phosphate buffer, pH 7.6, containing 60 μmol of [2,2-'H]-tyramine or in deuterated buffer, pD 7.1, with 60 μmol of [2,2-'H]-tyramine for the production of (2S)-['H]- and (2R)-['H]-p-hydroxyphenethyl alcohol, respectively. Coupling reagents included 150 μmol of NADH, 5 units of alcohol dehydrogenase, and 10 units of catalase. Incubations were carried out at 37°C and allowed to proceed for 3 to 6 h or overnight. After completion of the reaction, p-hydroxyphenethyl alcohols were isolated and analyzed by 1H NMR spectroscopy as described above.

Chemical Conversion of [2-'H]-p-Hydroxyphenethyl Alcohol to [2-'H]-Tyramine—The procedure for the chemical conversion of [2-'H]-p-hydroxyphenethyl alcohol to [2-'H]-tyramine was that of Farnum and Klinman (7) as modified by Scaman and Palcic (22).

Coupled Lysyl Oxidase-Alcohol Dehydrogenase Incubations—Coupled enzyme oxidation reactions were carried out as described by Bartterby et al. (14) with modification. A typical incubation included 1600 units of (2R)-['H]- or (2S)-['H]-tyramine Substrates—Two units of bovine plasma amine oxidase were incubated in 6 ml of 30 mM phosphate buffer, pH 7.6, containing 60 μmol of [2,2-'H]-tyramine or in deuterated buffer, pD 7.1, with 60 μmol of [2,2-'H]-tyramine for the production of (2S)-['H]- and (2R)-['H]-p-hydroxyphenethyl alcohol, respectively. Coupling reagents included 150 μmol of NADH, 5 units of alcohol dehydrogenase, and 10 units of catalase. Incubations were carried out at 37°C and allowed to proceed for 3 to 6 h or overnight. After completion of the reaction, p-hydroxyphenethyl alcohols were isolated and analyzed by 1H NMR spectroscopy as described above.

Formation of S-(+)-O-Acetylmandelate Esters—The derivatization and purification protocol were modified from Parker (26) for reaction on a scale of 1 mg or less. To a solution of 7 pmol of Tyrasylol, 15 pmol of S-(+)-O-acetylmandelic acid and 1 μmol of 4-dimethylaminopyridine in 1 ml of dichloromethane at -10°C was added 15 pmol of dicyclohexylcarbodiimide. The reaction was allowed to proceed for 2 h, stirred, while the temperature of the reaction gradually increased to room temperature. The reaction was followed by silica TLC with ethyl acetate:n-hexane (1:2) as solvent (Rf = 0.40). The solution was washed successively with 25-mi portions of 0.5 N HCl, 2 N NaOH, and a saturated salt solution. The sample was dried under reduced pressure, solubilized in ethyl acetate:n-hexane (1:2), and the product isolated by chromatography on a 2-g flash silica column with the same solvent. The ester product was analyzed by 1H NMR spectroscopy.

RESULTS AND DISCUSSION

Aromatic Alkaloids as Substrates for Lysyl Oxidase—Prior studies on the stereospecificity of copper-dependent amine oxidases have used tyramine stereoisomers as model substrates, analyzing for the retention or loss of deuterium in the oxidation reaction. The enzymatically produced aldehyde is reduced in situ by alcohol dehydrogenase to the alcohol which is then analyzed by NMR spectroscopy (13, 15). Thus, the substrate potential of tyramine and structurally related amines for lysyl oxidase was assessed. Comparing kcat/Km values (Table 1), tyramine...
proven to be one of the more efficient substrates among those listed. Indeed, it is of some interest that each of the amines bearing an aromatic moiety on carbons 1, 2, 3, or 4 are oxidized more efficiently by this enzyme than is n-hexylamine. The \( k_{cat}/K_m \) values decrease in nearly linear fashion with increasing chain length of the alkyl carbon chain, reflecting decreases in the reactivity of the aromatic ring to the oxidized carbon. Previous studies with lysyl oxidase have shown the decreasing substrate potential and the increasing inhibitor potency of benzylamines substituted in the para position with increasingly electrophilic substituents. This was postulated to reflect the inductive effect of the substituted benzene ring on the migration of the electron of the reduced enzyme by oxygen (29). The magnitude of primary kinetic isotope effects obtained with 1,1-dideuterated n-butylamine (9) predicts that additional slow step(s) occur in the mechanism after rate-contributing \( \alpha \)-proton abstraction with such alkylamine substrates. Taking advantage of the fact that the aldehyde derived from \( p \)-hydroxybenzylamine can be quantified spectrophotometrically (\( \tau_{1/2} = 22,000 \text{ M}^{-1} \text{ cm}^{-1} \)), an experiment was undertaken to determine which of the two half-reactions catalyzed by lysyl oxidase was the more rate-limiting. As shown (Fig. 2), the pattern of \( p \)-hydroxybenzaldehyde accumulation is consistent with that of a kinetic “burst,” in that a biphasic response is seen. The initial, pre-steady state rate of aldehyde release titers at 1.0 ± 0.1 mol of aldehyde/mol of active site with a rate (0.33 mol/min) which is 5.5 times faster than that of the second steady state phase of catalysis (0.06 mol/min). The slower steady state rate of aldehyde production argues for the rate-limiting reoxidation of the reduced enzyme by oxygen. Indeed, performing this assay under anaerobic conditions, a rapid pre-steady state phase of aldehyde release occurred which titered at 0.9 ± 0.1 mol of \( p \)-hydroxybenzaldehyde/mol of active site. The initial rate of this kinetic phase is in very good agreement with that observed in oxygen. However, the second slower phase of aldehyde production was essentially eliminated under the anaerobic conditions (Fig. 2), confirming that the oxidative half-reaction is the more rate-limiting of the two half-reactions in the oxidation of this substrate. It should also be noted that the same conclusion was derived from assays conducted in the absence of the urea component of this assay system (not shown). Urea (1.2 M) is included to enhance sensitivity by limiting polymerization of lysyl oxidase monomers to non-productive oligomers (24).

**Kinetic Isotope Effects**—Consistent with prior evidence for the rate contribution of \( \alpha \)-proton abstraction from primary amine substrates of lysyl oxidase, kinetic isotope effects are seen in both \( k_{cat} \) and \( K_m \) when comparing the oxidation of tyramine to its 1,1'-dideuterated derivative. The magnitudes of the effects seen with the 1,1'-dideuterated compound are comparable with those previously obtained with other amine substrates of lysyl oxidase (9, 16). Employing the nomenclature of Northrop (30), the predominance of the value for \( k_{cat}/K_m \) over that of \( {^2}k_{cat}/K_m \) is consistent with rate-limiting \( \alpha \)-proton abstraction occurring in the first half-reaction.

**Stereospecificity of \( \alpha \)- and \( \beta \)-Proton Abstraction**—To determine absolute \( \alpha \)-proton abstraction stereospecificity, \( ^{(R)} \), \( ^{(S)} \), and \( ^{(1S)^{(15)}H} \) tyramines were used as substrates of lysyl oxidase. \(^1\)H NMR spectra of the amines confirmed the presence of greater than 95% deuterium incorporation at the C-1 position, whereas the stereospecificity of the deuterium label has been confirmed by incubation of these amines with copper

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### Table I

<table>
<thead>
<tr>
<th>Amine</th>
<th>( K_m ) mM</th>
<th>( k_{cat} ) min(^{-1} )</th>
<th>( k_{cat}/K_m ) m(^{-1} \text{ M}^{-1} \text{ (X 10}^{14} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Diaminopentane</td>
<td>0.7 ± 0.1</td>
<td>5.8 ± 0.5</td>
<td>8.3 ± 1.2</td>
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<tr>
<td>n-Hexylamine</td>
<td>2.9 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>Benzylamine</td>
<td>0.5 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>Hydroxybenzylamine</td>
<td>0.3 ± 0.1</td>
<td>4.6 ± 0.3</td>
<td>14.8 ± 2.1</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.3 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>7.7 ± 2.3</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>0.7 ± 0.0</td>
<td>3.0 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Phenylpropylamine</td>
<td>1.2 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Phenybutylamine</td>
<td>2.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>
amine oxidases with known stereochemical courses of amine oxidation (13).

The deuterium content of the p-hydroxyphenethyl alcohol products compared with the substrates served as a marker for the steric course of the reaction. Retention of deuterium in product alcohols resulted in an 'H NMR spectrum consisting of a triplet attributed to the C-1 proton signals with a chemical shift near 3.7 ppm and a doublet for the C-2 proton signals with a chemical shift near 2.8 ppm. A loss of deuterium resulted in an alcohol with a signal of a triplet for both C-1 and C-2 protons, with shifts near 3.7 and 2.8 ppm, respectively.

Lysyl Oxidase Catalyzes the Abstraction of the Pro-S Proton from C-1 of Tyramine—Fig. 3, a and b, shows the 'H NMR spectra of the alcohol products obtained from incubation of lysyl oxidase with (1R)-[3H]- and (1S)-[2H]tyramine, respectively. The product isolated from an incubation with (1R)-tyramine exhibited a triplet near 3.7 ppm and a doublet near 2.8 ppm, indicating that the deuterium had been retained. The corresponding alcohol product derived from the (1S)-[1H]tyramine is fully protonated as the NMR spectrum is comprised of a triplet near 3.7 ppm and a triplet near 2.8 ppm, as expected for a reaction with pro-S proton abstraction specificity.

It has been proposed that substrate oxidation follows a transaminase-type mechanism, with the substrate amine forming a Schiff base intermediate with the carbonyl cofactor of the enzyme. Subsequent proton abstraction and migration of electrons from C-1 of the substrate would yield a reduced enzyme-imine intermediate from which the aldehyde product can be released. Molecular oxygen may then reoxidize the enzyme releasing hydrogen peroxide and ammonia (29). This mechanism has also been proposed for other copper-dependent amine oxidases and was recently confirmed for the bovine plasma amine oxidase (31-33). However, a subset of these copper amine oxidases, including those of bovine and porcine plasma, also catalyze the exchange of a proton at C-2 with solvent during the oxidation of phenethylamine derivatives (7, 13). This incorporation of a solvent proton at C-2 has been attributed to nonessential and reversible imine-enamine formation after C-1 proton abstraction (Scheme I). The ability of lysyl oxidase to catalyze the solvent exchange pathway at C-2 of tyramine was examined spectroscopically. Incubations with [2,2-3H]tyramine and deuterated buffer, and complementary experiments with [2,2-2H]tyramine and protonated buffer both demonstrated that lysyl oxidase catalyzed the exchange of a proton at C-2 of substrate with solvent. Fig. 4 shows the NMR spectra of the product alcohols isolated from these wash-in experiments. The complete exchange of protons at C-2 was observed with [2,2-2H]tyramine as substrate. The spectrum of the p-hydroxyphenethyl alcohol isolated from this solvent exchange incubation exhibited a doublet pattern near 3.7 ppm for proton signals at C-1 and a triplet pattern near 2.8 ppm for the proton signals at C-2, indicating that deuterium had been incorporated into the C-2 position. Moreover, variability in the amount of deuterium wash-in was noted as 1 and 1.2 deuterium atoms were found in separate experiments. Less complete exchange of solvent protons into C-2 of product alcohols was observed; however, when [2,2-2H]tyramine was used as the substrate. Approximately 50% of the product was C-2 deuterated alcohol, as a result of bypassing the solvent exchange pathway, whereas the remain-

\[
\begin{align*}
E_{ox} + RCH\_CH\_NH\_2 + \text{Solvent Exchange} + H_2O & \rightarrow E_{red}-NH=CH-CHR + H_2O + NH_3 \\
E_{ox} + RCH\_CH\_NH\_2 + H_2O & \rightarrow E_{red}-NH=CH-CHR + H_2O + NH_3
\end{align*}
\]

\text{SCHEME I}

![Fig. 3. Partial 360-MHz 'H NMR spectra of p-hydroxyphenethyl alcohols obtained from incubation of lysyl oxidase with (1R)-[3H]tyramine (a) and (1S)-[2H]tyramine (b).](image)

![Fig. 4. Partial 360-MHz 'H NMR spectra of p-hydroxyphenethyl alcohols obtained from incubation of lysyl oxidase with [1,1-2H]tyramine in H2O (a) and [1,1-2H]tyramine in H2O (b).](image)
ing product was monodeuterated. This is evident on the NMR spectrum from the reduced triplet pattern near 2.8 ppm for the C-2 signal which integrated to 0.5 protons and the uneven doublet pattern near 3.7 ppm for the protons at C-1.

The stereospecificity of these wash-in reactions was determined by derivatization of the alcohol products with S-(+)-O-acetylmandelic acid (27). This chiral derivatizing agent creates nonequivalence of the protons at both C-1 and C-2, allowing each to be observed by a distinct chemical shift in the NMR spectrum. The absolute chemical shift of the pro-R and pro-S proton at C-2 of p-hydroxyphenethyl alcohol was determined by derivatization of C-2 chirally deuterated alcohols. These were obtained from wash-in reactions with the bovine plasma amine oxidase. This copper amine oxidase is reported to catalyze the stereospecific solvent wash-in at C-2 to the pro-R position, and, therefore, the reaction carried out in a protonated buffer yielded (2S)-[2H]alcohol, whereas the reaction in deuterated buffer yielded (2R)-[2H]alcohol (7). The NMR spectra of the esters of the (2R)-[2H]- and (2S)-[2H]p-hydroxyphenethyl alcohols indicated that the chemical shifts of the C-2 signal occurred at 2.32 and 2.28 ppm, respectively (Fig. 5, a and b). Therefore, as observed with other aromatic and aliphatic alcohols, the pro-R proton resonated at the high field of the pro-S proton (27).

Although a limited amount of product alcohol was available from wash-in experiments for the derivatization reaction, the stereospecificity of the wash-in at C-2 was confirmed as being pro-R-specific. The NMR spectra of the S-(+)-O-acetylmandelate ester derivatives of the alcohols from the wash-in reactions in deuterated and protonated buffer are shown in Fig. 5, c and d, respectively. The ester product isolated from derivatization of the alcohol from the wash-in reaction in the deuterated buffer exhibited a C-2 signal at 2.32 ppm, as expected for a (2R)-[2H]alcohol. The corresponding ester derivative obtained by reacting the alcohol from the protonated buffer wash-in reaction exhibited a C-2 signal at 2.28 ppm, as expected for a (2S)-[2H]alcohol. Therefore, both the deuterated and protonated buffer wash-in reactions of the lysyl oxidase occurred with pro-R specificity.

The stereospecificity of the wash-out reaction at C-2 was investigated using C-2 chirally deuterated amines as substrates. These substrates were obtained from the wash-in reactions of the bovine plasma amine oxidase in protonated and deuterated buffer as described above, yielding (2S)-[2H]- and (2R)-[2H]p-hydroxyphenethyl alcohols, respectively. The alcohols were then chemically converted to amines, with an overall yield of 25-45%. No scrambling or loss of deuterium label occurred during the conversion, as determined from integration of the 1H NMR signals of C-1 and C-2 protons of starting alcohol compared with amine product.

Since the solvent exchange at C-2 is the result of two half-reactions of wash-out, followed by wash-in, it was anticipated that lysyl oxidase would catalyze the pro-R proton abstraction from C-2. This would suggest that the two processes are fully reversible, following an equivalent mechanism in both directions. The pro-R specificity of the wash-out reaction was confirmed, as evidenced from the 1H NMR spectra of the alcohols obtained from the oxidation of the (2S)-[2H]- and (2R)-[2H]tyramine (Fig. 6a). The spectrum of the alcohol product of (2S)-[2H]tyramine is comprised of a doublet near 3.7 ppm for the C-1 signal and a triplet near 2.8 ppm for the C-2 signals, indicating that deuterium had been completely retained during the reaction. This was consistent for duplicate incubations. The minor single peaks downfield from the doublet and triplet signals are the downfield signals of two triplets arising from fully protonated alcohol product and represent approximately 10% of the total product. A similar amount of diprotonated material was also present in the starting amine substrate. The major signals arising from the monodeuterated product are shifted 0.02 ppm upfield due to an α- and β-deuterium isotope effect (34).

The corresponding spectrum of the alcohol product of (2R)-[2H]tyramine (Fig. 6b) exhibited a triplet near 3.7 ppm for the C-1 signal and the triplet near 2.8 ppm for the C-2 signal, indicating loss of deuterium from the C-2 position. However,
integration of the signal for the C-2 protons was reduced from the expected value of 2 protons to 1.3 protons. This suggested that some C-2 monodeuterated alcohol product was present, to account for the reduced integration. A small peak, upfield from the triplet near 2.8 ppm, can be seen in the spectrum and is attributed to the upfield peak of a triplet arising from monodeuterated product. The expected doublet for the C-1 signal of monodeuterated alcohol near 3.7 ppm is masked under the two upfield peaks of the triplet at this chemical shift and therefore is not visible. Approximately 45% of the deuterium was abstracted from the (2R)-[^2H]tyramine by lysyl oxidase, whereas 55% of the deuterium was retained. Some variability in the amount of deuterium removed from the C-2 pro-R position was noted in replicate incubations, with up to 90% of the deuterium retained in some incubations. Variation in the amount of deuterium abstraction is the result of alteration in the competition between the main catalytic pathway, responsible for C-1 proton abstraction, and the alternate pathway of imine-enamine exchange. Although the reasons for the observed differences in the solvent exchange pathway are not clear, factors such as aging of the enzyme preparation may be important. From the solvent exchange experiments, it is apparent that the C-2 pathway is not a required step in catalysis for lysyl oxidase. This is supported by the observation that lysyl oxidase acts on substrates such as benzylamine (16).

The results of the wash-in and wash-out experiments suggested that there is an isotope effect on the pro-R hydrogen abstraction from C-2. This would account for the reduced amount of wash-in observed with [2,2-[^2H]tyramine in the protonated buffer, as well as the retention of deuterium observed in the wash-out experiments with (2R)-[^2H]tyramine as substrate. There is no apparent isotope effect on kcat and a modest effect on kcat/Km for [2,2-[^2H]tyramine (Table II), as suggested by the stereochemical studies.

Interesting comparisons can be made between the stereochemical course of tyramine oxidation and the solvent exchange characteristics of lysyl oxidase and that of another enzyme isolated from aortic tissue, semicarbazide-sensitive amine oxidase. Semicarbazide-sensitive amine oxidase is a detergent-extractable enzyme that is highly active in vascular tissue (35). It is distinguished from the copper amine oxidases by its high affinity for benzylamine and its activity on small aliphatic amines such as methylamine. The enzyme has not yet been assigned an Enzyme Commission classification, as the relationship between it and the copper amine oxidases has not been established. The reactivity of semicarbazide-sensitive amine oxidase with reagents such as semicarbazide and hydrazines suggests that it contains a carboxyl-type cofactor (36), but it is not clear if the cofactor is topa quinone which was recently found to be present in the copper amine oxidases (4, 5). Semicarbazide-sensitive amine oxidase is distinct from lysyl oxidase as semicarbazide-sensitive amine oxidase is extractable with detergents such as Triton X-100, has a molecular mass near 180 kDa, and does not act on tropoelastin, which is a substrate for lysyl oxidase (37, 38).

The stereospecificity of the bovine and porcine aortic semicarbazide-sensitive amine oxidase is the same as that of lysyl oxidase, as semicarbazide-sensitive amine oxidase preferentially abstracts the pro-S proton from C-1 of tyramine and exchanges solvent into C-2 of product aldehydes with pro-R specification (59). The stereochemical course of lysyl oxidase distinguishes them from the copper amine oxidases examined to date (13, 15). The copper amine oxidases which catalyze pro-S proton abstraction do not exhibit the solvent exchange pathway. This includes the plant seedling amine oxidases from pea, chickpea, and soybean and the porcine kidney diamine oxidase. Alternatively, the enzymes which do catalyze the exchange of a C-2 proton with solvent are either pro-R-specific, as in the case of the porcine plasma amine oxidase, or show an apparent nonstereospecificity at C-1, as for the bovine, rabbit, and sheep plasma amine oxidase. The case of the bovine plasma amine oxidase deserves closer examination, however, as this enzyme exhibits unusual mirror image binding of tyramine, giving rise to two separate and stereospecific modes of tyramine oxidation. One mode follows C-1 pro-R and C-2 pro-S abstraction, whereas the second preferred mode is that of C-1 pro-S and C-2 pro-R abstraction. This suggests that the mechanism of lysyl oxidase and semicarbazide-sensitive amine oxidase may be similar to the one preferred mode of the bovine plasma enzyme. However, there is poor sequence homology between the lysyl oxidase amino acid sequence predicted from the cDNA sequence (39, 40) and active site peptides of several copper amine oxidases, suggesting that the cofactor and mechanism of lysyl oxidase is distinct from these other enzymes (5, 41). Further information on the cofactor and the active site structure will be required to assess the similarity of these enzymes.

REFERENCES

**TABLE II**

Oxidation of tyramine by lysyl oxidase: deuterium kinetic isotope effects

<table>
<thead>
<tr>
<th>Amines</th>
<th>kcat (min⁻¹)</th>
<th>Km (mM)</th>
<th>kcat/Km</th>
<th>δkcat</th>
<th>δkcat/Km/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyramine</td>
<td>1.88 ± 0.38</td>
<td>0.27 ± 0.04</td>
<td></td>
<td>2.6</td>
<td>4.8</td>
</tr>
<tr>
<td>[1,1-[^2H]Tyramine</td>
<td>0.73 ± 0.10</td>
<td>0.52 ± 0.07</td>
<td>2.6</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>[2,2-[^2H]Tyramine</td>
<td>1.88 ± 0.38</td>
<td>0.38 ± 0.04</td>
<td>1.0</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>
Stereospecificity of Lysyl Oxidase