Stereochemical Course of Tyramine Oxidation by Semicarbazide-Sensitive Amine Oxidase†

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ABSTRACT: Two semicarbazide-sensitive amine oxidases (SSAO's) from bovine and porcine aortic tissue were partially purified and characterized, and the stereochemical course of amine oxidation was evaluated. The porcine and bovine SSAO's were membrane bound glycoproteins, with K_m values for benzylamine of 8 and 16 μM, respectively. The reactivity of SSAO with semicarbazide and phenylhydrazine suggests that the cofactor is a carbonyl type molecule. The stereochemical course of the bovine and porcine aortic semicarbazide-sensitive amine oxidase reaction was investigated using chiral tyramines, deuterated at C-1 and C-2, and ^{1}H-NMR spectroscopy to establish the loss or retention of deuterium in product *p*-hydroxyphenethyl alcohols. The preferred mode of tyramine oxidation was found to occur with the loss of *pro-S* proton at C-1, coupled with solvent exchange into C-2, a pattern which has not been observed for any copper amine oxidase examined to date. The solvent exchange reaction also occurred stereospecifically, with loss from and reprotonation to the *pro-R* position, suggesting that these two processes occur from the same face of the enamine double bond.

Amine oxidases that are inhibited by semicarbazide are active in a wide variety of animal and plant tissues, as well as in microorganisms (Mondovi, 1985). They encompass the copper-containing amine oxidases which fall into the EC 1.4.3.6 classification and a second group of enzymes in which copper dependence has not been shown, referred to simply as the semicarbazide-sensitive amine oxidases (SSAO's). Smooth muscle cells of vascular tissue are a particularly good source of SSAO (Lewinsohn, 1981), but the enzyme has been detected in diverse sources including ox dental pulp (Norqvist et al., 1982), rat anococcygeus tissue (Callingham, 1981), rat brown adipose tissue (Barrand & Callingham, 1984), horse liver (Callingham & Barrand, 1987), human umbilical artery (Precious & Lyles, 1988), and rat aorta (Coquil et al., 1973).

The physiological function of the SSAO is unknown, but its prevalence in vascular tissue suggests that the enzyme plays an as yet undefined role in the maintenance of cardiovascular function (Lewinsohn, 1981). SSAO is inhibited by the vasoactive drug hydrazaline (Lyles et al., 1983), although the efficacy of this compound has not been attributed to SSAO inhibition. As well, the activity of aortic SSAO has been associated with the formation of cardiovascular lesions, through the deamination of allylamine to acrolein (Boor et al., 1990).

The relationship between the SSAO and copper-containing amine oxidases is unclear. Very little mechanistic data are available for the SSAO that might clarify this situation. A comparison of the physiochemical characteristics of SSAO and copper amine oxidases has revealed both similarities and differences between the two groups of enzymes. For example, members from both groups are glycoproteins that show affinity for Concanavalin A or *Lens culinaris* lectin and appear to be dimers with subunit molecular masses of approximately 90 kDa (Callingham & Barrand, 1987). Both groups of these enzymes are inhibited by semicarbazide and phenylhydrazine, suggesting the cofactor contains a carbonyl group (Bardsley, 1985). Recently, the cofactor structure of the bovine plasma copper amine oxidase was conclusively established as TOPA, or 2,4,5-trihydroxyphenylalanine (Janes et al., 1990), but it would not be prudent to assume that this cofactor is present in other enzymes without further evidence.

There are several physiochemical properties that distinguish SSAO from copper amine oxidases. For example, the SSAO's are localized on the plasma membrane of smooth muscle cells (Wibo et al., 1980) while the copper amine oxidases are soluble enzymes. As well, SSAO's generally have a greater affinity for benzylamine, compared to the copper amine oxidases. SSAO's are characterized by a K_m value in the low micromolar concentrations (Barrand & Callingham, 1984; Lyles & Singh, 1985; Hysmith & Boor, 1988) although data for enzyme from human sources exhibit K_m values closer to those expected for the copper amine oxidases (Lyles et al., 1990).

Both SSAO and copper amine oxidases catalyze the oxidative deamination of amines to aldehydes, with the release of hydrogen peroxide and ammonia. Mechanistically, copper amine oxidases from bovine and porcine plasma are the best characterized. It was recently shown that the bovine plasma enzyme follows a transaminase type of mechanism via formation of an imine intermediate (Janes & Klinman, 1991).

$$E_{ox} + {}^{+}H_3N{-}CH_2{-}CH_2{-}R \rightleftharpoons E_{red}{-}N^{+}H{=}CH{-}CH_2{-}R + H_2O \rightarrow E_{red}{-}NH_2 + O{=}CH{-}CH_2{-}R + O_2 \rightarrow E_{ox} + H_2O_2 + NH_4^{+} \quad (1)$$

In addition to this main catalytic pathway, a side reaction of imine–enamine tautomerization has been found to occur in some of the copper-containing enzymes prior to imine hydrolysis (Lovenberg & Beaven, 1971; Coleman et al., 1991).

$$E_{red}{-}N^{+}H{=}CH{-}CH_2{-}R \rightleftharpoons E_{red}{-}NH{-}CH{=}CH{-}R \quad (2)$$

In this minor pathway, a proton at the β-position of the substrate may be exchanged with solvent.

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The copper amine oxidases are unique in that all possible modes of proton abstraction at C-1 (*pro-R*, *pro-S*, and an apparent nonstereospecificity) have been observed within this one class of enzyme (Coleman et al., 1989). The bovine plasma enzyme displays an unusual substrate-dependent stereochemistry as benzylamine is oxidized via abstraction of the *pro-S* proton at C-1 (Battersby et al., 1979) while dopamine and tyramine are oxidized with an apparent nonstereospecificity (Summers et al., 1979; Coleman et al., 1989). The pea seedling amine oxidase lacks the ability to bind substrates in alternate modes as benzylamine, tyramine, and dopamine are oxidized by abstraction of only the *pro-S* proton at C-1 (Battersby et al., 1976; Coleman et al., 1989; Summers et al., 1979). Conversely, the porcine plasma amine oxidase reaction with dopamine and tyramine occurs with the removal of the *pro-R* proton at C-1 (Coleman et al., 1989).

Such diversity within one formal classification of enzymes has not been observed with any other group of enzymes. While the mechanistic basis for this heterogeneity is not known, correlations in the stereochemical behavior at C-1 and C-2 have been observed for these enzymes (Coleman et al., 1991). Those that catalyze the abstraction of the *pro-S* hydrogen do not catalyze the imine–enamine tautomerization pathway, while enzymes which catalyze an apparent nonstereospecific or *pro-R* proton abstraction at C-1 do exchange a proton at C-2 with solvent.

Comparison of the stereochemistry of SSAO enzymes and copper amine oxidases is one approach to establish the mechanistic relatedness of the two groups of enzymes. Previously, the stereochemical course of dopamine and benzylamine oxidation by SSAO from rat aorta was described as being *pro-S* specific (Yu, 1987; Yu & Davis, 1988). However, these reports did not establish whether SSAO catalyzed the solvent exchange reaction. The present study reports on the isolation and partial characterization of bovine and porcine aortic SSAO. ^{1}H-NMR spectroscopy and chirally deuterated tyramines were used to determine the complete stereochemical course of reaction of these enzymes. A unique pattern of oxidation at C-1 and solvent exchange at C-2 were observed for both SSAO enzymes.

EXPERIMENTAL PROCEDURES

All chemicals were of reagent grade unless otherwise specified, and all protonated solvents were distilled before use. Deuterated solvents were obtained from Merck, Sharp and Dohme (99.95% ^{2}H), or General Intermediates of Canada (99.9% ^{2}H). [2,2-^{2}H]Tyramine hydrochloride (98 atom % ^{2}H) was obtained from Merck, Sharp and Dohme. Reverse osmosis water that was passed through anion-exchange, cation-exchange, carbon, and 0.45-μm cartridges (Milli-Q) was used for all chemical solutions and buffers. Thin-layer chromatography plates were Kieselgel 60 F_{254}, 0.2 mm, from E. Merck, Darmstadt. A Hewlett Packard 8451A diode array spectrophotometer was used for spectral work.

Enzymes

Equine liver alcohol dehydrogenase, catalase, horseradish peroxidase type II, tyrosine decarboxylase (*Streptococcus faecalis*), and bovine plasma amine oxidase were obtained from Sigma. Porcine and bovine semicarbazide sensitive amine oxidases (SSAO's) were isolated from aortic tissue of animals obtained from a local abattoir.

Activity and Protein Determination

All protein determinations were done using the Bio-Rad kit (Bradford, 1976) using bovine serum albumin as the standard protein. The activity of SSAO was determined at pH 7.6 by monitoring the production of benzaldehyde (ϵ = 11 500 M^{-1} cm^{-1}) from the oxidation of benzylamine at 252 nm. Benzylamine concentrations of 200 and 100 μM were used for bovine and porcine SSAO, respectively. A unit of activity was defined as the amount of enzyme which oxidized 1 μmol of substrate/min at 37 °C.

Solubilization and Isolation of Semicarbazide-Sensitive Amine Oxidase

After removal of the excess fat and tissue, aorta were rinsed in water and put through a meat grinder (4-mm sieve). Ground tissue was then used immediately as an enzyme source or stored frozen at −30 °C.

Enzyme was isolated from 200–500-g batches of tissue. The following protocol is for a 200-g sample of tissue. The tissue was washed in 1 L of 10 mM sodium phosphate, pH 7.6, by stirring for 15 min and straining through 4 layers of cheesecloth. The buffer was discarded and the tissue was blended using an Osterizer blender and 400 mL of the same buffer. The slurry was then stirred for 15 min and centrifuged at 10000*g* for 30 min. The supernatant was discarded and the pellet was resuspended in 400 mL of 30 mM sodium phosphate, pH 7.6, with 1% Triton X-100. The bovine aortic tissue was extracted by stirring for 2 h and then centrifuged at 10000*g* for 30 min. The supernatant was retained and the pellet was blended in 400 mL of the same buffer without detergent. Triton X-100 was then added to a final concentration of 1%, and the tissue was extracted a second time as above. Porcine aortic tissue was extracted with the same buffer as the bovine tissue but by stirring for 6 h for the first extraction and 18 h for the second extraction.

The detergent extract was loaded at 2 mL/min onto a DEAE-Sepharose CL-6B column (5 × 20 cm) that was equilibrated with 30 mM sodium phosphate, pH 7.6. The column was washed with approximately 500 mL of the same buffer until the unbound protein had eluted, followed by 100 mM sodium phosphate, pH 7.6, 100 mM NaCl. Activity was eluted in approximately 500 mL of this buffer. The enzyme was loaded at 1 mL/min on 75 mL of a Sepharose 4B *Lens culinaris* lectin (2 mg of lectin/mL of gel) which was equilibrated with 0.1 mM each $MgCl_2$, $CaCl_2$, and $MnCl_2$, and 0.5 M NaCl in 20 mM sodium phosphate, pH 7.6. The column was washed with approximately 200 mL of the equilibrium buffer until unbound protein had eluted. A sharp peak of activity was eluted by loading a total volume of 200 mL of 1 M methyl α-D-glucose in sodium phosphate, pH 7.6, onto the column. After one column volume of this buffer had passed into the column, flow was halted and the buffer was allowed to remain in contact with the column for 4 h or overnight. The active fractions eluted with the methyl α-D-glucose buffer were combined and concentrated using an Amicon ultrafiltration cell with a PM 30 membrane to less than 10 mL and chromatographed on S-200 Sephacryl (2.5 × 95 cm) equilibrated with 20 mM sodium phosphate, pH 7.6, 0.1 M potassium chloride. A flow rate of 0.75 mL/min was used, and 10-mL fractions were collected. Activity was eluted near 180 mL.

Inhibition of SSAO by Semicarbazide and Phenylhydrazine

An aliquot of enzyme was reacted with semicarbazide at 4 °C for 24 h. Residual activity was assayed directly, with a 100-fold dilution of excess inhibitor. For comparison, the inhibition of bovine plasma amine oxidase was also evaluated.

Reversibility of inhibition was tested by removing excess inhibitor with a PD-10 column (Pharmacia), concentrating the enzyme using a Centricon 10 unit (Amicon), and assaying for activity immediately after this treatment and after 24 h at 4 °C. Aliquots of enzyme were also reacted with phenylhydrazine, monitoring the increase in absorbance at 420 nm or determining residual activity after 10 min at 20 °C. The reversibility of inhibition was evaluated as noted above.

^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument at ambient temperature (22 + 1 °C). The spectra of *p*-hydroxyphenethyl alcohols were obtained in $(^2H_3C)_2CO$:^2H_2O (4:1), with the residual H^2H_2C signal set at 2.19 ppm as an internal reference standard. Spectra of tyramine hydrochlorides were obtained in ^2H_2O using the residual H^2HO signal at 4.81 ppm as an internal reference standard. Spectra of (*S*)-(+)-*O*-acetylmandelate esters were obtained in $C_6{}^2H_6$ using the residual $C_6{}^2H_5{}^1H$ signal at 7.15 ppm as an internal standard. Spectra were accumulated into 16K of computer memory using a 45° pulse with a 7-s relaxation delay between pulses for ^2H_2O-containing solvents and a 2-s relaxation delay for samples in organic solvents. Integrations of the signals of the NMR spectra are considered to be accurate to ±5%.

Synthesis of [1(R)-^{2}H]- and [1(S)-^{2}H]Tyramine

DL-[2-^{2}H]Tyrosine was obtained by reacting fully protonated tyrosine with KO^2H in ^2H_2O in the presence of pyridoxal hydrochloride (Fujihara & Schowen, 1984). After neutralization of the reaction, the deuterated amino acid was isolated as a solid and recrystallized from hot water by the addition of ethanol. The extent of deuteration at the C-2 position was greater than 98% as determined by ^{1}H-NMR spectroscopy, and isolated yields for the reaction were 20–30%. [1(*S*)-^{2}H]Tyramine was obtained by decarboxylation of DL-[2-^{2}H]tyrosine with *S. faecalis* tyrosine decarboxylase in 50 mM sodium acetate buffer, pH 5.8. The corresponding [1(*R*)-^{2}H]tyramine was obtained by decarboxylation of L-[2-^{1}H]tyramine with the same enzyme, with the reaction carried out in deuterated sodium acetate buffer, pD 6.0, where pD is the uncorrected pH meter reading. For both reactions, product amines were separated from unreacted amino acid by ion-exchange chromatography on Amberlite IRC 50 (H^+ form).

Coupled Amine Oxidase–Alcohol Dehydrogenase Incubations

Stereochemical Course of Tyramine Oxidation at C-1. Coupled amine oxidase reactions were carried out as described by Battersby and co-workers (1976) with modification. To this end, a typical incubation included 0.3 unit of amine oxidase in 3–5 mL of 30 mM phosphate buffer, pH 7.6, containing 10 μmol of tyramine, 25 μmols of NADH, 4 units of alcohol dehydrogenase, and 11 000 units of catalase. The reactions were allowed to proceed until 50% of the NADH had been oxidized, as determined by a decrease in the absorbance at 340 nm, or overnight. Samples were then extracted 3 times with 25 mL of ethyl acetate. Successive extracts were concentrated to dryness under reduced pressure, chromatographed on a 2-g flash silica column (60–100 mesh, BDH) using ethyl acetate:hexane (2:1) as a solvent. The column fractions containing the product were then concentrated to dryness under reduced pressure and used for ^{1}H-NMR analysis. An alternative workup procedure involved loading the enzyme incubation mixtures onto a C18 Sep-Pak cartridge (Waters), the cartridge was washed with 5 mL of water, and then product alcohols were eluted with 10 mL of methanol. The methanol eluate was concentrated to dryness under reduced pressure and chromatographed on a flash silica column as described above and analyzed by ^{1}H-NMR spectroscopy. Both alcohol and some residual amine and residual NADH were visible in ^{1}H-NMR spectra using this protocol.

[2(R)-^{2}H]- and [2(S)-^{2}H]-p-Hydroxyphenethyl Alcohols. Two units of bovine plasma amine oxidase was incubated in 6 mL of 30 mM phosphate buffer, pH 7.6, containing 60 μmol of [2,2-^{2}H]tyramine or in deuterated buffer, pH 7.1, with 60 μmol of [2,2-^{1}H]tyramine for the production of [2(*S*)-^{2}H]- and (2(*R*)-^{2}H]-*p*-hydroxyphenethyl alcohols, respectively. Coupling reagents, including 150 μmol of NADH, 11 units of alcohol dehydrogenase, and 80 000 units of catalase, were added. Incubations were carried out at 37 °C and allowed to proceed to 3–6 h, or overnight. After completion of the reaction, *p*-hydroxyphenethyl alcohols were isolated and analyzed by ^{1}H-NMR spectroscopy as described above.

Chemical Conversion of [2-^{2}H]-p-Hydroxyphenethyl Alcohol to [2-^{2}H]Tyramine

The following procedure for the chemical conversion of [2-^{2}H]-*p*-hydroxyphenethyl alcohol to [2-^{2}H]tyramine was modified from that of Farnum and Klinman (1986). The quantities of reagents given below are for the conversion of 5 mg of alcohol to tyramine.

Protection of [2-^{2}H]-p-Hydroxyphenethyl Alcohol. A 2 molar excess of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (11.6 μL) and benzyl chloride (9 μL) was added to a screw-cap test tube with the deuterated alcohol (5 mg) dissolved in 1 mL of methanol. The mixture was capped and stirred for 18 h at 65 °C. A second 2 molar excess of DBU and benzyl chloride was added if necessary. The reaction was followed by silica TLC using ethyl acetate:*n*-hexane:95% ethanol (10:10:1) as a solvent (R_f = 0.67). After cooling, 1 mL of 1 N NH_4OH was added and the reaction was stirred for 30 min. The benzylated alcohol was extracted into CH_2Cl_2, washed with water, 1 N HCl, and water and dried under reduced pressure. ^{1}H-NMR spectrum in $CDCl_3$ (internal reference $CHCl_3$, δ 7.24) showed the following (δ in ppm): δ 2.7 (t, 1 H), 3.8 (d, 2 H), 5.0 (s, 2 H), 6.9 (d, 2 H), 7.1 (d, 2 H), 7.4 (m, 5 H).

Conversion of 4-(Benzyloxy)phenethyl Alcohol to Toluenesulfonyl Ester. The benzylated adduct was dissolved in 0.5 mL of dry CH_2Cl_2 and 40 μmol of 4-dimethylaminopyridine (DMAP) (4.86 mg) in 250 μL of CH_2Cl_2 and 30 μmol of toluenesulfonyl chloride (5.73 mg) in 250 μL of CH_2Cl_2 was added. The reaction mixture was stirred at ambient temperature for several hours and a second addition of reagents was added if required. The reaction was followed by silica TLC using ethyl acetate:*n*-hexane:95% ethanol (10:10:1) as a solvent (R_f = 0.85). A 0.5-mL volume of pyridine:water (4:1) was added and stirred for 30 min. The tosylated product was extracted into CH_2Cl_2, washed with water, 1 N HCl, and water adjusted to pH 9.0 with bicarbonate. The adduct was filtered through paper and dried under reduced pressure. ^{1}H-NMR spectrum in $CDCl_3$ (internal reference $CHCl_3$, δ 7.24) showed the following (δ in ppm): δ 2.5 (s, 3 H), 3.0 (t, 1 H), 4.2 (d, 2 H), 5.4 (s, 2 H), 6.9 (d, 2 H), 7.1 (d, 2 H), 7.3 (d, 2 H), 7.4 (m, 5 H), 7.7 (d, 2 H).

Formation of Phthalimide Derivative. A 5-fold excess (w/w) of potassium phthalimide (25 mg) to tosylated adduct was added in 0.6 mL of dry *N,N*-dimethylformamide (DMF) to the tosylated adduct. The mixture was stirred at 60 °C overnight, and the phthalimide adduct was extracted into CH_2Cl_2 and washed with ice water, 1 N NaOH with ice, and then water until neutral. The product was filtered through paper and dried under reduced pressure. The product was isolated by chromatography on a 5-g silica flash column using CH_2Cl_2:95% ethanol (98:2) as solvent. ^{1}H-NMR spectrum in $CDCl_3$:CD_3OD (50:1) (internal reference $CHCl_3$, δ 7.24) showed the following (δ in ppm): δ 3.0 (t, 1 H), 4.0 (d, 2 H), 5.1 (s, 2 H), 6.9 (d, 2 H), 7.2 (d, 2 H), 7.4 (m, 5 H), 7.7 (m, 2 H), 7.9 (m, 2 H).

Hydrazinolysis of Phthalimide Derivative to Protected Amine. The phthalimide adduct was dissolved in 1 mL of 95% ethanol, with 100 μL of hydrazine hydrate, and the mixture was stirred overnight at 65 °C. The product was dried under reduced pressure, dissolved in CH_2Cl_2 and washed 2 times with 1 N NaOH and 2 times with water, and dried under reduced pressure. The reaction was followed by silica TLC using 2-propanol:H_2O:NH_4OH (7:1:1) as a solvent (R_f = 0.64). ^{1}H-NMR spectrum in CD_3OD (internal reference CD_2HOD, δ 4.78) showed the following (δ in ppm): δ 2.7 (t, 1 H), 2.9 (d, 2 H), 5.1 (s, 2 H), 6.9 (d, 2 H), 7.1 (d, 2 H), 7.3 (m, 5 H).

Deprotection of 4-(Benzyloxy)phenethylamine to Tyramine. The material was dissolved in 3 mL of 95% ethanol with 5 drops of 1 N HCl and 3–5 mg of 5% palladium on charcoal (BDH). Hydrogenolysis was carried out at ambient temperature and H_2 flow of 5 psi overnight. The reaction was followed by silica TLC using 2-propanol:H_2O:NH_4OH (7:1:1) as a solvent (R_f = 0.60). At completion of the reaction, the catalyst was removed by filtration with methanol and dried. No further purification was required. Overall yield of the reaction was 25–45%. ^{1}H-NMR spectrum showed retention of deuterium at C-2 with no loss or scrambling during the chemical conversion. ^{1}H-NMR spectrum in D_2O (internal reference HDO, δ 4.81) showed the following (δ in ppm): δ 3.1 (t, 1 H), 3.3 (d, 2 H), 6.9 (d, 2 H), 7.2 (d, 2 H).

Formation of (S)-(+)-O-Acetylmandelate Esters

The derivatization and purification protocol were modified from Parker (1983) and Whitesell and Reynolds (1983) for reaction on a scale of 1 mg or less. To a solution of 7 μmol of tyrasol, 15 μmol of (*S*)-(+)-O-acetylmandelic acid, and 1 μmol of 4-dimethylaminopyridine in 1 mL of dichloromethane at −10 °C was added 15 μmol of dicyclohexylcarbodiimide in 0.5 mL of dichloromethane. A white precipitate of dicyclohexylurea was observed to be formed within 1 min of addition of dicyclohexylcarbodiimide. The reaction was allowed to proceed for 2 h, stirring, while the temperature of the reaction gradually increase to room temperature. The reaction was followed by silica TLC with ethyl acetate:*n*-hexane (1:2) as solvent (R_f = 0.40). The solution was washed successively with 25-mL portions of 0.5 N HCl, 2 N Na_2CO_3, and a saturated salt solution. The sample was dried under reduced pressure, resulting in a white solid. The dry material was solubilized in ethyl acetate:*n*-hexane (1:2), and the product was isolated by chromatography on a 2-g flash silica column with the same solvent. The ester product, which was a liquid, was analyzed by ^{1}H-NMR spectroscopy.

Table I: Purification of Bovine Semicarbazide-Sensitive Amine Oxidase[a]

step	units	sp act. (units/mg of protein)	yield (%)
Triton X-100 extraction			
extraction 1	17.0	0.0053	100
extraction 2	5.3	0.0018	
DEAE-Sepharose CL6B	13.7	0.0081	48
lentil lectin	3.0	0.062	13
S-200	1.6	0.11	7
	0.4	0.04	2

[a] 500 g of starting material.

Table II: Purification of Porcine Semicarbazide-Sensitive Amine Oxidase[a]

step	units	sp act. (units/mg of protein)	yield (%)
Triton X-100 extraction			
extraction 1	2.5	0.00084	100
extraction 2	0.7	0.00044	
DEAE-Sepharose CL6B	2.2	0.0015	69
lentil lectin	0.5	0.0225	16
S-200	0.06	0.050	2
	0.27	0.027	8

[a] 475 g of starting material.

RESULTS

Isolation and Partial Characterization of Bovine and Porcine Semicarbazide-Sensitive Amine Oxidase

Semicarbazide-sensitive amine oxidase was partially purified from bovine and porcine aortic tissue (Tables I and II) with procedures previously reported for rat brown adipose tissue (Barrand & Callingham, 1984) and cultured porcine aortic smooth muscle cells (Hysmith & Boor, 1988). The loss of 15% of the enzyme activity in the preliminary washing was tolerated, since removal of possible contaminating plasma amine oxidase was necessary. Buffers containing 1 M sodium chloride had no solubilizing effect on the enzyme, indicating that SSAO may be an intrinsic membrane protein or a lipoprotein. Enzyme activity was greatest in the first Triton X-100 extraction, with the second extraction yielding only 30% of the first. The activity in crude extractions was stable for several days at 4 °C in the presence of Triton X-100, unlike monoamine oxidases which are unstable in the presence of this detergent (Salach, 1979). Detergent was only required for initial solubilization of the enzyme, and inclusion of Triton X-100 at 0.1% in buffers used for subsequent steps did not influence yields or stability.

The enzyme showed variability in its behavior during anion-exchange chromatography. Some activity was eluted with only 100 mM phosphate buffer while most activity was obtained with 100 mM phosphate buffer containing 150 mM sodium chloride.

Affinity chromatography was the most effective step in increasing the specific activity of the enzyme. Retention of SSAO activity on both Concanavalin A and *Lens culinaris* lectins established the glycoprotein nature of the enzyme. Low yields were obtained from Concanavalin A compared to *Lens culinaris*, indicating that the enzyme was highly glycosylated. While both lectins have an affinity for mannose and glucose moieties and their α-methyl derivatives, the binding constants for *Lens culinaris* lectin are approximately 50-fold lower than for Concanavalin A (Stein et al., 1971). SSAO was eluted

Table III: Relative Activity of Bovine and Porcine Semicarbazide-Sensitive Amine Oxidase with a Panel of Amines[a]

amine	% relative activity of SSAO		amine	% relative activity of SSAO	
	bovine	porcine		bovine	porcine
benzylamine	100	100	tryptamine	10	20
methylamine	81	127	histamine	9	17
β-phenethylamine	49	79	putrescine	4	11
tyramine	34	73	cadaverine	3	10

[a] 200 μM concentration.

from the *Lens culinaris* lectin using a 1 M solution of either methyl α-D-mannose or methyl α-D-glucose. Enzyme was also eluted with buffers containing lower concentrations of carbohydrate, which may reflect on variation in the levels of enzyme glycosylation. Such heterogeneity was suggested to be present in porcine plasma (Falk et al., 1985) and bovine plasma amine oxidases (Yasunobu et al., 1976).

The final preparations of porcine and bovine SSAO were pale yellow with a featureless spectrum and retained full activity for several months at 4 °C. Polyacrylamide gels of both the bovine and porcine SSAO showed several major bands were still present. Both enzymes displayed a high affinity for benzylamine with a K_m value for bovine SSAO of 16 μM (±2) and for the porcine SSAO of 8 μM (±1). Estimates of K_m values were obtained using a weighted least-squares linear regression program (Cleland, 1979). The bovine and porcine enzymes displayed similar levels of activity with a panel of amines (Table III). The enzymes had very low diamine oxidase activity with cadaverine and putrescine. Histamine and tryptamine were poor substrates while tyramine, β-phenethylamine, and methylamine were better, although they were oxidized at a rate lower than that of benzylamine. The higher turnover of methylamine by the porcine SSAO, relative to benzylamine, was due to substrate inhibition by benzylamine at 200 μM.

Porcine and bovine SSAO were inhibited by semicarbazide but the inhibition was less than observed with the commercially available copper-containing bovine plasma amine oxidase. The plasma enzyme was fully inactivated after 2 h at 10 μM semicarbazide, while only 30% of the bovine SSAO and 40% of the porcine SSAO activity was inhibited under identical conditions. Even after 24 h at 4 °C with 10 μM semicarbazide, approximately 20% of the bovine and porcine SSAO was still active. At 100 μM semicarbazide, both SSAO enzymes and the bovine plasma amine oxidase were almost completely inactivated (95–97%) after 2 h at 4 °C. The inhibition of SSAO was irreversible as demonstrated after removal of excess semicarbazide. Inhibition of SSAO's by phenylhydrazine was also irreversible. The formation of a yellow-colored phenylhydrazone adduct, with an absorption maximum near 420 nm, was concomitant with the inhibition of bovine SSAO.

Stereochemistry of Tyramine Oxidation

Proton Abstraction at C-1. Chiral C-1 deuterated tyramines were obtained enzymatically by decarboxylation of C-1 protonated and deuterated tyrosine using tyrosine decarboxylase from *S. faecalis*. Since the reaction of this enzyme is known to proceed with greater than 95% retention of configuration with tyramine (Battersby et al., 1980), parallel incubations can be used to obtain both 1(*R*)-^{2}H and 1(*S*)-^{2}H amines. The ^{1}H-NMR spectra of both [1(*S*)-^{2}H]- and [1(*R*)-^{2}H]tyramines obtained in this manner confirm the presence of greater than 95% deuterium incorporation at the C-1 position of each amine

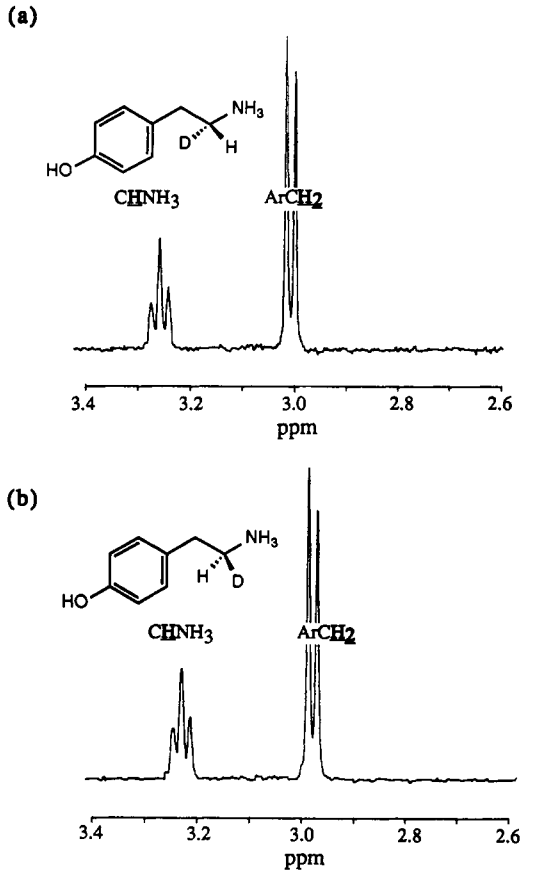
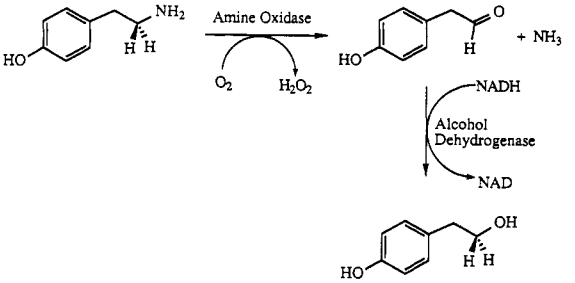


FIGURE 1: Partial 360-MHz ^{1}H-NMR spectra of (a) [1(*R*)-^{2}H]-tyramine and (b) [1(*S*)-^{2}H]tyramine derived from incubations of tyrosine decarboxylase.

Scheme I: Amine Oxidase Reaction Coupled to Alcohol Production via Alcohol Dehydrogenase



(Figure 1). The signal attributed to the C-1 proton is a triplet near 3.2 ppm, while the signal for the C-2 protons is a doublet near 3.0 ppm.

In the stereochemical studies, amines were converted to aldehyde products by amine oxidases. The aldehydes were then coupled directly to alcohol production using alcohol dehydrogenase and NADH to eliminate nonenzymatic oxidation and Schiff base formation (Scheme I). The deuterium content of the *p*-hydroxyphenethyl alcohol products compared to the substrates served as a marker for the steric course of the reaction. Retention of deuterium in product alcohols resulted in an ^{1}H-NMR spectrum consisting of a triplet attributed to the C-1 proton signals and a doublet for the C-2 proton signals, similar to the original amine but with chemical shifts near 3.7 ppm and 2.8 ppm, respectively. A loss of deuterium resulted in an alcohol with a triplet signal for both C-1 and C-2 protons, with shifts near 3.7 ppm and 2.8 ppm, respectively.

Tyramine oxidation occurred predominantly with the cleavage of the *pro-S* proton by both the bovine and porcine

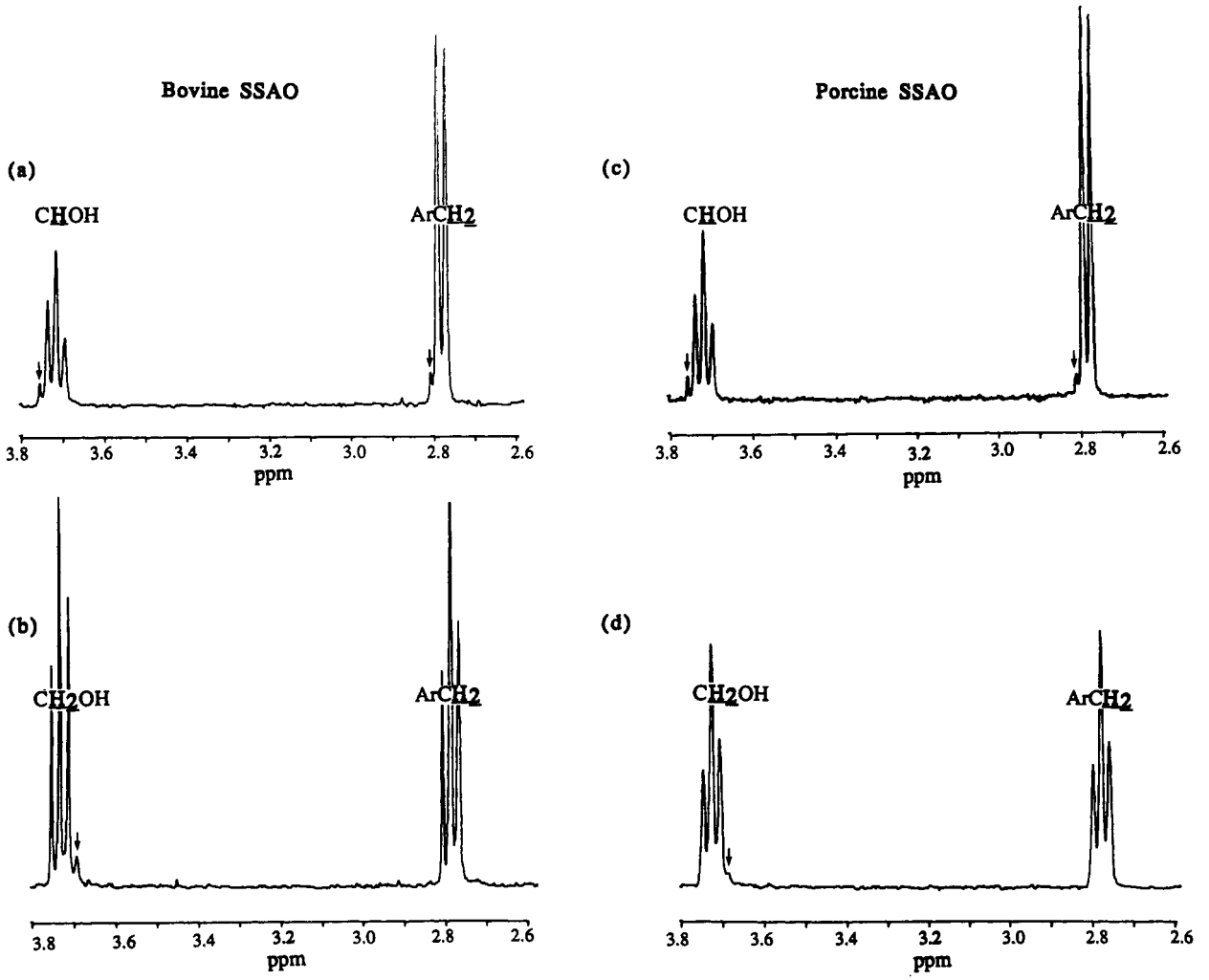


FIGURE 2: Partial 360-MHz ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols isolated from coupled incubations of [1(*R*)-^{2}H]tyramine [(a) and (c)] and [1(*S*)-^{2}H]tyramine [(b) and (d)] with bovine and porcine SSAO.

SSAO. The spectra of the alcohols obtained from [1(*R*)-^{2}H]tyramine exhibited mainly a triplet near 3.7 ppm and a doublet near 2.8 ppm indicating that the majority of the deuterium had been retained (Figure 2a,c). However, minor signals (marked by arrows) corresponding to the downfield peaks of two triplets from fully protonated product are also present at approximately the same chemical shifts. Assignment of these minor signals to fully protonated alcohol was confirmed via spin decoupling experiments. This indicates that a small amount of deuterium, and therefore *pro-R* specific abstraction, had occurred. The major signals arising from the monodeuterated alcohol are shifted 0.02 ppm upfield due to an α- and β-deuterium isotope effect (Bernheim & Batiz-Hernandez, 1966).

The NMR spectra of the alcohol products for each enzyme derived from [1(*S*)-^{2}H]tyramine are composed mainly of triplets near 3.7 ppm and near 2.8 ppm, indicating that the majority of the deuterium had been lost (Figure 2b,d). Again, the stereochemical course of the reaction was not absolute, as a small amount of monodeuterated product was isolated from the reaction. This minor product was more difficult to detect, since the doublet near 2.8 ppm is completely masked by the triplet; however, the upfield peak of the minor triplet near 3.7 ppm can be seen (marked by an arrow) as it is shifted 0.02 ppm upfield from the larger triplet of the major product.

Duplicate incubations of [1(*R*)-^{2}H]tyramine with bovine SSAO resulted in approximately 7% and 11% diprotonated product, while 91% and 89% of the product was diprotonated when [1(*S*)-^{2}H]tyramine was used as the starting material. Similarly, for the porcine SSAO, 13% and 11% of the product was diprotonated when [1(*R*)-^{2}H]tyramine was used as substrate while 90% and 86% of the product was diprotonated when [1(*S*)-^{2}H]tyramine was the substrate. While it is difficult to estimate accurately the amount of minor product formed in the reaction, the consistent occurrence of these minor products would appear to reflect on the enzymes stereospecificity. The retention and loss of deuterium in the products from the *S*- and *R*- modes is complementary for both enzymes, and the presence of an isotope in the scissile bond did not appear to affect the stereochemical course of the reaction.

Imine/Enamine Tautomerization. Incubations with protonated tyramine and deuterated buffer were used to establish whether the bovine and porcine SSAO exhibit the imine/enamine pathway. As initial experiments established that both enzymes catalyzed a nearly complete exchange of protons at C-2 of tyramine with deuterated solvent, the stereochemistry of the wash-out and wash-in reactions was examined.

[2(R)-^{2}H]- and [2(S)-^{2}H]Tyramine. The stereochemical course of the wash-out reaction of the SSAO was determined using chiral C-2 deuterated tyramines. These amines were obtained by exploiting the known specificity of the bovine plasma amine oxidase wash-in reaction which is reported to proceed with *pro-R* incorporation of solvent into the C-2 position of dopamine (Farnum & Klinman, 1986). From tritiated solvent, 95% of C-2 *pro-R* labeled alcohol was obtained. Similarly, bovine plasma amine oxidase was found to catalyze

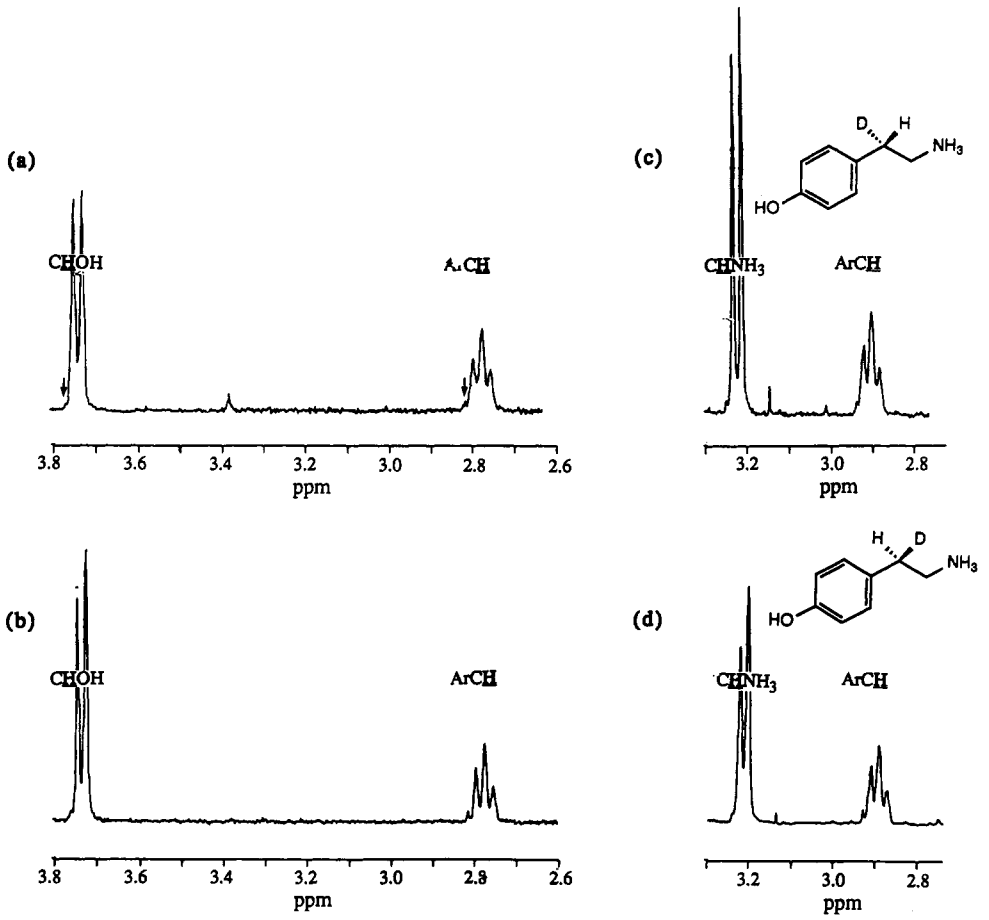


FIGURE 3: Partial 360-MHz ^{1}H-NMR spectra of [2(*R*)-^{2}H]-*p*-hydroxyphenethyl alcohol (a) and [2(*S*)-^{2}H]-*p*-hydroxyphenethyl alcohol (b) derived from coupled incubations with bovine plasma amine oxidase, and spectra of corresponding amines [(c) and (d)].

pro-R incorporation of 0.8 deuterium atom at C-2 of tyramine (Coleman et al., 1989). Therefore, the coupled reaction of bovine plasma amine oxidase in a deuterated buffer with [2,2-^{1}H]tyramine with alcohol dehydrogenase resulted in [2(*R*)-^{2}H]-*p*-hydroxyphenethyl alcohol while the reaction in protonated buffer with [2,2-^{2}H]tyramine resulted in [2(*S*)-^{2}H]-*p*-hydroxyphenethyl alcohol. In the present study, integration of the spectra of [2(*R*)-^{2}H]-*p*-hydroxyphenethyl alcohol indicated that approximately 1 deuterium atom had been washed into the C-2 position, yielding approximately 95% of the monodeuterated species, while 5% was diprotonated (Figure 3a). The doublet near 3.7 ppm and the large triplet near 2.8 ppm are attributed to the C-1 and C-2 signals originating from the monodeuterated product. These signals are shifted slightly upfield from two smaller triplets, attributed to the diprotonated species. Only the downfield peak of each triplet from the diprotonated alcohol is visible (marked with arrows). Integration of the spectra of [2(*S*)-^{2}H]-*p*-hydroxyphenethyl alcohol from the wash-in reaction in protonated buffer indicated that approximately 0.9 proton had been washed in to C-2; however, the C-1 doublet near 3.7 ppm was skewed, indicating that a significant amount of dideuterated alcohol was present (Figure 3b). It was estimated that about 70% of the product from the reaction was monodeuterated, 10% was diprotonated, and 20% was dideuterated. The enzyme, therefore, appears to be less stereospecific when the wash-out reaction involved the removal of deuterium from C-2, rather than incorporation of deuterium from solvent. It was noted that, in some cases, an altered ratio of dideuterated, monodeuterated, and diprotonated alcohols were produced in incubations left overnight, compared to incubations carried out for 3–6 h.

The C-2 chirally deuterated alcohols obtained from the bovine plasma amine oxidase reactions were chemically converted to amines using the protocol outlined in Experimental Procedures. Yields from the reaction varied from 25% to 45%. No scrambling or loss of deuterium label occurred during the conversion, as determined from integration of the ^{1}H-NMR signals of C-1 and C-2 protons of starting alcohol compared to amine product (Figure 3c,d). The signals for the C-1 and C-2 protons of the monodeuterated amine are attributed to the doublet near 3.2 ppm and the triplet near 2.9 ppm, respectively.

Wash-Out Reaction. The porcine aortic SSAO was found to exhibit a preference for abstraction of the *pro-R* proton from C-2 of tyramine. The spectrum of the alcohol product of [2(*R*)-^{2}-]tyramine is comprised of a triplet near 3.7 ppm and a triplet near 2.8 ppm, indicating that the deuterium had been lost during the reaction (Figure 4a). A slightly reduced integration for the C-2 signal at 2.8 ppm of 1.95 protons indicated that a small amount of monodeuterated product may be present; however, the expected signal from the triplet, shifted slightly upfield from the major product near 2.8 ppm, was not visible. As well, the doublet for the monodeuterated signal near 3.7 ppm would be completely hidden under the triplet, but integration of the individual peaks of the triplet indicated a deviation from the expected 1:3:1 ratio for the peaks. With these factors considered, it was estimated that 5% to 10% of the reaction proceeded with *pro-S* proton abstraction while the majority of the reaction occurred with removal of the *pro-R* hydrogen.

The corresponding spectrum of the alcohol products obtained from [2(*S*)-^{2}H]tyramine exhibited a doublet superimposed on a small triplet near 3.7 ppm for the C-1 signal

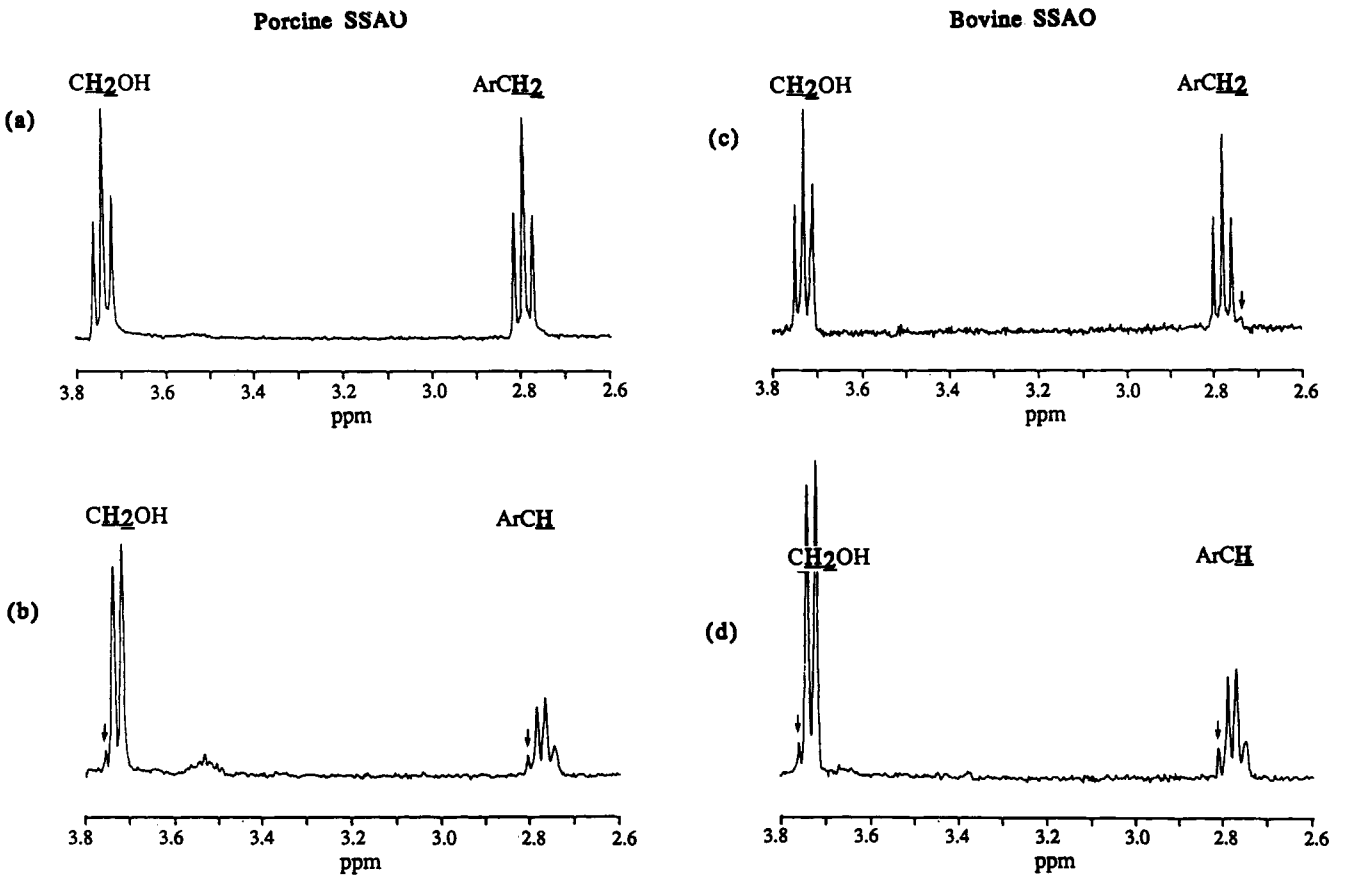


FIGURE 4: Partial 360-MHz ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols from coupled incubations of [2(*R*)-^{2}H]tyramine [(a) and (c)] and [2(*S*)-^{2}H]tyramine [(b) and (d)] with porcine and bovine SSAO.

and a large triplet overlapping a smaller triplet near 2.8 ppm for the C-2 signal (Figure 4b). Minor signals (marked by arrows), corresponding to the downfield peaks of two triplets arising from fully protonated product, indicate that a small amount of product was diprotonated. The large doublet near 3.7 ppm and the large triplet near 2.8 ppm arise from the monodeuterated alcohol and indicate that the majority of deuterium had been retained during the reaction. The major signals are shifted 0.02 ppm upfield. Integration of the signals indicated that 0.9 deuterium atoms were retained at C-2 and that 85% of the product was of the monodeuterated species. Approximately 15% of the product was the diprotonated species, while the amine substrate contained 10% diprotonated alcohol product, indicating that a small amount of the reaction occurred with removal of the *pro-S* hydrogen at C-2.

The bovine aortic SSAO showed the same stereochemical preference as the porcine enzyme, as mainly the *pro-R* proton was abstracted from C-2 of tyramine. The spectrum of the alcohol product of [2(*R*)-^{2}H]tyramine was composed of a triplet near 3.7 ppm and a triplet near 2.8 ppm, indicating that a significant portion of the deuterium had been lost, as expected (Figure 4c). The upfield peak of the triplet arising from the C-2 signal of monodeuterated alcohol is present near 2.8 ppm (marked with an arrow). Integration of the C-2 signal near 2.8 ppm of 1.9 protons indicated that approximately 10% of the product was monodeuterated while 90% of the product was diprotonated.

The spectrum of the alcohol product from the bovine SSAO reaction with [2(*S*)-^{2}H]tyramine was very similar to that of the porcine enzyme, exhibiting a doublet superimposed on a triplet near 3.7 ppm and a large triplet overlapping a smaller triplet near 2.8 ppm (Figure 4d). The large doublet and upfield triplet arise from the monodeuterated alcohol, indicating that the majority of deuterium had been retained. Minor signals (marked with arrows) corresponding to the downfield peaks of two triplets from fully protonated product indicate that a small amount (≅0.2 atom) of deuterium had been lost from C-2. Integration of the signals indicated that 85% of the product was the monodeuterated species while 15% was the diprotonated species, suggesting that a small amount of the reaction had occurred with removal of the *pro-S* hydrogen, as observed for the porcine SSAO.

Configurational Analysis of C-2 Protons. To establish the stereospecificity of the wash-in reaction, a methodology was required to distinguish between the *pro-R* and *pro-S* protons at C-2 of tyramine. Derivatizing the alcohols with a chiral agent, (*S*)-(+)-*O*-acetylmandelic acid, created nonequivalence of each proton at C-2 and allowed each to be observed by a distinct chemical shift in the NMR spectrum. While similar methodology has been used to establish the absolute configuration of carbon atoms immediately adjacent to the derivatizing agent at C-1, it may also be used to distinguish between protons of the C-2 carbon (Huang et al., 1986; Casati et al., 1987) and of polyhydroxyl compounds (Ohtani et al., 1991).

The method of derivatization and sample purification was simple and rapid, and it could be completed in approximately 3 h. The reaction was amenable to a small scale as typically 1 mg of alcohol was used, although the reaction was equally successful when 0.3–0.5 mg of alcohol was used. Two molar equivalents of (*S*)-(+)-*O*-acetylmandelic acid and dicyclohexylcarbidiimide were required as both the phenyl ring hydroxyl and the aliphatic C-1 hydroxyl were esterified. There was no evidence of preferential reactivity of one alcohol enantiomer compared to the other, similar to results reported for other aromatic and aliphatic alcohols (Parker, 1983; Whitesell & Reynolds, 1983).

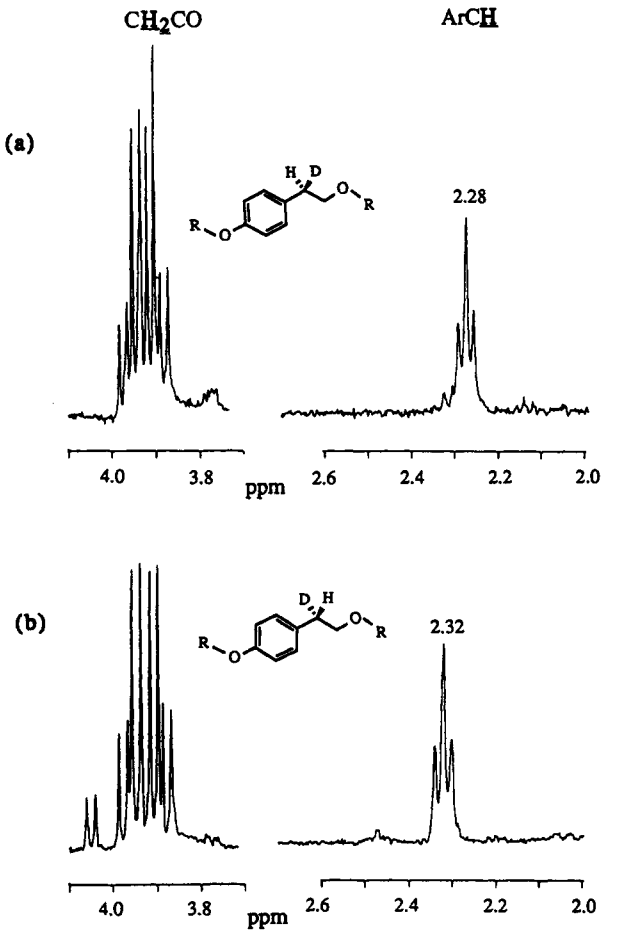


FIGURE 5: Partial 360-MHz ^{1}H-NMR spectra of (*S*)-(+)-*O*-acetylmandelic acid derivatives of (a) [2(*S*)-^{2}H]-*p*-hydroxyphenethyl alcohol and (b) [2(*R*)-^{2}H]-*p*-hydroxyphenethyl alcohol. The group R represents the acetylmandelate portion of the ester.

C-2 chiral alcohols obtained from the bovine plasma amine oxidase wash-in reaction in protonated and deuterated buffers were used to determine the absolute chemical shifts of the *pro-R* and *pro-S* protons of the (*S*)-(+)-*O*-acetylmandelate ester. From the spectra of the ester derivatives of [2(*R*)-^{2}H]- and [2(*S*)-^{2}H]-*p*-hydroxyphenethyl alcohol, the chemical shift of the C-1 signal of the alcohol occurred near 3.9 ppm while the C-2 signal occurred at 2.32 ppm and 2.28 ppm, respectively (Figure 5). Therefore, the C-2 *pro-R* hydrogen resonated to the high field of the *pro-S* hydrogen, as had been found for C-1 protons of a number of aliphatic and aromatic alcohols esterified with (*S*)-(+)-*O*-acetylmandelic acid (Parker, 1983). Derivatives with the (*R*)-(−)-*O*-acetylmandelic acid were also prepared (spectra not shown). The ^{1}H-NMR chemical shifts for the C-2 signal of the *pro-R* and *pro-S* deuterated derivatives were 2.28 and 2.32 ppm, respectively, which was the reverse of the (*S*)-(+)-*O*-acetylmandelic derivatives, as expected.

Wash-In Reaction. The solvent wash-in behavior of bovine and porcine SSAO was determined by reacting [2,2-^{1}H]-tyramine in deuterated buffer, or [2,2-^{2}H]tyramine in protonated buffer. The alcohol products were then derivatized with (*S*)-(+)-*O*-acetylmandelic acid, and the chemical shifts of the C-2 proton signals were compared to the standard 2(*R*)-^{2}H or 2(*S*)-^{2}H derivatives.

The wash-in reaction catalyzed by the bovine SSAO with deuterated buffer resulted in approximately 1 deuterium atom being incorportated, yielding 95% monodeuterated alcohol product with the remaining product being diprotonated (Figure 6a). This is seen on the ^{1}H-NMR spectra as a large doublet signal near 3.7 ppm for the C-1 protons and a large triplet near 2.8 ppm for the C-2 protons. The configurational analysis of the C-2 protons indicated that a solvent deuterium has been incorporated predominantly with *pro-R* specificity, resulting in a 2(*R*)-^{2}H derivative with a chemical shift for the signal of the C-2 proton occurring at 2.32 ppm (Figure 6b).

Alternatively, the products of the bovine wash-in reaction with protonated buffer and [2,2-^{2}H]tyramine were found contain approximately 1 proton at C-2, yielding 10% diprotonated and 90% monodeuterated alcohol (Figure 6c). Configurational analysis of the alcohol products indicated that the monodeuterated species was the 2(*S*)-^{2}H ester derivative with a chemical shift for the C-2 proton signal occurring at 2.28 ppm, again indicating a preference for *pro-R* specific wash-in (Figure 6d). As well, the small amount of the ester of the diprotonated product was visible in the NMR spectrum (marked by an arrow).

The wash-in reaction of the porcine SSAO was very similar to that of the bovine SSAO (data not shown). The reaction in deuterated buffer with [2,2-^{1}H]tyramine yielded 95% monodeuterated alcohol product, with the residual product being diprotonated. Again, as with the bovine SSAO, configurational analysis indicated that the monodeuterated alcohol was due to incorporation of deuterium to the *pro-R* position. The chemical shift of the ^{1}H-NMR signal for the C-2 protons was at 2.32 ppm, as seen for the 2(*R*)-^{2}H standard.

The protonated buffer wash-in reaction catalyzed by the porcine SSAO yielded 5% dideuterated, 85% monodeuterated, and 10% diprotonated alcohol product. Nonetheless, the C-2 ^{1}H-NMR signals from the (*S*)-(+)-*O*-acetylmandelic ester of the monodeuterated alcohol occurred at a chemical shift of 2.28 ppm, indicating that it was the 2(*S*)-^{2}H derivative and that wash-in had been mainly *pro-R*, as expected. Again, a small amount of diprotonated alcohol ester was visible in the NMR spectrum.

DISCUSSION

Partial Purification and Characterization of Aortic SSAO

A partial physiochemical characterization of the bovine and porcine SSAO indicated that these aortic enzymes displayed characteristics typical of other SSAO enzymes. The majority of the activity was membrane bound and required detergent in the extraction buffer to release significant amounts of activity. The bovine and porcine enzymes adhered strongly to Concanavalin A and less so to *Lens culinaris* lentil lectin, indicating that they are glycoproteins, as observed for other SSAO's.

Typically, both the bovine and porcine SSAO displayed low K_m values for benzylamine of 16 and 8 μM, respectively. Such values are similar to those for SSAO from several sources and significantly lower than the K_m value for the plasma enzyme of each animal, which is 1.69 mL for bovine plasma (Palcic & Klinman, 1983) and 90 μM for porcine plasma amine oxidase (Gorkin, 1983). A comparison of various substrates indicated that SSAO had the highest affinity and turnover rate with benzylamine. Benzylamine, however, does not occur naturally in mammalian metabolism. It has been suggested that methylamine, a byproduct of bacterial or endogenous metabolism of creatinine, lecithin, sarcosine, or choline, may be one substrate of the enzyme in vivo (Precious et al., 1988).

One of the most controversial areas in amine oxidase research concerns the nature of the cofactor of the nonflavin enzymes. The nonflavin amine oxidases have been suggested to contain a carbonyl cofactor, possibly pyridoxal phosphate,

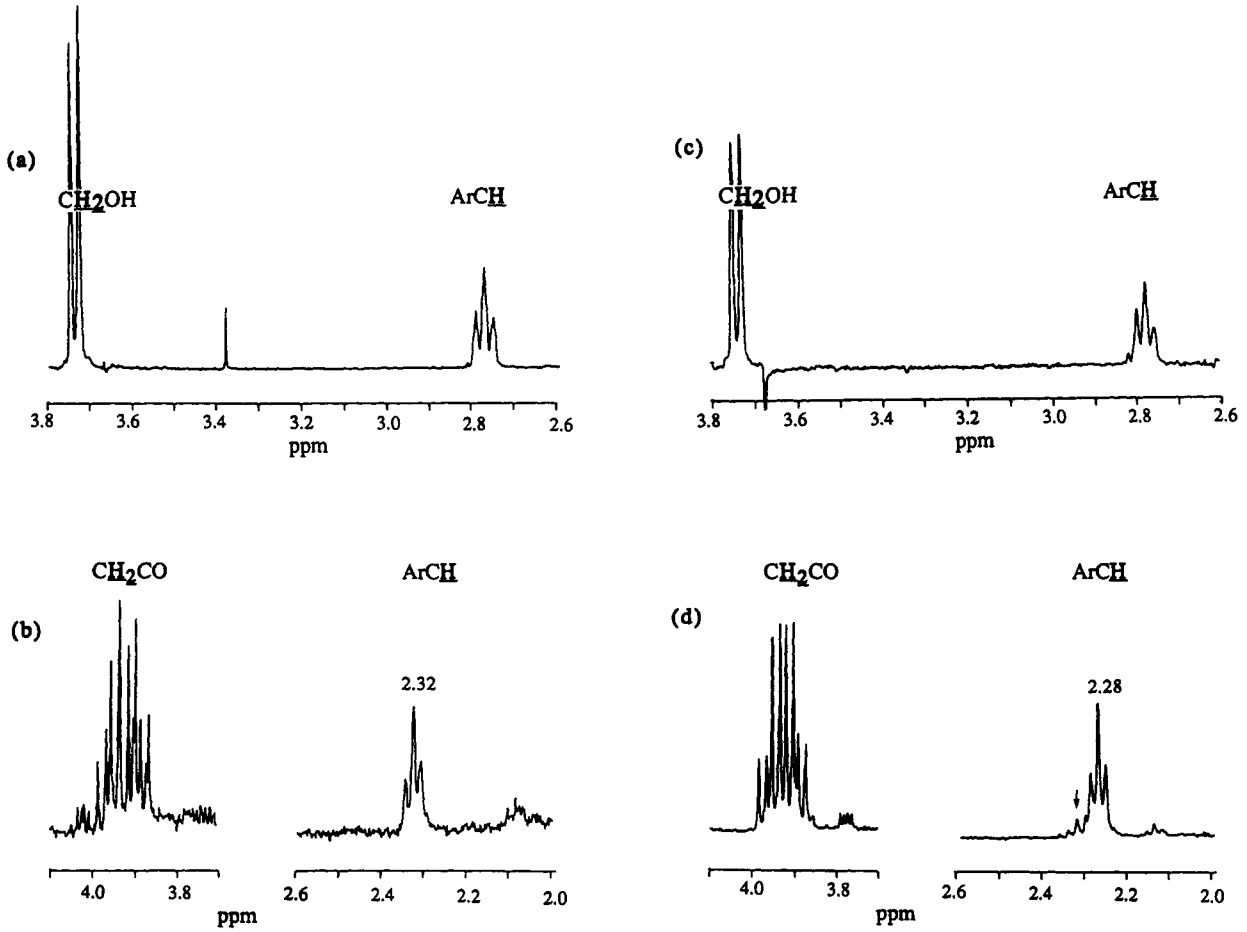


FIGURE 6: Partial 360-MHz ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols isolated from coupled incubation of bovine SSAO with [1,1-^{1}H]tyramine in ^2H_2O (a) and [1,1-^{2}H]tyramine in H_2O (c), and spectra of the (*S*)-(+)-*O*-acetylmandelic acid derivatives of the alcohols [(b) and (d)].

since they were sensitive to the carbonyl reagents semicarbazide and phenylhydrazine. Pyrroloquinoline quinone was also suggested to be the cofactor of several oxidases, including lysyl oxidase (Van der Meer & Duine, 1986), pig kidney diamine oxidase (Van der Meer et al., 1986), methylamine oxidase (Van Ierel et al., 1986), and pea seedling amine oxidase (Glatz et al., 1987) on the basis of indirect evidence. However, these assignments appear to be incorrect, as conclusive identification of the cofactor of bovine plasma amine oxidase as TOPA was recently obtained (Janes et al., 1990). Spectral properties of enzyme–phenylhydrazone adducts suggested that TOPA may also be present in other copper amine oxidases, including porcine kidney and pea seedling amine oxidase (Brown et al., 1991). While identification of the cofactor of the SSAO's has not been established, the preliminary characterization of SSAO in this work suggests that the cofactor is a carbonyl-containing molecule.

Phenylhydrazine and semicarbazide were both irreversible inhibitors of SSAO, confirming the carbonyl nature of the cofactor. However, the reactivity of the SSAO enzymes with semicarbazide was distinct from that of the bovine plasma amine oxidase. The bovine and porcine SSAO were inhibited by 100 μM semicarbazide within 2 h at 4 °C, or by 10 μM after 24 h at 4 °C. It is of interest to note however, that despite the nomenclature of "semicarbazide sensitive" for the aortic enzyme, the bovine plasma amine oxidase reacted much more quickly with the semicarbazide than did the SSAO enzymes. This would suggest that the nature or the accessibility of the cofactors of the two enzymes are not equivalent.

Copper amine oxidases are characterized by a pink or peach color, with an absorption maximum near 480 nm attributable to the organic cofactor (Yamada & Yasunobu, 1962). In the present work, both SSAO enzymes exhibited a featureless spectrum and were pale yellow in color. Since the enzymes were not homogeneous, the true spectral properties of the enzyme may have been masked, but the lack of the absorbance at 480 nm may reflect an altered environment for the cofactor in the active site or the presence of a modified cofactor.

Stereochemical Course of Tyramine Oxidation

Both the bovine and porcine SSAO were found to exhibit a preference for abstraction of the *pro-S* proton at C-1 of tyramine, although a limited amount of the reaction (5–10%) occurred with removal of the *pro-R* proton. This stereochemistry is consistent with that reported for the oxidation of benzylamine (Yu & Davis, 1988) and dopamine (Yu, 1987) by SSAO from rat aorta. Therefore, all aortic SSAO enzymes examined thus far exhibit a consistent stereochemical reaction course regardless of species, and for substrates with and without a β-methylene group.

The minor amount of *pro-R* proton abstraction at C-1 of tyramine may have been due to the presence of a second amine oxidase activity, but this is unlikely since similar results were obtained with enzyme preparations at differing levels of purity. It is also unlikely that incomplete coupling of aldehyde to alcohol production could have been responsible for the results, since at least a 10-fold concentration of alcohol dehydroge-

nase and a 3-fold excess of NADH was used. Rather, the amount of *pro-R* specificity observed was consistent between incubations, suggesting that the behavior was intrinsic to the enzyme.

There is precedence in the amine oxidase literature which suggests that the small amount of scrambling observed in our stereochemistry studies is not unusual. The pea seedling copper amine oxidase catalyzed the oxidation of [1(*R*)-^{3}H,^{14}C]-benzylamine with 100 ± 3% ^{3}H retention and [1(*S*)-^{3}H,^{14}C]-benzylamine with 2 ± 0.5% retention of ^{3}H (Battersby et al., 1976). Similarly, the bovine plasma amine oxidase displayed a small amount of stereochemical variability in the oxidation of benzylamine (Battersby et al., 1979). With [1(*S*)-^{3}H,^{14}C]-benzylamine as a substrate, 4.6% of the ^{3}H was retained in the product while 96% of the ^{3}H was retained when [1(*R*)-^{3}H,^{14}C]benzylamine was the substrate. As well, a more significant lack of absolute stereochemistry was noted for the oxidation of (*R*)- and (*S*)-3-[1-^{2}H]methylbutylamines by the bovine plasma enzyme (Shibuya et al., 1990). In this case, the enzyme was found to mainly catalyze the removal of the *pro-S* hydrogen; however a significant loss of the *pro-R* hydrogen was observed. With the 1(*S*)-deuterated methylbutylamine as a substrate, deuterium was lost from 66% of the product and retained in 34%. The products isolated from the 1(*R*) deuterated substrate had essentially the opposite ratio, with deuterium being lost from 32% and retained in 68%. The stereochemical course of the reaction of bovine plasma amine oxidase was not influenced by the presence of an isotope, and the ratios of monodeuterated and diprotonated products obtained from the 1(*R*)- and 1(*S*)-deuterated methylbutylamines were complementary.

The limited amount of nonstereospecificity in the SSAO reaction can be accounted for in different ways. The substrate may be able to bind in two productive modes, such that either of the C-1 protons of tyramine may be abstracted, with the ratio of *pro-R* to *pro-S* proton removal being dependent on the populations of the two modes. While the presence of such dual binding modes has been suggested for bovine plasma amine oxidase to account for its apparent nonstereospecific behavior with dopamine (Summers et al., 1979) and tyramine (Coleman et al., 1989), this is a rare phenomenon in enzymology. Moreover, the mode of reaction of the bovine plasma amine oxidase is strongly influenced by the presence of the isotope in the scissile bond, while we have shown that the mode of the SSAO reaction is independent of the presence of deuterium.

Alternatively, only a single binding mode for tyramine may be available in the SSAO active site, and the base responsible for C-1 abstraction may be positioned for efficient abstraction of only the *pro-S* proton. The *pro-R* proton may face away, allowing only limited access. This theory has been put forward to explain the stereochemical behavior of serine hydroxymethyltransferase and tryptophan synthase (Malthouse et al., 1991). These enzymes have been shown to lack absolute stereospecificity for the abstraction of the α-protons of glycine. Serine hydroxymethyltransferase was found to abstract the *pro-S* proton 7400 times faster than the *pro-R* proton while tryptophan synthase preferentially catalyzed the exchange of the *pro-R* proton of glycine 380 times faster than the *pro-S* proton. Recent work with serine hydroxymethyltransferase using 2-aminomalonic acid as a substrate has confirmed the presence of a single binding mode in this enzyme (Thomas et al., 1991).

The stereochemical preference of SSAO to abstract the C-1 *pro-S* proton of a substrate is similar to that exhibited by the majority of the copper amine oxidases studied to date. Copper amine oxidases have been found to catalyze the removal of the *pro-S* hydrogen at C-1 more than any other reaction course. Examples of these include the pea, chickpea, and soybean seedling enzymes, the pig kidney diamine oxidase and the bovine plasma amine oxidase for benzylamine, *p*-hydroxybenzylamine and 3-methylbutylamine (Coleman et al., 1991; Battersby et al., 1979; Suva & Abels, 1978; Shibuya et al., 1990). Even in the case of the apparent nonstereospecific proton abstraction from C-1 of dopamine catalyzed by the bovine plasma amine oxidase, removal of the *pro-S* proton was suggested to be the preferred stereochemical course of the reaction (Farnum & Klinman, 1986).

Table IV: Stereochemistry at C-1 of Tyramine and Solvent Exchange Characteristics at C-2 Catalyzed by Semicarbazide-Sensitive and Copper Amine Oxidases

enzyme source	C-1 proton abstraction	C-2 solvent exchange
Semicarbazide-Sensitive Amine Oxidases		
porcine aorta	*pro-S*[a]	yes
bovine aorta	*pro-S*[a]	yes
Copper Amine Oxidases (EC 1.4.3.6)		
pea seedling	*pro-S*[b]	no
porcine kidney	*pro-S*[c]	no
soybean seedling	*pro-S*[c]	no
chick pea seedling	*pro-S*[c]	no
porcine plasma	*pro-R*[b]	yes
bovine plasma	nonstereospecific[b]	yes
sheep plasma	nonstereospecific[c]	yes
rabbit plasma	nonstereospecific[c]	yes

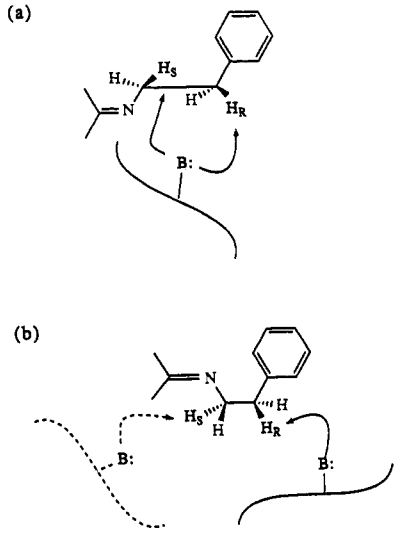
[a] This work. [b] Coleman et al. (1989). [c] Coleman et al. (1991).

The SSAO's are distinguished from the FAD-dependent monoamine oxidases, which catalyze the same type of reaction as the copper-containing and semicarbazide-sensitive enzymes. The FAD-containing enzymes have been found to oxidize amines with the abstraction of the *pro-R* proton at C-1 (Belleau et al., 1960). This is true of FAD monoamine oxidases that have been isolated from a variety of tissues and different animal species (Yu et al., 1986) as well as for aliphatic and aromatic substrates (Battersby et al., 1979). In addition, the monoamine oxidases do not catalyze the exchange of protons at C-2 of dopamine with solvent protons (Lovenberg & Beaven, 1971).

Imine/Enamine Tautomerization

Both SSAO's were found to preferentially catalyze the *pro-R* stereospecific wash-out and wash-in of protons at C-2 of tyramine with solvent. The presence of the solvent exchange pathway, together with the *pro-S* specificity of proton removal at C-1, is sufficient to distinguish the SSAO enzymes from the copper amine oxidases (Table IV). To date, the copper amine oxidases, which have exhibited *pro-S* proton abstraction at C-1 of tyramine, have not catalyzed the imine/enamine tautomerization reaction with tyramine. For copper amine oxidases showing an alternate stereochemical mode of proton abstraction at C-1 but which do catalyze the C-2 proton exchange, different amounts of deuterium are incorporated compared to the SSAO enzymes. For example, 0.8 deuterium atom was incorporated by bovine plasma amine oxidase while 1.2 atoms were incorporated by the enzyme from porcine plasma (Coleman et al., 1989). The efficiency of the C-2 exchange process may be a function of the orientation of substrate in the enzyme active site. In particular, the position of the base(s) necessary for the proton abstractions, in the SSAO enzymes, may be altered from those of the copper amine oxidase enzymes.

Scheme II: Schematic Representation of the Active Site of the Semicarbazide-Sensitive Amine Oxidase for (a) syn or (b) anti Proton Abstraction from C-1 and C-2



Although the wash-out reaction occurred mainly via abstraction of the *pro-R* proton at C-2, a small amount of the reaction occurred by an alternate mode with abstraction of the *pro-S* proton, irrespective of the position of the deuterium label. The small amount of *pro-S* proton abstraction at C-2 corresponds well to the small amount of *pro-R* proton removal observed at C-1, suggesting that the 1-*S*, 2-*R* and 1-*R*, 2-*S* modes may be linked. However, further work with substrates chirally deuterated at both C-1 and C-2 will be required to establish this.

It is of interest to compare the stereochemical course of dopamine oxidation by the bovine plasma amine oxidase to that of the SSAO enzymes. While the bovine plasma amine oxidase exhibits nonstereospecific wash-out of protons at C-2 of dopamine (Summers et al., 1979), isotope labeling at C-1 was found to introduce a stereochemical selectivity in the abstraction of the C-2 proton (Farnum & Klinman, 1986). For example, abstraction of the 1(*R*) and 2(*S*) protons occurred together, as did the abstraction of the 1(*S*) and 2(*R*) protons. However, the latter stereochemical mode was suggested to be the preferred binding mode of dopamine as determined from isotope effects. A fully expressed isotope effect for catalysis of the [1(*S*)-^{2}H]dopamine indicated that the 1(*S*) and 2(*R*) protons of the substrate were correctly aligned in the active site for catalysis and required no reorientation. As this is the same stereochemical mode followed by the SSAO enzymes in this work, it suggests some degree of relatedness between the two enzymes.

This stereochemical course of proton abstraction and exchange at C-1 and C-2 catalyzed by SSAO may be realized with either a syn or anti conformation, utilizing a single base or two bases, respectively (Scheme II). A syn relationship is suggested if assumptions about the active site conformation of the bovine plasma enzyme, described by Farnum and Klinman (1986), are valid for SSAO. These researchers constructed a model which established a fixed geometry for the hydrogen and nitrogen bonds at C-1 that undergo cleavage, and accounted for the *pro-S* proton abstraction from C-1 of benzylamine and the dual binding modes of dopamine. Their model also limited interactions between the aromatic ring of the substrate and the pyridine ring of the cofactor, then suggested to be PQQ. However, our work with the SSAO enzymes cannot distinguish between distinct binding modes or a single binding mode with some loss of hydrogen from the *pro-R* position. As well, the nature of the cofactor of the SSAO is not known. Therefore, an anti conformation is also possible to account for the proton abstractions at C-1 and C-2.

For both the bovine and porcine SSAO, the wash-in reaction in protonated and deuterated buffer occurred predominantly with *pro-R* specificity, just as wash-out had been *pro-R* specific. This suggests that wash-in and wash-out occur from the same side of the imine double bond and that the two processes are the reverse of each other, unlike the reaction catalyzed by the bovine plasma amine oxidase which is nonstereospecific for C-2 proton abstraction with reprotonation occurring to the *pro-R* position (Farnum & Klinman, 1986). These results strongly suggest that a single base is involved in the C-2 exchange processes of the SSAO's. Whether this base is also responsible for proton abstraction at C-1 remains to be determined.

Summary

The pattern of C-1 and C-2 proton abstraction by the SSAO's is unique when compared to the stereochemical courses reported for all other amine oxidases examined to date. The SSAO reaction occurs preferentially with removal of the *pro-S* hydrogen from C-1 of tyramine. Tyramine oxidation occurs with solvent exchange into C-2 of product *p*-hydroxyphenethyl alcohol, and both loss of a proton from substrate and reprotonation occur mainly with *pro-R* specificity. The copper amine oxidases, which also catalyze *pro-S* proton abstraction at C-1, lack the solvent exchange pathway at C-2. The copper amine oxidases from bovine and porcine plasma, which do catalyze the solvent wash-in reaction, exhibit an apparent nonstereospecific proton removal at C-1 or *pro-R* specificity, respectively. The SSAO's are also distinct from the flavin-dependent amine oxidases, which exhibit a consistent stereochemical reaction course of *pro-R* proton abstraction from C-1 with no solvent exchange into C-2. This mechanistic information, in conjunction with the preliminary characterization of the cofactor of the SSAO enzymes, suggests that the SSAO enzymes are distinct from the flavin amine oxidases but show some similarities to the copper amine oxidases. Work is in progress to elucidate the cofactor structure of the SSAO enzymes.

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