Catalytic Turnover of Substrate Benzylamines by the Quinone-Dependent Plasma Amine Oxidase Leads to H_2O_2-Dependent Inactivation: Evidence for Generation of a Cofactor-Derived Benzoxazole†

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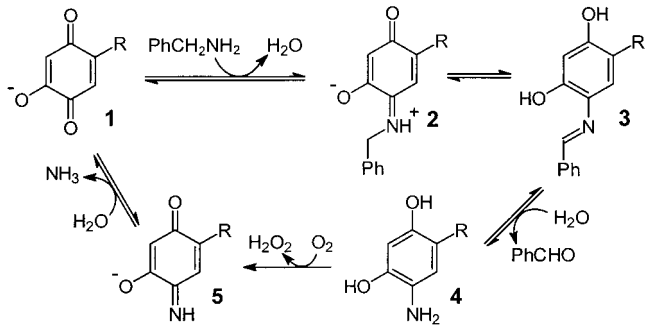
ABSTRACT: Incubation of bovine plasma amine oxidase (BPAO) with benzylamine and various *p*-substituted analogues results in a time-dependent inactivation that is attributable to buildup of the H_2O_2-turnover product on the basis of protection afforded by coincubation with catalase. The mechanism of inactivation is distinct from that effected by H_2O_2 itself, which requires higher concentrations. Solution studies using models for the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor reveal a loss of catalytic activity arising from oxidation of the dihydrobenzoxazole tautomer of the product Schiff base, that competes with hydrolytic release of benzaldehyde product. The resulting stable benzoxazole exhibits a characteristic absorption depending on the nature of the benzylamine *p*-substituent. For benzylamine itself, the model benzoxazole absorbs at 313 nm, in an area of strong absorption by the enzyme, whereas for 4-nitrobenzylamine, the absorption of the model benzoxazole is sufficiently red-shifted (at 365 nm) to be discerned above the background enzyme absorption. Inactivation of BPAO by 4-nitrobenzylamine is accompanied by loss of the resting TPQ anion absorption at 480 nm concomitant with generation of a new absorption near 360 nm. Resonance Raman spectra of the inactivated enzyme show a close correspondence with those for the model 4-nitrobenzylamine-derived benzoxazole. Substrate-dependent inactivation is also observed for the other two mammalian enzymes examined, equine plasma amine oxidase and human kidney amine oxidase. Catalase provides complete protection in these instances as well. Benzoxazole formation may constitute a common mechanism of inactivation of quinone-dependent amine oxidases by normal substrates in vitro if the product H_2O_2 is permitted to accumulate. More importantly, the results suggest that the benzoxazole inactivation pathway may be important physiologically and may have influenced the distribution of amine oxidases and catalase in cells.

Plasma amine oxidase is a member of a family of copper-containing mammalian and nonmammalian enzymes that convert unbranched primary amines to aldehydes by way of a quinone-dependent transamination mechanism (*1*, *2*). The quinone cofactor for most of these enzymes is a 2,4,5-trihydroxyphenylalanine quinone (TPQ), derived postranslationally from an active-site tyrosine residue (*2*). In the case of lysyl oxidase, the quinone cofactor is further derivatized by nuclear substitution of a nearby active-site lysine ϵ-amino group, giving the so-called lysine tyrosylquinone (LTQ) cofactor (*3*). During catalytic turnover, the primary amine is converted to aldehyde concomitant with reduction of O_2 to H_2O_2. A transamination mechanism has been confirmed by many studies and has been successfully simulated using

Scheme 1



models for the TPQ cofactor (*4*−*6*). As illustrated in Scheme 1 for benzylamine, condensation with quinone **1** generates substrate Schiff base **2**. Tautomerization of substrate Schiff base to product Schiff base **3** followed by hydrolysis affords the reductively aminated form **4**, which, concomitant with reduction of O_2 to H_2O_2, is oxidized to quinoneimine **5**, the latter undergoing hydrolysis to release ammonia and regenerate **1**. The role of the tri-histidine-bound active-site copper is to mediate biogenesis of the TPQ cofactor following ribosomal production of the apoenzyme (*7*, *8*) and to facilitate the O_2-dependent regeneration of the quinone cofactor (*9*).

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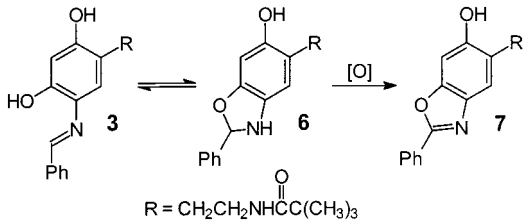
Scheme 2



Although secondary amines are not substrates of the enzyme, we reported that 3-pyrroline and its 3-aryl derivatives effect time-dependent mechanism-based inactivation of bovine plasma amine oxidase (BPAO)[1] (*10*). One criterion for a mechanism-based inactivator is that its interaction with the enzyme must be competitive with normal substrate processing (*11*), so that coincubation with, e.g., benzylamine, must exhibit a concentration-dependent protection against inactivation. However, in our initial efforts to demonstrate protection against inactivation by selected 3-aryl-3-pyrrolines, coincubation with increasing concentrations of benzylamine did not relieve the inhibition. In fact, we soon found that preincubation with benzylamine alone resulted in time-dependent loss of activity, at the relatively high concentrations being used. We first suspected that inhibition in the latter case arose from product inhibition by the turnover product benzaldehyde that accumulated during the incubation period. If this were the case, then protection by increasing [$PhCH_2NH_2$] should be observable if the enzyme assays were performed after gel chromatographic separation of enzyme from benzaldehyde and other small-molecule solutes. Since gel filtration did not lead to restoration in enzyme activity, we concluded that substrate benzylamine must itself be a time-dependent inactivator of BPAO.

In this paper, we describe experiments that implicate benzylamine and its ring-substituted analogues as mechanism-based inactivators of BPAO. We show that loss of activity is dependent on the buildup of H_2O_2 in the solution, but that H_2O_2 is not itself an inactivator at such concentrations. In thinking about possible explanations for enzyme inactivation by substrate, we considered the possible relevance of prior model studies, where the TPQ catalyst was lost irreversibly over time due to draining off the product Schiff base **3** through oxidation of its dihydrobenzoxazole tautomer to a benzoxazole (Scheme 2), which competes with catalytic recycling (*4*). Logically, the time-dependent inactivation of BPAO by benzylamine may reflect such conversion at the enzyme active site. As described herein, direct spectroscopic comparison between the modified enzyme and the corresponding synthetic benzoxazole models provides direct evidence that benzoxazole formation underlies the H_2O_2-dependent irreversible loss of activity during incubation of BPAO with substrate.

EXPERIMENTAL PROCEDURES

General Methods. NMR spectra were obtained on a Varian Gemini 300 instrument (^{13}C NMR at 75 MHz), with chemical shifts referenced to the solvent peak. High-resolution mass spectra (HRMS, electron impact) were obtained at 20−40 eV on a Kratos MS-25A instrument. UV−vis spectra were obtained using a jacketed (temperature-controlled) cell compartment and Perkin-Elmer PECSS software. Doubly distilled water was used for all experiments. All solvents, reagents, and organic fine chemicals were the most pure available from commercial sources. Bovine plasma amine oxidase (100 units/g of protein) used for the kinetics studies, bovine liver catalase (2700 units/mg of protein), and PDX G.F. 25 were purchased from Sigma. Bovine plasma amine oxidase used for spectroscopic and some kinetic studies was purified as described previously (*12*) and displayed properties consistent with high purity. Equine plasma amine oxidase (EPAO) was purified by a revised protocol (to be published elsewhere) based on that initially specified in ref *13*. Human kidney amine oxidase (HKAO) was obtained as described in ref *14*. During synthetic operations, all evaporations were conducted at reduced pressure using a rotary evaporator. Preparation and characterization of 6-hydroxy-2-phenyl-5-(2-pivalamidoethyl)benzoxazole (**9a**) (*4*) and 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone (**8**) (*5*, *6*) were described previously.

Table 1: Inactivation of BPAO by Benzylamine and Products of Benzylamine Oxidation[a]

compound	conc	incubation time (h)	% remaining activity[b]
benzylamine	3 mM	0.8	50 (47.5)[c]
benzylamine	3 mM	4	24 (6.1)[c]
benzylamine	3 mM	24	22 (6.0)[c]
benzylamine	4 mM	19	12
H_2O_2	3 mM	4	81
H_2O_2	6 mM	20	43
H_2O_2/catalase	3 mM/600 units/mL	4	99
benzylamine/catalase	3 mM/600 units/mL	4	79 (100)[d]
H_2O_2/PhCHO	1.5 mM/1.5 mM	24	88
H_2O_2	1.5 mM	24	75

[a] Incubations were at 30 °C in pH 7.2 sodium phosphate buffer with 4.4 μM BPAO (Sigma), unless specified otherwise. [b] Remaining activity was measured after gel filtration. Numbers listed are the average of two measurements that varied by less than 10%. [c] Using the homogeneous preparation of BPAO (5.0 μM) employed for spectroscopic studies and 3.35 mM benzylamine at 25 °C. [d] Using the homogeneous preparation of BPAO (3.5 μM), 5.0 mM benzylamine at 25 °C, and 200 units of catalase.

Determination of the Partition Ratio for Inactivation of BPAO by Benzylamine and Its Substituted Analogues. BPAO (4.4 μM) was incubated with 10 different concentrations of benzylamine or its substituted analogues (see Table 2), varying from 0.3 to 5 mM for 24 h at 30 °C in pH 7.2 sodium phosphate buffer. In each case, the enzyme was assayed for activity after the incubation solution was passed through a column of PDX G.F. 25 (1 × 7.8 cm). The control enzyme solution was prepared as described above except that the identical volume of distilled water was used instead of benzylamine solution. Remaining activity was determined by the ratio of the measured activity of the inactivated enzyme to the activity of the control enzyme. A plot of the remaining activity (%) vs [$ArCH_2NH_2$]/[BPAO] was constructed. Extrapolation of the linear portion of the data at lower [$ArCH_2NH_2$] gave the partition ratios [turnover number minus one (*11*)] listed in Table 2. Representative plots for benzylamine and 4-nitrobenzylamine are shown in Figures

[1] Abbreviations: BPAO, bovine plasma amine oxidase; EPAO, equine plasma amine oxidase; HKAO, human kidney amine oxidase; LTQ, lysine tyrosylquinone; PBN, α-phenyl-*N*-*tert*-butylnitrone; TPQ, 2,4,5-trihydroxyphenylalanine quinone.

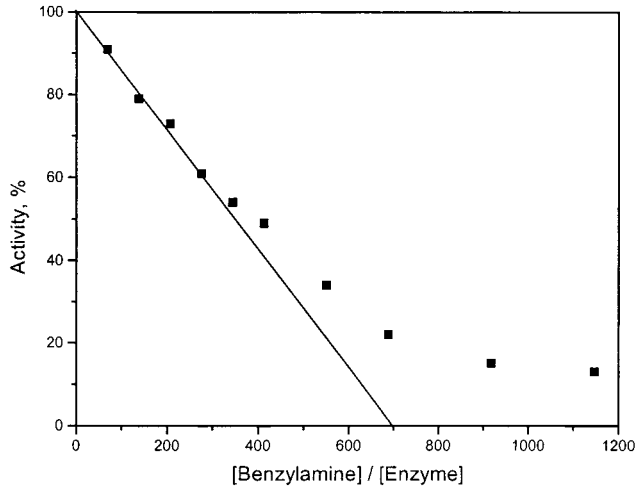


FIGURE 1: Partition ratio plot for inactivation of BPAO (4.4 μM) by benzylamine at 30 °C. See Experimental Procedures for details.

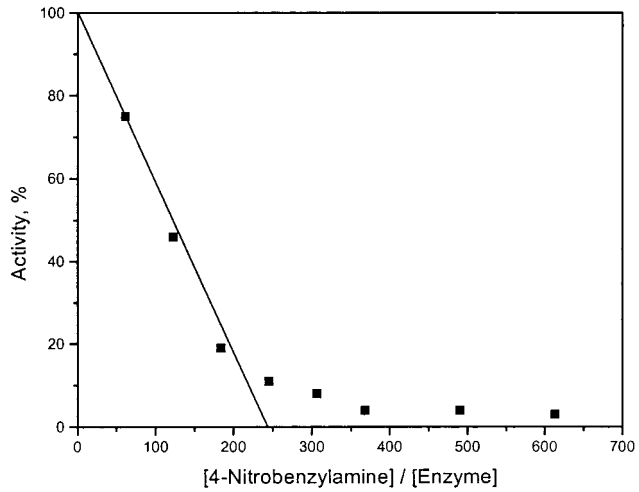


FIGURE 2: Partition ratio plot for inactivation of BPAO (4.4 μM) by 4-nitrobenzylamine at 30 °C. See Experimental Procedures for details.

1 and 2. Especially in the case of benzylamine, the points corresponding to higher [$ArCH_2NH_2$] deviate from a straight line probably because the enzyme is protected against further inactivation by the increasing concentrations of product ArCH═O forming under this higher partition ratio condition.

Phenylhydrazine Titration after Inactivation of BPAO with Benzylamine. Incubation of the enzyme (3.5 μM) with benzylamine (4 mM) in 100 mM phosphate buffer, pH 7.2, for 19 h at 30 °C resulted in 88% loss of activity after gel filtration. The collected enzyme fraction was titrated with phenylhydrazine and the absorbance at 450 nm was measured. About a 90% decrease in the absorbance at 450 nm was observed as compared to the control experiment.

Preparation of 5-tert-Butyl-6-hydroxy-2-arylbenzoxazoles **9.** A mixture of 4-substituted-benzylamine hydrochloride (4 mmol), K_2HPO_4 (522 mg, 3 mmol), $Cu(ClO_4)_2$ $6H_2O$ (74 mg, 0.2 mmol), dipyridyl (31 mg, 0.2 mmol), and **8** (36 mg, 0.2 mmol) in 200 mL of 30% aqueous CH_3CN was adjusted to pH 10 with KOH. The red solution was stirred for 15 h in an open 250 mL Erlenmeyer flask at room temperature. The solution was adjusted to pH 3 by addition of 1 N HCl and concentrated to half its original volume. The aqueous layer was extracted with ethyl acetate (150 mL × 2). The organic layer was dried (Na_2SO_4) and concentrated to afford crude solids **9**.

5-*tert*-Butyl-6-hydroxy-2-(4-trifluoromethylphenyl)benzoxazole (**9b**) was obtained in 72% yield after flash column chromatography (EtOAc/hexane = 1:3): mp 257 °C; λ_{max} (CH_3CN) 323 nm; ^{1}H NMR ($CDCl_3$) δ 1.49 (s, 9H), 6.97 (s, 1H), 7.73 (s, 1H), 7.77 (d, J = 8.12 Hz, 2H), 8.31 (d, J = 8.12 Hz, 2H); ^{19}F NMR ($CDCl_3$, CF_3COOH internal standard) δ 12.53; HRMS calcd for $C_{18}H_{16}F_3N_1O_2$ 335.1133, found 335.1120 (58%). 5-*tert*-Butyl-6-hydroxy-2-(4-nitrophenyl)-benzoxazole (**9c**) was recrystallized from EtOAc to give 26 mg of yellow crystals (46% based on **8**): mp 266 °C; λ_{max} (CH_3CN-H_2O) 365 nm (ϵ = 16147 M^{-1} cm^{-1}); ^{1}H NMR (DMSO-d_6) δ 1.41 (s, 9H), 7.14 (s, 1H), 7.56 (s, 1H), 8.29 (d, J = 8.91 Hz, 2H), 8.36 (d, J = 8.91 Hz, 2H), 10.15 (s, OH, 1H); ^{13}C NMR (DMSO-d_6) δ 29.4 (3C), 34.7, 97.4, 117.2, 123.5, 124.4 (2C), 127.6 (2C), 131.2, 132.4, 133.7, 134.9, 148.4, 149.5, 156.0, 158.4; HRMS calcd for $C_{17}H_{16}N_2O_4$ 312.1110, found 312.1122 (57%). 5-*tert*-Butyl-6-hydroxy-2-(4-phenylazophenyl)benzoxazole (**9d**) was recrystallized from EtOAc to obtained 28 mg (39% based on **1b**): mp 270 °C; λ_{max} (DMSO) 385 nm; ^{1}H NMR (DMSO-d_6) δ 1.42 (s, 9H), 7.14 (s, 1H), 7.55 (s, 1H), 7.59 (3H), 7.91 (m, 2H), 8.02 (d, J = 7.61 Hz, 2H), 8.27 (d, J = 7.61 Hz, 2H), 10.03 (s, OH, 1H); ^{13}C NMR (DMSO-d_6) δ 29.5 (3C), 34.7, 97.4, 116.9, 122.7 (2C), 123.3 (2C), 127.6 (2C), 129.1, 129.5 (2C), 131.9, 133.9, 134.4, 149.4, 151.9, 152.8, 155.5, 159.5.

Spectroscopic Characterization of Modified Enzyme. A 21.8 μM stock solution of the homogeneous preparation of BPAO was brought to 10.7 mM in 4-nitrobenzylamine (~500-fold excess) and the mixture was incubated at 30 °C for 2 h. Protein solution was then run over a Sephadex G-25M PD-10 column using 0.1 M phosphate buffer, pH 7.2, and concentrated. Absorption spectra were taken using a Hewlett-Packard 8453 photodiode array spectrophotometer. Raman spectra were collected on a Spex Triplemate 1877 (1800 groove setting), using a liquid-N_2 cooled Spex Spectrum One CCD detector, and Coherent Ar ion laser. Raman spectra were collected at room temperature for 15 min using 457.9 nm excitation and a power of 40 mW. The model compound **9c**, only slightly soluble in double deionized H_2O, was dissolved in both acetonitrile and EtOH. Analysis was taken using a 2-fold dilution of model compound to double deionized H_2O.

RESULTS

Inactivation of BPAO by Benzylamine. In our initial experiments, where we observed time-dependent inhibition by concentrations of benzylamine as low as 0.5 mM, we suspected that this could reflect product inhibition by benzaldehyde building up in solution. In fact, when evaluated directly, incubation of BPAO (4 μM) with varying concentrations of benzaldehyde (0.5, 1, and 1.5 mM) in 100 mM phosphate buffer, pH 7.2, at 30 °C resulted in a non-time-dependent inhibition of activity by 27, 60, and 75%. However, the activity was fully recovered after PDX G.F. 25 gel filtration. These data are consistent with the previous description of noncompetitive inhibition of BPAO by benzaldehyde with a K_m of 1.5 mM (at 25 °C) (*15*). Thus, to estimate any permanent inactivation due to benzylamine processing, all further activity assays were performed after gel filtration to remove all noncovalently bound substances.

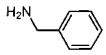
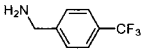
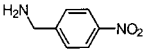
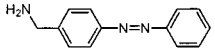
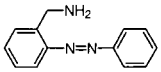
Data for inactivation of BPAO by benzylamine and products formed by enzymatic processing of benzylamine are listed in Table 1. The loss of enzyme activity upon incubation with benzylamine was rapid at the early stage of incubation (up to 4 h) but slowed thereafter as evidenced by the remaining activity after 24 h incubation being only slightly lower than that after 4 h incubation (Table 1, entries 2−3). The data obtained using the commercial and purified BPAO preparations differed somewhat, in part due to differing conditions of the experiments, but a parallel trend was observed. Complete inactivation of enzyme by benzylamine was not achieved, presumably because the product benzaldehyde protected the enzyme against further inactivation. The partition ratio, the number of molecules leading to product per each inactivation event, was determined to be 700 for benzylamine (Figure 1). Incubation of enzyme with H_2O_2 resulted in loss of activity that was prevented by catalase, but the degree of inactivation was far less than that produced by equal concentrations of benzylamine. This suggested that the inactivation of interest did not primarily arise from action of H_2O_2 formed in 1:1 stoichiometry by benzylamine turnover.

Comparing entries 2 and 8 in Table 1, it can be seen that addition of catalase to the benzylamine incubation reduced the inactivation significantly, suggesting that maximal inactivation required both benzylamine and H_2O_2. To check for the possibility that inactivation arose from combined action of the two products H_2O_2 and benzaldehyde, a mixture of H_2O_2 (1.5 mM) and benzaldehyde (1.5 mM) was coincubated with enzyme. However, the finding that the loss of activity in this case was less than that using the same concentration of H_2O_2 alone (1.5 mM), showed that inactivation of enzyme was not due to the accumulation of both H_2O_2 and benzaldehyde. In fact, benzaldehyde seemed to protect the enzyme from inactivation by H_2O_2. On the basis of these experimental results, it seemed clear that inactivation of enzyme depended on both the catalytic processing of benzylamine and the presence of H_2O_2.

Because the stoichiometric reaction of the amine oxidase quinone cofactor with phenylhydrazine yields a stable 450 nm-absorbing phenylhydrazone, spectral titration of the enzyme with phenylhydrazine has become the classical approach to evaluating irreversible modification of the quinone cofactor (*12*). Titration of the enzyme inactivated by 4 mM benzylamine (12% remaining activity after 19 h) with phenylhydrazine revealed a 90% decrease in absorbance at 450 nm as compared to the control enzyme, suggesting that inactivation of enzyme by benzylamine results in modification of the active site quinone cofactor.

Inactivation by Ring-Substituted Benzylamines. If inactivation of BPAO by benzylamine involves cofactor modification, one would expect to see changes in the absorption spectrum relative to the native form of the cofactor (λ_{max} = 480 nm for the TPQ anion). Conversion of the active site cofactor to a benzoxazole should be accompanied by a shift from 480 nm to the wavelength characteristic of the conjugated benzoxazole. Unfortunately, benzoxazole **7** derived from benzylamine and TPQ model **1** (R = CH_2CH_2-NHC(=O)CMe_3) has a maximum absorption at 314 nm, which would be nonresolvable in the enzyme case owing to the very strong absorption of the protein in this region.

Table 2: Properties of Benzylamine Analogs and Their Benzoxazole Derivatives **9**

substituted benzylamine	partition ratio[a]	λ_{max} of benzoxazole derivative (solvent)
	700	**9a** 313 nm (CH_3CN)
	460	**9b** 322 nm (CH_3CN)
	245	**9c** 365 nm (CH_3CN-H_2O)
	[b]	**9d** 384 nm (DMSO)
	[c]	[d]

[a] From plots of % activity vs [$ArCH_2NH_2$]/[BPAO], measured after gel filtration following incubations of 0.3 to 5.0 mM $ArCH_2NH_2$ with 4.4 μM BPAO (Sigma) for 24 h at 30 °C in pH 7.2 sodium phosphate buffer. [b] No inhibition (10 mM) of BPAO after incubating 24 h. [c] Complete inhibition (2.5 mM) of BPAO after incubating 20 h. However, enzyme activity was partly recovered after dialysis. [d] Benzoxazole derivative not synthesized.

However, since para-substituted benzylamine analogues were reported as good substrates of BPAO (*16*), we expected that they would behave like benzylamine with regard to inactivating the enzyme and might give rise to benzoxazoles with more discernible absorption bands.

Several benzylamine analogues (see Table 2) and their benzoxazole derivatives of the *tert*-butyl TPQ model **8** were synthesized in an effort to determine a suitable substrate that would permit spectroscopic visualization of the inactivated enzyme. The 4-CF_3 derivative was of interest in regard to a possible determination of covalent binding to the enzyme by ^{19}F NMR (to be studied at a later time). The two phenylazo-derived benzylamine analogues were expected to form benzoxazole derivatives displaying absorptions at very long wavelength because of the built-in chromophore (λ_{max} = 385 nm for the 4-phenylazobenzylamine-derived benzoxazole **9d**). Neither the 2- or the 4-phenylazo compounds were found to be an irreversible inactivator of BPAO, but both 4-CF_3- and 4-NO_2-substituted benzylamine analogues inactivated BPAO irreversibly in the same manner as did benzylamine itself. Further study revealed that 4-nitrobenzylamine inactivated the enzyme more efficiently than benzylamine, with a partition ratio of 245 (Figure 2). Most importantly, the synthetic benzoxazole **9c** isolated from reaction of **8** with 4-nitrobenzylamine exhibited an absorption at 365 nm that should be discernible above the background absorption of the protein (Scheme 3).

Substrate-Dependent Inactivation of other Amine Oxidases. Clearly, any mechanistic inferences or implications for the physiological significance of the substrate-inactivation pathway depend in part on the generality of the phenomenon. We therefore examined two other mammalian amine oxidases. Benzylamine is an excellent substrate for the equine plasma amine oxidase (EPAO). When EPAO is incubated with benzylamine (15 μM protein, 5 mM benzylamine, 25 °C in 0.1 M KPO_4 buffer, pH 7.2), only 4% of the control activity remains after 4 h without catalase, but 100% remains

Scheme 3

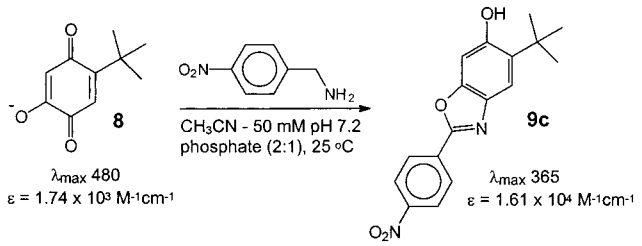


Table 3: Time-Dependent Inactivation of BPAO by 4-Nitrobenzylamine[a]

time (h)	control activity (u/mg BPAO)	experimental activity (u/mg BPAO)	% of control
0.5	1028	661	62.4
1.0	1129	113	10.7
2.0	1055	37	3.5
4.0	1022	21	2.0

[a] A homogeneous preparation of BPAO (4.83 μM) was incubated with or without 3.3 mM 4-nitrobenzylamine at 30 °C in pH 7.2 sodium phosphate buffer, followed by gel filtration.

after 4 h if catalase is included. The human kidney amine oxidase (HKAO) displays its highest activity toward diamines. When HKAO is incubated with *p*-(dimethylamino)-benzylamine (16 μM protein, 5.4 mM substrate, 25 °C in 0.1 M KPO_4 buffer, pH 7.2) HKAO was completely inactivated in less than 2 h in the absence of catalase, but 100% of the HKAO activity remained if catalase was present.

Absorption and Resonance Raman Spectroscopic Studies. The spectroscopic evaluation of BPAO inactivation was carried out using a homogeneous enzyme preparation, initial studies with which reproduced the time-dependent inactivation by benzylamine observed with the commercial enzyme (Table 1). Incubation with 4-nitrobenzylamine exhibited a more rapid loss of enzyme activity (Table 3). The sample selected for spectroscopic evaluation involved incubating BPAO with a 500-fold excess of 4-nitrobenzylamine at 30 °C for 2 h, resulting in loss of 90% of the control activity, assayed following gel chromatographic separation. The absorption spectrum as compared to that obtained for a control enzyme sample is shown in Figure 3. It is clearly evident that the native absorption at 480 nm has been replaced by one near 360 nm, consistent with conversion to the benzoxazole. Although the concentrations of enzyme in this figure are not exactly matched, the much stronger absorption at 360 nm relative to 480 nm is consistent with the relative difference in extinction coefficient noted in the model study (Scheme 3), suggesting that benzoxazole formation can account for the bulk of the inactivated enzyme.

Additional evidence to support formation of a cofactor-based benzoxazole was obtained through comparison of resonance Raman (RR) data on the inactivated enzyme with that for the 4-nitrobenzylamine-derived model benzoxazole **9c** (Figure 4). In native BPAO, the C5=O and C2=O stretching modes are found at 1678 and 1587 cm^{-1}, respectively (*17*−*19*). Comparison of RR spectra of the 4-nitrobenzylamine-BPAO adduct and model compound **9c** shows several congruent peaks, with the absence of the C5=O and C2=O stretches. The absence of the C5=O frequency suggests that this bond has been altered upon the addition of 4-nitrobenzylamine to C5. Additionally, the

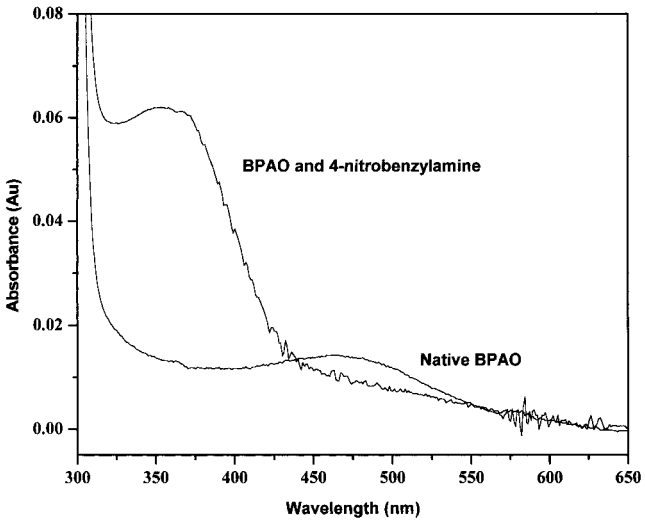


FIGURE 3: Absorption spectra for native BPAO (3.4 μM) and for BPAO isolated by gel filtration following inactivation by excess 4-nitrobenzylamine for 2 h at 30 °C as described in Experimental Procedures. The final concentration of the modified BPAO spectrum shown was 2.7 μM.

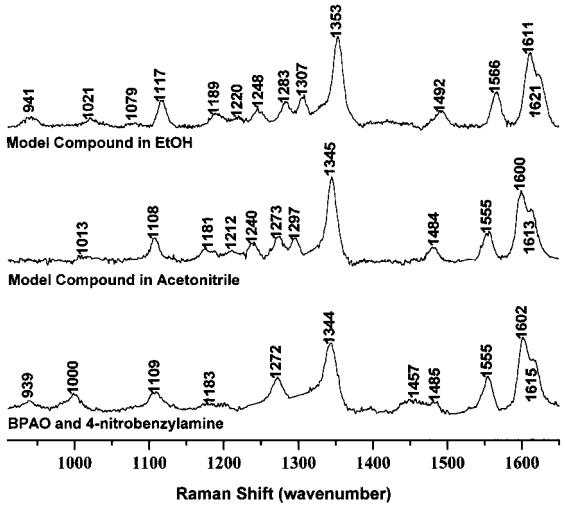


FIGURE 4: Resonance Raman spectra of BPAO inactivated with 4-nitrobenzylamine and the model 4-nitrobenzylamine-derived benzoxazole **9c** in two different solvents (the spectra represent solvent subtraction; laser excitation was at 457.9 nm).

absence of the C2=O frequency suggests that this bond has also changed, consistent with conversion of C2=O to C2−OH in the benzoxazole. We can tentatively assign either the 1181 or the 1273 cm^{-1} frequency to the C2−O stretch of the adduct, as stretching modes for phenols range approximately between 1180 and 1275 cm^{-1} (*20*). Aromatic ethers, such as $Ar-O-CH_2$, have a characteristic C−O stretching frequency approximately between 1210 and 1310 cm^{-1}, so it is possible that the band at 1273 cm^{-1} present in the adduct is due to C−O stretching of the ether linkage.

In the RR spectrum of the BPAO-methylamine adduct, a feature at 1616 cm^{-1} was assigned to the C=N stretch of the product imine (*19*). Derivatization of the cofactor to generate the benzoxazole equivalent to **9c** would contain C=N bonded to the C5 of TPQ. The RR spectrum of the adduct and the model compound show modes at 1615 cm^{-1} (adduct), 1621 cm^{-1} (model in EtOH), and 1613 cm^{-1} (model in acetonitrile). The RR spectrum of the model compound in

EtOH displayed a shift of 8−10 cm^{-1} as compared to the model compound in acetonitrile. Taking this into account, we can tentatively assign this frequency as the C═N stretch of the product imine. Overall, the striking similarities in the spectra of **9c** and the 4-nitrobenzylamine-BPAO adduct indicate that the oxazole structure of **9c** is an excellent candidate for the structure of the adduct.

DISCUSSION

The physiological function of plasma amine oxidase is not entirely clear, but it is believed to be responsible for metabolism of blood-borne endogenous and exogenous amines, with a K_m preference for arylalkylamines as opposed to short alkylamines. Some of the mammalian plasma enzymes in this class were originally referred to as benzylamine oxidase, owing to the apparent selectivity for this amine. The fact that the oxidation of benzylamine to benzaldehyde can be conveniently monitored by the increase at 250 nm has led to its use as the standard activity assay for the enzyme. Such assays are typically run for 30−60 s, where the increase in A_{250} is linear. The studies described here demonstrate that incubation of BPAO with relatively high concentrations of benzylamine for longer periods of time results in time-dependent loss of activity that was not regained after gel filtration. It was concluded that H_2O_2 produced by normal enzyme turnover of substrate participated in inactivation, based on the observation that catalase (which removes H_2O_2) protected against inactivation by benzylamine. When the enzyme was incubated with H_2O_2 alone at a concentration equal to what would be generated by complete turnover of benzylamine, there was irreversible loss of activity but to a much lower extent. Thus, although H_2O_2 itself inactivates the enzyme, the mechanism for this must be distinct from the more potent inactivation by H_2O_2 that occurs during substrate processing. A control study ruled out the possibility that this inactivation reflected the combined action of H_2O_2 and benzaldehyde product.

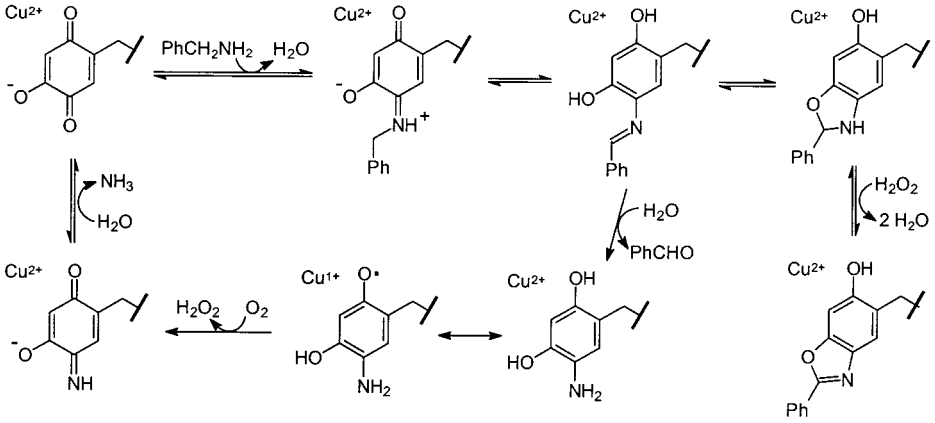
There are limited reports of substrate turnover-dependent inactivation for certain members of the copper amine oxidase family. Time-dependent inactivation of diamine oxidase by H_2O_2 was reported to be greatly potentiated by the copresence of its typical diamine substrates (*21*, *22*). Also, lysyl oxidase exhibits a time-dependent inactivation by diamine substrates (*23*). In the latter case, inactivation has been ascribed at least in part to hydroxyl radical-induced damage to the enzyme, since radical scavengers such as DMSO afforded significant protection. Support for hydroxyl radical formation in the case of diamine oxidase was provided by spin-trapping with α-phenyl-*N*-*tert*-butylnitrone (PBN) (*24*). Since the PBN-hydroxyl radical adduct was observed only in the presence of substrate and was abrogated by the copresence of catalase, it was proposed that inactivation might reflect a Fenton-like generation of hydroxyl radical by reaction of H_2O_2 with the reduced enzyme [reacting either with Cu(I) or the semiquinoneimine] (*24*). In a recent study using the *E. coli* amine oxidase, an observed fractional loss of the ability to regenerate the starting quinone spectrum (at 480 nm) with repetitive cycles of reduction by substrate in the presence of H_2O_2, was also ascribed to possible damage of the enzyme by H_2O_2-derived hydroxyl radical (*25*). Nonetheless, a hydroxyl radical-dependent inactivation of the enzyme alone would not explain why inactivation of lysyl oxidase by radiolabeled diamine substrates was accompanied by covalent binding of radiolabel to the protein (*23*).

In the case of BPAO, time-dependent inactivation by substrate has *not* been an issue receiving direct attention, although one can find hints for such in the literature. For example, in one of the first papers describing purification of BPAO, the rate of oxidation of subsequently added aliquots of substrates appeared to be less than the rate of oxidation of the first aliquot (*26*). Also, in studies aimed at reductive trapping (by [^{3}H]$NaBH_3CN$) of substrate-reduced BPAO, the control incubation with 20 mM benzylamine exhibited a 25% loss of activity in 2 h at pH 6, assayed following Sephadex G-25 gel filtration (*27*). Substrate-dependent inactivation of BPAO may not have been documented previously because of the short time periods of typical activity assays, where the H_2O_2 concentration does not build up to inactivating levels. We first detected the time-dependent inactivation phenomenon in our efforts to show benzylamine protection against certain mechanism-based inactivators of BPAO, which required long term coincubation with relatively high concentrations of benzylamine. In fact, previous reports of an apparent inhibition of plasma amine oxidase by very high concentrations of benzylamine could reflect the generation of significant levels of H_2O_2 even during the short time frame of the rate assay used in this case (*28*).

Our finding that the H_2O_2-dependent inactivation of BPAO during processing of benzylamine is accompanied by irreversible modification of the quinone cofactor, documented spectroscopically and by phenylhydrazine titration, suggests to us that inactivation reflects mainly an H_2O_2-dependent oxidative "derailment" of some reduced intermediate in the normal substrate turnover cycle. Our previous finding that catalytic aerobic turnover of benzylamine by TPQ models eventually ceased due to irreversible conversion of the TPQ catalyst to a benzoxazole (*4*), led us to consider this same possibility also for the enzyme. Using 4-nitrobenzylamine, we showed that inactivation of enzyme is accompanied by replacement of the normal 480 nm TPQ anion absorption by a stronger absorption near 360 nm (Figure 3). This spectral change and relative change in extinction coefficient matches that seen for the model study (Scheme 2). Confirmation of the structure of the modified enzyme was obtained by resonance Raman comparison of the inactivated enzyme with the model 4-nitrophenyl benzoxazole **9c** (Figure 4).

Incorporation of our suggested mode of inactivation into the consensus mechanism for catalytic turnover of benzylamine is shown in Scheme 4. It shows that the product Schiff base is in equilibrium with a dihydrobenzoxazole, which can be oxidized by H_2O_2 to the benzoxazole, thus explaining the protective effect of catalase. In our model study (Scheme 2), catalase did not inhibit benzoxazole formation, and we found that the oxidant in this case was the starting quinone (*4*). Lack of an effect of catalase in that model study probably reflects the fact that H_2O_2, generated during oxidative recycling (by O_2) of the aminoresorcinol form of the TPQ model, is rapidly reduced by a second molecule of the aminoresorcinol and thus does not build up in solution. Nonetheless, in unpublished studies, we have found that H_2O_2

Scheme 4



can readily oxidize dihydrobenzoxazoles, establishing precedent for the proposed oxidation in Scheme 4.

During enzymatic deamination of benzylamine, the molecule of H_2O_2 generated upon reduction of O_2 at the O_2-binding site during cofactor regeneration could potentially be internally transferred so as to effect oxidation of the dihydrobenzoxazole in the next round of catalysis, but this clearly does not transpire, as the enzyme would then always become rapidly inactivated. The finding that catalase protects against inactivation shows that it is H_2O_2 released into bulk solution that is responsible for the inactivating oxidation. In this regard, it is likely that H_2O_2 gains access to the dihydrobenzoxazole in the same way that water gains access to the tautomeric product Schiff base to affect its hydrolysis. The H_2O_2 may be able to enter the active site from either the substrate channel, or possibly the interior solvent-filled cavity. Although there are probably multiple potential mechanisms of substrate-dependent inactivation of copper amine oxidases, our evidence suggests that benzoxazole formation represents the major mechanism occurring in vitro in the case of benzylamine.

It should be noted that the mechanism in Scheme 4 may be generally applicable to amine oxidases from a variety of sources, regardless of their substrate specificity. This expectation is supported by the results with two other mammalian enzymes, EPAO and HKAO. For all three amine oxidases tested, substrate-dependent inactivation occurs, and catalase affords complete protection. Since only the dihydrobenzoxazole and not the product Schiff base tautomer is oxidizable, clearly the equilibrium formation of the former must be sterically allowed for those enzymes where this type of inactivation has been observed. If any particular active site were to sterically preclude dihydrobenzoxazole formation for the particular substrate amine in question [benzylamine for BPAO and EPAO, and *p*-(dimethylamino)benzylamine for HKAO], then such enzyme should not show inactivation by substrate.

Collectively, the results suggest that amine oxidase inactivation via benzoxazole formation may be significant in physiological settings where locally high concentrations of H_2O_2 are available. Certain amine oxidases, particularly yeast enzymes that may have a broad degradative or catabolic role, appear to be targeted to peroxisomes (*29*−*31*) where the catalase concentration is high and hence may be protected from this inactivation pathway. However, recent data indicated that diamine oxidase and catalase are located in different subcellular compartments in kidney cells (*32*), so substrate-dependent inactivation via benzoxazole formation may be physiologically significant in this case or for those extracellular amine oxidases that are anchored to the membrane or bound to an extracellular matrix.

In our in vitro studies, a relatively high concentration of H_2O_2 is reached due to turnover of relatively high concentrations of amine, but the inactivation depicted in Scheme 4 reflects strictly a partitioning of the product Schiff base/dihydrobenzoxazole between H_2O-mediated hydrolysis (productive turnover) and H_2O_2-mediated oxidation (inactivation). Thus inactivation in vivo would not require a high concentration of amine substrate as long as H_2O_2 concentrations were maintained independently. For the inactivation pathway to potentially come into play, H_2O_2 and amine need only be present concurrently; although the rate is dependent on $[H_2O_2]$, some inactivation can take place even at relatively low concentrations of both.

Knowledge of the potential inactivation described here is crucial for any in vitro enzymologic experiments where $[H_2O_2]$ is allowed to build up in solution. Such is the case in attempts to demonstrate substrate protection against mechanism-based inactivators, where a large extent of substrate turnover could occur during the preincubation period. In these cases, the addition of catalase should effectively suppress the substrate-dependent inactivation so that an effect of substrate concentration on inhibitor-dependent inactivation can be judged. In fact, with the inclusion of catalase, we were able to show that increasing concentrations of benzylamine afforded increasing protection against inactivation by a particular inhibitor rather than the increasing levels of inactivation observed in the absence of catalase (*10*).

The potential for amine oxidases to be inactivated by substrates raises interesting evolutionary questions. Our results show that the inactivation chemistry is an intrinsic part of the reactivity of TPQ. Perhaps under numerous physiological settings, other enzymes would remove the H_2O_2 produced during turnover, or diffusion may limit the extent of enzyme inactivation. However, one may also see possible advantages to the inactivation reaction because several of the possible product aldehydes are actually more toxic to cells than the amine substrates. Thus, it may be advantageous to limit or decrease the metabolic flux to prevent oxidative damage to the cell by the accumulating products.

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