A Comparative Study of the Binding and Inhibition of Four Copper-Containing Amine Oxidases by Azide: Implications for the Role of Copper during the Oxidative Half-Reaction†

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ABSTRACT: Copper amine oxidases (CuAOs) catalyze the oxidative deamination of primary amines operating through a ping-pong bi-bi mechanism. In this work, azide (an exogenous monodentate ligand) was used to probe the role of copper during the oxidative half-reaction of CuAO catalysis. The effects of azide on both the reductive and oxidative half-reactions of pea seedling amine oxidase (PSAO), the recombinant human kidney diamine oxidase (rhDAO), *Arthrobacter globiformis* amine oxidase (AGAO), and *Pichia pastoris* amine oxidase (PPLO) have been examined. For the reductive half-reaction, defined as the oxidation of amine substrate to an aldehyde, azide was discovered to exhibit either noncompetitive or competitive inhibition with respect to the amine, depending on the enzyme source. With regard to the oxidative half-reaction, defined as the reoxidation of the enzyme via reduction of O_2 to H_2O_2, azide has been determined to exhibit competitive inhibition with respect to O_2 in PSAO with a calculated K_i value that is in excellent agreement with the experimentally determined K_d value for the Cu(II)−N_3^- complex. Azide was found to exhibit mixed-type/partially competitive inhibition with respect to substrate O_2 in rhDAO, with an apparent K_i that is similar to the K_d value for the Cu(II)−N_3^- complex. The competitive inhibition for PSAO and the partially competitive inhibition for rhDAO are consistent with O_2 interacting directly with copper during enzymatic reoxidation. For the enzymes AGAO and PPLO, pure noncompetitive and mixed-type/partially competitive inhibition is observed. K_i values for reductive and oxidative half-reactions are equivalent and are lower than measured K_d values for the Cu(II)−N_3^- complexes in oxidized and substrate-reduced forms of these enzymes. Given these observations, it appears that substantial inhibition of the reductive half-reaction occurs at the concentrations of azide used for the oxidative half-reaction experiments, thereby complicating kinetic interpretation. At this time, the data do not permit us to distinguish between two possibilities: (1) inhibition by azide with respect to O_2 is intrinsically competitive in CuAOs, but this effect cannot always be deconvolved experimentally from the effects of azide on the reductive half-reaction; or (2) CuAOs differ in some steps of their reoxidation mechanisms.

Amine oxidases catalyze the oxidative deamination of primary amines to the corresponding aldehydes, utilizing O_2 as the oxidant with concomitant production of H_2O_2 and NH_3. The two classes of amine oxidases present in mammals are the quinone and copper-containing amine oxidases (CuAOs)[1] and the flavin-dependent monoamine oxidases (MAO enzymes, EC 1.4.3.4). Quinone copper-containing amine oxidases principally oxidize primary amines and can be divided into two subclasses based on the type of cofactor present in the active site: 2,4,5-trihydroxyphenylalanine quinone (TPQ, EC 1.4.3.6) or lysyl tyrosylquinone (LTQ, EC 1.4.3.13). The TPQ cofactor is formed via the post-translational processing of a conserved tyrosine residue, while LTQ is formed by means of the cross-linking of a conserved tyrosine and lysine. Formation of both cofactors is a self-processing event requiring only copper and molecular oxygen (*1*−*4*).

CuAOs are known to be widely distributed in nature, having a multitude of physiological roles (*5*−*7*), and may be involved in diverse processes such as hormone biosynthesis, wound healing, detoxification, cell growth, signaling, apoptosis, lymphocyte adhesion, adipocyte maturation, and possibly glucose homeostasis (*5*, *8*−*13*). Over the past decade, the availability of the first three-dimensional structures of CuAOs has provided a wealth of information ultimately needed to understand substrate specificity, biogenesis of the TPQ cofactor, and the enzyme mechanism (*5*, *14*−*18*). The structurally characterized CuAOs are homodimers ranging from 70 to 120 kDa per monomer, each

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[1] Abbreviations: AGAO, *Arthrobacter globiformis* amine oxidase; APAO, *Arthrobacter* P1 amine oxidase; BPAO, bovine plasma amine oxidase; CuAO, quinone copper-containing amine oxidase; ECAO, *Escherichia coli* amine oxidase; HRP, horseradish peroxidase; LSAO, lentil seedling amine oxidase; LTQ, lysyl tyrosylquinone; MAO, monoamine oxidase; PSAO, pea seedling amine oxidase; PKAO, porcine kidney amine oxidase; PPAO, pig plasma amine oxidase; PPLO, *Pichia pastoris* lysyl oxidase; SSAO, semicarbazide sensitive amine oxidase; TPQ, 2,4,5-trihydroxyphenylalanine quinone; TPQ_{AMR}, TPQ aminoresorcinol; TPQ_{IMQ}, TPQ iminoquinone; TPQ_{OX}, oxidized TPQ; TPQ_{SQ}, TPQ semiquinone.

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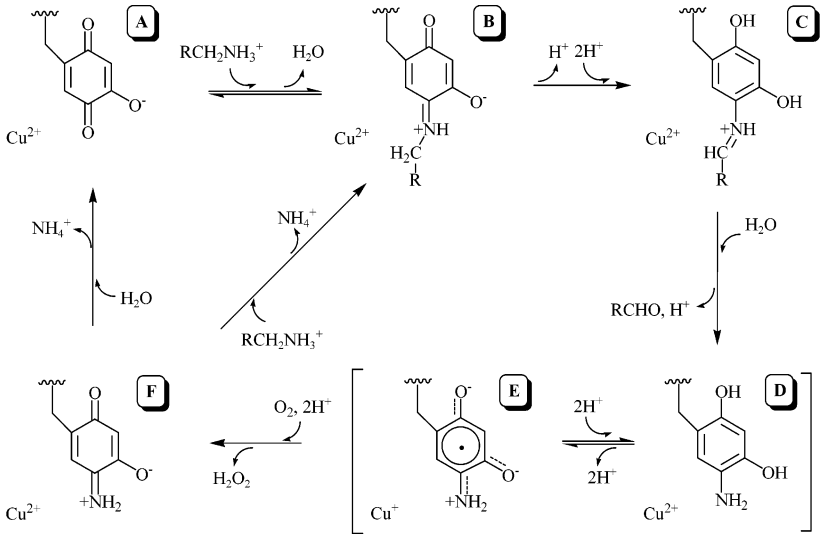
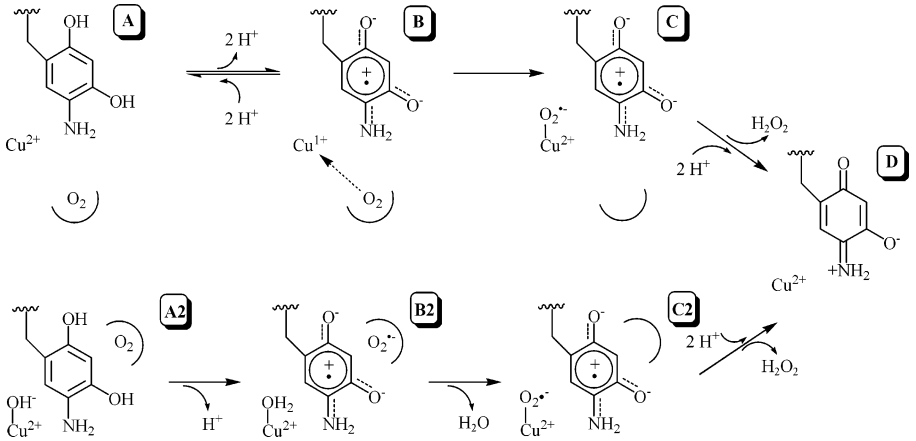
Scheme 1: Proposed Mechanism of CuAO Catalysis



Scheme 2: Proposed Mechanisms for CuAO Reoxidation[a]



[a] Both pathways (**A** → **D** and **A2** → **D**) begin with the TPQ_{AMR} moiety (**D** in Scheme 1). The assignments of the protonation states for the intermediates outlined in the **A2** → **C2** step are not certain.

containing a single active site composed of a mononuclear type II copper ion and the TPQ cofactor (*19*−*21*).

CuAO catalysis proceeds through a ping-pong bi-bi mechanism divided into two half-reactions:

$$E_{ox} + RCH_2NH_2 \leftrightarrow E{-}RCH_2NH_2 \rightarrow E_{red} + RCHO \quad (1)$$

$$E_{red} + O_2 + H_2O \rightarrow E_{ox} + H_2O_2 + NH_3 \quad (2)$$

The first half-reaction is conventionally known as the reductive half-reaction and is detailed in Scheme 1 (**A** → **D**). The crucial step is the conversion of the initial quinoneimine "substrate Schiff base" (**B**, TPQ_{SSB}) to a quinoladimine "product Schiff base" (**C**, TPQ_{PSB}), facilitated by abstraction of a proton from the α-carbon of the amine substrate by an absolutely conserved aspartate acting as a general base (*22*, *23*). Product aldehyde is then released by hydrolysis, generating the reduced cofactor (TPQ_{AMR}). The second half-reaction involving reoxidation of the organic cofactor is known as the oxidative half-reaction and is diagrammed in the steps from **D** to **A**. When aldehyde is released, the reduced enzyme may exist as an equilibrium between a Cu(II)−aminoresorcinol species (**D**, TPQ_{AMR}) and a Cu(I)−semiquinone species (**E**, TPQ_{SQ}). The K_{eq} for the **D** ⇌ **E** step depends on the temperature and the source of the enzyme (*24*). Reduction of O_2 to H_2O_2 yields an iminoquinone species (**F**, TPQ_{IMQ}), which hydrolyzes to liberate NH_3 and the resting cofactor (**A**, TPQ_{OX}) (*5*). The release of NH_3 may also occur through a transimination reaction between the substrate and the iminoquinone, generating the substrate Schiff base (**F** → **B**) (*25*).

The principal unresolved issue in CuAO catalysis is the precise role of copper during enzymatic reoxidation. Two possible reoxidation mechanisms are outlined in Scheme 2. We have suggested that the first electron reduction of dioxygen occurs by reaction with the Cu(I)−semiquinone species (Scheme 2, **B**), resulting in a Cu(II)-bound superoxide species (Scheme 2, **A** → **C**) (*24*, *26*). This proposal circumvents the well-known spin conversion problem associated with two-electron reductions of oxygen (*27*) and is supported by the ample precedence for the reactivity of three-coordinate Cu(I) sites with dioxygen in copper-containing metalloproteins (*28*−*31*). Subsequently, a detailed kinetic study of the catalytic intermediates in lentil seedling amine oxidase (LSAO) provided direct support for this mechanism (*32*, *33*). Recently, a kinetic and structural study of the metal-substituted CuAO from *Arthrobacter globiformis* suggested two possible reoxidation mechanisms (*34*). In one, the initial

electron transfer to O_2 comes directly from the TPQ_{AMR} species, and the reduction of Cu(II) is not required. The role of Cu(II) in this suggested mechanism is to bind the superoxide anion and facilitate the second electron transfer from the one-electron-reduced cofactor. However, this study also points out that the rate enhancement for reoxidation observed in the native, copper-containing enzyme, compared to metal-substituted forms, is consistent with reduction of Cu(II) to Cu(I), thereby implicating the Cu(I)−TPQ_{SQ} species as a catalytically viable intermediate (*34*).

Substantial research on the CuAO from *Hansenula polymorpha* (HPAO) has led to the proposal that copper reduction is not essential for regeneration of the resting cofactor, leading to the suggestion of an alternative reaction pathway (Scheme 2, **A2** → **C2**) (*35*−*37*). These studies suggest that dioxygen receives the first electron directly from TPQ_{AMR} (**A2** → **B2**, rate-limiting step), with the superoxide species subsequently migrating to Cu(II) (**B2** → **C2**) (*25*). An essential feature of this mechanism is that the copper-bound axial water serves as a proton source in TPQ reduction, resulting in a Cu(II)−OH^- species (**A2**). It is believed that following the first transfer of an electron to O_2, the pK_a of TPQ_{SQ} is perturbed in a way that allows for rapid proton transfer back to the metal-bound hydroxide species forming H_2O. The metal-bound water would then be expected to undergo rapid substitution with superoxide anion (*25*). In addition to these proposals, a theoretical study utilizing density functional theory has implicated the paramagnetic copper center in the spin transitions necessary to produce singlet TPQ from the reaction of singlet TPQ_{AMR} with triplet O_2 (*38*).

Finally, a structural study of the steady-state intermediate that accumulates in *Escherichia coli* amine oxidase (ECAO) upon aerobic exposure to excess β-phenylethylamine indicated the presence of a reduced dioxygen species bound axially to copper and equidistant from the copper and TPQ cofactor, consistent with electron transfer to dioxygen occurring from either Cu(I) or TPQ_{AMR} (*39*, *40*). Collectively, the available data suggest that the reoxidation mechanism may depend on the identity of the CuAO. Consequently, detailed kinetics and spectroscopic experiments with structurally characterized CuAOs are required to distinguish among these mechanistic possibilities.

N_3^- and CN^- are known to replace the equatorial water ligand of copper and are effective inhibitors of multiple CuAOs (*41*−*44*). Therefore, investigation of the electronic and mechanistic effects of ligand substitution reactions provides insight into both the active site structure and the catalytic mechanism of copper-containing amine oxidases (*43*−*46*). A previous study demonstrated that the effects of azide and cyanide on catalysis of several CuAOs can be rationalized in terms of copper coordination by these exogenous ligands and their effects on the TPQ_{AMR}−Cu(II) ⇌ TPQ_{SQ}−Cu(I) internal redox equilibrium (*44*). Recently, we have published a detailed spectroscopic and kinetic report on the interaction of cyanide with two CuAOs, supporting the catalytic viability of the semiquinone (*26*).

It may be anticipated that if substrate dioxygen interacts directly with the copper site during enzymatic reoxidation, inhibition by azide should appear competitive with respect to O_2. Although the results from inhibition experiments conducted under turnover conditions are not unambiguous, such data provide an important foundation for assessing possible mechanisms and in this case can provide an informative test of hypotheses for the role of copper in the reaction of reduced CuAOs with O_2. Herein, we present a detailed spectroscopic and kinetic characterization of the interaction of azide with respect to both the reductive and oxidative half-reactions in copper-containing amine oxidases AGAO, rhDAO, PPLO, and PSAO.

MATERIALS AND METHODS

Enzyme Purification and Isolation. AGAO, rhDAO, PPLO, and PSAO were purified as described previously (*7*, *47*−*49*). Protein concentrations were calculated using extinction coefficients at 280 nm as previously reported for AGAO (*50*), rhDAO (*7*), PPLO (*48*), and PSAO (*49*) and using an extinction coefficient of 2500 M^{-1} cm^{-1} for TPQ (*25*). All experiments with AGAO and PSAO were carried out in 100 mM potassium phosphate buffer (pH 7.2), while those with rhDAO (pH 7.2) and PPLO (pH 7.0) were performed in 50 mM HEPES buffer.

Azide Titrations of Oxidized Amine Oxidases. Samples of AGAO (85 μM) and PSAO (15.3 μM) were titrated with a 1.5−2.5 M stock of NaN_3 (Fisher Chemicals), and the LMCT bands for the Cu(II)−N_3^- complexes were monitored at 388 and 389 nm. Samples of rhDAO (33 μM) and PPLO (39.3 μM) were titrated with 610 mM and 5 M NaN_3 stock solutions, respectively. LMCT bands were monitored at 395 nm for rhDAO and 385 nm for PPLO.

All sodium azide stocks were made fresh immediately prior to use and were adjusted to the respective experimental pH values listed above. UV and visible absorption data were acquired utilizing a Cary 6000i UV/vis/NIR spectrophotometer (Varian) connected to a Cary dual-cell Peltier accessory for temperature control. Absorbance data were analyzed using Origin version 7.0 (Microcal). The ΔA values of the respective LMCT bands were plotted versus azide concentration, and the data were fit to a titration curve to determine respective K_d values for the Cu(II)−N_3^- complex. Titrations of PSAO, AGAO, and PPLO were performed at 30 °C, while that of rhDAO was performed at 37 °C.

Azide Titrations of Substrate-Reduced Amine Oxidases. To determine if the affinity of azide for copper was altered in the substrate-reduced form of the enzymes, samples of PPLO (50 μM) and AGAO (25 μM) were purged with argon passed through a vanadium bubbler, HCO_3, H_2O setup (*26*, *51*) and then reduced with a slight excess of substrate. Fresh solutions of benzylamine and azide were made anaerobic by purging with argon. As previously described, a glucose/glucose oxidase/catalase solution was added to the protein sample prior to addition of substrate to ensure that anaerobic conditions were maintained throughout the titration (*26*). The ΔA values of the respective LMCT bands were determined from difference spectra and plotted versus azide concentration to determine respective K_d values for the Cu(II)−N_3^- complex using a titration binding curve (Origin version 7.0).

Azide-Mediated Inhibition of HRP. Since azide is a known H_2O_2-dependent mechanism-based inhibitor of HRP with a k_{inact} of 0.69 min^{-1} (*52*), it was critical to show that azide-mediated inactivation of HRP was not occurring during the time frame of the coupled assays (≤1 min), as described below. A series of control assays performed with PSAO [the

Table 1: Properties of Copper Amine Oxidases

		K_M (μM)		k_{cat}(dimer) (s^{-1})		K_d[Cu(II)−N_3^-] (mM)	
enzyme	TPQ[a]	amine	O_2	amine	O_2	oxidized	reduced
PSAO	1.8	424 ± 30[b]	46 ± 3	252 ± 3	308 ± 9	53 ± 5	59 ± 10[c]
rhDAO	1.4	26 ± 4[d]	14 ± 2	7.9 ± 0.2	4.0 ± 0.1	38 ± 3	ND[g]
AGAO	1.4	1.5 ± 0.2[e]	33 ± 2	44 ± 2	53 ± 2	213 ± 18	34 ± 5
PPLO	1.6	39 ± 2[f]	41 ± 2	11.2 ± 0.3	12.6 ± 0.3	153 ± 16	87 ± 21

[a] Values are reported as TPQ content per dimer. [b] Amine substrate putrescine, in 100 mM KPO_4 at pH 7.2 and 30 °C with an *I* of 540 mM (KCl). [c] Value reported in ref *44*. [d] Amine substrate putrescine, in 50 mM HEPES at pH 7.2 and 37 °C with an *I* of 250 mM (KCl). [e] Amine substrate β-phenylethylamine, in 100 mM KPO_4 at pH 7.2 and 30 °C with an *I* of 440 mM (K_2SO_4). [f] Amine substrate benzylamine, in 50 mM HEPES at pH 7.0 and 30 °C with an *I* of 150 mM (KCl). [g] Not determined.

CuAO that exhibits the fastest turnover (Table 1)] at 250 mM azide indicated there was no azide-mediated effect on the ability of HRP to turn over the H_2O_2 that was generated during the oxidation of putrescine. In addition, control assays with PPLO showed that there was no difference between the experimentally determined rate of benzylamine oxidation using the direct assay and the ABTS/HRP coupled assay in the presence of 40 mM azide (data not shown).

Azide Inhibition of the Reductive Half-Reaction. To determine the effects of azide upon the first half-reaction (eq 1), kinetic characterization was carried out at three different azide concentrations for AGAO, rhDAO, PPLO, and PSAO. Substrate O_2 was kept at near-saturating levels (~185 μM dissolved O_2), while the level of substrate amine was varied below and above the K_M values for each enzyme. The preferred amine substrate varied depending on the enzyme source, with putrescine being used for PSAO and rhDAO, β-phenylethylamine for AGAO, and benzylamine for PPLO. Ionic strength was controlled for all kinetic assays (those for both the reductive and oxidative half-reactions) by addition of either KCl (rhDAO, PPLO, and PSAO) or K_2SO_4 (AGAO). KCl and K_2SO_4 were determined to have no inhibitory effect on the respective CuAO activity, as determined through a series of control assays conducted prior to experimentation (*26*). Assays (including those described below for the oxidative half-reaction) were performed at controlled ionic strengths by varying the ratio of azide to salt (AGAO, 440 mM; rhDAO, 250 mM; PPLO, 150 mM; PSAO, 540 mM). The factor controlling ionic strength was determined by the amount of azide required to achieve adequate levels of inhibition.

The rate of β-phenylethylamine oxidation by AGAO was monitored using a horseradish peroxidase (HRP)−ABTS [2,2′-azinobis(3-ethyl)benzthiazoline-6-sulfonic acid] coupled assay. The peroxidase-catalyzed reduction of H_2O_2 to H_2O is coupled to the one-electron oxidation of ABTS, forming a metastable radical cation with a λ_{max} of 414 nm (*53*). For PPLO, the amine oxidase activity was determined by monitoring benzaldehyde production over the course of 3 min at 250 nm using an extinction coefficient of 12 800 M^{-1} cm^{-1} (*54*). Catalytic activities of PSAO and rhDAO were monitored via the 4-aminoantipyrine/vanillic acid coupled assay of Holt et al. utilizing an extinction coefficient of 6000 M^{-1} cm^{-1} at 498 nm for the quinoneimine dye (*1*, *55*).

Kinetic analysis involved first equilibrating each enzyme with a given amount of azide for 2 min under magnetic stirring, followed by addition of substrate amine to initiate each assay. Assays were run at least in duplicate, most often triplicate, at a particular substrate concentration. Error bars on each plot represent the standard deviation of the rate at a particular substrate concentration (Origin version 7.0). Steady-state kinetic data were collected on a Hewlett-Packard 8453 diode-array spectrophotometer equipped with a thermostated cell chamber connected to an Endocal RTE-5 circulating water bath.

Azide Inhibition of the Oxidative Half-Reaction. To determine the effects of azide on the oxidative half-reaction of amine oxidation (eq 2), levels of substrate amine were kept at saturating amounts (at least 10K_M) while the level of substrate O_2 was varied. Rates of oxygen uptake in the presence of varying amounts of azide were determined using an Instech (Plymouth Meeting, PA) model 125/05 Clark-type oxygen electrode interfaced with a single-channel Instech model 103 oxygen electrode amplifier. All assays were conducted in a thermostated, 680 μL sealed batch cell equipped with an Instech model 103 magnetic stirring mechanism. The ionic strength of all assays was kept consistent as described above. Concentrations of amine substrates varied given the respective K_M values for the different enzymes with 200 μM β-phenylethylamine used for AGAO, 2 mM putrescine used for rhDAO, 460 μM benzylamine utilized for PPLO, and 6 mM putrescine used for PSAO.

The following procedure was used for each enzyme. Buffer was made at the appropriate ionic strength and allowed to equilibrate overnight in a 30 °C H_2O bath. Substrate amine was then freshly added to the temperature and air-equilibrated buffer, which was subsequently divided into two flasks. The first flask was left exposed to air, while the second flask was purged with argon passed through a vanadium bubbler, HCO_3, H_2O setup (*51*) for at least 1 h. Mixtures of air-equilibrated and anaerobic buffer were then injected into the cell chamber via gastight syringes to achieve a desired level of dissolved O_2, as monitored using the calibrated Clark-type oxygen electrode. When desired levels of dissolved O_2 were reached, the cell chamber was sealed and the output reading was monitored for a period of 10 s to ensure there was no electrode drift. Assays were then initiated by addition of 2−3 μL of the respective enzyme via a gastight syringe. Initiation of kinetic assays by addition of enzyme could be accomplished due to the fact that azide binding in CuAOs has been shown to be rapid (*26*, *37*, *44*).

Substantial efforts were made to ensure the reproducibility of experimental rates. For each kinetic curve, a minimum of 14−20 data points were collected. Each data point represents the rate of oxygen depletion from the assay chamber, determined from a linear fit of the change in dissolved oxygen levels over time. Reported error bars arise from the standard error associated with the linear fits to the initial reaction rate data (Origin version 7.0). In several cases,

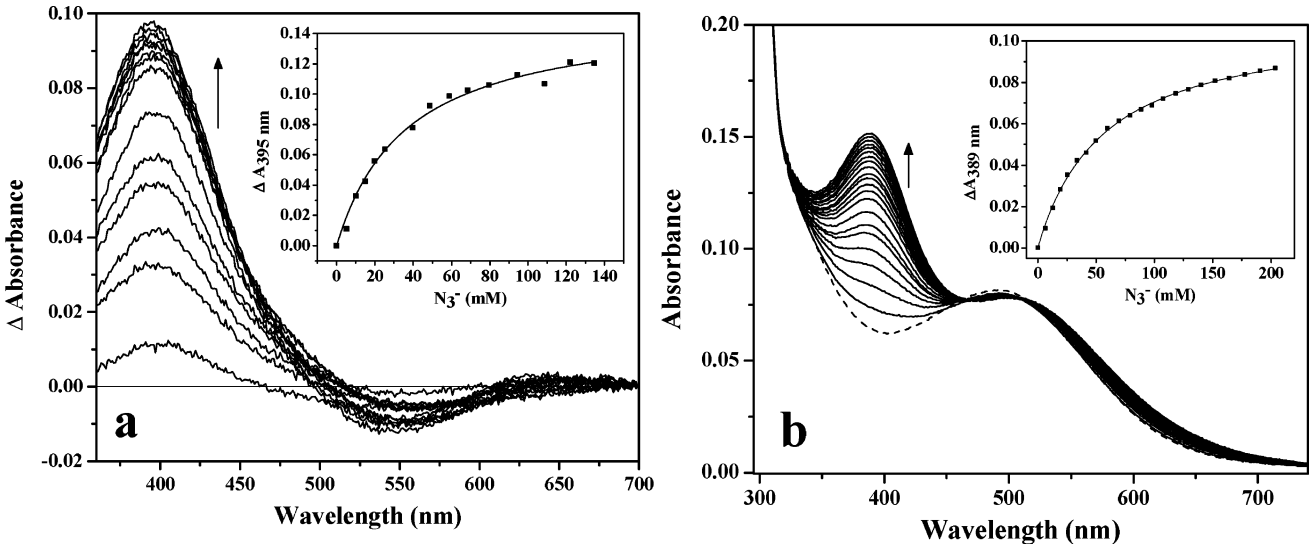


FIGURE 1: Azide coordination to Cu(II) in oxidized amine oxidases. (a) Difference spectra observed in the azide titration of rhDAO. (b) Azide titration in PSAO. The dashed spectrum (- - -) is the spectrum of the wild-type protein prior to addition of azide. Insets depict changes in absorbance of respective LMCT bands fit to a titration curve, yielding K_d values for respective Cu(II)−N_3^- complexes (see the text).

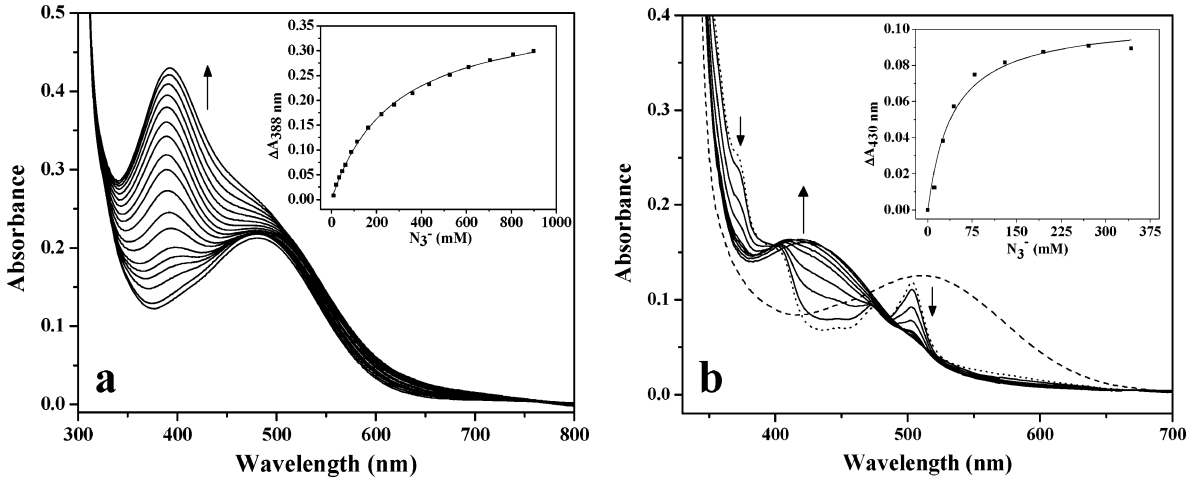


FIGURE 2: Azide coordination to Cu(II) in oxidized (a) and substrate-reduced (b) AGAO. The dashed spectrum (- - -) in panel b is the spectrum of the oxidized, resting protein sample, while the dotted spectrum (···) is that of the substrate-reduced protein prior to addition of azide. Arrows represent the spectral changes associated with the addition of azide. Insets depict the second-order fits used for the determination of K_d values for Cu(II)−N_3^- complexes.

rates of O_2 consumption were obtained on consecutive days at the same concentrations of dissolved oxygen, and in each case that was examined, the respective rates were within error.

Data Analysis. For each enzyme, kinetic data for both the reductive and oxidative half-reaction were analyzed using a statistical software package (GraphPad Prism version 4.03, GraphPad Software, San Diego, CA). Data were fit using global nonlinear regression analysis. Competitive, uncompetitive, noncompetitive, and mixed-type ($1 < \alpha < \infty$ and $\beta = 0$) inhibition models were entered into GraphPad as user-defined models according to Segel (*56*). In each case, the appropriate mode of inhibition was judged by the model that best fit the experimental data as determined by analysis of the statistical parameters built into GraphPad (R^2, absolute sum of squares, runs test, residuals). During the curve fitting process, the inhibition constant was established as a global parameter. Therefore, the K_i values reported in the Results represent the best fit value for respective data sets. Lineweaver−Burk plots are shown to allow visual comparisons of the inhibition models. These plots were not used to assign modes of inhibition for respective data sets which were determined by the global fitting analysis.

RESULTS

Azide as a Cu(II)-Directed Ligand in Oxidized Amine Oxidases. Respective λ_{max} values for the LMCT bands for Cu(II)−N_3^- complexes were found to be 395 nm for rhDAO ($\epsilon_{395} \cong 2500\ M^{-1}\ cm^{-1}$; Figure 1a), 385 nm for PPLO, 389 nm for PSAO ($\epsilon_{389} \cong 3100\ M^{-1}\ cm^{-1}$; Figure 1b), and 388 nm for AGAO ($\epsilon_{388} \cong 2000\ M^{-1}\ cm^{-1}$; Figure 2a). The experimentally determined K_d values for these enzymes are reported in Table 1.

Titrations with rhDAO proved to be more difficult as slight protein precipitation was observed upon the initial addition of azide. As a result, it was necessary to preincubate rhDAO with a small amount of azide, followed by a centrifugation step prior to the titration. Furthermore, during the course of the titration, a slight increase in the 700 nm baseline was

presumably a result of azide-mediated photodamage to the protein sample, for which there is precedence in the literature (*37*). For this reason, spectral changes associated with the addition of azide were monitored over a wavelength range of 310−700 nm using a monochromatic incident light source, and difference spectra were baseline corrected prior to fitting absorbance changes (Figure 1a).

Azide titrations for PPLO did not display the typical saturation behavior observed for the other CuAO enzymes (data not shown). Once azide levels reach ∼200 mM, ionic strength effects complicated analysis of the titration data. These effects are most likely a consequence of the greater degree of solvent accessibility in the active site environment of PPLO (*17*). Subsequently, the reported K_d value was estimated by fitting only the absorbance changes associated with azide additions up to 200 mM.

Azide Titrations of Substrate-Reduced Amine Oxidases. To examine the affinity of azide for Cu(II) in the substrate-reduced forms of AGAO and PPLO, the enzymes were reduced with benzylamine under anaerobic conditions and the azide titrations were carried out as previously described for PSAO (*26*) (Table 1). Notably, there is a 6-fold increase in the affinity of azide for Cu(II) in the substrate-reduced form of AGAO, and the λ_{max} of the LMCT band for the Cu(II)−N_3^- complex is shifted to the red by 42 nm with respect to that of the oxidized, resting enzyme ($\epsilon_{430} \cong 2000$ M^{-1} cm^{-1}) (Figure 2b). For substrate-reduced PPLO, the affinity of azide for Cu(II) was also observed to increase, but no significant shift in the λ_{max} of the LMCT band was observed to occur (data not shown). It is important to note that the ionic strength effects described above for PPLO are also apparent in this case. A titration of the substrate-reduced form of rhDAO was not attempted due to azide-mediated precipitation as reported above.

Kinetic Parameters. K_M and k_{cat} values for substrates amine and O_2 in the absence of inhibitors were generated through global model nonlinear regression analysis (GraphPad Prism) for each CuAO and are listed in Table 1. The values in Table 1 are in excellent agreement with the kinetic parameters generated by regression analysis using the Michaelis−Menten equation for control curves (Origin version 7.0; data not shown).

Azide Inhibition of the Reductive Half-Reaction. The effects of azide on the reductive half-reaction (eq 1) were determined by analyzing the kinetic parameters of each enzyme for a given amine substrate in the presence of increasing amounts of azide, at saturating O_2 levels. Nonlinear regression analysis revealed that a noncompetitive model best described the nature of azide's effects on amine oxidation for PSAO, AGAO, and PPLO (Table 2). Figure 3 shows azide's effects on amine oxidation in PSAO and PPLO. Lineweaver−Burk replots of the nonlinear regression global fits (Figure 3b,d) clearly show these lines converging on the *x* axis, as expected for noncompetitive inhibition (*56*).

For rhDAO, azide inhibition with respect to substrate putrescine was found to be competitive with a K_i value of 37 ± 8 mM (Table 2). The finding that a small copper ligand directly affects binding of substrate amine in this class of enzymes is unusual, and possible reasons for this are presented below. Additionally, it was found that the increase in ionic strength abolished the substrate inhibition previously reported for rhDAO with putrescine (*7*).

Table 2: Copper Amine Oxidase Azide Inhibition Parameters

enzyme	half-reaction	mode of inhibition	K_i (mM)
PSAO	reductive	noncompetitive	250 ± 18
	oxidative	competitive	31 ± 2
rhDAO	reductive	competitive	37 ± 8
	oxidative	partially competitive	92 ± 7
AGAO	reductive	noncompetitive	18 ± 2
	oxidative	noncompetitive	19 ± 1
PPLO	reductive	noncompetitive	26 ± 1
	oxidative	partially competitive[a]	23 ± 1

[a] The mode of inhibition is dependent upon azide concentration. At low azide levels (5−15 mM), a pure noncompetitive model best describes the data, whereas a mixed-type/partially competitive model best fits the data at higher azide levels (30−45 mM).

Azide Inhibition of the Oxidative Half-Reaction. The effects of azide on the oxidative half-reaction (eq 2) were determined by analyzing the kinetic parameters of each enzyme with saturating amine substrate (at least $10K_M$) at a series of azide concentrations, while O_2 levels were varied from ∼10 to 180 μM. Nonlinear regression analysis revealed that either competitive, noncompetitive, or mixed-type inhibition models best described the nature of azide's effects on O_2 reduction, depending on the enzyme source (Table 2). Figure 4 shows azide's effects on O_2 reduction in PSAO and PPLO. The Lineweaver−Burk representation of the nonlinear regression global fits for PSAO (Figure 4b) clearly illustrates a competitive mode of inhibition. It should be noted that the K_i value of 31 ± 2 mM generated from the nonlinear regression analysis is in very good agreement with the experimentally determined K_d value for the Cu(II)−N_3^- complex in this enzyme (Tables 1 and 2). This result can readily be rationalized in terms of azide and substrate dioxygen directly competing for binding to copper during the oxidative half-reaction.

In the case of PPLO (Figure 4c), the global fitting analysis of the data revealed that a noncompetitive model of inhibition best describes the data at low azide concentrations (5 and 15 mM), with the mode of inhibition shifting to the mixed type at higher azide concentrations (30 and 45 mM). The Lineweaver−Burk replot of the nonlinear regression global fits (Figure 4d) clearly shows this shift, as the 0, 5, and 15 mM lines have a common *x* intercept, whereas the 30 and 45 mM lines intercept the control off both axes. Linear mixed-type inhibition is defined as a mixture of pure noncompetitive inhibition and partially competitive inhibition (*56*). Consequently, neither a pure noncompetitive, a pure competitive, nor a mixed-type inhibition model adequately fit all the data curves shown in Figure 4c. The fits displayed in the figure represent two inhibition models, with the 0, 5, and 15 mM curves being fit with a pure noncompetitive model (generating a K_i of 41 ± 2 mM) and the 30 and 45 mM curves being fit with a mixed-type model. When the 0, 30, and 45 mM curves are fit, values of K_i (23 ± 1 mM) and αK_i (112 ± 5 mM) are obtained, which are in very good agreement with the calculated K_i from the reductive half-reaction (26 ± 1 mM) and the K_d (87 ± 21 mM) for the Cu(II)−N_3^- complex in substrate-reduced PPLO.

Azide's effects on the oxidative half-reaction for AGAO were found to be purely noncompetitive as judged by the global fitting analysis (Table 2); a common *x* intercept was observed in the Lineweaver−Burk replot of fits (data not shown). For rhDAO, azide displayed a mixed-type/partially

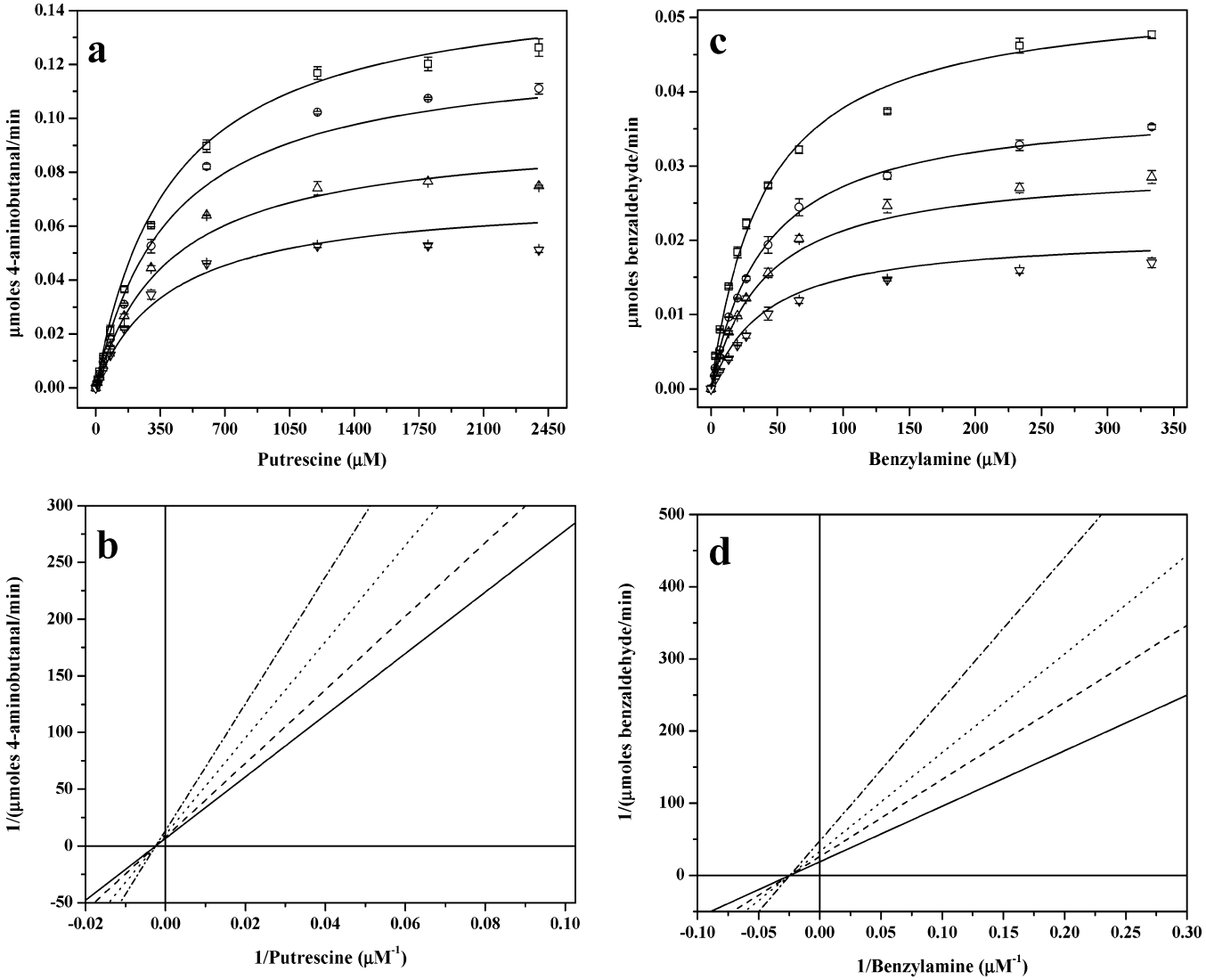


FIGURE 3: Azide inhibition of the reductive half-reaction in PSAO and PPLO. (a) PSAO experimental data curve fits obtained through global model nonlinear regression analysis. Data were collected in the presence of 0 (□), 46 (○), 133 (△), and 250 mM azide (▽). (b) Lineweaver−Burk plot generated using the inverse of the hyperbolic fits in panel a. Lines correspond to 0 (—), 46 (− − −), 133 (- - -), and 250 mM azide (−•−). (c) PPLO experimental data curve fits obtained through global model nonlinear regression analysis. Data were collected in the presence of 0 (□), 10 (○), 20 (△), and 40 mM azide (▽). (d) Lineweaver−Burk plot generated using the inverse of the hyperbolic fits in panel c. Lines correspond to 0 (—), 10 (− − −), 20 (- - -), and 40 mM azide (−•−).

competitive mode of inhibition. Quantitative analysis of the oxygen electrode data should be regarded as somewhat tentative because of variation among preparations of the recombinant enzyme in these experiments. The K_i value reported in Table 2 from nonlinear regression analysis must be considered an approximate value.

DISCUSSION

The monodentate ligands azide and cyanide are known to displace the equatorial water ligand of Cu(II) in amine oxidases and inhibit both half-reactions (eqs 1 and 2) (*26*, *44*, *57*, *58*). Although these ligands have been used previously for the study of CuAOs, significant variations in the methodology, as well as the quality and reproducibility of the data, have led to ambiguities in interpretation. Furthermore, systematic and direct comparisons among CuAOs are necessary, as it has become increasingly clear that significant differences may exist in the interaction of CuAOs with these ligands (*26*, *44*, *45*). To interpret the effects of azide on CuAO mechanism, kinetic parameters were determined for both the reductive and oxidative half-reactions in the presence and absence of azide. Specifically, we wished to test the previously articulated hypothesis that azide competes with O_2 for binding to copper during enzymatic reoxidation. Because of an intrinsic property of the amine oxidase ping-pong bi-bi mechanism, the two half-reactions (i.e., substrate amine oxidation and substrate O_2 reduction) are kinetically independent (*59*−*62*). In an attempt to cleanly dissect the effects of azide during each half-reaction, kinetic experiments were performed with variable concentrations of one substrate (either amine or O_2) while the other substrate was kept at saturating concentrations. This report is the first comparative study that analyzes the effects of azide on both half-reactions of multiple amine oxidases (AGAO, PPLO, PSAO, and rhDAO), including bacterial, yeast, plant, and mammalian enzymes.

Azide as a Cu(II)-Directed Ligand. Variations in the binding affinity of azide for Cu(II) among resting, oxidized CuAOs have been previously described (*37*, *41*, *43*, *44*, *63*, *64*). The data reported herein support these findings as K_d

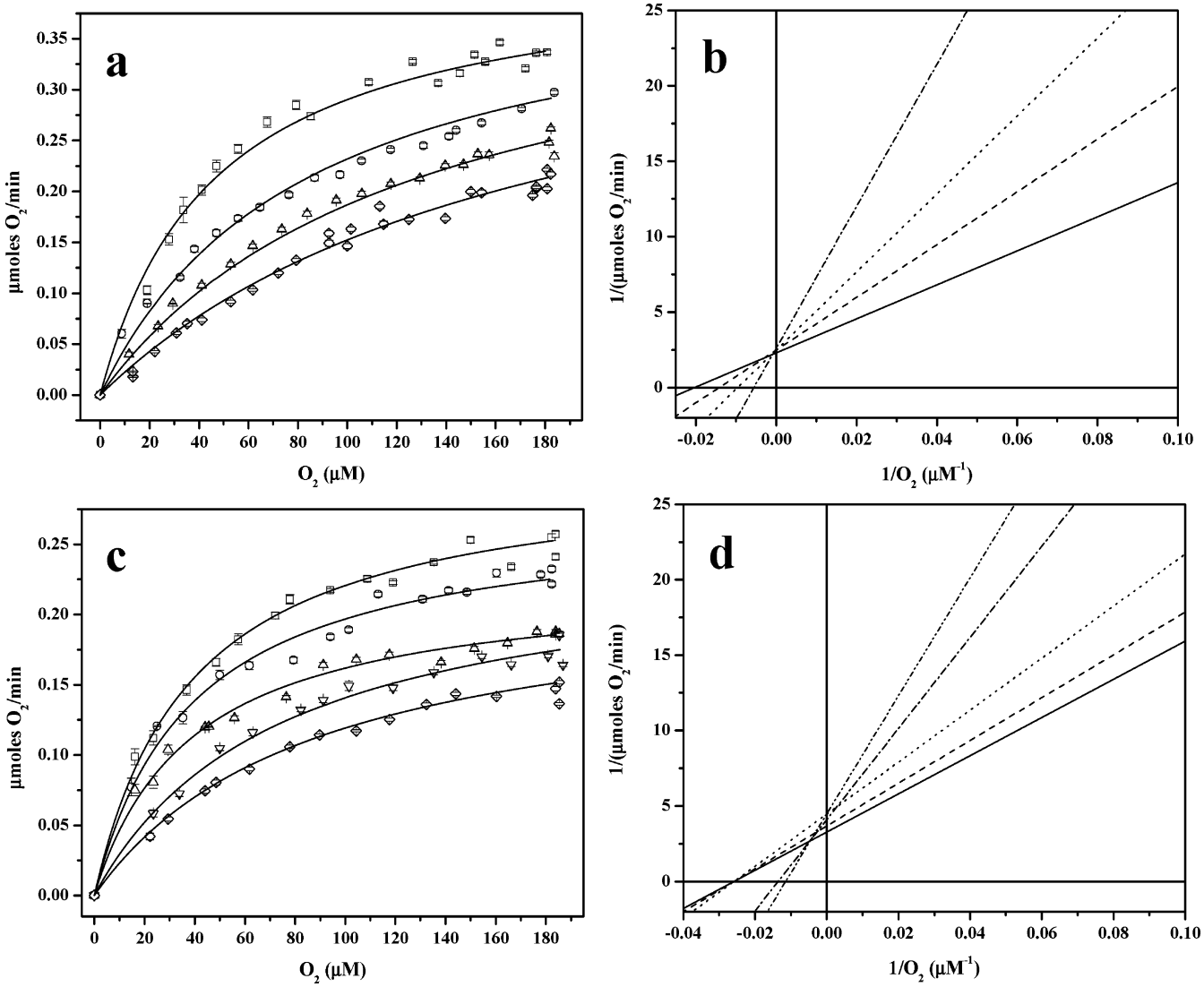


FIGURE 4: Azide inhibition of the oxidative half-reaction in PSAO and PPLO. (a) PSAO experimental data curve fits obtained through global model nonlinear regression analysis. Data were collected in the presence of 0 (□), 25 (○), 55 (△), and 90 mM azide (◇). (b) Lineweaver−Burk plot generated using the inverse of the hyperbolic fits in panel a. Lines correspond to 0 (—), 25 (− − −), 55 (- - -), and 90 mM azide (−•−). (c) PPLO experimental data curve fits obtained through global model nonlinear regression analysis. Data were collected in the presence of 0 (□), 5 (○), 15 (△), 30 (▽), and 45 mM azide (◇). (d) Lineweaver−Burk plot generated using the inverse of the hyperbolic fits in panel c. Lines correspond to 0 (—), 5 (− − −), 15 (- - -), 30 (−•−), and 45 mM azide (−••−).

values for Cu(II)−N_3^- complexes in the oxidized enzymes range from 38 to 213 mM (Table 1). In addition, it has been reported that the oxidation state of the quinone cofactor in some CuAOs may affect the affinity of azide for Cu(II). A marked increase in the affinity of N_3^- for Cu(II) has been reported for APAO when TPQ is in the reduced aminoquinol state, relative to the oxidized state; however, no difference in affinity was noted between these two enzymatic states in PKAO, PPAO, or PSAO (Table 1) (*41*, *44*, *63*, *65*). Results obtained for BPAO indicated a slightly higher binding affinity for azide in the resting form of the enzyme (*43*). These results indicate that N_3^- binds to Cu(II) with a higher affinity in the substrate-reduced forms of AGAO and PPLO than in the oxidized (resting) states (Table 1). Moreover, the LMCT λ_{max} shifts to the red by ~42 nm in substrate-reduced AGAO relative to that of the oxidized enzyme (Figure 2). The shift in the energy of the LMCT transition suggests that the redox state of the TPQ cofactor perturbs the electronic structure of the Cu(II) center in AGAO.

Azide Inhibition of the Reductive Half-Reaction. The inhibition of pig plasma amine oxidase (PPAO) by azide with respect to substrate amine has previously been described as uncompetitive (*63*, *64*), while inhibition patterns for BPAO have been reported as mixed at low amine concentrations with a shift to uncompetitive at higher substrate concentrations (*43*). In this study, azide was discovered to exhibit noncompetitive inhibition of the reductive half-reaction, with one exception (Table 2). The result that azide acts as a competitive inhibitor in rhDAO with respect to substrate putrescine is a novel result among CuAOs. The most straightforward interpretation is that azide binding to copper in the free enzyme perturbs the active site in a way that prevents substrate amine binding. For example, binding of azide to Cu(II) at the equatorial position could disrupt the active site hydrogen bonding network and thereby alter the orientation of the reactive TPQ C5 carbonyl, rendering it inaccessible to substrate amine. Hydrogen bonding has been shown to play an essential role in positioning the TPQ

cofactor during both biogenesis and amine oxidation (*66*−*70*). This hypothesis is further supported by the nearly identical values determined for the K_d (38 ± 3 mM) of the Cu(II)−N_3^- complex in the free enzyme and the calculated K_i (37 ± 8 mM) for azide inhibition with respect to substrate putrescine.

In the case of PSAO, azide displays noncompetitive inhibition toward substrate amine (Figure 3a,b). This indicates that azide binds randomly and reversibly to Cu(II) at different stages of catalysis in a manner independent of the binding of substrate. Current data show that azide binds to copper in the free enzyme (TPQ_{OX}) and the substrate-reduced enzyme (TPQ_{AMR}) with the same affinity (Table 1); therefore, the noncompetitive nature of azide inhibition may simply represent azide complexing Cu(II) at these two stages of catalysis. Azide's inhibition of amine oxidation in PSAO with the relatively high K_i value of 250 ± 18 mM indicates that significant reduction in the velocity of the reductive half-reaction does not occur at the concentrations of azide used during the oxidative half-reaction experiments (see below). Thus, PSAO represents a favorable case for cleanly dissecting the effects of azide on the oxidative half-reaction.

The inhibition of AGAO and PPLO by azide with respect to substrate β-phenylethylamine and benzylamine, respectively, was also found to be noncompetitive. For AGAO, the determined inhibition constant of 18 ± 2 mM (Table 2) is significantly lower than the calculated K_d for the oxidized enzyme (213 ± 18 mM) but close to that of the substrate-reduced form (34 ± 5 mM) (Figure 2). For PPLO, the K_i of 26 ± 1 mM (Table 2) is significantly lower than the experimentally determined K_d values for the Cu(II)−N_3^- complex in both the oxidized and substrate-reduced forms of the enzyme (Table 1). Thus, the affinity of azide for Cu(II) may be higher in one of the three catalytic states for which this complex is not easily characterized (TPQ_{SSB}, TPQ_{PSB}, or TPQ_{IMQ}; see Scheme 1). Given the relatively potent noncompetitive nature of azide binding in AGAO and PPLO, it can be expected that once azide levels approach or exceed K_i values (vs substrate amine) a substantial decrease in reaction velocity [$V_{max(i)}$] may result during the reductive half-reaction of these enzymes. This subject is readdressed during the discussion of azide's effects on O_2 reduction in AGAO and PPLO.

Azide Inhibition of the Oxidative Half-Reaction. Considerable disagreement about the interpretation of the effects of exogenous Cu(II) ligands on the oxidative half-reaction exists (eq 2). Two independent studies of PPAO concluded that azide was a competitive inhibitor with respect to substrate O_2, with calculated inhibition constants being consistent with measured K_d values for the Cu(II)−N_3^- complex (*41*, *65*). These results implicated the copper site as having a direct role in O_2 activation. A subsequent study with HPAO found the inhibition by azide to be noncompetitive with respect to O_2 during the oxidative half-reaction (*37*). The results of this latter study are viewed as being consistent with the proposal that O_2 binds to an off-metal site in the proximity of TPQ prior to its reduction, with the initial electron transfer to O_2 coming directly from TPQ_{AMR} (Scheme 2) (*25*, *35*, *37*, *61*, *71*, *72*). In HPAO, this hypothetical O_2 binding site is ~3 Å from TPQ and ~4.7 Å from the copper and is lined by residues Y407, L425, and M639 (*61*, *73*). Recently, we have experimentally identified a hydrophobic pocket in AGAO, PPLO, and PSAO by utilizing xenon as a probe for potential dioxygen-binding sites (*73*). The crystal structures of these enzymes revealed one common xenon binding pocket in the proximity of the active site (~7 Å from Cu and ~9 Å from TPQ). Xenon was subsequently shown to bind in this exact site in BPAO (*18*). It is important to note that the potential O_2 binding site identified in these Xe−CuAO structures is distinct from the site previously proposed by Klinman et al. for BPAO and HPAO. While the experimental identification of a consensus Xe binding site does not rule out the possibility of O_2 binding in the site proposed by Klinman et al. (*61*), it is clear that the most direct trajectory for O_2 from the observed Xe binding site toward the active site would involve initial close approach to the copper center (Figure 5). This hypothesis is supported by the results presented herein for the oxidative half-reaction in PSAO.

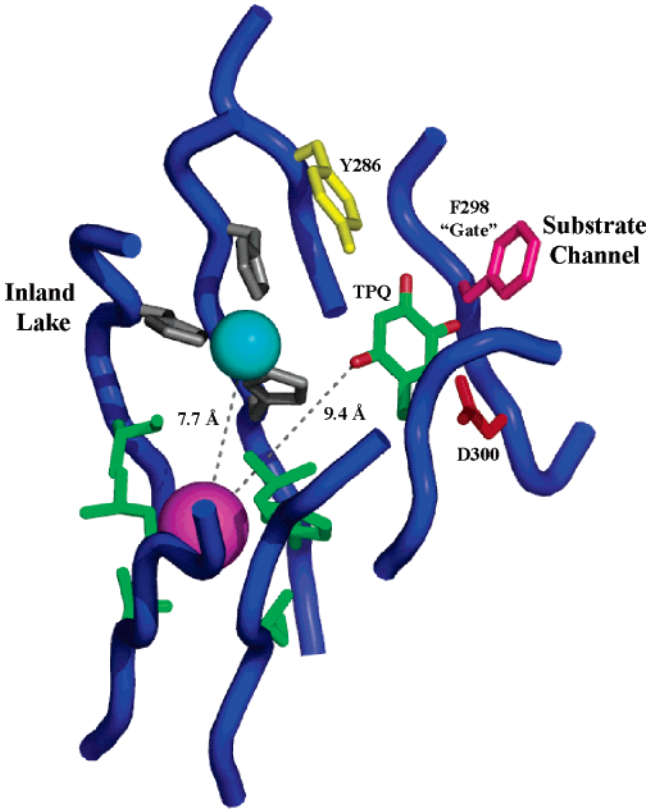


FIGURE 5: Xenon binding pocket in PSAO (PDB entry 1W2Z). Both Xe (purple) and Cu(II) (cyan) are represented as van der Waals spheres. Residues of interest are labeled. The productive orientation of TPQ is colored by atom (red for oxygen and green for carbon). The copper ligands (H442, H444, and H603; gray) and the residues forming the xenon binding pocket (I405, L407, Y446, I601, L616, and T618; green) are not labeled for clarity. The approximate locations of the substrate channel and the inland lake are given (see ref *17*). Xenon−Cu(II) and xenon−TPQ(O2) distances are also given (see ref *73*). This figure was generated using PyMOL (*77*).

Our results clearly show that azide exhibits competitive inhibition against substrate O_2 in PSAO with a K_i value of 31 ± 2 mM (Figure 4a,b). This value is in very good agreement with the measured K_d value of 53 ± 5 mM for the Cu(II)−N_3^- complex in this enzyme (Figure 1b). We believe these results are most consistent with mechanistic proposals placing Cu(I) on-pathway in PSAO, with the Cu(I)−TPQ_{SQ} moiety principally involved in O_2 activation (Scheme 2, **A** → **D**). Despite the fact that azide binds Cu(II) and O_2 reacts with Cu(I), ligation of Cu(II) by azide should

appear to be competitive toward O_2 for the following reasons: (1) the Cu(II)−TPQ_{AMR} moiety is in direct equilibrium with the Cu(I)−TPQ_{SQ} moiety with an intramolecular electron transfer rate in PSAO of ∼20000 s^{-1} (*24*, *74*); and (2) tetragonal Cu(II) complexes are known to display facile ligand substitution, and therefore, the reversible coordination by N_3^- should be rapid (*43*, *58*). In addition, azide is known to stabilize Cu(II) in substrate-reduced CuAOs and has been found to bind to copper in reduced PSAO with the same affinity as in the oxidized enzyme (Table 1) (*44*). It can be expected that during the oxidative half-reaction the binding of azide to Cu(II) shifts the Cu(II)−TPQ_{AMR} ⇋ Cu(I)−TPQ_{SQ} equilibrium in favor of the former species, thereby preventing electron transfer from TPQ_{AMR} to Cu(II), in a manner consistent with competitive inhibition. Further evidence of this equilibrium shift was observed during the azide titration of substrate-reduced AGAO, with the rapid disappearance of the Cu(I)−TPQ_{SQ} bands occurring upon addition of azide (Figure 2 and Table 1).

PSAO is an excellent enzyme system for cleanly observing azide's effects on O_2 reduction, as the calculated K_i value for the noncompetitive inhibition of substrate amine oxidation by azide is ≈5 times greater than the affinity of azide for Cu(II) in either the oxidized, resting, or substrate-reduced TPQ_{AMR} state (Table 1). This means that the reaction velocity [$V_{max(i)} \approx V_{max}$] during the reductive half-reaction is largely unaltered at the concentrations of azide used in the oxidative half-reaction experiments, even though at relatively high concentrations, azide may reduce the rates of one or more steps in the reductive half-reaction. Kinetic studies of PPAO provide a useful precedent (*65*). In this case, azide was discovered to inhibit both ES* → E_{red} and E_{red} → EO conversions, with azide's effects primarily credited to the inhibition of the E_{red} → EO step through direct competition with O_2 (*65*). Given our current understanding of the amine oxidase mechanism, these steps would correspond to the **C** → **D** and [**D** ⇋ **E**] → **F** steps in Scheme 1. We have experimentally confirmed that for PSAO saturating concentrations of amine ($14K_M$) overcome any effects of azide on the reductive half-reaction such that azide exhibits clean competitive inhibition toward substrate O_2 in the oxidative half-reaction experiments (*75*).

Azide was found to exhibit mixed-type/partially competitive inhibition with respect to substrate O_2 in rhDAO. Given the competitive nature of azide inhibition toward amine oxidation, kinetic effects on the reductive half-reaction could easily be overcome by using concentrations of amine in the oxidative half-reaction experiments that would cause $V_{max(i)}$ to approximate V_{max} ($77K_M$). Therefore, the observed partially competitive mode of inhibition observed with regard to O_2 reduction can most easily be explained by azide competing with O_2 for copper. However, further studies are required to confirm the reported K_i value (Table 2).

Azide was observed to exhibit noncompetitive inhibition with respect to O_2 in AGAO (Table 2). In PPLO, the inhibition shifts from noncompetitive at low azide levels (5−15 mM) to the mixed type at higher concentrations (30−45 mM) (Figure 4c,d). In both these cases, K_i values for reductive and oxidative half-reactions are equivalent (Table 2) and are lower than measured K_d values for the Cu(II)−N_3^- complexes (Table 1). Given this observation, we expected that substantial inhibition in the rate of the reductive half-reaction may occur at the concentrations of azide used for the oxidative half-reaction experiments and that this would complicate kinetic interpretation. This hypothesis was tested through theoretical curve simulations using GraphPad Prism. By utilizing the equations provided by Cleland for a simple ping-pong bi-bi system (*76*) with an inhibitor that reacts with free and substrate-reduced enzyme only, simulations demonstrated that reaction rates would depend on the magnitude of the K_i terms relative to each other and the inhibitor concentrations. Systematic variation of the K_i for the first half-reaction, relative to the K_i for the second half-reaction, reveals substantial effects on $V_{max(i)}$ for a family of theoretical *V* versus *S* curves (data not shown). In fact, these effects on $V_{max(i)}$ could directly affect which inhibition model best fit the data (*75*). While these simulations do not exactly represent the kinetic behavior of amine oxidase inhibition by azide, which can be expected to be much more complex because azide may bind at multiple kinetic stages and affect multiple rate constants, it does illustrate how the relative K_i values may influence the kinetic curves observed for the two half-reactions. Hence, it is quite possible that the noncompetitive mode of inhibition observed for azide in the oxidative half-reaction in AGAO and PPLO simply represents the relatively low K_i value for azide inhibition of the reductive half-reaction. In this context, the shift in the inhibition of PPLO by azide to the mixed type at higher azide levels can best be understood as the onset of partially competitive inhibition between azide and O_2 for copper. The reasonable agreement between αK_i (112 ± 5 mM) and the K_d (87 ± 21 mM) for the Cu(II)−N_3^- complex in substrate-reduced PPLO supports this hypothesis. The fact that this behavior is not observed for AGAO is consistent with the interpretation offered by Klinman for similar results with HPAO (*37*) or would be consistent with azide affecting multiple kinetic steps in the reductive half-reaction. At this time, the data do not permit us to distinguish between two possibilities: (1) inhibition by azide with respect to O_2 is intrinsically competitive in CuAOs, but this effect cannot always be deconvolved experimentally from the effects of azide on the reductive half-reaction; or (2) CuAOs differ in some steps of their reoxidation mechanisms.

Mechanistic Conclusions. Although it is not possible to unambiguously determine the effects of azide on the oxidative half-reaction in AGAO and PPLO, it is worthwhile to address the mechanistic implications of the competitive nature of this inhibition in PSAO and PPAO, with regard to the different mechanistic proposals presented in the introductory section. Competitive inhibition by azide in the oxidative half-reaction cannot readily be reconciled in terms of O_2 prebinding to the off-metal proteinaceous site proposed by Klinman and co-workers (Scheme 2, **A2**), with subsequent electron transfer occurring directly from TPQ_{AMR} to O_2. However, if the recently identified xenon binding pocket in AGAO, BPAO, PPLO, and PSAO truly represents a transient O_2 binding site in these enzymes, then our results can be rationalized by the fact that migration of O_2 toward the active site would result in an initial close approach to copper, from which electron transfer should readily occur (Scheme 2, **A** → **B**; Figure 5).

Kishishita et al. (*34*) consider their results in terms of mechanisms in which copper reduction is not required [O_2 is reduced via TPQ_{AMR} with Cu(II) simply acting as a site

for superoxide binding] and in which the Cu(I)−TPQ_{SQ} moiety is a catalytic intermediate. For the former mechanism, azide would exert competitive inhibition toward O_2 only if it affected the rate constant for binding of superoxide to Cu(II) in a manner that would be experimentally detected as an increase in the apparent K_M for O_2, assuming that no other step becomes rate-limiting in the presence of azide. This does not seem likely given the fact that both azide and superoxide are anions, and Cu(II) is known to undergo facile ligand substitution (*43*, *58*).

If PSAO and PPAO operated through the scheme proposed by Klinman and co-workers (Scheme 2, **A2** → **D**) (*25*), the most straightforward explanation for competitive inhibition is that azide coordination to copper perturbs the affinity of O_2 for the off-metal, hydrophobic dioxygen binding pocket as described for HPAO and BPAO (*61*, *71*). In this context, it has been argued that the increased net charge at the metal center has a direct effect on the affinity of O_2 for this hydrophobic binding site, which was manifested as an increase in $K_M(O_2)$ in Co(II)-substituted HPAO, relative to that in native HPAO in which the Cu(II)−OH^- species is believed to be present (*36*). Given that binding of azide to the copper center would result in the same net charge exhibited by copper-bound hydroxide, it would seem that other factors would need to be invoked to rationalize the reduced affinity of the hydrophobic pocket for O_2.

CONCLUSIONS

Despite the fact that azide ligand substitution reactions have been previously used for the study of CuAO catalysis, no direct, systematic comparison of the reductive and oxidative half-reaction kinetics among multiple CuAOs has been performed. One issue complicating kinetic interpretation is that for several CuAOs, azide has varying affinities for Cu(II), depending on the redox state of the quinone cofactor. We have found that a comparative study of azide's effects on the reductive and oxidative half-reactions is essential for the clear interpretation of the inhibition results for each half-reaction. Not surprisingly, our results with PSAO illustrate the critical importance of maintaining steady-state conditions in one half-reaction of an enzyme operating through a ping-pong bi-bi mechanism while performing kinetic inhibition studies on the opposite half-reaction. Generally, this can be easily performed for enzyme systems in which the inhibitor of interest has an effect with respect to only one substrate; however, when an inhibitor induces substantial inhibition in both half-reactions, cleanly dissecting the effects of the inhibitor against only one substrate may be difficult. This is illustrated in the cases of AGAO and PPLO for which azide is a potent noncompetitive inhibitor with respect to substrate amine.

Collectively, the competitive interaction of azide with respect to O_2 reported herein for PSAO and previously for PPAO (*41*, *65*) provides strong support for a direct interaction of dioxygen with the metal center during enzymatic reoxidation in these enzymes. This conclusion is most easily rationalized in terms of O_2 reacting with the Cu(I)−TPQ_{SQ} moiety, with azide stabilizing the Cu(II)−TPQ_{AMR} species. Although data with rhDAO suggest the inhibition by azide with respect to O_2 is at least partially competitive, additional studies to establish the catalytic viability of the Cu(I)−TPQ_{SQ} moiety in this enzyme are warranted. We suspect a similar mechanism to be operative in AGAO and PPLO as well. Given the large body of evidence in support of a nonredox role of copper in HPAO, the data collectively suggest that copper amine oxidases from different sources may in fact utilize discrete mechanisms to reoxidize the reduced quinone species, despite the similarities of their active site structures in the resting state.

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