Evidence Against Reduction of Cu^{2+} to Cu^{+} during Dioxygen Activation in a Copper Amine Oxidase from Yeast

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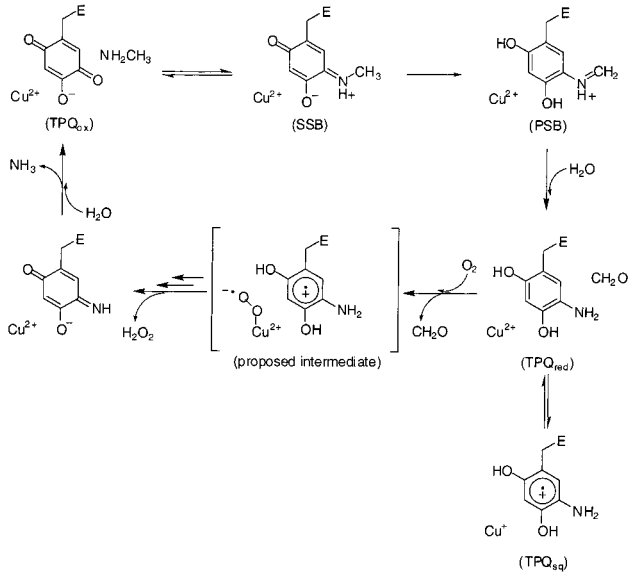
Abstract: The role of metal in copper amine oxidases was studied by removal and replacement of the copper in the enzyme from *Hansenula polymorpha*. The active-site cupric ion was reduced with the substrate, methylamine, and removed by anaerobic dialysis against cyanide and EDTA. Metal-free protein was found to have little or no activity in oxidizing methylamine and was unreactive toward phenylhydrazine. Addition of cobaltous ion to the metal-free protein restored 19% of the native specific activity and 75% of the native reactivity toward phenylhydrazine. A detailed kinetic study of the cobalt-substituted amine oxidase revealed that the major cause of the reduced specific activity was a 68-fold increase in the apparent K_m for oxygen. Saturation of the cobalt enzyme with oxygen gave a k_{cat} equivalent to the native k_{cat}. This study of a cobalt-substituted amine oxidase supports the mechanism proposed by Su and Klinman [Su; Klinman *Biochemistry* **1998**, *37*, 12513−12525] in which electrons are passed directly from the reduced TPQ cofactor into pre-bound oxygen without the need for a prior reduction of the metal. Instead of transferring electrons, copper is proposed to provide electrostatic stabilization during a rate-limiting reduction of molecular oxygen to superoxide anion.

The copper amine oxidases (CAOs[1]) are a class of enzymes found in organisms ranging from bacteria and fungi to plants and animals. These enzymes carry out the oxidative deamination of primary amines, forming the corresponding aldehyde and ammonia, with subsequent reduction of oxygen to hydrogen peroxide. To carry out this reaction, CAOs contain two cofactors: an organic cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), and a cupric ion.

The role of the TPQ cofactor in amine oxidation is well-established and summarized in Scheme 1. To start the reductive half-reaction,[2,3] TPQ_{ox} reacts with the substrate amine to form the substrate Schiff base (SSB), activating the C-1 proton of the substrate for abstraction. Proton abstraction results in the product Schiff base (PSB), with reduction of the cofactor. Hydrolysis of this intermediate releases the product aldehyde, leaving the enzyme in an aminoquinol form (TPQ_{red}). In the oxidative half-reaction,[4] oxygen interacts with TPQ_{red} to produce hydrogen peroxide and iminoquinone. Finally, hydrolysis of the iminoquinone releases ammonia and regenerates TPQ_{ox}. In previous studies of bovine serum amine oxidase (BSAO), it was shown that TPQ_{red} accumulates during turnover, in support of a partial rate limitation of the overall reaction by cofactor oxidation.[4] Significant rate limitation by the oxidative half-reaction is also implicated for HPAO, which shows no deuterium isotope effect on k_{cat} with methylamine.[5]

Scheme 1. Mechanism of CAOs[2−4] (SSB, Substrate Schiff Base; PSB, Product Schiff Base)



The role of the copper cofactor during amine oxidation is less well understood. Initial mechanistic studies of the copper amine oxidases assumed that the copper was essential for transferring electrons from the reduced TPQ to oxygen. In support of this assumption, Turowski et al.[6] showed that an

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(1) Abbreviations: CAO, copper amine oxidase; HPAO, *Hansenula polymorpha* amine oxidase; BSAO, bovine serum amine oxidase; TPQ, 2,4,5-trihydroxyphenylalanine quinone; TPQ_{ox}, fully oxidized TPQ; TPQ_{sq}, semiquinone form of TPQ; TPQ_{red}, fully reduced TPQ; SSB, substrate Schiff base; PSB, product Schiff base; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MeAm, methylamine; CuDep-HPAO, copper-depleted HPAO; M-HPAO, copper-depleted HPAO which has been reconstituted with metal M^{2+}.

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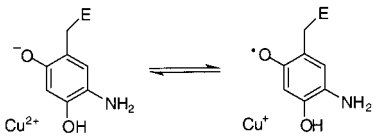
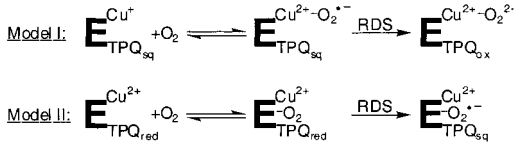
Scheme 2. Formation of Cu^{+} by Electron Transfer from TPQ_{red}[6]



Scheme 3. Proposed Mechanism for Dioxygen Binding and Reduction in BSAO[4,a]



[a] Model I: dioxygen binds to Cu^{+} to form superoxide. Model II: dioxygen binds at a non-metal site and receives an electron directly from TPQ_{red} (RDS; rate-determining step).

electron can be transferred at a catalytically competent rate from TPQ_{red} to the cupric ion in the copper amine oxidase from pea seedling, generating TPQ_{sq} and cuprous ion, Scheme 2. The resulting TPQ_{sq}/Cu^{+} species was proposed to bind and activate oxygen as the first step in the oxidative half-reaction. In opposition to the assumed electron-transfer role for copper, a detailed kinetic study by Su and Klinman[4] demonstrated that the rate-limiting step in the oxidative half-reaction of BSAO is the single electron reduction of a pre-bound form of O_2. Since it was expected that the transfer of an electron from Cu^{+} to pre-bound oxygen would be a fast process, two other possible rate-limiting steps were considered, Scheme 3. In model I, oxygen bound at copper (as Cu^{2+}-$O_2^{\bullet-}$) undergoes a rate-limiting electron transfer from TPQ_{sq} to form Cu^{2+}-O_2^{2-}. In model II, O_2 bound initially at a non-metal site undergoes rate-limiting electron transfer from TPQ_{red} to form the superoxide anion. Three pieces of evidence were presented in favor of the second model, which included (i) the detection of E-TPQ_{red} but not E-TPQ_{sq} under enzymatic turnover, (ii) the very positive redox potential[7] (and hence larger driving force) for reaction of E-TPQ_{sq}·Cu^{2+}-$O_2^{\bullet-}$ in relation to E-TPQ_{red}·Cu^{2+}·O_2, and (iii) the magnitude of the measured O-18 kinetic isotope effect.[4] One conclusion from Model II of Scheme 3 is that the copper does not transfer electrons from the cofactor to oxygen and that the Cu^{+}·TPQ_{sq} species is off the normal reaction pathway.

Another suggestion about the role of copper in CAOs comes from the chemistry of the TPQ model compounds. The last step of the synthetic preparation of 2-hydroxy-5-(5′-hydantoin methyl)-1,4-benzoquinone (a TPQ_{ox} model compound) is oxidation of 5-(2,4,5 trihydroxybenzyl)hydantoin (a model compound for the reduced cofactor).[8] This reaction is accomplished under slightly basic conditions in the presence of air, but with no added metal, showing that the reduced cofactor is sufficiently active to reduce molecular oxygen without the assistance of a metal.

To address the role of metal during catalytic turnover of the copper amine oxidases, a number of previous workers have investigated metal replacement in CAOs from bovine serum (BSAO),[9−11] lentil seedling,[12,13] and pig plasma.[14] Recent studies by Agostinelli et al.[10,11] and Padiglia et al.[13] characterized the cobalt reconstituted enzyme. In the former study, the cobalt-containing enzyme contained significant copper contamination, precluding unambiguous conclusions. In the latter study by Padiglia et al.,[13] where the cobalt enzyme is reported to give low activity, it is unclear whether the copper content was analyzed following reconstitution with cobalt. In neither instance were reactions analyzed with regard to their dioxygen dependence.

As described herein, methodology has been developed, using the amine oxidase from *Hansenula polymorpha* (HPAO), for metal removal and reconstitution which preserves enzyme activity and removes ambiguity of interpretation due to contaminating copper. Consistent with earlier work,[11,15] metal is found to play a structural role in the reductive half-reaction. More significantly, the nativelike activity of the cobalt-containing enzyme at high oxygen concentration allows us to rule out an obligatory electron-transfer role for metal during the conversion of O_2 to H_2O_2. This provides strong support for the earlier proposal by Su and Klinman[4] that the first electron from TPQ_{red} is transferred directly to dioxygen bound at a non-metal site. The degree to which the active site copper provides charge stabilization in the rate-limiting formation of superoxide anion has been estimated by comparison of rates for the enzymatic and model reactions.

Experimental Section

Materials and General Methods. All chemicals were purchased from commercial providers and used without further purification, except as noted. Oxygen was scrubbed from nitrogen by passing the gas stream through a basic solution of pyrogallol. Solutions were degassed by bubbling scrubbed nitrogen through the solutions for at least 6 h while stirring. All glassware was washed with nitric acid prior to use. Trace metal analysis was performed on a Perkin-Elmer 3000DV ICP-AES using commercially available metal standard solutions. 6-Amino-4-ethylresorcinol·HCL (TPQ_{red} model compound) was provided by Dr. Minae Mure.[8]

Protein Expression and Purification. HPAO was expressed and purified as described by Plastino et al.[16] with a few modifications. First, the growth media was supplemented with 10 μM $CuSO_4$. This was found to increase the yield of protein and reduce zinc contamination. Second, the cells were lysed using a Bead-Beater (Biospec Products) fitted with a stainless steel chamber and an ice−water cooling jacket. Cell disruption was performed by turning the Bead-Beater on for 5 min and then off for 5 min to allow the system to cool. This procedure was repeated 5 times for complete disruption of the cells. Eighteen liters of culture yielded 130 mg of purified protein as determined by the Bradford protein assay (Bio-Rad).

General Protein Characterization. Phenylhydrazine derivatization was performed by adding a 5-fold molar excess of a freshly prepared solution of phenylhydrazine·HCl to a sample of the protein. Spectra were recorded at 30 °C until the absorbance at 448 nm stopped increasing (typically 15 min). An extinction coefficient[17] of 40 500 M^{-1} cm^{-1} at 448 nm and a subunit molecular weight[18] of 75 700 were used to determine the percentage of active TPQ cofactor in the protein. For

(7) $O_2 \rightleftarrows O_2^{\bullet-}$, −0.16 V vs SHE at pH 7 [Sawyer *Oxygen Chemistry*; Oxford University Press: New York, 1991]; $HO_2^{\bullet} \rightleftarrows HO_2^{-}$, 0.74 V vs SHE at pH 7 [Hore *Standard Potentials in Aqueous Solution*; M. Decker: New York, 1985].

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TPQ_{ox}, an extinction coefficient of 1850 M^{-1} cm^{-1} at 480 nm was used.[19] For TPQ_{sq}, an extinction coefficient of 7100 M^{-1} cm^{-1} at 464 nm was used.[20]

Enzyme activity was measured by monitoring the rate of oxygen consumption using a Clark electrode at 25 °C with 1.5 mM methylamine in 100 mM potassium phosphate buffer, pH 7.1. The ionic strength was adjusted to 300 mM with KCl.

Copper Removal from HPAO. All buffers were degassed prior to use. Potassium phosphate buffer was passed through a 10 cm column of chelating resin (Sigma). In an anaerobic chamber, HPAO was diluted to 0.4 mL with a degassed solution of 1 mM MeAm·HCl in 100 mM potassium phosphate buffer at pH 7.2 to give a final protein concentration of about 2 mg/mL. This solution was introduced into a 0.5 mL Slide-a-Lyzer cassette (10 kDa cutoff, Pierce) with a syringe. The Slide-a-Lyzer was placed in 200 mL of 100 mM potassium phosphate buffer, pH 7.2, containing 1 mM MeAm·HCl, 1 mM KCN, and 1 mM Na_2-EDTA which had been cooled to 4 °C. The buffer chamber was kept at 4 °C and stirred under a constant flow of scrubbed nitrogen. The dialysis buffer was changed in the anaerobic chamber after 6 to 16 h. The protein was kept in this buffer for 4 changes of buffer, then transferred to 200 mL of 100 mM potassium phosphate buffer, pH 7.2, containing 1 μM EDTA. The protein was dialyzed against the second buffer for two changes, and finally dialyzed against 200 mL of 50 mM NaHEPES, pH 7.2. The protein was then removed from the Slide-a-Lyzer with a syringe to give copper-depleted enzyme (CuDep-HPAO). To obtain larger quantities of CuDep-HPAO, this procedure was scaled up by using a larger volume of protein solution, while maintaining the protein concentration at 2 mg/mL; this necessitated the use of a 3 mL Slide-a-Lyzer cassette.

Oxidation of TPQ_{sq}. CuDep-HPAO was placed in a hand-crafted cuvette having a bulbous top and a ground glass joint. After adding the sample, in an anaerobic chamber, the cuvette was sealed with a rubber septum. The sealed cuvette was placed in a Hewlett-Packard 8452A diode-array spectrophotometer fitted with a thermostated cell holder, set to 25 °C, and an initial spectrum recorded. If metal was to be added to the sample, this was achieved via a gastight syringe (Hamilton) and another spectrum was recorded. The solution was then oxygenated by transferring it to the bulbous part of the cuvette and removing the septum. The decrease in absorbance, due to the loss of the semiquinone, was monitored at 466 nm.

Activity Measurements. To prepare CuDep-HPAO for metal reconstitution and kinetic assays, the Slide-a-Lyzer was removed from the last dialysis buffer in air and the protein removed from the Slide-a-Lyzer. As phenylhydrazine only reacts with TPQ in HPAO when a metal is in the active site, vide infra, the protein was reacted with excess phenylhydrazine for 15 min at 30 °C to inactivate any active sites from which the copper had not been removed. The resulting solution was transferred to a Microcon microconcentrator (30 kDa MW cutoff, Amicon), washed 3 times with 50 mM NaHEPES, pH 7.2, and concentrated. The protein concentration was determined by the Bradford assay (Bio-Rad) and a molar equivalent of the appropriate metal was added to the protein. The protein was kept at 4 °C until use (at least overnight and not more that 1 week).

Steady-state kinetic measurements were performed as previously described[5] using a Clark electrode. Assays were performed in 100 mM potassium phosphate buffer, pH 7.1, containing 1.5 mM MeAm·HCl. The ionic strength was maintained at 300 mM with KCl. The data were fit to the Michaelis−Menten equation by nonlinear regression using Kaleidagraph (Abelbeck Software).

Cobalt EPR. Glycerol was added 1:1 v/v to a sample of Co-HPAO (21.2 mg/mL) to form a glass upon freezing. This sample was placed in a quartz EPR tube and frozen in liquid N_2. The spectra were collected using a Varian E-109 system equipped with a standard TE102 cavity and a Heli-tran liquid helium cryostat (Air Products). Quantitation was done using Co^{2+}-EDTA standards made from a cobalt ICP standard solution diluted into 20 mM ammonia/ammonium sulfate buffer (pH 9.0) containing excess EDTA.[21] Double integration of the spectra was done using Kaleidagraph (Abelbeck Software).

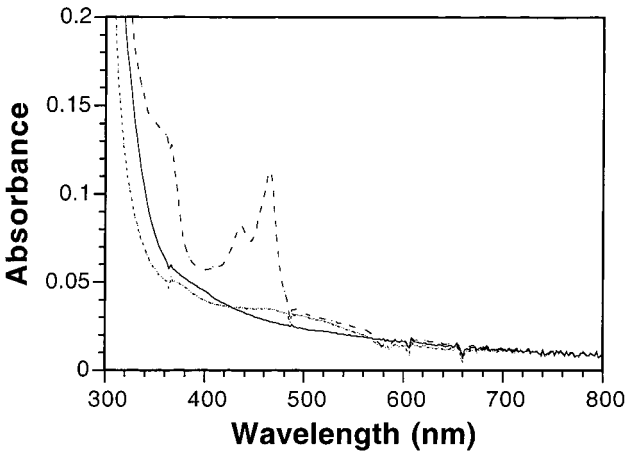

Figure 1. UV−vis spectra of CuDep-HPAO (1 mg/mL). The dashed line is CuDep-HPAO, as recovered from the dialysis slide (TPQ_{sq}). The solid line is CuDep-HPAO, which has been reduced with 1 equiv of dithionite (TPQ_{red}). The dotted line is CuDep-HPAO after exposure to air (TPQ_{ox}).

Model Compound Oxidation. A solution of the HCl salt of 6-amino-4-ethylresorcinol was prepared by dissolving 20.58 mg (108.5 μmol) in 10.0 mL of 10 mM HCl. The reaction buffer (1 mL, 100 mM potassium phosphate, pH 7.1, ionic strength = 300 mM) was equilibrated to the concentrations of oxygen shown in Figure 7 by passing mixtures of N_2 and O_2 over the solution while stirring at 25 °C. The model compound solution was added to the equilibrated buffer solution to give a final concentration of 10.85 μM model compound and the consumption of oxygen was followed. The observed rate constant (k_{obs}) was determined by fitting the change in oxygen concentration over time to a single-exponential equation.

Results

Copper Removal from HPAO. Treatment of HPAO with MeAm, KCN, and EDTA results in protein that has had most of the copper removed from its active site. The amount of residual copper was found to vary slightly, ranging from 10 to 20% of the total copper, but was usually less than 15%. Attempts to decrease the amount of residual copper by increasing the dialysis time or the number of times the buffer was changed did not improve the results. (Increasing the concentration of protein in the dialysate above 2 mg/mL resulted in CuDep-HPAO with greater residual copper, up to 30%.) Omission of the methylamine resulted in protein with 89% copper. The UV−vis spectrum of the CuDep-HPAO, Figure 1, is the same as that reported by Dooley et al. for the semiquinone form of TPQ_{sq}.[22] The intensity of the absorbance at 464 nm in the CuDep-HPAO indicates a stoichiometric amount of TPQ_{sq} has been formed.

Oxidation of TPQ_{sq} to TPQ_{ox}. Shown in Table 1 are the oxidation rates for TPQ_{sq} with various metals and in the absence of metal at 25 °C. In the absence of metal bound to HPAO, the TPQ_{sq} is quite stable in air, taking over 11 h to fully oxidize. Addition of Cu^{2+}, Zn^{2+}, or Co^{2+} to the active site accelerates the loss of absorbance 220-, 63-, or 15-fold, respectively.

Characterization of CuDep-HPAO. In the absence of metal bound to HPAO, the TPQ_{ox} and TPQ_{sq} forms of cofactor are

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Table 1. Rate of TPQ_{sq} Oxidation at 25 °C

enzyme form	rate[a] (min^{-1})	$t_{1/2}$ (min)	relative rate
CuDep	1.23×10^{-3}	564	1
Cu^{2+}	273×10^{-3}	2.55	221
Zn^{2+}	78×10^{-3}	8.9	63
Co^{2+}	19.2×10^{-3}	36.1	15.6

[a] In 50 mM NaHEPES, pH 7.2.

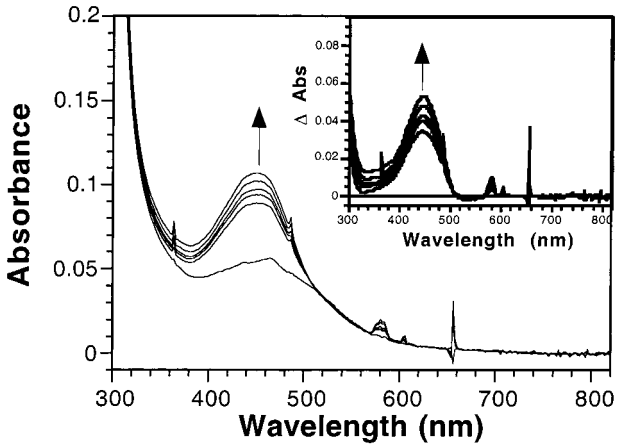

Figure 2. Reaction of phenylhydrazine with fully oxidized CuDep-HPAO (2.2 mg/mL, 13.8% Cu) at 30 °C. Spectra were recorded at 0, 1, 2.5, 5, 10, and 15 min. The lowest trace is the enzyme before addition of phenylhydrazine. The final phenylhydrazone concentration is 9.0% of the total protein. Inset: Difference spectra.

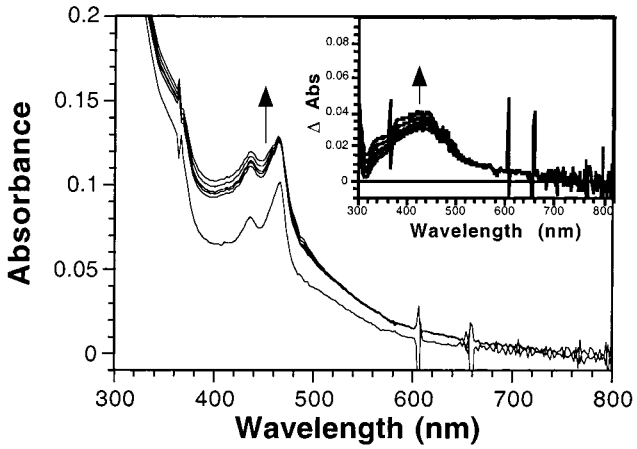

Figure 3. Reaction of phenylhydrazine with the semiquinone form of CuDep-HPAO (2.0 mg/mL, 14.2% Cu) at 30 °C. Spectra were recorded at 0, 1, 2.5, 5, 10, and 15 min. The lowest trace is the enzyme before addition of phenylhydrazine. The final phenylhydrazone concentration is 11.9% of the total protein. Inset: Difference spectra.

unreactive toward phenylhydrazine. Figure 2 shows the spectra recorded over 15 min during the formation of the phenylhydrazone derivative of a sample of fully oxidized CuDep-HPAO containing 29.4 μM protein, 26.5 μM TPQ_{ox}, and 13.8% residual copper. The absorbance at 448 nm after 15 min, due to the phenylhydrazone derivative, corresponds to 9.0% of the total protein. Even after 2 days at 4 °C, the absorbance at 448 nm only corresponds to 14.6% of the total protein. Shown in Figure 3 are the spectra recorded during the reaction of the semiquinone form of CuDep-HPAO with phenylhydrazine. As in the case with TPQ_{ox}, there is a small amount of reaction (12%) of the sample with phenylhydrazine, which correlates with the amount of residual copper (14%) in the sample. In the difference spectra can be seen a very slight loss of absorbance at 466 nm, probably

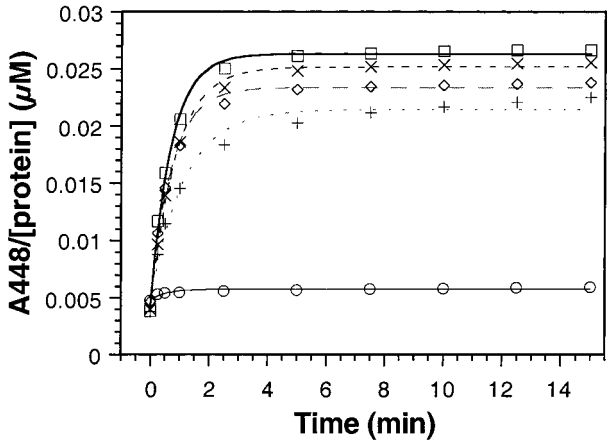

Figure 4. Time course of the reaction of phenylhydrazine, at 30 °C with CuDep-HPAO (○), Cu-HPAO (□), Co-HPAO (×), Zn-HPAO (◇), and Ni-HPAO (+). Absorbance values have been normalized to the protein concentration in each sample and then fit to a single expontential increase at 466 nm. The final absorbance values correspond to 10% (CuDep-HPAO), 65% (Cu-HPAO), 62% (Co-HPAO), 58% (Zn-HPAO), and 53% (Ni-HPAO) of the total protein.

due to air oxidation of TPQ_{sq}. These observations provide a method for the phenylhydrazine inactivation of sites which retain copper prior to the reconstitution of the active site with metal. Typically, the inactivation was performed with the enzyme in the TPQ_{sq} state.

Metal Reconstitution. The metal-binding site of CuDep-HPAO can be reconstituted with either Cu^{2+}, Zn^{2+}, Co^{2+}, or Ni^{2+}. Addition of 1 molar equiv of any of these metals restores reactivity with phenylhydrazine. Shown in Figure 4 are the time courses for reaction of CuDep-HPAO and the four different metal forms of HPAO with phenylhydrazine. The lowest trace is for CuDep-HPAO (11.2% residual copper) and corresponds to a final phenylhydrazone content of 10%. The top trace is for Cu-HPAO and corresponds to 65% phenylhydrazone. Co-, Zn-, and Ni-HPAO correspond to 62%, 58%, and 53%, respectively. It can also be seen that the rate of reaction with phenylhydrazine is not significantly affected by the nature of the metal in the active site.

Activity of Metal-Substituted HPAO. After inactivating the active sites of CuDep-HPAO which contain residual copper with phenylhydrazine and adding an equivalent of either Cu^{2+}, Zn^{2+}, Co^{2+}, or Ni^{2+}, the specific activity of the different metal forms of HPAO toward methylamine was measured at 25 °C, 258 μM O_2 (Table 2). It can been seen in the second row that native protein that has been reacted with phenylhydrazine has lost more than 99% of its activity. CuDep-HPAO initially shows 5% of its original activity, which is attributed to the residual copper in this protein. Addition of Cu^{2+} to CuDep-HPAO-PHZ restores activity to 79% of native. The inability to restore full activity is due, in part, to the fact that ca. 10% of the protein has been inactivated with phenylhydrazine before addition of metal ion. If the TPQ content of Cu-HPAO is taken into account, full activity is restored. Addition of Co^{2+} to CuDep-HPAO-PHZ leads to 19% of native activity. Zn^{2+} and Ni^{2+} each yield 1.7% of native activity.

Steady-State Kinetics. The significant increase in activity of Co-HPAO relative to CuDep-HPAO facilitated further characterization of this form of the enzyme. Figure 5 shows a plot of three determinations of V/E vs $[O_2]$ determined for three separate preparations of Co-HPAO. The kinetic parameters obtained from a single fitting of all of the data are $k_{cat} = 2.08$

Table 2. Specific Activity for Different Forms of HPAO with Methylamine

HPAO form	spec activ[a] (U/mg)	rel activ (%)	TPQ/subunit	Cu/subunit	M^{2+}/subunit
native	1.13	100	0.69	1.0	
+ PHZ[b]	0.00368	0.33	–	n.d.	
CuDep	0.0571	5.05	0.10	0.112	
+ PHZ[b]	0.0106	0.94	–	n.d.	
+ Cu^{2+}	0.894	79	0.54	1.23	1.23 (Cu)
+ Co^{2+}	0.213	19	0.52	0.162 (0.05)[c]	0.93 (Co)
+ Ni^{2+}	0.0195	1.7	0.41	0.163 (0.05)[c]	0.71 (Ni)
+ Zn^{2+}	0.0193	1.7	0.45	0.156 (0.04)[c]	0.86 (Zn)

[a] In 100 mM potassium phosphate, ionic strength of 300 mM, 1.5 mM methylamine, pH 7.1, 25 °C, 258 μM O_2 (1 U = 1 μmol/min). [b] Phenylhydrazine was added to a 5-fold molar excess over protein as described in the Experimental Section. [c] The parentheses indicate the increase in copper following addition of Co^{2+}, Ni^{2+}, or Zn^{2+}.

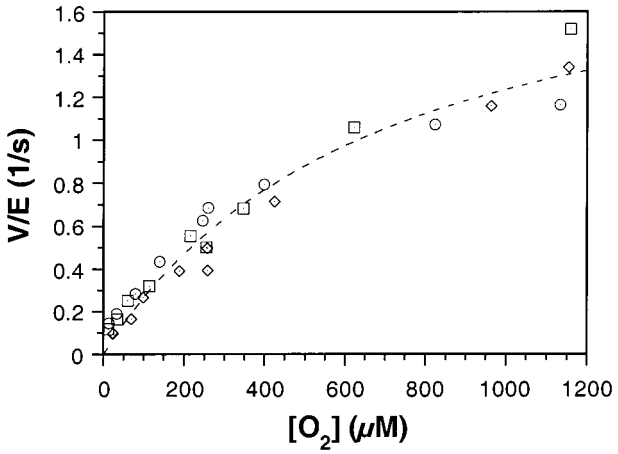

Figure 5. Effect of oxygen concentration on the rate of oxygen consumption for Co-HPAO at 25 °C using methylamine (1.5 mM) as the other substrate. Data are from three separate determinations using three separate preparations of Co-HPAO. All three data sets were used in the curve fitting. Conditions: 100 mM potassium phosphate, pH 7.1, ionic strength = 300 mM.

Table 3. Kinetic Parameters for Native and Co-HPAO[a]

enzyme	k_{cat} (s^{-1})	$k_{cat}/K_m(O_2) \times 10^3$ (M^{-1} s^{-1})	K_m (O_2) (μM)
native HPAO	2.12 ± 0.04	219 ± 20	10 ± 1
Co-HPAO	2.08 ± 0.2	3.06 ± 0.30	680 ± 110

[a] In 100 mM potassium phosphate, 1.6 mM MeAm (native), 1.5 mM MeAm (Co-HPAO), pH 7.1, ionic strength = 300 mM, 25 °C.

± 0.20 s^{-1}, $k_{cat}/K_m(O_2) = (3.06 \times 10^3) \pm (0.30 \times 10^3)$ M^{-1} s^{-1} and $K_m(O_2) = 680 \pm 110$ μM. To compare with native HPAO, $k_{cat} = 2.12 \pm 0.04$ s^{-1}, $k_{cat}/K_m(O_2) = (2.19 \times 10^5) \pm (0.20 \times 10^5)$ M^{-1} s^{-1} and $K_m(O_2) = 10 \pm 1$ μM. Table 3 shows a comparison of the kinetic parameters for native and Co-HPAO. The major difference is seen in the apparent K_m for oxygen, which is 68-fold larger in Co-HPAO than in the native enzyme.

Oxidation State of Co in Co-HPAO. To interpret the kinetic data, it was necessary to determine the oxidation state of the cobalt in Co-HPAO. Therefore, the X-band EPR spectrum of Co-HPAO at 8 K was recorded and is shown in Figure 6. The spectrum has two broad features centered at $g = 6.0$ and 2.7, as well as a broad and a sharp feature near $g = 2$. The $g = 6$ feature shows fine-structure which can be attributed to the ^{59}Co hyperfine interaction. Six of the predicted eight features are readily observed with a spacing of 80 G between these hyperfine lines. The sharp feature at $g = 2$ is attributed to an unidentified $S = {}^1/_2$ species, while the more broad feature is attributed to the Co^{2+}. Using Co^{2+}-EDTA as a standard, double integration

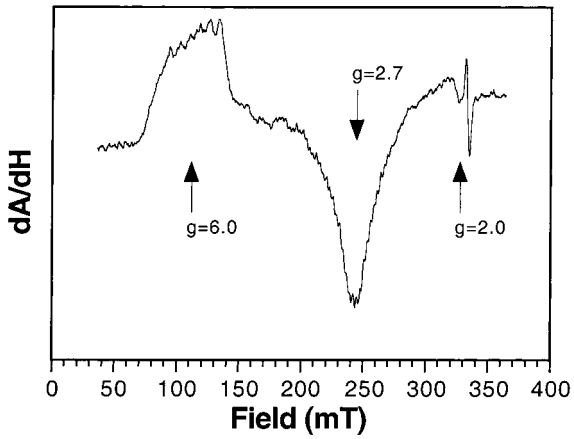

Figure 6. X-band EPR spectrum of Co-HPAO (10.6 mg/mL) in 50% glycerin at 8 K, 4 mW power, 9.24 GHz, and 32 G modulation amplitude.

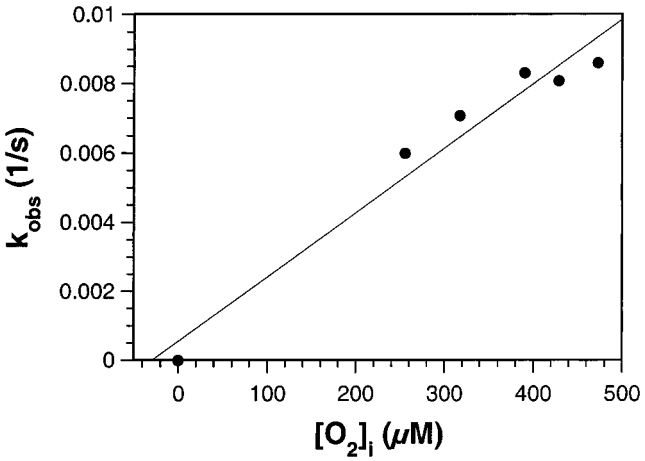

Figure 7. Rate of oxidation of TPQ_{red} model compound (6-amino-4-ethylresorcinol·HCL) vs oxygen concentration at 25 °C. Conditions: 100 mM potassium phosphate, pH 7.1, ionic strength = 300 mM, 258 μM O_2.

of the spectrum indicates that the spectrum accounts for ca. 90% of the total cobalt in the sample.

Model Compound Oxidation. The psuedo-first-order rate of oxidation of 6-amino-4-ethylresorcinol·HCl (a TPQ_{red} model compound) was determined by following the rate of oxygen consumption at different oxygen concentrations at 25 °C, Figure 7. The psuedo-first-order rate constants of oxygen consumption were plotted against the initial concentration of oxygen in each assay and fit to a linear equation. The slope of this line gives a second-order rate constant of 18.6 M^{-1} s^{-1} for model compound oxidation.

Discussion

Copper Removal. The active site cupric ion in amine oxidases is very tightly bound, making its removal for metal substitution studies difficult. Previous workers[9,14,23] have removed the copper from bovine serum amine oxidase by reducing the protein with dithionite and dialyzing the reduced protein against cyanide, a cuprous ion chelator. This is effective for HPAO as well; however, the harsh reducing conditions damage HPAO, such that reconstitution with copper does not restore enzyme activity (data not shown). In this study, methylamine (a substrate for HPAO) was used to reduce HPAO instead of dithionite. After dialysis against cyanide, more than 80% of the original copper was removed from HPAO and, importantly, the resulting protein could be reconstituted with copper, restoring full activity to the enzyme. Agostinelli et al.[10,11] recently described a similar approach to copper removal from BSAO obtaining protein with 35% residual copper.

A possible cause for the residual copper in this study may be the presence of nonfunctional proteins, either inactivated amine oxidase proteins due to partial denaturation of the protein, or other copper-containing, noncatalytic proteins due to variations in the protein preparation procedure. Another source of nonfunctional protein could be the incomplete processing of TPQ in HPAO or the cofactor being in an inactive conformation. Evidence against all of these possibilities is that the protein, which retains copper, oxidizes methylamine and reacts with phenylhydrazine. Denatured or nonfunctional contaminating protein would not oxidize methylamine or react with phenylhydrazine. The lack of activity would also be observed with incompletely processed TPQ or TPQ in an inactive conformation. A more likely explanation for the residual copper in the protein is that CuDep-HPAO has a high affinity for trace copper in the buffers.

Although methylamine is not usually considered a reductant, in combination with TPQ it forms a "mechanism-based" reducing agent. Dooley et al.[22] showed that in anaerobic, substrate-reduced amine oxidases there is a temperature-dependent electron transfer from TPQ_{red} to the cupric ion in the active site of the protein resulting in TPQ_{sq} and cuprous ion. They also showed that the position of equilibrium between these two species is drawn toward the TPQ_{sq}/Cu^{+} by addition of cyanide.

While it was expected that TPQ would be converted to its semiquinone form after reduction with methylamine, it was not known if TPQ_{sq} would be stable enough to isolate. Dooley et al.[22] observed a sub-stoichiometric amount of TPQ_{sq} in BSAO formed by anaerobic reduction of TPQ with benzylamine in the presence of cyanide. They attributed the loss of the semiquinone species to a disproportionation of the semiquinone. However, if kept anaerobic, the TPQ_{sq} in HPAO is obtained as a stable form of the cofactor after metal removal, Figure 1. Thus, methylamine is not only an effective reducing agent for copper removal from HPAO, but it also provides an opportunity to characterize TPQ_{sq} in the protein.

TPQ_{sq} Reactivity. Since the TPQ_{sq}/Cu^{+} was previously proposed to be the species which interacts with oxygen during turnover,[6] it was of interest to characterize how the metal free and metal-substituted forms of HPAO with TPQ_{sq} would react with oxygen. While it was expected that the semiquinone radical would react quickly with oxygen forming TPQ_{ox}, the metal-free, semiquinone form of HPAO was observed to react very slowly with oxygen, Table 1. In light of the inability of TPQ_{ox} to react with phenylhydrazine in the absence of divalent metal ion, vide infra, the low reactivity of TPQ_{sq} is likely due to a misalignment of the cofactor such that it is inaccessible to O_2.

Addition of metals to the active site of CuDep-HPAO increases the rate of TPQ_{sq} oxidation. Cupric ion increases the oxidation rate anaerobically, presumably due to an electron transfer from TPQ_{sq} to Cu^{2+} forming TPQ_{ox} and Cu^{+}. Since this is off the normal reaction pathway (there is an extra electron in the system), a more complete characterization of this electron transfer was not pursued. Addition of Zn^{2+} or Co^{2+} to CuDep-HPAO also increases the rate of TPQ_{sq} oxidation by oxygen, Table 1, with Zn^{2+} a better catalyst than Co^{2+}. This is opposite to the trend observed for TPQ_{red} oxidation, where Co^{2+} supports rapid turnover while Zn^{2+} appears to be a very poor catalyst, Table 2. The cause of this trend reversal is not currently understood. Similarly, the detailed mechanism whereby metal ion catalyzes TPQ_{sq} oxidation to TPQ_{ox} is unclear. One plausible pathway may involve electrostatic stabilization of a reduced oxygen species by M^{2+} (see the detailed discussion of the normal catalytic reaction below).

Phenylhydrazine Reactivity. Catalysis of the oxidation of TPQ_{sq} by zinc and cobalt suggested that copper may not be the only metal capable of supporting turnover in HPAO, but a more complete analysis of catalytic activity was needed to confirm this suggestion. From the work by Suzuki et al. with BSAO,[9] substitution of the active site Cu^{2+} with other metals was expected to result in enzyme with a significantly reduced activity relative to the native enzyme. In such an instance, residual activity due to copper complicates the interpretation of observed activity. More recent reports with BSAO[10,11] have dealt with the problem of residual copper by noting the difference in activity between the metal-depleted and the reconstituted forms of enzyme. To simplify the analysis in this study, a protocol was developed to remove any activity due to contaminating copper prior to metal reconstitution.

Phenylhydrazine was chosen as an inhibitor for active sites which retained Cu^{2+} after the metal removal procedure. Phenylhydrazine is a reagent that reacts specifically with carbonyl moieties. The large extinction coefficient of the hydrazone formed upon reaction of phenylhydrazine with TPQ is used to quantitate the amount of TPQ in a sample of HPAO.[17] The first two rows of Table 2 show the effectiveness of phenylhydrazine as an inactivator of HPAO. The specific activity of HPAO drops to less than 1% of its original activity upon reaction with phenylhydrazine. In the absence of metal, however, phenylhydrazine does not react with TPQ, Table 2, row 3. This is not due to the TPQ being in its reduced state, since the spectrum of TPQ_{ox} is clearly seen, Figure 2. The small reaction of phenylhydrazine with CuDep-HPAO, which is observed, corresponds to the amount of copper contamination in the sample.

A similar observation of metal-dependent hydrazine reactivity was made by De Matteis et al. using BSAO,[15] where they interpreted the unreactivity of TPQ toward hydrazine inhibitors as being due to a change of the pK_a of a water molecule which donates a proton to the C-2 oxygen of TPQ upon reduction. In the native enzyme, the water would be coordinated to copper and have a much lower pK_a than water in the metal-free enzyme. This is a possible explanation; however, no supporting experiments have been reported. An alternative explanation is that the metal, probably through its axial water molecule, is an important factor for correct orientation of the TPQ ring. Several recent studies[5,16,24,25] have shown that mobility of the TPQ ring

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(24) Cai, D.; Dove, J.; Nakamura, N.; Sanders-Loehr, J.; Klinman, J. P. *Biochemistry* **1997**, *36*, 11472−11478.

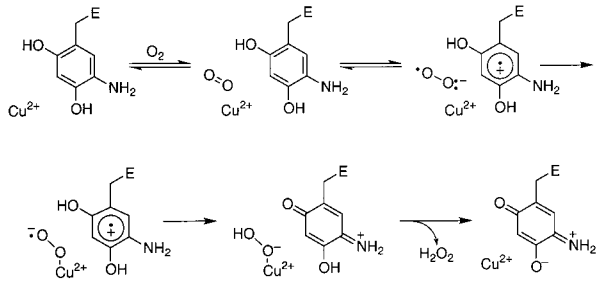
has significant effects on the activity of the enzyme. Further, these studies have suggested that residues in the active site are arranged to hold the TPQ ring in very precise orientations for efficient catalysis. The results presented here, with CuDep-HPAO, suggest that the copper is another "residue" that plays an important role in orienting the TPQ ring correctly.[26]

Enzymatic Turnover. One molar equivalent of metal appears sufficient to reconstitute nearly stoichiometric metal to HPAO, Table 2. After reconstitution, the activity of the metal-substituted forms of HPAO was studied. Restoring copper into the active site leads to full activity, after correction for the concentration of reactive TPQ, indicating that the protein has not been irreversibly damaged by the metal removal process. As seen previously,[9,23] cobalt also restores some activity to the enzyme. Zinc and nickel may provide some catalytic activity, but this activity is low enough that it could be from copper contamination during the reconstitution procedure. The low activity of Zn-HPAO and Ni-HPAO is unlikely to be due to the cofactor being in an inactive orientation, as shown by the reactivity of these forms of the protein toward phenylhydrazine, but could be due to a very large K_m for oxygen.

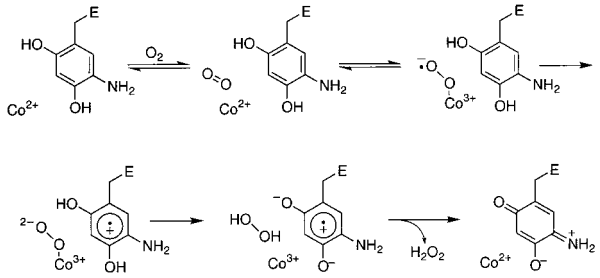
Since the purpose of these studies was to gain a better understanding of the interaction between the metal site in HPAO and molecular oxygen, it was important to conduct a detailed kinetic study of the oxidative half-reaction of Co-HPAO. As shown in Figure 5 and Table 3, the large observed difference between Co-HPAO and native HPAO, at atmospheric O_2, is due to an increase in the apparent K_m for oxygen. Significantly, under conditions of oxygen saturation, Co-HPAO shows a k_{cat} that is almost identical to that for native enzyme. The magnitude of k_{cat} for native HPAO, using methylamine as the substrate, has been proposed to be limited by reoxidation of TPQ_{red}.[5] In the case of Co-HPAO, where additional steps may also contribute to turnover, we conclude that the rate of reoxidation is likely to be $\geq k_{cat}$. That the k_{cat} values for both forms of the enzyme are so similar suggests that both WT-HPAO and Co-HPAO reduce dioxygen by the same mechanism and that Co^{2+} and Cu^{2+} must be comparable catalysts in this system, i.e., that the precise nature of the metal is not critical for enzymatic turnover. The difference in K_m between these two metallo-forms of HPAO suggests that the metal may, however, play a role in the formation of a viable O_2 binding site.

Before concluding a mechanism for oxygen reduction in HPAO from this study, the oxidation state of the cobalt in Co-HPAO had to be confirmed. High-spin Co^{2+} is an $S = {}^3/_2$ ion and has an observable EPR spectrum, while high-spin Co^{3+} is an integer spin ion and is not observable under standard EPR conditions. The EPR spectrum of Co-HPAO is shown in Figure 6, and exhibits features at $g = 6.0$, 2.7, and 2.0. Spectra similar to this have been observed previously in several other Co^{2+} reconstituted proteins,[27−29] and have been interpreted as arising from Co^{2+} in an asymmetric tetrahedral or five-coordinate environment having significant rhombicity.[27] Since the crystal structure of HPAO shows the copper in a distorted tetrahedral

Scheme 4. Proposed Mechanism for Dioxygen Reduction in HPAO



Scheme 5. An Alternative Mechanism for Co-HPAO that Involves a Change of Valence at Cobalt



or square-pyramidal liganding geometry,[30] we conclude that this EPR spectrum confirms the presence of Co^{2+} in the resting form of Co-HPAO.

A Proposed Mechanism. The data in this study are most consistent with a mechanism for dioxygen reduction that does not involve prior reduction of the copper in the active site. As shown in Scheme 4, the first electron from TPQ_{red} is transferred directly to oxygen forming a superoxide anion. Once formed, the superoxide anion is stabilized by the metal, allowing further reduction by a second electron from TPQ_{sq}. This mechanism was initially proposed by Su and Klinman[4] based on kinetic arguments and is further supported by a recent crystal structure by Wilmot et al.,[31] showing a dioxygen species, likely peroxide, bound to the Cu^{2+}.

By replacing Cu^{2+} with Co^{2+}, this study provides further evidence that an electron transfer from TPQ_{red} to copper in HPAO is not necessary for turnover. If a Cu^{2+}/Cu^{+} valence change were on the reaction pathway for native HPAO, an analogous valence change, either Co^{3+}/Co^{2+} or Co^{2+}/Co^{+}, would be necessary for Co-HPAO. On the basis of the reduction potential of Co^{2+}/Co^{+} (−400 to −500 mV vs SHE in methionine synthase[32]) and other species in the active site of HPAO, a Co^{2+}/Co^{+} valence change can be eliminated as a viable mechanism. If the cobalt in the resting Co-HPAO were Co^{3+}, a Co^{3+}/Co^{2+} valence change would be analogous to the Cu^{2+}/Cu^{+} mechanism proposed by Turowski et al.[6] However, the Co^{3+}/Co^{2+} mechanism can be ruled out since the EPR data show that the cobalt in the resting Co-HPAO is Co^{2+}.

An alternative Co^{2+} mechanism is shown in Scheme 5. In this mechanism, the first electron transferred to dioxygen comes from Co^{2+}, forming Co^{3+} and superoxide. A second electron is transferred from TPQ_{red} to the Co^{3+}/superoxide species, forming TPQ_{sq} and Co^{3+}/peroxide. The last electron transfer is from TPQ_{sq} to Co^{3+} to form TPQ_{ox} and $Co^{2+}$with release of H_2O_2.

(25) Schwartz, B.; Green, E. L.; Sanders-Loehr, J.; Klinman, J. P. *Biochemistry* **1998**, *37*, 16591−16600.

(26) It is possible that all of the TPQ in the active site does react with phenylhydrazine but the extinction coefficient of the phenylhydrazone derivative is significantly decreased in the absence of the active site metal. However, the presence of TPQ which reacts with phenylhydrazine after reconstitution of the active site with metal suggests that this is not the case.

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(31) Wilmot, C. M.; Hajdu, J.; McPherson, M. J.; Knowles, P. F.; Phillips, S. E. V. *Science* **1999**, *286*, 1724−1728.

(32) Drummond, J. T.; Matthews, R. G. *Biochemistry* **1994**, *33*, 3732−3741.

Although this mechanism is possible, and cannot be ruled out, it does not involve an initial electron transfer from TPQ_{red} to the metal. Furthermore, the Co^{2+}/Co^{3+} mechanism is quite different from the possible Cu^{2+} mechanisms. Most of the intermediates shown in Scheme 5 have the metal with an increased charge and the cofactor and oxygen species reduced by one electron when compared to Scheme 4. The only intermediate in common between the two mechanisms is the species with oxygen bound to the enzyme prior to electron transfer. This species is required in both mechanisms due to the lack of an observable viscosity effect on *V*/*K* for oxygen in both the WT HPAO and Co-HPAO.[33] It would be expected that the enzyme would not be optimized for the different charge localization required by the mechanism in Scheme 5, and although the enzyme may be able to accommodate the difference, we find it highly unlikely that the enzyme could carry out both reactions at equal rates. The fact that the rate of turnover (k_{cat}) for both the cobalt and copper-containing enzymes is the same suggests that both metallo-forms are using the same mechanism. This makes the mechanism of Scheme 5 very unlikely. With possible mechanisms which involve electron transfer from TPQ_{red} to the metal ruled out, we are led to the conclusion that copper does not undergo formal cycling from Cu^{2+} to Cu^{+} during catalysis and must, therefore, serve a different purpose.

An alternate role for the metal is as a Lewis acid, serving to stabilize superoxide formed from direct electron transfer from TPQ_{red} to O_2. If the metal is simply acting to stabilize superoxide, Zn^{2+} and Ni^{2+} should also be able to support catalysis; however, these forms of HPAO have very little activity. One possible explanation for the variation in reactivity among the metallo-forms of HPAO is a difference in the pK_a of water bound to different metals. A change of pK_a would explain the difference in activity between Cu^{2+} (pK_a = 7.5) and Co^{2+} (pK_a = 9.6) but does not explain the difference between Co^{2+}, Zn^{2+} (pK_a = 9.6) and Ni^{2+} (pK_a = 9.4).[34] Another possible explanation for the difference between Co^{2+}, Zn^{2+}, and Ni^{2+} could lie in the relative K_m for oxygen for each of these metals. The kinetic data show that Co-HPAO has a K_m for oxygen which is 68-fold higher than the wild-type enzyme (Table 3), so it is possible that the Zn^{2+} and Ni^{2+} forms are active but have a K_m for oxygen which is even higher. A final possibility for the differences between Zn^{2+}/Ni^{2+} and Cu^{2+}/Co^{2+} is the possibility of a charge-transfer interaction between Cu^{2+} or Co^{2+} with superoxide anion, after the one-electron reduction of O_2. Cu^{2+} could stabilize the superoxide anion by partially accepting an electron, forming a species with some Cu^{+}/O_2 character, whereas Co^{2+} could stabilize a superoxide anion by partial donation of an electron, forming a species with some Co^{3+}/O_2^{2-} character. Neither of these processes is possible with either Zn^{2+} or Ni^{2+}. While superoxide stabilization in the manner described above does involve, at least, a partial valence change of the metal, this valence change occurs only after dioxygen has been reduced by one electron.

Finally, we wished to investigate the rate acceleration provided by the enzyme in dioxygen reduction. Comparison of the second-order rate constant for the model compound oxidation (18.6 M^{-1} s^{-1}) with the second-order rate constant for the enzymatic oxidative half reaction with HPAO (2.19×10^5 M^{-1} s^{-1}) shows that the enzyme increases the rate of oxidation by more than 4 orders of magnitude at pH 7.1.[35] Electrostatic stabilization of superoxide anion formation has been described recently for glucose oxidase.[36] In this case, protonation of an active site histidine leads to an observed rate acceleration of 100-fold, which after correction for pH-dependent changes in redox potential for the active site flavin gives a net rate acceleration of ca. 10^5 fold. The surprising result from studies of this copper amine oxidase is that the active site divalent metal ion not only plays an analogous role of electrostatic stabilization in superoxide anion formation, but that the degree of rate acceleration arising from charge stabilization may be similar as well.

Conclusions

By examining the kinetics of oxygen consumption for Co-HPAO, we find that an electron transfer from the cofactor to the metal is not required during catalytic turnover in HPAO. We propose, instead, that the metal acts as an electrostatic center in stabilizing the formation of superoxide anion. An important corollary of these studies is the requirement for dioxygen binding to a non-metal site on the protein. Both kinetic and spectroscopic studies are in progress to address the molecular nature of O_2 binding. Finally, there is the question of why a protein would select copper as an active site metal if electron transfer to the metal were unnecessary. In this context, it is important to remember that the copper amine oxidases are dual function proteins, catalyzing both cofactor biogenesis and catalytic turnover. It appears that the unique redox properties of copper are required for cofactor biogenesis[37] but not catalytic turnover.

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