The Hydrogen Peroxide Reactivity of Peptidylglycine Monoxygenase Supports a Cu(II)-Superoxo Catalytic Intermediate

Andrew T. Bauman, Erik T. Yukl, Katsiaryna Alkevich, Ashley L. McCormack, and Ninian J. Blackburn

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From the Department of Environmental and Biomolecular Systems, OGI School of Science and Engineering at Oregon Health and Sciences University, Beaverton, Oregon 97006-8921 and the Mass Spectrometry Laboratory, Vaccine and Gene Therapy Institute, Oregon Health and Sciences University, Beaverton, Oregon 97006-3448

We have investigated the reaction of peptidylglycine monoxygenase (PHM) catalyzes the hydroxylation of peptidylglycine substrates at the Cα position, the first step in the amidation of peptides by the bifunctional enzyme peptidylglycine α-amidating monoxygenase (1, 2). The enzyme requires two copper(I) and undergoes redox cycling during catalysis via the intermediacy of both dicopper(II) and dicopper(I) forms (3). Structural (5–7), spectroscopic (8–12), and theoretical (13, 14) studies have provided a detailed description of the ligand environment of the copper centers, which are bound in separate domains, about 11 Å apart (Fig. 1). One copper (CuM, also termed Cuα) is bound to three histidines (His107, His108, and His172) in domain 1. The other copper (CuH, also termed Cuβ) is bound to two histidines (His242 and His244) and a methionine (Met239) in domain 2. X-ray absorption spectroscopy studies have identified large changes in coordination between Cu(II) and Cu(I) states (11). In the oxidized enzyme, CuH is further ligated by at least one solvent molecule, whereas CuM coordinates two histidines and two water molecules in the equatorial plane with the methionine in an axial position undetectable by EXAFS (11, 12). Reduction causes the water ligands to dissociate and the methionine to move close (2.25 Å) to the CuM center (9, 11). In contrast, crystallographic studies have failed to detect large changes in metal coordination during redox (6).

The detailed mechanism of substrate hydroxylation has been the subject of much debate. In general, it is accepted that the enzyme cycles through a reductive phase in which the two copper centers are reduced to Cu(I) and an oxidative phase in which O2 is activated by binding at one of the copper centers and subsequently hydroxylates the substrate. How this chemistry occurs is still unclear. Crystal structures of substrate-bound forms of PHM have located a di-iodo-YG substrate bound in the vicinity of the CuM center (2, 5, 6) (Fig. 1A), whereas a precatalytic complex of PHM with the slow substrate tyrosyl-D-threonine and dioxygen shows O2 bound at CuM but rotated away from the Cu-Cu(sb)ubstrate) vector (7). These structures strongly support the premise that the CuM center but provide no information on the chemical identity of the reactive species. If the reactive oxygen species is a CuM-peroxo or hydperoxo complex, an electron must be transferred from CuH to CuM to complete the 2-electron reduction of O2 to peroxide, but this itself presents a mechanistic challenge, since the two coppers are separated across an 11-Å solvent-filled cavity, and the shortest through-bond pathway is >80 Å. To overcome this problem, Prigge et al. (6) identified a potential electron transfer (ET) pathway involving the CuH ligand His108, Glu179, a hydrogen-bonded water molecule, and the peptide substrate, which reduced the ET pathway to ~20 Å. Invoking a different strategy, Jaron and Blackburn (10) suggested that O2 might react initially at CuM and that the superoxide so formed could channel to CuM providing a carrier for the electron and possibly a proton.

Neither of these mechanisms is consistent with all of the available data. Glutamine 170, a critical residue in the substrate-mediated ET pathway, can be mutated to alanine without loss of catalytic activity (15), whereas in the related enzyme dopamine β-monooxygenase, oxygen reduction and substrate hydroxylation remain tightly coupled even in the case of extremely slow substrates, apparently ruling out superoxide channeling, where some leakage of superoxide into bulk solution would expected (16). These results led Klinman and co-workers (16–18) to argue against peroxide as a viable intermediate in both PHM and dopamine β-monooxygenase and to propose that the reactive oxygen species is a Cu(II)-superoxo species, which abstracts a hydrogen atom from substrate prior to the electron transfer step.

If a CuM(II)-peroxo or hydperoxo species is an intermediate, then it...
should be possible to generate product by reacting the oxidized enzyme with hydrogen peroxide and substrate as depicted schematically in the oxidative phase of the mechanism shown in Fig. 1B. This “peroxide shunt” has been shown to occur in other oxygenase systems such as cytochrome P450 (19, 20), methane monooxygenase (21), and naphthalene 1,2-dioxygenase (22). Peroxide shunt reactions have the characteristic that when labeled peroxide is used as the source of the hydroxylating oxygen atom, the label is quantitatively transferred to the products (22). In this paper, we have investigated the reactivity of hydrogen peroxide with the oxidized form of the PHM catalytic core (residues 42–356, PHMcc) and find that hydroxylated product is indeed formed from size exclusion chromatography was loaded in 20 mM Tris acetate buffer, pH 8.0, was placed in a 50-ml conical centrifuge tube to which 100 mM copper sulfate was added at 1 ml. The resulting precipitate was centrifuged at 12,000 x g for 1 h. The enzyme began elution at 0.6 column volumes and continued to elute over 45 ml. At this stage of purification, SDS-PAGE revealed a purity level of 90–95%. The enzyme was further purified by anion exchange chromatography on a Biocad 700E perfusion chromatography system (Applied Biosystems), using a 10 × 100-ml Peek column packed with Poros 20-μm HQ anion exchange resin. Partially purified PHM from size exclusion chromatography was loaded in 20 mM Tris acetate buffer, pH 8.2, and then washed with 2 column volumes of the buffer. The column was eluted by a 0–300 mM NaCl gradient in loading buffer, over 10 column volumes. Purified PHM eluted close to 100 mM NaCl. Yields of pure PHMcc for 5 days of harvest ranged from 30 to 50 mg. Copper Reconstitution—The purified protein in 20 mM sodium phosphate, pH 8.0, was placed in a 50-ml conical centrifuge tube to which 100 mM copper sulfate was added at 1 μl/min on ice with gentle stirring until the molar ratio of copper added per protein was 2.5:1. The protein was then concentrated in an Amicon ultrafiltration device (10,000 Da cut-off) from 30 to 1 ml. This was followed by three wash sequences to remove excess and/or adventitiously bound copper. During each wash, 10 ml of 20 mM sodium phosphate buffer, pH 8, containing 25 μM Cu(II) (as Cu(SO₄)₂) was added to the ultrafiltration device, and the solution was concentrated to 1 ml. A final concentration step adjusted the pure PHMcc to 1 mM. The protein was then flash frozen in cryotubes and stored in aliquots at −80 °C. The final Cu/protein ratio was in the region of 2.0–2.2:1.

Copper and Protein Concentration—Protein concentration was determined using the A₄₃₅₀ and an extinction coefficient (1 mg/ml) of 0.98 as previously described (10). A₄₃₅₀ measurements were recorded on a Shimadzu UV-265 spectrophotometer at ambient temperature. Copper concentrations were determined using a PerkinElmer Optima 2000 DV inductively coupled plasma optical emission spectrometer.

HPLC Separation and Detection of Product and Substrate—Reverse-phase HPLC was performed with a Varian Pro Star solvent delivery module equipped with a Varian Pro Star model 410 autosampler (250-μl syringe, 100-μl sample loop), on a 250 × 4.6-mm Varian Microsorb-MV 100-5 C18 column. Substrate (dansyl-YVG; American Peptide Co.) and product (produced by the PHM-catalyzed reaction) were monitored by their dansyl fluorescence using a Waters 474 scanning fluorescence detector (λₑₓ = 365 nm, λₑᵐ = 558 nm). Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. Product was separated from substrate via isocratic loading and elution at 25% B in A.

Steady State Kinetic Measurements—The kinetics of the peroxide reaction were determined by following the rate of substrate consumption (or product formation) as a function of time. The reaction was performed in a water-jacketed glass reaction vessel, with stirring, in 100 mM MES buffer, pH 5.5, at 25 °C. All reagents except for hydrogen peroxide were added to the following final concentrations: dansyl-YVG (50–400 μM), Cu²⁺ as copper sulfate (5 μM), and PHM (2.5–5 μM). After the reagents were allowed to incubate for 2 min, the reaction was initiated by adding H₂O₂ from a 15 mM stock, to a final concentration of 0.5–4.0 mM. In a typical experiment using 1 mM H₂O₂, 330-μl aliquots were removed every 30 s, transferred to a 1.5-ml microcentrifuge tube containing 10 μl of 10% trifluoroacetic acid, and vortexed for 10 s. Substrate and product were separated by HPLC, and their concentrations were determined using a standard curve built from chromatograms of 10–200 μM samples of dansyl-YVG run under identical conditions. Kinetic constants were extracted from the raw data by fitting to the Michaelis-Menten equation using nonlinear regression in Sigmaplot 8.0.

Measurement of the Dansyl-YVG Dissociation Constant Kᵥ—For the oxidized enzyme, an Amicon (5-ml) ultrafiltration device was first pre-incubated overnight with 300 μM Cu(II)-loaded PHM (2.0 copper:protein) in 100 mM MES, pH 5.5, containing 1 mM dansyl-YVG. The protein solution was then washed repeatedly with buffer until the substrate concentration in the filtrate had fallen to a low level as determined by measurement of dansyl fluorescence of the filtrate. This conditioning procedure ensured that all irreversible substrate and/or protein binding sites on the membrane were occupied. Next, dansyl-YVG was titrated into the PHM solution, and aliquots of the filtrate were extracted for analyses as follows. A septum was placed over the ultrafiltration cell, and the cell was pressurized by injecting 0.6 ml of air over the solution. After a small amount of filtrate had been collected, it was returned to the concentrator, and the process was repeated three times. On the fourth pressurization, 10 μl of filtrate was saved for analysis of the concentration of “free” substrate by fluorimetry (λₑₓ = 365 nm; λₑᵐ = 558 nm). The remaining filtrate was returned to the concentrator along with the next titration aliquot (30 μl) of 10 mM substrate for a net volume gain of 20 μl. This procedure was repeated until a total substrate concentration of 1 mM was reached.
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For the reduced protein, the following modifications to the procedure were used. All solutions were first purged with argon and placed in an anaerobic chamber. Cu(II)-loaded PHM (2.0 copper/protein) in 100 mM MES, pH 5.5, was reduced with a 5-fold excess of buffered ascorbate and then titrated with dansyl-YVG in a conditioned ultrafiltration cell in the anaerobic chamber using an identical protocol.

The data were analyzed by constructing plots of fractional binding, \( f = ((S_T) - (S_F))/([S_T]) \) versus free substrate \( S_F \) (where the subscripts \( T \) and \( F \) represent total and free concentrations, respectively). These data were fit by nonlinear regression (SigmaPlot 8.0) to the equation,

\[
f = \frac{nS_F}{K_D + S_F}
\]

where \( K_D \) is the dissociation constant for binding of dansyl-YVG to PHM, and \( n \) is the number of binding sites. Values of \( K_D \) and \( n \) were refined in the fits.

\( ^{18}O \) Incorporation Experiments—\( ^{18}O \)-Labeling reactions were carried out similarly to those for kinetic analysis, except that all reactions were carried out in an anaerobic chamber. \( ^{18}O_2 \) reactions were performed by first flushing the enzyme solution with argon for 15 min. The argon headspace was replaced with \( ^{18}O_2 \), and the reaction mixture was equilibrated for 2 min. \( H_2^{18}O \) experiments were identical to those completed with \( H_2^{16}O \). All reactions were quenched with trifluoroacetic acid (10% in water) and purified on HPLC prior to mass spectrometry measurements.

Mass Spectrometry—Samples for mass spectrometry were purified on HPLC and then diluted 250 times for infusion with 50:50 acetonitrile/water in 0.1% formic acid. Samples were directly infused into the electrospray ionization source of a LCQ Deca XP Plus (Thermo, San Jose, CA) ion trap mass spectrometer at 3 \( \times 10^{4} \) microscans) over a range of \( m/z \) 500–1500. The standard isotope distribution for substrate and product was calculated using MS-Isotope in Protein Prospector 4.0.5 (R. Baker and K. Clausner; available on the WorldWide Web at prospector.ucsf.edu/ucsfhtml4.0/msiso.htm).

Measurement of Peroxide Concentration—Hydrogen peroxide concentrations were determined by using a BIOXYTECH \( H_2O_2 \)-560 quantitative peroxide formulation kit (OXIS International Inc.). 25 \( \mu \)l of quenched reaction mixture was diluted to 2 ml and mixed thoroughly. 100 \( \mu \)l of this mixture was then added to 1 ml of working reagent. The \( A_{560} \) was recorded for the samples and a series of standards, after incubating for 1 h at ambient temperature.

Peroxide Degradation Monitored by Oxygen Production—Oxygen production was monitored using a Rank Brothers oxygen electrode at 25 °C. 100 mM MES (pH 5.5) was added to a stirred cell until a stable baseline was achieved. The stirred cell was capped, and \( H_2O_2 \) was then added with a Hamilton syringe, through a small opening in the cap, to a final concentration of 43 \( \mu \)M. Once all the oxygen was converted to hydrogen peroxide, substrate and PHM were added with a Hamilton syringe to final concentrations of 200 and 5 \( \mu \)M, respectively. Each reaction was performed in a total volume of 2 ml. The reaction was quenched at the desired time, by the addition of 70 \( \mu \)l 10% trifluoroacetic acid. The quenched reaction was immediately analyzed for \( H_2O_2 \), product, and substrate concentrations.

Stoichiometry of Peroxide Consumption to Product Formation Using the Glucose/O Glucose Oxidase (GO) Reaction to Generate Hydrogen Peroxide—These experiments were performed in the same manner as the glucose/GO reactions above, with the exception that substrate was added to 400 \( \mu \)M, and only 21% oxygen saturation was used. The initial \( H_2O_2 \) concentration (formed by conversion of the dissolved oxygen to peroxide by glucose/GO) was assayed prior to the addition of substrate and PHM and again after the reaction was quenched with trifluoroacetic acid.

EPR Spectroscopic Quantitation of the Reduction of the Copper Centers in PHM by Hydrogen Peroxide—EPR spectra were obtained from PHM samples with \([\text{Cu(II)}] = 250 \mu\text{M}\), on a Bruker E500-X-band EPR spectrometer equipped with a SuperX microwave bridge, a superhigh Q cavity, and a nitrogen flow cryostat (Helitran; Advance Research Systems). The following experimental conditions were used: temperature,
80 K; microwave frequency, 9.4 GHz; microwave power, 20 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 10 G. EPR signals were quantified by double integration under nonsaturating conditions and by comparison with 100, 200, and 300 μM Cu(II) (EDTA) standards. Titration with hydrogen peroxide was performed by adding 10-μl aliquots of a 30% hydrogen peroxide stock solution to a 200-μl initial sample volume.

RESULTS

The reaction cycle of PHM (Fig. 1B) involves a reductive phase in which the enzyme is reduced by ascorbate to a dicopper(I) reduced intermediate and an oxidative phase in which the reduced enzyme reacts with peptide substrate and dioxygen to generate the hydroxylated product. As depicted in Fig. 1, many of the proposed mechanisms have suggested a Cu(I) (II)-peroxide or hydroperoxide as the hydroxylating species, formed from the reaction of PHM-Cu(I) with O2 and subsequent transfer of one electron from each Cu(I) center to dioxygen. If such an intermediate exists, then it should also be generated directly from the reaction of hydrogen peroxide with the oxidized (dicopper(II)) enzyme in the presence of substrate.

Measurement of Peptidyl-α-hydroxyglycine Product Using HPLC—To test whether this peroxide shunt chemistry occurred in PHMcc, we measured the reaction of the oxidized enzyme with dansyl-YVG and hydrogen peroxide. Initial concentrations of reagents were 200 μM substrate, 1 mM peroxide, and 5 mM PHM in 100 mM MES buffer, pH 5.5. Aliquots were sampled at 30-s time intervals, and product was separated from substrate by HPLC, using the fluorescence of the dansyl group for detection (Fig. 2a). Under these conditions, all of the substrate was converted into product within 2–3 min. Further, since the substrate was present in 40-fold excess over the enzyme, at least 40 enzyme turnovers occurred, implying that the reaction was catalytic. Control experiments where peroxide was incubated with substrate and 5 μM Cu2+ in the absence of PHM or in the presence of PHM that had been heated at 90 °C for 30 min gave no product (data not shown), demonstrating that the reaction was enzymatic.

**TABLE 1**

Comparison of kinetic and thermodynamic parameters for the PHM-catalyzed hydroxylation of dansyl-Tyr-Val-Gly by the ascorbate/dioxygen and peroxide pathways

| Parameter | Ascorbate/O2 | Peroxide reaction | Reduced PHM | Oxidized PHM |
|-----------|--------------|------------------|-------------|---------------|
| K_m       | 2780 ± 260   | 800 ± 400        | 22.5 ± 2.7  | 145 ± 16      |
| K_cat     | 9.2 ± 0.4    | 5 ± 3            |             |               |
| K_D       | 5.1 ± 0.6    |                  |             |               |

Fig. 2 shows rate data for the peroxide reactivity over a range of H2O2 concentrations (Fig. 2c) or dansyl-YVG concentrations (Fig. 2d). Kinetic parameters extracted from these plots are listed in Table 1. Table 1 also lists kinetic parameters for the ascorbate/O2-dependent reaction (Fig. 2b). These data show that although the rate of the peroxide reaction is much slower than the ascorbate/O2 reaction under the standard reaction conditions of 1 mM H2O2 and 200 μM dansyl-YVG, this is due primarily to a large increase in the value of K_m for the dansyl-YVG substrate, which is 2 orders of magnitude larger than for the ascorbate reaction. A modest decrease in k_cat from 9.2 to 5 s⁻¹ is observed for the peroxide reaction.

The large increase in K_m for the peroxide pathway suggests that the dansyl-YVG binds to a different form of the enzyme than in the ascorbate/O2-dependent pathway, consistent with reactivity occurring within the oxidized rather than the reduced form of the enzyme. To gain further insight into this possibility, we determined the dissociation constant for dansyl-YVG to PHM in its Cu(I) and Cu(II) forms. PHM was titrated with aliquots of dansyl-YVG in an ultrafiltration cell at 23 °C.
and a small volume of filtrate was extracted for measurement of the free substrate concentration from its fluorescence signal. Plots of fractional formation of enzyme-dansyl-YVG versus free substrate were fit to theoretical curves for substrate binding. The results are shown in Fig. 3. The $K_D$ values were 22.5 and 145 μM for the Cu(I) and Cu(II) forms of PHM, respectively. The $K_D$ for Cu(II)-PHM is in the same range as $K_M$ for the ascorbate/O$_2$-dependent pathway, consistent with the dansyl-YVG binding to the Cu(I) form as predicted by the general mechanism of Fig. 1. The $K_D$ for Cu(II)-PHM is about 7 times larger than the reduced $K_D$ and indicates that structural factors in the oxidized enzyme weaken the binding. Thus, the increased $K_M$ measured for the peroxide pathway, although larger than $K_M$ for Cu(II)-PHM, is more consistent with substrate binding to the Cu(II) than the Cu(I) form of the enzyme.

We also examined the possibility that hydrogen peroxide does not interact directly with the dicopper(I) enzyme but instead reduces it to the dicopper(I) form and that product forms by reaction of the dicopper(II) enzyme but instead reduces it to the dicopper(I) form, which would appear to rule out a simple reductive role for peroxide. However, subsequent experiments showed that products of the PHM catalytic reaction are able to accelerate the spontaneous disproportionation of peroxide to dioxygen and water, such that anaerobiosis could never be achieved by the above procedure. A more sophisticated method of obtaining anaerobic conditions utilizing the glucose/ST

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FIGURE 3. The dissociation constant $K_D$ was determined by titrating dansyl-YVG into a solution of either reduced (Cu(I)) (a) or oxidized (Cu(II)) PHM (b) in an ultrafiltration cell and analyzing the concentration of free substrate from the fluorescence of the filtrate after each addition. The data were analyzed by nonlinear regression of plots of fractional binding $f = ([S] - [S_f])/(E_f)$ versus free substrate $S_f$ (where the subscripts $T$ and $F$ represent total and free concentrations, respectively) fit to Equation 1.

FIGURE 4. Mass spectrometric determination of the distribution of labeled oxygen in the product of the PHM/dansyl-YVG/H$_2$O$_2$ reaction in unlabeled water. Top, solid trace, m/z for dansyl-YV-α-(OH)-Gly formed from H$_2$O$_2$ under atmospheric $^{18}$O$_2$ (solid trace) or H$_2^{18}$O$_2$ under anaerobic conditions (dashed trace). The minor bands in both sets of spectra arise from the natural abundance of $^{18}$H and $^{13}$C and represent 34.4 and 11.8% of the main peak at 587.1 for the $^{18}$O-labeled product. Middle, representative mass spectrum for dansyl-YV-α-(OH)-Gly formed from H$_2^{18}$O$_2$ under atmospheric $^{18}$O$_2$. The spectrum clearly shows the large contribution from the $^{18}$O-labeled product at m/z = 589.1. The peak at m/z = 589.1 is the $^{18}$O-labeled product and must be corrected for the 11.8% of $^{18}$O labeled product present at this mass. Bottom, percentage of $^{18}$O in the dansyl-YV-α-(OH)-Gly product formed from H$_2^{18}$O$_2$ under atmospheric $^{18}$O$_2$ (a), H$_2$O$_2$ under atmospheric $^{18}$O$_2$ (b), H$_2^{18}$O$_2$ under anaerobic conditions (c), and H$_2$O$_2$ under atmospheric $^{18}$O$_2$ (d). Calculated isotope ratios are listed in Table 1.

glucose oxidase reaction needed to be developed to test the effect of anaerobiosis on the PHM peroxide reactivity (see below).

It has been shown (25) that the $K_M$ for peptidylsubstrates is unaffected by the nature of the reductant. To test this with the dansyl-YVG substrate, we determined $K_M$ (dansyl-YVGC) using N,N’-dimethylphenylenediamine as reductant. The measured $K_M$ was 4.1 μM as compared with 5.5 μM for ascorbate. These data further suggest that peroxide is not merely fulfilling a reductive role, since if this were the case, $K_M$ (dansyl-YVGC) would not be expected to increase.

Isotope Distribution in the Peptidyl-α-hydroxyglycine Product—Electrospray ionization mass spectrometry was used to determine the oxygen isotope composition in the hydroxylated product. In previous studies, it was shown that when PHM reacts by the ascorbate/O$_2$ pathway, the oxygen atom incorporated into the α-hydroxy group of the α-hydroxyglycine product is derived entirely from molecular oxygen (26). If the peroxide pathway represents a peroxide shunt, then the α-hydroxy oxygen atom should similarly be derived from peroxide (22). Accordingly, we carried out reactions using H$_2$O$_2$ and H$_2^{18}$O$_2$ under anaerobic or aerobic ($^{18}$O$_2$) conditions. The results are shown in Fig. 4 and Table 2. When H$_2$O$_2$ was used, the product corresponded to the peptidyl-(α-18O)-glycine with m/z of 587 Da. H$_2^{18}$O$_2$ reacted under...
an aerobic conditions gave peaks corresponding to $\alpha-^{18}$OH (m/z 589, 90%) and $\alpha-^{16}$OH (m/z 587, 10%), as expected for the 90% enrichment of $^{18}$O in the labeled peroxyde. This confirmed that the oxygen atom was derived from peroxide rather than from solvent. However, when $H_2^{18}$O reacted under anaerobic conditions, only 35% of the product was labeled with $^{18}$O, and the remainder had exchanged with $^{16}$O. Since solvent exchange could be ruled out from the previous experiment, this result implied that the oxygen in the product was derived in part from molecular oxygen. An experiment where $H_2^{16}$O was reacted with substrate in the presence of 99 atom % $^{18}$O yielded both the $^{16}$-OH and $^{18}$-OH products in the ratio 40:60. These results indicate that, as expected for a monooxygenation reaction, the oxygen atom at the $\alpha$-OH group is derived from peroxyde but that a pathway exists for this oxygen to exchange with oxygen from molecular oxygen. Two mechanisms appear plausible for this: (i) reaction of peroxyde with the dicopper(II) enzyme may lead to an intermediate that is in rapid equilibrium with a Cu(I)-dioxygen species that can subsequently exchange with atmospheric O$_2$, or (ii) like ascorbate, peroxyde is acting as a reducing agent and forms the dicopper(I) intermediate, which itself generates product through the dicopper(I)-dioxygen route. As discussed above, the latter scenario would predict that product formation should not occur (or should be dramatically slower) under anaerobic conditions, contrary to observation. However, nonenzymatic peroxyde disproportionation could potentially generate sufficient oxygen to drive the reaction even under formally anaerobic conditions. To test this possibility, we measured the ratio of peroxyde consumed to product formed.

**Stoichiometry of the Peroxyde Reaction**—Table 3 compares peroxyde consumed with peptidyl-$\alpha$-hydroxylamine product formed for a number of different determinations. It is clear that peroxyde consumption exceeds product formation by 2--3-fold. Oxygen electrode measurements showed that peroxyde was decomposed to molecular oxygen and water in a nonenzymatic chain (Haber-Weiss) reaction:

$$H_2O_2 + HO^\cdot \rightarrow O_2 + H_2O + OH^\cdot$$

$$H_2O_2 + OH^\cdot \rightarrow H_2O + HO_2^\cdot$$

**Reactions 1 and 2**

This reaction was accelerated in the presence of the components of the PHM catalytic reaction, suggesting that PHM catalysis generates a species capable of initiating and/or propagating nonenzymatic peroxyde decomposition (for further details, see supplemental Fig. S1 and accompanying discussion). The presence of oxygen from the decomposition of peroxyde may explain why the rate of the PHM-peroxyde reaction does not decrease under “anaerobic” conditions, since O$_2$ is continuously generated by peroxyde decomposition. Also in mixed isotope experiments such as the reaction of PHM with $H_2^{18}$O under $^{16}$O$_2$, the product would still contain $^{18}$O even if peroxyde acted solely as a reductant due to $^{16}$O$_2$ generation from nonenzymatic peroxyde decomposition.

We therefore sought to monitor product formation under conditions where the nonenzymatic reaction was suppressed or eliminated. We used the reaction of glucose oxidase in the presence of 50 mM glucose and O$_2$-saturated buffer to generate a 1.2 mM solution of anaerobic hydrogen peroxyde. This reaction was allowed to proceed in a sealed oxygen electrode cell with no head space until all of the oxygen had been consumed (Fig. 5). Then PHM and dansyl-YVG were introduced into the cell, and the rate of peptidyl-$\alpha$-(OH)-glycine was determined by HPLC. This system had the advantage that any oxygen produced by peroxyde decomposition was rapidly recycled to hydrogen peroxyde by the GO reaction without releasing any superoxide into solution. The system was able to absorb oxygen entering the cell even when the cap was removed, and the cell was exposed to atmospheric O$_2$. With the cell sealed, the peroxyde concentration after all oxygen had been consumed was found to be 1.2 mM, and it remained unchanged, providing that no further oxygen was introduced into the system.

**Fig. 5 shows oxygen electrode traces corresponding to the GO reaction.**

**Fig. 5** shows the rates of product formation from the PHM/GO system compared with appropriate controls. Of great significance, the rate of product formation did not decrease when peroxyde was kept strictly anaerobic. Furthermore, peroxyde consumption was now strictly coupled to product formation, with the ratio of peroxyde consumed to product formed equal to unity (Table 3). This result suggests that peroxyde reacts with PHM to generate product by a pathway that does not rely on simple reduction to the dicopper(I) form and reaction of the latter with oxygen to form water and hydrogen peroxyde.

**Table 3** Stoichiometry of dansyl-YV-(OH)- Gly production to hydrogen peroxyde consumed for the standard PHM peroxyde reaction (200 $\mu$M dansyl-YVG + 5 $\mu$M Cu$^{2+}$ + 5 $\mu$M PHM + 1 mM hydrogen peroxyde, pH 5.5) and for the reaction carried out anaerobically in the presence of 50 mM glucose + 45 $\mu$g/ml glucose oxidase.

The latter reaction, the $H_2O_2$ was produced in situ from the reaction of the glucose/glucose oxidase with oxygen-saturated MES buffer and was measured to be 1.2 mM.
have a common Cu(I)-O₂ intermediate, peroxide must reduce the Cu(II) to Cu(I) within the PHM cavity at one of the copper centers. To test this hypothesis, we measured the ability of peroxide to reduce the copper centers of PHM at pH 5.5 using EPR spectroscopy as shown in Fig. 6. The EPR integrated intensity dropped to 75% of the fully oxidized Cu(II)-peroxo entity were the reactive species, it could produce peptidyl α-hydroxyglycine and water without the need for electron transfer from the other copper center (Cu₃). This would imply that the ascorbate/O₂ pathway might be abrogated in mutants with impaired function at Cu₃, whereas the peroxide reactivity was unaffected. Accordingly, we measured the ability of H172A, a Cu₃ mutant known to retain less than 1% of wild type activity (23), to generate product via the peroxide pathway. The H172A mutant was incubated with 1 mM peroxide and either 400 μM or 1 mM dansyl-YVG. The reaction was allowed to proceed for up to 60 min, 20 times longer than the time required by the wild type protein to convert 200 μM of substrate completely into product. The results showed that H172A produced no observable product.

We also tested the peroxide reactivity of CuM site deletion mutants. H242A has been shown to bind copper only at the Cu₄ site (11). The H242A mutant was also unable to produce product, indicating that the peroxide reactivity is not centered at Cu₄, since it does not proceed when only Cu₄ is occupied.

DISCUSSION

We have demonstrated that PHM is able to catalyze the hydroxylation of peptidylglycine substrates starting from the oxidized enzyme and using hydrogen peroxide as the only source of oxygen. When peroxide labeled with ¹⁸O was reacted with PHM and substrate in the presence of ¹⁶O₂, only 35% of the label was incorporated into the product. This scrambling of the label was not due to solvent exchange, since full incorporation of ¹⁸O occurred when both the peroxide and the ambient oxygen were labeled with ¹⁸O or when labeled peroxide was reacted under anaerobic conditions. Hence, the peroxide pathway must generate an increased time of incubation and represented only a fraction of the total copper in the protein. Thus, it is likely that an equilibrium exists between Cu(II)-peroxo and Cu(I)-superoxo and/or Cu(I)-dioxygen species, which must be accounted for in any mechanism for peroxide reactivity.

H172A and H242A Mutants—A true peroxide shunt requires no additional electrons to complete the monoxygenase reaction. Thus, if a CuM(II)-peroxo and CuI(II)-superoxo and/or CuI(II)-dioxygen species, which must be accounted for in any mechanism for peroxide reactivity.

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We have demonstrated that PHM is able to catalyze the hydroxylation of peptidylglycine substrates starting from the oxidized enzyme and using hydrogen peroxide as the only source of oxygen. When peroxide labeled with ¹⁸O was reacted with PHM and substrate in the presence of ¹⁶O₂, only 35% of the label was incorporated into the product. This scrambling of the label was not due to solvent exchange, since full incorporation of ¹⁸O occurred when both the peroxide and the ambient oxygen were labeled with ¹⁸O or when labeled peroxide was reacted under anaerobic conditions. Hence, the peroxide pathway must generate an increased time of incubation and represented only a fraction of the total copper in the protein. Thus, it is likely that an equilibrium exists between Cu(II)-peroxo and Cu(I)-superoxo and/or Cu(I)-dioxygen species, which must be accounted for in any mechanism for peroxide reactivity.

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intermediate that is capable of exchange with atmospheric dioxygen. We demonstrated that peroxide is decomposed in a nonenzymatic process to dioxygen and that the decomposition is accelerated when PHM is catalyzing substrate hydroxylation, suggesting that PHM catalysis generates a species capable of initiating and/or propagating nonenzymatic H₂O₂ decomposition. However, we were able to fully suppress the nonenzymatic peroxide decomposition by carrying out the PHM/peroxide reaction in the presence of glucose and glucose oxidase, which recycled any free oxygen back to peroxide and thus provided a strictly anaerobic environment. Using this system, we were then able to show that the PHM/peroxide reaction was tightly coupled and that its rate did not decrease when oxygen was totally excluded from the bulk solution.

Mixed labeling of the product with O from both peroxide and atmospheric dioxygen could occur if peroxide acted primarily to reduce the Cu(II) centers to Cu(I). In this scenario, it would be fulfilling the same role as ascorbate and hence would produce a dicopper(I) intermediate that would be poised for reaction with dioxygen in solution. Although the Michaelis constant for binding of O₂ to the enzyme-dansyl-YVG complex has not been measured, Kₘ,O₂ for dansyl-Gly-Gly-Ser is 73 μM at pH 6 and 37 °C (18). Since the PHM reaction is known to be equilibrium-ordered with O₂ binding to the enzyme-peptidylglycine complex (24), the Kₘ,O₂ will vary with substrate, but it is unlikely that Kₘ,O₂ for dansyl-YVG would vary substantially from that for dansyl-GGS. Using this assumption, we would predict that kₘ,O₂ for the reductive pathway should decrease dramatically when the O₂ concentration in the bulk solution decreased to undetectable levels as measured by the O₂ electrode in the glucose/glucose oxidase system. Since we observed no decrease in the rate of the PHM-peroxide reaction, we conclude that peroxide cannot be simply fulfilling the role of reductant and must be generating reactive oxygen species within the PHM active site cavity.

The increase in Kₘ,dansyl-YVG observed in the peroxide reaction and mirrored in the Kₐ for dansyl-YVG binding to the Cu(I) and Cu(II) forms of PHM provides strong corroborating evidence that the peroxide reactivity resides primarily within the oxidized enzyme.

The observation that isotopic molecular oxygen is able to exchange into product generated from hydrogen peroxide and oxidized enzyme implies that an intermediate exists along the reaction pathway that is electronically equivalent to metal-bound dioxygen. The most likely candidate for this intermediate would be a Cu₃(II)-dioxygen complex. Thus, for Cu(II)-peroxo to be a viable intermediate, it must be in equilibrium with Cu(I)-dioxygen. This would require that the long range electron transfer from Cu₃(I) to Cu₃(II) be reversible. As discussed in the Introduction, a number of novel suggestions have been necessary to explain the absence of a direct (through-bond) ET pathway from Cu₃(I) to Cu₃(II), including substrate mediation (6), superoxide channeling (10), or oriented solvent (16). Given these constraints on the available ET pathways, we consider reversible electron transfer to be highly improbable, as has also been argued by Klinman and co-workers (16).

Another test of the viability of a Cu(II)-peroxo would be the formation of peptidyl α-(OH)-glycine from the reaction of mutants that lacked a functional Cu₄ center with peroxide and Cu(II)-PHM, since the Cu(II)-peroxo species does not require additional ET for activity. We found that the Cu₄, mutant H172A was unable to catalyze product formation from peroxide and oxidized enzyme. This mutant has been shown to be less than 1% as active as wild type PHM in the ascorbate dioxygen pathway (23), and it has been suggested that the decrease in activity is due to impaired ET from the modified Cu₄ site. The complete absence of product formation even after 60 min may suggest that H172A abrogates the peroxide pathway in some additional way, perhaps by impairing the ability of peroxide to reduce the Cu₄ center. As a control, we also tested the Cu₃ site mutant H242A, which we have previously shown causes loss of copper binding at the M center (11). If this mutant was active in the peroxide reaction, it would suggest that peroxide binding and reactivity could occur at Cu₄. This mutant was similarly inactive in the peroxide reaction. These data provide compelling evidence that electron transfer from Cu₄ to Cu₃ is still obligatory in the peroxide pathway and hence that a Cu₃(II)-peroxo cannot be the catalytic intermediate.

A mechanism for the peroxide reactivity that is in accord with all of our data is shown in Fig. 7. We propose that hydrogen peroxide reacts initially at Cu₄(II), reducing the copper atom and forming a species that is electronically equivalent to Cu(I)-superoxide but may also be protonated. The superoxide formed can either react further within the PHM cavity or be lost to the bulk solution. The superoxide reacts inside the cavity with Cu₃(III), which captures the superoxide as a Cu₃(II)-superoxide species electronically equivalent to or in equilibrium with Cu₃(II)-dioxygen, providing a mechanism for oxygen exchange with atmospheric molecular oxygen.

FIGURE 7. Suggested mechanism for the production of hydroxylated product in the PHM/dansyl-YVG/H₂O₂ reaction. A, the peroxide reacts initially at Cu₄(II), reducing the copper atom and forming a species that is electronically equivalent to Cu(I)-superoxide. The superoxide formed can either react further within the PHM cavity or be lost to the bulk solution. B, the superoxide reacts inside the cavity with Cu₃(III), which captures the superoxide as a Cu₃(II)-superoxide species electronically equivalent to or in equilibrium with Cu₃(II)-dioxygen, providing a mechanism for oxygen exchange with atmospheric molecular oxygen.
the superoxo as a Cu(II)-superoxide, a species electronically equivalent to or in equilibrium with Cu(I)-dioxygen, providing a mechanism for oxygen exchange with atmospheric molecular oxygen. The superoxo that leaks from the active site into the bulk solution would act as a chain carrier in the nonenzymatic decomposition of peroxide and would explain why the latter is accelerated during PHM-peroxide catalysis.

Our data lead us to a conclusion similar to that of Klinman and coworkers (16, 18), that the CuM(II)-superoxide is probably the catalytically competent reduced oxygen intermediate. The proposed mechanism implies that the peroxide and ascorbate/O2 pathways intersect in a common intermediate, which can be formulated as a Cu(I)-superoxide. This is corroborated by the similar kcat values measured for the two pathways. The large difference in overall rate is due to the striking difference in substrate affinity between oxidized and reduced CuM centers to which the substrate must bind in the peroxide and ascorbate/O2 reactions, respectively. EXAFS has shown that Met314 binds more tightly to hydroperoxo and CuM(II)-superoxo intermediates, respectively (13, 14). Hydrogen atom abstraction by CuM-superoxo was found to be energetically neutral and to proceed with a lower activation barrier than the hydroperoxo pathway. Geometry optimization of the putative CuM(II)-superoxo intermediate suggested a square pyramidal structure for the intermediate, with superoxo bound side-on in the equatorial plane, H242 and H244 as additional equatorial ligands, and M314 making a long axial interaction.

The individual redox potentials of the copper centers in PHM are unknown, but the mechanism in Fig. 7 predicts that peroxide should reduce the CuM site preferentially to CuM. We used EPR to monitor the reaction of oxidized PHM with hydrogen peroxide at pH 5.5. We observed a maximum of about 25% EPR-undetectable Cu(II), which could be derived from either CuM reduction or spin-coupled CuM(II)-superoxo (or both). These numbers indicate that a maximum of half of the CuM centers can be reduced by peroxide, but this may be sufficient to support the rates that we measure. Further work will be required to fully elucidate the reaction chemistry of peroxide with the individual copper centers in PHM.

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