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Differential Reactivity between Two Copper Sites in Peptidylglycine α-Hydroxylating Monooxygenase

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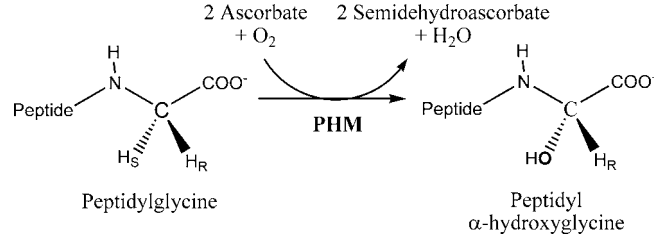
Abstract: Peptidylglycine α-hydroxylating monooxygenase (PHM) catalyzes the stereospecific hydroxylation of the Cα of C-terminal glycine-extended peptides and proteins, the first step in the activation of many peptide hormones, growth factors, and neurotransmitters. The crystal structure of the enzyme revealed two nonequivalent Cu sites (Cu_M and Cu_H) separated by ~11 Å. In the resting state of the enzyme, Cu_M is coordinated in a distorted tetrahedral geometry by one methionine, two histidines, and a water molecule. The coordination site of the water molecule is the position where external ligands bind. The Cu_H has a planar T-shaped geometry with three histidines residues and a vacant position that could potentially be occupied by a fourth ligand. Although the catalytic mechanism of PHM and the role of the metals are still being debated, Cu_M is identified as the metal involved in catalysis, while Cu_H is associated with electron transfer. To further probe the role of the metals, we studied how small molecules such as nitrite (NO_2^-), azide (N_3^-), and carbon monoxide (CO) interact with the PHM copper ions. The crystal structure of an oxidized nitrite-soaked PHM*cc*, obtained by soaking for 20 h in mother liquor supplemented with 300 mM $NaNO_2$, shows that nitrite anion coordinates Cu_M in an asymmetric bidentate fashion. Surprisingly, nitrite does *not* bind Cu_H, despite the high concentration used in the experiments (nitrite/protein > 1000). Similarly, azide and carbon monoxide coordinate Cu_M but not Cu_H in the PHM*cc* crystal structures obtained by cocrystallization with 40 mM NaN_3 and by soaking CO under 3 atm of pressure for 30 min. This lack of reactivity at the Cu_H is also observed in the reduced form of the enzyme: CO binds Cu_M but not Cu_H in the structure of PHM*cc* obtained by exposure of a crystal to 3 atm CO for 15 min in the presence of 5 mM ascorbic acid (reductant). The necessity of Cu_H to maintain its redox potential in a narrow range compatible with its role as an electron-transfer site seems to explain the lack of coordination of small molecules to Cu_H; coordination of any external ligand will certainly modify its redox potential.

Introduction

Many peptides, such as hormones, growth factors, and neurotransmitters, require α-amidation of their carboxy terminus to be fully biologically active.[1−3] The α-amidation is carried out in the trans-Golgi network and in secretory granules of neural and endocrine tissues by two sequential reactions. The first reaction is the stereospecific hydroxylation of the Cα of a C-terminal peptidylglycine (see Scheme 1), a reaction catalyzed by peptidylglycine α-hydroxylating monooxygenase (PHM). The second reaction, an *N*-dealkylation that yields the amidated peptide and glyoxylate, is catalyzed by peptidyl-α-hydroxyglycine α-amidating lyase (PAL).[4,5] Although the two enzymes can be expressed and can function independently, in most species they are expressed as a bifunctional single-chain enzyme, peptidylglycine α-amidating monooxygenase (PAM).

Scheme 1. Stereospecific Reaction Catalyzed by PHM



PHM is a binuclear copper protein with two nonequivalent type-2 Cu sites, Cu_M and Cu_H, separated by 11 Å.[6] It catalyzes the stereospecific Cα hydroxylation of the terminal glycine by

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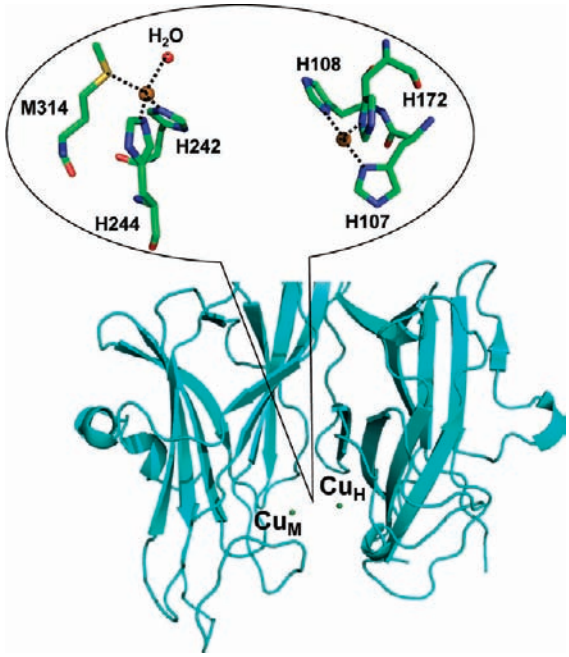

Figure 1. Structure of Cu_M and Cu_H sites of PHM*cc*. Cu_M is the catalytic site, and Cu_H is the electron-transfer site. The two copper centers are separated 11 Å by an interdomain cleft fully accessible to substrates, solvent, and small molecules.

incorporating one oxygen atom from dioxygen, using ascorbate as the physiological reductant. In the resting state of the enzyme, Cu_M has a distorted tetrahedral geometry, with one methionine, two histidines, and a water ligand that can be substituted by an external ligand (Figure 1). Cu_H has a planar T-shaped geometry, with three histidine residues and a vacant ligand position that could potentially bind a fourth ligand (Figure 1). Although the chemical structures of the copper centers Cu_M and Cu_H are clearly dissimilar, ligand field magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) studies on the resting oxidized PHM did not find any measurable differences between the two Cu sites.[7] Interestingly, when nitrite was used as a ligand, MCD and EPR measurements showed the presence of the two metal centers.[7]

Although the catalytic mechanism of PHM is still being investigated, there is agreement about the main roles of the metals: Cu_M is the metal involved in catalysis, while Cu_H is associated with electron transfer.[8−10] As mentioned above, these two copper centers can be classified as type-2 on the basis of their structural and spectroscopic properties: (i) both Cu_M and Cu_H are coordinated by histidines (Cu_M also includes a methionine in its coordination sphere) with a vacant position (Cu_H) or a position occupied by an exogenous ligand as water (Cu_M) and (ii) the EPR spectrum show typically a large A value ($A_z = 157 \times 10^{-4}$ cm^{-1})[7] for the unique copper signal.[11] Further, the two Cu(II) signals in the EPR spectrum of the nitrite-perturbed PHM have large A values ($A_z = 160 \times 10^{-4}$ and 165×10^{-4} cm^{-1}).[7] Therefore, there is a question about the type-2 characteristics of Cu_H, which are typical for catalytic sites, and its postulated role as an electron-transfer site. Single-electron-transfer reactions are usually carried out by type-1 copper centers (blue copper sites), characterized by a cysteine residue coordinated to the copper.[11]

To probe further into the role of the metals and to provide additional insight into the results of the MCD and EPR experiments, we studied how small molecules such as nitrite (NO_2^-), azide (N_3^-), and carbon monoxide (CO) interact with the copper active sites. Nitrite is the natural substrate of copper-containing nitrite reductases and thus can bind to biological copper sites.[12] Nitrite also forms metal complexes with Cu(II), adopting different geometries,[13−17] and with Cu(I), usually as an *N*-monodentate ligand.[18,19] Azide has also been used as a ligand for copper proteins in the oxidized state,[20−22] and there are several examples in the literature of Cu(II) and Cu(I) complexes with azide.[23,24] Carbon monoxide is a well-known π-acceptor ligand that can stabilize low oxidation states such as Cu(I), making it a very attractive ligand for reactivity studies of the reduced form of the enzyme. We found that nitrite, azide, and carbon monoxide bind Cu_M but, unexpectedly, do not bind Cu_H, despite the presence of a vacant position in the Cu_H coordination sphere and the high concentration of the ligands used in the experiments. The lack of coordination of small molecules thus seems to be an essential feature of the Cu_H site and is probably related to its proposed functional role as an electron-transfer site.

Results and Discussion

ox-PHM*cc*−Nitrite Complex. Crystals of the PHM*cc*−nitrite complex were prepared by soaking protein crystals for 20 h in mother liquor supplemented with 300 mM $NaNO_2$. The 2.35 Å resolution structure revealed that the nitrite anion coordinates Cu_M in an asymmetric bidentate fashion with copper-to-nitrite oxygen distances of 1.9 and 2.6 Å (Figure 2). It displaces the water ligand found in the oxidized form of the enzyme, with one of its oxygens occupying the approximate position of the oxygen of the water molecule. The Cu_M environment changes significantly from tetracoordinated $N_{2(His)}S_{(Met)}O_{(water)}$ to pentacoordinated $N_{2(His)}S_{(Met)}O_{2(nitrite)}$. This particular coordination

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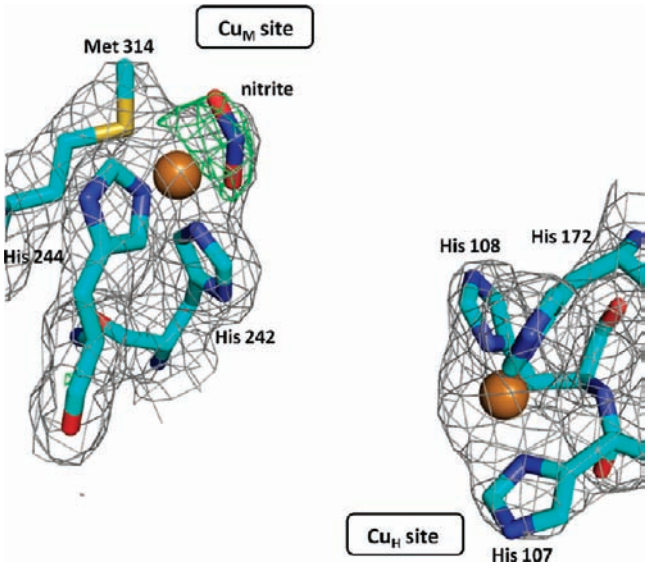

Figure 2. Structure of Cu_M and Cu_H sites of a nitrite-soaked PHM*cc*. The nitrite is shown bound to Cu_M in an asymmetric bidentate fashion (Cu−O1 = 1.9 Å; Cu−O2 = 2.6 Å). The gray mesh shows the σ-weighted $2mF_o - DF_c$ (contoured at 1.0σ) and the green mesh the $mF_o - DF_c$ omit maps (contoured at 2.8σ). The omit maps were computed with Refmac5. Carbons are colored cyan, nitrogens blue, oxygens red, sulfur yellow, and copper gold.

mode of a nitrite ligand has been found in nitrite-soaked crystals of nitrite reductases (CuNRs), enzymes for which nitrite is the natural substrate.[25−29] In CuNRs, the catalytic type-2 copper ion is coordinated by three histidine residues, suggesting that the presence of methionine in Cu_M, at least in the oxidized form of the enzyme, is not playing a critical role in the binding and coordination mode of nitrite. Nitrite has also been found coordinated to the same type-2 Cu of CuNRs as an *O*-monodentate ligand, but only when substitutions are introduced in residues surrounding the nitrite binding site that render the enzyme inactive.[30] In PHM, the Cu_M site is open, without any neighboring residue that could force nitrite to adopt a different coordination mode. The PHM structure also revealed that the plane defined by the Cu_M and the two O atoms of nitrite and the plane defined by the N and the two O atoms of nitrite form a 30° angle (Figure S1, Supporting Information). This feature for Cu−(NO_2) binding was also observed in several nitrite-soaked CuNR enzymes.[25] However, small-molecule compounds having the Cu−(NO_2) motif with nitrite bound to Cu as a bidentate ligand usually exhibit the four atoms (Cu, O1, O2, N) in the same plane. Out of more than 40 structures deposited in the Cambridge Structural Database (CSD),[31] only two have the nitrite bent out of the plane, and even in those cases the angle is small (less than 30°).[32,33]

Two more nitrite anions were also present in the structure. One is located at a protein−nickel−protein crystal contact,[34] coordinated to Ni(II) in an asymmetric bidentate fashion ($Ni-O_{nitrite}$ = 1.95 and 2.65 Å), in a manner similar to that observed in Cu_M. However, in contrast to what was observed for Cu_M, the Ni and the N and two O atoms of nitrite are all in the same plane (Figure S2, Supporting Information). The other nitrite was found also in the periphery of the protein, hydrogen-bonded to the −NH of Gly^{258} (2.8 Å) through one of its oxygens.

Remarkably, Cu_H does not bind nitrite, as evidenced by the lack of density at the fourth coordination site of Cu_H in a $2F_o - F_c$ electron density map (Figure 2). This is surprising in view that the concentration of nitrite used in the experiments, 300 mM, was very high (nitrite/protein > 1000). Usually, soaking in a 5 mM $NaNO_2$ solution for 1 h is enough for binding nitrite to copper(II) in protein crystals.[30] There is clearly something intrinsic to the Cu_H site structure that prevents nitrite binding, despite the presence of a vacant coordination position. Azide was similarly found coordinated to Cu_M but not Cu_H in the PHM*cc*−azide crystal structures (see below).

To enable comparisons between spectroscopic and X-ray structural data, PHM*cc*−nitrite crystals were prepared using the same concentration of sodium nitrite used in the spectroscopic studies carried out by Solomon and co-workers.[7] Six transitions were observed in the MCD spectrum of nitrite-perturbed PHM*cc* [~6900 (+), ~8900 (−), 10 700 (+), 12 350 (−), 14 200 (+), and 17 000 (−) cm^{-1}], while only two [~12 200 (+) and 16 700 (−) cm^{-1}] were found in the spectrum of the resting PHM*cc*.[7] As the Cu_H site appears unperturbed in the PHM*cc*−nitrite structure, the new spectroscopic features found for the nitrite-perturbed PHM*cc*, the absorptions at ~6900 (+), ~ 8900 (−), ~10 700 (+), and ~12 350 (−), must be assigned to the Cu_M−(NO_2) site of the enzyme. The Cu_M^{2+} is in a five-coordinate environment with two histidines, one methionine, and nitrite acting as an asymmetric bidentate ligand. The stereochemistry around the copper(II) ion is best described as a distorted square-pyramidal geometry (SPY) rather than trigonal bipyramidal (TBPY). The geometric parameter $\tau = (\beta - \alpha)/60$ (where β and α are the largest basal angles; $\tau = 0$ corresponds to a perfect SPY, while $\tau = 1$ indicates a perfect TBPY) is used to characterize the stereochemistry around the Cu_M^{2+} ion.[35] The basal plane is defined by $N_{His242}-S_{Met}-O1_{nitrite}-O2_{nitrite}$, where the largest angles are S−Cu−O1 = 152° (β) and N−Cu−O2 = 139° (α). Thus, the value of $\tau = 0.22$ indicates a geometry for Cu_M better described as SPY. The apical position is occupied by the Nε donor of His^{244}. It is interesting to note that all four possible copper(II) d−d transitions are observed in the MCD spectrum of PHM for Cu_M when it is coordinated by nitrite. Spectral assignments to specific orbital transitions require a more detailed ligand field analysis, which is beyond the scope of the present work. The difference between the 14 200 (+) (nitrite-perturbed) MCD absorption band assigned to Cu_H and the band at ~12 200 (+) (native) could be due to the noticeable broadness of the band and the fact that the native ~12 200 (+) band is formed by contributions from both Cu_H and Cu_M electronic transitions. Also, binding of ligand to Cu_M produces in most cases small but significant changes in the

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histidines coordinating Cu_H. These involve mainly changes in the conformations of the coordinating histidines in the form of changes in $\chi 2$ but sometimes also in $\chi 1$. These changes are sometimes as large as 90°. This type of changes will affect, in a direct and significant way, the interactions between the metal orbitals and those of the ligands and may result in significant spectral changes.

Further spectroscopic characterization was provided by X-band EPR studies.[7] The spectrum of PHM shows only one set of Cu(II) hyperfine couplings ($g_x = 2.050$, $g_y = 2.060$, $g_z = 2.288$), making it impossible to distinguish Cu_M from Cu_H. The nitrite−PHM*cc*, on the other hand, shows two sets of Cu(II) hyperfine couplings in the g_z region (2.265 and 2.298), thus distinguishing the two copper sites. The features at $g_x = 2.060$, $g_y = 2.060$, $g_z = 2.265$ can be assigned to the $Cu^{II}_M-(NO_2)$ site, while the ones at $g_x = 2.060$, $g_y = 2.060$, $g_z = 2.298$ can be assigned to the Cu_H, because only the Cu_H was found unperturbed with respect to the native enzyme. The distorted SPY geometry found for $Cu_M-(NO_2)$ is consistent with the EPR spectrum ($g_z > g_x, g_y > 2.0$), which clearly shows that the ground-state orbital for the Cu_M^{2+} unpaired electron is $d(x^2-y^2)$.

ox-PHM*cc*−Azide Complex. PHM*cc* crystals soaked in mother liquor supplemented with 350 mM NaN_3, the azide concentration used in the spectroscopic studies reported by Solomon and co-workers,[7] were seriously damaged after 1 h and completely dissolved a few hours later. Crystals soaked for 1 h were tested for X-ray diffraction, but the resolution of the data was very low (>3.5 Å). Crystals that diffracted to an acceptable resolution were prepared by cocrystallization using 40 mM NaN_3. Final cocrystallization conditions were 0.5 mM $CuSO_4$, 1.25 mM $NiCl_2$, 100 mM sodium cacodylate, pH = 5.5, 5% glycerol, and 40 mM NaN_3 at room temperature. After 11 days, data were collected from a crystal that diffracted to 2.4 Å resolution. The structure determined using these data revealed an azide anion coordinated to the Cu_M (Cu−N = 2.0 Å) (Figure 3), replacing the water ligand found in the oxidized state of the native enzyme or the O_2 bound in the reduced form in the presence of substrate, and keeping the same tetrahedral geometry around copper. Kinetic and spectroscopic studies of azide binding to dopamine β-hydroxylase (DBH), a tetrameric PHM analogue, were interpreted as indicating that azide binds to a single copper per monomer, in agreement with the crystallographic data on PHM.[36]

Electron density maps also showed a sodium ion bound to the azide ligand, behaving as a $\mu(1,1)$-bridging bidentate ligand connecting the Cu_M^{2+} to the Na^+ (Figure 4). This $Cu^{2+}-(N_3^-)-Na^+$ motif has not been previously observed in proteins and is very rare, even in synthetic metal complexes.[37] In addition, the loop $Asp^{127}-Glu^{128}-Gly^{129}-Thr^{130}$ moves closer to the Cu_M site, in comparison to its position in the free enzyme (Figure 4). The sodium ion binds to the azide, attracts the carboxylate groups of Asp^{127} and Glu^{128}, and changes the conformation of the loop. The residues flanking the loop, Cys^{126} and Cys^{131}, are held by disulfide bridges to Cys^{81} and Cys^{114}, respectively, preventing a large-scale propagation of the new conformation. However, this loop is not moved in PHM*cc*−azide crystals obtained by soaking (see Supporting Information), suggesting that this conformational change cannot take place within the native crystals upon binding of azide to the Cu_M site.

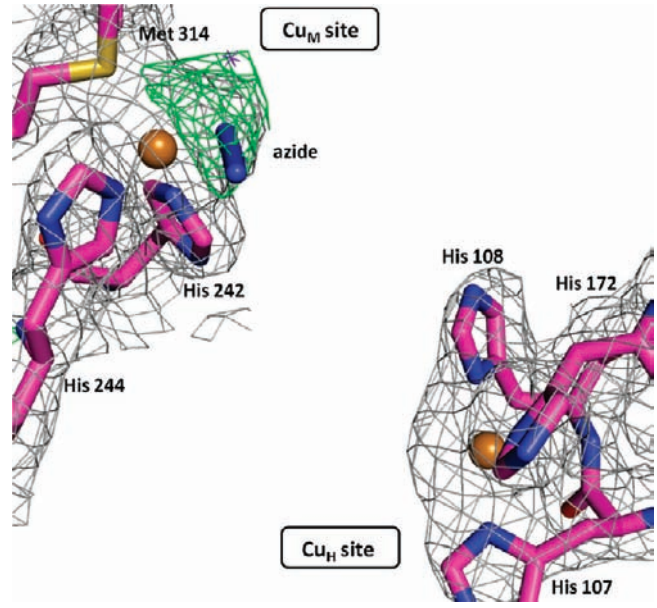

Figure 3. Structure of Cu_M and Cu_H sites in PHM*cc*−azide crystals. The azide is shown bound to Cu_M in a monodentate fashion (Cu−N = 2.0 Å). The gray mesh shows the σ-weighted $2mF_o - DF_c$ (contoured at 1.0σ) and the green mesh the $mF_o - DF_c$ omit maps (contoured at 2.8σ). The omit maps were computed with Refmac5. Carbons are colored magenta, nitrogens blue, oxygens red, sulfur yellow, and copper gold.

The coordination of the Cu_H site in the structure of the PHM*cc*−azide crystal is also intriguing. In the free enzyme, Cu_H has a vacant coordination site that could potentially be occupied by a fourth ligand. However, Cu_H does not bind azide (Figure 3). This is true not only in the cocrystallized sample at lower azide concentration. Despite the low resolution (3.0−3.3 Å) of the X-ray data sets obtained in soaking experiments at (i) 100 mM NaN_3 for 1.5 h, (ii) 50 mM NaN_3 for 26 h, and (iii) 50 mM NaN_3 for 6 h, the structures of these crystals all revealed azide bound to Cu_M but not to Cu_H (see Table 1 and Supporting Information), consistent with the observations in the higher resolution structure obtained by cocrystallization. Further, the high B factor exhibited for Cu_H in all data sets (80−105 Å^2) in comparison to the protein average B factor (50 Å^2) may be indicative of an occupancy lower than 1.0, suggesting that azide may remove Cu_H after long exposure of crystals to high NaN_3 concentration.

Differences observed between the X-ray structures of PHM*cc*−azide complexes determined here and the published spectroscopic data (MCD, EPR) obtained on PHM*cc* solution with 350 mM NaN_3[7] may be a consequence of the different NaN_3 concentration used in the experiments.

red-PHM*cc*−CO Complex. The ability of metal ions to bind ligands and stabilize their coordination sphere depends, among other factors, on their oxidation state. Both copper ions in PHM alternate between Cu(II) and Cu(I) in the catalytic cycle.[38] Thus, the behavior of reduced PHM in the presence of carbon monoxide—a very good ligand for copper(I)—was investigated (see Table 2 for crystallographic data). A PHM*cc* crystal in 5 mM ascorbic acid was exposed to 3 atm CO for 15 min and then analyzed by X-ray crystallography (see Experimental Section for further details). The structure shows CO bound to Cu_M as an "end-on, bent" ligand; the Cu−C distance is 1.8 Å,

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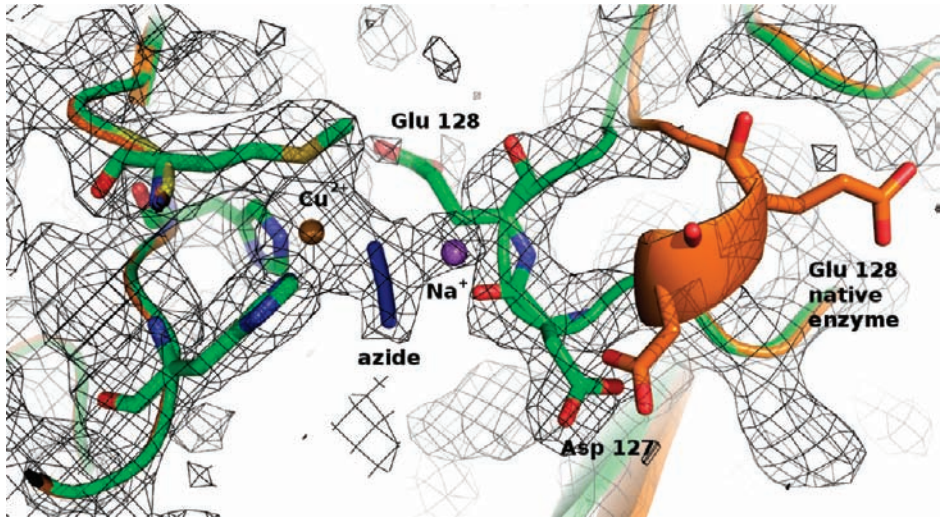

Figure 4. The loop Asp[127] to Thr[130] is closer to the Cu_M site in the PHM*cc*−azide than in the native enzyme. The gray mesh represents the $2F_o - F_c$ electron density contoured at 1.0σ. Carbons are colored green, nitrogens blue, oxygens red, sulfur yellow, copper gold, and sodium purple. The original position of the loop when PHM is crystallized with just 3 mM NaN_3 (instead of 40 mM) is shown in orange.

Table 1. Statistics for Crystallographic Data Collection and Refinement for Oxidized PHM*cc* Complexes under Different Conditions[a]

	ox-PHM*cc*−NO_2^- 300 mM 20 h soak	ox-PHM*cc*−N_3^- 40 mM cocrystal	ox-PHM*cc*−N_3^- 100 mM 1.5 h soak	ox-PHM*cc*−N_3^- 50 mM 26 h soak	ox-PHM*cc*−CO 3 atm 15 min soak	ox-PHM*cc*−subs-nitrite	ox-PHM*cc*−subs-azide
unit cell parameters (Å)							
a	68.6	68.5	68.9	68.7	69.2	68.4	68.1
b	68.6	68.6	69.3	69.0	69.7	68.8	68.8
c	80.3	81.4	81.5	81.3	83.1	80.0	79.8
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
source	X6A	rotating anode	rotating anode	rotating anode	X25	X4C	X4C
wavelength (Å)	1.00	1.54	1.54	1.54	1.10	0.98	0.98
resolution (Å)	52−2.35	52−2.40	53−3.05	53−3.25	35−2.00	52−2.70	52−2.75
unique reflections	16 345	15 128	7716	6295	37 462	10 776	8970
redundancy	7.1 (6.8)	5.0 (5.1)	5.3 (5.4)	5.6 (5.8)	5.9 (6.7)	7.0 (5.6)	4.8 (3.0)
completeness (%)	99.7 (100.0)	99.5 (99.9)	99.8 (100.0)	98.7 (99.5)	97.0 (100.0)	99.0 (91.4)	87.3 (49.3)
$\langle I\rangle/\sigma\langle I\rangle$	49.4 (3.7)	25.2 (3.1)	22.1 (4.1)	20.9 (4.3)	4.6 (1.5)	35.7 (2.1)	21.1 (1.7)
R_{sym} (%)	4.6 (49)	11.1 (59)	10.0 (51)	10.3 (54)	8.0 (41)	8.5 (53)	8.9 (41)
refinement							
R_{cryst}/R_{free} (%)	20/24	20/26	20/25	19/24	21/24	22/27	24/30
stereochemistry							
rms bond lengths (Å)	0.007	0.008	0.011	0.012	0.009	0.012	0.006
rms angles (°)	1.06	1.07	1.15	1.20	1.10	1.22	0.97
model composition							
amino acids	308	309	311	310	310	309	307
Cu/Ni/Na	2/1/−	2/1/2	2/1/−	2/1/1	2/1/−	2/1/−	2/1/4
nitrite	3	−	−	−	−	2	−
azide	−	2	3	3	−	−	6
CO	−	−	−	−	1	−	−
glycerol	3	3	3	3	4	1	−
water	168	215	16	12	163	18	18
total atoms	2597	2677	2471	2461	2692	2443	2437
PDB ID code	3MIB	3MIC	3MID	3MIE	3MIF	3MIG	3MIH

[a] Numbers in parentheses correspond to the last resolution shell.

and the Cu_M−C−O angle is 110° (Figure 5). This geometry is the same as that adopted by dioxygen in the precatalytic intermediate formed by soaking PHM*cc* crystals with slow substrate and ascorbate in the presence of dioxygen.[34] Again, no electron density is found at the open coordination position of Cu_H, indicating that $Cu(I)_H$ is not reactive against carbon monoxide. A water molecule is found interacting very weakly with the Cu_H (Cu_H-O_{water} = 3.3 Å, not shown in Figure 5). The lack of binding of CO at the Cu_H site is consistent with the lack of reactivity against molecular oxygen observed for the precatalytic intermediate of PHM*cc* (see above). In the final refinement, Cu_H refined to an occupancy of 0.7, a fact that reflects that part of the Cu_H is displaced under the conditions of the experiment. Binding of CO to Cu_M and the lack of reactivity of Cu_H are fully consistent with the previous results of EXAFS and FTIR studies[39] as well as with studies of DBH, a PHM analogue.[40,41]

ox-PHM*cc*−CO Complex. Although CO is not as good a ligand for Cu(II) as for Cu(I), CO-soaking experiments were

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Table 2. Statistics for Crystallographic Data Collection and Refinement for Reduced PHM*cc* Complexes under Different Conditions[a]

	red-PHM*cc*−nitrite 300 mM	red-PHM*cc*−azide 50 mM	red-PHM*cc*-CO 3 atm
unit cell parameters (Å)			
a	69.0	68.1	69.2
b	69.3	69.1	68.9
c	81.1	79.9	81.7
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
source	X4C	X4C	rotating anode
wavelength (Å)	0.98	0.98	1.54
resolution (Å)	53−3.10	52−3.25	53−2.15
unique reflections	7468	6287	21 690
redundancy	7.0 (6.0)	5.7 (4.6)	4.5 (4.4)
completeness (%)	99.9 (99.0)	99.1 (91.9)	98.1 (99.4)
$\langle I\rangle/\sigma\langle I\rangle$	23.4 (2.2)	25.3 (2.2)	29.7 (2.6)
R_{sym} (%)	8.1 (57)	8.8 (47)	4.6 (66)
refinement			
R_{cryst}/R_{free} (%)	22/28	24/30	21/26
stereochemistry			
rms bond lengths (Å)	0.009	0.007	0.009
rms angles (°)	1.11	1.06	1.11
model composition			
amino acids	306	304	310
Cu/Ni/Na	1/1/−	1/1/−	2/1/−
nitrite/azide/CO	2/−/−	−/1/−	−/−/2
acetate	−	−	1
glycerol	−	−	3
water	12	2	153
total	2408	2376	2667
PDB ID code	3MLK	3MLL	3MLJ

[a] Numbers in parentheses correspond to the last resolution shell.

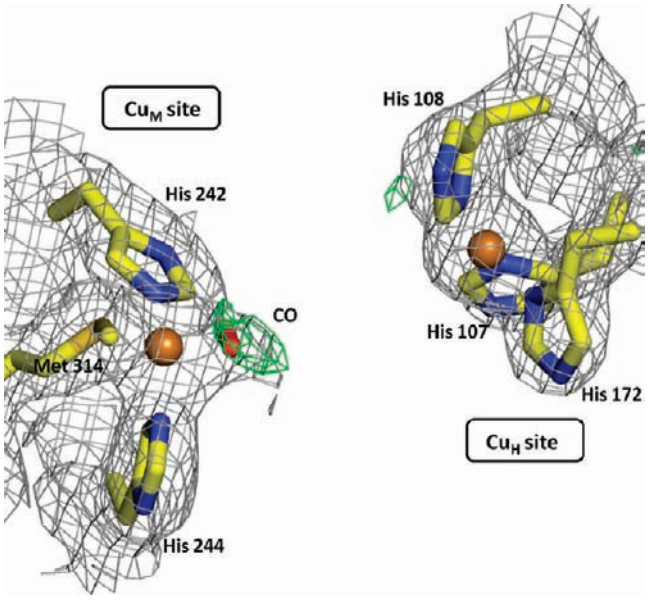

Figure 5. Structure of Cu_M and Cu_H sites of the reduced PHM*cc*−CO complex. The CO is shown bound to Cu_M in a bent configuration (Cu−C = 1.8 Å; Cu−C−O = 110°). The gray mesh shows the σ-weighted $2mF_o - DF_c$ (contoured at 1.0σ) and the green mesh the $mF_o - DF_c$ omit maps (contoured at 2.8σ). The omit maps were computed with Refmac5. Carbons are colored yellow, nitrogens blue, oxygens red, sulfur yellow-orange, and copper gold.

carried out with the oxidized form of the enzyme to further probe the reactivity of $Cu(II)_H$ and to compare it with the structure of the reduced PHM*cc*-CO. The structure revealed that CO coordinates $Cu(II)_M$ with the same geometry observed in the complex with the reduced form of the enzyme. However, in addition to its regular conformation, the loop spanning residues 127−130 is found also in an alternative conformation (occupancy = 0.5), similar to that found in the ox-PHM*cc*−azide structure obtained by cocrystallization (see above). In the CO case, the carboxylate of Glu[128] of the new conformation coordinates Cu_M as a monodentate ligand. Even though pressure unfolding usually requires much higher pressures (e.g., on the order of 10 000 psi)[42] than that used in these investigations (50 psi), one tentative explanation for this double conformation is that it was caused by pressure.

red-PHM*cc*−Nitrite and red-PHM*cc*−Azide Complexes. The behavior of reduced PHM in the presence of high concentrations of nitrite and azide was also investigated (see Table 2 for crystallographic data). As in the oxidized state of the enzyme, nitrite and azide bind $Cu(I)_M$ (Figure S4, Supporting Information). Surprisingly, $Cu(I)_H$ is completely removed from its site in the presence of high concentrations of nitrite or azide (300 mM $NaNO_2$ or 40 mM NaN_3). Also, in the azide complex, the $Cu(I)_M$ site is only partially occupied; the structure was refined with half occupancy for the $Cu(I)_M$−azide moiety. The low resolution of these crystals (>3 Å) in comparison with the typical resolution of reduced PHM crystals (around 2 Å) is probably related to structural deterioration caused by the loss of Cu_H.

Binding Experiments in the Presence of Substrate. We carried out experiments to investigate whether the presence of substrate has any effect at the copper centers that could modify their chemical behavior toward molecules such as nitrite or azide. Our experiments had to be carried out in the oxidized state of the enzyme because Cu_H is removed from the protein in the reduced state (see above). Using the substrate (*N*-α-acetyl-3,5-diiodotyrosylglycine, Ac-DiI-YG) and the experimental conditions reported previously,[8,43] crystals of PHM−substrate were prepared by soaking native crystals of PHM*cc* for 1 h in mother liquor supplemented with 1 mM substrate, followed by soaking in mother liquor supplemented with 1 mM substrate and sodium nitrite or sodium azide (see Table 1 for crystallographic data). The X-ray structures did not show bound substrate in either case, but, as found in the experiments without substrate, Cu_M binds nitrite or azide while Cu_H does not (Figure S5, Supporting Information). Binding of nitrite or azide and/or high concentration of sodium nitrite or sodium azide in the media seems to prevent substrate binding to PHM.

Why Does Cu_H *Not* Bind Small Molecules? We have shown that, under the conditions used, azide, nitrite, and carbon monoxide do not bind to Cu_H. We have previously shown that Cu_H does not bind dioxygen under conditions in which it binds to Cu_M. Blackburn and co-workers showed that isocyanides bind to Cu_M but not to Cu_H.[44] They also showed that, in DBH, CO binds to a single copper per monomer.[40] All these results point to Cu_H as a metal site with unusual reactivity.

The catalytic α-hydroxylation reaction uses dioxygen as the oxygen source. The two electrons required for the dioxygen reduction are provided by ascorbate via one-electron reduction of Cu_M and Cu_H. In this manner, PHM starts its catalytic cycle in the $Cu^I_M\cdots Cu^I_H$ stage. The substrate binds close to the Cu_M site, and a reactive oxygen species bound to Cu_M is responsible for substrate hydrogen abstraction. In this scenario, Cu_H is an electron-transfer site, providing the second electron required to complete the catalytic cycle. The driving force for this step is

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given by the difference in redox potential between Cu_H and the acceptor species, which includes an oxygen-bound Cu_M. The lack of coordination of small molecules to Cu_H is probably related to the necessity for Cu_H to maintain its redox potential in a narrow range to maintain its function as an electron donor. It is known that alterations in the coordination sphere (number and type of ligand donor atoms) and stereochemistry at the metal center can produce great differences in the potential at which electron-transfer reactions occur.[45] The change from three histidines in a T-shape geometry to, for instance, three histidines and one small ligand (e.g., monodentate nitrite, azide, or carbon monoxide) in a square-planar geometry would surely change $Cu(I)/(II)_H$ redox potential. Were this site accessible to small-molecule binding, metabolites present in the physiological environment would change the enzyme kinetics.

Binding of O_2 to $Cu(I)_H$ would be particularly detrimental, as it could be reduced to superoxide and released. The X-ray structure of reduced PHM*cc* with dioxygen bound to the Cu_M in the presence of a slow substrate revealed that Cu_H does not bind dioxygen.[34] This is essentially the same reactivity we observe with carbon monoxide. These results are consistent with the proposal presented here: the architecture of the Cu_H site prevents small-molecule binding to avoid any redox potential change.

From a chemical point of view, it is very difficult to understand why very good copper ligands such as nitrite, azide, and carbon monoxide do not bind Cu_H, since there is a vacant position on its coordination sphere. Apparently, the bis-histidines His^{107} and His^{108}, in concert with His^{172}—all three histidines binding through the Nδ donor—form a particular geometry and electronic structure for Cu_H that prevents binding at the fourth vacant position. One characteristic of the Cu_H coordination that may account for its lack of reactivity is its unusual geometry: the three histidines are in a shape that we described in general as a T-configuration. However, the coordination of Cu_H is more unusual than that. The three His−Cu−His angles in the 13 complexes (10 reported in this manuscript) are the following: $H^{107}-Cu_H-H^{108}$ ranging between 138.6 and 151.4°, $H^{107}-Cu_H-H^{172}$ between 107.2 and 111.9°, and $H^{108}-Cu_H-H^{172}$ between 94.3 and 110.9°. This arrangement is somewhere between square-planar with one empty position and planar-trigonal. It is possible that this coordination, imposed by the protein environment, prevents binding to a fourth site. In addition, in all three cases the histidines coordinate the copper with their Nδ the more positionally restricted of the two histidine nitrogens, making it more difficult for the coordination to rearrange to accommodate a fourth ligand. A detailed quantum mechanical analysis of Cu_H could provide insights on this matter.

The metal coordination in outer-sphere electron-transfer sites is found fully occupied with ligands provided by the protein structure. Type-1 copper sites in blue copper proteins, which almost exclusively function as electron-transfer molecules, are coordinated by two His and one Cys in a distorted trigonal plane with an axial protein residue (usually Met) in an overall distorted tetrahedral geometry; no vacant/open position is present in their coordination sphere.[46,47] The binuclear Cu_A center, as in cytochrome *c* oxidase, is another example.[48] Recently, a red copper protein with a copper site with an open position in the reduced form (a water molecule occupies that position in the oxidized form) has been spectroscopically and structurally characterized.[49,50] It had been proposed that this red copper protein (absorption band at 390 nm), isolated from *Nitrosomonas europaea*, might be involved in electron transfer because it shares significant sequence homology to blue copper proteins and has a typical Cu−thiolate bond. However, later investigations suggested that electron transfer is accomplished by an inner-sphere mechanism.[51]

The same feature (absence of an open coordination position) is observed for iron electron-transfer proteins that work via an outer-sphere mechanism. From the simple tetrahedral iron−sulfur center such as is found in rubredoxin to the very complex iron−sulfur clusters as in other ferredoxins,[52] and even the six-coordinated heme in cytochromes,[53] in all cases, efficient outer-sphere electron transfer is guaranteed with a fully saturated coordination sphere for the metal site. The only exception is the heme at cytochrome *c*′, which has an open coordination position at the active site;[54] however, its physiological role as an electron-transfer site is still unclear.[55,56]

In summary, all very well known outer-sphere electron-transfer metal sites have the attribute of being fully saturated in their coordination sphere by ligands provided by the protein structure. Surprisingly, the Cu_H outer-sphere electron-transfer site in PHM has an apparent open coordination position that remains empty at high concentrations of small anions/molecules such as nitrite, azide, and carbon monoxide. Something essential with the typical architecture of Cu_H creates an electronic structure for Cu_H that impedes the binding of an extra ligand. This property ensures that Cu_H maintains its redox potential in a narrow range, compatible with its role in efficient electron transfer in the catalytic cycle of PHM.

Experimental Section

Preparation of Crystals and Soaking Experiments. Stably transfected Chinese hamster ovary (CHO) cell lines secreting PHM*cc* (residues 42−356) were constructed using the pCIS vector system[57] and purified as described previously.[6] The native protein was crystallized by the hanging-drop diffusion method at 293 K. Thus, PHM*cc* (1 μL; concentration = 16 mg/mL) was mixed with an equal volume of mother liquor (0.1−0.5 mM $CuSO_4$, 1.0−1.25 mM $NiCl_2$, 100 mM sodium cacodylate, pH = 5.5, 3.08 mM NaN_3, 5% glycerol), and crystals appeared in 3−4 days. As reported before, nickel(II) ion is incorporated into the PHM*cc* structure as a crystal contact site.

Oxidized PHM*cc*−Nitrite Crystals. Nitrite-soaked PHM*cc* crystals were obtained by placing native PHM*cc* crystals in mother liquor supplemented with 300 mM $NaNO_2$ for 20 h at room temperature. The crystals were then transferred to fresh mother liquor supplemented with 300 mM $NaNO_2$ and 30% glycerol as a

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cryoprotectant, prior to flash-freezing. Data collection and refinement statistics are reported in Table 1.

Oxidized PHM*cc*−Azide Crystals. ox-PHM−azide crystals were obtained by cocrystallization at 293 K, using the same methodology as for crystallizing native PHM*cc*, but the mother liquor contained 40 mM NaN_3 instead of 3.08 mM. Crystals appeared in 3−4 days.

Oxidized PHM*cc*−CO Crystals. A pressure chamber built by Kas Kumar was used for CO soaking into PHM (unpublished work). The reservoir of the pressure chamber was filled with mother liquor containing 25% glycerol and saturated with CO. Native PHM*cc* crystals were soaked into the CO-containing cryoprotectant solution for less than 1 min and transferred with a loop to the pressure chamber. The chamber was purged several times with low pressure of CO and then gradually filled with 50 psi CO and finally maintained at this pressure for 30 min. After the chamber was brought back to atmospheric pressure, the crystal was rapidly removed and cryo-frozen in liquid nitrogen.

Oxidized PHM*cc*−Nitrite Crystals in the Presence of Substrate. PHM*cc* crystals, grown as described above, were first soaked in mother liquor supplemented with 1 mM substrate (Ac-3,5-diI-YG) for 1 h at room temeprature. The crystals were then soaked in a solution containing 300 mM $NaNO_2$, 1 mM substrate, and the rest of the mother liquor components for 14 h at room temperature.

Oxidized PHM*cc*−Azide Crystals in the Presence of Substrate. Crystals of PHM*cc* complexed with substrate were obtained as described above (1 h soaking in mother liquor with 1 mM substrate). The crystals were then soaked in a solution containing 40 mM NaN_3, 1 mM substrate, and the rest of the mother liquor components for 8 h at room temperature.

Reduced PHM*cc*−CO Crystals. Reduced CO-soaked PHM*cc* crystals were prepared following the same pressure chamber and protocol described for the oxidized CO-soaked PHM*cc* crystals (see above) but adding 5 mM ascorbic acid to the cryoprotectant solution, and the crystal was in the pressure chamber filled with 50 psi CO for 15 min.

Reduced PHM*cc*−Nitrite Crystals. PHM*cc* crystals, grown as described above, were reduced with ascorbic acid by soaking native crystals for 1 h in mother liquor with 5 mM ascorbic acid (1.85 mM $NiCl_2$, 100 mM sodium cacodylate, pH = 5.5, 3.08 mM NaN_3, 5% glycerol). The reduced crystals were then soaked in a solution containing 300 mM $NaNO_2$, 5 mM ascorbic acid, and the rest of the mother liquor components for 13 h at room temperature.

Reduced PHM*cc*−Azide Crystals. Reduced PHM*cc* crystals were obtained as described above and then soaked in a solution containing 40 mM NaN_3 and 5 mM ascorbic acid in mother liquor components for 6 h at room temperature.

Data Collection, Structure Determination, and Refinement. Diffraction data were collected on single frozen crystals, either at a home source (Rigaku RU-200 rotating anode and R-AXIS IV image plate detector) or at beamline X6A, X4C, or X25 at the National Synchrotron Light Source at Brookhaven National Laboratory. Frames were processed with the HKL2000 software package.[58] All crystals belong to the orthorhombic space group $P2_12_12_1$. All structures were determined by molecular replacement with the program AMoRe,[59] using the coordinates of the native enzyme (1PHM.pdb) as the search model. The models were built interactively with program O[60] and refined using REFMAC 5.0 as implemented in the CCP4 suite of programs.[61,62] Anisotropic refinement using translation, libration, and screw rotation (TLS) of rigid bodies was carried out using four groups (residues 46−65, 66−185, 186−302, and 303−354) or each domain (46−197 and 198−354) as a TLS group.[63,64] Solvent molecules were added automatically using the program ARP/WARP[62,65] and visually inspected with the program O.[60] Refinement was monitored by calculating R_{free} values using 5% of the reflections set aside for cross-validation. Crystallographic data collection and refinement statistics are summarized in Tables 1 and 2.

Accession Numbers. Atomic coordinates and structure factors have been submitted to the Protein Data Bank; PDB ID codes are indicated in Tables 1 and 2.

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Supporting Information Available: Figures showing the angular orientation of the Cu_M-O_2N moiety of the nitrite-soaked PHM*cc* crystal, structure of the Ni crystal contact of a nitrite-soaked PHM*cc*, structure of Cu_M and Cu_H sites of an azide-soaked PHM*cc*, structure of the Cu_M site of a reduced nitrite-soaked PHM*cc*, and structure of Cu_M and Cu_H sites of a nitrite-soaked PHM*cc* in the presence of substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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