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Substrate-Induced Carbon Monoxide Reactivity Suggests Multiple Enzyme Conformations at the Catalytic Copper M-Center of Peptidylglycine Monooxygenase

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Abstract

The present study uses CO as a surrogate for oxygen to probe how substrate binding triggers oxygen activation in peptidylglycine monooxygenase (PHM). Infrared stretching frequencies ($\nu(\text{C}\equiv\text{O})$) of the carbonyl (CO) adducts of copper proteins are sensitive markers of Cu(I) coordination, and are useful in probing oxygen reactivity since the electronic properties of O₂ and CO are similar. The carbonyl chemistry has been explored using PHM WT and a number of active site variants in the absence and presence of peptidyl substrates. We have determined that upon carbonylation (i) a major CO band at 2092 cm⁻¹ and a second minor CO band at 2063 cm⁻¹ are observed in the absence of peptide substrate Ac-YVG; (ii) the presence of peptide substrate amplifies the minor CO band and causes it to partially interconvert with the CO band at 2092 cm⁻¹; (iii) the substrate induced CO band is associated with a second conformer at CuM; and (iv) the CuH-site mutants which are inactive, fail to generate any substrate-induced CO bands. The total intensity of both bands is constant suggesting that the Cu(I)M site partitions between the two carbonylated enzyme states. Together, these data provide evidence for two conformers at CuM, one of which is induced by binding of the peptide substrate, with the implication that this represents the conformation that also allows binding and activation of O₂.

Graphical abstract

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INTRODUCTION

Peptidylglycine monooxygenase (PHM) catalyzes C α hydroxylation of glycine-extended pro-peptides, the first step in the biosynthesis of their C-terminally amidated bioactive forms.^{1–3} PHMcc contains two nonequivalent, mononuclear Cu sites, termed CuM and CuH that are separated by 11 Å of solvent. CuM is coordinated by His242, His244 and Met314, while CuH is bound to His107, His108, and His172. The catalytic reaction requires both copper ions to cycle between Cu(II) and Cu(I) oxidation states, but mechanistic aspects such as substrate-induced oxygen activation, and/or the nature of the catalytic intermediates are not well understood. Previous research has led to the consensus that the role of CuM involves catalysis, reacting with oxygen and hydroxylating the nearby substrate, while CuH is responsible for electron transport.

Detailed kinetic and thermodynamic studies of PHM have revealed an equilibrium ordered mechanism in which substrate binds prior to oxygen.^{4–7} The first step in the catalytic cycle involves reducing the two copper centers, followed by substrate binding. Oxygen is then capable of reacting to form an oxygen intermediate species thought to be a cupric superoxo^{7–10} responsible for C–H atom abstraction (HAT) and subsequent radical rebound leading to hydroxylation of substrate. The equilibrium ordered mechanism can be differentiated from an equilibrium random mechanism because the deuterium kinetic isotope effects (KIEs) have shown that the off-rate for the E-S complex is greater than the rate for either oxygen binding or subsequent catalysis⁴. Based on the large value of the intrinsic KIE for the chemical step, Francisco and coworkers⁶ suggested a H atom tunneling mechanism in which the activated state is gated by molecular motions which transiently orient the reactive superoxo species in the optimal conformation for H atom transfer. McIntyre and coworkers extended this idea to include two types of motions that generated a pre-organizational state and a reorganizational state⁴. The *pre-organizational* state was proposed to be formed by motions of enzyme and substrate required to attain an E-S complex while the *reorganizational* state was deemed to require gating motions or vibrations within the protein required for optimizing reactivity. The identities of these motions are unknown but conceptually we may speculate that substrate binding is expected to induce subtle conformational dynamics which leads to oxygen activation, a phenomenon often referred to as “substrate triggering”.

A number of crystal structures are available for PHM including structures of the Cu(II) and Cu(I) oxidations states,¹¹ oxidized enzyme in the presence of the dipeptide substrate diiodo-tyrosylglycine,¹² a pre-catalytic oxygen complex,¹⁰ and adducts with small molecules.¹³ In the oxidized structure, the peptidylglycine substrate is bound close to the CuM center and makes a salt bridge between the C-terminal carboxylate of the substrate glycine residue with the protein residue Arg240. Additional interactions occur between the glycine carboxylate and the phenolate-OH of Y318, as well as a H-bond with the amide of N316, but the substrate does not interact directly with Cu(II)M or its ligands. Adding complexity, no structure of a reduced, substrate-bound form is available making it difficult to visualize any structural perturbations that might occur between the substrate and the CuM center in its reactive Cu(I) form. The presence of two Cu(I) centers, with similar ligand sets but distinct chemistry introduces additional challenges for determining the individual reactivity of the

sites, and particularly how their coordination and electronic structure is perturbed by substrate binding in the nearby pocket. X-ray absorption spectroscopy (XAS) has been used extensively to derive structural information on oxidized, reduced and substrate-bound forms^{14, 15} including single site H and M derivatives^{16–18}, adducts with exogenous ligands such as CO^{19, 20}, changes induced by pH^{21, 22}, and active site mutants^{21–24}. These studies failed to detect any variation in the metrical parameters of the reduced proteins in the presence of substrate.

The one approach that *has* indicated substrate perturbation is infrared spectroscopic studies of CO binding. In the absence of substrate, FTIR spectroscopy detected a PHM-CO complex with a single CO stretching frequency of 2092 cm⁻¹.^{15, 16, 19, 22, 24} Results from the homologous dopamine β - monooxygenase (D β M) established that this CO is bound at the CuM, coordinated by two histidines and one methionine. D β M shares mechanistic, sequence, and structural homology to PHM and has a very similar CO stretching frequency at 2089 cm⁻¹.²⁰ Half apo derivatives of both PHM and DBM were capable of maintaining CO reactivity and were observed via XAS to coordinate a sulfur ligand from the M-site M314 residue, providing further evidence for the assignment of a CuM-site carbonyl.^{17, 18} Additionally, PHM CuM site mutants H242A and M314I which have an empty M site in the reduced state, did not react with CO in the presence or absence of peptide substrate.^{16, 19, 24}

In PHM WT, the presence of substrate induces a second CO band at 2063 cm⁻¹.^{19, 24} Since the frequency of this second carbonyl species is identical to the CO stretching frequency found for molluscan hemocyanin it was originally assigned to a CO complex at the CuH-site.^{19, 24, 25} Hemocyanin is an oxygen-binding dicopper protein with each copper coordinated by three histidine residues^{26, 27} and can have CO stretching frequencies ($\nu(\text{CO})$) ranging from 2043 cm⁻¹ to 2063 cm⁻¹ depending on species²⁵. These data demonstrate that slight changes in the local molecular environments rather than a change in ligand sets can affect the CO stretching frequency, and therefore leave this original assignment open to question.

In this study we further explored the CO reactivity of PHM and its variants as a surrogate for oxygen binding and reactivity. We have determined that upon carbonylation (i) a major CO band at 2092 cm⁻¹ and a second minor CO band at 2063 cm⁻¹ is observed in the absence of peptide substrate Ac-YVG; (ii) the presence of peptide substrate amplifies the minor CO band and partially inter-converts with the CO band at 2092 cm⁻¹; (iii) the substrate induced CO band is associated with a second conformer at CuM; and (iv) the CuH-site mutants which are inactive, fail to generate any substrate-induced CO bands. These data provide evidence for two conformers at CuM, one of which is induced in the presence of peptide substrate, with the implication that this represents the conformation that allows binding and activation of O₂.

Materials and methods

Chemicals

Buffers and ascorbate were purchased from Sigma-Aldrich at a minimum purity of 99%. Substrate Ac-Tyr-Val-Gly (Ac-YVG) was purchased from Peptide International.

PHMcc WT and Mutant Enzymes

PHM WT and variants were constructed using the PHM catalytic core sequence (PHMcc) comprised of residues 42–356. PHMcc WT and variants (H107A, H108A, H107H108A, M314H and M109I) were previously constructed in the pCIS vector²⁸ and grown using hollow-fiber bioreactor technology as described previously.^{16, 19, 22, 24, 29} All proteins utilized in these experiments were purified and reconstituted with approximately 2 coppers per protein, except the double His mutant H107H108A which binds copper only in the M-site and therefore contained about 1 equivalent copper per protein, determined using a Perkin-Elmer Optima 2000 DV inductively coupled plasma optical emission spectrometer (ICP OES). These variants have been characterized in previous studies from our laboratory via steady state kinetics and spectroscopic approaches (XAS and EPR).^{16, 19, 21, 22, 24, 30} After purification, protein was stored at -80°C . Protein was determined using $\text{OD}_{280}(1\%) = 0.980$ on a Cary 50 spectrophotometer, while copper was determined by ICPOES.

CO Binding

Purified PHMcc was concentrated to approximately 4 mM in copper in 20 mM phosphate pH 8.0 and adjusted with 4 volumes of 50 mM MES/HEPES/CHES/Formate to pH 7.5 (unless stated otherwise) and transferred to an airtight conical vial at room temperature.^{16, 22, 24} Samples were purged with a gentle stream of CO before the addition of a 5-fold excess (5 mM) of anaerobic ascorbate, then incubated again with CO for five 5 minutes. In experiments with peptide substrate, Ac-YVG was added prior to ascorbate at a ratio of 2 molar equivalents of substrate per protein. Titration of Ac-YVG with PHM WT was performed over a range of mole equivalents of peptide substrate Ac-YVG from 0.5 to 4.0 equiv. Protein solutions of 70–80 μL were loaded into the IR cell (50 micron pathlength) at a final concentration of approximately 1 mM in copper. After the protein data were collected, the IR cell was flushed with buffer under the same conditions and re-measured for baseline correction. FTIR data were recorded on a Bruker Tensor 27 FTIR spectrophotometer continuously purged with CO_2 -free dry air as previously described.^{16, 22} Samples were equilibrated inside the IR chamber at room temperature for 15 minutes to purge water vapor and CO_2 prior to data collection. One thousand scans were collected for both protein sample and buffer blank from $2250\text{--}1900\text{ cm}^{-1}$ at a resolution of 2 cm^{-1} . Spectral analysis including subtraction of the buffer-blank was performed using GRAMS AI Spectroscopy Software (Thermo).

CO band intensities were calculated using the peak height normalized to the total protein concentration measured by OD_{280} of the 50 micron sample. This approach gave better reproducibility than one using peak areas determined by Gaussian simulation and deconvolution of overlapping peaks. The approach can be justified by the following arguments. To isolate the peaks, our experimental protocol requires the measurement of a separate buffer blank which must be subtracted from the sample dataset to remove the intensely-sloping water signal on which the weak FTIR CO peaks lie. Unlike more traditional FTIR experiments on Cu(I)-CO binding which have used photodissociation to generate the background under conditions where the sample characteristics remain unchanged, our experiments require that the buffer blank be measured as a *separate* sample, introducing small differences in path-length, sample filling, and presence of microscopic gas

bubbles (due to IR heating during spectral acquisition) all of which contribute to differences in background subtraction between samples. Aware of these issues we also undertook control experiments to compare the reproducibility of intensities determined by peak area versus those determined by peak height and found the peak height method to be much more reproducible.

Results

FTIR Spectroscopy of CO Binding to PHM WT

Carbon monoxide is used to model the oxygen binding sites of copper proteins due to its electronic similarities with O₂. Previous X-ray crystallography data has shown that CO interacts with the CuM, but not the CuH center (Fig. 1). In a previous study of PHM WT and the variant H172A, CO stretching frequencies were measured in the presence and absence of peptide substrate Ac-YVG (reported in Table 1).^{17, 19, 24} When CO was reacted with the fully reduced di-Cu(I) form of the enzyme a band at 2092 cm⁻¹ was observed and assigned to a carbon monoxide adduct bound at the M-center. However in the presence of substrate a second band of similar intensity was observed at 2063 cm⁻¹ which was assigned to a CO adduct at the H-center on the basis of the similarity of the $\nu(\text{C}\equiv\text{O})$ to that of Cu(I) carbonyls with three histidine ligands.¹⁹

With the availability of a number of additional variant forms, we have reinvestigated the CO binding chemistry in order to obtain a better picture of how the CO reactivity is influenced by binding of substrate. The new FTIR data on PHM WT demonstrate that in addition to the 2092 cm⁻¹ reported previously, a weak CO band is observed at 2063 cm⁻¹ even in the absence of substrate Ac-YVG (Fig 2a). This evidence suggests an alternative explanation, that two populations of CO-bound species at CuM exist in equilibrium, one of which is favored upon substrate binding. In order to further probe the CO assignments, we titrated PHM WT with Ac-YVG, and carefully normalized the CO intensities to the protein concentration (Fig. 2b). Increasing equivalents of peptide substrate Ac-YVG (0.5, 2, and 4) added to PHM WT increased the peak intensity observed at 2063 cm⁻¹, with a maximum intensity reached after 2 equivalents. Careful quantitation indicates that the CO band at 2092 cm⁻¹ decreases as the intensity at 2063 cm⁻¹ increases, although not at a 1 to 1 ratio.

Figure 3 shows CO band intensities plotted as a function of added substrate for both bands. In Fig 3(b), the intensity of 2092 cm⁻¹ is compared with that of the 2063 cm⁻¹ peak as black and red bars respectively, while in Fig 3(c) the intensity of each peak is plotted in an additive fashion. The sum of both peaks demonstrates a remarkably constant total intensity (Fig. 3c). This data shows that although a portion of the 2092 cm⁻¹ CO band is converted into the 2063 cm⁻¹ band, both species still remain at equilibrium. This behavior is more consistent with two conformations of the M-site CO complex interconverting in response to substrate binding.

FTIR Spectroscopy of CO Binding to M314H

To distinguish between assignment of the 2062 cm⁻¹ band as an H-site or M-site carbonyl, previous attempts were made using mutants M314I and H242A, neither of which were found

to bind CO. We therefore turned to the M314H mutant where the S(Met) donor is replaced with a N(His) donor. The increased sigma donor capacity of the histidine ligand is expected to decrease the stretching frequency of a CO ligand bound at the M-center by increasing the back-bonding interaction, but to leave an H-site CO complex unchanged.^{15, 20, 31, 32} Results for PHM WT and variant M314H in the presence and absence of peptide substrate Ac-YVG are shown in Figure 4. As predicted, in the absence of substrate, PHM M314H produced a carbonyl species that downshifted approximately 20 cm⁻¹ relative to that of WT with a $\nu(\text{CO})$ at 2075 cm⁻¹ (Figure 4b, Table 1). In the presence of peptide substrate Ac-YVG, a second peak appeared at 2052 cm⁻¹. This peak also shifted from the original 2063 cm⁻¹ observed for WT PHM in the presence of Ac-YVG. The strong dependence of frequency on CuM-site ligands unambiguously assigns the substrate-induced CO band as a CuM-carbonyl in a different chemical environment than the 2092 cm⁻¹ band.

FTIR Spectroscopy of CO Binding to CuH Site Mutants with and without Substrate

Kline *et al.* showed that histidine mutants at the CuH center decrease the catalytic activity by > 95% and yet still maintain coupling of oxygen and product.²² This profound effect indicates that the native CuH scaffold is required for full activity, and raises the possibility that H-site ligation and conformation might be important for substrate activation of CO/O₂. Therefore we also investigated the FTIR spectra of the variants H107A, H108A, H107AH108A, and M109I in the presence and absence of peptide substrate Ac-YVG to document the effects on CO stretching frequency.

FTIR shows that CO binds to the mutants with a similar frequency to that observed for PHM WT at ~2092 cm⁻¹, but fails to generate the substrate-induced peak (Fig. 5 & Fig. 6). Each of the CuH-site mutants have an intact copper at both the CuM and CuH center except for the double mutant H107AH108A in which the H-site is empty,^{16, 22, 24} but cannot reproduce WT-CO chemistry in the presence of substrate. In the absence of peptide substrate, single mutants H107A and H108A do not perturb the $\nu(\text{CO})$ at 2092 cm⁻¹, while the double mutant H107AH108A decreases the CO stretching frequency by 3 cm⁻¹ (Figure 5a–c, with reported values listed in Table 1 as $\nu(\text{CO})$ 1). Interestingly upon peptide substrate addition, these mutants fail to generate the second carbonyl species, and in some cases actually cause a downward shift in the original $\nu(\text{CO})$ of 2092 cm⁻¹ (Figures 5a–c, again listed as $\nu(\text{CO})$ 1 in Table 1). In the presence of substrate, PHM mutant H107A exhibited an identical IR spectrum to that without substrate, whereas H108A and H107AH108A decreased the original $\nu(\text{CO})$ by 5 cm⁻¹ and 9 cm⁻¹, respectively. The substrate activated CO band could not be rescued in H107A or H108A using imidazole as an exogenous ligand (data not shown). A weak shoulder is observed in H107AH108A upon peptide substrate addition.

Next, we wanted to know if non-coordinating residues at the H-center would affect the CO stretching frequencies, and if so, to what extent? PHM M109I was used because we previously reported that it does not coordinate CuH at neutral pHs.^{21, 22} PHM M109I reacted with CO to produce a stretching frequency at 2092 cm⁻¹, and in the presence of peptide substrate a second CO band appeared at 2063 cm⁻¹, (Fig. 6 a and Table 1). However, unlike WT, M109I perturbs the equilibrium of the two peaks, causing more of the 2092 cm⁻¹ CO band to interconvert to 2063 cm⁻¹ in the presence of peptide substrate.

Intensity Ratios

The overall total intensities of the CO peaks in the presence and absence of peptide substrate remained relatively constant for PHM WT and its variants (Fig. 7). Carbonylation of reduced PHM WT in the absence of peptide substrate produced an intense CO band at 2092 cm^{-1} , with a minor peak intensity found at 2063 cm^{-1} (Figure 7, labeled $\nu(\text{CO})$ 1 and 2 respectively). The addition of substrate causes the $\nu(\text{CO})$ at 2063 cm^{-1} to become more apparent while 2092 cm^{-1} decreases. The sum of these two peak intensities is similar to the sum of the two CO peak intensities in the experiments excluding substrate. The total CO intensities of the H-site mutants where the substrate-induced band is not observed are also similar to the sum of the two bands in WT/M314H/M109I and to each other. If the band intensities reflect the sum of all M-site CO species, this suggests that the H-site variants generate a single conformation at M, all of which reacts with CO to form the carbonyl.

Discussion

X-ray crystallography structures are available for PHM WT in the oxidized and reduced,¹¹ oxidized plus substrate,¹² an inhibitor-bound pre-catalytic oxygen complex,¹⁰ and in the presence of small molecules.¹³ However, no structure of a reduced, substrate-bound form is available. XAS studies have been reported for the reduced and reduced plus substrate but do not indicate any observable changes in structure when substrate binds to the reduced form.¹⁴ Despite the lack of spectroscopic evidence upon substrate addition in PHM, kinetic data have established that substrate binding precedes oxygen binding and activation.⁴ Strict coupling of oxygen consumption and product formation also suggests that oxygen is activated and ready to commit to catalysis only after substrate binds.^{21, 22} FTIR spectroscopy has been the only technique to detect a change upon substrate addition¹⁹ and thus has been utilized here as a probe of substrate-induced structural and electronic perturbations at the catalytic M-center.

PHM WT was found previously to exhibit a major CO band at 2092 cm^{-1} unambiguously assigned to the CuM center,^{15, 17, 19, 20, 22, 24, 31} and upon substrate addition a second band appeared at 2063 cm^{-1} .^{19, 24} The present study highlights the fact that a minor CO band at 2063 cm^{-1} is apparent *prior* to substrate addition suggesting that it may relate to a catalytically competent state. The new data indicate that this state is favored when substrate binds, and may thus represent a spectroscopic marker for substrate triggering of reactivity. The results from PHM variant M314H absolutely assigns the substrate induced CO band to the CuM center, suggesting a second conformer. The additional histidine ligand causes increased sigma donation, which allows more back bonding with the metal and decreases the CO stretching frequency. Upon carbonylation of M314H, both CO bands downshift to 2075 cm^{-1} and 2052 cm^{-1} respectively, where the effect of the substrate likewise lowers the CO frequency, albeit by a smaller amount than in the WT protein. The downshift suggests a second conformation at the CuM center, which if applied to O_2 would imply an activated state.

The new IR data shows that both IR bands are actually present in the absence of substrate, but the lower frequency band is of much lower intensity. Upon the addition of substrate the two bands partially inter-convert, with a maximum intensity reached after 2 equivalents (a

relatively constant total CO intensity is maintained throughout the titration experiments). CuH site variants which exhibit extremely low levels of catalytic activity fail to generate the substrate-induced band, suggesting a correlation between substrate-induced M-site perturbation and activation of oxygen chemistry. These mutants appear to have a single CO band at 2092 cm^{-1} whether or not substrate is present, and the overall peak intensity is similar to the sum of the two peak intensities observed for PHM WT. These observations on the relative intensities of the CO bands are consistent with M-site reactivity towards CO where all conformations are reactive, where the distribution of CO-bound conformers is proportional to the fractional occupancy, and where the total intensity of all CO bands is determined by the total M-site copper occupancy. Furthermore, the absence of substrate-induced bands in H-site variants implies that the two copper centers work together to provide a precise geometric scaffold necessary for M-site CO/O₂ activation. These data further support the argument that the multiple CO stretching frequencies do not represent CO binding at two independent copper sites, rather that the CO bands are in equilibrium and partially interconvert with two varying populations at the CuM center. The X-ray structures corroborate this evidence additionally demonstrating that only the CuM center is reactive towards small molecules and illustrating that CO binds in an end-on, bent configuration (Fig. 1).¹³ It is unclear why substrate-induced interconversion does not proceed to completion, but it is possible that the high concentrations required to observe the IR bands lead to a fraction of aggregated PHM molecules that cannot undergo substrate-induced activation. This suggestion is supported by published³³ data and by unpublished observations from our laboratory that PHM becomes less active at higher concentrations.

The factors likely to contribute to this second CO conformational state could include (i) a second CO ligand coordinated at the M-site; (ii) changes in the M-site ligand set; (iii) a reorganization of the electronics at the CuM center; or (iv) alterations in the hydrogen-bonding network which affect the orientation of the CO ligand. Taking each of these possibilities in turn, a second CO bound to CuM can be discarded since both CO frequencies would change when the second molecule of CO became ligated. Changes in the M-center ligand set are also unlikely. Karlin and co-workers have reported model complexes built using the HH and HGH copper-binding motif containing either Cu-N δ or Cu-N ϵ imidazole coordination. These complexes form 3-coordinate carbonyls with frequencies ranging from 2092 cm^{-1} to 2112 cm^{-1} for the four complexes studied.³⁴ In addition, the ϵ -HGH complex that most resembles the CuM-site coordination has an IR frequency at 2092 cm^{-1} and is coincidentally reported as the only complex reacting with O₂. These model studies might suggest that the PHM 2092 cm^{-1} band could represent a Met-off configuration while the lower frequency band could arise from a Met-on state. This would imply that coordination of the Met ligand generated a significant increase in back bonding from Cu(I) to CO. Two observations argue against this interpretation. First a similar mechanism applied to the M314H variant would predict no downshift in the absence of substrate (His314 in an “off” conformation) but a larger downshift in the presence of substrate due to the expected increase in sigma donor power of N(His) over S(Met) in the “His-on” conformation. Second, previous XAS studies of WT CO complexes in the absence of substrate show clear evidence for Cu-S coordination¹⁹. Therefore it is probable that both conformations contain (His)₂S(Met)CO ligand sets.

On the other hand, it is certainly conceivable that substrate binding perturbs the electronic interaction between Cu(I) and one or more ligands in a fashion that is not directly observable by XAS. In particular, changes in orientation of the S orbitals with respect to Cu(I) might influence the overall electronic structure of the carbonyl complex. This is an attractive idea, since the Met ligand is essential to catalysis^{21, 35, 36}. A recent X-ray emission study of PHM³⁷ has emphasized an important role for d-p mixing between the filled d¹⁰ manifold and both S-Met and N-His ligands, with metal-d:ligand-S overlap predominating. Therefore differences in Cu-S(Met) orientation could have significant effects on back-bonding interactions.

Other studies have established an important role for thioether coordination in C–H bond activation, but one that is very dependent on the electronic state and architecture of the other ligands in the complex. A variety of different classes of inorganic cupric superoxo species have been characterized as models for the putative superoxo intermediate in PHM and DBM, and include the side on “Kitajima” complex^{38, 39}, the end-on “Sundermeir/Karlin” complex^{40–46}, and the end-on “Itoh”^{47–49} complex. Unlike the PHM M-center, these are built on 3- or 4-coordinate *N-donor* scaffolds and do not contain thioether coordination. Attempts to incorporate thioether ligands into the complexes have met with mixed success. In one study, a N₂S containing model complex deemed to be a good model for PHM M-center (Cu–S distance of 2.203 Å, close to the 2.24 Å reported for the Cu–S(M314) PHM distance) was reacted with oxygen and hydrogen peroxide.⁵⁰ Reaction with O₂ led to oxidation of the thioether, with no observable Cu(I)–O₂ intermediate. Studies on related Cu(II)-⁵¹ and Cu(III)-peroxo⁵² species also showed little or no stabilization of reduced oxygen species via thioether coordination. However, Karlin and coworkers have recently reported a new N₃S(thioether)–Cu(I) complex which reacts with oxygen to form a superoxo intermediate at –125°C with $\lambda_{\text{max}}=425$ nm and $\nu(\text{O}=\text{O})=1117$ cm^{–1}, and undergoes further reaction with the Cu(I) precursor to form *trans*- μ -peroxo di-Cu(II)⁵³. Studies on the reactivity of the superoxo species suggest that the thioether moiety in this complex *increases* reactivity towards hydroxylation of C–H bonds through an increase in electrophilic character. The differences in reactivity between these various thioether-containing models indicate the need for careful tuning of Cu–S electronic structure in order to turn on oxygen reactivity.⁵⁴

Electronic tuning of metal-thioether reactivity has also been reported in radical-SAM containing enzymes, where S-adenosyl methionine is bound to one of the Fe atoms of a 4Fe-4S cluster.⁵⁵ The radical-mediated catalysis is required for important biological processes such as DNA repair, biotin synthesis, and glycl radical formation, among others. XAS data and density functional theory (DFT) calculations revealed that substrate binding alters the degree of metal-to-sulfur back bonding, leading to cleavage of the C–S bond. This study demonstrates that non-coordinating interactions such as substrate binding in an active site pocket can influence metal-methionine electronic interactions, although the positive charge residing on the S-adenosylmethionine ligand may profoundly influence the metal-sulfur electronic interactions.

Interestingly, the substrate hippuric acid (N-benzoylglycine) appears to induce a second CO band not at 2063 cm^{–1} (observed for peptide substrate Ac-YVG) but at 2075 cm^{–1} and does

not appear to affect the $\nu(\text{CO})$ at 2092 cm^{-1} (reference¹⁹ and Table 1). The observed differences in CO stretching frequencies could be due to the different modes of substrate binding near the CuM-site that have different effects on the electronics of the CuM center as argued above. A change in the orientation of bound CO could also induce a second stretching frequency especially if the CO enters into hydrogen bonding interactions either with the substrate or with ordered solvent. The crystal structure of PHM-CO measured in the absence of substrate shows CO bound in an unusual bent, end-on configuration at the CuM center,¹³ in marked contrast to the expected linear binding mode. Inspection of the structure reveals that the O of the CO moiety interacts with the amide of Gly308, on the opposite side from where substrate would bind. Thus, reorientation of the CO towards the substrate when the latter is bound could result in disruption of the interaction with the glycine main chain amide, and the formation of a new interaction leading to an altered stretching frequency.

Multiple CO bands arising from different populations of active site conformers were also observed in flash photolysis studies of CO binding to cytochrome c oxidase.^{56, 57} Cytochrome c oxidase (CcO) is the terminal respiratory enzyme in the mitochondrial electron transfer chain responsible for reduction of molecular oxygen to water. In strongly visible light, CO is dissociated from the heme_{a3} pocket and rebinds to the Cu_B with at least two CO stretching frequencies between 2060 cm^{-1} and 2035 cm^{-1} . The frequency difference was attributed to having a highly ordered versus a more flexible Cu-CO complex. The behavior of the CcO system emphasizes the fact that changes in CO frequency due to conformational complexity can exist without any change in ligand set around the coordinating metal ion.

The multiple absorption bands observed via IR may also reflect dynamics important in controlling PHM catalysis. The PHM catalytic reaction is known to proceed via a H-tunneling mechanism in which the enzyme must reach a critical configuration for H-atom transfer from substrate to the developing oxygen intermediate.^{2, 4, 6, 58} The enzyme is able to sample many dynamical states via specific vibrational modes along the reaction coordinate which couple the local motions of the metal-oxygen activated intermediates with critical substrate modes such as the pro-S C-H bond, until the optimal configuration for H-atom abstraction is achieved. It is likely from the effects of H-site mutations on M-site CO chemistry that dynamics at the H-center are also important for M-site preorganization. For example the conformational flexibility of the CuH center has previously been demonstrated via its ability to engage in a pH-dependent conformational switch²¹⁻²³ where a histidine ligand protonation induces coordination of Met109 to the CuH center. It is clear that cross talk between the two copper centers represents an important element of the catalytic process. We note that a recent structure of the sister enzyme dopamine β -monooxygenase has provided support for interdomain movement as a component of catalysis since the unit cell includes molecules in both an open (Cu-Cu $\sim 14\text{ \AA}$) and closed (Cu-Cu $\sim 4\text{--}5\text{ \AA}$) conformations⁵⁹ indicating that the catalytic domain is capable of significant conformational dynamics. While it is still unclear whether interdomain movement is relevant with respect to oxygen activation by substrate, this new evidence suggests that it should remain in consideration.

Conclusion

The multiple CO stretching frequencies observed in this study most likely represent two conformations at CuM in which the second CO band depends on the mode of substrate binding and may thus report on a substrate-induced catalytically active configuration. Upon substrate addition, the lower (ν_{CO}) band is amplified while the other decreases, but not at a 1:1 ratio, indicating an equilibrium which is shifted in the presence of substrate. The multiple CO stretching frequencies observed for PHM WT and variants exhibit a constant intensity in the presence and absence of peptide substrate. While the precise nature of substrate activation remains elusive, our data suggests electronic perturbation, re-orientation of bound diatomic and/or changes in H-bonding interactions are the most likely contributors. Finally, the failure of H-site variants to generate the putative active configuration implies that the CuH-site scaffold is essential not only for the electron transfer, but also for specifically tuning the CuM-site reactivity.

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ABBREVIATIONS

MES	2-(N-morpholino)ethanesulfonic acid
HEPES	<i>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</i>
CHES	N-cyclohexyl-2-aminoethanesulfonic acid
Acyl-YVG	Acyl-Tyr-Val-Gly
PHMcc	peptidylglycine- monooxygenase catalytic core
EXAFS	extended X-ray absorption fine structure
XAS	X-ray absorption spectroscopy
ICP-OES	inductively coupled plasma optical emissions spectrometry
DBM	dopamine β -monooxygenase
WT	wild-type
PAM	peptidylglycine α -amidating monooxygenase
CHO DG44	Chinese hamster ovary
CO	carbon monoxide
Hc	hemocyanin

Hc-CO	carboxyhemocyanin
HA	hippuric Acid
FTIR	Fourier transform infrared
KIE	kinetic isotope effect
DFT	density functional theory

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Figure 1.

Crystal structure of CuM and CuH sites of reduced WT PHMcc-CO complex at 2.15 Å (PDB ID code 3MLJ). PHM CuM is shown bound to CO in an end-on, bent configuration with additional coordination from His242, His 244, and Met314. CuH is coordinated by His107, 108, and 172.

Figure 2.

FTIR spectra of CO complexes formed with reduced PHM WT. (a) CO complexes of reduced PHM WT in the presence (green) and absence (black) of peptide substrate Ac-YVG. (b) PHM WT-CO complexed with increasing ratios of peptide substrate Ac-YVG; 0.5 equivalents in red; 2 equiv in purple; 4 equiv in black. The IR data were normalized to the protein concentration.

Figure 3.

Comparison of the raw CO band intensities of PHM WT with increasing amounts of peptide substrate Ac-YVG at 2092 cm⁻¹ and 2063 cm⁻¹. (a) Zero equivalents of peptide substrate added in gray; 0.5 equivalents in red; 2 equiv in purple; 4 equiv in black. (b–c) The IR data in the bar graphs were plotted without normalizing the CO peak intensities. (b) Bar graph comparing the CO intensities at 2092 cm⁻¹ (black) and 2063 cm⁻¹ (red) of reduced PHM WT as increasing amounts of substrate are added. (c) The total peak intensity for both 2092

cm^{-1} (black) and 2063 cm^{-1} (red) was plotted as a stacked bar graph for WT PHM-CO with increasing peptide substrate ratios.

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Figure 4.

FTIR spectra comparing the CO complexes formed for PHM WT and variant M314H in the presence and absence of peptide substrate Ac-YVG. (a) WT PHM-CO complexed with substrate (green) and without substrate (black). (b) PHM M314H-CO complexed with substrate (red) and without substrate (black). Spectra are normalized to the intensity of the CO band at 2092 cm⁻¹ and 2075 cm⁻¹ for WT and M314H respectively in the absence of substrate. In the presence of substrate, WT was normalized to 2063 cm⁻¹ and M314H normalized to 2052 cm⁻¹.

Figure 5.

FTIR spectra of the CO complexes of PHM CuH site histidine variants, H107A, H108A, and H107AH108A in the presence and absence of substrate Ac-YVG. (a) H107A with substrate (teal) and without substrate (black); (b) H108A with substrate (yellow) and without substrate (black); (c) H107AH108A with substrate (blue) and without substrate (black). Spectra are normalized to the intensity of the only CO band observed, $\sim 2092 \text{ cm}^{-1}$.

Figure 6.

FTIR spectra of the CO complexes of PHM CuH site variant M109I in the presence and absence of substrate Ac-YVG. M109I with substrate (magenta) and without substrate (black). In the absence of peptide substrate, spectra are normalized to the intensity of the CO band at 2092 cm⁻¹. In the presence of substrate, M109I was normalized to 2063 cm⁻¹.

Figure 7.

Stacked bar graph comparing the total CO band intensities of PHM WT and variants in the absence and presence of peptide substrate Ac-YVG. The main CO intensity observed in the absence of substrate at $\sim 2092\text{ cm}^{-1}$ is denoted $\nu(\text{CO})\ 1$ and is represented in black. In the presence of substrate, if a second $\nu(\text{CO})$ is observed it is denoted $\nu(\text{CO})\ 2$ and is in red.

Table 1

Infrared Frequencies and Copper Stoichiometries for Carbonyl Complexes of PHMcc WT and Variants

protein	$\nu(\text{CO})$ 1	$\nu(\text{CO})$ 2	Cu:Protein
PHM WT	2092		2.02 ± 0.15
PHM WT + Ac-YVG	2092	2063	
PHM WT + Hippuric Acid	2093	2075	
H172A ²⁴	2092		1.4 ± 0.3
H172A + Ac-YVG	2092	2065	
M314H	2075		1.6 ± 0.28
M314H + Ac-YVG	2075	2052	
M109I	2092		2.08 ± 0.14
M109I + Ac-YVG	2092	2063	
H107A	2092		2.01 ± 0.01
H107A + Ac-YVG	2092		
H108A	2093		2.04 ± 0.23
H108A + Ac-YVG	2087		
H107AH108A	2090		1.09 ± 0.15
H107AH108A + Ac-YVG	2083		
H242A ²⁴	no peak		1.11 ± 0.03
M314I ¹⁹	no peak		1.2 ± 0.38