Effects of copper occupancy on the conformational landscape of peptidylglycine \(\alpha\)-hydroxylating monooxygenase

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The structures of metalloproteins that use redox-active metals for catalysis are usually exquisitely folded in a way that they are prearranged to accept their metal cofactors. Peptidylglycine \(\alpha\)-hydroxylating monooxygenase (PHM) is a dicopper enzyme that catalyzes hydroxylation of the \(\alpha\)-carbon of glycine-extended peptides for the formation of des-glycine amidated peptides. Here, we present the structures of apo-PHM and of mutants of one of the copper sites (H107A, H108A, and H172A) determined in the presence and absence of citrate. Together, these structures show that the absence of one copper changes the conformational landscape of PHM. In one of these structures, a large interdomain rearrangement brings residues from both copper sites to coordinate a single copper (closed conformation) indicating that full copper occupancy is necessary for locking the catalytically competent conformation (open). These data suggest that in addition to their required participation in catalysis, the redox-active metals play an important structural role.

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Secreted peptides function as hormones, neurotransmitters, and growth factors. In the animal kingdom, many of these peptides—most of them produced by enzymatic cleavage of large precursors—must be amidated at their carboxy terminus to exhibit full biological activity. Surprisingly, these amidases are not generated by an amination reaction. Instead, precursors or intermediates with a glycine at their C-terminus are transformed into active, terminally amidated des-glycine hormones by oxidative cleavage of the glycine N-Ca bond (1–3). Two enzymes, peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) and peptidyl-α-hydroxylglycine α-amidating lyase (PAL; EC 4.3.2.5) working sequentially are the only proteins known to catalyze this reaction. A gene encoding active, bifunctional, integral membrane peptidylglycine α-amidating monooxygenase (PAM) was recently identified in Chlamydomonas reinhardtii, a unicellular green alga, raising the possibility that PAM existed in the last eukaryotic common ancestor (4–7). In C. reinhardtii, as in placozoa and sponges—organisms that lack both nervous and endocrine systems—and in most animals, the PHM catalytic core follows an N-terminal signal sequence and is followed by the PAL catalytic core, a single transmembrane domain and an unstructured cytosolic domain (5,7–10). The PAM gene appears to have duplicated and in some organisms, including Drosophila melanogaster, PHM and multiple PAL proteins are encoded by separate genes (7). It is now clear that a PAM protein that requires ascorbate, copper, and molecular oxygen appeared before the evolution of the nervous system.

PHM, a two Cu enzyme, catalyzes the stereospecific hydroxylation of the glycine Ca of peptidylglycine substrates. PAL, a Zn-containing enzyme, completes the reaction, yielding the α-amidated peptide product plus glyoxylate. Both domains have broad substrate specificity; peptides with all 20 amino acid amides have been isolated (3). The cloning and successful expression of PHM, PAL, and bifunctional PAM have made detailed structural and functional studies possible (11–15). The structures of the PHM and PAL domains of rat PAL, alone and in complexes with substrates, inhibitors, and other ligands, have been determined (11–15). Other techniques used to study PHM have ranged from kinetics and kinetic isotope effect measurements (16–20) to inhibitor design (21–24), spectroscopy (25–29) including X-ray absorption spectroscopy (XAS) (17,30–33), and computational studies (17,34–36). These studies have provided significant insights into the mechanism of both domains, especially of PHM.

The reaction carried out by PHM—hydroxylation of an aliphatic carbon—is chemically highly sophisticated. The two copper atoms in PHM, CuH and CuM (located in the N- and C-subdomains, respectively), each use a single reducing equivalent from ascorbate to catalyze the reduction of molecular oxygen for the hydroxylation of the Ca of glycine at the carboxy-terminus of the peptide substrate (37,38). PHM is one of a limited number of enzymes that require copper for catalytic function; the residues that bind CuH and CuM are perfectly conserved from C. reinhardtii to human PHM. The transporters and chaperones needed to utilize copper in the secretory pathway are also highly conserved, suggesting an equally sophisticated metalation mechanism. The catalytic chemistry must therefore proceed within the confines of a complex cellular transport machinery, with the overall functioning of the system needed to balance the requirements of selective metalation with the structural determinants of catalytic function.

The studies enumerated above, especially X-ray diffraction, revealed numerous ancillary properties of PHM that may or may not be necessary for catalysis: CuH and CuM are 11 Å apart in both the reduced and the oxidized states; the peptide substrate provides the path for the electron transfer; CuH has an empty coordination site that remains empty even in the presence of high concentrations of small-molecule copper ligands; O₂ binds only to CuM; O₃ binds with end-on geometry and only in the presence of substrate; H₂O₂ can bypass the requirement for O₂ and for an additional source of reducing equivalents but binds CuM with side-on geometry. It is unknown whether these highly specific features are absolute mechanistic requirements or instead only evolutionary events that accompanied organismic specialization. A mechanism as sophisticated as that of PHM provides a unique platform for uncovering the differences among the evolution of the chemical requirements for product production, the constraints on metal ion coordination imposed by the metalation machinery, and those resulting from subsequent evolutionary events. The highly conserved CuH and CuM sites in PHM suggest that many of these features may be essential for function.

Information necessary to address these questions includes the effect of missing copper in one or both sites and the effect of modifying the coordination of CuH on the structure, activity and other properties of PHM. CuH is coordinated by three highly conserved histidine residues: H107, H108, and H172. Mutation of any one of these residues to alanine results in an inactive enzyme (39–40). This loss of activity may be due to different consequences of the mutations. For example, do the mutations weaken or prevent Cu binding to the modified CuH site, with loss of one of the two required electrons for O₂ reduction? Or, do the mutant proteins, even though they have an intact CuM site—the site of substrate binding—fail to bind substrate? In this work, we describe structural studies on rat PHM aimed at addressing some of these important questions. Crystallographic data on apo wild-type PHM and on several crystal forms of three CuH mutants (H107A, H108A, and H172A) presented here show that the copper ions are required not only for the catalytic steps but also for substrate binding and for locking the overall conformation of PHM in a configuration that is catalytically active. Absence of copper, or modified copper coordination, has a major effect on the overall flexibility of PHM, allowing the molecule to adopt conformations that have not been observed with wild-type PHM.

Results

Structure of rat apo-PHM. All of the PHM structures published to date contain two copper ions. To assess the possible structural role of copper, the structure of apo-PHM (PDB ID: 5WKW), which lacks bound copper, was determined to a resolution of 1.8 Å (Table 1). The final R-values are Rwork = 20.9% and Rfree = 27.3%. As expected, there was no density for either of the two cop- pers—CuH or CuM (Fig. 1). Other than this, the structure is strikingly similar to the wild-type holoenzyme (PDB ID: 1PHM) (RMSD 1.6 Å for 1214 main chain atoms), with the largest differences restricted to the loops connecting the β-strands (Supplementary Figure 1A).

Structures of the PHM mutants (H107A, H108A, and H172A). The structures of each of the CuH site PHM mutants were determined by X-ray diffraction in the absence and in the presence of 1–3 mM citrate, used as an additive to improve resolution. None of the crystallization conditions included Cu²⁺. Only the structures of crystals that diffracted to the highest resolution were fully refined and are reported here. All structures were determined without the addition of ascorbate and it is therefore expected that all observed copers are in the oxidized state (Cu²⁺).

Structure of the H107A-PHM mutant. The crystals of the CuH mutant, H107A-PHM (PDB ID: 6ALV), grown in the conditions used for wild-type PHM, diffract to only 3.5 Å resolution. Nevertheless, the structure was determined by molecular replacement and refined to an R-value of 27.6% (R-free = 29.7%) with
excellent geometry (Table 1). The structure, except for the lack of Cu$^{2+}$ in the CuH site, is very similar to that of wild-type PHM (RMSD = 0.42 Å for 283 Ca carbons) (Fig. 1c). Despite lacking its Cu$^{2+}$, the rest of the CuH site shows little change except for the absence of the side chain of residue 107. The mutant does have Cu$^{2+}$ in the CuM site and the geometry of the coordination is almost identical to that of wild-type PHM (Fig. 1c). The main difference is a small (<0.5 Å) narrowing of the space between the two domains (Supplementary Fig. 1B).

Crystals of H107A-PHM (PDB ID: 5WJA) obtained from solutions containing 1–3 mM citrate as an additive diffracted to 2.3 Å resolution and contained two molecules in the asymmetric

Table 1 Data collection and refinement statistics

|                     | Apo-PHM PDB ID: 5WKW | H107A-PHM PDB ID: 6ALV | H108A-PHM PDB ID: 6AO6 | H172A-PHM PDB ID: 6AMP | H107A-PHM - citrate PDB ID: 5WJA |
|---------------------|-----------------------|------------------------|------------------------|------------------------|----------------------------------|
| **Data collection**  |                       |                        |                        |                        |                                  |
| Space group         | P2$_1$/c              | P2$_1$/c               | P2$_1$/c               | P2$_1$/c               | P2$_1$/c                         |
| a, b, c (Å)         | 58.72, 66.56, 70.18   | 69.04, 68.85, 81.53    | 69.12, 69.84, 81.99    | 59.42, 66.36, 69.79    | 59.54, 100.61, 101.58            |
| α, β, γ (°)         | 90, 90, 90            | 90, 90, 90             | 90, 90, 90             | 90, 90, 90             | 90.00, 89.98, 90.00              |
| Resolution (Å)      | 50.00–1.79            | 50.0–3.50              | 52.85–3.30             | 50.0–2.48              | 50.0–2.3 (2.34–2.30)             |
| $R_{	ext{sym}}$    | 0.078 (0.387)         | 0.12 (0.70)            | 0.062 (0.671)          | 0.068 (0.037)          | 0.082 (0.853)                    |
| I/σI                | 38.39 (2.58)          | 13.43 (2.19)           | 10.1 (1.10)            | 25.32 (2.43)           | 21.34 (2.00)                     |
| Completeness (%)    | 99.1 (92.0)           | 92.2 (88.9)            | 99.3 (45.5)            | 99.1 (95.5)            | 100 (100)                        |
| Redundancy          | 7.4 (4.2)             | 3.6 (2.7)              | 2.95 (2.51)            | 4.5 (3.7)              | 4.2 (4.2)                        |
| No. unique reflections | 26454                | 4848                   | 8535                   | 10124                  | 52994                            |
| No. total reflections| 195741               | 17348                  | 25247                  | 45581                  | 223549                           |
| Source              | FR-E Superbright/     | FR-E Superbright/      | APS Beamline 23-ID-B   | FR-E Superbright/      | APS Beamline 23-ID-B             |
|                     | Saturn944             | Saturn944              | B                      | Saturn944              |                                  |
| Wavelength (Å)      | 1.54                  | 1.54                   | 1.033                  | 1.54                   | 1.033                            |
| **Refinement**      |                       |                        |                        |                        |                                  |
| Resolution (Å)      | 48.30–1.79 (1.83–1.78) | 52.69–3.50 (3.59–3.50) | 53.17–3.30 (3.38–3.30) | 48.09–2.48 (2.54–2.48) | 71.48–2.30 (2.36–2.30)           |
| No. reflections     | 24960 (1691)          | 4343 (187)             | 7507 (267)             | 9583 (650)             | 26271 (1815)                     |
| $R_{	ext{work}}$/ $R_{	ext{free}}$ | 0.20/0.27 (0.29/0.39) | 0.27/0.29 (0.52/0.61)  | 0.18/0.24 (0.25/0.40)  | 0.21/0.29 (0.32/0.40) | 0.20/0.27 (0.29/0.39)            |
| No. atoms           | 2489                  | 2402                   | 2428                   | 2394                   | 4916                             |
| Protein             | 245                   | —                      | 22                     | 12                     | 140                              |
| Water               |                       |                        |                        |                        |                                  |
| B-factors           | 47.62                 | 45.35                  | 80.2                   | 90.4                   | 58.90                            |
| Protein             | 29.02                 | —                      | 85.81                  | 50.7                   | 53.15                            |
| Ligand/ion          | 45.85                 | 45.35                  | 80.2                   | 90.4                   | 58.90                            |
| Water               | 36.20                 | —                      | 61.82                  | 39.8                   | 49.70                            |
| R.m.s deviations    |                       |                        |                        |                        |                                  |
| Bond lengths (Å)    | 0.015                 | 0.031                  | 0.010                  | 0.013                  | 0.012                            |
| Bond angles (°)     | 1.81                  | 1.73                   | 1.46                   | 1.69                   | 1.78                             |

Fig. 1 Geometry of the CuH and CuM sites in the structures of apo-PHM and PHM mutants-H107A, H108A and H172A. a Cu coordination in wild-type PHM (PDB ID: 1PHM), shown in orange. Cu$_{\text{CuI}}$ and Cu$_{\text{CuII}}$ of wild-type PHM are shown in orange spheres. Water molecule is shown as red crossmark. b Alignment of Cu coordinating residues of wild-type PHM with those of apo-PHM (cyan). Cu$_{\text{CuI}}$ and Cu$_{\text{CuII}}$ are absent in apo-PHM. c Alignment of wild-type PHM with H107A-PHM (magenta). Cu$_{\text{CuI}}$ is absent in H107A-PHM and CuM is shown as magenta sphere. d Alignment of wild-type PHM with H108A-PHM (blue). Cu$_{\text{CuI}}$ is absent in H108A-PHM and CuM is shown as blue sphere. e Alignment of wild-type PHM with H172A-PHM (yellow). Cu$_{\text{CuI}}$ is absent in H172A-PHM and CuM is shown as yellow sphere.
The two molecules (Fig. 2a, b, molecules A and D, respectively) have significantly different conformations. In molecule A, both copper sites contain Cu as in wild-type PHM (Fig. 2a). However, even though no citrate is bound, a conformational change of the loop Cys126-Thr130 brings Glu128 in close proximity to the CuM site and unlike in wild-type PHM, the copper in the CuM site in this molecule is coordinated by Met314, His242, His244, and E128 takes the place of H2O as the fourth ligand (Fig. 2c). Furthermore, in this monomer, the Cu2+ present at the CuH site has a coordination different from that of the wild-type PHM. (The possibility of this being another metal can be ruled out based on the preparation of the crystals and the results of previous experiments: neither Ca, Mg, Zn, Cd, Co, Fe, Mn, Ni, Mo, Ba, V, Se, nor Si can replace Cu41–45.) The geometry is tetrahedral and is composed of the remaining two histidines (H108 and H172) plus two water molecules (Fig. 2c). In contrast, in molecule D, no density is found for CuH (Fig. 2b). Instead, the two histidine residues from the former CuH site interact with a bound citrate which, in turn, has a weak interaction with CuM mediated by H2O (Fig. 2d). Citrate binds close to the empty site of CuH and interacts directly with H108 and H172; the carboxylate of the citrate partially occupies the space that in wild-type PHM forms the peptide binding site (Fig. 2d). In addition, there is a movement of the loop spanning residues 45–53.

### Structure of the H108A-PHM mutant

PHM H108A (PDB ID: 6AO6) crystallizes with the same unit cell and space group as wild-type PHM. In the structure, determined to 3.0 Å resolution, H108A-PHM shows no major conformational changes compared to wild-type PHM (Supplementary Fig. 1C) even though there is no electron density for copper at the CuH site (Fig. 1d, Table 1). Crystals of H108A-PHM (PDB ID: 6ALA) obtained in a buffer containing citrate (3 mM) diffracted to 2.5 Å resolution and have a different space group and cell dimensions from those of wild-type PHM and H107A-PHM-cit (Fig. 3, Table 2). In these crystals there are two identical molecules with a bound citrate in the asymmetric unit present in a conformation significantly different from that of wild-type PHM. When the N-subdomain of the H108A-PHM-cit structure is aligned with that of wild-type PHM, the C-subdomain displays a rotation of ~18° toward the N-subdomain, compared to its position in the wild-type PHM structure. As a consequence of this hinge movement, several secondary elements of the C-subdomain move as much as 15 Å closer to the N-subdomain (Fig. 3b). These changes result in a complete reorganization of the coordination of the copper in CuM. The two histidines, His 242 and His 244, remain coordinated to the copper, but Met 314 and the fourth ligand in wild-type PHM (a water molecule) do not bind to the ion. Of the two vacated Cu2+ coordination positions, one is occupied by the 2-carboxylate of citrate, and the other, surprisingly, by the Nε of His 107, originally a copper ligand of the CuH site (Fig. 3c). In the wild-type PHM structure, the distance between His242 Nε and His107 Nε is 11.7 Å, while it is 3.9 Å in the H108A-PHM-cit structure (Fig. 3). The participation of His 107 in the coordination of Cu2+ at the CuM site brings the two domains of PHM closer together by approximately 7.8 Å.

Although citrate was added to the crystallization medium just to promote diffraction to higher resolution46,47, binding of citrate to the mutant PHMs could be related to the binding of fumarate to dopamine β-hydroxylase (DBH), a known DBH enhancer48. Four of the carbons of citrate, besides the absence of the double bond and the hydroxyl, are isosteric with those of fumarate.

### Structure of the H172A-PHM mutant

Crystals of the H172A-PHM mutant (PDB ID: 6AMP) have the same space group as wild-type PHM and highly similar cell dimensions and structure (RMSD 0.57 Å for 271 α-carbons). Even though the molecule...
does not contain copper in the Cu_H site (Fig. 1e), copper at the Cu_M site has the same coordination and geometry as the wild-type. The Cu_H site displays only the minimum changes compatible with the mutation and the absence of its copper (RMSD 0.61 Å) (Fig. 1e, Table 1). The N- and C-subdomains come closer by a small displacement (<0.5 Å) (Supplementary Fig. 1D).

**Binding of peptide substrate.** The structure of oxidized wild-type PHM with the substrate N-acetyl-di-iodotyrosyl glycine (N-Ac-di-I-YG) bound has been determined previously. To assess whether the copper ions are required for substrate binding, crystals of apo-PHM (PDB ID: 5WM0) were soaked in solutions containing 1 mg/mL of N-Ac-di-I-YG for up to 12 h. Data were collected from these crystals which diffracted to 2.4 Å resolution and the structure, refined to an R_{factual}/R_{free} = 0.20/0.28, shows no density for the bound peptide (Fig. 4a, Table 2).

Similarly, the refined structures of crystals of the H108A (PDB ID: 6AY0) and H172A (PDB ID: 6AN3) mutants grown without citrate and soaked in solutions of mother liquor containing 1 mg/mL of N-Ac-di-I-YG for 2–12 h showed no density corresponding to the bound peptide (Fig. 4b, c, Table 2), suggesting that the presence of both copper ions is required for substrate binding. Co-crystallization with solutions containing 1 mg/mL peptide resulted in crystals that again did not show density for the bound peptide (Supplementary Fig. 2).

**Comparison of DBH structure with PHM.** DBH contains a catalytic domain (DBH-cat; residues 209 to 507) that is related in sequence (human DBH to rat PHM—29% identity and 44% similarity), copper content, structure, and mechanism to PHM. Similar to PHM, DBH-cat has two subdomains—N-terminal (residues 209 to 356) and C-terminal (residues 357 to 507)—each containing a copper in the active form of the enzyme (also called Cu_H and Cu_M). The reported crystal structure of DBH (PDB ID 4ZEL; 2.9 Å resolution) contains two molecules in the asymmetric unit, referred to as molecule A and molecule B. Only one of the four possible copper ions (Cu_M in molecule A) is reported in the PDB, albeit with low occupancy (reflected by the high B = 151.88). The structures of the individual DBH-cat subdomains are highly similar between the two molecules in the asymmetric unit (RMSD: N-terminal 0.73 Å for 141 Ca, C-terminal 0.21 Å for 150 Ca) even though the conformations of the DBH-cat domains in the two molecules are quite different (RMSD 4.10 Å for 260 Ca). In DBH molecule A, the two subdomains come closer together (closed arrangement), shortening significantly the distance between the putative Cu_H and the Cu_M sites (distance between DBH Cu_H and Cu_M sites is about 4–5 Å while in wild-type PHM, this distance is 11.7 Å). In DBH molecule B, the arrangement of the two subdomains is similar to that of wild-type PHM, making the distance between the putative Cu_H–Cu_M sites ~11 Å (Supplementary Fig. 3).
mediated radical chemistry. However, this catalytic reactivity is likely that a component of this copper transfer mechanism may involve shared ligand complexes at the H-center where one or more of its histidine copper-ligands do not coordinate the copper.

To address these issues, it is necessary to determine (a) the structural elements at the active site that facilitate the catalytic chemistry and (b) the conformational landscape which enables the enzyme to mature from its apo to the fully metalated catalytically competent form.

Since the Cu$_{H}$ site mutants exhibit dramatically reduced activity, their structures may provide clues to both of these objectives. As a step toward this goal, we have determined the structures of the apo-enzyme and three His variants at the H-center-H107A, H108A, and H172A, all without the addition to the overall functioning of the system requires a balance between the requirements for selective metalation and the structural determinants of catalytic function. It has been suggested that ATP7A, a P-type ATPase, is a major required component of the system that metalates the PHM catalytic center. It is likely that a component of this copper transfer mechanism may involve shared ligand complexes at the H-center where one or more of its histidine copper-ligands do not coordinate the copper.

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### Discussion

The structures of metalloenzyme active sites are exquisitely tailored to their catalytic function. PHM is an example of a copper enzyme that utilizes highly evolved reaction chemistry to catalyze a difficult hydroxylation believed to proceed via cupric-superoxo-mediated radical chemistry. However, this catalytic reactivity must proceed within the confines of equally sophisticated cellular transport mechanisms designed to ensure that the enzymes are metalated selectively and in response to cellular signals. In mammalian cells, the copper homeostatic machinery utilizes a pathway comprised of importers, chaperones, and energy-driven membrane pumps that eventually results in metalation of the catalytic metal centers via transporter-enzyme complexes. Thus, the overall functioning of the system requires a fine balance between the requirements for selective metalation and the structural determinants of catalytic function. It has been suggested that ATP7A, a P-type ATPase, is a major required component of the system that metalates the PHM catalytic center. It is likely that a component of this copper transfer mechanism may involve shared ligand complexes at the H-center where one or more of its histidine copper-ligands do not coordinate the copper.

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### Table 2 Data collection and refinement statistics

| Apo-PHM + Peptide PDB ID: 5WMO | H108A-PHM + Peptide PDB ID: 6AY0 | H172A-PHM + Peptide PDB ID: 6AN3 | H108A-PHM + Citrate PDB ID: 6ALA |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Data collection**             |                                 |                                 |                                 |
| Space group                     | P2$_1$2$_1$2$_1$                | P2$_1$2$_1$2$_1$                | P2$_1$2$_1$2$_1$                |
| Cell dimensions                 |                                 |                                 |                                 |
| a, b, c (Å)                     | 59.09, 65.93, 69.80             | 58.80, 66.53, 70.21             | 59.02, 66.84, 70.24             |
| α β γ (°)                       | 90, 90, 90                     | 90, 90, 90                     | 90, 90, 90                     |
| Resolution (Å)                  | 50.2-2.4 (2.44-2.40)            | 50.2-2.50 (2.54-2.50)           | 48.42-2.05 (2.10-2.05)          |
| R$_{wp}$                        | 0.075 (0.329)                  | 0.063 (0.129)                  | 0.051(0.145)                   |
| I/σ                           | 35.75 (2.77)                   | 48.10 (13.64)                  | 45.98 (5.83)                   |
| CC(1/2)                         | NA                             | NA                             | NA                             |
| Completeness (%)               | 99.2(88.1)                     | 94.5(69.0)                     | 95.6(70.6)                     |
| Redundancy                      | 5.5(2.6)                       | 6.2(5.5)                       | 5.4(2.0)                       |
| No. unique reflections         | 11068                          | 9437                           | 17975                          |
| No. total reflections           | 60812                          | 58408                          | 93871                          |
| Source                          | FR-E Superbright               | FR-E Superbright               | FR-E Superbright               |
| Wavelength (Å)                  | 1.54                           | 1.54                           | 1.54                           |
| Refinement                      |                                 |                                 |                                 |
| Resolution (Å)                  | 47.93-2.4 (2.46-2.00)           | 48.30-2.6 (2.66-2.60)           | 48.43-2.05 (2.10-2.05)          |
| No. reflections                 | 10468 (682)                    | 8153 (554)                     | 16297 (909)                    |
| R$_{wp}$/R$_{free}$             | 0.20/0.28 (0.25/0.27)          | 0.19/0.26 (0.20/0.27)          | 0.20/0.28 (0.20/0.27)          |
| No. atoms                       | 2418                           | 2418                           | 2397                           |
| Protein                         |                                 |                                 |                                 |
| Ligand/ion                      | 27                             | 31                             | 176                            |
| Water                           |                                 |                                 |                                 |
| B-factors                       |                                 |                                 |                                 |
| Protein                         |                                  |                                  |                                 |
| Ligand/ion                      |                                 |                                 |                                 |
| Water                           | 41.2                           | 29.12                          | 51.0                           |
| R.m.s deviations               | 0.017                          | 0.014                          | 0.016                          |
| Bond lengths (Å)                | 1.93                           | 1.81                           | 1.87                           |
| Bond angles (°)                 | 0.016                          | 0.016                          | 0.016                          |

The structures of the subdomains of DBH-cat are similar to the equivalent subdomains of wild-type PHM. The N-terminal subdomains of DBH molecules A and B have RMSDs of 1.33 Å and 1.86 Å with the N-terminal subdomain of PHM, respectively (Fig. 5b); the RMSDs for the C-terminal subdomains are 0.85 Å (85 Ca) and 0.86 Å (85 Ca) (Fig. 5c), respectively. Nevertheless, the overall conformation of both DBH-cat molecules in the crystal structure is different from that observed in the structure of wild-type PHM and the highly similar apo-PHM. Molecule A is the most different (RMSD 3.21 Å for 219 Ca). Although, the DBH-cat of molecule B is more similar to wild-type PHM. Molecule A is the most different (RMSD 3.21 Å for 219 Ca). Although, the DBH-cat of molecule B is more similar to wild-type PHM, it still shows significant differences (RMSD 2.49 Å for 213 Ca).

The arrangement of subdomains in the DBH-cat of molecule A (closed) is highly similar to that of the H108A-PHM-cit structure (RMSD 1.19 Å for 195 Ca), including the position of the single copper ion of each structure (difference in position of the copper atom in the two aligned structures <1.6 Å). The similarity of these two structures, which contain a single copper, suggests that they may represent one-copper intermediates in the assembly of the two-copper competent PHM.
their di-copper forms with $K_m$ values between 3 and 18 μM (wild-type $K_m$ which is 8.3 μM) using dansyl-tyr-val-gly as substrate. The decrease in activity was thus almost entirely due to the large (>150 fold) decrease in $k_{cat}$ for this substrate. In the structures with one or both coppers missing, no substrate binding was observed, either by soaking or co-crystallization.

It is clear that in solution, H-site mutants can exist in forms that retain copper and substrate binding capacity, and the finding that crystal forms exist for H108A and H172A that are isostructural with wild-type broadly confirms these findings. However, the crystallographic determination of other conformers that either lack copper at the H-site, or that exhibit different conformations and/or binding modes, suggests that the mutations stabilize alternative conformers that may be intermediates in catalysis or metal transfer chemistry. The crystal forms that lack copper at the H-site may be stabilized by crystal-packing constraints or have lost copper to the copper-free crystallization media because of the weaker affinity of Cu(II) for the histidine-depleted site. Notwithstanding the fact that this result was unexpected, and recognizing that the mutants in solution may retain their di-copper structures, the crystal structures reported herein provide an unprecedented window into the role of metal occupancy in modulating the conformational landscape of PHM.

While loss of both coppers in the structure of apo PHM has no significant effect on the conformation of the molecule, mutations that cause loss of copper from a single site appear to have significant effects. We have previously shown that PHM mutations at the CuM (M314I) site result in conformational changes and a reduction in thermal stability. The modifications of the CuH site reported in the present paper also result in significant structural effects. In wild-type PHM, this site has an unusual geometry: Cu$^2^+$ is only coordinated by three histidines (His 107, His 108 and His 172) with a T-shaped geometry. The fourth coordination position has been shown to remain unoccupied even in the presence of high concentrations of strong copper liganding small molecules (nitrite, azide, CO) which is unusual, given the strong preference of cupric centers to adopt square or tetragonal geometry. Here, insights into the CuH site were gained by studying the effects of substituting each of the three histidine ligands by alanine, one at a time.

The structure of the H107A mutant crystallized in the same conditions as the wild-type is highly similar to that of wild-type PHM. The coordination of the copper at the CuM site remains unchanged and even the two remaining histidines at CuH site show only minor changes (Fig. 1c). Inclusion of 1–3 mM citrate, which produced crystals (H107A-PHM-cit) that diffracted to higher resolution, had unexpected effects on the structure of H107A-PHM and provided information about alternative conformations that can be adopted by this mutant (Fig. 2). H107A-PHM-cit has two molecules in the asymmetric unit. Interestingly, the copper sites of these two molecules (A and D) present in the same unit cell have very different configurations (Fig. 2), probably because citrate is only present in one of the molecules (molecule D). In molecule A, copper binds at the CuH site despite the absence of His 107 by retaining the coordination with His 108 and His 172 and adding two water molecules as ligands. Even though molecule A does not have a bound citrate, it shows a change in the conformation of the loop containing residues 126 to 130 that brings Glu 128 into the proximity of the CuM site (Fig. 2a). Molecule D, in contrast, has no copper at the CuH site and contains a bound citrate that bridges the two remaining histidines of the copperless CuH site to the CuM site (Fig. 2b). It is clear that these changes are a consequence of the combination of the two modifications: the substitution of His 107 and the presence of citrate. Loss of coordination by H107 allows the CuH site to adopt a tetrahedral coordination by the inclusion of two water molecules, implying that square planar geometry at the H site is destabilized relative to the tetrahedral alternative. This observation strengthens the case for a functional requirement for a non-reactive open coordination site in CuH that allows electron transfer but prevents binding of small molecules that could modify the electrochemical potential of the copper. How the particular coordination of wild-type PHM accomplishes this feat remains unexplained.

Crystals of H108A-PHM, despite crystallizing in the same space group as wild-type PHM with similar cell dimensions, diffract only to 3.0 Å resolution and the structure shows no density for the copper in the CuH site. In every other respect, the structure of this mutant is highly similar to that of the wild-type (RMSD 0.57 Å for 1220 atoms of the main chain). The situation is different for the crystals obtained in the presence of 1–3 mM citrate: they diffract to 2.5 Å, the space group and cell dimensions are different from those of the wild-type and contain two identical molecules in the asymmetric unit with bound citrate. Neither molecule contains copper in the CuH site. Compared to wild-type PHM, the C-subdomain shows an ~18° rotation around an axis that goes through residue 201. The 2-carboxylate of the bound citrate coordinates the copper at the CuM and leads to a rearrangement of the copper coordination sphere (Fig. 3). The two histidines, His 242 and His 244, remain liganded to the copper but Met 314 does not coordinate the copper any longer. The water that occupies the fourth ligand position in wild-type PHM is replaced, surprisingly, by the N$_\text{ε}$ of His 107—which in wild-type PHM coordinates the copper at CuM. This interaction requires that the N$_\text{ε}$ and the C-domains come closer together. The changes in conformation that result in this approach are extensive. The most salient feature is the flattening of the $β$-sheet of the N-terminal domain closest to the central cavity, which involves straightening of strand 5, the strand that starts with residue 107. It is difficult to quantitate these changes but one possible measure of the effect of this straightening may be the change in the distance between the $α$-carbons of residues Met 314 and His 107. In
wild-type PHM, the distance is 19.6 Å and it is reduced to 12.5 Å in the H108A-PHM citrate complex. A measure of the effects that these changes in coordination have in other sections of the β-sheet involved in the CuH site—i.e., strand 9—is reflected in the distance between Met 314 and His 172; this distance is 17.9 Å in wild-type PHM and 13.8 Å in H108A-PHM-cit. Other notable changes are those in the loop spanning residues 129 to 136. This loop connects strands 5 and 7, and although its local conformation remains unchanged from that in wild-type PHM, its overall position is shifted away from the C-terminal domain. Again, these changes seem to be due as much to the mutation as to binding of citrate. In any case, they reflect the range of possible conformations that the molecule can adopt.

The H172A-PHM mutant is highly similar in structure to the oxidized wild-type PHM (PDB ID: 1YIP). More surprising, although it has no copper in the CuH site, the conformation of the two remaining histidines in this site, His 107 and His 108, is almost unmodified from that of wild-type PHM and the CuM site retains the same coordination of the copper observed in wild-type PHM.

To determine whether copper at both sites is required to bind peptide substrate, crystals of apo-PHM were both soaked and co-crystallized in mother liquor containing N-Ac-di-I-YG. This peptide binds to wild-type holo-PHM in both the oxidized and reduced forms41. In contrast, the structure of apo-PHM determined with data collected from the N-Ac-di-I-YG soaked or co-crystallized crystals showed no additional density corresponding to the bound peptide, indicating that copper at both sites is required not only for the later steps of the catalysis but also for binding substrate. Furthermore, two water molecules that are a part of a network formed by Q170, Q272, H108, and the peptide substrate are present in wild-type PHM + peptide structure (PDB ID: 1OPM) but absent in H108A-PHM and H172A-PHM (Fig. 6a, b). These water molecules are possibly required for the peptide to bind to the enzyme. In the H108A and H172A mutants, lack of copper in the CuH site could prevent the two water molecules from forming the network required for peptide binding. In the wild-type enzyme, substrate binding has been shown to induce a new mode of CO binding at the M-center which lowers the C≡O infrared stretching frequency and suggests electronic activation of the diatomic ligand. This process does not occur in the mutants, consistent with a lack of substrate binding although clearly other factors could also be responsible57.

The recently published 2.9 Å resolution structure of apo human dopamine β-hydroxylase (hDBH, PDB ID: 4ZEL)49 has two monomers in the asymmetric unit (molecules A and B) with different conformations. In chain A, the two subdomains display a closed conformation in which the C-subdomain is closer to N-subdomain (hinge rotation of ~18°) resulting in a structure very similar to that observed in our H108A-PHM structure in complex with citrate (Fig. 5f, e). Furthermore, the individual active site residues of chain A of hDBH align closely with those of H108A-PHM in complex with citrate (Fig. 5f). The single copper ions present in both structures have similar coordination and are in approximately the same positions in the aligned structures (distance ≤1.6 Å). The other chain of the hDBH crystals (molecule B), however, shows an open active site similar to that of wild-type PHM (Fig. 5a). Christensen and co-workers49 modeled a copper in the CuM site of chain A and used the positions of the Cu-liganding residues in chains A and B to infer the position of the three other copper ions. Based on this, the authors suggested the possibility that CuH and CuM could come as close as 4–5 Å to each other during the catalytic cycle, close enough to form a binuclear copper center49. However, the fact that this
conformation was only seen in structures with a single copper ion (H108A-PHM-cit and hDBH chain A) is more compatible with the conclusion that having two bound coppers has an important structural role: locking PHM (and probably DBH) in the catalytically competent form. This role is unusual for a catalytic redox active metal. The closed structures observed in hDBH and H108A-PHM-cit may represent intermediates in the loading of copper ions during the final assembly. This is an attractive hypothesis since the molecule with only one copper could have access to a number of alternative conformations which may assist in loading the second copper, and/or may be required for recognition of its cognate transfer partner.

All PHM structures determined previously (reduced, oxidized, with and without ligands, and in a precatalytic complex) have had copper ions in both sites including the CuII mutant M314. In all these structures, the distance between the two coppers is approximately ~11 Å. This observation, together with the fact that PHM crystals can carry out sustained catalysis, supports a mechanism that does not require a large conformational change to bring the copper ions into close enough proximity for a direct electron transfer between the ions.

The structures of the CuII site mutants (H107A, H108A, and H172A), even those with unoccupied copper sites, are all found in the open conformation. The incorporation of citrate in the crystallization media as an additive had a positive effect on the resolution but, surprisingly, resulted in structures that show significant changes with respect to wild-type, providing a glimpse into the changed conformational landscape that results from the absence of one copper.

**Methods**

**Protein expression, mutagenesis, and purification.** Stable cell lines secreting PHM and its mutants, H107A, H108A, and H172A, were created using our established methods. Briefly, CHO cell lines were transfected with a vector encoding the protein of interest and dhfr. The cells were selected by growth in αMEM plus 10% dialyzed fetal bovine serum, subcloned and tested for enzyme expression and clonality until stable, clonal lines were obtained. Typically, it took several months to obtain a cell line in which at least 1–2% of total protein synthesis was devoted to the protein of interest, and in which the cell doubling time remained less than a day. Wild-type and mutant cell-lines were thawed from cryovials and grown until the cells were 80% confluent, typically for 2–3 weeks, after which the serum level was lowered to 0.5% FCS serum. Thereafter, the bioreactors were fed with 0.5% serum-containing medium every other day and spent medium (20 mL) from the ECS was harvested and frozen at −20 °C for subsequent purification.

**Preparation of samples for apo and mutant proteins.** Purified enzymes (wild-type and mutants) were dialyzed against 20 mM sodium phosphate buffer, pH 8.0. The apo-protein isolated from recombinant CHO cells contained no copper, and was used without further addition of copper. Mutant proteins, H107A, H108A, and H172A-PHM, were also devoid of copper as isolated and were subsequently reconstituted with cupric sulfate by slow addition of 2.5 molar equivalents Cu(II) per protein followed by two cycles of dialysis to remove unbound cupric ions. Protein Concentrations were determined using OD280(1%) = 0.980 on a Cary spectrophotometer. Copper concentrations were determined using a Perkin-Elmer Optima 2000 DV inductively coupled plasma optical emission spectrometer.

**Crystallization.** Crystals of apo-PHM and PHM mutants, H107A, H108A, and H172A, were prepared using hanging drop vapor diffusion. Conditions for crystallization were similar to those used for wild-type PHM. In brief, drops were made by mixing 1 µL of PHM (14–17 mg/mL) with 1 µL of 0.1 M Tris HCl pH 8.5, 0.54 M MgCl₂, and 19–24% PEG 4000 by vapor diffusion. Prior to data collection, apo-PHM, H108A, and H172A mutant crystals were soaked with 1 mg/mL N-Acetyl-di-iodophenylnalanyl-glycine or N-Ac-di-I-tyrosyl-glycine peptide for 2 h. Crystals of H107A-PHM and H108A-PHM in complex with citrate were grown from the condition described above using 1–3 mM citrate.

**Data collection, structure determination, and refinement.** Data for apo-PHM, H108A-PHM, and H172A-PHM crystals were collected on an FR-E Super-Bright Rigaku (Americas Corporation, The Woodlands, TX) copper rotating anode x-ray generator as the source with a DECSTRIS Pilatus 3R 280K-A detector at 100 K. Data for H107A-PHM were collected at beam line 17-ID-1, NSLSII, BNL on a DECSTRIS Eiger 6M detector. Data for H107A-PHM in complex with citrate were collected on beam line 23-ID APS. Data were indexed, integrated, and scaled with HKL3000, XDS, and Fast DP. H172A-PHM structure was determined by molecular replacement using wild-type PHM (PDB ID: 1PHM) as a template. All the other structures were determined by Fourier synthesis using the initial structure. Each of the models was rebuilt and refined using alternate cycles of Coot and restrained refinement with Refmac5 in the CCP4 suite. Final models were validated using Coot and Molprobity. Figures for the structures were prepared with PyMOL. RMSD between models was calculated using the LSQ function in Coot.

**Data availability.** Atomic coordinates and structure factors are available at the Protein Data Bank with the following PDB IDs for the corresponding proteins: apo-PHM (PDB ID: 5WKW), H107A-PHM (PDB ID: 6ALV), H108A-PHM (PDB ID: 6A06), H172A-PHM (PDB ID: 6AMP), H107A-PHM in complex with citrate (PDB ID: 5WJA), H108A-PHM in complex with citrate (PDB ID: 6ALA), apo-PHM in complex with peptide (no peptide observed, PDB ID: 5WMO), H108A in complex with peptide (no peptide observed, PDB ID: 6AY0), and H172A in complex with peptide (no peptide observed, PDB ID: 6AN3) were deposited in the Protein Databank. All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Author contributions
S.M., C.S., K.R., and C.D.K. collected and analyzed the data. B.A.E., R.E.M., S.B.G., N.B., and L.M.A. conceived and designed the project. The manuscript was written with contribution of all authors. All authors have given approval to the final version of the manuscript.

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