Spectroscopic and Crystallographic Evidence for the Role of a Water-Containing H-Bond Network in Oxidase Activity of an Engineered Myoglobin

Igor D. Petrov,†,§ Roman Davydov,‡,§ Matthew Ross,‡,§ Xuan Zhao,† Brian Hoffman,*,†‡ and Yi Lu*,†‡

†Department of Chemistry, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States
‡The Department of Chemistry, Northwestern University, Evanston, Illinois 60201, United States

ABSTRACT: Heme-copper oxidases (HCOs) catalyze efficient reduction of oxygen to water in biological respiration. Despite progress in studying native enzymes and their models, the roles of non-covalent interactions in promoting this activity are still not well understood. Here we report EPR spectroscopic studies of cryoreduced oxy-F33Y-Cu3Mb, a functional model of HCOs engineered in myoglobin (Mb). We find that cryoreduction at 77 K of the O2-bound form, trapped in the conformation of the parent oxyferrous form, displays a ferric-hydroperoxo EPR signal, in contrast to the cryoreduced oxy- wild-type (WT) Mb, which is unable to deliver a proton and shows a signal from the peroxo-ferric state. Crystallography of oxy-F33Y-Cu3Mb reveals an extensive H-bond network involving H2O molecules, which is absent from oxy-WT Mb. This H-bonding proton-delivery network is the key structural feature that transforms the reversible oxygen-binding protein, WT Mb, into F33Y-Cu3Mb, an oxygen-activating enzyme that reduces O2 to H2O. These results provide direct evidence of the importance of H-bond networks involving H2O in conferring enzymatic activity to a designed protein. Incorporating such extended H-bond networks in designing other metalloenzymes may allow us to confer and fine-tune their enzymatic activities.

Activation of O2 is important in biology because O2, despite its high abundance in the Earth’s atmosphere and high oxidizing power, is kinetically inert. A prominent example of O2 activation is aerobic respiration, in which the oxidizing potential of O2 is used to generate a proton gradient, driving synthesis of adenosine triphosphate, the energy source for most biological processes. This reaction is efficiently catalyzed by terminal oxidases, e.g., heme-copper oxidases (HCOs) containing a heme and Cu site central to the enzymatic activity. While most HCO and related enzymes have been proposed,1–3 an understanding of structural features responsible for O2 activation is still being sought. For example, while a number of recent advances in biology, biosynthetic models of complex metal-binding sites that can complicate spectroscopic studies, biosynthetic models are simpler to synthesize and contain only the metal-binding site central to the enzymatic activity. While most HCO models use organic molecules as ligands, our biosynthetic approach uses small, stable, and well-characterized proteins, such as sperm whale myoglobin (Mb), as the scaffold. Thanks to recent advances in biology, biosynthetic models of complex metal-binding sites can be prepared more easily and with higher yields than using organic molecule ligands. Furthermore, the structurally rigid scaffold protein allows defining long-range non-covalent interactions and probing their roles more precisely. Using this approach, we previously reported the design of an HCO mimic by introducing two His and one Tyr residues in the active site of wild-type (WT) Mb (named F33Y-Cu3Mb) and found that, unlike WT Mb, which only binds O2 reversibly, this protein achieves O2 reduction to H2O with >500 turnovers.9d Thus, this minimal functional mimic, much simpler than native oxidases while still functioning under physiological conditions, provides a platform for elucidating structural features responsible for O2 reduction that bridges the gap between native enzymes and small molecular mimics of native enzymes.

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We hypothesize that F33Y-CuB Mb operates by heterolytic O−O bond cleavage (Scheme 1), analogous to the accepted mechanism for native oxidases and monooxygenases, but the detailed structural features and interactions that promote this reaction in F33Y-CuB Mb are still unclear. Here we use cryogenic EPR spectroscopy and high-resolution X-ray crystallography to show that an extensive H-bond network involving H2O molecules exists in the active site of F33Y-CuB Mb and plays an important role in facilitating proton delivery to the oxygen and activating it for reduction, thus, imparting oxidase activity.

EPR has been widely used to probe the environment of O2-activating iron enzymes.10 Although oxy-heme is an electronically free closed-shell system with no EPR signal, EPR spectroscopy has been used to examine its interactions with nearby residues and its intermediates upon reduction, by a method called cryoreduction.11 Brieﬂy, the oxy-heme protein, trapped at 77 K, is exposed to CuB Mb in Comparison with WTMb activating iron enzymes.10 Although oxy-heme is an electronically free closed-shell system with no EPR signal, EPR spectroscopy has been used to o

Figure 1. EPR spectra of oxy-WTMb and oxy-F33Y-CuB Mb after radiolytic reduction with a ∼3 Mrad dose of γ radiation from 60Co, and after subsequent stepwise annealing for 1 min at indicated temperatures (sharp signal marked by asterisk is due to radiolytically generated H-atoms at 77 K in quartz EPR tube).

Table 1. g-Values of Cryoreduced Oxy-ferrous Myoglobins

| protein          | g1    | g2    | g3    |
|------------------|-------|-------|-------|
| WTMb peroxo      | 2.22  | 2.12  | 1.965 |
| hydroperoxo      | 2.32  | 2.18  | 1.943 |
| F33y CuB Mb peroxo | 2.24  | 2.13  | 1.964 |
| hydroperoxo      | 2.29  | 2.16  | 1.950 |
| hydroperoxo      | 2.34  | 2.19  | 1.937 |

*Hydroperoxo product formed upon annealing. †Primary hydroperoxo product formed at 77 K.

myoglobins and monooxygenases.10a−c,11,12 Because WTMb, the scaffold for F33Y-CuB Mb, was among the first and most studied proteins by the above method, the abundance of results facilitates understanding of the latter by direct comparison. In this study, F33Y-CuB Mb contained heme but no metal ion in the CuB site, because a previous report showed that this protein exhibits high oxidase activity without a metal ion at CuB,9d mimicking cyt bd-oxygen reductases, which lack this site.2

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To gain further insight into O₂ reduction by F33Y-Cu₃Mb, we monitored the decay of the above cryoreduced species by annealing. As previously reported, annealing the cryoreduced oxy-WTMb at 190 K converts the peroxo signal with gₛₘₐₓ = 2.22 to the more rhombic EPR signal of a ferric-hydroperoxo intermediate, with g = [2.32, 2.18, 1.93] (Figures 1 and S1), through protonation of the peroxo moiety. Upon annealing of cryoreduced oxy-F33Y-Cu₃Mb at 173–175 K for at least 1 min, a signal with g = [2.34, 2.19, 1.94] begins to appear and continues to grow in at 190 K (Figures 1, S1, and S2). These EPR parameters are very similar to those of ferric-hydroperoxo-WTMb. Appearance of this signal during annealing is interpreted to arise from both protonation of the ferric-peroxo centers with gₛₘₐₓ = 2.24 to form ferric-hydroperoxo, and relaxation/repositioning of the hydroperoxo ligand of the gₛₘₐₓ = 2.29 species of the heme iron (Scheme 2).

Based on the known reactivity of the (hydro)peroxo-hemes, these intermediates are expected to thermally decompose to compound II (CpdII), a ferryl species that is EPR silent. Consistent with this expectation, further annealing both samples at 210 and 220 K (Figures 1 and S1) results in obvious decay of the (hydro)peroxo signals to an EPR-silent state. It has been shown that further cryoreduction of the EPR-silent CpdII (Fe(IV)=O) at 77 K generates a low-spin rhombic EPR signal from the Fe(III)=O− or Fe(III)=O2− species, depending on the protonation state of ferryl precursor. Subsequent cryoreduction of the EPR-silent species in WTMb resulted in a signal with g = [2.43, −1.93] (Figure S3), characteristic of Fe(III)=O− (ferric oxo) species observed in cryoreduced CpdII (g = [2.43, 2.12, 1.93]), confirming the EPR-silent state arising after annealing of (hydro)peroxo-WTMb as the Fe(IV)=O≡O− CpdII. A minor signal with g = [2.52, −1.90] (Figure S3) was also observed, previously assigned as protonated ferric-oxo, i.e., ferric-hydroxy (Fe(III)=OH−). In the case of F33Y-Cu₃Mb, further cryoreduction of the EPR-silent annealed species yielded a dominant EPR signal with g = [2.52, 2.15, 1.90] (Figure S3), likewise assigned as ferric-hydroxy. This result supports the hypothesis that the hydroperoxo-F33Y-Cu₃Mb converts to CpdII. This assignment is confirmed by comparison with the same signals observed in cryoreduced samples of CpdII, generated in both proteins by reaction with H₂O₂ at 21–25 °C (Figure S4).

Observation of two distinct signals in cryoreduced oxy-F33Y-Cu₃Mb at 77 K suggests the presence of two structural states of the oxy-ferrous precursor. The peroxo signal with gₛₘₐₓ = 2.24 is nearly identical to the signal observed in WTMb and well within the range of those of other oxygen-binding proteins such as hemoglobin, suggesting that the oxy-ferrous population leading to this state has similar interactions and behaves in a similar manner. On the other hand, the observation of a hydroperoxo species with gₛₘₐₓ = 2.29 at 77 K requires a configuration in which the oxygen can be easily protonated at 77 K (Scheme 2). The observation of protonated Fe(III)=OH− in F33Y-Cu₃Mb upon subsequent cryoreduction of annealed species provides further support for the facile protonation of intermediates. Previous cryoreduction studies of a variety of oxy-heme proteins showed that proton transfer to a cryoreduced oxy-heme at 77 K requires the presence of an extended H-bonded proton delivery network in the oxy-ferrous protein that includes protic residues and at least one ordered H₂O molecule H-bonded to the terminal O-atom of the O₂ ligand. Based on these observations, we hypothesized that the F33Y-Cu₃Mb may contain a similar H-bond network for faster proton delivery to the oxy-heme than in WTMb. To test this, we obtained the crystal structure of oxy-F33Y-Cu₃Mb.

The oxy-F33Y-Cu₃Mb crystal was prepared by soaking ferric-Mb crystals in dithionite, followed by exposure to O₂, and the oxy state was confirmed by single-crystal UV/vis spectroscopy before diffraction (Figure S5). The structure, refined at 1.27 Å resolution, is compared with the structure of oxy-WTMb in Figure 2. An omit-map showing the electron density of the oxygen ligand is shown in Figure S6. Due to reduced oxygen affinity of F33Y-Cu₃Mb, refinement of this structure yielded an incomplete occupancy for the oxygen ligand, but it nevertheless supports the above interpretation of the EPR data. As suggested by the EPR results, an extended H-bond network involving two H₂O molecules is observed, with W1 stabilized by H-bonds with the engineered His43 and Tyr33 residues, and W2 stabilized by H-bonds to W1 and the engineered His29 (Figure 2). Together, this H-bond network links the designed residues and the distal O-atom of bound O−O. As a result of these changes in the active-site pocket, the O−O in F33Y-Cu₃Mb is rotated from that in WTMb by ~13° about the Fe−O bond, apparently to maximize its ability to accept H-bonds from His64 and W2. Such an interaction of the oxygen with the W2 is expected to enable the facile protonation, by W2, of the peroxo intermediate formed on cryoreduction at 77 K, yielding the hydroperoxo observed by the cryogenic EPR.

Given the spectroscopic and crystallographic evidence for an H-bond network that interacts with the O₁ in oxy-F33Y-Cu₃Mb, and its absence in oxy-WTMb, we conclude that this H-bond network is the important structural feature that transforms WTMb, a protein that can activate O₂ and reduce it to H₂O, into F33Y-Cu₃Mb, a protein that can only bind O₂ reversibly, into F33Y-Cu₃Mb, a protein that can activate O₂ and reduce it to H₂O. We propose that this activation is achieved by polarization of the O−O bond by the H-bonded H₂O, allowing facile reduction and protonation of the oxygen and intermediates. While H-bond networks involving water have been observed in native heme enzymes such as cyt P450, and a similar network has been proposed to occur in HCOs-based computational and isotope studies, design of metalloenzymes incorporating this important structural feature has been difficult to perform and confirm. The combination of cryoreduction EPR spectroscopy and high-resolution crystallography presented in this work provides a clear example of the importance of H-bond networks involving water in conferring activity to designed metalloenzymes. We believe this structural feature will be critical to enhancing the future success of high-activity metalloenzyme design.

**Figure 2.** Crystal structure of oxy-F33Y-Cu₃Mb determined at 1.27 Å resolution (PDB: 5HAV), compared with that of oxy-WTMb (1A6M).
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