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Enzyme Design

A Designed Functional Metalloenzyme that Reduces O_2 to H_2O with Over One Thousand Turnovers**

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Much progress has been made in designing metalloproteins with structures similar to native enzymes[1] and advances in computational biology have allowed for rational design of enzyme function as well.[2] Despite these achievements, most designed enzymes have simple active site structures and low activities, with limited turnover numbers. Designing artificial enzymes with higher complexity and number of turnovers is not only an important measure of success in the field, but can also reveal the structural features responsible for tuning enzymatic activities and may result in artificial enzymes for practical applications. A prime example of a complex metalloenzyme with an important function is the family of terminal oxidases, such as heme–copper oxidases (HCOs)[3] and *bd* oxygen reductases,[4] which catalyze the kinetically difficult reduction of O_2 to water and, in doing so, generate the transmembrane proton gradient that drives important processes such as ATP synthesis. While there are many catalysts that reduce oxygen to water,[5] a long-standing challenge has been to carry out this reaction under mild conditions without the production of reactive oxygen species (ROS), such as superoxide and peroxide. ROS not only damage biomolecules in cells and fuel cell components, but also decrease energy efficiency, because ROS are a result of incomplete catalysis. In addition, efficient catalysts that avoid the use precious metal ions and instead use iron or copper will greatly decrease costs in practical applications.[6] Therefore, designing enzymes that catalyze complex reactions, such as oxygen reduction, at mild pH values that are based on small, stable, and easy to produce scaffold proteins, would yield ideal models for studying the fundamentals of this reaction and produce useful catalysts for applications such as fuel cells.

In HCOs the active site responsible for O_2 reduction is a heterobinuclear metal center containing a heme Fe and a His-ligated Cu (Cu_B). We have reported the introduction of two histidine residues in the heme pocket of sperm whale myoglobin (swMb) through Leu29His and Phe43His mutations,[7] which, together with the native distal His64 residue formed a copper-binding site (this mutant is called Cu_BMb, see Supporting Information, Figure S1 a). As purified, Cu_BMb has no metal in the Cu_B site (designated as E-Cu_BMb; that is, empty). Introducing the copper-binding site into myoglobin transformed it from a simple oxygen carrier into a copper-dependent heme oxygenase at pH 8,[8] which degrades the heme cofactor to verdoheme. Since the active site Tyr residue in native HCOs has been shown to be critical to the function of these enzymes,[9] we hypothesized that the lack of a tyrosine next to one of the histidine ligands in copper-loaded Cu_BMb (Cu-Cu_BMb) may limit conversion of O_2 to water. Herein, we report the introduction of a Tyr residue at two different positions close to the histidines of the putative copper-binding site of Cu_BMb and demonstrate that these artificial enzymes are able to efficiently reduce O_2 to water (one enzyme with over 1000 turnovers) with minimal release of superoxide or peroxide. These results demonstrate that both the presence and positioning of the tyrosine are important for terminal oxidase activity.

Since the design and activity of the Cu_BMb mutant were first reported,[7,8] the crystal structure of E-Cu_BMb has been solved; the histidine ligands in this structure overlay well with those of bovine HCO[10] (PDB 1V54; Figure S1 a) and this structure was used to guide the placement of a Tyr residue in the active site pocket of Cu_BMb. Since Tyr244 is four residues away from His240 in the primary sequence of bovine HCO, we first placed an analogous Tyr four residues from His29, a Phe33Tyr mutation (called F33Y-Cu_BMb). The crystal structure of E-F33Y-Cu_BMb shows excellent agreement with the computer model (Figure S1 b). A crystal structure of Cu-F33Y-Cu_BMb has also been obtained (Figures S1 and S2). However, although Tyr33 points into the Cu-binding site and is next to His29, Tyr33 does not overlay well with Tyr244 of bovine HCO (Figure 1 a). To find a better structural overlay, a recent crystal structure of a cbb_3 HCO[11] revealed that the Tyr residue can be near the His ligand spatially, while not necessarily close in the primary sequence. Therefore, we modeled Tyr next to His29 through a Gly65Tyr mutation (called G65Y-Cu_BMb). The energy-minimized computer

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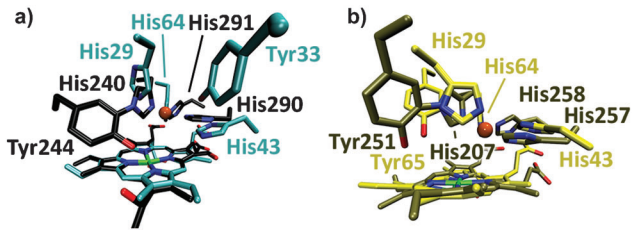

Figure 1. Structures and computer models of native and designed oxidases. a) Overlay of the crystal structures of bovine *Cc*O[10] (black) and E-F33Y-Cu_BMb (cyan); b) Overlay of the crystal structure of cbb_3 HCO from *Pseudomonas stutzeri*[11] (tan) and E-G65Y-Cu_BMb computer model (yellow); The Cu_B copper is represented as an orange sphere. Nitrogen = blue; Oxygen = red; Iron = green.

model of E-G65Y-Cu_BMb overlays very well with that of cbb_3 HCO (Figure 1b).

The spectral properties of the oxidized and reduced states of these variants are similar to those of wild type swMb (WTswMb;[12] Figure S4). However, upon exposure of the ferric states (Fe^{3+}) of both E-F33Y-Cu_BMb and E-G65Y-Cu_BMb to excess reductant [ascorbate with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as a mediator] in air-saturated buffer in a sealed cuvette, a transition from ferric through oxy (O_2 bound to Fe^{2+}) to deoxy (Fe^{2+}) myoglobin was observed (Figure S4e,f), which is in contrast to WTswMb (Figure S4d) and suggested that O_2 dissolved in solution.

To determine whether the product of oxygen reduction is water, ^{17}O NMR spectroscopy was carried out. Production of $H_2{}^{17}O$ above the background level (natural abundance) of $H_2{}^{17}O$ in water was monitored in a sealed vessel containing $^{17}O_2$, the myoglobin variant, and excess reductant, using ^{17}O-labeled Tyr as an external standard. A typical ^{17}O NMR spectrum is shown in the Supporting Information, Figure S5. Figure 2 shows the ratios of the $H_2{}^{17}O$ signal area to ^{17}O-Tyr signal area at various time points after addition of $^{17}O_2$ to initiate its reduction by WTswMb, E-F33Y-Cu_BMb, and E-G65Y-Cu_BMb, normalized to the area of the signal before initiating the reaction. The normalized ratio of water in the WTswMb sample remains close to one, suggesting that the protein did not produce any new $H_2{}^{17}O$. In contrast, E-F33Y-Cu_BMb and E-G65Y-Cu_BMb produced up to 10 mM $H_2{}^{17}O$ at 120 min, as indicated by an increase in the normalized ratio, confirming the production of water from O_2.

The rates of oxygen reduction were measured quantitatively using an O_2 electrode to monitor the concentration of O_2 over time in the presence of reductant, similar to methods reported for native HCO.[13] The rate of O_2 disappearance was measured for WTswMb and the Cu_BMb variants (Figure S6). A hallmark of native terminal oxidases is the clean reduction of O_2 to water with minimal release of superoxide or peroxide.[14] To identify the product (O_2^-, O_2^{2-}, or H_2O) we added superoxide dismutase (SOD) and catalase which selectively react with superoxide and peroxide, respectively, producing O_2 as one of their products, which would slow the apparent rate proportional to the amount of ROS released. By comparing the rates of reduction in the absence of and in the presence of SOD and catalase, the portion of O_2 reduction that is due to water formation (in white) versus superoxide or peroxide formation (in gray) can be calculated (see Figure 3a and Table S5 and Supporting Text). Not surprisingly, most of the O_2 consumption by WTswMb is due to superoxide or peroxide formation, consistent with autoxidation,[15] and these rates remain unaffected in the presence of Zn^{2+}, Ag^+, or Cu^{2+} ions. Interestingly, introducing two histidine residues (Cu_BMb) into the distal pocket of WTswMb results in substantial inhibition of superoxide/peroxide formation in comparison to WTswMb, but does not significantly contribute to water formation, and therefore results in a decrease in the

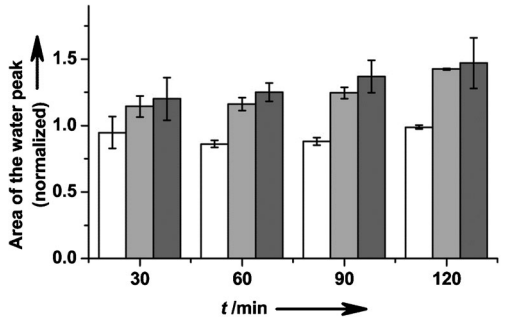

Figure 2. Quantitation of $H_2{}^{17}O$ product by ^{17}O NMR spectroscopy: area of the $H_2{}^{17}O$ signal normalized to an external standard for WTswMb (white), E-F33Y-Cu_BMb (light gray) and E-G65Y-Cu_BMb (dark gray) at 30, 60, 90, and 120 min. Error bars indicate standard deviation.

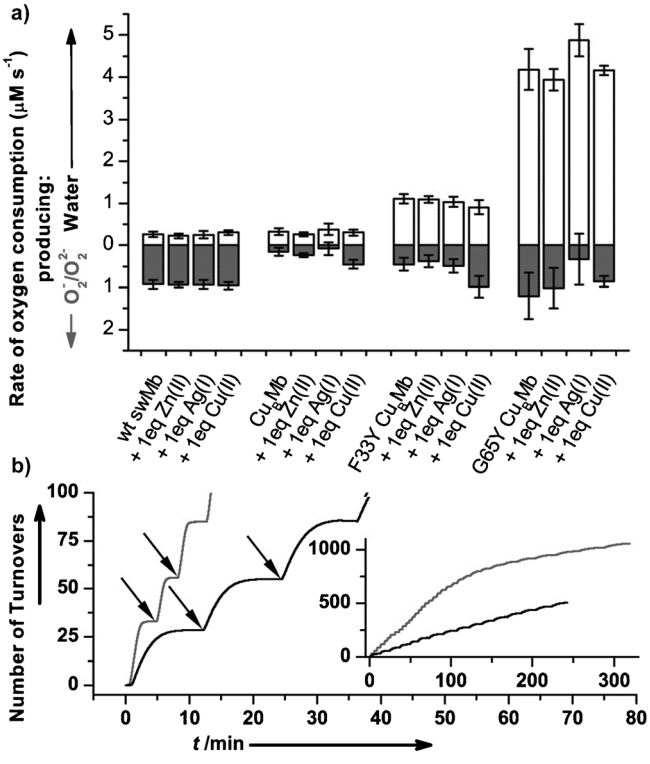

Figure 3. Characterization of the O_2 reduction; a) Rates of O_2 reduction to form either water (white) or superoxide/peroxide (gray) with 18 μM WTswMb, Cu_BMb, F33Y-Cu_BMb, or G65Y-Cu_BMb; b) Number of turnovers of O_2 reduction by E-F33Y-Cu_BMb (black) and E-G65Y-Cu_BMb (gray); arrows indicate addition of approximately 28 equivalents O_2. Inset, region of a high number of turnovers.

overall rate of O_2 consumption. Similar to WTswMb, addition of Zn^{2+}, Ag^{+}, or Cu^{2+} ions to E-Cu_BMb does not perturb the rate or product of O_2 reduction. In contrast to both WTswMb and Cu_BMb, introducing a Tyr next to the one of the His ligands at either position 33 or 65 results in a dramatic increase in water formation and overall rate of O_2 consumption. The rate of water production increases up to 4.2 $\mu M s^{-1}$ for E-G65Y-Cu_BMb and 1.1 $\mu M s^{-1}$ for E-F33Y-Cu_BMb when using 18 μM enzyme. To our knowledge, this is the first time that Tyr and its specific positioning has been shown to play an important role in directing the product of O_2 reduction to water formation, instead of release of superoxide or peroxide. Under further optimized conditions (with a higher concentration of reductant), the rate of O_2 reduction to water by E-G65Y-Cu_BMb is 28 min^{-1} (Figure S10), which is within approximately 150-fold of native HCOs.[16] Additionally, we show in the Figure S7 that 1.8 mM and 18 mM cyanide, a known inhibitor of HCO activity, inhibits the activity of E-G65Y-Cu_BMb and E-F33Y-Cu_BMb, respectively.

Surprisingly, the O_2 reduction activity is independent of the presence and identity of the metal in the engineered Cu_B site (Figure 3a). To further validate this finding, we repeated the studies with varying equivalents of Cu^{2+} ions (0–2 equivalents) as well as a strong metal chelator, ethylenediaminetetraacetate (EDTA), but no further change was noticed (Figure S8). As E-G65Y-Cu_BMb is still around 150-fold lower in activity than native HCOs,[16] our results are not intended to rule out the role of the copper ion native HCOs. However, these results do indicate that a copper center is not strictly necessary for oxygen reductase chemistry and are consistent with the recent discovery of *bd* oxygen reductases[4] that lack the Cu_B center and yet can still perform the oxidase activity.

More importantly, this study demonstrates the importance of the presence and positioning of tyrosine in the active site. In both types of native oxidases the reaction is either known to or proposed to proceed by the mechanism of two electron reduction of the oxygen to a peroxo intermediate, followed by rapid protonation and heterolytic O–O bond cleavage, leading to a transient ferryl intermediate; it has also been proposed that the conserved tyrosine in HCOs is involved in donating an electron.[3,4] To elucidate the potential role of the Tyr, we have obtained the crystal structure of E-F33Y-Cu_BMb. When comparing E-Cu_BMb and E-F33Y-Cu_BMb with WTswMb, we found that introducing two histidine residues and then an additional tyrosine residue has led to stabilization of two and three water molecules, respectively, in the distal pocket (Figure S11), in comparison to one water molecule in the distal pocket of WTswMb. In addition, where WTswMb has only one hydrogen bond partner available to interact with the bound oxygen (H64), two and three more hydrogen bonding capable residues are available in E-Cu_BMb and E-F33Y-Cu_BMb, respectively. Studies of crystal structures of various states of HCOs have suggested a role for water molecules and an extended hydrogen bonding network in the oxygen reduction step,[17] and computational studies[18] of HCOs have supported the role of similar interactions in oxidase activity. Based on these studies, we propose that tyrosine and its associated hydrogen-bonding network activates the ferric–superoxo state of our designed enzymes and this activation allows it to accept a second electron from the exogenous reductant, for complete reduction to water. Further studies are underway to determine the exact role of Tyr in our model protein system.

Finally, to test the extent of the functional activities of these enzymes, we have carried out multiple turnover reactions. To a solution containing E-F33Y-Cu_BMb or E-G65Y-Cu_BMb and excess reductant, approximately 500 μM (28 equivalents) O_2 was added repeatedly and each time the total O_2 consumption was monitored using an O_2 electrode. These stepwise additions resulted in the multiple plateaus shown in Figure 3b. We calculate that E-F33Y-Cu_BMb and E-G65Y-Cu_BMb achieved more than 505 and 1056 turnovers, respectively (Figure 3b, inset).

In summary, we have demonstrated the first successful design of a functional protein capable of cleanly reducing oxygen to water with minimal release of superoxide or peroxide, similar to the activity of terminal oxidases, with more than 1000 turnovers. Through these designed functional proteins, we have also shown that Tyr next to one of the copper coordinating His ligands plays a critical role in directing O_2 reduction to water formation. Furthermore, the positioning of this Tyr is critical in affecting the rate of catalysis. This work complements a recent report of a genetically incorporated cross-linked tyrosine–histidine in F33Y-Cu_BMb that increases the oxidase activity significantly.[19] Even though the designed proteins are still less active than terminal oxidases, it is remarkable that oxygen reduction to water was conferred to a much smaller protein, myoglobin, with only three mutations of the distal pocket. Given their high enzymatic turnovers, smaller size, higher stability, and higher expression yield, these designed enzymes will serve as ideal models for a more detailed understanding of terminal oxidases and allow for potential applications in biology and alternative energy.

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