Enzyme Models

DOI: 10.1002/anie.201108756

Significant Increase of Oxidase Activity through the Genetic Incorporation of a Tyrosine–Histidine Cross-Link in a Myoglobin Model of Heme–Copper Oxidase**

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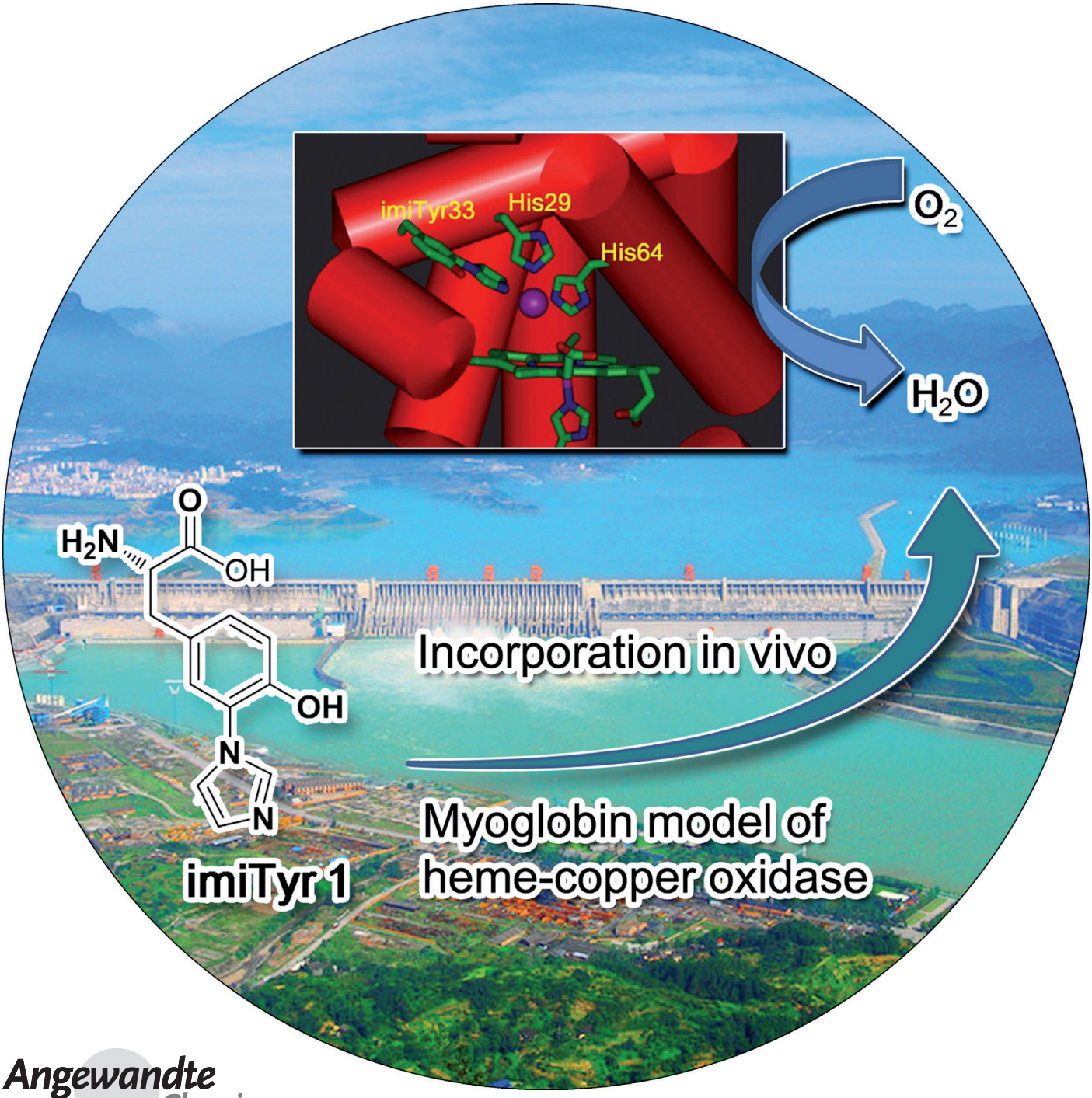

In aerobic respiration, heme–copper oxidase[1] (HCO) performs efficient four-electron reduction of oxygen to water without releasing toxic, reactive oxygen species (ROS) such as superoxide and peroxide.[2] Because of its importance in biogenetics, fascinating chemistry, and potential applications in fuel cells, HCO has been intensively investigated by structural biologists,[3] enzymologists,[1,4] and synthetic chemists.[5] While tremendous progress has been made from the above studies, several important questions about the structural features and mechanism of HCO still remain. Of particular interest is the unique post-translationally modified covalent cross-link between carbon atom C6 of a tyrosine residue and nitrogen atom Nε2 of a nearby histidine residue (Tyr–His cross-link, Figure 1 A), which was first revealed by a high-resolution X-ray structure[3a] and then confirmed by biochemical and biophysical studies.[6] Importantly, the Tyr–His cross-link is conserved in bacterial and mammal HCOs,[5b] thus implicating a strong evolutionary connection and the functional significance of the cross-link.

The presence of a covalent Tyr–His cross-link is firmly established in HCOs; however, its exact role in HCO function is still not fully understood. A major limitation of our understanding is that such a post-translational modification cannot be directly probed through traditional site-directed mutagenesis methods. To overcome this limitation, synthetic model compounds have been made, and studies of these model compounds have suggested that the cross-link between Tyr and His residues significantly decreased the pK_a values of phenol and imidazole side chains. The decreased pK_a value of phenol is believed to facilitate proton delivery and tyrosyl-radical formation.[5b,7] Despite these advances, questions about the roles of the Tyr–His cross-link still remain, because no study has been able to compare the functions of His and Tyr residues at the same positions in the same protein with and without cross-linking.

To answer the above question and to gain new insights into the HCO catalytic process,[7c] we report herein the genetic incorporation of the unnatural amino acid imiTyr (**1**; Figure 1 C), which mimics the cross-linked Tyr–His ligand,

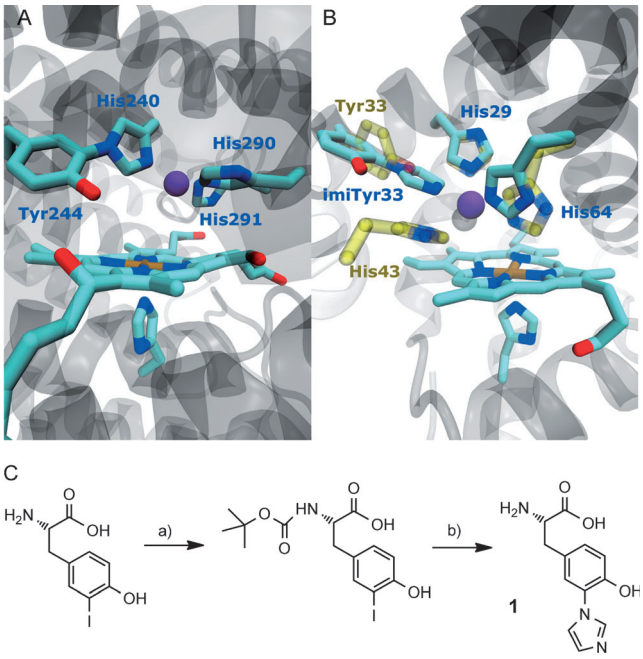

Figure 1. A) Cu_B site of cytochrome c oxidase (bovine numbering). B) Structure model overlay of imiTyrCu_BMb (cyan) and F33YCu_BMb (yellow). C) Synthesis of imiTyr (**1**). a) Di-*tert*-butyl dicarbonate, THF, 1 N NaOH, RT, 4 h. b) Imidazole, Cs_2CO_3, CuI, DMF, reflux, 16 h. Color code in (A) and (B): Cu pink, N blue, O red, Fe brown.

into a functional model of HCO in sperm whale myoglobin[8] (Figure 1 B). The obtained enzyme model with the incorporated Tyr–His ligand imiTyr was termed imiTyrCu_BMb. We then compared the activity of imiTyrCu_BMb with a mutant that has His and Tyr residues at the same positions in the same protein but lacks such a cross-link (F33YCu_BMb; F = Phe, Y = Tyr). Our results show that imiTyrCu_BMb recapitulated important features of HCO; imiTyrCu_BMb exhibited selective O_2-reduction activity while generating less than 6% ROS at more than 1000 turnovers. Importantly, incorporation of imiTyr in the HCO model resulted in eightfold increase in selectivity and threefold increase in catalytic turnover, thereby supporting that the Tyr–His cross-link is indeed essential for HCO function.[5b] The increase in catalytic turnover provides for the first time direct evidence that the covalently cross-linked Tyr–His moiety can dramatically enhance the oxidase activity.

We chose myoglobin for genetic incorporation of the unnatural amino acids and to carry out functional studies, because the large (ca. 200 kDa) HCO membrane proteins consist of multiple subunits that are difficult to produce homogeneously in large amounts, whereas myoglobin is a much smaller (18 kD) water-soluble protein that can be expressed and purified easily in gram quantities.[9] Previous biochemical and spectroscopic studies have indicated that the designed heme–copper center in myoglobin can be a functional model of HCOs.[8]

We first synthesized (*S*)-2-amino-3-(4-hydroxy-3-(1*H*-imidazol-1-yl)phenyl)propanoic acid (**1**; hereafter termed imiTyr), which contains the *ortho*-imidazole–phenol linkage (Tyr–His ligand), and then we genetically encoded it into

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[**] We gratefully acknowledge the Major State Basic Research Program of China (2010CB912301, 2009CB825505), National Science Foundation of China (30870592, 90913022, Y2JY141001) to J.W. and X.L., and National Institutes of Health (GM062211) to Y. L. We thank Z. Xie and X. Peng for help in protein mass spectroscopy, and Kyle D. Miner and Arnab Mukherjee for developing the activity assay
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201108756.

myoglobin. The unnatural amino acid imiTyr (**1**) was synthesized by first protecting the amine group of 3-iodotyrosine using di-*tert*-butyl dicarbonate[10] to give Boc-L-3-iodotyrosine (Boc = *tert*-butoxycarbonyl), which was then heated to reflux with imidazole in the presence of Cs_2CO_3 and CuI in DMF to afford **1** in 50% yield[7c] (Figure 1C).

To selectively incorporate imiTyr (**1**) at defined sites in proteins in *E. coli*, a mutant *Methanococcus jannaschii* tyrosyl amber suppressor tRNA (MjtRNA$^{Tyr}_{CUA}$)/tyrosyl-tRNA synthetase (MjTyrRS) pair was evolved that uniquely specifies **1** in response to the TAG codon. An MjTyrRS library, pBK-lib-jw1, in which six residues (Tyr32, Leu65, Phe108, Gln109, Asp158, and Leu162) were randomized, and any one of the six residues (Ile63, Ala67, His70, Tyr114, Ile159, Val164) was either mutated to Gly or kept unchanged, was used to screen for active and selective mutants of tRNA synthetase MjTyrRS for **1**, as reported.[11] Nine MjTyrRS clones emerged after three rounds of positive selections and two rounds of negative selections (Table S1 in the Supporting Information). The best clone grew at a concentration of 120 μg mL^{-1} of chloramphenicol in the presence of 1 mM **1**, but only at a concentration of 20 μg mL^{-1} chloramphenicol in the absence of **1**, and the clone was named imiTyrRS. Sequencing of this clone revealed five mutations: Tyr32Glu, Leu65Ser, His67Gly, Asp158Tyr, and Leu162Asn. Among the five mutations, the His67Gly mutation was required to create additional space to accommodate the imidazole group. The other four mutations may make specific hydrogen-bonding interactions to stablize the imiTyr sidechain. Work is underway to obtain a structure of the imiTyrRS protein to gain insight into these interactions.

To determine the efficiency and fidelity for the incorporation of unnatural amino acid **1** into proteins, an amber stop codon was substituted for Ser4 in sperm whale myoglobin containing a C-terminal His_6 tag. Protein expression was carried out in *E. coli* in the presence of the selected synthetase (imiTyrRS), the tyrosyl amber suppressor tRNA MjtRNA$^{Tyr}_{CUA}$, and 1 mM **1**; as a negative control protein expression was also carried out in the absence of **1**. Analysis of the purified protein by SDS-PAGE showed that full-length myoglobin was expressed only in the presence of unnatural amino acid **1** (Figure 2A), thus indicating that tRNA synthetase imiTyrRS is specifically active for **1** but does not utilize any natural amino acid. The yield of the mutant myoglobin was 10 mg L^{-1}. For comparison, the yield of wild-type sperm whale myoglobin (wtMb) under similar conditions was 50 mg L^{-1}. ESI-MS spectrometric analysis of the mutant myoglobin gave an observed average mass of 18497 Da, which is in close agreement with the calculated mass of 18496 Da for the Ser4→**1** myoglobin mutant.

After we demonstrated the efficient genetic incorporation of imiTyr (**1**) into wtMb, we expressed myoglobin double mutant Leu29His/Phe33→**1** (called imiTyrCu$_B$Mb) as a functional HCO model[8] with a yield of 5 mg L^{-1}. The selective incorporation of imiTyr at position 33 was again confirmed by SDS-PAGE (Figure 2A) and ESI-MS (Figure S2 in the Supporting Information). Together with distal residue His64 in sperm whale Mb, the newly introduced residues His29 and imiTyr33 constitute a Cu_B binding site according to our model and place the copper ion in proximity to heme iron (Figure 1B). The UV/Vis spectrum of imiTyrCu$_B$Mb at pH 7.4 is similar to that observed for wtMb and is typical of a six-coordinate, H_2O-bound, high-spin heme with a Soret band absorption maximum at 408 nm (Figure S3 in the Supporting Information). The deoxy-imiTyrCu$_B$Mb was obtained by reducing the purified met-imiTyrCu$_B$Mb with excess dithionite. The UV/Vis spectrum (Figure S3 in the Supporting Information) of deoxy-imiTyrCu$_B$Mb exhibits a Soret band at 430 nm and a visible absorption band at 562 nm. This spectrum is nearly identical to that of deoxy-wtMb, thus indicating that mutation in imiTyrCu$_B$Mb did not cause major structural changes of the protein. Addition of Cu^{2+} ions at pH 7.4 to imiTyrCu$_B$Mb resulted in changes of the spectrum in the Soret region (Figure S4 in the Supporting Information), which was fitted to a double reciprocal plot and a Hill plot.[8b] These results indicate that a single Cu^{2+} binding site was present in imiTyrCu$_B$Mb, with a K_D value of 1.6 μM.

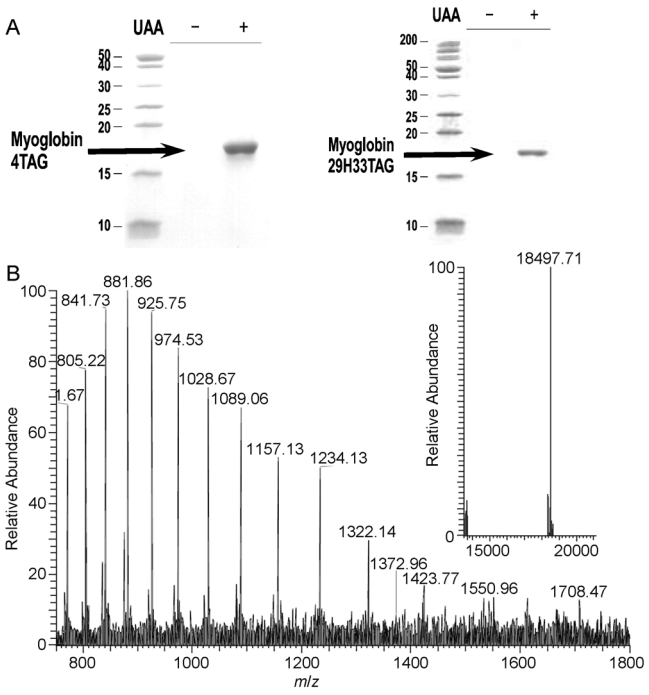

Figure 2. A) Coomassie-stained SDS-PAGE gel of expression of two myoglobin mutants in the presence and absence of 1 mM unnatural amino acid (UAA) **1**; TAG4 mutant (left) and 29His33TAG mutant (right; both indicated by black arrows). In the presence of the UAA **1**, imiTyr is introduced at the position of the TAG codon, leading to the Ser4→**1** mutant (left) and to the Leu29His/Phe33→**1** double mutant (called imiTyrCu$_B$Mb, right). B) ESI-MS spectra of the TAG4 mutant. The inset shows the deconvoluted spectrum; expected mass: 18496 Da, found: 18497 Da.

Since the hallmark of native HCOs is the clean catalytic reduction of O_2 to water without releasing ROS, we then measured the rate of oxygen reduction catalyzed by imiTyr-Cu$_B$Mb by using an O_2 electrode in the presence of 1000 equivalents of ascorbic acid as the reductant and 100 equivalents of TMPD (tetramethyl-*p*-phenylenediamine dihydrochloride) as a redox mediator. A similar method was used to measure enzymatic activity of native HCO.[12] To differentiate between ROS and water products, we employed

catalase, which selectively reacts with H_2O_2 to produce O_2, and superoxide dismutase (SOD), which selectively reacts with superoxide to produce H_2O_2 and O_2. If O_2 consumption results in release of ROS but not in formation of water, the apparent O_2-reduction rate will be lower in the presence of catalase and SOD, because they will convert ROS back into O_2. By comparing the rates of reduction in the absence and presence of catalase and SOD, the portion of O_2 reduction that is due to water formation (light gray, Figure 3A) and due to ROS formation (dark gray, Figure 3A) can be calculated. Not surprisingly, most of the O_2 consumed by wtMb lead to ROS, both in the presence and absence of Cu^{2+} ions. By contrast, imiTyrCu_BMb catalyzed O_2 reduction without generating more than 6% H_2O_2 in the presence of one equivalent Cu^{2+} ions, with an oxygen-consumption rate of 12 μM min^{-1} (enzyme activity = 2 oxygen molecules/min). In the absence of Cu^{2+} ions, however, more than 30% of O_2 was converted into ROS, thus indicating that Cu_B is important for the catalytic turnover of O_2 into H_2O.

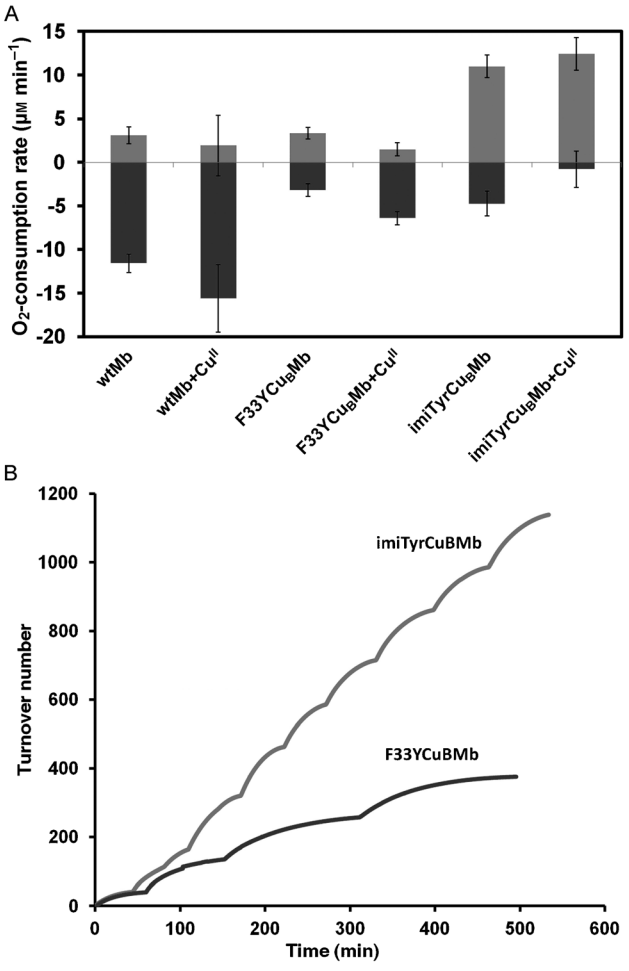

Figure 3. A) Rates of oxygen reduction to form either water (light gray) or ROS, peroxide, and superoxide, (dark gray) catalyzed by 6 μM wtMb, F33YCu_BMb, or imiTyrCu_BMb, in the presence or absence of Cu^{2+} (6 μM), with TMPD (0.6 mM) and ascorbic acid (6 mM) as reductants. B) O_2-reduction turnover number measured during the stepwise addition of O_2. The reduction was catalyzed by imiTyrCu_BMb or F33YCu_BMb.

The myoglobin system described herein provides a unique possibility to directly compare the effect of His and Tyr residues with and without cross-linking. To take advantage of this unique feature, we also measured O_2 consumption for the myoglobin mutant Leu29His/Phe33Tyr/Phe43His (F33YCu_BMb). This mutant harbors a Cu_B site consisting of residues His29, His43, and His64, as we have shown previously,[8b] and a tyrosine residue in position 33. While His43 and Tyr33 sidechains are in similar positions as the imidazole group and phenol group of imiTyr33, respectively (Figure 1B), no cross-link is present between them. Not only was the overall activity of the F33YCu_BMb lower (ca. 40%) than that of imiTyrCu_BMb, but more than 50% of the O_2 was also converted into ROS by F33YCu_BMb with or without Cu^{2+} ions added, thus confirming that the cross-linked Tyr–His ligand plays a critical role for the selective reduction of O_2 into H_2O.

To test if imiTyrCu_BMb is a robust O_2-reduction catalyst that is capable of performing many turnovers without self-destruction, we then measured O_2 consumption under multiple turnover conditions. TMPD (0.6 mM) and ascorbic acid (6 mM) were added to a solution containing imiTyrCu_BMb (6 μM), $CuSO_4$ (6 μM), and O_2 (800 μM). O_2 reduction was then monitored by using an oxygen electrode until all the oxygen was consumed. Subsequently, a further addition of O_2 (800 μM) was carried out by purging the solution with pure oxygen, and then oxygen reduction was again measured until all the oxygen was reduced. These stepwise additions resulted in the multiple plateaus observed in the traces shown in Figure 3B, and these processes were repeated until imiTyr-Cu_BMb achieved more than 1000 turnovers. The turnover number reported here is an underestimate, because the extra turnovers occurring during oxygen addition were not included in the calculation. Notably, little decrease in the O_2-consumption rate was observed after the catalyst reached 1000 turnovers, thereby indicating that at this point the imiTyr-Cu_BMb catalyst has remained intact. By contrast, F33YCu_BMb, the mutant without the cross-link, underwent less than 400 turnovers under similar conditions. These results again support that the cross-linked Tyr–His ligand is required for optimal O_2 reduction to H_2O. Here, the difference in catalytic activity between imiTyrCu_BMb and F33YCu_BMb can also be attributed to the different geometries of the copper center. To further investigate the mechanism, we are currently working to solve the crystal structure of imiTyrCu_BMb and examine the enzymatic property of another mutant having a Phe–His-type cross-linkage at the position 33.

In conclusion, by directly incorporating the unnatural amino acid imiTyr (**1**) into myoglobins in *E. coli* in response to the amber codon TAG, we have successfully designed a functional HCO model imiTyrCu_BMb that catalyzes selective and efficient oxygen reduction to water. The HCO model imiTyrCu_BMb bearing the Tyr–His cross-link is eightfold more selective with threefold more turnovers than F33YCu_BMb, which does not contain the cross-link but harbors His and Tyr residues at the same positions in the same protein. Since the synthesis of imiTyr contains only two steps with 50% overall yield, and mutant proteins bearing imiTyr (**1**) at any site can be easily obtained and purified in

milligram quantities through site-directed mutagenesis and recombinant protein expression, further systematic investigation of the function of the Tyr–His cross-link is now possible. While imiTyrCu_BMb exhibits lower enzymatic activity (2 O_2/min)[12] in comparison to native heme–copper oxidase (ca. 300 O_2/s), it is possible to rapidly improve our HCO model to achieve higher activity through directed evolution and incoporation of unnatural amino acids. Our designed enzyme harbors the unnatural amino acid imiTyr, which is highly analogous to the post-translationally modified tyrosine–histidine ligand found in the Cu_B site of HCO; this designed enzyme serves as an ideal model for a more detailed understanding of HCOs and allows for potential applications in synthetic biology and alternative energy.[5b,9,13]

Experimental Section

To express mutant myoglobin protein imiTyrCu_BMb, plasmid pBAD-JYAMB-His29TAG33 was cotransformed with pBK-imiTyrRS into GeneHog-Fis *E. coli* cells. Cells were amplified in LB (lysogeny broth) media (5 mL) supplemented with kanamycin (50 μg mL^{-1}) and tetracycline (15 μg mL^{-1}). A starter culture (1 mL) was used to inoculate 100 mL of liquid LB supplemented with appropriate antibiotics and **1** (1 mM). Cells were then grown at 37 °C to OD600 (optical density at 600 nm) of 0.5, and protein expression was induced by the addition of 0.2 % arabinose. After 12 h of growth at 37 °C, cells were harvested by centrifugation. Wild-type or mutant myoglobin was then purified by Ni-NTA affinity chromatography under denaturing conditions and reconstituted with heme as previously described.[9]

Oxygen consumption was measured by using an Oxygraph Clark-type oxygen electrode (Hansatech Instruments) at 25 °C in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer, pH 7.4. All experiments were performed three times to obtain standard deviations. The electrode was calibrated against air-saturated buffer and O_2-depleted buffer prior to use.The protein was exchanged into 20 mM Tris buffer (pH 7.4), which was previously treated with chelex beads overnight to remove metal ions in the buffer. . The final protein concentration was adjusted to 6 μM. For the experiment with Cu^{2+}, $CuSO_4$ was added to a final concentration of 6 μM and stirred for 10 min. For the experiment with catalase and SOD, catalase (Sigma–Aldrich) was added to a final concentration of 7.3 U L^{-1}, and SOD (Sigma–Aldrich) was added to a final concentration of 0.5 U μL^{-1}. The reaction was initiated by adding TMPD and ascorbic acid to a final concentration of 0.6 mM and 6 mM, respectively. The oxygen concentration was read immediately after adding reductant. The turn-over experiment described in Figure 3B was performed similarly except that after the O_2 in the reaction chamber was exhausted, the chamber was vented with pure O_2 to boost O_2 concentration to 800 μM. The concentration of ascorbic acid was kept at 6 mM.

Received: December 12, 2011
Published online: March 12, 2012

Keywords: enzyme catalysis · enzyme models · oxygen reduction · post-translational modifications · protein design

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