A Designed Metalloenzyme Achieving the Catalytic Rate of a Native Enzyme

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ⓢ *Supporting Information*

ABSTRACT: Terminal oxidases catalyze four-electron reduction of oxygen to water, and the energy harvested is utilized to drive the synthesis of adenosine triphosphate. While much effort has been made to design a catalyst mimicking the function of terminal oxidases, most biomimetic catalysts have much lower activity than native oxidases. Herein we report a designed oxidase in myoglobin with an O_2 reduction rate ($52\ s^{-1}$) comparable to that of a native cytochrome (cyt) cbb_3 oxidase ($50\ s^{-1}$) under identical conditions. We achieved this goal by engineering more favorable electrostatic interactions between a functional oxidase model designed in sperm whale myoglobin and its native redox partner, cyt b_5, resulting in a 400-fold electron transfer (ET) rate enhancement. Achieving high activity equivalent to that of native enzymes in a designed metalloenzyme offers deeper insight into the roles of tunable processes such as ET in oxidase activity and enzymatic function and may extend into applications such as more efficient oxygen reduction reaction catalysts for biofuel cells.

Metalloenzymes normally exhibit much higher activities under milder conditions than small inorganic catalysts, even though the metalloenzymes use only a limited number of physiologically available metal ions or metal-containing cofactors. Rational design of metalloenzymes that mimic native enzymes allows the elucidation of structural features and mechanisms responsible for the high activity and efficient use of earth-abundant metal cofactors and facilitates the design of more robust and cost-effective catalysts for many applications.[1] A key measure of success in this endeavor is the activity of the designed enzymes compared with the native enzymes that they model. While tremendous progress has been made in the field, most reported metalloenzymes display activities that are far below those of the native enzymes that they mimic.

Excellent examples of metalloenzymes of interest are terminal oxidases in the respiratory chain, in which O_2 is reduced to H_2O in a four-electron ($4e^-$) process and the energy harvested from this reduction is converted into a proton gradient that drives the synthesis of adenosine triphosphate, the universal source of energy for most biological processes.[2] In addition to their important role in bioenergetics, terminal oxidases are also of interest to biofuel cell research because the oxidases are among the most efficient oxygen reduction reaction (ORR) catalysts, having the lowest overpotentials and using earth-abundant metal ions such as iron and copper, unlike the best industrial ORR fuel cell catalysts, which require precious platinum.[6] To understand the efficiency of this important class of metalloenzymes, many synthetic models have been made to mimic their structures and functions, with particular focus on the heme-copper center at the active site of most oxidases.[7] Despite years of effort by many groups, the activities of the biomimetic compounds in homogeneous solution are still far below those of native oxidases.[8]

As an alternative approach to biomimetic studies of native enzymes using small organic molecules, we use small, stable proteins such as myoglobin (Mb) as scaffolds to design structural features that mimic those of native enzymes.[4,9] Mb functions as an O_2 storage protein in biology. To transform Mb into an oxidase, we have introduced conserved structural elements of heme-copper oxidases, one Tyr and two His residues, into the distal pocket of Mb. One such variant, L29H/F43H/G65Y Mb (called G65Y-Cu$_B$Mb), was able to reduce O_2 to water with a catalytic oxygen reduction rate of $0.30\ s^{-1}$ (Figure S1) and >1000 turnovers using ascorbate as a reductant and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as a redox mediator.[4]

While the above results demonstrate that we can rationally design a functional enzyme with a high number of turnovers, the catalytic rate ($0.30\ s^{-1}$) is still far below that of native enzymes (e.g., cytochrome (cyt) cbb_3 oxidase from *Rhodobacter sphaeroides* has an O_2 reduction rate of $52\ s^{-1}$).[10] Our preliminary investigations of G65Y-Cu$_B$Mb and similarly designed Mb model enzymes suggest that electron transfer (ET) into the catalytic center may be rate-limiting. This finding is consistent with studies of native cyt c oxidase[11] and some synthetic models showing that electron delivery into the active site might be a rate-limiting step.[7b,12] In searching for new redox partners of Mb to increase the ET rate and potentially the catalytic activity, we were excited to learn of the work by Hoffman and co-workers, who replaced three negatively charged amino acids (Asp44, Asp60, and Glu85) in myoglobin with three positively charged lysines to improve electrostatic interactions between the Mb and its redox partner, cyt b_5, which is negatively charged.[13] Such a D44K/D60K/E85K

variant, called Mb(+6), enhanced the rate of ET from the triplet state of Zn-deuteroporphyrin-substituted Mb to ferric cyt b_5 by >2 orders of magnitude (from 5.5×10^3 to $\sim 1.0 \times 10^6$ s^{-1}).[13a] Inspired by this work, we report herein that the D44K/D60K/E85K mutant of G65Y-Cu$_B$Mb (named G65Y-Cu$_B$Mb-(+6) hereafter; see Figure 1a) exhibited dramatically enhanced oxidase activity, from 0.30 to 52 s^{-1}, making it a designed metalloenzyme with activity comparable to its target native enzymes.
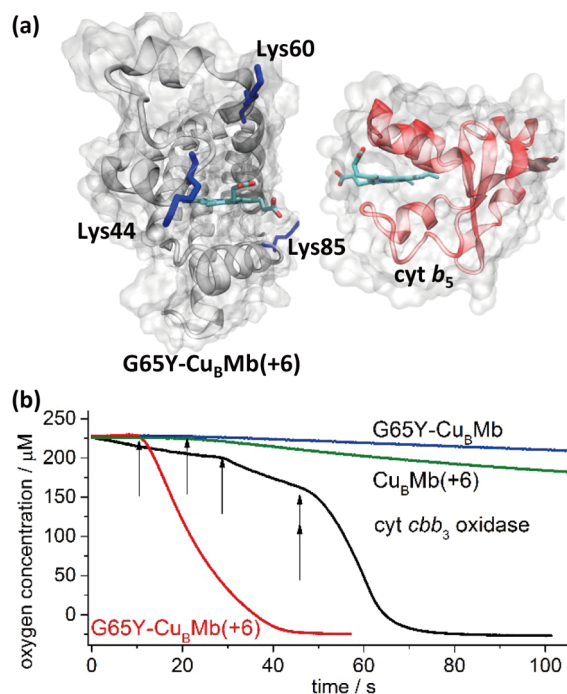

Figure 1. (a) Structures of G65Y-Cu$_B$Mb(+6), showing the engineered lysines in blue, and cyt b_5 (PDB IDs 1CYO for cyt b_5[3] and 4FWY for F33Y-Cu$_B$Mb;[4] rendered through VMD[5]). (b) Oxidase activity of G65Y-Cu$_B$Mb(+6) in comparison with those of native cyt cbb_3 oxidase and G65Y-Cu$_B$Mb at the same concentration under the typical conditions of: NADH, 2 mM; cyt b_5 reductase, 80 nM; cyt b_5, 5 μM; G65Y-Cu$_B$Mb(+6), 50 nM. The black arrows indicate the addition of reductant and the double arrow shows the injection of native cyt cbb_3 oxidase.

The construction, expression, and purification of G65Y-Cu$_B$Mb(+6) were carried out using a protocol reported previously.[4] The mutations were confirmed by DNA sequencing, and the corresponding protein containing the mutations was corroborated by mass spectrometry (calculated MW = 17 477; observed MW = 17 476). The UV−vis electronic absorption spectra of G65Y-Cu$_B$Mb(+6) in both the ferric and ferrous forms are similar to those of G65Y-Cu$_B$Mb (Figure S2a) and the EPR spectrum of ferric G65Y-Cu$_B$Mb(+6) is similar to that of G65Y-Cu$_B$Mb (Figure S3), suggesting that the mutations cause minimal perturbation of the overall protein structure and the structure of the heme active site.

To determine the oxidase activity as measured by the rate of O$_2$ reduction, we used an O$_2$-selective electrode commonly employed to monitor the catalytic activity in native oxidases.[10,14] In the presence of 80 nM NADH-cyt b_5 reductase, the physiological redox partner of cyt b_5,[15] and 5 μM cyt b_5, the concentration of O$_2$ in the presence of 50 nM G65Y-

Cu$_B$Mb(+6) decreased rapidly (Figure 1b), and the rate of O$_2$ reduction was 52 s^{-1}. The reaction was carried out in 5 mM potassium phosphate buffer (pH 6), which has been shown to be the optimal pH for O$_2$ reduction in this and other Cu$_B$Mb variants.[4] The activity decreased at higher ionic strength (Figure S4), suggesting that the ionic strength affects the electrostatic interactions between Mb and cyt b_5, which is consistent with the results reported by Hoffman and co-workers in the similar system.[16] Therefore, low ionic strength (5 mM) was used in this study. Similar to G65Y-Cu$_B$Mb,[4] which has been shown to mimic copper-independent cyt bd oxidase,[17] the addition of copper to G65Y-Cu$_B$Mb(+6) did not increase the oxidase activity.[4] As a result, no copper was added to the reaction mixture. In contrast, G65Y-Cu$_B$Mb without the D44K/D60K/E85K mutations exhibited a rate of 0.3 s^{-1} under the same conditions. Furthermore, the protein without the G65Y mutation, Cu$_B$Mb(+6), also exhibited a low O$_2$ reduction rate of 8.1 s^{-1}, which affirms the importance of the G65Y mutation for oxidase activity.[4] Remarkably, the O$_2$ reduction rate of G65Y-Cu$_B$Mb(+6) (52 s^{-1}) is so high that it is similar to that of native cbb_3 oxidase (50 s^{-1}) measured under the same conditions.

To confirm that the dramatic increase in the oxidase activity of G65Y-Cu$_B$Mb(+6) is due to enhanced ET, we first repeated the above experiment by systematically removing each protein component involved in the O$_2$ reduction. As shown in Figure S5, minimal O$_2$ reduction occurred in the absence of either NADH-cyt b_5 reductase, cyt b_5, or G65Y-Cu$_B$Mb(+6), suggesting that all three proteins are essential for the enzymatic activity. Furthermore, we monitored the oxidation of cyt b_5 in the presence of either G65Y-Cu$_B$Mb or G65Y-Cu$_B$Mb(+6) through stopped-flow UV−vis spectroscopy in order to estimate the rates of ET between cyt b_5 and the two Mb variants (Figure 2 and Figure S6). We first found that the
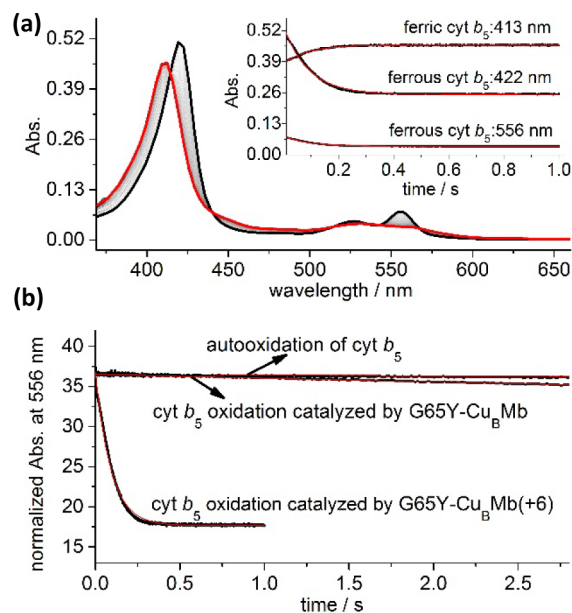

Figure 2. Studies of ET between cyt b_5 and G65Y-Cu$_B$Mb or G65YCuBMb(+6). (a) Representative stopped-flow UV−vis spectra of cyt b_5 oxidation catalyzed by G65Y-Cu$_B$Mb(+6) and (inset) time traces at characteristic wavelengths. (b) Time traces of cyt b_5 oxidation represented by the absorption at 556 nm, overlaid with global spectroscopic fitting (red lines).

pseudo-first-order rate constant for ET between 5 μM cyt b_5 and G65Y-Cu$_B$Mb(+6) increased linearly as the concentration of G65Y-Cu$_B$(+6) increased (Figure S7),[13] consistent with a bimolecular reaction as observed previously. Then we used the same concentration of cyt b_5 as in the oxidase activity assay (5 μM) but increased the concentration of the Mb variant from 50 nM to 0.5 μM, which is still a fraction of the cyt b_5 concentration. The pseudo-first-order rate constant for ET between cyt b_5 and G65Y-Cu$_B$Mb(+6) was determined to be $(1.2 \pm 0.2) \times 10^1$ s^{-1}, which is ~400-fold faster than that of G65Y-Cu$_B$Mb $((3.0 \pm 0.5) \times 10^{-2}$ s$^{-1})$. This 400-fold increase is on the same order of magnitude as that reported by Hoffman and co-workers (180-fold increase).[13] The slight difference is attributable to the differences in the Mb mutations and redox cofactors (heme vs Zn porphyrin) used in the two studies. More importantly, this ET rate enhancement is similar to the rate enhancement of O$_2$ reduction, which strongly suggests that the enhanced ET rate is responsible for the increased oxidase activity. The reduction potentials of G65Y-Cu$_B$Mb(+6) and G65Y-Cu$_B$Mb were determined to be (115 ± 11) and (129 ± 5) mV, respectively, by spectroelectrochemical methods (Figure S8). Since the reduction potentials of the two mutants are similar, these results suggest that the major contributor to the increased ET rate is the enhanced electrostatic interaction between the negatively charged cyt b_5 and positively charged G65Y-Cu$_B$Mb(+6) rather than a difference in the ET driving force.

To investigate whether O$_2$ reduction in the Mb variant is a 4e$^-$ process, as in native oxidases, we measured the amount of H$_2$O$_2$ produced from the reaction using an amperometric H$_2$O$_2$ sensor. Under the same conditions as in the O$_2$ reduction experiment shown in Figure 1b, G65Y-Cu$_B$Mb(+6) generated H$_2$O$_2$ at a rate of 3.5 s^{-1} (Figure 3a), or 7% of the total O$_2$ reduced, while G65Y-Cu$_B$Mb produced 22% H$_2$O$_2$ (Figure S1).[4] Furthermore, since the NADH used in the O$_2$ reduction experiment is a two-electron reductant, the ratio of the rate of

NADH consumed in the reaction to that of the O$_2$ reduced can be used to determine whether the O$_2$ reduction is a 4e$^-$ process, as demonstrated in other heme enzymes.[18] The NADH oxidation was monitored using the absorption at 352 nm (see Figure 3b).[18] The rate of the reaction was compared with that of O$_2$ reduction using an O$_2$-selective electrode under the same conditions as in Figure 3b. As shown in the Figure 3b inset, the ratio of the rate of NADH oxidation to that of O$_2$ reduction was 1.91 ± 0.05 for G65Y-Cu$_B$Mb(+6). This result is consistent with the low percentage of H$_2$O$_2$ directly probed by the above electroanalytical method. Together, these two results strongly suggest that O$_2$ reduction by G65Y-Cu$_B$Mb(+6) is a 4e$^-$ process that produces H$_2$O as in native oxidases. Finally, 50 nM G65Y-Cu$_B$Mb(+6) was able to reduce 220 μM O$_2$ in the reaction system (Figure 1b), corresponding to >4400 turnovers.

The rational design of proteins that mimic native enzymes both structurally and functionally is both exciting and challenging because nature has had millions of years to evolve enzymes to carry out complex reactions like O$_2$ reduction. Therefore, any progress in protein design that moves closer to the properties of native enzymes is encouraging, with the ultimate goal being to match the activities of native enzymes while using the models to understand and explore potential applications. While most biochemical studies of native enzymes use site-directed mutagenesis to find residues that lower the activity of the enzyme, biosynthetic modeling seeks to uncover residues that result in a gain of function and increase the activity; the two approaches therefore complement each other. For example, while ET is known to play a critical role in oxidase activity, the exact mechanism is not well understood. A report by Brunori and co-workers indicated that the internal ET rate accounts for the turnover number and that the ET rate and turnover number display the same pH and temperature dependence.[19] Another report by Fabian and co-workers provided evidence that the rate-limiting step is the initial electron transfer to the catalytic site.[20] The present results using biosynthetic models strongly suggest that the rate of ET into the active site plays a critical role in increasing the enzymatic activity in a mechanism like that of a native oxidase. A related and exciting area of research is to immobilize native enzymes and their variants onto electrodes to understand the protein–protein and protein–electrode interactions for efficient ET in applications such as biofuel cells.[1m,21] Since our biosynthetic models are much smaller and more robust that native oxidases and their variants, the ability to design biosynthetic models that match the O$_2$ reduction activity of native enzymes may open a new avenue to explore applications in biofuel cells.

In conclusion, we have succeeded in engineering Mb, which natively binds O$_2$ reversibly, into an oxidase that displays the O$_2$ reduction activity of a native oxidase with a similar rate and 4e$^-$ reduction process. We achieved the goal by enhancing the ET rate through engineering of more favorable electrostatic interactions between Mb and its redox partner cyt b_5 following the introduction of similar structural elements (conserved His and Tyr) from the native enzyme into Mb. By demonstrating our ability to design a protein whose activity matches that of the native enzyme, we have obtained a deeper understanding of structural features that are important for oxidase activity, which may allow engineering of artificial enzymes for biochemical and biotechnology applications such as more efficient ORR catalysts for biofuel cells.
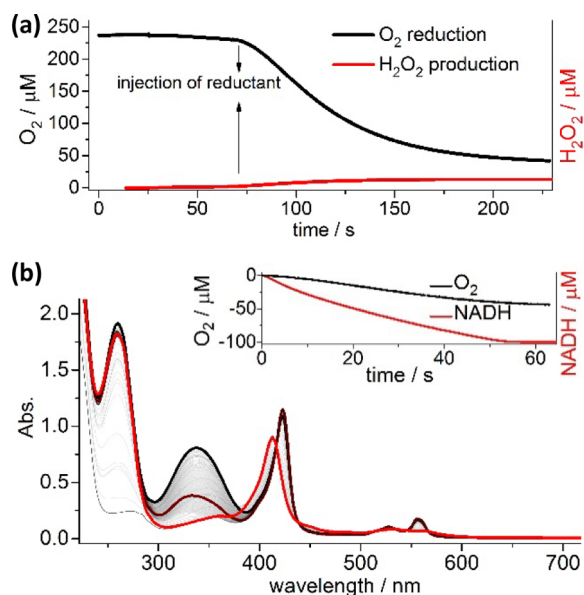

Figure 3. (a) Measurement of O$_2$ reduction and H$_2$O$_2$ production in the O$_2$ reduction reaction. (b) Representative kinetic UV–vis spectra of reaction solution with 100 μM NADH, sampled at 0.5 s intervals over 100 s. The inset shows NADH oxidation and oxygen reduction catalyzed by G65Y-Cu$_B$Mb(+6).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07119.

> Experimental details of protein expression, purification, and spectroscopic characterization (PDF)

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Notes

The authors declare no competing financial interest.

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