Communication

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.5b07119 • Publication Date (Web): 28 Aug 2015

A Designed Metalloenzyme Achieving the Catalytic Rate of a Native Enzyme

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Supporting Information Placeholder

ABSTRACT: Terminal oxidases catalyze four-electron reduction of oxygen to water, and the energy harvested is utilized to drive ATP synthesis. While much effort has been made to design a catalyst mimicking the function of terminal oxidases, most biomimetic catalysts have much lower activity than a native oxidase. We herein report a designed oxidase in myoglobin with an O_2 reduction rate (52 s^{-1}) comparable to that of a native 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 the native redox partner of terminal oxidases, cyt b_5, resulting in a 400 fold ET rate enhancement. Achieving high activity equivalent to native enzymes in a designed metalloenzyme offers deeper insight into the roles of tunable processes like 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 elucidation of structural features and mechanisms responsible for 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 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.

An excellent example 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 over-potentials and using

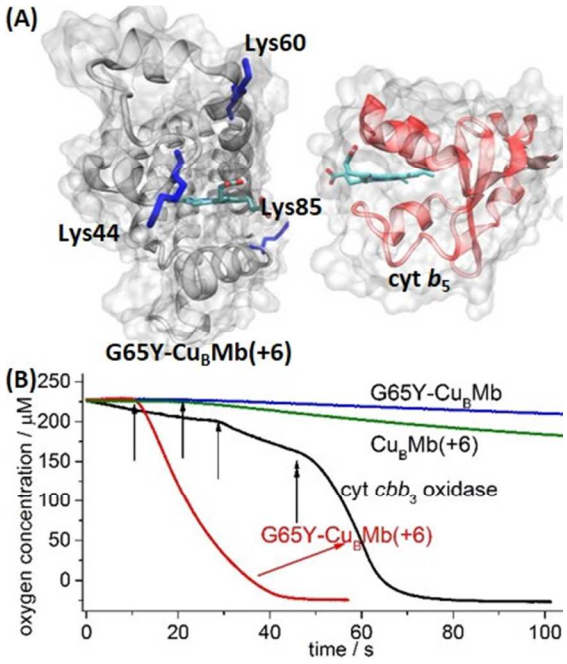

Figure 1. (a) Structures of G65Y-Cu$_B$Mb, showing the engineered lysines in blue and cyt b_5 (PDB ID: cyt b_5 1CYO [3]; F33Y-Cu$_B$Mb 4FWY[4], rendered through VMD[5]) (b) Oxidase activity of G65Y-CuBMb in comparison with that of native cyt cbb_3 oxidase and G65YCu$_B$Mb with the same concentration, under the typical condition. Typical condition: 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.

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 homogenous 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 and stable proteins such as myoglobin (Mb) as scaffolds to design structural features that mimic native enzymes.[4, 9] Mb functions as an O_2 storage protein in biology. To transform the 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_BMb) was able to reduce O_2 to water, with catalytic oxygen reduction rate of 0.30 s^{-1} (Figure S1) and >1,000 turnovers, using ascorbate as a reductant and 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., cyt cbb_3 oxidase from *Rhodobacter sphaeroides* has O_2 reduction rate of 52 s^{-1}).[10] Our preliminary investigations of the G65Y-Cu_BMb and similarly designed Mb model enzymes suggest that electron transfer (ET) into the catalytic center may be rate limiting. This finding is consistent with the studies of native cyt *c* oxidase[11] and some synthetic models that showed that the 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 catalytic activity, we were excited to learn the work by Hoffman and coworkers, 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 5.5x10^3 s^{-1} to ~ 1.0x10^6 s^{-1}).[13a] Inspired by this work, we report herein that introducing the D44K/D60K/E85K mutations into G65Y-Cu_BMb (named G65Y-Cu_BMb(+6) hereafter, see Figure 1a) resulted in dramatically enhanced oxidase activity, from 0.30 s^{-1} to 52 s^{-1}, making a designed metalloenzyme with activity comparable to its target native enzymes.

Construction, expression and purification of G65Y-Cu_BMb(+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: 17477; observed MW: 17476). The electronic absorption spectra in the ultraviolet and visible region (UV-vis) of G65Y-Cu_BMb(+6) in both ferric and ferrous forms are similar to those of G65Y-Cu_BMb (Figure S2a), and the EPR spectrum of ferric G65Y-Cu_BMb(+6) is similar to that of G65Y-Cu_BMb (Figure S3), suggesting that the mutations cause minimal perturbation to the overall structure and the structure of the heme active site.

To determine the oxidase activity measured by the rate of O_2 reduction, we used an O_2-selective electrode commonly used in monitoring 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 of cyt b_5, the concentration of O_2 in the presence of 50 nM G65Y-Cu_BMb(+6) decreased rapidly (Figure 1b), with the rate of the O_2 reduction being 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_BMb variants.[4] The activity decreased with higher ionic strength (Figure S4), suggesting that the ionic strength affects the electrostatic interaction between Mb and cyt b_5, which is consistent with those reported by Hoffman and coworkers in the similar system.[16] Therefore the low ionic strength (5 mM) was used in this study. Similar to G65Y-Cu_BMb,[4] which has been shown to mimic copper-independent cyt *bd* oxidase,[17] addition of copper to G65Y-Cu_BMb(+6) did not increase oxidase activity.[4] As a result, no copper was added in the reaction. In contrast, the G65Y-Cu_BMb without the D44K/D60K/E85K mutations exhibited a rate of 0.3 s^{-1} under the same condition. Furthermore, the protein without the G65Y mutation (Cu_BMb(+6)) also exhibited a low O_2 reduction rate 8.1 s^{-1}, which affirms the importance of the G65Y mutation in oxidase activity.[4] Remarkably, the O_2 reduction rate of G65Y-Cu_BMb(+6) (52 s^{-1}) is so fast that it is similar to that of native cbb_3 oxidase (50 s^{-1}) measured under the same condition.

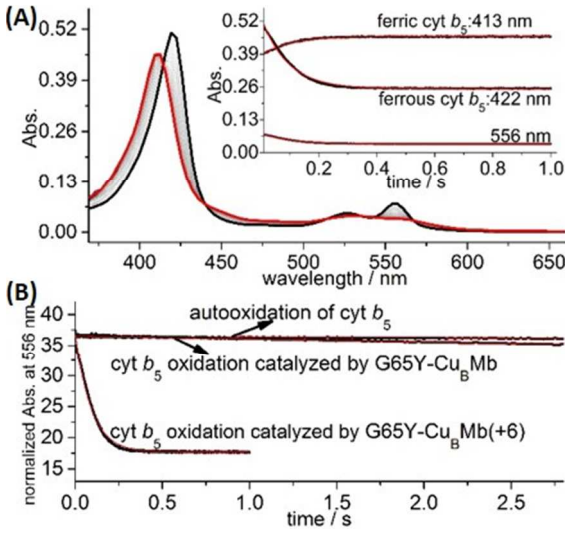

Figure 2. ET studies between cyt b_5 and G65Y-Cu_BMb and G65YCuBMb(+6). (a) Representative stopped-flow UV-visible spectra of cyt b_5 oxidation catalyzed by G65Y-Cu_BMb(+6) and time trace at characteristic wavelength (inset). (b) Time trace of cyt b_5 oxidation represented by absorption at 556 nm, overlaid with global spectroscopic fitting (red lines).

To confirm that the dramatic increase in the oxidase activity of G65Y-Cu_BMb(+6) is due to the 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_BMb(+6), suggesting 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_BMb or G65Y-Cu_BMb(+6) through stopped-flow UV-vis spectroscopy, in order to estimate the ET rate between cyt b_5 and the two Mb variants (Figure 2 and Figure S6). We first found that the pseudo first order ET rate constants between 5 μM cyt b_5 and G65Y-Cu_BMb(+6) increased linearly as the concentration of G65Y-Cu_B(+6) increased (Figure S7),[13] consistent with bimolecular reactions as observed previously. Then we used the same concentration of cyt b_5 (0.5 μM) as in the oxidase activity assay, but we increased concentration of Mb variants from 50 nM to 0.5 μM, which is still a fraction of cyt b_5. The pseudo first order rate constant between cyt b_5 and G65Y-Cu_BMb(+6) was determined as (1.2 ± 0.2) x 10^1 s^{-1}, which is ~400 fold faster than that of G65Y-Cu_BMb ((3.0 ± 0.5) x 10^{-2} s^{-1}). This 400-fold increase is in the same order of magnitude as those reported by Hoffman and coworkers (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_BMb(+6) and G65Y-Cu_BMb were determined to be (115 ± 11) and (129 ± 5) mV by spectroelectrochemical methods (Figure S8). Since the reduction potentials of both 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_BMb(+6), not due to a difference in ET driving force.

To investigate whether the O_2 reduction in the Mb variant is a $4e^-$ process, as in native oxidases, we measured H_2O_2 produced from the reaction using an amperometric H_2O_2 sensor. Under the same conditions as in the O_2 reduction experiment shown in Figure 1b, the G65Y-Cu_BMb(+6) generates peroxide at 3.5 s^{-1} (Figure 3a), or 7% of total O_2 reduced, while the G65Y-Cu_BMb produced 22% of peroxide (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 over 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 by absorption at 352 nm (see Figure 3b).[18] The rate of the reaction is compared to that of O_2 reduction using an O_2-selectvie electrode under the same conditions as in Figure 3b. As shown in Figure 3b (inset), the ratio between the rate of NADH oxidation and that of O_2 reduction is (1.91 ± 0.05) for G65Y-Cu_BMb(+6). This result is consistent with the low percentage of H_2O_2 directly probed by the above electroanalytical method. Together, both results strongly suggest that the O_2 reduction carried out by the G65Y-Cu_BMb(+6) is a $4e^-$ process producing H_2O like in native oxidases. Finally, 50 nM G65Y-Cu_BMb(+6) was able to reduce 220 μM of O_2 in the reaction system (Figure 1b), corresponding to > 4,400 turnovers.

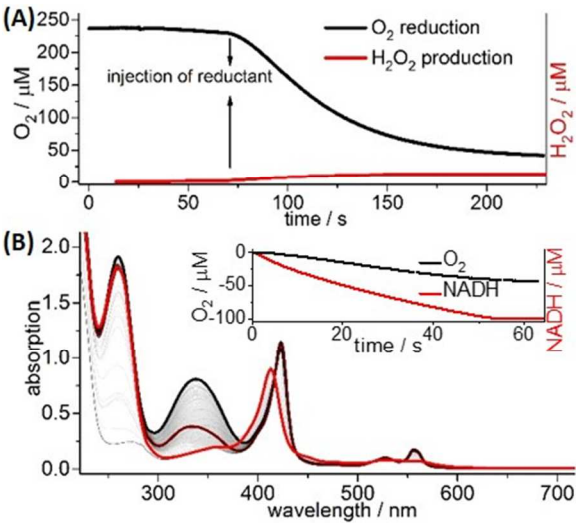

Figure 3. (a) Measurement of O_2 reduction and H_2O_2 production in the O_2 reduction reaction. (b) Representative kinetic UV-visible spectra of reaction solution with 100 μM NADH, sampled at 0.5 s interval over 100 s. NADH oxidation and oxygen reduction catalyzed by G65Y-Cu_BMb(+6) (inset).

Rational design of proteins that mimic native enzymes both structurally and functionally is both exciting and challenging because nature has millions of years to evolve enzymes to carry out complex reactions like O2 reduction. Therefore, any progress in protein design that moves closer to properties of native enzymes is encouraging, with the ultimate goal being to match the activity of native enzymes while using the model 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 coworkers indicated the internal ET rate accounts for the turnover number, and both ET rate and turnover number display the same pH and temperature dependence.[19] Another report by Fabian and coworkers provided evidence that the rate-limiting step is the initial electron transfer to the catalytic site.[20] Our results here using biosynthetic models strongly suggest that ET rate into the active site plays a critical role in increasing 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, being able to design biosynthetic models that match the O2 reduction

activity of native enzymes may open a new avenue to explore application 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 rates through engineering 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 proteins that match the activities of native enzymes, we have obtained deeper understanding of structural features important for oxidase activity, which may allow engineering artificial enzymes for biochemical and biotechnology applications such as more efficient ORR catalysts for biofuel cells.

ASSOCIATED CONTENT

Supporting Information

Experimental detail of protein expression, purification and spectroscopic characterization is described as supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

We gratefully acknowledge the US National Institutes of Health (GM06221) to Y.L, Major State Basic Research Program of China (2015CB856203), National Science Foundation of China (21325211, 91313301, and 81302687), Tianjin Municipal Grant (14ZCZDSY00059, 14JCYBJC43400) and Innovation Fund For Technology Based Firms (14C26211100178) to J.W.. National Science Foundation of China (31270859), and the Youth Innovation Promotion Association of Chinese Academy of Sciences to X.L.. We also thank Prof. Robert B. Gennis and Prof. Alexander Scheeline for helpful discussion, Dr. Hanlin Ouyang for providing native cyt cbb_3 oxidase for control experiments, and Mr. Evan Mirts for proof reading.

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