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Systematic Tuning of Heme Redox Potentials and Its Effects on O_2 Reduction Rates in a Designed Oxidase in Myoglobin

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Supporting Information

ABSTRACT: Cytochrome *c* Oxidase (C*c*O) is known to catalyze the reduction of O_2 to H_2O efficiently with a much lower overpotential than most other O_2 reduction catalysts. However, methods by which the enzyme fine-tunes the reduction potential ($E°$) of its active site and the corresponding influence on the O_2 reduction activity are not well understood. In this work, we report systematic tuning of the heme $E°$ in a functional model of C*c*O in myoglobin containing three histidines and one tyrosine in the distal pocket of heme. By removing hydrogen-bonding interactions between Ser92 and the proximal His ligand and a heme propionate, and increasing hydrophobicity of the heme pocket through Ser92Ala mutation, we have increased the heme $E°$ from 95 ± 2 to 123 ± 3 mV. Additionally, replacing the native heme *b* in the C*c*O mimic with heme *a* analogs, diacetyl, monoformyl, and diformyl hemes, that posses electron-withdrawing groups, resulted in higher $E°$ values of 175 ± 5, 210 ± 6, and 320 ± 10 mV, respectively. Furthermore, O_2 consumption studies on these C*c*O mimics revealed a strong enhancement in O_2 reduction rates with increasing heme $E°$. Such methods of tuning the heme $E°$ through a combination of secondary sphere mutations and heme substitutions can be applied to tune $E°$ of other heme proteins, allowing for comprehensive investigations of the relationship between $E°$ and enzymatic activity.

Developing catalysts for fuel cells is a major focus of the current search for alternative energy sources, including catalysts for O_2 reduction, which remains the least efficient part of the fuel cells.[1a] The catalysts currently used for this reaction use precious platinum possessing relatively high overpotentials, which renders the catalyst energy inefficient. Thus, a major challenge in this area is to design efficient catalysts for O_2 reduction that use earth-abundant metal ions and employ low overpotentials.[1b,c] In a recent DFT study, Kjaergaard et al. developed a method for direct comparison between the best platinum-based catalysts with cytochrome *c* oxidase (C*c*O),[1d] a biocatalyst that also reduces O_2 to H_2O under physiological conditions,[2] and concluded that C*c*O is a better catalyst in terms of possessing much lower overpotential than current platinum-based catalysts. In addition, the C*c*O uses earth abundant metal ions (iron and copper) for catalysis. However, C*c*O is a large, (e.g., MW ≈ 200 kDa for bovine C*c*O) membrane protein that is not very stable and has much lower site density on electrochemical surfaces than small molecular catalysts.[3] Therefore, an active area of research is to make biomimetic models of C*c*O that are much smaller, robust, and efficient in O_2 reduction.[4]

The catalytic site of C*c*O, where O_2 binding, activation, and reduction occurs, is a binuclear heme-copper center, which consists of a high spin heme and a copper (Cu_B) coordinated to three histidines, one of which is cross-linked to a tyrosine residue.[5a,b] The thermodynamic efficiency of O_2 reduction reaction in C*c*O's is determined by the reduction potential ($E°$) of the catalytic heme iron.[2d] In order to maximize the energy efficiency of this reaction and reduce its overpotential, it is desirable to tune the heme $E°$ such that it is as high and as close to that of O_2 (810 mV at pH7 vs SHE; all $E°$ values reported in this work are also vs SHE).[1a] However, while the $E°$ of the heme (Fe^{3+}/Fe^{2+} couple) is known to vary from −59 mV in *cbb*$_3$ oxidase to +365 mV in bovine heart C*c*O, the structural features responsible for such tuning of the $E°$ is not well understood.[5c−e] In addition, while the role of heme $E°$ in controlling electron transfer rates has been investigated extensively,[5f,g] its impact on O_2 reduction reactions, as in the case of C*c*Os, remains unexplored. Understanding how the heme $E°$ in C*c*Os is tuned is important not only for designing efficient O_2 reduction catalysts with a lower overpotential but also for gaining insight into how different oxidases are able to tune their heme $E°$'s to match with those of their redox partners so that the electron flow is in the right direction for efficient O_2 reduction. A slight change of heme $E°$ in terminal oxidases could potentially reverse the electron transfer direction, resulting in no or partial O_2 reduction. Such investigations are difficult in the native C*c*O due to the presence of multiple metal cofactors, which renders the spectroscopic and electrochemical characterization of the heme-copper center challenging. To overcome these difficulties, synthetic models have been made.[4] However, to our knowledge, no study has investigated ways to fine-tune the $E°$ of the catalytic heme and its effects on O_2 reduction activity in C*c*O or its synthetic models.

In a complementary approach to studying native C*c*Os and synthetic models, we have engineered a heme-copper center in a much smaller (MW = 17.4 kDa) O_2-binding protein sperm whale myoglobin (called Cu_BMb),[6a,b] and showed that the presence of a Tyr in the active site through F33Y mutation (called F33Y-Cu_BMb) resulted in a catalyst that reduces O_2 to

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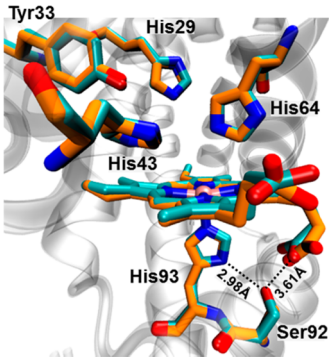

Figure 1. Overlay of the X-ray crystal structures of F33Y-Cu_BMb (4FWX, cyan) and S92A-F33Y-Cu_BMb (4TYX, orange). Heme *b* and side chains of His29, His43, His64, Tyr33, His93, and Ser/Ala92 are shown in licorice. Ser92 forms H-bonds with heme propionate and His93.

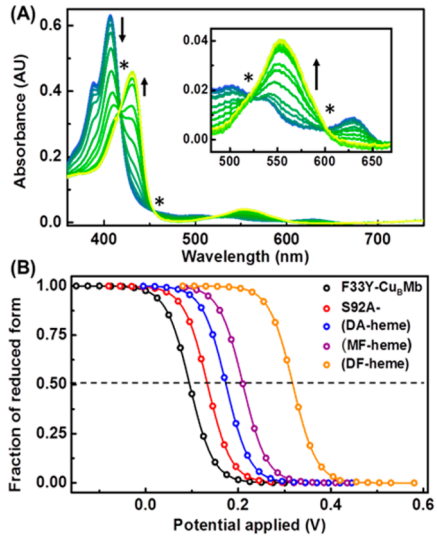

Figure 2. (A) Spectra obtained for F33Y-Cu_BMb starting from the oxidized form (green) going to the reduced form (yellow) in 100 mM phosphate buffer, pH6. Isosbestic points are indicated by a star (*). Inset shows a magnified view of the same spectra in 500−650 nm wavelength range. (B) Nernst fit of the spectral plot for F33Y-Cu_BMb and its variants. Dotted lines represent the point where the fraction of protein reduced is equal to that oxidized and the corresponding potential value is the $E°$ of the protein.

H_2O with hundreds of turnovers and minimal release of other reactive oxygen species (ROS) (Figure 1).[6c−e] In addition to being smaller, this functional mimic of C*c*O in Mb is much easier to purify and is free of other metal-binding sites as in C*c*O and, thus, represents an ideal candidate for investigating how to tune the $E°$ of heme in a C*c*O-like environment and elucidating its impact on the O_2 reduction rates. Herein, we report the successful tuning of heme $E°$ in F33Y-Cu_BMb either through introducing a hydrophobic residue that perturbs hydrogen bonding (H-bonding) interactions in the proximal side of heme or by replacing the native heme *b* with heme *a* analogs with high $E°$, resulting in O_2 reduction catalysts with $E°$ values within ∼215 mV range. Additionally, we have also obtained a direct correlation between heme $E°$ and oxidase activity of the C*c*O mimics.

The F33Y-Cu_BMb was expressed and purified without a copper ion at the Cu_B site.[6c−e] No copper ion was added in this work, as previous studies have shown that the presence of copper has little influence on the oxidase activity of F33Y-Cu_BMb,[6c−e] similar to the Cu_B-independent cytochrome *bd* oxidase.[2c] The $E°$ of F33Y-Cu_BMb was determined using a UV−vis spectroelectrochemical method as described previously;[6f] such a method was shown to be able to maintain the integrity of the protein while providing the benefit of direct monitoring of spectral transition from the oxidized form of the enzyme (Fe^{3+}) to its reduced form (Fe^{2+}) upon sweeping the potential (Figure 2A). The $E°$ of F33Y-Cu_BMb was obtained by global fitting of the spectral transition to the Nernst equation (black curve, Figure 2B; see Supporting Information (SI) for details). The $E°$ of 95 ± 2 mV determined from this process lies between the −59 mV in cbb_3 oxidase and +365 mV in bovine C*c*O. This finding motivated us to explore ways to increase $E°$ of F33Y-Cu_BMb to match that of C*c*O in order to lower the overpotential for the O_2 reduction reaction.

A previous study has shown that replacing the neutral Val68 residue in the distal pocket of human Mb with negatively charged Glu or Asp can decrease the $E°$ by up to ∼200 mV.[7a] Since this work, most mutations in Mb have resulted in a change of $E°$ of much less than 200 mV; even fewer reports of mutations have been shown to increase the $E°$.[7b−d] More importantly, since the distal pocket plays a critical role in binding and reducing O_2, it is even more difficult to tune the $E°$ without affecting its oxidase activity. Under such constraints, we directed our attention to modifications on the proximal side of heme. Previous studies have demonstrated that increasing the hydrophobicity and tuning H-bonding around the secondary coordination sphere of the metal-binding site can increase the $E°$ of metalloproteins.[7e] Applying the same methodologies toward Mb, we began to look for possible sites to alter on the proximal side of heme. NMR studies on WTMb have suggested that the Nε proton of the proximal His93 ligand forms a weak H-bond with the lone pair of Ser92 (Figure 1).[7f,g] Therefore, disrupting this H-bond may increase the positive character of the Nε proton of His93, thereby reducing the overall electron donating ability of the imidazole ring towards the heme iron, leading to an increase in the heme $E°$. Based on this hypothesis, we replaced the Ser92 with Ala, which being more hydrophobic may also raise the heme $E°$ through preferential stabilization of Fe^{2+} over Fe^{3+}.[7e] The UV−vis spectra of both oxidized and reduced S92A-F33Y-Cu_BMb are very similar to those of F33Y-Cu_BMb (Table S1), suggesting that the S92A mutation exerted minimal perturbation on the protein structure. A 1.64 Å resolution crystal structure of this mutant indicates that the S92A mutation did indeed eliminate the H-bonding interactions between Ser92, His93, and heme propionate, resulting in the negatively charged propionate moving away from the mutation site of Ser92 and heme iron (Figures 1, S1). The $E°$ of S92A-F33Y-Cu_BMb was found to be 123 ± 3 mV (Figures 2B, S2), suggesting that introducing the hydrophobic residue and the associated changes in the H-bonding interactions are responsible for the observed enhancements in the heme $E°$. This finding correlates well with a recent study in cbb_3 oxidase, in which perturbing H-bonding interactions of proximal histidine ligand of catalytic heme b_3 lead to a variation in $E°$ values by ∼40 mV.[5c]

While the increase of $E°$ from 95 to 123 mV is encouraging, it is still much less than the 365 mV of bovine C*c*O. To increase the $E°$ further, we noticed a major difference between the heme in our protein model in Mb and that in bovine CcO: while heme *b* is present in our Mb model system, various A- and B-

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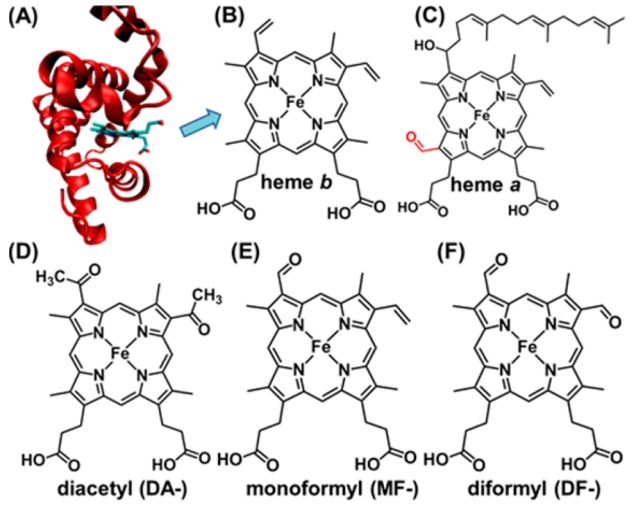

Figure 3. (A) Protein scaffold of F33Y-Cu_BMb. (B) Heme *b* cofactor present in F33Y-Cu_BMb. (C) Heme *a* present in the catalytic site of bovine C*c*O. (D) Diacetyl heme (E) Monofomyl heme and (F) Diformyl heme incorporated in F33Y-Cu_BMb apo-protein.

type C*c*Os with high $E°$ values use heme *a* (Figure 3).[5e] The presence of an electron-withdrawing formyl group conjugated to the porphyrin ring in heme *a* can destabilize the oxidized form of the heme and thus increase the heme $E°$.[8a,b] For example, Gibney and co-workers replaced the heme *b* in a *de novo* designed α-helical bundle protein with heme *a* and observed an increase in $E°$ value by 157 mV.[8b] While this result is exciting in the *de novo* designed protein, replacing the heme *b* with heme *a* in Mb resulted in a misfolded protein,[8c] due to the lack of space in the Mb to accommodate the C14 farnesyl chain (Figure 3).

Based on the above comparison and findings, we embarked on a systematic study of increasing heme $E°$ by using a different strategy of replacing the native heme *b* in Mb with heme *a* analogues of high $E°$. Mb is an ideal system for such a strategy because the heme *b* can be readily extracted and replaced with non-native heme cofactors of similar structures and sizes.[9a,b] We therefore decided to replace heme *b* in F33Y-Cu_BMb with diacetyl (DA-), monoformyl (MF-), and diformyl (DF-) derivatives of heme that are similar in structure to that of heme *b* in Mb but resemble heme *a* in electronic properties (Figure 3). The extraction of heme *b* and incorporation of the above three heme analogs into F33Y-Cu_BMb were carried out using the protocol reported previously with minimal modification (see SI).[9c] The UV−vis absorption spectra of the three F33Y-Cu_BMb variants reveal an intense Soret band around 400 nm, along with α and β transitions in the visible region (Table S1), which are typical of the oxidized form of Mb. The secondary structures of the heme substituted proteins were confirmed by circular dichroism spectra, which showed minima at 222 and 209 nm, consistent with well folded α helical protein containing native heme *b* (Figure S3).[9d]

Having confirmed that the F33Y-Cu_BMb variants were well-folded in the presence of non-native heme cofactors, we carried out UV−vis spectroelectrochemical measurements to determine their $E°$'s. The spectroelectrochemical reduction of each of these three variants exhibited a clean transition from its oxidized to reduced form (Figure S2), and the global fit of data to the Nernst equation (Figure 2B) revealed $E°$ values of 175 ± 5, 210 ± 6, and 320 ± 10 mV for F33Y-Cu_BMb (DA-heme), (MF-heme), and (DF-heme), respectively. Thus, by incorporation of non-native heme cofactors, we have been able to increase the $E°$ for F33Y-Cu_BMb by approximately 80, 115, and 215 mV respectively, with the $E°$ of the F33Y-Cu_BMb with DF-heme (320 ± 10 mV) being very close to that of bovine C*c*O (365 mV).

Finally, having achieved rational tuning of the heme $E°$ in F33Y-Cu_BMb, we explored the effect of heme $E°$ on its oxidase activity. The rates of O_2 reduction by these F33Y-Cu_BMb variants in the presence of the reductant ascorbate ($E°$ = 96 mV) and redox mediator, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) ($E°$ = 270 mV) were measured using an O_2 electrode and a protocol reported previously for both native C*c*O's and their models.[6d,e] For the oxidase to function efficiently, it is required to perform a complete $4e^-$ reduction of O_2 into H_2O; any incomplete reduction, such as $1e^-$ or $2e^-$ reduction of O_2 to superoxide (O_2^-) or peroxide (O_2^{2-}) will result in not only lower efficiency in O_2 reduction, but also ROS that is detrimental to the biomolecules in living cells or components in fuel cells. Therefore, we measured the ratio of H_2O formation with respect to O_2^- and O_2^{2-} as previously reported,[6d,e] by repeating the O_2 reduction experiments in the presence of superoxide dismutase and catalase, which selectively react with O_2^- and O_2^{2-}, respectively (see SI for details). Interestingly, the oxidase activity of the F33Y-Cu_BMb variants increase with increasing $E°$ of the heme, while the ratio of H_2O produced with respect to the ROS remains almost constant (Figure 4A). The F33Y-Cu_BMb (DF-heme), with an $E°$ value of 320 mV, exhibits an over 5-fold increase in oxidase activity as compared to F33Y-Cu_BMb with heme *b*. These results strongly suggest that increasing the heme $E°$ makes the heme iron a better acceptor of electrons from the redox mediator, resulting in a higher driving force for a faster O_2 reduction reaction.

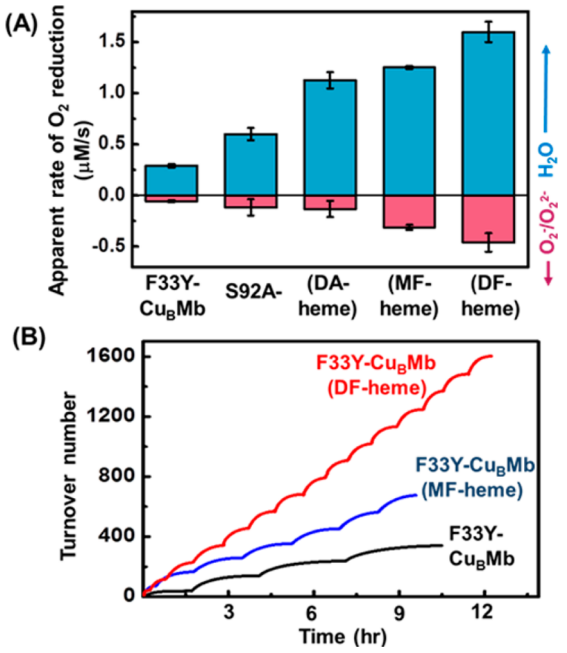

Figure 4. (A) Rates of O_2 reduction to form either H_2O (blue) or ROS (red) catalyzed by 6 μM F33Y-Cu_BMb variants in 100 mM phosphate buffer (pH6) containing 258 μM O_2, 1 mM TMPD, and 10 mM ascorbate. (B) O_2 reduction turnover measured during the stepwise addition of O_2 for F33Y-Cu_BMb and its (MF-heme) and (DF-heme) variants.

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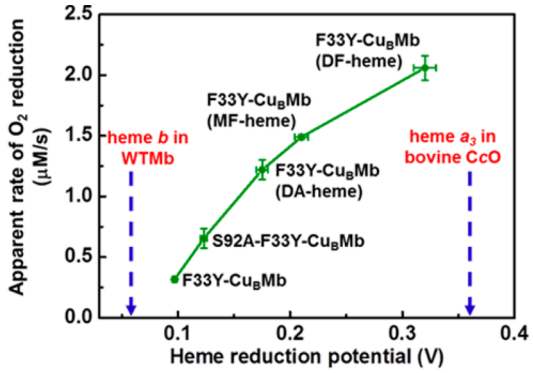

Figure 5. Variation of O_2 reduction activity with heme $E°$ for F33Y-Cu_BMb variants. Dotted blue line indicates $E°$ of WTMb and bovine CcO.

Furthermore, all the three tested enzymes (F33Y-Cu_BMb, F33Y-Cu_BMb (MF-heme), and F33Y-Cu_BMb (DF-heme)) are capable of performing hundreds of turnovers, with the more active enzyme possessing the highest $E°$ (F33Y-Cu_BMb (DF-heme)), performing more than 1000 turnovers under similar conditions and over the same time (Figure 4B). In control experiments, we also replaced the heme *b* in WTMb with DA-heme and DF-heme and found that such a replacement increased the $E°$ value of WTMb from 61 ± 2 mV to 154 ± 3 mV and 268 ± 7 mV respectively (Figure S5). Thus, the increase in heme $E°$ in WTMb shows parallel results with the F33Y-Cu_BMb mutant. Interestingly, even though the increase in heme $E°$ leads to an increase in overall O_2 reduction rates for both WTMb and F33Y-Cu_BMb, the percentage of complete O_2 reduction to H_2O shows opposite trends. While, for WTMb variants, higher heme $E°$ results in higher ROS formation, for F33Y-Cu_BMb variants, the higher heme $E°$ results in more H_2O formation (Figure S5). This comparison strongly suggests the importance of the H-bonding network formed by distal mutations (that form the Cu_B site and Tyr) and associated H_2O molecules (Figure S1, crystal structure of F33Y-Cu_BMb) to control the protonation/deprotonation of O_2 for its reduction to H_2O.

In summary, we have demonstrated effective methods to systematically increase the $E°$ of the heme in a functional model of CcO in myoglobin, by up to ~215 mV. The methods include removing H-bonding interactions and increasing hydrophobicity in the proximal side of the heme and replacing the native heme *b* with heme *a* analogs that possess electron-withdrawing groups that possess higher $E°$. A correlation of the heme $E°$ with the O_2 reduction activity is also established (Figure 5). The methods of tuning $E°$ shown in this study can be applied to other heme proteins allowing systematic investigation of the relationship between heme $E°$ and other enzymatic activities.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, spectroelectrochemical measurements, and X-ray crystallography of F33Y-Cu_BMb and its variants. 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 interest.

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