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A Designed Functional Metalloenzyme that Reduces O₂ to H₂O with Over One Thousand Turnovers

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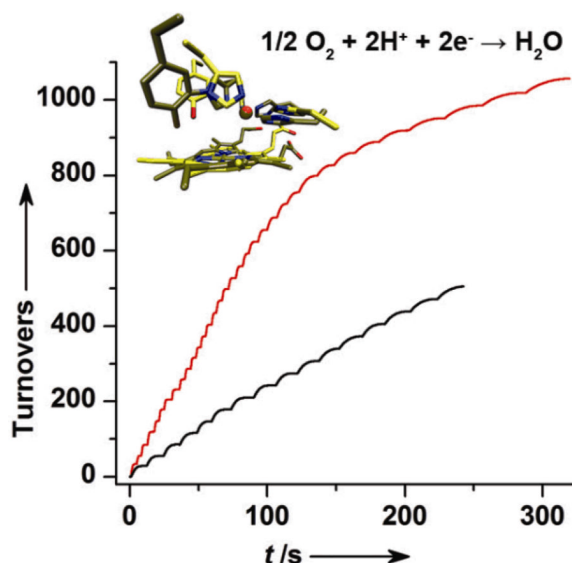
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Summary

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Rational design of functional enzymes with high turnovers is a significant challenge, especially those with complex active site and difficult reactions, such as in respiratory oxidases. Introducing 2 His and 1 Tyr into myoglobin resulted in designed enzymes that reduce O₂ to H₂O with > 1000 turnovers and minimal release of reactive oxygen species. This also showed that presence and positioning of Tyr, not Cu, are critical for activity.

Keywords

Heme Copper Oxidases; Metalloenzymes; Metalloprotein Design; Oxidoreductases; Protein Design

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 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 numbers of turnovers is not only an important measure of success in the field, but can also reveal structural features responsible for tuning enzymatic activities and may result in artificial enzymes for practical applications. A primary 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 O₂ to water and, in doing so, generate the transmembrane proton gradient that drives important processes such as ATP synthesis. While many catalysts that reduce oxygen to water have been reported^[5], a long-standing challenge is to carry out the reaction without the production of reactive oxygen species (ROS), such as superoxide and peroxide, under mild conditions. ROS not only damage biomolecules in cells and components in fuel cells, but also decrease energy efficiency, as ROS are a result of incomplete catalysis. In addition, efficient catalysts using non-precious metal ions such as iron or copper will greatly decrease costs in practical applications^[6]. Therefore, designing enzymes that catalyse complex reactions, such as oxygen reduction, at mild pH that are based on small, stable, and easy to produce scaffold proteins, would yield ideal models for studying the fundamentals of this chemistry and produce useful catalysts for applications such as fuel cells.

In HCOs the active site responsible for O₂ reduction is a heterobinuclear metal center containing a heme-Fe and a His-ligated Cu (Cu_B). We have previously reported the introduction of two histidines in the heme pocket of sperm whale myoglobin (swMb) through Leu29His and Phe43His mutations^[7], which, together with the native distal His64 formed a copper binding site (this mutant is called Cu_BMb, see Supporting Information, Figure S1A). As purified, Cu_BMb has no metal in the Cu_B site (designated as E-Cu_BMb; i.e. 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 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 O₂ to water. Herein, we report the introduction of a Tyr 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 O₂ to water, one with over 1000 turnovers, and with minimal release of superoxide or peroxide. These results demonstrate that both the presence and positioning of the tyrosine is 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 (PDB 1V54) (see Supporting Information, Figure S1A) and this structure was used to guide the placement of Tyr in the active site pocket^[11]. 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 via a Phe33Tyr mutation (called F33Y-Cu_BMb). The crystal structure of E-F33Y-Cu_BMb shows excellent agreement with the computer model (Figure S1B). 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 1A). To find a better structural overlay, a recent crystal structure of a *cbb*₃ HCO^[10] revealed that the Tyr can be near the His ligand spatially, while not necessarily close in the primary sequence. Therefore, we alternatively modelled Tyr next to His29 through a Gly65Tyr mutation (called G65Y-Cu_BMb). The energy-minimized computer model of E-G65Y-Cu_BMb overlays very well with that of *cbb*₃ HCO (Figure 1B).

The spectral properties of oxidized and reduced states of these variants are similar to those of wild type swMb (WTswMb)^[12] (Supporting Information, Figure S4). However, upon exposure of the ferric- states 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 to deoxy was observed (Supporting Information, Figure S4E–F), which is in contrast to WTswMb (Supporting Information, Figure S4D), and prompted an investigation of the product of this reduction.

To confirm that the product of oxygen reduction is water, ¹⁷O NMR was carried out. Production of H₂¹⁷O above the background level (natural abundance) of H₂¹⁷O in water was monitored in a sealed vessel containing ¹⁷O₂, the myoglobin variant, and excess reductant, using ¹⁷O-labeled Tyr as an external standard. A typical ¹⁷O NMR spectrum is shown in Supporting Information, Figure S5. Figure 2 shows the ratios of the H₂¹⁷O peak area to ¹⁷O-Tyr peak area at various time points after addition of ¹⁷O₂ to initiate its reduction by WTswMb, E-F33Y-Cu_BMb, and E-G65Y-Cu_BMb, normalized to the area of the peak before initiating the reaction. The normalized ratio of water in the WTswMb sample remains close to 1, suggesting that the protein did not produce any new H₂¹⁷O. In contrast, E-F33Y-Cu_BMb and E-G65Y-Cu_BMb produced up to 10 mM H₂¹⁷O at 120 min, as indicated by an increase in the normalized ratio, confirming the production of water.

The rates of oxygen reduction were measured quantitatively using an O₂ electrode to monitor the concentration of O₂ over time in the presence of reductant, similar to protocols reported for native HCO^[13]. The rate of O₂ disappearance was measured for WTswMb and the Cu_BMb variants (Supporting Information, Figure S6). A hallmark of native terminal oxidases is the clean reduction of O₂ to water with minimal release of superoxide or peroxide^[14]. To identify the product (O₂⁻, O₂²⁻, or H₂O) we employed superoxide dismutase (SOD) and catalase which selectively react with superoxide and peroxide, respectively, producing O₂ as one of the 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 O₂ reduction due to water formation (in blue) and due to superoxide or peroxide formation (in red) can be calculated (see Figure 3A, and Supporting Information Table S5 and Supporting Text). Not surprisingly, most of the O₂ consumption rate by WTswMb is due to superoxide or peroxide formation, consistent with autoxidation^[15] and these rates remain unaffected in the presence of Cu²⁺, Zn²⁺ or Ag⁺. Interestingly, introducing two histidine residues 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 overall rate of O₂ consumption. Similar to WTswMb, addition of Cu²⁺, Ag⁺ or Zn²⁺ to E-Cu_BMb does not perturb the rate and product of O₂ 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 O₂ consumption. The rate of water production increases up to 4.2 and 1.1 μM/s when using 18 μM E-G65Y-Cu_BMb and E-F33Y-Cu_BMb, respectively. 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 O₂ reduction to water formation instead of release of superoxide or peroxide. Under further optimized conditions (with a higher concentration of reductant), the rate of O₂ reduction to water by E-G65Y-Cu_BMb is 28 min⁻¹ (Supporting Information, Figure S10) – within ~150 fold of native HCOs^[16]. Additionally, we show in Supporting Information, 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 O₂ reduction activity is independent of the presence and identity of the metal in the engineered Cu_B site (Figure 3A). To further support this finding, we repeated the studies with varying equivalents of copper (0–2 equivalent) as well as a strong metal chelator, ethylenediaminetetraacetate (EDTA), but no further change was noticed (Supporting Information, Figure S8). As E-G65Y-Cu_BMb is still ~150 fold lower in activity than HCOs,^[16] our results are not intended to rule out the role of copper ion native HCOs. Alternatively, these results do indicate that a copper center is not strictly necessary for oxygen reductase chemistry and are consistent with 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 or proposed to proceed by the critical steps 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 is also 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 with 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 (Supporting Information, 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-CuBMb and E-F33Y-CuBMb, 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 works to activate the ferric-superoxo state of our designed enzymes and this activation allows it to accept a second electron from the exogenous reductant towards complete reduction to water. Further studies are underway to elucidate the exact role of Tyr in our model protein system.

Finally, to test the extent of the functional activities, we have carried out multiple turnover reactions. To a solution containing E-F33Y-Cu_BMb or E-G65Y-Cu_BMb and excess reductant, ~500 μ M (28 equivalents) O₂ was added repeatedly and each time the total O₂ consumption was monitored using an O₂ electrode. These stepwise additions resulted in the multiple plateaus observed in Figure 3B. We calculate that E-F33Y-Cu_BMb and E-G65Y-Cu_BMb achieved >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 O₂ reduction to water formation. Furthermore, the positioning of this Tyr is critical for affecting the rate of catalysis. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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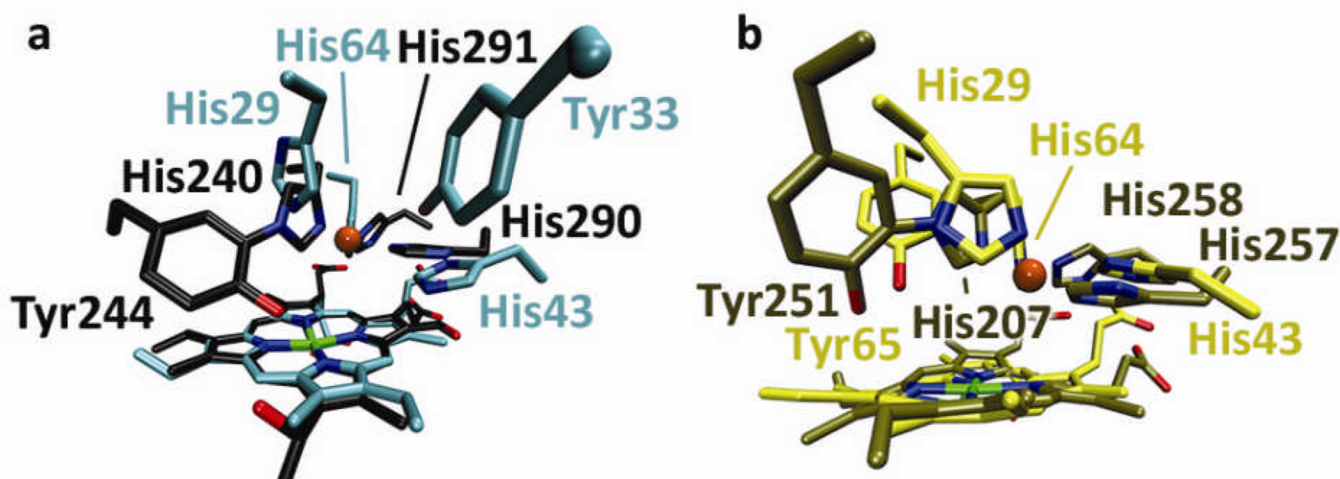


Figure 1.

Structures and computer models of native and designed oxidases. a) Overlay of the crystal structures of bovine CcO (grey) and E-F33Y-Cu_BMb (cyan); b) Overlay of the crystal structure of *cbb*₃ HCO from *Pseudomonas stutzeri*^[10] (tan) and E-G65Y-Cu_BMb computer model (yellow); The Cu_B copper in CcO is represented as an orange sphere.

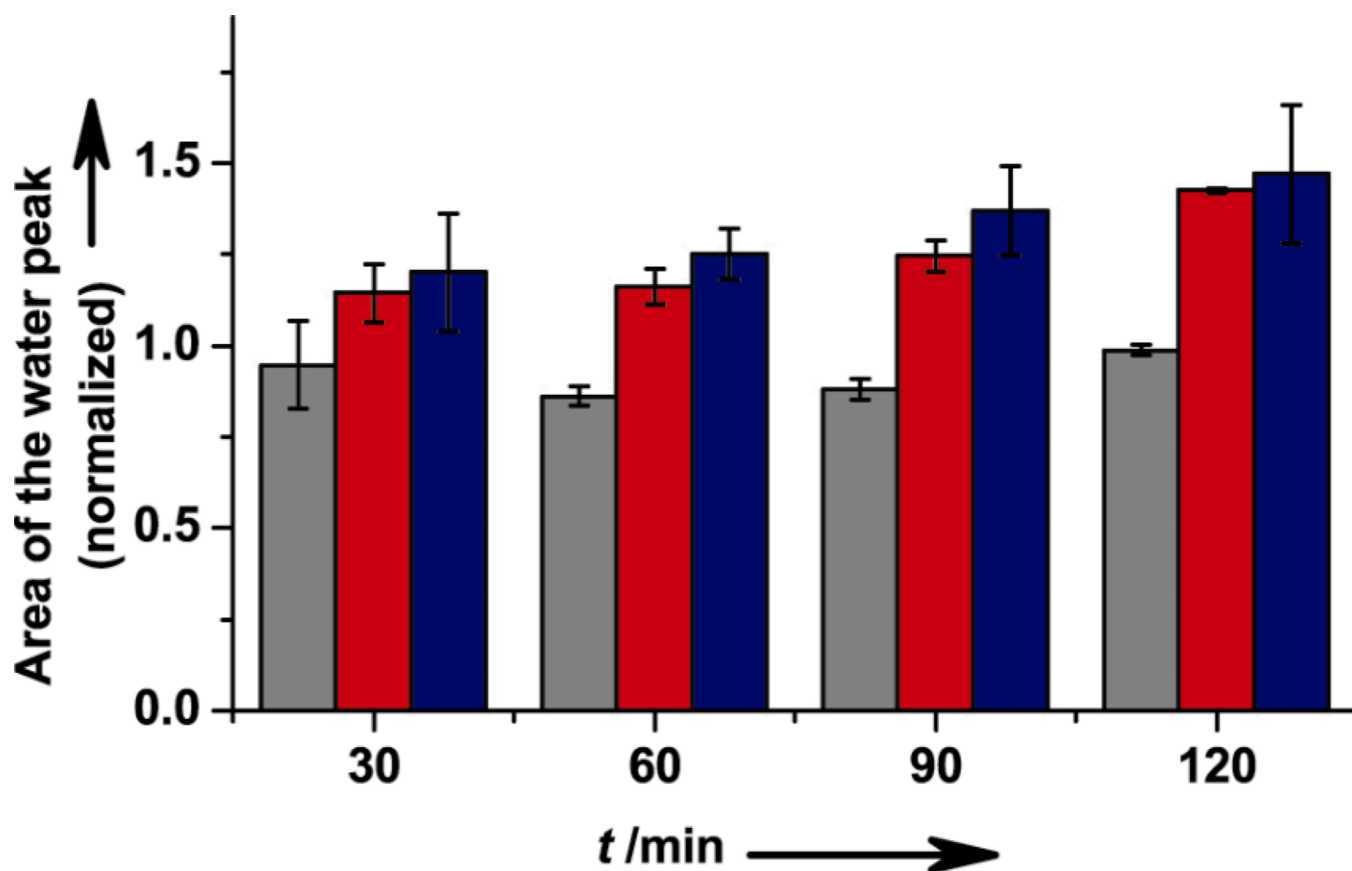


Figure 2. Characterization of product by ^{17}O NMR spectroscopy; normalized area of the H_2^{17}O peak for WTswMb (gray), E-F33Y-Cu_BMb (red) and E-G65Y-Cu_BMb (blue) at 30, 60, 90, and 120 minutes. Error bars indicate standard deviation.

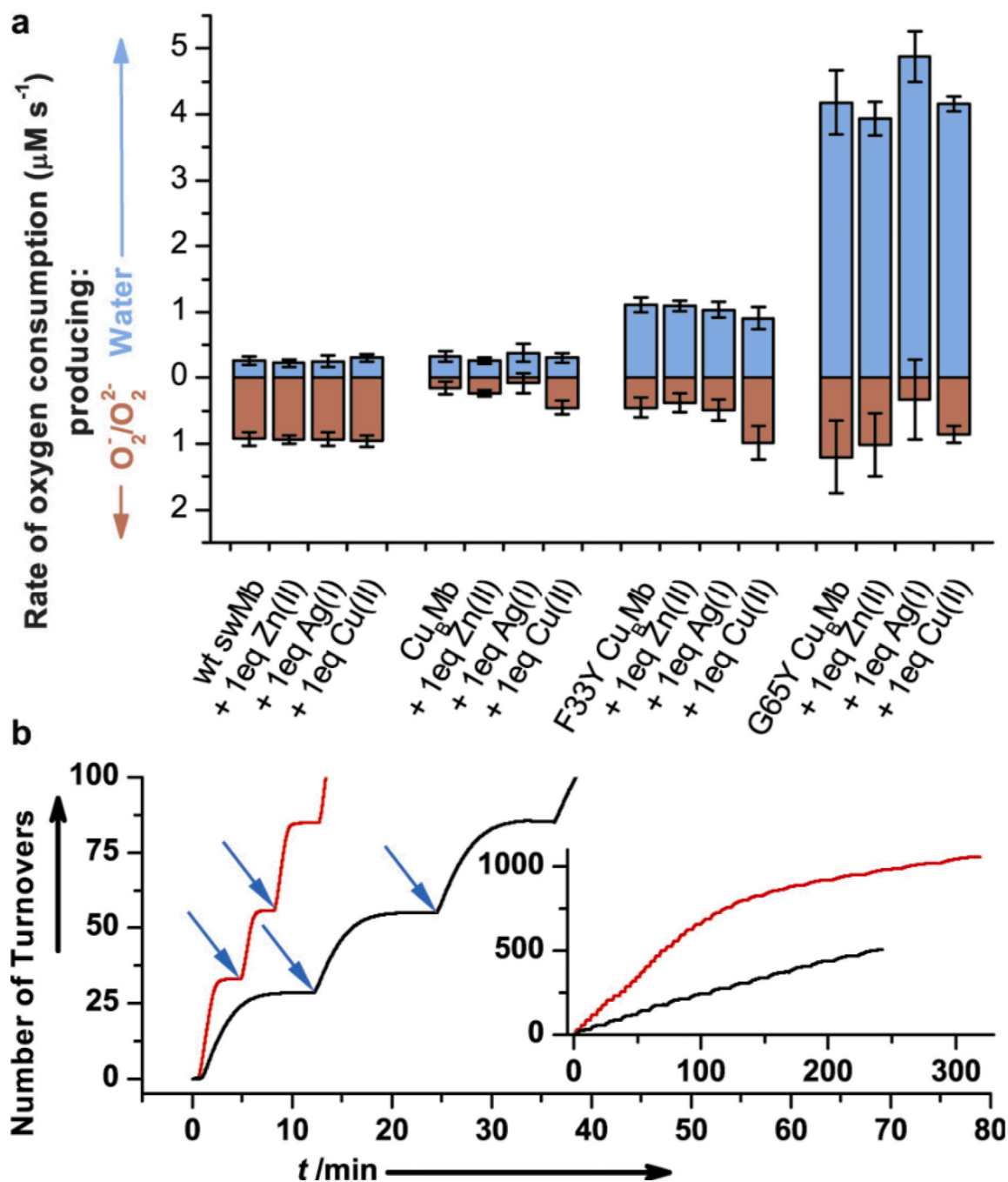


Figure 3.

Characterization of the O_2 reduction; a) Rates of oxygen reduction to form either water (blue) or superoxide/peroxide (red) with $18 \mu\text{M}$ 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 (red); blue arrow indicates addition of ~ 28 equivalents O_2 . Inset, region of high number of turnovers.