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Defining the Role of Tyrosine and Rational Tuning of Oxidase Activity by Genetic Incorporation of Unnatural Tyrosine Analogs

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S *Supporting Information*

ABSTRACT: While a conserved tyrosine (Tyr) is found in oxidases, the roles of phenol ring pK_a and reduction potential in O_2 reduction have not been defined despite many years of research on numerous oxidases and their models. These issues represent major challenges in our understanding of O_2 reduction mechanism in bioenergetics. Through genetic incorporation of unnatural amino acid analogs of Tyr, with progressively decreasing pK_a of the phenol ring and increasing reduction potential, in the active site of a functional model of oxidase in myoglobin, a linear dependence of both the O_2 reduction activity and the fraction of H_2O formation with the pK_a of the phenol ring has been established. By using these unnatural amino acids as spectroscopic probe, we have provided conclusive evidence for the location of a Tyr radical generated during reaction with H_2O_2, by the distinctive hyperfine splitting patterns of the halogenated tyrosines and one of its deuterated derivatives incorporated at the 33 position of the protein. These results demonstrate for the first time that enhancing the proton donation ability of the Tyr enhances the oxidase activity, allowing the Tyr analogs to augment enzymatic activity beyond that of natural Tyr.

Tyrosine (Tyr) is unique among all natural amino acids, as it is capable of donating both an electron and a proton in enzymatic reactions. As a result, a Tyr is found in the active sites of a number of enzymes such as galactose oxidases, ribonucleotide reductase, and cytochrome *c* oxidase (CcO).[1−8] For instance, Stubbe and Nocera et al. utilized a series of Tyr analogs as probes to study the proton-coupled electron transfer in enzymes using Tyr radicals.[9−12] However, the exact role of Tyr in many enzymatic activities remains to be understood. A primary example is the Tyr found in the active site of CcO.

As the terminal oxidase in the respiration chain, CcO catalyzes efficient reduction of O_2 to H_2O, harvests the released chemical energy, and converts it into the proton gradient that drives the synthesis of adenosine triphosphate, the energy form for most cellular processes.[13−16] This reaction requires rapid transfer of four electrons and four protons to molecular oxygen in a highly controlled manner in order to prevent the release of toxic reactive oxygen species (ROS), such as superoxide and peroxide, which not only damage proteins through radical reactions but also lower the energy conversion efficiency due to incomplete O_2 reduction.[8] Critical to controlling this important reaction is the presence of a highly conserved Tyr, found by X-ray crystallography to be adjacent and crosslinked to one of the histidine ligands to the Cu_B center.[17,18] It is suggested that this Tyr donates one electron and one proton during the reaction.[15,19] In addition, it is proposed that the pK_a of the phenol ring may play an important role in modulating oxidase activity,[8,20] but experimental support for such a proposal remains elusive despite decades of investigations on many oxidases and their variants. CcO is a large (∼200 kDa for bovine CcO) membrane protein consisting of many other metal-binding sites (e.g., Cu_A and heme *a* as electron donors) and tyrosines at other locations, making it difficult to focus on the O_2 reduction site and the conserved Tyr. More importantly, because mutations of this conserved Tyr to other amino acids abolished the oxidase activity,[21,22] it has not been possible to address the role of the phenol pK_a in oxidase activity at molecular details. These issues represent the remaining major challenges in our understanding of the oxidase mechanism in bioenergetics, and addressing them could lead to the design of more efficient biocatalysts for applications such as catalysts for fuel cells using earth abundant metal ions (instead of platinum) with low overpotentials.

In previous studies, we have prepared functional models of oxidases by introducing two histidines (His29 and His43) and one Tyr (Tyr33) into the distal pocket of sperm whale myoglobin (Mb) (Figure 1).[23−31] Together with the His64 present in Mb, this protein, called Phe33Tyr-Cu_BMb, mimics CcO not only structurally but also functionally, reducing O_2 to H_2O with more than 500 turnovers.[28] In contrast to CcO, the Phe33Tyr-Cu_BMb model is much smaller (18 kDa) and is free of other metal-binding sites, making it much easier to study the roles of the active site Tyr using common spectroscopic methods. The crystal structure of Phe33Tyr-Cu_BMb showed a water molecule (Wat1) in the heme pocket within 2.5 Å of the hydroxyl group of Tyr33 (Figure 1), which is potentially involved in the
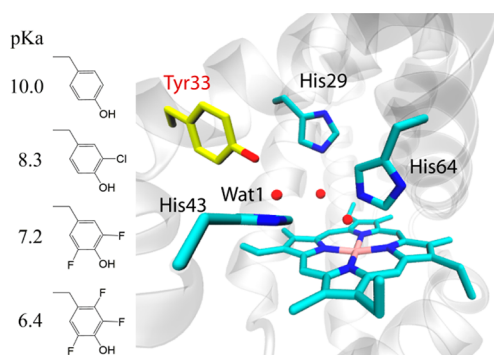
Figure 1. Crystal structure of the Phe33Tyr/Leu29His/Phe43His sperm whale myoglobin (Phe33Tyr-Cu$_B$Mb, PDB ID: 4FWX) and pK_a of Tyr and Tyr analogs. From top to bottom: Tyr, 3-chlorotyrosine (ClTyr), 3,5-difluorotyrosine (F$_2$Tyr), and 2,3,5-trifluorotyrosine (F$_3$Tyr).

putative proton delivery from Tyr33 to the heme-bound oxygen. The other atoms in Tyr33, however, do not appear proximal enough to directly interact with the O$_2$ in the active site. More importantly, unnatural amino acids such as imiTyr that mimics the cross-linked His-Tyr can be readily introduced into the Phe33Tyr-Cu$_B$Mb.[29] This approach complements synthetic modeling, by which a Tyr has been introduced into models of heme-copper centers.[32−34] Herein we report introduction of a series of unnatural amino acid analogs of Tyr with different pK_a's and reduction potentials of the phenol ring into the Cu$_B$Mb at position 33 to provide conclusive evidence for the roles of the pK_a and reduction potential in regulating oxidase activity.

We chose three unnatural amino acid analogs of Tyr with progressively lower pK_a of the phenol group than that of the native Tyr (pK_a = 10.0): 3-chlorotyrosine (ClTyr, pK_a = 8.3), 3,5-difluorotyrosine (F$_2$Tyr, pK_a = 7.2), and 2,3,5-trifluorotyrosine (F$_3$Tyr, pK_a = 6.4) (Figure S1). The ClTyr was synthesized through a one-step chemical transformation as described previously,[35] while F$_2$Tyr and F$_3$Tyr were synthesized through an enzymatic reaction catalyzed by Tyr phenol lyase.[10] These Tyr analogs were incorporated into the protein in response to a TAG amber stop codon by evolved tyrosyl amber suppressor tRNA (*Mj*tRNA$^{Tyr}_{CUA}$)/tyrosyl-tRNA synthetase (*Mj*TyrRS) pairs from *Methanocaldococcus jannaschii*.[29,36,37] The successful preparation of the proteins was confirmed by electrospray ionization mass spectrometry (Table S1). The as-purified proteins do not contain any metal ion in the Cu$_B$ site. No copper ion was added in this work, as previous studies have shown that the presence of copper does not have any influence on the oxidase activity of Phe33Tyr-Cu$_B$Mb.[28] The UV−vis absorption spectrum of the Phe33ClTyr-Cu$_B$Mb as purified showed a Soret band at 407 nm and visible absorption bands at 501, 538, and 629 nm (Figure S2). Upon reduction by adding excess dithionite, the deoxy form of the protein exhibited a Soret band at 431 nm and a visible absorption band at 558 nm. Both UV−vis spectra of the met and deoxy forms of the Phe33ClTyr-Cu$_B$Mb are almost identical to the corresponding spectra of WTMb or Phe33Tyr-Cu$_B$Mb, as do the UV−vis spectra of the proteins containing the F$_2$Tyr and F$_3$Tyr (Figure S2). Furthermore, the EPR spectrum of met-Phe33Tyr-Cu$_B$Mb is very similar to that of WTMb, while the spectra of met-Phe33ClTyr-Cu$_B$Mb and met-Phe33F$_2$Tyr-Cu$_B$Mb display a small rhombic signal at *g* ≈ 6 (Figure S3). These results suggest that introducing the Tyr analogs has minimal perturbation on the structures of the protein models.

In a previous study, a Tyr-based radical was observed in the reaction of the Mb-based oxidase with O$_2$ and H$_2$O$_2$, stressing the central role of Tyr in oxidase reaction.[38] Removing the three other tyrosines in Phe33Tyr-Cu$_B$Mb (Tyr 103, 146, and 151) resulted in an almost identical EPR signal, providing support that Tyr33 may be the location of the tyrosyl radical.[38] Inspired by the work of Stubbe and coworkers,[10−12] we used EPR to characterize the reaction between H$_2$O$_2$ and ferric Phe33Tyr-Cu$_B$Mb and its variants containing unnatural Tyr analogs to provide a direct evidence of the location of the tyrosyl radical. As shown in Figure 2, upon reaction with stoichiometric amount of H$_2$O$_2$, the
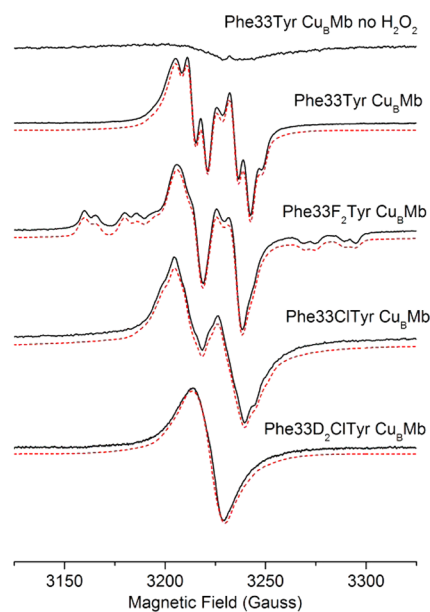
Figure 2. From top to bottom: X-band EPR spectra of Phe33Tyr-Cu$_B$Mb and the radicals formed in the reactions of Phe33Tyr-Cu$_B$Mb, Phe33F$_2$Tyr-Cu$_B$Mb, Phe33ClTyr-Cu$_B$Mb, and Phe33D$_2$ClTyr-Cu$_B$Mb with H$_2$O$_2$. Spectra simulated by SIMPOW6 are drawn in red dotted lines.[39]

Phe33Tyr-Cu$_B$Mb exhibited a free radical signal typical of a tyrosyl radical, distinctive from those from WTMb or Cu$_B$Mb (without the Phe33Tyr mutation).[10] As shown by Stubbe et al.,[10] fluorine substitution on the Tyr's phenol ring can introduce a unique splitting pattern for the tyrosyl radical signal. Indeed, simulation of the radical in Phe33F$_2$Tyr-Cu$_B$Mb gave three species with slightly different *g* values, with the major species (59%) having a *g* tensor of 2.013, 2.007, 2.004 and splitting from the fluorine nucleus of 44, 27, 161 MHz and 35, 17, 144 MHz, respectively (Table S2). In comparison, simulation of the radical from Phe33Tyr-Cu$_B$Mb gave a *g* tensor of 2.009, 2.004, 2.002 and splitting from ring protons of 26, 11, 19 MHz. This difference observed in Phe33F$_2$Tyr-Cu$_B$Mb arises from replacement of the Tyr33 with F$_2$Tyr and strongly suggests that the radical in this reaction originates at position 33.

To provide further support for this assignment, we replaced the Phe33 with 3-chlorotyrosine (Phe33ClTyr-Cu$_B$Mb) and obtained its EPR spectrum under the same conditions. Since the chloro-substitution can introduce quadruple splitting with a weak coupling constant that is then further broadened by the presence of both ^{35}Cl and ^{37}Cl isotopes, its contribution to the radical hyperfine splitting should be minimal. Indeed, much less hyperfine splitting was observed in the EPR spectrum of Phe33ClTyr-Cu$_B$Mb than those of either Phe33Tyr-Cu$_B$Mb or

Phe33F$_2$Tyr-Cu$_B$Mb (Figure 2). The EPR spectrum from Phe33ClTyr-Cu$_B$Mb was simulated by removing a ring proton. Finally, in addition to the halogen on the phenol ring, substitution of the protons with deuterons could also induce a change in the hyperfine splitting pattern.[40,41] To eliminate the splitting from the protons, we synthesized β,β-D$_2$-3-ClTyr (D$_2$ClTyr) from β,β-D$_2$Tyr in one step with 45% yield. This unnatural amino acid was then incorporated into myoglobin using the same tRNA/aaRS system for the Phe33ClTyr-Cu$_B$Mb to give Phe33D$_2$ClTyr-Cu$_B$Mb. Compared to the spectra from Phe33ClTyr-Cu$_B$Mb, Phe33D$_2$ClTyr-Cu$_B$Mb gives a different splitting pattern that can be simulated by changing β protons to deuterons. Together, these results provide conclusive evidence that the tyrosyl radical is located at residue Tyr33.

Having identified the location of Tyr radical, we then investigated the relationship between the oxidase activity and physical properties of the Tyr, such as pK_a of the phenol ring and the reduction potential of the Tyr analogs. The rates of oxygen reduction catalyzed by 6 μM myoglobin mutants containing Tyr33 or its unnatural amino acid analogs were measured by a Clark-type O$_2$ electrode in 20 mM tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 7.4, using a protocol reported previously for both HCOs and their models.[28,29,42] The rates of O$_2^-$ and O$_2^{2-}$ generation were determined by comparing the rates of oxygen consumption in the presence and absence of superoxide dismutase (SOD) and catalase, respectively, as described previously.[28] Since the SOD and catalase will convert O$_2^-$ or O$_2^{2-}$ to O$_2$ and H$_2$O, lowering apparent O$_2$ consumption rates, the fraction of O$_2^-$, O$_2^{2-}$, and H$_2$O generation can be calculated based on the known stoichiometry of the above reactions. Remarkably, the oxidase activities of these models are inversely proportional to the pK_a of the phenol ring of Tyr33 and its analogs (Figure 3A), supporting the proposed role of Tyr in oxidase activity by providing a proton to facilitate O−O bond cleavage and formation of H$_2$O. Furthermore, the percent water formation is also inversely proportional to the pK_a of the phenol rings (Figure 3B). In addition, to investigate the role of the reduction potential of these tyrosines in the oxidase activity, we measured the reduction potential of the Tyr and its analogs at pH 13, the pH at which the phenol is fully deprotonated so that the potential is independent of the pK_a (Figure S4).[10] Since oxidation of Tyr is an irreversible one-electron process on the electrode,[43] we only obtained the anodic peak potential (E_p). As the halogenation decreases phenol pK_a's from 9.8 to 6.4, the E_p increases from 672 to 850 mV in the same order, making the two factors difficult to deconvolute. Interestingly, the oxidase activity of these proteins increases as the E_p (at pH 13) of the Tyr and its analogs increases (Figure 3C). However, we found poor correlation between the reduction potentials of the Tyr and its analogs at pH 7 and their oxidase activity ($R^2 = 0.46$, Figure S5).

To further demonstrate the robustness of our oxidase models, we carried out multiple turnover experiments with a previously established protocol.[28] As shown in Figure 3D, the Phe33ClTyr-Cu$_B$Mb mutant was able to catalyze O$_2$ reduction for more than 1200 turnovers without significant reduction of catalytic rate. Under similar conditions, the Phe33Tyr-Cu$_B$Mb could catalyze the reaction for only less than 500 turnovers.[28] Therefore, introducing the unnatural amino acids has allowed us not only to clarify the role of Tyr but also to increase the activity and turnovers of the enzyme.

In summary, through the genetic incorporation of a series of unnatural amino acid analogs of Tyr with progressively decreasing pK_a's of the phenol ring and increasing reduction
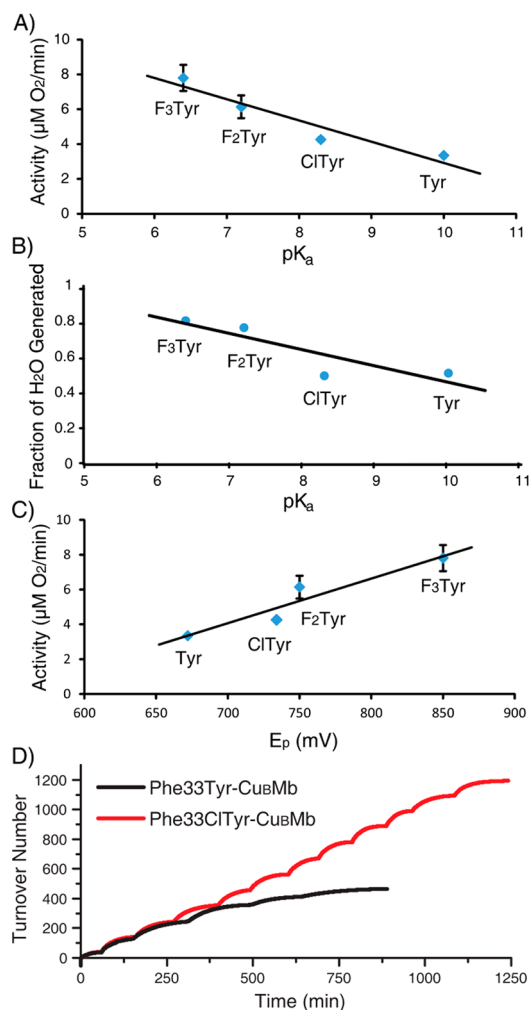
Figure 3. pK_a of phenols on the Tyr and its analogs affects oxidase activity. (A) Correlation of oxidase activity of Phe33Tyr-Cu$_B$Mb, Phe33ClY-Cu$_B$Mb, Phe33F$_2$Y-Cu$_B$Mb, and Phe33F$_3$Y-Cu$_B$Mb vs pK_a of phenols on the Tyr and its analogs, $R^2 = -0.98$. (B) Correlation of water produced in oxygen reduction reaction performed by these proteins vs the pK_a of phenols on the Tyr and its analogs, $R^2 = 0.94$. (C) Correlation of oxidase activity of Phe33Tyr-Cu$_B$Mb, Phe33ClY-Cu$_B$Mb, Phe33F$_2$Y-Cu$_B$Mb, and Phe33F$_3$Y-Cu$_B$Mb vs peak potential at pH 13 (E_p) of the corresponding Tyr and Tyr analogs, $R^2 = 0.95$. (D) O$_2$-reduction turnover number by Phe33ClTyr-Cu$_B$Mb and Phe33Tyr-Cu$_B$Mb measured during the stepwise addition of O$_2$.

potentials into the active site of a functional model of an oxidase in myoglobin, an inversely proportional correlation was found between pK_a of the phenol ring in the Tyr and Tyr analogs and oxidase activity and selectivity of the reaction toward water production in these models. Using unnatural amino acids as spectroscopic probes, the radical generated in the reaction of myoglobin-based functional oxidase models with H$_2$O$_2$ is unambiguously assigned to the active site Tyr33 evidenced by hyperfine splitting patterns of the halogenated tyrosines and one of its deuterated derivatives.

Multi-electron processes occur in numerous essential chemical and biological reactions such as respiration and solar energy conversions. While chemists have often achieved these functions using metal ions such as Pd, Ru, and Rh that can undergo multi-electron processes, nature uses redox-active Tyr residues in conjunction with earth abundant transition metals such as Mn, Fe, and Cu. More importantly, protons are often involved in such

multi-electron processes, a role that Tyr can also fulfill. Our ability to genetically encode unnatural amino acids with tunable pK_a and reduction potential into specific sites of designed proteins should greatly improve our ability to elucidate the precise roles of Tyr in enzymatic reactions and to design redox-active enzymes.

Indeed, by directly incorporating the unnatural amino acids into a functional oxidase model in response to the amber codon TAG in *E. coli*, we have successfully provided evidence, for the first time in a metalloprotein, that the enhanced proton donating ability of the phenol ring of the Tyr is responsible for the high oxidase activity and selectivity for water production. Since the syntheses of these unnatural amino acids are facile with high yields and the mutant proteins can be easily obtained at milligram quantity, incorporation of these unnatural amino acids offers exciting new opportunities to design metalloenzymes with functional properties even better than those of native enzymes and to address a variety of unresolved questions regarding the activity in these enzymes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details about F_2Tyr synthesis, pK_a measurement, protein expression and purification, UV−vis spectra, and oxidase activity assay. 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.

■ ACKNOWLEDGMENTS

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