Significant improvement of oxidase activity through the genetic incorporation of a redox-active unnatural amino acid†

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While nature employs various covalent and non-covalent strategies to modulate tyrosine (Y) redox potential and $pK_a$ in order to optimize enzyme activities, such approaches have not been systematically applied for the design of functional metalloproteins. Through the genetic incorporation of 3-methoxytyrosine (OMeY) into myoglobin, we replicated important features of cytochrome c oxidase (CcO) in this small soluble protein, which exhibits selective O$_2$ reduction activity while generating a small amount of reactive oxygen species (ROS). These results demonstrate that the electron donating ability of a tyrosine residue in the active site is important for CcO function. Moreover, we elucidated the structural basis for the genetic incorporation of OMeY into proteins by solving the X-ray structure of OMeY specific aminoacyl-tRNA synthetase complexed with OMeY.

Introduction

Designing artificial enzymes with higher activity and selectivity can reveal important features responsible for tuning enzymatic activities, and result in efficient catalysts for practical applications.1-9 One key mechanism which accounts for the high activity of many natural enzymes is the fine-tuning of the redox potential of tyrosine residues. In order to optimize the electron transfer rate to enable enzymatic turnover with high efficiency and selectivity, nature has exploited various strategies, such as post-translational modifications including topa quinone in copper amine oxidases, the Tyr-His crosslink in CcO, the Tyr-Cys crosslink in galactose oxidases (GO), and histidine base association in photosystem II (PSII).10-16 Such strategies are highly effective in modulating the redox potential (from 0.1 V to 1.1 V vs. NHE) and $pK_a$ of specific tyrosine residues to suit the specific needs of various reactions, thereby greatly enhancing enzyme activity.10-16 However, it remains difficult to perform rational tuning of the redox potential and $pK_a$ of specific tyrosine residues in designed metalloproteins. Here we show that, through the genetic incorporation of redox-active unnatural amino acids with desirable redox potential, a significant improvement in oxidase activity can be achieved.

During the final stage of aerobic respiration, CcO catalyzes the efficient reduction of O$_2$ to H$_2$O, which requires rapid transfer of four electrons and four protons to the oxygen substrate, preventing the release of toxic reactive oxygen species (ROS).13 The key step in oxygen reduction is the scission of the O–O bond in the ferric-superoxo intermediate, leading to the formation of an intermediate P in the heme $a_3$/Cu$_B$ binuclear active site.17 The donor of a proton and electron for this reaction has been suggested to be a unique tyrosine residue covalently cross-linked to one of the histidine ligands of Cu$_B$. This Tyr-His crosslink is thought to lower the $pK_a$ and redox potential of the tyrosine residue, thus facilitating proton and electron donation to the oxygen substrate.15,18

In previous studies, we have reported the introduction of various tyrosine analogs into a myoglobin-based functional oxidase,19-20 including imiTyr,19 which mimics the Tyr-His crosslink in CcO, and a series of halogenated Tyr analogous with decreasing $pK_a$.21 By replacing Tyr33 with imiTyr and halogenated tyrosine analogs, the activity and selectivity of the functional oxidase increases. Moreover, the oxidase activity is correlated with the $pK_a$ of the phenol ring of Tyr or its halogenated analogs, indicating the active role of Tyr in the oxidase reaction. However, since the reduction potentials of halogenated Tyr analogous are closely related to their $pK_a$, we have not yet addressed whether fine-tuning the redox potential of the tyrosine residue influences oxidase activity. Herein we report genetic incorporation of a tyrosine analog, 3-methoxy tyrosine...
results of this study are consistent with the general principle that OMeY, as a substrate of TPL, can specifically bind to the active site of the enzyme with high affinity, facilitating the catalytic reaction. The catalytic efficiency of TPL with OMeY was found to be higher than with Tyr, as evidenced by the higher specific activity observed with OMeY compared to Tyr. This enhanced catalytic activity is likely due to the lower redox potential of OMeY compared to Tyr, which facilitates the two-electron oxidation reaction required for the synthesis of 3,4-dihydroxy-L-phenylalanine (Dopa), a key intermediate in the melanogenesis pathway.

To selectively incorporate OMeY at defined sites in proteins, a mutant Methanocaldococcus jannaschii tyrosyl amber suppressor tRNA (Mj tRNA_{ACU}^{TYR})/tyrosyl-tRNA synthetase (Mj TyrRS) pair was evolved that uniquely specifies OMeY in response to the TAG codon, as previously reported. The evolved TyrRS (Fig. S7–S8†), named OMeYRS, has six mutations: Tyr32Glu, Leu65Ser, His70Gly, Tyr109Gly, Asp158Asn, and Leu162Val.

To determine if OMeY could be incorporated into proteins with high efficiency and fidelity, an amber stop codon was substituted for Ser4 in sperm whale myoglobin (Mb). Protein production was carried out in E. coli in the presence of the selected synthetase (OMeYRS), Mj tRNA_{ACU}^{TYR} and 1 mM OMeY, or in the absence of OMeY as a negative control. Analysis of the purified protein by SDS-PAGE showed that full-length myoglobin was expressed only in the presence of OMeY (Fig. 1A), indicating that OMeYRS was specifically active with OMeY but inactive with natural amino acids. The yield for this mutant myoglobin was 10 mg L⁻¹. By comparison, the yield of wild-type sperm whale myoglobin (WTMb) was 50 mg L⁻¹. ESI-MS analysis of the Ser4 OMeY mutant myoglobin gave an observed average mass of 18 461.6 Da, in agreement with the calculated mass of 18 461.1 Da (Fig. 1B).

To test whether the catalytic activity could be improved through the genetic incorporation of unnatural amino acids, we replaced Phe33 in CuMb with OMeY, generating Phe33OMeY-CuMb (Fig. 2). This mutant showed a similar UV-vis spectrum to that of Phe33Tyr-CuMb (Fig. S9†), indicating that the overall environment around the heme center should also be similar.
We then measured the rates of oxygen reduction catalyzed by 6 μM myoglobin mutants with an O2 electrode in 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4. Ascorbate (1000 equivalents) and tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 100 equivalents) were used as reductant and redox mediator, respectively.29 To differentiate between reactive oxygen species (ROS) and water products, we used catalase and superoxide dismutase (SOD), which catalyze the disproportionation of hydrogen peroxide or superoxide into oxygen and water. If O2 consumption results in the formation of ROS but not water, the O2 reduction rate should decrease in the presence of catalase and SOD, because they will convert ROS to O2. By comparing the rates of reduction in the presence and absence of ROS scavenger, the portions of O2 reduction due to water formation (in blue) and due to ROS formation (in red) can be calculated (Fig. 3A and Table S2†). Our results show that Phe33Tyr-CuMBb was able to reduce O2 at a rate of 6.5 μM min⁻¹, with 51% of O2 being converted into water. In contrast, Phe33OMeY-CuMBb exhibited significantly higher oxidase activity at 15.0 μM min⁻¹ for O2 reduction, with 82% conversion of O2 into water. Similarly to the case of Phe33Tyr-CuMBb, addition of copper to Phe33OMeY-CuMBb did not increase oxidase activity.26 Since the pKₐ of OMeY (Fig. S6†) is similar to that of Tyr, the lower redox potential of OMeY is likely responsible for the increased oxidase activity and O2 reduction selectivity of Phe33OMeY-CuMBb.

To further demonstrate the robustness of the best oxidase-mimicking enzyme, Phe33OMeY-CuMBb, we carried out multiple turnover experiments (Fig. 3B). Phe33OMeY-CuMBb was able to catalyze O2 reduction for more than 1100 turnovers without significant reduction of catalytic rate. Under similar conditions, Phe33Tyr-CuMBb could only catalyze the reaction for fewer than 500 turnovers.20 Previous studies with various halogenated Tyr analogs in an oxidase model have shown that oxidase activity is correlated with the pKₐ of Tyr or its analogs, however, correlation between oxidase activity and reduction potential at pH 7 is weak. Since Tyr oxidation at neutral pH, when Tyr is protonated, is a process coupled with the loss of a proton, the reduction potential of Tyr is influenced by pKₐ. It is hard to separate the effect of pKₐ from the reduction potential of Tyr. As OMeY has a much lower reduction potential but similar pKₐ to Tyr, it is clear that decreasing the reduction potential, similar to decreasing the...
pKₐ, also enhances oxidase activity (Fig. 3C). The correlation of
reduction potential/pKₐ with oxidase activity is consistent with
the active role of Tyr in the oxidase reaction, as previous studies
have shown that a tyrosyl radical is formed during the oxygen
reduction reaction of the Mb-based functional oxidase.29

Unnatural Tyr analogs as spectroscopic or functional probes
have been developed to study the function of Tyr in different
enzymes. Halogenated Tyr analogs have different pKₐ values, as
well as distinct EPR signals,24,25 making them useful for pin-
pointing the location of the tyrosyl radical intermediate and the
proton donating ability of Tyr. Dopa and NH₂Y have decreased
reduction potential.22,23 They are used in reductive enzymes as
they are susceptible to oxidative damage. OMeY has a lower
reduction potential than Tyr, yet is relatively stable under
oxidative conditions, making it suitable for studying oxidative
enzymes, such as cytochrome c oxidase, galactose oxidase, and
lytic polysaccharide monooxygenase.

Conclusions

In summary, by incorporation of OMeY, an analog with a 179
mV lower reduction potential and similar pKₐ to Tyr, into a Mb-
based functional oxidase, we found that the oxidase activity of
the protein is correlated with the reduction potential of active
site Tyr or its analogs. This further reveals the active role of Tyr
in the oxidase reaction.

Tyr is an important residue for electron transfer31 as well as
catalysis,14 due to its redox activity and proton-coupled electron
transfer ability. Nature has evolved different ways of conducting
post-translational modifications,32–34 along with manipulating
hydrogen bonding and π–π stacking to fine-tune the properties
of Tyr. OMeY, with its low reduction potential while being
relatively stable to O₂, has been added as a unique member to
the toolbox of Tyr analogs35–37 for studying and engineering Tyr-
containing enzymes.

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