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Post-Translational Modification

Probing the Function of the Tyr-Cys Cross-Link in Metalloenzymes by the Genetic Incorporation of 3-Methylthiotyrosine**

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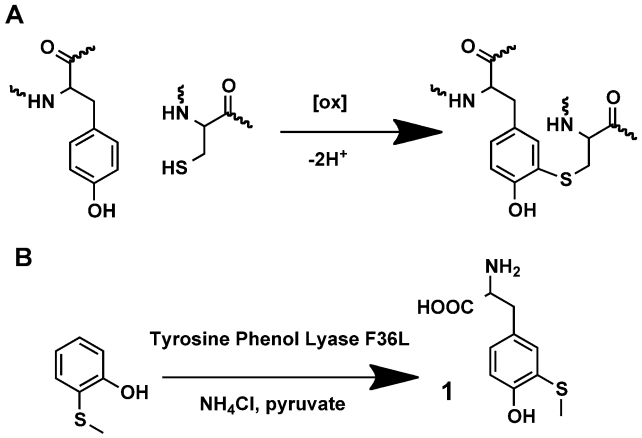
Dedicated to Professor Chih-chen Wang on the occasion of her 70th birthday

The thioether-bonded tyrosine–cysteine cofactor (Tyr-Cys, Scheme 1 A) is ubiquitous for diverse metalloenzymes, including the copper-dependent galactoase oxidase[1] (GO)



Scheme 1. A) The autocatalytic post-translational modification that yields the Tyr-Cys cofactor in various metalloenzymes, including GO, CDO, NirA, and TvNiR. B) A biosynthetic route to MtTyr **1**, catalyzed by the TPL mutant F36L.

and glyoxal oxidase, iron-dependent cysteine dioxygenase[2] (CDO), siroheme- and $[Fe_4S_4]$-dependent sulfite reductase[3] (NirA), and *T. nitratireducens* cytochrome c nitrite reductase[4] (TvNiR) (Supporting Information, Figures S4–S7). In all of the aforementioned enzymes, a covalent bond is formed autocatalytically between the C3 ring carbon atom of a tyrosine residue and the Sγ atom of a neighboring cysteine residue, without requiring exogenous proteins[5] (Scheme 1 A). Because of the fascinating chemistry and potential industrial applications[6] of these enzymes, the functional significance of the essential Tyr-Cys cofactor has been intensively investigated by synthetic chemists,[7] physical chemists,[8] enzymologists,[1,9] and structure biologists,[2–4,10] and tremendous progress has been made in this area.

While the presence of the "home-made" cofactor[11] Tyr-Cys is firmly established in GO, CDO, NirA, and TvNiR,[2–4,10] its exact role in metalloenzyme function is still not fully understood. A major limitation of our understanding is that such a post-translational modification (PTM) cannot be directly probed through traditional site-directed mutagenesis methods. To overcome this limitation, synthetic model compounds have been made,[7] and studies of these model compounds have suggested that the cross-link between the Tyr and Cys residues significantly decreases the pKa value and reduction potential of the phenol side chain,[5,8f,g] and therefore facilitates proton-coupled electron transfer[12] (PCET) between the substrate and the Tyr-Cys cofactor, which is critical for optimizing enzyme activity. Despite these advances, questions about the roles of the Tyr-Cys cross-link still remain, because no study has been able to directly probe the functions of the thioether substitution at the tyrosine residue without introducing extra mutations. While mutation of the cysteine residue that takes part in the Tyr-Cys cofactor formation invariably leads to disruption of the Tyr-Cys cross-link and significant decrease of enzyme activity,[2–4,10] the extent to which the thioether substitution participates in the catalytic process has not been determined, and the possibility of a purely structural role for the Tyr-Cys cross-link has been proposed.[13]

To answer the above questions and to gain new insights into the Tyr-Cys cofactor function, we report herein the genetic incorporation of a new unnatural amino acid (UAA) 2-amino-3-(4-hydroxy-3-(methylthio)phenyl)propanoic acid **1** (hereafter termed MtTyr; Figure 1), which mimics the Tyr-Cys cofactor, into a functional model of TvNiR in sperm whale myoglobin (Mb). This enzyme model with the incorporated UAA MtTyr was termed MtTyrMb. We then compared the activity of MtTyrMb with a mutant that has a Tyr residue at the same position in the same protein (termed TyrMb). Our

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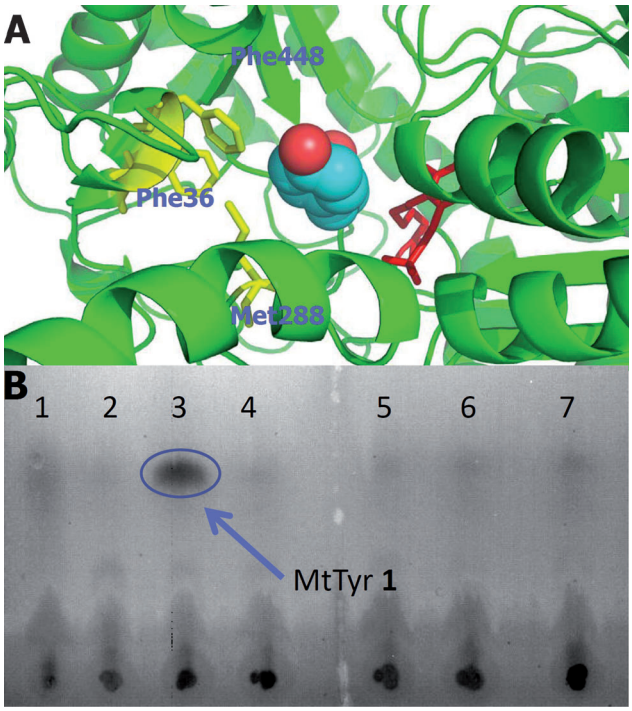

Figure 1. A) View of the active-site structure of tyrosine phenol lyase (TPL) complex (pdb code 2TPL) with inhibitor 3-(4′-hydroxyphenyl)propionic acid and cofactor pyridoxal-5′-phosphate (PLP). Residues Phe36, Phe448, and Met288 are shown as yellow sticks. The PLP cofactor is shown as red sticks. 3-(4′-hydroxyphenyl)propionic acid is shown as spheres. B) Thin-layer chromatography (TLC) assay for TPL catalyzed MtTyr synthesis. Lane 1: wild-type TPL. Lanes 2–7: TPL mutants bearing random mutations in residues 36, 288, or 448.

results show that MtTyrMb exhibited much better activity than TyrMb. The increase in catalytic activity provides for the first time direct evidence that the thioether substitution on the tyrosine residue can enhance enzyme activity. Our ability to directly encode MtTyr, combined with state of the art computational design,[14] rational design,[15] and directed evolution[6c,d,16] methods, should allow us to take full advantage of the unique redox and proton donor properties of the MtTyr ligand, and to rapidly expand the substrate scope and utility of existing Tyr-Cys dependent multi-electron oxidase and reductases.

To synthesize MtTyr **1**, we first attempted to transform 2-(methylthio)phenol to **1** by using wild-type *Citrobacter Freundii* (ATCC8090) tyrosine phenol lyase (TPL), as it has been previously reported that wt TPL has a relatively broad substrate scope.[17] However, we could not detect any UAA **1** formation, by using a ninhydrin thin-layer chromatography (TLC) assay (Figure 1B). Close inspection of the TPL structure reveals that residues Phe448, Phe36, and Met288, which together form a hydrophobic pocket and stabilize the tyrosine substrate through van der Waals interactions, need to be mutated to better accommodate **1** as substrate. Indeed, molecular modeling shows that the thioether substitution on the tyrosine substrate may cause substantial steric clash with these residues (Supporting Information, Figure S8). To evolve a TPL mutant that can efficiently catalyze the synthesis of MtTyr **1**, a TPL library, pEt-TPL1 was constructed. Residues Phe448, Phe36, or Met288 were randomized individually through an overlapping extension PCR by using synthetic oligonucleotide primers in which the intended randomization sites was encoded by NNK ($N = A + T + C + G$; $K = T + G$), affording pEt-TPL1 with a library size of 96. Ninety-six clones expressing different TPL mutants were then picked from a petri dish and transferred to liquid minimal medium in a microtiter plate and allowed to grow to saturation. Cells were then lysed by adding lysozyme, and then 2-(methylthio)phenol, NH_4Cl, and pyruvate were added. The plate was then incubated at 37 °C for 4 h. Each well of the microtiter plate was then analyzed for amino acid formation by the ninhydrin TLC assay (Figure 1B). To our delight, we found that one clone (Lane 3, Figure 1B) efficiently catalyzed the synthesis of an amino acid. Sequencing reveals that this clone has the Phe36 Leu mutation. Molecular modeling indicates that the Phe36 Leu results in significant enlargement of the enzyme pocket to allow for optimal interaction between the enzyme and the MtTyr substrate (Supporting Information, Figure S9). Experiments are underway to elucidate the structural basis for the TPL Phe36Leu mutant and substrate interaction. We then scaled up TPL Phe36Leu mutant protein production and performed 2-(methylthio)phenol transformation in gram quantities. The resulting amino acid was separated by HPLC (Supporting Information, Figure S2), and was confirmed to be MtTyr by NMR and mass spectrometry (Supporting Information, Figure S3). The yield after HPLC purification was 40%. To the best of our knowledge, this is the first report of the directed evolution of TPL to expand its substrate scope, and efficient UAA synthesis by using a TPL mutant. Moreover, by using ^{15}N-enriched NH_4Cl or ^{13}C-enriched pyruvate as precursors, ^{15}N or ^{13}C labeled MtTyr can be conveniently synthesized. Genetic incorporation of ^{15}N or ^{13}C labeled MtTyr could be extremely valuable for elucidating Tyr-Cys cofactor function in various metalloenzymes.[5,8a]

To selectively incorporate MtTyr at defined sites in proteins, a mutant *Methanococcus jannaschii* tyrosyl amber suppressor tRNA (MjtRNA$^{Tyr}_{CUA}$)/tyrosyl-tRNA synthetase (MjTyrRS) pair was evolved that uniquely specifies **1** in response to the TAG codon, as previously reported.[18] One MjTyrRS clone emerged after positive and negative selections, which grew at 100 $\mu g\,mL^{-1}$ of chloramphenicol in the presence of 1 mM **1**, but only at 20 $\mu g\,mL^{-1}$ chloramphenicol in its absence, and was named MtTyrRS. This clone had four mutations: Tyr32Glu, His70Gly, Asp158Ala, and Leu162Pro. The His70Gly and Asp158Ala mutations are introduced to create additional space to accommodate the methylthio group, and Tyr32 is mutated to a smaller hydrophilic amino acid, creating a larger binding pocket while providing the required hydrogen bonding interaction to stabilize the MtTyr side chain. The Leu162Pro mutation was previously observed for an *o*-methyl tyrosine specific MjTyrRS mutant.[19] When Leu162 is mutated to Pro, the α-helix spanning from residue 146 to 164 retreats significantly, and leaves ample space for the methylthio group of the unnatural amino acid MtTyr.

To determine if **1** is incorporated into the protein with high efficiency and fidelity, an amber stop codon was substituted for Ser4 or Phe33 in sperm whale myoglobin

(Mb). Protein production was carried out in *E. coli* in the presence of the selected synthetase (MtTyrRS), *Mj*tRNA$^{Tyr}_{CUA}$, and 1 mM **1**, or in the absence of **1** as a negative control. Analysis of the purified protein by SDS-PAGE showed that full-length myoglobin was expressed only in the presence of **1** (Figure 2A), indicating that MtTyrRS was

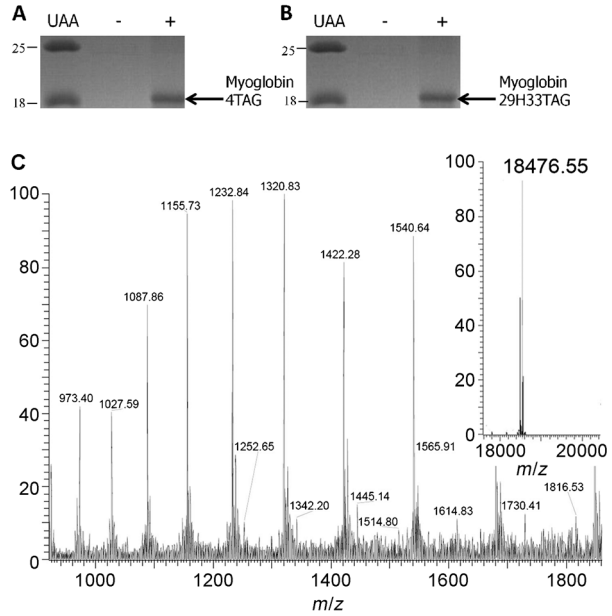

Figure 2. Coomassie-stained SDS-PAGE of TAG4 mutant myoglobin (A) or MtTyrMb mutant myoglobin (B) expression in the presence and absence of 1 mM **1**. C) ESI-MS spectrum of the myoglobin TAG4 mutant. The insert shows the deconvoluted spectrum; expected mass: 18477.2 Da, found: 18476.5 Da.

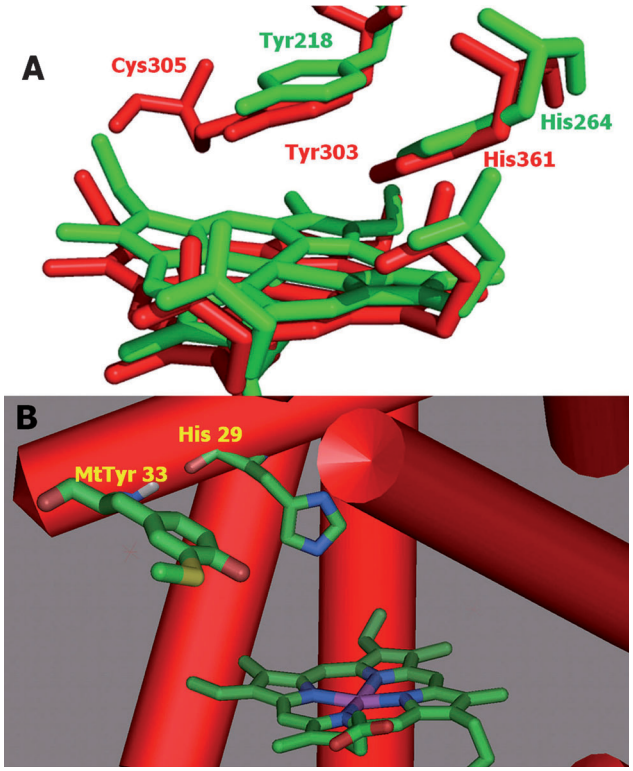

Figure 3. A) Structural alignment of *Wolinella succinogenes* cytochrome c nitrite reductase (green, pdb code 1FS7) and TvNiR (red, pdb code 3F29). Note the presence of a Tyr-Cys cross-link between Tyr303 and Cys305 in TvNiR. B) Structural model of MtTyrMb, constructed based on the crystal structure of the F33Y/L29H/F43H mutant myoglobin (pdb code 4FWX). Residues MtTyr33 and His29 occupy similar positions in the MtTyrMb active site as Tyr303 and His361 in TvNiR.

specifically active for **1** but inactive for any natural amino acids. The yield for mutant myoglobin was 10 mg L^{-1}. For comparison, the yield of wild-type sperm whale myoglobin (wtMb) was 50 mg L^{-1}. ESI-MS analysis of the Ser4→**1** mutant myoglobin gave an observed average mass of 18476.5 Da, in agreement with the calculated mass of 18477.2 Da.

Cytochrome c nitrite reductase (NiR) catalyzes the six-electron reduction of nitrite to ammonia in a three step process [Supporting Information, Figure S10; Eq. (1)]. The presence of hydroxylamine as an intermediate in the reaction cycle of NiR is demonstrated by the experimental results that NiR also catalyzes hydroxylamine reduction to ammonia with high efficiency [Eq. (2)].[10,20]

$$NO_2^- + 6\,e^- + 8\,H^+ \rightarrow NH_4^+ + 2\,H_2O \quad (1)$$

$$NH_2OH + 2\,e^- + 2\,H^+ \rightarrow NH_4^+ + H_2O \quad (2)$$

It has been proposed that a conserved tyrosine residue situated close to the substrate-binding heme is responsible for donating an electron and a proton to the hydroxylamine intermediate[21] (Figure 3; Supporting Information, Figure S10), to yield ammonia and a tyrosyl radical, which persists until a further electron arrives.[20,22] Recent X-ray structural studies revealed that a Tyr-Cys cross-link between residues Tyr303 and Cys305 is present in TvNiR.[4a] While this cross-link does not cause significant structural changes in the TvNiR active site in comparison to NiRs that do not contain this PTM (Figure 3A), formation of the tyrosine–cysteine covalent bond results in significant lowering of the pK_a and reduction potential of Tyr303. It was proposed that these changes are responsible for the higher overall activity of TvNiR compared to NiR, which does not harbor the Tyr-Cys cofactor.[4a]

To directly test the above hypothesis, we constructed a functional model of TvNiR, called MtTyrMb, using myoglobin as a scaffold protein. As Figure 3 shows, MtTyrMb harbors the unnatural amino acid mutation MtTyr at position 33, and a histidine mutation at position 29. Residues MtTyr33 and His29 of MtTyrMb are situated in similar locations relative to heme as residues Tyr303 and His316 of TvNiR (Figure 3). These conserved residues are known to be important for the catalytic activity of NiR.[4a] We then measured the rate of $^{15}NH_2OH$ reduction catalyzed by MtTyrMb by ^{15}N NMR.[22b] We first incubated 50 mM $^{15}NH_2OH$ with 100 mM sodium dithionite in 500 mM sodium phosphate buffer, pH 7.0. We found that the presence of dithionite alone does not result in $^{15}NH_2OH$ reduction, as there was no change in the ^{15}N NMR spectrum (Supporting

Information, Figure S11). Once 5 μM of MtTyrMb was added to the solution, the signal for $^{15}NH_2OH$ at 90 ppm disappeared within 30 min, and a new peak at 21 ppm emerged, indicating that hydroxylamine had been reduced to ammonium (Supporting Information, Figure S11). In the presence of 50 μM tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), however, the reaction was completely inhibited (Supporting Information, Figures S4, S12). CORM-3 is stable in water at acidic pH but in physiological buffers rapidly liberates carbon monoxide,[22c] which then binds to the ferrous iron in MtTyrMb (Supporting Information, Figure S13). These results indicate that hydroxylamine reduction requires binding to the heme iron in MtTyrMb.

To measure the reaction rate, the reactions were allowed to proceed in the presence of 0.5 μM enzyme for different amounts of time, and then terminated by the addition of 50 μM CORM-3, followed by ^{15}N NMR spectrum acquisition. To measure the amount of hydroxylamine conversion to ammonium, 20 mM ^{15}N-urea was added prior to NMR spectrum acquisition, as an internal standard. The amount of hydroxylamine remaining in the solution, and the amount of ammonium having formed, was calculated by measuring peak areas at 90 ppm and 21 ppm, respectively (Supporting Information, Figures S11, S12). Our results show that MtTyrMb catalyzed hydroxylamine reduction to ammonium with more than 95% selectivity (Figure 4; Supporting Information, Figure S12), with a hydroxylamine consumption rate of 400 μM min^{-1} ($k_{cat} = 800\ min^{-1}$). By contrast, TyrMb, which does not bear 3-methylthio substitution at residue Tyr33, catalyzed the reaction four times slower ($k_{cat} = 200\ min^{-1}$). MtTyrMb and TyrMb exhibited similar K_m values at 7 mM and 6 mM, respectively. Since myoglobin is much easier to produce, and easier to characterize spectroscopically than NiR, which is a multiple-heme periplasmic protein requiring numerous protein factors for maturation,[23] MtTyrMb serves as an ideal model to further elucidate the mechanism of NiR activity.

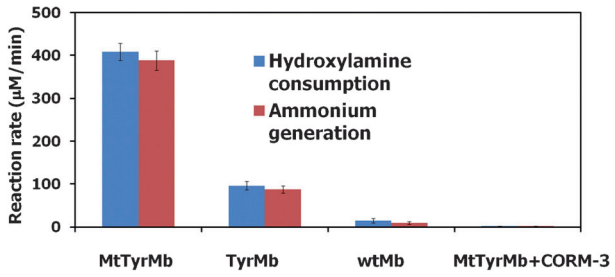

Figure 4. Relative rates of hydroxylamine reduction by different myoglobin mutants. The reactions were carried out between 50 mM $^{15}NH_2OH$ and 100 mM sodium dithionite, catalyzed by 0.5 μM myoglobin mutants, in 500 mM sodium phosphate buffer, pH 7.0.

In conclusion, by directly incorporating the unnatural amino acid MtTyr **1** into myoglobin in *E. coli* in response to the amber codon TAG, we have successfully designed a functional NiR model which catalyzes efficient and selective hydroxylamine reduction to ammonia. The MtTyrMb NiR model bearing a modified tyrosine residue MtTyr is four-fold more active than TyrMb, which does not contain the 3-methylthio substitution at residue Tyr33. These results provide for the first time direct evidence that 3-methylthio substitution on a tyrosine residue can enhance enzyme activity. As the synthesis of MtTyr requires only one step with 40% overall yield, mutant proteins bearing MtTyr **1** at any site can be easily obtained and purified in milligram quantities by site-directed mutageneis and recombinant gene expression. Our designed enzyme harbors the unnatural amino acid MtTyr, which is highly analogous to the post-translationally modified Tyr-Cys cofactor found in the active sites of GO, CDO, NirA, and TvNiR, serves as an ideal model for a more detailed understanding of these metalloenzymes, and allows for potential applications in synthetic biology and industrial catalysis. There is currently strong interest in the directed evolution of galactose oxidase to obtain mutants with desirable activity towards monosaccharides other than galactose.[6] Our new method for the genetic incorporation of MtTyr should allow for the rapidly evolution of metalloenzymes with specific activity towards various monosaccharides by using well-characterized protein maquettes as a copper ligand and by applying rational and computational design methods.[6,14–16]

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