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Genetic Incorporation of a Metal-Chelating Amino Acid as a Probe for Protein Electron Transfer**

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Dedicated to Professor Kenneth S. Suslick on the occasion of his 60th birthday

Electron transfer (ET) governs many important biochemical processes, including photosynthesis and cytochrome P450-mediated oxidation.[1] While tremendous progress has been made towards understanding the mechanism of electron transfer in proteins,[2] ET experiments in proteins have commonly relied on ligation of redox-active probes to uniquely reactive surface-accessible histidine (His) or cysteine (Cys) residues, and are in general limited to small soluble proteins.[1] Efficient photoinduced electron transfer (PET) typically requires close proximity[2c,3] (less than 14 Å) of the donor and acceptor. The PET method nicely complements FRET studies[4] (which are ideal for the study of conformational dynamics in the 20–80 Å range) for the investigation of small-scale conformation changes in biopolymers.[5] While fluorescence quenching by PET is a powerful method for uncovering macromolecular conformation dynamics,[5] such experiments are usually performed by using natural amino acids, such as tryptophan and tyrosine, as electron donors and fluorophores as electron acceptors, and they are therefore limited to relatively simple biological systems.

To circumvent these limitations, we report here a novel strategy for the genetic incorporation of the metal-chelating amino acid (*S*)-2-amino-3-[4-hydroxy-3-(1*H*-pyrazol-1-yl)phenyl]propanoic acid **1** (hereafter termed pyTyr) into proteins produced in *E. coli* in response to the amber stop codon, TAG. By solving the crystal structure of the *Aequorea victoria* green fluorescent protein (GFP) bearing pyTyr at a specific site (Figure 1), we demonstrate that Cu^{II} selectively binds to pyTyr in proteins, forming a pyTyr/Cu^{II} complex (termed pyTyrCu) and that the Cu^{II} ion, pyrazole ring, and phenol ring form a coplanar complex. In contrast to natural amino acids[5b] or other genetically encoded non-natural amino acids such as dopa, 3-aminotyrosine, or difluorotyrosine, which have been used as electron donors in ET measurements,[6] pyTyrCu is an electron acceptor. These unique properties and the ability to place pyTyrCu at any position in a protein should allow for ET measurements in complex biological systems that were previously unattainable. GFP is the most popular marker for protein localization and interaction, although its true function in jellyfish remains

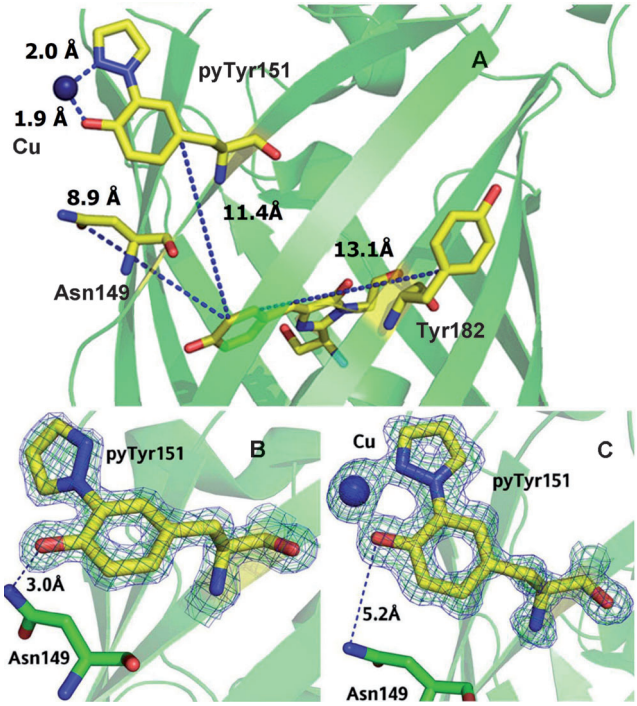

Figure 1. A) Overall structure of GFP-151pyTyrCu complex. Cu^{II} ion is blue sphere; GFP chromophore, and residues 151, 149, and 182 are sticks; C yellow, N blue, O red. B) pyTyr151 F_o-F_c omit electron density map (contoured at 4.5σ) in the absence of Cu^{II} ions. C) Electron density map of the pyTyr151Cu complex.

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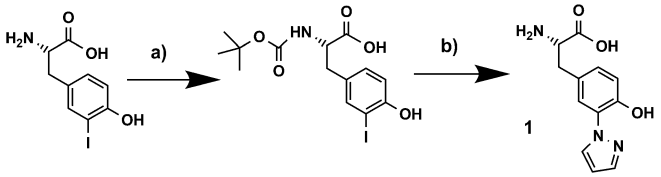
unknown after forty years of investigation.[7] Recently, it was discovered that GFP is a light-induced electron donor and therefore may have a light sensing function in vivo.[7b,8] However, because of a lack of methods for the site-specific placement of electron acceptors in proteins, the ET mechanism of GFP has not been thoroughly investigated. Using our method, we demonstrate herein that PET between the GFP chromophore and pyTyrCu occurs rapidly, within one nanosecond, and in a distance-dependent manner.

While the genetic incorporation of metal-chelating non-natural amino acids containing a bipyridine or 8-hydroxyquinoline group has been previously described,[9] their application in probing electron transfer in proteins has not been demonstrated. The use of these non-natural amino acids has been limited because of their complicated synthesis; current synthetic routes require more than five steps starting from commercially available materials to make a racemic mixture, require heavy-metal catalysts, carcinogenic solvents, strong acids and bases, and demand multiple column purification steps. Herein, we describe a highly efficient strategy for the synthesis of enantiomerically pure metal-binding non-natural amino acid **1** in two convenient steps with high yield and demonstrate its genetic incorporation into proteins.

The metal-chelating pyTyr **1** was synthesized by first protecting the amine group of 3-iodotyrosine using di-*tert*-butyl dicarbonate, producing Boc-L-3-iodotyrosine, which was then allowed to react with pyrazole in the presence of Cs_2CO_3 and CuI in dimethylformamide (DMF) and heated at reflux to give **1** with an overall yield of 50% (Scheme 1).



Scheme 1. Synthesis of pyTyr **1**. a) Di-*tert*-butyl dicarbonate, tetrahydrofuran, 1 N NaOH, RT, 4 h. b) pyrazole, Cs_2CO_3, CuI, DMF, heated to reflux, 16 h.

After removing the DMF on a rotary evaporator, the mixture was extracted with ethyl acetate and distilled water. We found that pyTyr **1** was contained in the aqueous phase and could be used directly for all subsequent screening and protein expression experiments without further purification. As all reagents used for the synthesis of **1** are environmentally benign, and no column purification is necessary, this new metal-binding non-natural amino acid **1** can be easily prepared in any biology or chemistry laboratory.

To selectively incorporate pyTyr at defined sites in proteins produced in *E. coli*, a mutant *Methanococcus jannaschii* tyrosyl amber suppressor tRNA (MjtRNA$^{Tyr}_{CUA}$)/tyrosyl-tRNA synthetase (MjTyrRS) pair was evolved that uniquely incorporates **1** in response to the TAG codon, as previously reported.[10] One MjTyrRS clone emerged after three rounds of positive selection and two rounds of negative selection (Supporting Information, Table S1). This clone grew at 120 μg mL^{-1} of chloramphenicol in the presence of 0.5 mM **1**, but only at 20 μg mL^{-1} chloramphenicol in its absence, and was named pyTyrRS. Sequencing of this clone revealed four mutations: Tyr32Asp, Leu65Thr, His70Gly, and Asp158Ala. The His70Gly and Asp158Ala mutations were created additional space to accommodate the pyrazole ring, and Tyr32 and Leu65 were each mutated to smaller hydrophilic amino acids, creating a larger binding pocket while providing the required hydrogen-bonding interaction to stabilize the pyTyr side chain.

To determine if **1** is incorporated into the protein with high efficiency and fidelity, an amber stop codon was substituted for Ser4 in sperm whale myoglobin or Tyr151 in GFP (GFP-151pyTyr). Protein expression was carried out in *E. coli* in the presence of the selected synthetase (pyTyrRS), MjtRNA$^{Tyr}_{CUA}$, and 0.5 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 and GFP were expressed only in the presence of **1** (Figure 2A), indicating

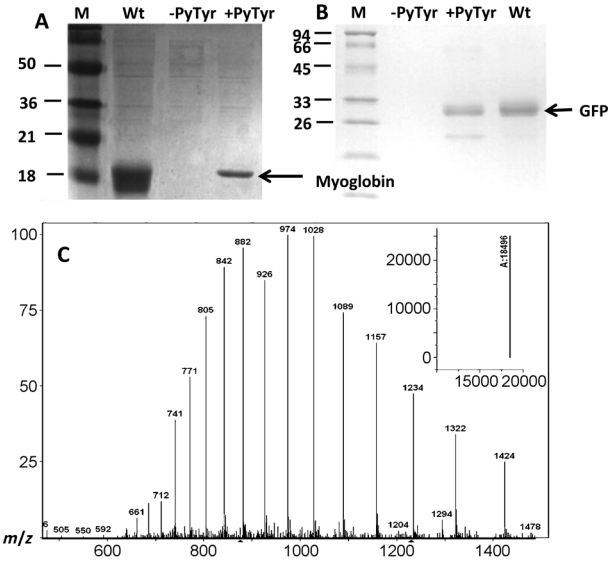


Figure 2. Coomassie-stained SDS-PAGE of TAG4 mutant myoglobin (A) or TAG151 GFP (B) expression in the presence and absence of 0.5 mM **1**. C) ESI-MS spectra of the myoglobin TAG4 mutant. Insert: deconvoluted mass spectrum; expected mass: 18496 Da, found: 18496 Da.

that pyTyrRS was specifically active for **1** but inactive for any of the natural amino acids. The yield for mutant myoglobin and GFP was 10 mg L^{-1} and 20 mg L^{-1}, respectively. For comparison, the yields of wild-type sperm whale myoglobin (wtMb) and GFP were 50 mg L^{-1} and 100 mg L^{-1}, respectively. ESI-MS analysis of the Ser4→**1** mutant myoglobin gave an average mass of 18496 Da (H^+), in agreement with the calculated mass of 18496 Da.

To examine how pyTyr binds a Cu^{II} ion, we determined the high-resolution crystal structure of GFP-151pyTyr, in both the absence and presence of Cu^{II} ions (Figure 1 and Tables S2–S3). Replacement of Tyr151 by pyTyr caused little perturbation to the overall structure of GFP. In the

absence of a Cu^{II} ion, pyTyr adopted a twisted conformation, with a dihedral angle of 30 degrees between its pyrazole and phenol rings (Figure 1B). We then soaked the GFP-151pyTyr crystal in 25 mM $CuCl_2$, and again determined its structure. A Cu^{II} ion binds specifically to pyTyr, coordinating to the phenol oxygen atom and pyrazole nitrogen atom, with a Cu–O and Cu–N distance of 1.9 Å and 2.0 Å, respectively (Figure 1C). The pyrazole ring, phenol ring, and copper atom were essentially coplanar.

We then measured the relative fluorescence intensity of 5 μM wild-type GFP, GFP-149pyTyr, GFP-151pyTyr, and GFP-182pyTyr in the presence of varying concentrations of Cu^{II} ions. As shown in Figure 3, addition of 5 μM Cu^{II} ions

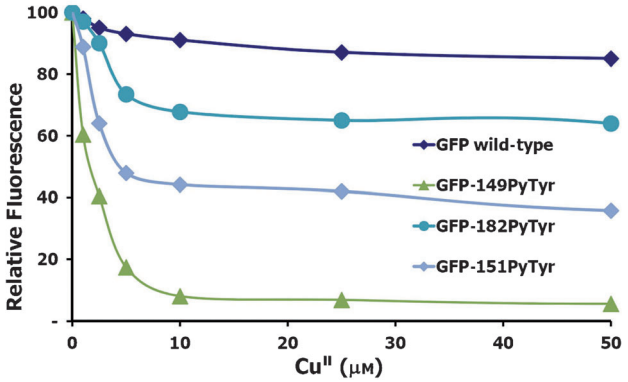


Figure 3. Relative fluorescence of 5 μM wild-type GFP, GFP-149pyTyr, GFP-151pyTyr, and GFP-182pyTyr in the presence of varying concentrations of Cu^{II} ions. These experiments were performed in 60 mM Tris-HCl pH 7.0 buffer. Fluorescence intensity was measured using $\lambda_{ex} = 397$ nm and $\lambda_{em} = 508$ nm.

resulted in 85%, 50%, and 25% quenching of GFP-149pyTyr, GFP-151pyTyr, and GFP-182pyTyr fluorescence, respectively. In contrast, wild-type GFP showed less than 5% quenching after treatment with 5 μM Cu^{II} ions. Substitution by pyTyr alone did not significantly affect the fluorescence quantum yield (Figure S3). We performed competitive metal-capture analysis[11a] by measuring the fluorescence intensity of GFP-149pyTyrCu in the presence of various Cu^{II} chelators with different binding affinity (methionine, ethylenediamine, His, bipyridine, and *N*-(2-hydroxyethyl)iminodiacetic acid (HIMDA)). A similar method was used to determine the binding affinity between amyloid beta peptides and Cu^{II}.[11a] Our results indicate that there is a high affinity Cu^{II} binding site in GFP-149pyTyr, with a K_D of 0.9 nM, higher affinity than that of bipyridine ($K_D = 3$ nM).[11a] Addition of the reductant potassium ferrocyanide to GFP-149pyTyrCu resulted in full recovery of GFP fluorescence (Figure S7). Reductive titration[11b] with potassium ferrocyanide revealed that GFP-149pyTyrCu had a reduction potential of 168 mV (Figure S8), close to that of $Cu^{2+} + e^- \rightarrow Cu^{+}$, which is 153 mV. These results indicate that pyTyr binds to Cu^{II} and Cu^{I} with similar binding affinities, according to the Nernst equation.

If there is light-induced electron transfer from GFP to a Cu^{II} ion in GFP-149pyTyrCu, then a Cu^{I} ion should be detected upon photoirradiation of GFP-149pyTyrCu. We detected Cu^{I} ions by using a Cu^{I} specific chelator, bathocuproine disulfonate (BCS).[11c] The stability constants for $Cu^{II}(BCS)_2$ are $\log K_1 = 6.1$ and $\log \beta_2 = 11.9$,[11c] indicating that BCS cannot compete with GFP-149pyTyr for Cu^{II} binding. BCS forms a specific-colored complex (2:1) with Cu^{I} ions, whose molar extinction coefficient at 483 nm is 12500, and has an overall association constant $\log \beta_2 = 19.8$.[11c] Therefore, BCS can effectively compete for Cu^{I} ions from GFP-149pyTyr. As shown in Figure S9, upon photoirradiation of 2 μM GFP-149pyTyr with a 405 nm laser in the presence of 5 μM Cu^{II} ions and 10 μM BCS, the absorbance at 480 nm rapidly increased then reached a plateau within ten minutes. Small molecules were then separated from protein using a PD-10 desalting column, confirming that the increase in 480 nm absorbance was due to the formation of 4.1 μM $Cu^{I}(BCS)_2$. Interestingly, for each mole of GFP-149pyTyr, two equivalents of Cu^{I} ions were produced, consistent with a previous report that GFP can undergo light-induced two-electron oxidation.[10] Dynamic fluorescence quenching was excluded since the quenching efficacy decreased as temperature increased, as shown in the Stern–Volmer plot (Figure S5). Fluorescence quenching through resonance energy transfer (RET) was also excluded, because there was no spectral overlap between the emission spectrum of GFP and the absorption spectrum of pyTyrCu (Figure S6). Collectively, these results suggest that the fluorescence quenching of the GFP mutants was caused by efficient PET from the GFP chromophore to pyTyrCu.

To resolve the rate and distance dependence of photoinduced electron transfer between the GFP chromophore and protein-bound Cu^{II} ions, the fluorescence lifetime was measured.[12] The fluorescence decay of GFP, with or without Cu^{II} ions, fit well with a single-exponential decay component. The fluorescence lifetime of GFP pyTyr mutants, but not wild-type GFP, decreased significantly in the presence of Cu^{II} ions (Figure 4 and Table 1), which allowed for calculation of the rate of ET (k_{ET}).[12] Remarkably, addition of Cu^{II} ions to GFP-149pyTyr caused a substantial reduction of the fluorescence

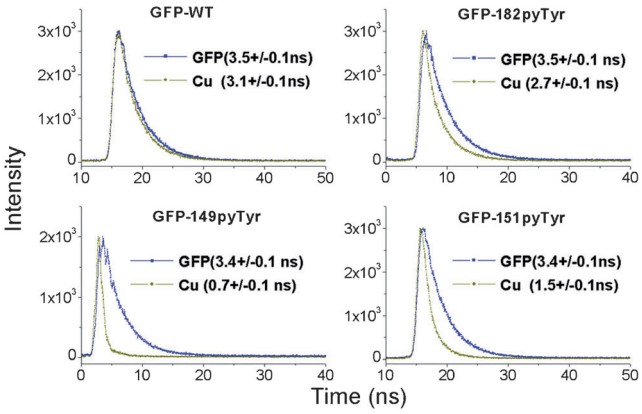


Figure 4. Fluorescence decay curves of 5 μM GFP-pyTyr in the absence or presence of 5 μM Cu^{II} ions. These experiments were performed in 60 mM Tris-HCl pH 7.0 buffer. The fluorescence lifetime measurements were carried out on a time-correlated single-photon-counting (TCSPC) spectrofluorometer (FL900 Edinburgh Instruments, Ltd.) using $\lambda_{ex} = 375$ nm and $\lambda_{em} = 510$ nm.

Table 1: GFP-pyTyr mutant fluorescence lifetimes and electron-transfer rate calculation.

Mutant	τ_1 [ns] without Cu^{II}	τ_2 [ns] with Cu^{II}	Distance [Å]	k_{ET}[a] [s^{-1}]
149pyTyr	3.4	0.7	8.9	1.13×10^9
151pyTyr	3.4	1.5	11.4	0.37×10^9
182pyTyr	3.5	2.7	13.1	0.08×10^9

[a] k_{ET} values were calculated from the fluorescence lifetime: $k_{ET}={\tau_2}^{-1}$(with Cu^{II})$-{\tau_1}^{-1}$ (without Cu^{II}).

lifetime from 3.4 ns to 0.7 ns, which corresponds to an electron transfer rate of $1.13\times10^9\ s^{-1}$. The ET rates of GFP-151pyTyrCu and GFP-182pyTyrCu were slower, at 0.37×10^9 and $0.08\times10^9\ s^{-1}$, respectively. The edge-to-edge distance from the GFP chromophore to the pyTyr151 residue was 11.4 Å, according to the crystal structure of GFP-151pyTyrCu (Figure 1). Edge-to-edge distances from the GFP chromophore to pyTyr149 or pyTyr182 residues (Table 1) was estimated by measuring the shortest distance between the GFP chromophore and the β-carbon of the Asn149 or Tyr182 residues, respectively. Together, these results demonstrate that PET from the GFP chromophore to pyTyrCu can occur rapidly, in less than one nanosecond, and that the PET rate decreases exponentially as distance increases (Figure S10 and Table 1).[1] The distance decay factor ($\beta=0.7\ Å^{-1}$) is smaller than those in the single-step tunneling reactions. Multistep electron tunneling (hopping) may be responsible for the smaller distance decay factor.[1i,2c] As electron transfer in β-strands occurs much faster than in other protein secondary structures,[1c] we propose that GFP, which consists predominantly of β-strands, may be optimized for ET reactions. Notably, the electron transfer rate from the GFP chromophore to a protein-bound Cu^{II} ion is only 10–100-fold less than the rate for the primary ET reactions of photosynthesis,[1g] and faster than that of a protein mimic of photosystem II.[12]

To estimate PET efficiency, the change in free energy for charge separation can be calculated using the Rehm–Weller formula given in Equation (1)[5b]

$$\Delta G(\mathrm{eV}) = E_{ox}(D^+/D) - E_{red}(A/A-) - E_{0,0} + C \quad (1)$$

where E_{ox} is the first one-electron oxidation potential of the electron donor, E_{red} is the first one-electron reduction potential of the electron acceptor, $E_{0,0}$ is the energy of the zero–zero transition to the lowest-excited singlet state, C is the solvent-dependent Coulombic attration energy and is often neglected in polar solvents. E_{ox} of the GFP chromophore has not been experimentally determined, but it should be close to the E_{ox} of tyrosine (0.94 V). E_{red} of pyTyrCu is 0.168 V (Figure S8). The driving force of PET from the GFP chromophore to pyTyrCu was estimated to be around −2 eV.

In conclusion, we have demonstrated the highly efficient synthesis and site-specific incorporation of a new metal-chelating non-natural amino acid, pyTyr **1**. As the synthetic route of **1** consists of only two steps using only environmentally benign reagents, and no column purification is necessary, it can easily be prepared in any biology or chemistry laboratory. We have solved the crystal structure of GFP with pyTyr incorporated at a specific position, in the presence and absence of Cu^{II} ions, which revealed the structural basis for the strong binding of pyTyr to Cu^{II}. We showed that PET from the GFP chromophore to pyTyrCu can occur rapidly, within one nanosecond, suggesting that the GFP β-barrel may be optimized for the ET reaction. In vivo, GFP may form complexes with other proteins and exert photocontrol over cellular processes. This new genetically encoded metal-chelating amino acid could also aid the rational design of metalloproteins,[1e,13,14] as well as protein NMR spectroscopy using paramagnetic ions.[14c]

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