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ARTICLE

Intra-electron transfer of amicyanin from newly derived active site to redox potential tuned type 1 copper site

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Abstract Amicyanin, one of the type I copper proteins which has been used for the study, mediates the electron transfer reaction between methylamine dehydrogenase and cytochrome c-551i in *Paracoccus denitrificans* for energy production. The 6×Histidine-tag site which has been widely used in purification of a recombinant protein was introduced at the N-terminus of amicyanin to make the complex of 6×His-tagged plus cobalt functioning as a newly derived redox cofactor in amicyanin. In this study, Pro94 of amicyanin was substituted to Ala and Phe to tune up the midpoint potential (E_m) value of amicyanin 100 mV more positive and then intra-electron transfer rates were measured to examine whether the E_m value of the type 1 copper site in amicyanin affects intraprotein electron transfer or not. By the addition of H_2O_2, the Co^{2+}-loaded 6×His-tagged site was activated, and then electron was transferred from Cu^{1+} of type 1 copper site of amicyanin to Co^{3+} plus 6×His-tagged site. Electron transfer rates of cobalt loaded P94A and F amicyanin were much slower than that of native amicyanin. These results suggest that the communication between the newly protein-derived redox cofactor, 6×His-tagged site plus cobalt, and type 1 copper site is truly occurred and that the strength of electron transfer reaction between them is able to be controlled by an E_m value.

Keywords Amicyanin · Cofactor · Protein engineering · Redox potential · 6×His-tag

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Introduction

Protein engineering techniques have revolutionized many areas of the biological sciences such as chemical processing, bioremediation, and pharmaceuticals. Antibody drugs, fluorescence fused protein, and new enzymes with new or desirable functions are good examples of entities created by protein engineering. In a previous study, we tried to craft a protein to derive a new redox active cofactor for enzymatic catalysis and electron transfer [1]. The 6×-Histidine tag was introduced into the N-terminus amicyanin (Fig. 1A) mediating electron transfer from the methylamine dehydrogenase (MADH) to the cytochrome c-551i in *P.denitrificans* for bioenergetic processes [2–4]. Adding cobalt to a 6×-Histidine tag, which is generally used for recombinant protein purification, plus H_2O_2 mediates intra-electron transfer in amicyanin [1]. The idea is that the Co^{2+}-loaded 6×-Histidine tag is oxidized by the H_2O_2 addition and then it transiently starts to oxidize the reduced copper of amicyanin.

Amicyanin is a type 1 copper protein containing a single copper in its active site. Type 1 copper site mainly functions as mediators for electron transfer in wide ranges of biological systems in human, bacteria, and plants.

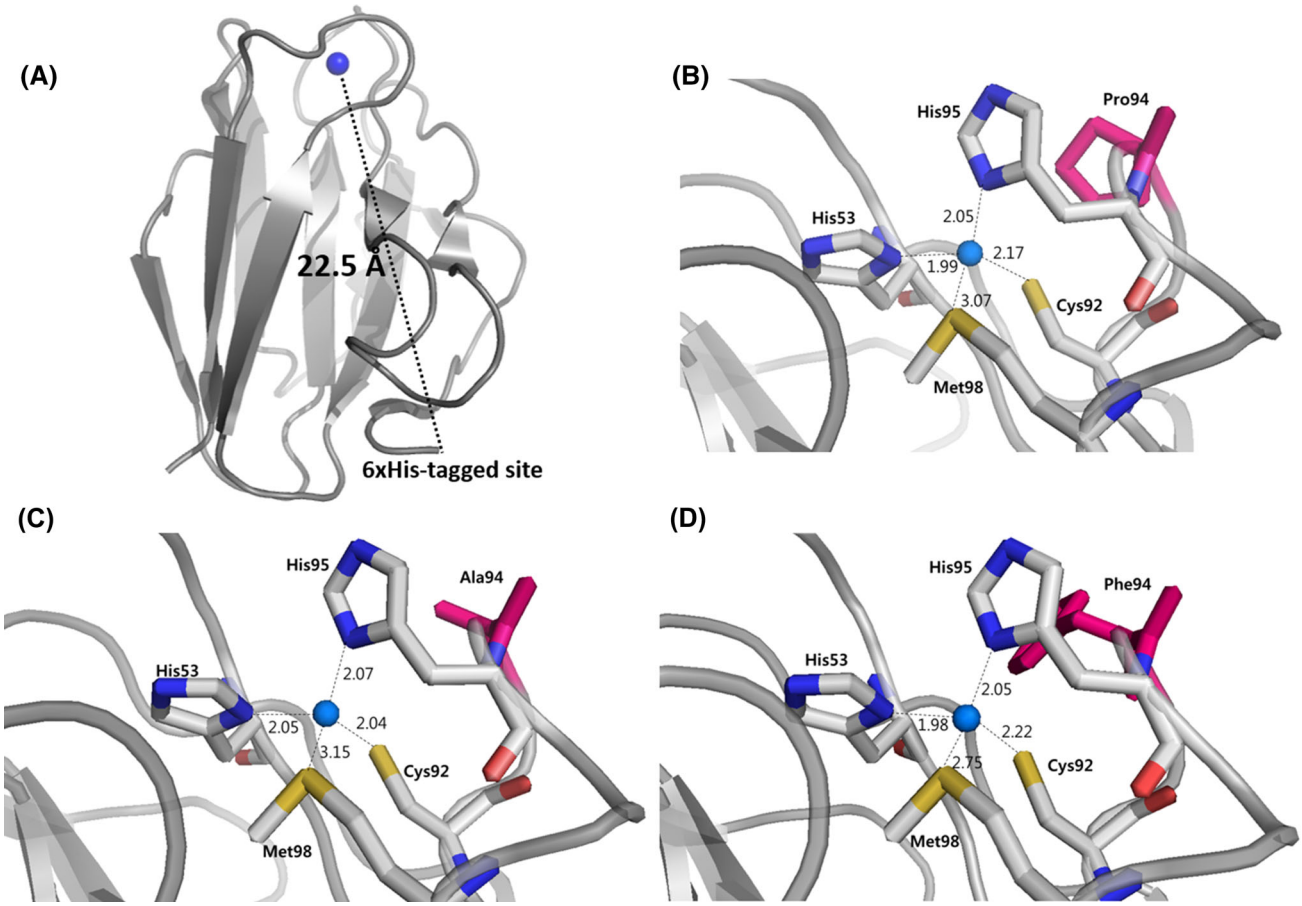

Fig. 1 Structure of amicyanin with 6×His-tagged site and stereoview near the copper site. Copper coordination distances are indicated for wild type amicyanin (PDB: 2OV0) in (**B**), P94A amicyanin (PDB:1SF5) in (**C**), and P94F amicyanin (PDB:1SFD) in (**D**). Atom colors: oxygen is red, nitrogen is blue, and sulfur is yellow. This figure was produced using PyMol (https://pymol.org/2/)

Amicyanin [2], azurin [5], and rusticyanin [6] from bacteria, plastocyanin [7] and stellacyanin [8] from plants and algae, as well as ceruloplasmin [9] from mammals are examples of members of type 1 copper proteins (Table 1). Even though most of type 1 copper proteins exhibit highly conserved structures containing mainly 8–9 β-strands, the oxidation–reduction midpoint potential (E_m) values of type 1 copper site in various proteins are very different ranging from + 184 mV to over + 700 mV (Table 1) due to structural constrains for preference of Cu^{2+} and Cu^{1+} status of copper in proteins [10], axial ligand bond distance [11], and hydrophobic environment [12] around copper. Copper in amicyanin is ligated by a Cys, two His, and a Met residues and it emits an intense blue color and strong absorption spectrum centered at 595 nm caused by a $S(Cys)\pi \rightarrow Cu(II)d_{x2-y2}$ ligand-to-metal charge transfer transition [13]. Oxidation–reduction midpoint potential (E_m) value of amicyanin is + 294 mV which is more positive than that of the Cu^{2+}/Cu^{+} aqua couple.

Table 1 Examples of type 1 copper proteins and their E_m values

	Examples (Source)	E_m (mV)
Type 1 copper proteins	Amicyanin (bacteria)	+ 294
	Azurin (bacteria)	+ 276
	Rusticyanin (bacteria)	+ 680
	Plastocyanin (plants)	+ 370
	Stellacyanin (plants)	+ 285

The E_m value of amicyanin is finely tuned by site-directed mutagenesis studies of specific residues in amicyanin. Pro94 is one of the target residues to be changed E_m values. Pro94 lies within the hydrophobic patch and the ligand loop of amicyanin. When Pro94 was substituted to Ala and Phe, P94A and F mutant amicyanins exhibited an increased E_m value by 150 and 120 mV at pH 7.5 (Table 2), respectively, due to changing hydrogen bonds patterns around the thiolate of the Cys ligand of copper and copper coordination distances which influenced the E_m value (Fig. 1) [14]. In this study, P94A and F mutant amicyanins are used to see how E_m value affects the intraprotein

Table 2 E_m values of amicyanins

Protein	pH 7.5 pH 8.0	
	E_m (mV)	E_m (mV)
Amicyanin	+ 294	+ 265
P94A amicyanin	+ 444	+ 380
P94F amicyanin	+ 414	+ 415

electron transfer over 20 Å distance. It illustrates additional proof that the Co^{2+}-loaded 6×His-tagged site functioned as redox center and suggests the possibility of controlling the efficiency of redox active site for the reaction. It provides insight into the understanding of the underlying physics and chemistry of electron flow through intra- or inter-proteins which is very important in photosynthetic and respiratory machinery in advance.

Materials and methods

Protein preparation The *mauC* gene [15] encoding amicyanin was cloned into the pUC19 vector containing the Lac promotor system and a 6×His-tag was inserted by Phusion site-directed mutagenesis between the sequence of N-terminus amino acid and the native signal sequence of the gene which directs an expressed protein into the periplasmic space. P94A and F mutation also were generated by Phusion site-directed mutagenesis. The *mauC*-containing plasmid was sequenced to ensure that there was not any presence of additional mutations. Primers are listed in Table 3. 6×His-tagged amicyanins were expressed in BL-21(DE3) *E. coli*. 1-L flask was wet up to let the cells grow overnight at 37 °C. The cells in 1-L flask were induced with 1 mL of 1 M isopropyl-β-D-1-thiogalactopyranoside (IPTG from Fisher Scientific–Hampton, NH, USA) for 4 h and then, the cells were harvested by centrifugation at 10,000 rpm for 25 min, at 4 °C. Cell pellets were suspended in SET Buffer (200 mM Trizma from Biorad–Hercules, CA, USA, at pH 8.0, 0.5 M sucrose from Sigma-Aldrich–St. Louis, Missouri, USA, and 0.25 g/L EDTA from Sigma-Aldrich). The cell suspension was treated with 0.1 g lysozyme(Sigma-Aldrich) that was predissolved in 10 mL distilled water, 0.2 mg DNAse I (Sigma-Aldrich), and a few drops of 1 M $MgSO_4$ (Sigma-Aldrich) or $MgCl_2$ (Sigma-Aldrich). The cells were centrifuged at 13,000 rpm for 30 min, and the supernatant was collected. The supernatant containing the periplasm was incubated in a 30 °C waterbath for 5 min. Slowly, 1 M $CuSO_4$ (Sigma-Aldrich) was added to the supernatant until the final $CuSO_4$ concentration reaches 100 μM [16]. This periplasmic fraction was subjected to fast protein liquid chromatography (FPLC, Biorad NGC system) with nickel nitrilotriacetic acid column (Biorad, Bio-Scale™ Mini Nuvia™ IMAC Cartridges). The column was equilibrated at 0.5 mL/min with 0.05 mM potassium phosphate (Biosolution–Seoul, South Korea) at pH 7.5. The 6×His-tagged wild type (WT) amicyanin and P94A and F mutant amicyanins were eluted from the column with 0.05 M potassium phosphate buffer (Biosolution) at pH 7.5 containing 100 mM imidazole (Sigma-Aldrich) and 300 mM NaCl (Sigma-Aldrich). The average yield of purified 6×His-tagged amicyanin was about 8.2 mg/g for WT, 4.7 mg/g for mutant amicyanins wet weight cells. The proteins were subjected to 12% SDS-PAGE to check expression and purity of them (Fig. 2).

Single-turnover kinetic experiments The redox state of the copper of amicyanin was monitored by a Perkins Elmer UV–Vis Spectrophotometer Systems. The Cu^{2+} in amicyanin possesses an $\varepsilon = 4600\ M^{-1}\ cm^{-1}$, otherwise the Cu^{1+} in amicyanin exhibits no color. The electron transfer reaction from cobalt bound to 6×His tag site to copper in the active site of amicyanin was measured in 0.05 M Tris–HCl buffer at pH7.5. 100 μM of $CoCl_2$ (Sigma-Aldrich) was used to treat 40 μM of 6×His-tagged and non-tagged amicyanins for 30 min, and absorbance changes were monitored at 330 nm. Any excess amount of cobalt was removed by a buffer change using centrifugal filter (Millipore–Burlington, MA, USA). 40 μM of amicyanins in cuvette were reduced by addition of the same equivalent of ascorbate (Sigma-Aldrich). 1 mM of hydrogen peroxide (Sigma-Aldrich) was added on cobalt attached amicyanins to initiate the reaction. The reactions were monitored between 500 and 700 nm to observe the oxidation of the Cu^{1+} to the Cu^{2+} of amicyanin. The observed rates (k_{obs}) at 595 nm were best fit to a single exponential increase.

Table 3 Primers for site-directed mutagenesis

	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Product size (GC)
P94A	F: TGCACC**GCA**CATCCCTTC	69.0	18nt (61%)
	R: GTGATAGTCATAGGTGCCGGCC	68.8	22nt (59%)
P94F	F: CACTGCACC**TTT**CATCCCTTC	66.1	21nt (53%)
	R: ATAGTCATAGGTGCCGGCCTC	66.5	21nt (57%)

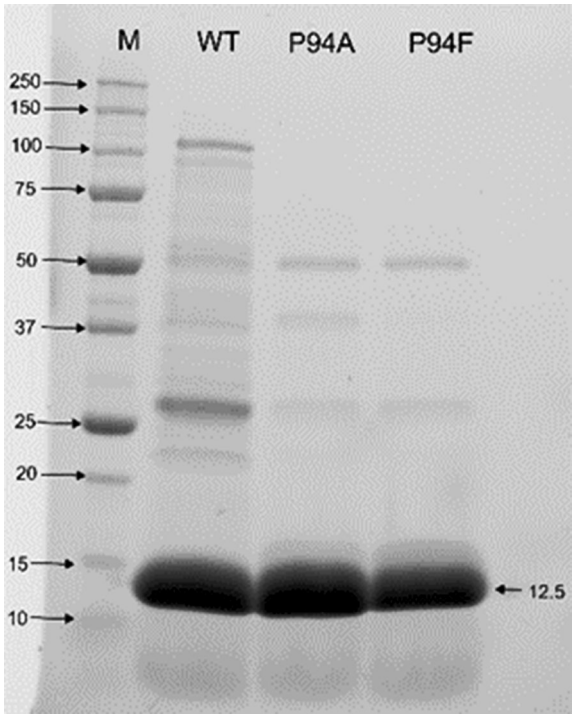

Fig. 2 SDS-PAGE results of amicyanins. The samples of WT, P94A, and P94F were loaded to electrophoresis on 12% gel containing sodium dodecyl sulfate(SDS). Amicyanins were observed between 10 kDa and 15 kDa. Image is exported by ChemiDoc from Biorad. M: Molecular weight marker

Results and discussion

Co^{2+}-loaded on 6×His-tagged site of wild type, P94A, P94F amicyanins

The reactivity of the 6×His-tagged amicyanins toward the natural electron donor, MADH [3], and the electron acceptor, cytochrome *c*-551i [17], was identical to that of non-6×His-tagged amicyanins (data not shown). To check the redox properties of 6×His-tagged P94A and P94F amicyanins, the reductive titration was performed and measured redox potentials. To fully reduce P94A and P94F amicyanin, approximately 2–3 folds less reductant ascorbate was required rather than wild type amicyanin (Fig. 3). These results suggest that redox potential of P94A and P94F amicyanin is more positive than that of wild type amicyanin so that it is easy to reduce P94A and P94F amicyanin with small amount of ascorbate. The measured E_m values of 6×His-tagged P94A and P94F amicyanins were 402 ± 4 and 435 ± 5 mV at pH 7.5, respectively. These values are almost identical to the non-tagged P94A and P94F amicyanin [14, 18].

The 6×His-tagged wild type, P94A, and P94F amicyanins were incubated with an excess amount of $CoCl_2$ to load Co^{2+} on the 6×His-tagged site to generate Co^{2+}-6×His-tagged complex. The incubation led to an absorbance increase around 330 nm region (Fig. 4A). The absorbance around 600 nm that corresponded to the Cu^{2+} site on amicyanin was not affected at all. Co^{2+} binding to histidine might be comparable to the coordination that is seen when His-tagged proteins bind to the metal of the Ni–NTA affinity resin using for protein purification [19]. Meaning of this spectral change is an assessment of Co^{2+} binding on the 6×His-tagged site of amicyanins [20]. A similar spectral evidence of dinuclear Co^{2+}–Co^{2+} engineering protein having the ligands provided by His residues was already presented [21, 22]. The amount of binding Co^{2+} on all three amicyanins is likely to be similar (Fig. 4B). The unbound Co^{2+} was completely excluded by buffer exchange.

The intra-electron transfer from the Co^{2+}-loaded on 6×His-tagged site to the Cu^{1+} of wild type, P94A, and P94F amicyanins

Cu^{1+} state of amicyanins is formed by addition of ascorbate [23]. It is pretty stable, so it is hard to be reoxidized to Cu^{2+} by air. When ascorbate was added to Co^{2+}-loaded 6×His-tagged wild type, P94A, and P94F amicyanins, the

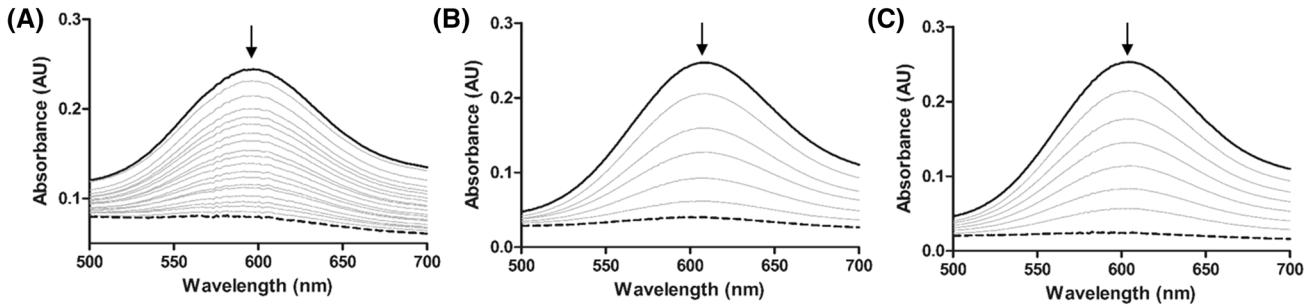

Fig. 3 Reductive titration of amicyanins by ascorbate. Absorption spectra corresponding to reduction of 60 μM of wild type (**A**), P94A (**B**), P94F (**C**) were monitored by each addition of 5 μM of ascorbate in 0.05 M Tris–HCl buffer at pH 7.5. The buffer solution containing amicyanin was titrated by addition of incremental amounts (1 μL of 10 mM ascorbate) of reductant and then the corresponded absorption spectrum was recorded each time. The arrows indicate the direction of reduction

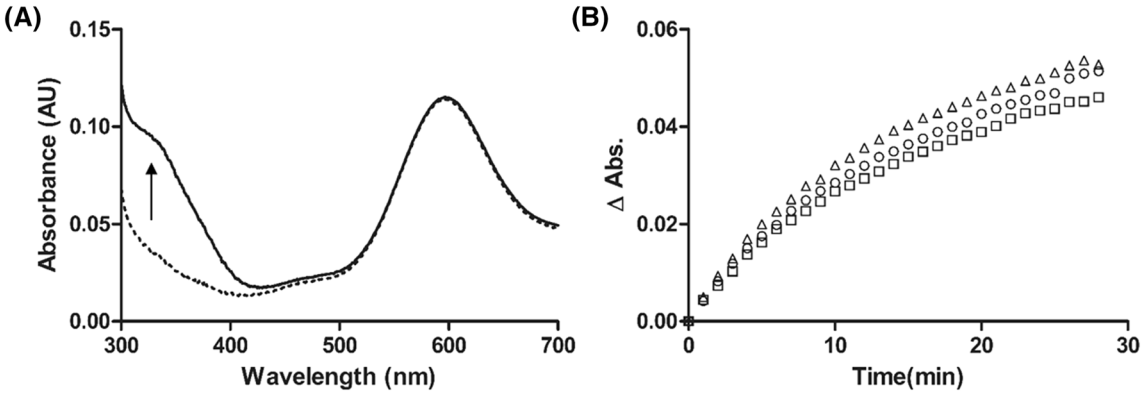

Fig. 4 Spectral changes corresponding to cobalt addition on 6×His-tagged amicyanins. Overall spectral changes before and after adding $CoCl_2$ on 6×His-tagged amicyanins were indicated in (**A**). The arrow indicates the direction of absorption increase. 40 μM of 6×His wild type, P94A, P94F was mixed with 100 μM of $CoCl_2$ in 0.05 M Tris–HCl buffer at pH 7.5, Spectral changes associated with cobalt loading to amicyanin around 330 nm was recorded every 1 min for 30 min. Direct absorbance changes at 330 nm of amicyanins 6×His-tagged wild type (open-triangle), P94A (open-circle), and P94F (open-square) are shown in (**B**)

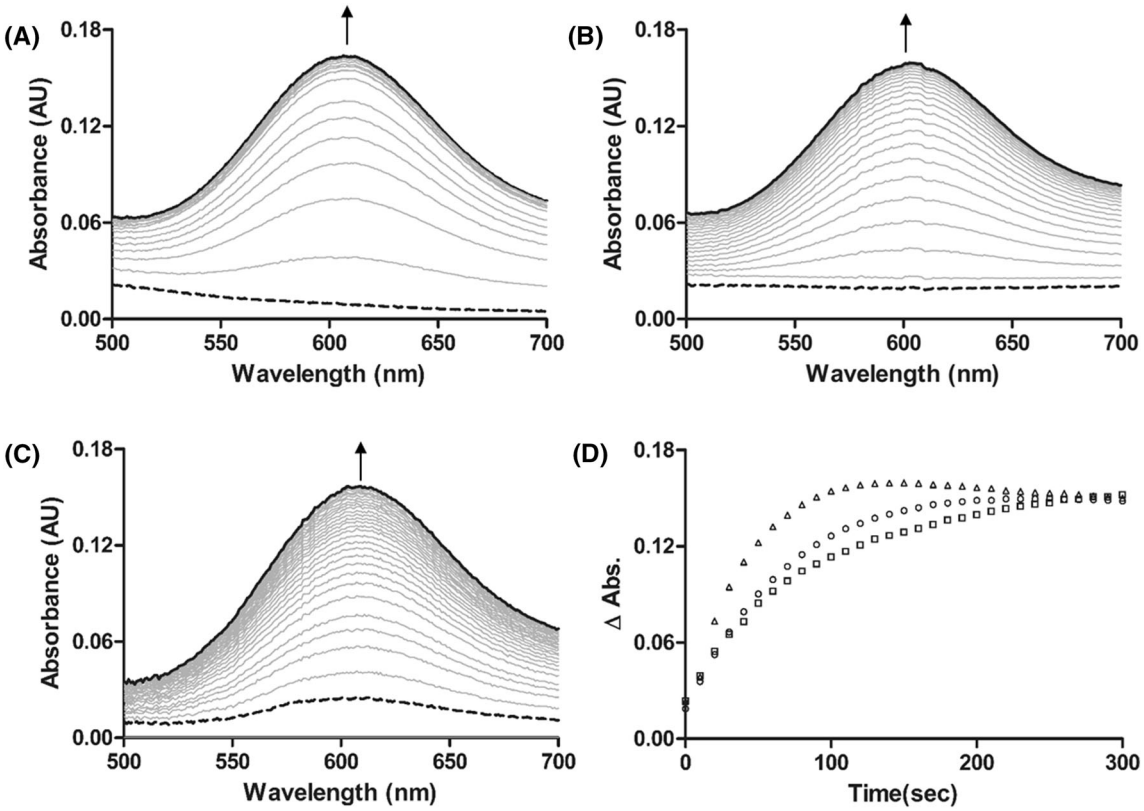

Fig. 5 Spectral changes of Cu^{1+} oxidation by Hydrogen Peroxide. 1 mM H_2O_2 was added on the reduced cobalt loaded 6×His-tagged wild type (**A**), P94A (**B**), P94F (**C**) amicyanins. Direct changes in absorbance at 595 nm of wild type (open-triangle), P94A (open-circle), and P94F (open-square) amicyanins taken from the data shown in (**A**), (**B**), and (**C**) were indicated in (**D**). The data were obtained with 40 μM amicyanin in 0.05 M Tris–HCl buffer at pH 7.5

strong absorption at 600 nm due to the Cu^{2+} state of amicyanins decreased and finally was completed bleached out (Fig. 5). These results indicate that the Cu^{1+} state of the Co^{2+}-loaded 6×His-tagged amicyanins is formed properly. It was observed that the absorbance around 600 nm reappeared by the addition of H_2O_2 to the reduced Co^{2+}-loaded 6×His-tagged wild type, P94A, and P94F amicyanins indicating that intra-electron transfer occurred from Cu^{1+} in type 1 copper site to the oxidized cobalt (Co^{3+}) induced by H_2O_2 in 6×His-taged amicyanins. The

Table 4 Observed electron transfer rate constant of Cu^{1+} oxidation

	Wild type	P94A	P94F
k_{obs} (s^{-1})	0.0283 ± 0.0014	0.0159 ± 0.0003	0.0117 ± 0.0003

absorbance changes at 600 nm for Co^{2+}-loaded 6×His-tagged wild type, P94A, and P94F amicyanins after adding H_2O_2 are shown in Fig. 5. The data fit very well to a single exponential increase to give electron transfer rate constant. The intraprotein electron transfer rate of the Co^{2+}-loaded 6 × His-tagged wild type amicyanin is the fastest exhibiting a k_{obs} of 0.028 s^{-1} for the WT amicyanin as compared to 0.016 and 0.012 s^{-1} for the P94A and P94F amicyanins, respectively (Table 4). A previous study illustrated the two step mechanism of H_2O_2 interaction with Co^{2+}-loaded 6×His-tagged site [1]. It is proposed that the initial step is the formation of a high-valent peroxo-cobalt (Co^{3+}) and the second step is the rate-limiting intraprotein electron transfer from Cu^{1+} to Co^{3+}. The E_m values for the Cu^{1+}/Cu^{2+} for wild type [24], P94A, and P94F amicyanin are 294, 402, and 435 mV, respectively. Even if an exact E_m value of Co^{3+}/Co^{2+} is not known, the ΔE_m providing driving force of intraprotein electron transfer reaction from Cu^{1+} to Co^{3+} was getting smaller in the order of P94F, P94A, and wild type amicyanins resulting in slower intraprotein electron transfer rate of P94F and P94A amicyanin. This result directly indicates that finely tuned E_m value of type 1 copper center of amicyanin affects intraprotein electron transfer. It suggests that the Co^{2+}-loaded 6×His-tagged site works well functionally for an electron transfer over 20 Å in distance and that the efficiency of the electron transfer was able to be controlled by tuning the redox potential of type 1 copper site.

In conclusion, to introduce the redox active species at specific sites in proteins, it requires large amount of energy and efforts. The results represented here illustrate the usage of simple and less time-consuming method that can be used to introduce a redox site on protein. This study provides additional proof that the Co^{2+}-loaded 6×His-tagged site functioned as a redox center and further underscores the possibility of controlling the efficiency of the redox active site for the reaction by tuning E_m value. This study must be meaningful for the understanding of the underlying physics and chemistry of electron flow through intra- or inter-proteins which is very important in photosynthetic and respiratory machinery in advance.

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