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ORIGINAL PAPER

Spectroscopic Analysis of the Cu^{2+}-Induced Fluorescence Quenching of Fluorescent Proteins AmCyan and mOrange2

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Abstract

Fluorescent proteins show fluorescence quenching by specific metal ions, which can be applied towards metal biosensing applications. In order to develop metal-biosensor, we performed spectroscopic analysis of the fluorescence quenching of fluorescent protein AmCyan and mOrange2 by various metal ions. The fluorescence intensity of AmCyan was reduced to 48.54% by Co^{2+} and 67.77% by Zn^{2+}; Cu^{2+} reduced the fluorescence emission of AmCyan to 19.30% of its maximum. The fluorescence intensity of mOrange2 was quenched by only Cu^{2+}, to 11.48% of its maximum. When analyzed by Langmuir equation, dissociation constants for AmCyan and mOrange2 were 56.10 and 21.46 μM, respectively. The Cu^{2+} quenching of AmCyan and mOrange2 were reversible upon treatment with the metal chelator EDTA, indicating that the metal ions were located on the protein surface. Their model structures suggest that AmCyan and mOrange2 have novel metal-binding sites.

Keywords Metal-biosensor · AmCyan · mOrange2 · Fluorescence · Quenching · Emission

Introduction

The detection and quantification of heavy metals are important for environmental monitoring and biological and clinical toxicology [1]. Metals such as copper (Cu) and zinc (Zn) are essential for biological functions, but if they exceed safe levels, they adversely affect human health [2, 3]. A variety of biomolecules (e.g., peptides, proteins, enzymes, antibodies, and nucleic acids) and whole cells have been proposed as heavy metal biosensors [1].

Fluorescent proteins, which have also been proposed as biosensors, change fluorescence intensity based on cues in the surrounding environment, such as pH and the concentration of metal ions [4, 5]. Metal ions induce fluorescence quenching by static quenching, energy transfer between the binding metal and the chromophore, or perturbations of the protein structure [6–8]. For this reason, the fluorescence intensity of fluorescent proteins may be reduced, depending on the specific transition metal environment, during in vivo experiments [8], which may cause experimental errors. On the other hand, this phenomenon can be applied for the development of fluorescent protein-based metal biosensor materials. In fact, several fluorescent proteins, including *Discosoma* sp. red fluorescent protein (DsRed), iq-Emerald, Dronpa, and green fluorescent protein (GFP), have been investigated for metal-induced fluorescence quenching and suggested as optical biosensors for metal quantitation [5, 8–11].

AmCyan is engineered from the *Anemonia majano* cyan fluorescent protein with two amino acid substitutions (N34S and K68M) that enhance its fluorescence emission (λ_{ex} = 458 nm and λ_{em} = 489 nm) [12]. The fluorescent protein mOrange is an orange fluorescent protein derived from DsRed [13], and mOrange2 was engineered from mOrange with four amino acid substitutions (Q64H, F99Y, E160K, and G196D). The photostability of mOrange2 under arc-lamp illumination is over 25-fold greater than that of mOrange [14]. Since the two fluorescent proteins are widely used together in various molecular and cell biology

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experiments, such as protein localization and fluorescence resonance energy transfer studies, it is necessary to study their metal-induced fluorescence quenching in combination. Furthermore, their utility as metal biosensors has not been studied.

To investigate the effect of metals on the fluorescence of AmCyan and mOrange2, we performed spectroscopic analysis of AmCyan and mOrange2 in the presence of various metal ions. In order to investigate their applicability as metal biosensors, we performed metal titration experiments and analyzed the ability of their fluorescence to recover after quenching. To better understand their metal-binding sites, we performed molecular modeling, which we compared with previously reported fluorescent protein–metal structures. Our result will be useful for future applications in molecular biology and for the development of metal biosensors.

Materials and Methods

Protein Preparation

Expression vector encoding pAmCyan (Clontech) and pmOrange2 (Clontech) was transformed separately into *Escherichia coli* BL21(DE3). We grew the cultures under shaking at 37 °C until the OD_{600} reached 0.6–0.8, and then added 0.5 mM isopropyl β-D-thiogalactopyranoside to induce protein expression at 18 °C for 18 h. After centrifugation at 3515×*g* for 30 min, the harvested cells were suspended in lysis buffer containing 50 mM Tris–HCl (pH 8.0) and 100 mM NaCl. After sonication, the supernatant was heat-treated at 50 °C for 30 min to denature the soluble protein from the expression host [15], and then centrifuged at 17,364×*g* for 30 min. We concentrated the supernatants and performed size-exclusion chromatography in the buffer containing 10 mM Tris–HCl (pH 8.0) and 100 mM NaCl using the Sephacryl 100-HR column (GE Healthcare). The eluted fractions were concentrated with Amicon Ultra-15 filters (Merck Millipore). The purities of AmCyan and mOrange2 were verified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the protein concentrations were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific).

Spectroscopic Analysis

All fluorescence intensities were measured in 96-well plates using the Synergy™ H1 microplate reader (BioTek). AmCyan and mOrange2 were fluorescently excited at 435 and 505 nm, respectively. The fluorescence emissions of AmCyan and mOrange2 were measured at 535 and 574 nm, respectively. All experiments were performed in triplicate at 25 °C.

Metal Ion Screening

We determined the effects of metals on the fluorescence emissions of AmCyan and mOrange2 by adding 1 mM of various metal ions (LiCl, NaCl, $MgCl_2$, KCl, $CaCl_2$, $MnCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$, and $ZnSO_4$) to 10 μM of each fluorescent protein in 10 mM of Tris–HCl buffer (pH 8.0) and 5 mM NaCl. We incubated the mixtures for 10 min, and then measured the fluorescence intensities of the samples.

Analysis of Time-Resolved Fluorescence Intensities

We measured the time-resolved fluorescence intensities of 10 μM fluorescent proteins mixed with 1 mM Cu^{2+} for 20 min at 1-min intervals. The first fluorescence intensity was recorded after 1 min. Orbital-shaking was performed for 10 s before each measurement.

Cu^{2+} Titration and Langmuir Equation

For the titration experiments, we mixed 10 μM fluorescent proteins with 0–1.0 mM $CuSO_4$ solution. The relative fluorescence quenching is defined as 1-Fluorescence intensity at an indicated concentration of Cu^{2+}/Fluorescence intensity without Cu^{2+}. The relative fluorescence quenching of AmCyan or mOrange2 by Cu^{2+} was analyzed by fitting of Langmuir equation to experimental data using SigmaPlot software (version 12. 3, Systat), as follows [16].

$$\text{Relative fluorescence quenching} = \frac{B_{\max}[Cu^{2+}]}{K_d + [Cu^{2+}]},$$

where B_{max} is the maximum registered fluorescence, K_d is the dissociation constant (μM), and $[Cu^{2+}]$ is the concentration of Cu^{2+} (μM). Theoretically, the strength of interactions between protein and a ligand is inversely correlated with the value of K_d.

Reversibility of Fluorescence Quenching

The reversibility of the fluorescence quenching of the fluorescent proteins was assessed using the metal-chelating agent ethylenediaminetetraacetic acid (EDTA). We mixed 50 μL of 10 μM fluorescent proteins with 50 μL of 1 mM $CuSO_4$ solution, and incubated the mixtures for 10 min. Next, we added 50 μL of EDTA (final concentration 0.25, 1.0, 5.0, and 50 mM) to the sample and incubated for an additional 10 min.

Structural Modeling of AmCyan and mOrange2

We developed structural models of AmCyan and mOrange using the SWISS-MODEL server (http://swissmodel.expas

y.org/) in automated mode. The homology models for AmCyan and mOrange2 were constructed from the crystal structures of the GFP-like fluorescent chromoprotein amFP486 (PDB code: 2A46) and PAmCherry1 protein (PDB code: 3NED), respectively. We compared the putative metal-binding sites with Dronpa–Cu^{2+} (5HZT) [5] and iq-Emerald–Zn^{2+} (4KW9) [8]. Visual representations of the modeled structures were created with the molecular graphics software PyMOL (http://www.pymol.org).

Results and Discussion

Spectroscopic Analysis of Quenchable Metal Ions

We conducted spectroscopic studies to investigate if AmCyan and mOrange2 were fluorescently quenched by various metal ions. The fluorescence intensity of AmCyan was unaffected by Li^+, Na^+, Mg^{2+}, K^+, Ca^{2+}, Mn^{2+}, and Ni^{2+}. Na^+ increased its fluorescence emission by 10%, whereas Co^{2+} and Zn^{2+} reduced its fluorescence intensity by 40 and 50%, respectively (Fig. 1A). Cu^{2+} was the most effective metal for quenching the fluorescence of AmCyan, as it caused an 80% reduction in intensity. Cu^{2+} quenched the fluorescence intensity of mOrange2 by 89%. Ni^{2+} and Zn^{2+} reduced its fluorescence intensity by 7%, whereas the other metal ions did not affect its fluorescence intensity (Fig. 1B). Thus, Cu^{2+} most effectively quenched the fluorescence of AmCyan and mOrange2, and Co^{2+} and Zn^{2+} reduced AmCyan intensity to a lesser extent.

Next, to confirm that the fluorescence quenching persisted over time, we performed time-resolved fluorescence measurements in the presence of Cu^{2+} at 1-min intervals for 20 min. We found that AmCyan fluorescence decreased to 9.5%, and to then 8.8%, of the maximum after 1 and 5 min, respectively, before gradually increasing to 9.5% (Fig. 1C). These findings indicate that Cu^{2+} and AmCyan reached equilibrium. In the case of mOrange2, Cu^{2+} caused quenching to 14.2% of the maximum fluorescence after 1 min, followed by continuous decreases to 11.6% of the maximum after 20 min (Fig. 1D). Although the concentration of Cu^{2+} was 100× higher than that of the fluorescent proteins, complete fluorescence quenching did not occur within 20 min. As a result, AmCyan and mOrange2 were mostly fluorescence-quenched

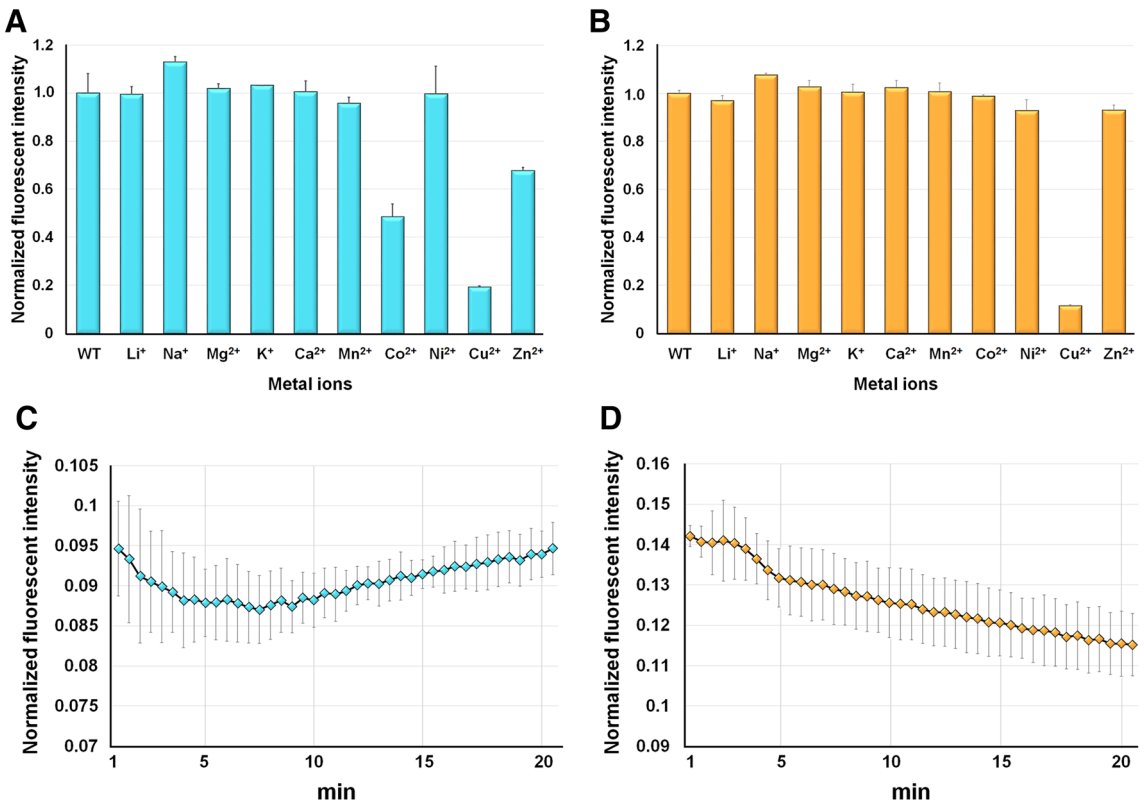

Fig. 1 Screening of AmCyan and mOrange2 for fluorescence quenching by metal ions. Fluorescence quenching effects of various metal ions (1 mM) on 10 µM (**A**) AmCyan and (**B**) mOrange2. Cu^{2+} ions significantly induced the fluorescence intensity for both fluorescent proteins. The measurement of the time-resolved fluorescence intensity of (**C**) AmCyan and (**D**) mOrange2 by Cu^{2+} ions. The concentration of metal was 100× higher than that of the fluorescent protein, but complete fluorescence quenching did not occur within 20 min. Data represent means ± standard deviations of three replicates

within 1 min. However, afterwards, AmCyan was observed to have slightly increased fluorescence intensity after 5 min, while mOrange2 was steadily decelerated. We consider this difference to be due to the binding affinity between the fluorescent protein and the Cu^{2+}.

Cu^{2+} Titration and Langmuir Equation

AmCyan and mOrange2 were titrated with 0–1 mM Cu^{2+}. AmCyan was quenched by approximately 10% when a similar concentration of Cu^{2+} was added, and by approximately 50% in the presence of 10× Cu^{2+} (Fig. 2A). When we added approximately 100× Cu^{2+}, we observed more than 90% quenching. In the case of mOrange2 (Fig. 2B), approximately 25% of its fluorescence was quenched by a similar concentration of Cu^{2+}, 50% by approximately 3× Cu^{2+}, and more than 80% by 100× Cu^{2+}. Therefore, at low concentrations of Cu^{2+}, mOrange2 was more sensitive to quenching than AmCyan.

These observations were further analyzed by Langmuir equation to obtain the values of K_d and B_{max} for the fluorescence quenching of AmCyan and mOrange2 by Cu^{2+} (Fig. 2). As a result, the values of K_d for AmCyan and mOrange2 were 56.10 and 21.46 µM, respectively, and those of B_{max} for AmCyan and mOrange2 were 1.02 and 0.86, respectively. According to the K_d values determined in this study, which indicate the binding affinity of Cu^{2+} to AmCyan or mOrange2, mOrange2 showed a higher affinity for Cu^{2+} than AmCyan. This is consistent with the experimental observance presented above. On the contrary, from the values of B_{max}, which indicates the maximum registered fluorescence, AmCyan was found to possess a higher fluorescence capacity than mOrange2, resulting in a higher relative quenching when saturated by Cu^{2+}.

Reversibility of the Alterations to the Fluorescence Intensity by EDTA

The fluorescence recovery of a fluorescent protein quenched by metal ions is important for the cost-effective application of biosensor materials. Based on the fluorescent protein crystal structures, two types of metal-induced fluorescent protein-quenching have been reported [5, 8, 9]. One is the direct binding of the metal to the chromophore [9]; in this type of binding, fluorescence is not restored by the metal chelator EDTA, due to the inaccessibility of the chromophore. The other type is fluorescence quenching by metal binding to the surface of the fluorescent protein [5, 8], which can be reversed by the metal chelator EDTA. To confirm that the fluorescence quenching we observed was reversible, we treated mixtures of 10 µM AmCyan or mOrange2 and 1 mM Cu^{2+} with 0.25–50 mM EDTA, and then measured the fluorescence of the samples. We found that the fluorescence intensity of the quenched fluorescent proteins increased when high concentrations of EDTA were added (Fig. 3). AmCyan showed fluorescence intensities of 12.75, 42.14, 54.89, and 89.40% of the maximum in the presence of 0, 0.25, 1.0, and 5.0 mM EDTA, respectively (Fig. 3A). When 50 mM EDTA was added, the fluorescence intensity increased by 30.38% over the unquenched control. We found that the fluorescence intensity of mOrange2 recovered to 12.18, 22.32, and 63.72% of the maximum, respectively, when 0, 0.25, and 1.0 mM EDTA were added (Fig. 3B). When 5 mM EDTA was added, the fluorescence intensity of mOrange2 was similar to that of the unquenched fluorescent

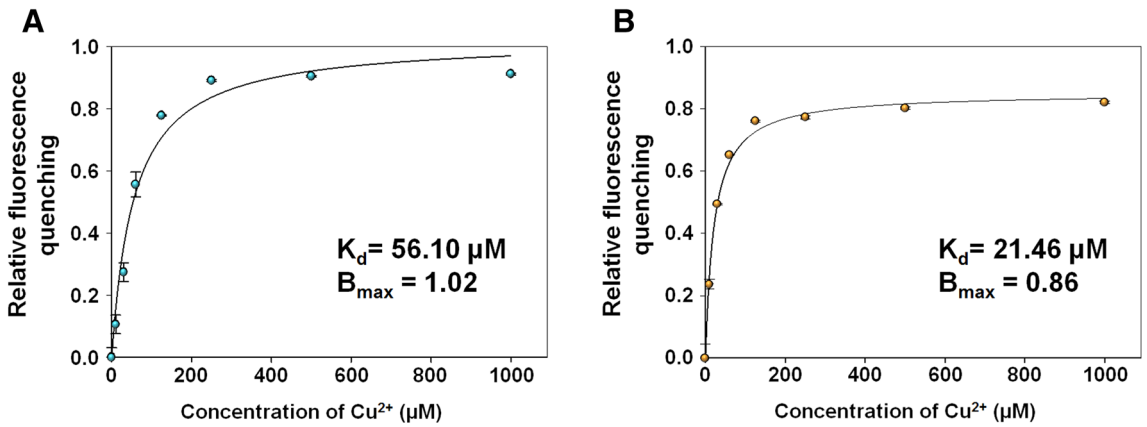

Fig. 2 Analysis of Cu^{2+}-mediated quenching of AmCyan and mOrange2. Titration of the Cu^{2+} ions into solutions containing (**A**) AmCyan and (**B**) mOrange2. The fluorescence emissions of AmCyan and mOrange2 were obtained at 535 and 574 nm, respectively, in the presence of 0, 10, 30, 60, 125, 250, 500, and 1000 µM Cu^{2+}. The relative fluorescence quenching is defined as 1-Fluorescence intensity at an indicated concentration of Cu^{2+}/Fluorescence intensity without Cu^{2+}. By curve fitting of Langmuir equation to experimental data, the values of K_d and B_{max} for AmCyan and mOrange2 were estimated to analyze the quenching of the fluorescence of AmCyan and mOrange2 by Cu^{2+}. Data represent means ± standard deviations of three replicates

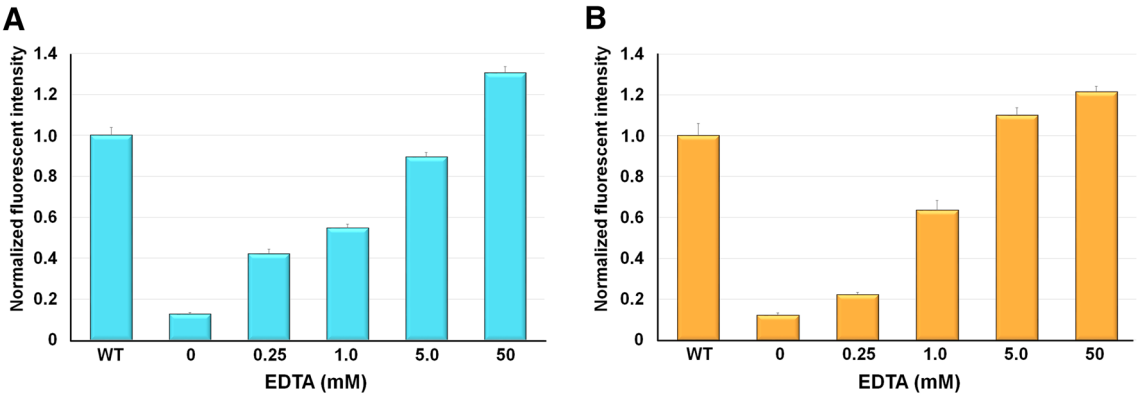

Fig. 3 Assessment of the reversibility of the Cu^{2+}-mediated quenching of AmCyan and mOrange2. We incubated 10 μM (**A**) AmCyan and (**B**) mOrange2 with 1 mM Cu^{2+} for 10 min. Next, we added 0.25, 1.0, 5.0, and 50 mM ethylenediaminetetraacetic acid (EDTA) to the quenched fluorescent protein solutions. The fluorescence of both of the quenched fluorescent proteins recovered after treatment with the metal chelator EDTA. The fluorescence of mOrange2 was more reversible than that of AmCyan, upon the addition of 1 mM EDTA. Data represent means ± standard deviations of three replicates

protein, whereas the fluorescence intensity increased by 20% over the unquenched in the presence of 50 mM EDTA. Thus, AmCyan and mOrange2 are reversibly quenched by Cu^{2+}. Comparative analysis revealed that mOrange2 had a higher reversibility by EDTA than AmCyan. Since EDTA cannot enter the inner space of a β-barrel fold, it is believed to chelate the metal from the β-barrel surface or the metal solution. Given that the fluorescence intensities increased beyond the unquenched controls in the presence of ≥ 50 mM EDTA, we hypothesize that EDTA is removing the quenching metal ions that bound to the fluorescent proteins during protein expression and purification.

Comparison of the Metal-Binding Site Using Modeling

To understand the fluorescence-quenching, metal-binding sites of the fluorescent proteins, we generated model structures of AmCyan and mOrange2 using amFP486 (sequence identity = 98.23%) and PAmCherry1 (sequence identity = 92.27%), respectively, as templates (Fig. 4A, B). These model structures were compared with the recently reported metal-bound Dronpa (PDB code 5HZT) and iq-Emerald (4KW9). In Dronpa, there are two metal-binding sites at His210–Hi212 (Cu^{2+}-binding site) and His212–His194 (Co^{2+}- or Ni^{2+}-binding site) (Fig. 4C). In iq-Emerald, Zn^{2+} or Ni^{2+} can bind at His202–His204 (Fig. 4D). These metal ions are commonly coordinated by 2 histidine residues, and each metal was apart the chromophore by ~ 24 Å [5, 8]. Superimposition of the model structures with the reported fluorescent protein–metal coordinates revealed that the His218 residue in AmCyan is positionally identical with His212 in Dronpa, but has no partner histidine residue to coordinate the metal ion on the β-barrel surface (Fig. 4A). On the other hand, in mOrange2, there are no histidine residues at the same positions as previously known metal-binding sites (Fig. 4B). Although the amino acid sequence of the histidine residues (i and $i+2$) on the β-strand of a fluorescent protein can facilitate robust transition metal-binding in a β-sheet mode [8], neither AmCyan nor mOrange2 contain the same binding mode in the amino acid sequence. Based on their modeling structures, we anticipate that AmCyan and mOrange2 interact with quenching metal ions via different binding sites than those in the previously reported fluorescent protein–metal bonds. In our spectroscopy experiments, AmCyan exhibited different binding affinities for Co^{2+}, Zn^{2+}, and Cu^{2+}, and we consider that each metal will have different binding affinities depending on the coordination preference of each metal at the expected metal binding sites. For example, in Dronpa, Cu^{2+} was tetrahedrally coordinated by two histidine residues on the β-can surface and two water molecules [5]. On the other hand, Co^{2+} and Zn^{2+} ions bind octahedral coordination with two histidine and four water molecules on the β-can surface [5]. As a result, AmCyan has a stable binding site that can maintain the favorable coordination of Cu^{2+}, but Co^{2+} and Zn^{2+} are considered to have binding sites that do not maintain stable coordination or to bind nonspecifically. On the other hand, since mOrange2 exhibits fluorescence quenching only for Cu^{2+}, it can be considered that mOrange2 can bind only Cu^{2+} and there is no binding site for other quenchable metals. In order to understand the exact metal binding site, further studies with AmCyan and mOrange2 are required for identifying its crystal structure in complex with Cu^{2+}. Nevertheless, our modeling structures of AmCyan and mOrange2 will provide insights into understanding the new metal binding site of FPs.

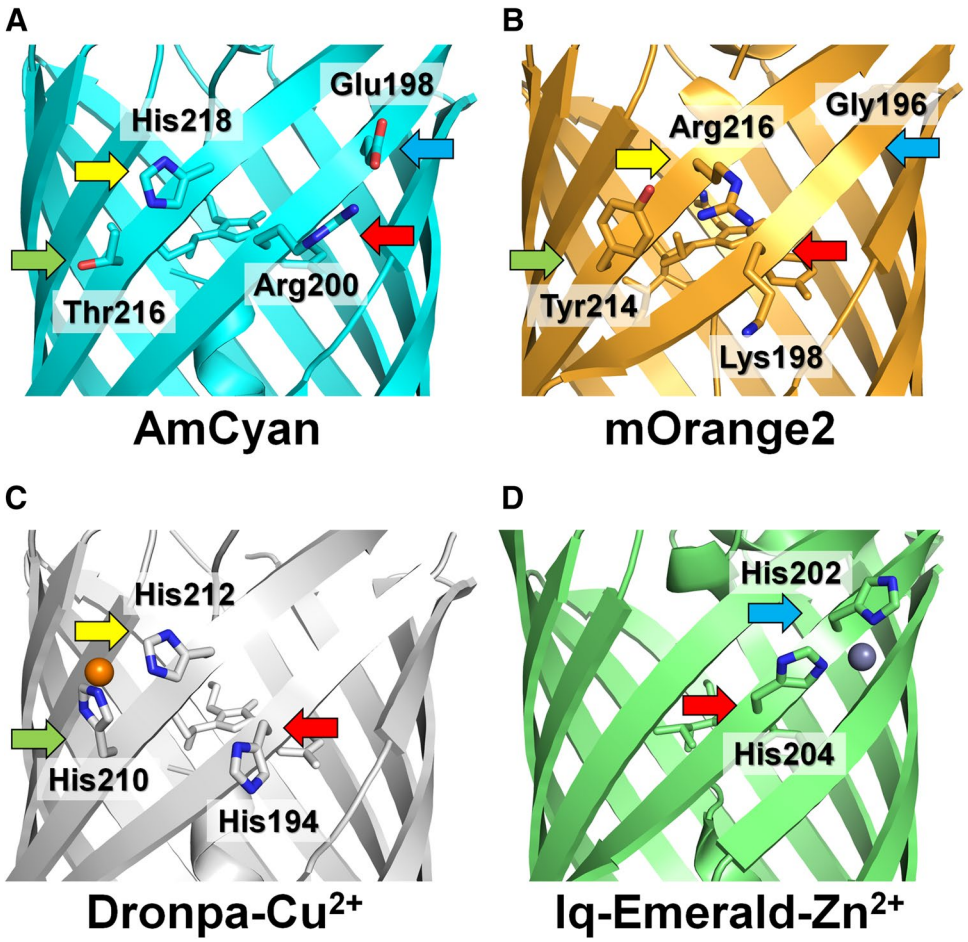

Fig. 4 Structural comparison of the modeled structures of (**A**) AmCyan and (**B**) mOrange2 with the previously reported metal-bound (**C**) Dronpa (PDB code 5HZT) and (**D**) iq-Emerald (4KW9). Arrows of the same color (red, blue, yellow, and green) indicate the same position in the superimposed fluorescent protein structures. Structural comparisons indicated that AmCyan and mOrange2 do not share the previously reported fluorescent protein metal-binding sites. (Color figure online)

Conclusion

We found that AmCyan and mOrange2 experienced fluorescence quenching with high sensitivity to Cu^{2+}, as compared with other metal ions. Co^{2+} and Zn^{2+} also quenched the fluorescence intensity of AmCyan, though not as effectively as Cu^{2+}. Thus, AmCyan would not be limited to use as a specific biosensor for Cu^{2+}, but could be used for the detection of heavy metals, such as Co^{2+} or Zn^{2+}. On the other hand, only Cu^{2+} quenched mOrange2, so mOrange2 could be used as a specific Cu^{2+}-biosensor material. The quenching of both fluorescent proteins was reversed by EDTA, and therefore, it can be reused in other applications. Our structural modeling showed that AmCyan and mOrange2 may have distinct metal-binding sites from those previously reported. Prior to use as biosensors, additional engineering of AmCyan and mOrange2 would be required to decrease the dissociation constants of Cu^{2+}. To solve this problem, the crystal structure of Cu^{2+}-bound AmCyan and mOrange2 should be clarified. Our results provide a molecular framework for the development of fluorescent protein-based metal biosensors using AmCyan and mOrange2.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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