Biochemical and Biophysical Research Communications 370 (2008) 57–61

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mechanism of copper induced fluorescence quenching of red fluorescent protein, DsRed

Yasmeen Rahimi, Ann Goulding, Suresh Shrestha, Sweetie Mirpuri, Sapna K. Deo *

Department of Chemistry & Chemical Biology, Indiana University Purdue University Indianapolis, 402 N. Blackford Street, LD326C, Indianapolis, IN 46202, USA

ARTICLE INFO

Article history:
Received 27 February 2008
Available online 17 March 2008

Keywords:
DsRed
Copper-binding
Fluorescence quenching
Static quenching
DsRed-Monomer
DsRed-Express

ABSTRACT

The red fluorescent protein, DsRed, and a few of its mutants have been shown to bind copper ions resulting in quenching of its fluorescence. The response to Cu^{2+} is rapid, selective, and reversible upon addition of a copper chelator. DsRed has been employed as an *in vitro* probe for Cu^{2+} determination by us and other groups. It is also envisioned that DsRed can serve as an intracellular genetically encoded indicator of Cu^{2+} concentration, and can be targeted to desired subcellular locations for Cu^{2+} determination. However, no information has been reported yet regarding the mechanism of the fluorescence quenching of DsRed in the presence of Cu^{2+}. In this work, we have performed spectroscopic investigations to determine the mechanism of quenching of DsRed fluorescence in the presence of Cu^{2+}. We have studied the effect of Cu^{2+} addition on two representative mutants of DsRed, specifically, DsRed-Monomer and DsRed-Express. Both proteins bind Cu^{2+} with micromolar affinities. Stern–Volmer plots generated at different temperatures indicate a static quenching process in the case of both proteins in the presence of Cu^{2+}. This mechanism was further studied using absorption spectroscopy. Stern–Volmer constants and quenching rate constants support the observation of static quenching in DsRed in the presence of Cu^{2+}. Circular dichroism (CD)-spectroscopic studies revealed no effect of Cu^{2+}-binding on the secondary structure or conformation of the protein. The effect of pH changes on the quenching of DsRed fluorescence in the presence of copper resulted in pK_a values indicative of histidine and cysteine residue involvement in Cu^{2+}-binding.

A variety of red fluorescent proteins have been isolated from reef corals. These red fluorescent proteins form chromophores internally and share structural similarities with green fluorescent proteins (GFP) [1–3]. Isolation and characterization of these GFP-like red proteins has expanded the possible applications of fluorescent proteins into multi-color labeling, resonance energy transfer, and intracellular tracking studies [2,4–6]. Like GFP, red fluorescent proteins have been mainly employed as genetically encoded fluorescent probes for cellular applications. However, GFPs have also been employed in other novel applications. For example, GFP was employed as an intracellular calcium indicator, as a chloride indicator, as a pH indicator, and in ligand monitoring using receptor inserted GFPs [7–12]. Only recently, we and others have found that red fluorescent proteins, namely native DsRed and its variants bind Cu^{2+} selectively in the presence of other divalent cations resulting in the quenching of their fluorescence [13–16]. The DsRed variants shown to bind Cu^{2+} include, DsRed-Express, DsRed-Monomer, DsRed2, and Rmu13. By relating fluorescence quenching of DsRed with Cu^{2+} concentration, *in vitro* biosensing systems for Cu^{2+} determinations have been developed [16,17]. Moreover, this ability to bind copper ions can now be utilized in the intracellular detection of Cu^{2+} based on the fluorescence of DsRed. Cu^{2+} is an important co-factor of several enzymes and plays a significant role in several cellular pathways and disease pathogenesis [18–20]. Therefore, the availability of genetically encodable probes such as DsRed, which can be targeted to specific organelles for detection of Cu^{2+}, would prove highly beneficial. In that regard, it is essential to understand the binding of Cu^{2+} to DsRed, its mechanism of quenching, and the spectral changes in the DsRed proteins in the presence of Cu^{2+}. In the work presented here, we have characterized the mechanism of fluorescence quenching, in the presence of Cu^{2+}, of DsRed-Express and DsRed-Monomer using spectroscopic tools. Additionally, we have performed studies to identify possible amino acid residues involved in the binding of Cu^{2+} to DsRed proteins. The two mutants of DsRed, DsRed-Monomer and DsRed-Express were selected for the study because our laboratory has developed biosensor for Cu^{2+} detection based on these two proteins. In addition, other Cu^{2+}-binding DsRed proteins, specifically, DsRed2, native DsRed, and Rmu13 are about 94–97% homologous with DsRed-Express. DsRed-Monomer shares 87% homology with DsRed-Express. Therefore, the two proteins selected in our study should serve as representative members of the Cu^{2+}-binding DsRed proteins.

* Corresponding author.
E-mail address: deo@chem.iupui.edu (S.K. Deo).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.03.034

DsRed-Express and DsRed-Monomer are variants of DsRed originally isolated from reef coral *Discosoma* sp. [21]. DsRed-Express is a fast-maturing tetramer of DsRed, that has an excitation and emission wavelength maximum of 558 nm and 583 nm, respectively [22]. This protein, which is commercially available through Clonetech, was obtained through nine amino acid mutations in native DsRed. The commercially available monomer of DsRed, DsRed-Monomer, was obtained after 45 amino acid substitutions in the native DsRed and possesses similar spectral properties to that of the DsRed-Express [22]. In the work presented here, we performed a variety of spectroscopic studies to understand the mechanism of fluorescence quenching upon Cu^{2+}-binding with DsRed variants. The results obtained provided an insight into the mechanism of fluorescence quenching, as well as possible amino acid residues involved in binding Cu^{2+}.

Materials and methods

Protein expression and purification

Protein expression and purification of DsRed-Express and DsRed-Monomer were performed using previously established protocols employing an immobilized copper column and using pDsRed-Express and pDsRed-Monomer plasmids (Clonetech, Palo Alto, CA) [15,17]. The purity was determined by SDS–PAGE using coomassie staining solution. DsRed protein concentration was determined using BioRad assay.

Determination of dissociation constant for Cu^{2+}

The purified proteins were dialyzed against 20 mM MOPS buffer, pH 7.4, to remove imidazole. The imidazole-free protein solutions were passed through Chelex-100 column to remove any trace levels of Cu^{2+}. To perform the Cu^{2+}-binding study, 100 μL of different concentrations of copper solution were added to a 100 μL of 1 μM of DsRed proteins. Fluorescence readings were obtained and buffer corrected. [F/F_0] were plotted against Cu^{2+} concentration to obtain dissociation constants. The fluorescence was measured (Varian Cary Eclipse Fluorescence Reader, Palo Alto, CA) by exciting the samples at 558 nm and recording the resulted emission at 591 nm and 581 nm for DsRed-Monomer and DsRed-Express proteins, respectively.

Stern–Volmer plot

A volume of 100 μL of different concentrations of Cu^{2+}solution was added to 100 μL of 1 μM protein in 20 mM MOPS buffer, pH 7.4, in an individual microtiter well. After the addition of Cu^{2+}, the solution was incubated at different temperatures (25, 30, and 16 °C). A fluorescence reading was taken by exciting the sample and measuring the emission, at the appropriate wavelength for each protein and was buffer corrected.

Spectroscopic studies

CD spectra. A volume of 250 μL of 3.3 μM protein in 20 mM MOPS buffer, pH 7.4, was placed in a 0.2 cm cell, and the CD absorption spectrum was obtained at room temperature (Jasco J-720 Spectropolarimeter, Tokyo, Japan). A volume of 10 μL of Cu^{2+} was added to the protein to get a final concentration of 0.5 mM and the absorbance spectrum was recorded. Spectra were corrected for the contribution of the buffer.

UV-visible absorption spectra. A volume of 1 mL proteins (3.4 μM) in 10 mM MOPS buffer, pH 7.4, was placed in a cuvette, and the absorption spectra of the protein were recorded at room temperature (Perkin-Elmer UV/vis/NIR LAMBDA). To this 10 μL of Cu^{2+} was added to yield a final concentration of 0.5 mM and the absorbance spectrum was recorded. The absorbance spectra were buffer corrected.

pH study

MOPS buffers ranging from pH 5.5 to 10.5 were prepared. Protein solutions (3 μM) in 10 mM MOPS buffer, pH 7.4, were mixed with different pH buffers to obtain a final concentration of 1 μM and the required pH. Fluorescence intensity was monitored at the characteristics wavelengths without and with the addition of Cu^{2+} (300 μM) and was buffer corrected.

Results and discussion

Recent studies in both our laboratory and others, found that DsRed variants bind Cu^{2+} selectively, resulting in quenching of the fluorescence emission of the proteins [13,16,17,23]. Copper ion-binding by DsRed has shown greater than 90% fluorescence quenching reversibility with the addition of a metal ion chelator, such as EDTA [16]. This selectivity and reversibility increases the usability of these proteins in a variety of sensing applications. DsRed proteins can also serve as genetically encodable copper ion-binding fluorescent probes. Genetic encoding of an ion-sensitive probe that can be targeted to a specific subcellular compartment would open up a new avenue of research. The copper-binding property of DsRed-Monomer and DsRed-Express were also utilized for affinity purification of these proteins using metal-chelating columns [15]. The copper-binding selectivity observed for these RFPs is interesting because GFP does not show any inherent metal-binding properties. A comparison of amino acid sequences between these proteins shows that GFP and DsRed share 24.2% sequence identity.

Fluorescence quenching seen for DsRed proteins in the presence of copper has been shown to have no effect on the emission wavelength maximum of the proteins. A variant of DsRed, DsRed2, has been examined as a highly selective and sensitive copper biosensor with a reported dissociation constant of 0.54 ± 0.09 μM [16]. Copper ion concentrations of 2.5 μM have shown upwards of 90% fluorescence quenching, with this protein. In another study, mutants of DsRed were constructed that showed moderate quenching with copper concentrations of 10 μM [13]. Dissociation constants for both drFP583 (native DsRed) and a mutant of drFP583, Rmu13 were reported as 14.80 ± 1.68 and 10.90 ± 1.74 μM, respectively, varying greatly from those of DsRed2 [13]. In studies, performed by our laboratory, we observed that the mutants of DsRed, DsRed-Express, and DsRed-Monomer were able to bind Cu^{2+}, selectively. DsRed-Monomer showed greater than 50% quenching at concentrations of 3 μM copper [24], whereas for DsRed-Express copper concentrations of 250 μM were needed to obtain a similar result. A detection limit, for copper, of 7.9×10^{-7} M was obtained using DsRed-Monomer as the binding element [17]. All of the studies performed thus far were targeted toward the construction of biosensors for copper, based on the copper selectivity of DsRed and the observed fluorescence quenching effect. None of these studies have characterized the effect of copper-binding on fluorescent proteins in terms of the changes in spectral properties or the mechanism of fluorescence quenching. To investigate these properties we have performed a series of spectroscopic studies using DsRed-Monomer and DsRed-Express in the presence of Cu^{2+}.

We first calculated the copper dissociation constants of the proteins, as a measure of the affinity for binding Cu^{2+}. These dissociation constants were calculated using the equation below.

$$\Delta F/\Delta F_{\max} = (K_d + [P] + [Cu] \pm \sqrt{[(K_d + [P] + [Cu])^2 - (4[P][Cu])}]/2[P]$$

where ΔF is the change in the measured fluorescence, $\Delta F_{\max}$ the maximum fluorescence change, [P] total protein concentration, K_d is the dissociation constant of the copper-binding site, and [Cu] the total concentration of copper. The curve of $\Delta F/\Delta F_{\max}$ against copper concentration was fitted using the equation above, Fig. 1. Dissociation constants of 5.4 ± 1.7 and 1.7 ± 0.3 μM for DsRed-Express and DsRed-Monomer, respectively, were measured. Based on these K_d values, DsRed-Monomer shows somewhat higher affinity for copper ion-binding than DsRed-Express. This dissociation constant for Cu^{2+} is similar to that obtained using DsRed2 [16]. Other naturally available copper-binding proteins and peptides have reported K_d values ranging from 10^{-6} to 10^{-15} M for proteins and up to 10^{-17} M for peptides [25,26]. In comparison, DsRed-Express and DsRed-Monomer appear to be relatively weak copper-binding proteins. However, the inherent fluorescence of these proteins and high selectivity for copper can be an advantage over other copper-binding proteins for sensing applications.

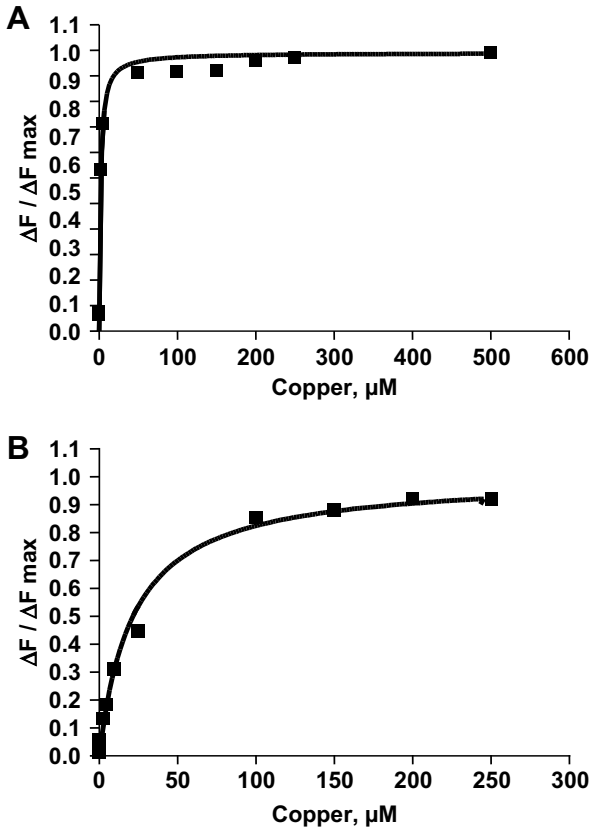

Fig. 1. Plot of $\Delta F/\Delta F_{max}$ against copper concentration, where ΔF is the change in measured fluorescence and ΔF_{max} is the maximum fluorescence change. Plot A corresponds to results obtained using DsRed-Monomer and plot B corresponds to results obtained with DsRed-Express.

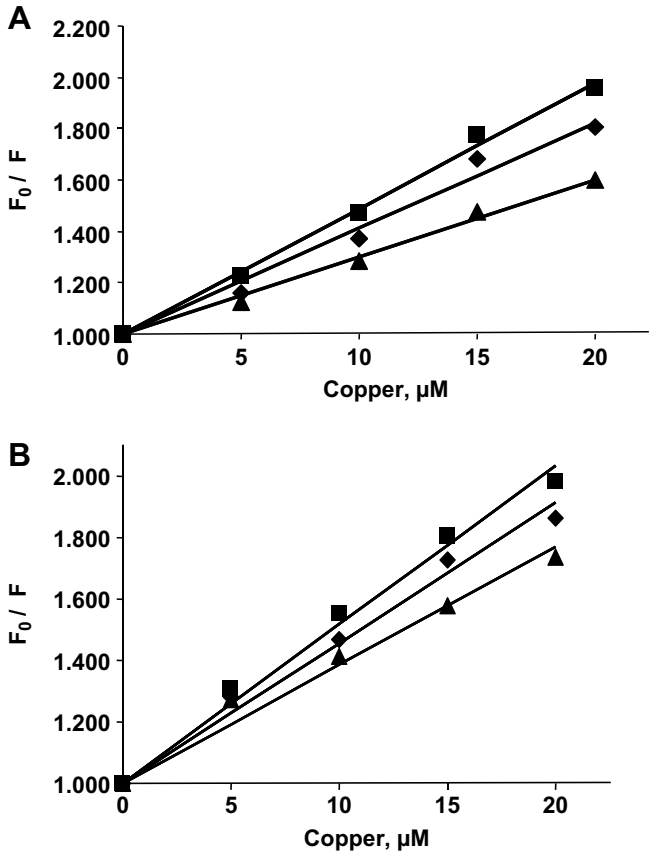

Fig. 2. Stern–Volmer plots generated by adding Cu^{2+} to DsRed proteins followed by incubation at (■) 16 °C, (♦) 25 °C, and (▲) 30 °C. Data obtained for DsRed-Monomer is presented as plot A and data obtained for DsRed-Express is presented as plot B.

A quenching in fluorescence was observed with DsRed proteins in the presence of Cu^{2+}. To investigate whether the mechanism of quenching is a dynamic or static process we generated Stern–Volmer plots [27]. These plots were generated by measuring the fluorescence of each protein upon addition of Cu^{2+}, at different temperatures (Fig. 2). For both DsRed-Express and DsRed-Monomer linear plots were generated, indicating that only one type of quenching was occurring. From the slopes of these plots the Stern–Volmer constant (K_{sv}) was calculated for each protein, which are displayed in Table 1 [27]. These constants showed an increase with a decrease in temperature. This observation indicates static quenching interactions between the proteins and copper ions, such that Cu^{2+} forms a complex with the protein at a defined site on the protein [27]. To further define the quenching seen between the proteins and Cu^{2+}, the K_{sv} values, obtained from the Stern–Volmer plots, were used to calculate the quenching rate constant, K_q, by the following equation

$$K_q = K_{sv}/\tau_0$$

where τ_0 represents the lifetime of the fluorophore of the protein, which was reported to be 3.3 ns for DsRed [28]. The K_q values obtained in our study ranged from 10^{12} to $10^{13}\ M^{-1}\ s^{-1}$, for both DsRed proteins in the presence of Cu^{2+}. These values are 100-fold larger than the maximum scatter collision quenching constant for quenchers ($2.0 \times 10^{10}\ M^{-1}\ s^{-1}$) [29]. Therefore, the K_q values obtained for the DsRed proteins indicate that the quenching seen for DsRed proteins in the presence of Cu^{2+} must be due to complex formation between the proteins and Cu^{2+}, as observed in static quenching.

To further validate that the mechanism, of the fluorescence quenching, in the presence of copper is static, we performed UV absorption scans of the two proteins in the presence and absence of Cu^{2+}. Dynamic quenching affects only the excited states of the fluorophore whereas the static quenching process affects the ground state of the fluorophore [27]. Therefore, the static quenching process leads to a change in absorption spectra. The absorption spectrum of DsRed-Express and DsRed-Monomer in the presence of Cu^{2+} showed that the absorbance intensity was affected by the addition of Cu^{2+} compared to the same concentration of protein without Cu^{2+} (Fig. 3). With a single equivalence of bound Cu^{2+}, the absorbance and the extinction coefficient of the proteins decreased at its characteristics absorption wavelength of 556 nm (Fig. 3, Table 2). The overall shape and pattern of peaks was retained for each of the proteins in the presence of Cu^{2+} compared to the peaks obtained with protein alone. Static quenching process typically yields changes in the absorption profile [27,29], however, this is not observed in the case of DsRed. The anomaly observed in

Table 1
Stern–Volmer constants (K_{sv}) determined from the slope of the Stern–Volmer plot and quenching rate constants determined using the equation $K_q = K_{sv}/\tau_0$, where K_{sv} is Stern–Volmer constant and τ_0 is the lifetime of the fluorophore

T (°C)	K_{sv} (M^{-1})	K_q ($M^{-1}\ s^{-1}$)
DsRed-Monomer		
16	48,800	$1.47 \times 10^{+13}$
25	41,200	$1.24 \times 10^{+13}$
30	30,100	$9.12 \times 10^{+12}$
DsRed-Express		
16	51,600	$1.56 \times 10^{+13}$
25	44,600	$1.35 \times 10^{+13}$
30	38,500	$1.16 \times 10^{+13}$

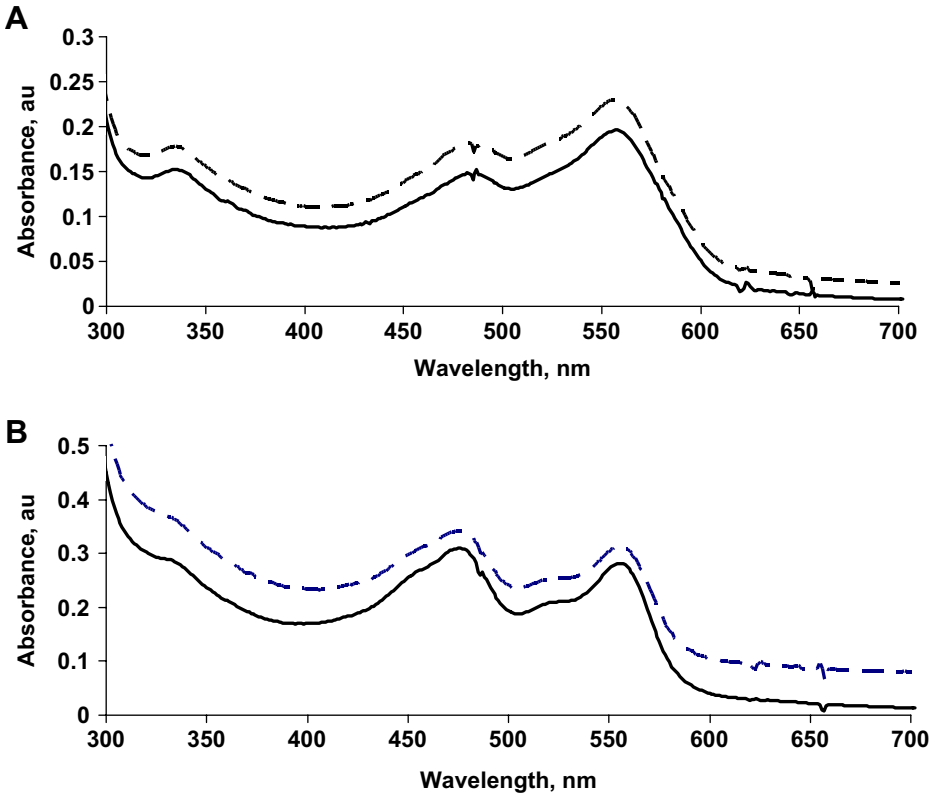

Fig. 3. UV–visible absorption spectra of DsRed-Monomer (plot A) and DsRed-Express (plot B) in the presence (—) and absence (---) of Cu^{2+}.

Table 2
UV–visible and CD spectral characteristics of DsRed-Monomer and DsRed-Express in the presence and absence of Cu^{2+}

Molar extinction coefficient M^{-1} cm^{-1}		% Secondary structure of proteins			% Secondary structure of Cu^{2+}-bound proteins		
No Cu^{2+}	With Cu^{2+}	α-Helix	β-Sheet	Random coil	α-helix	β-sheet	Random coil
DsRed-Monomer							
62,700	57,600	5	47	48	5	47	48
DsRed-Express							
91,400	82,350	4	48	48	5	47	48

Secondary structure content was calculated using K2d software [30].

this study can be explained as follows. It is known that in the case of proteins only the fluorophores located at the surface can be dynamically quenched whereas the internal fluorophores are quenched by static process due to higher hydrophobicity in the interior. In the case of DsRed, the chromophore is well shielded inside a β-barrel and hence is not accessible for dynamic or collisional quenching. Taking this factor into consideration and the Stern–Volmer constants obtained in our study, we hypothesize that the fluorescence quenching of DsRed in the presence of Cu^{2+} follows a sphere of action static quenching model. This indicates that the quencher forms a contact with the fluorophore leading to the formation of non-fluorescent species. This is further supported by the observation that the absorption spectral profile of DsRed does not change in the presence of Cu^{2+}, only the absorbance intensities have changed as expected for a sphere of action static mechanism [27,29]. We hypothesize that, in DsRed, Cu^{2+} forms a complex with specific amino acids on the protein. This complex formation can lead to formation of non-fluorescent species in three ways, (i) by affecting hydrogen-bonding network of the chromophore, (ii) by bringing Cu^{2+} close to chromophore such that it contacts the excited-state of the chromophore, (iii) by causing conformation/structural changes in the protein.

We investigated if the binding of Cu^{2+} to the DsRed proteins caused any structural or conformational changes within the protein, leading to quenching of fluorescence. To achieve this we monitored the far-UV CD spectra of DsRed-Express and DsRed-Monomer, with and without the presence of copper. The spectra, and the percent of the secondary structure (Table 2), were identical both in the presence and absence of copper. This result indicates that the observed fluorescence quenching in DsRed proteins is not due to any structural or conformational changes in the protein, upon Cu^{2+}-binding.

From the Stern–Volmer study and CD spectroscopy results we can deduce that Cu^{2+} forms a complex with specific amino acid residues of DsRed proteins while the structural/conformational integrity of the protein is maintained. To identify possible amino acids residues involved in Cu^{2+}-binding we studied the effect of pH on Cu^{2+}-binding by monitoring change in fluorescence intensity of DsRed proteins with a change in pH, both in the presence and absence of Cu^{2+}. In the absence of Cu^{2+} the two proteins showed no pH dependence across a range from pH 5 to 12, showing no change in fluorescence intensity. However, in the presence of Cu^{2+}, these plots showed a dramatic shift in fluorescence intensity from pH 6 to 8.5. The pK_a values of 7.3 and 7.0 for DsRed-Monomer

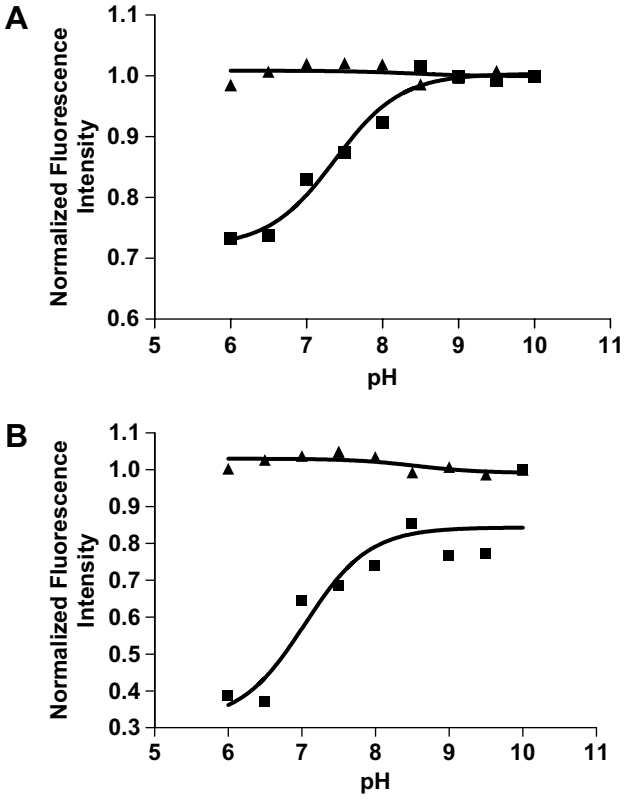

Fig. 4. The plot represents the effect of pH change on fluorescence intensity of DsRed-Monomer (plot A) and DsRed-Express (plot B) in the presence ((■) and absence of Cu^{2+} (▲).

and DsRed-Express, respectively, were obtained from these plots. These pK_a values suggest that either Cys or His residues may be involved in the Cu^{2+}-binding properties in DsRed proteins (see Fig. 4).

In summary, the DsRed proteins studied in this work have previously shown a unique selectivity for copper-binding. This copper-binding has been used in the development of affinity-based purification of DsRed and copper sensing applications. The work presented here has focused on spectroscopic studies to determine the mechanism of the observed fluorescence quenching in the presence of Cu^{2+}. The binding of copper to DsRed appears to have no effect on the overall structure of these proteins, as seen by far-UV CD spectral analysis. Deviations in the UV–visible absorbance values were seen in the presence of Cu^{2+}. However, no shift in absorption peaks was seen for the two proteins in the presence of Cu^{2+}. Both the UV–visible spectra and the Stern–Volmer constants suggest that the fluorescence quenching seen for DsRed proteins follows a sphere of action static quenching mechanism, indicating the formation of a complex between specific residues of the protein and the Cu^{2+}. Based on the pH study, either cysteine or histidine residues appear to be involved in binding copper ions in DsRed proteins. The results presented in this manuscript provide a ground work for future investigations in identifying Cu^{2+}-binding site in DsRed. In that regard, site-directed mutagenesis and X-ray crystallographic analysis are envisioned.

Acknowledgments

We appreciate funding from the National Institutes of Health (EB008231-01) and National Science Foundation (CHE-0748648). This work was supported by funding from IUPUI Research Support Funds Grant.

References

[1] G.S. Baird, D.A. Zacharias, R.Y. Tsien, Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral, Proc. Natl. Acad. Sci. USA 97 (2000) 11984–11989.
[2] V.V. Verkhusha, K.A. Lukyanov, The molecular properties and applications of Anthozoa fluorescent proteins and chromoproteins, Nat. Biotechnol. 22 (2004) 289–296.
[3] D. Yarbrough, R.M. Wachter, K. Kallio, M.V. Matz, S.J. Remington, Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-A resolution, Proc. Natl. Acad. Sci. USA 98 (2001) 462–467.
[4] M.G. Erickson, D.L. Moon, D.T. Yue, DsRed as a potential FRET partner with CFP and GFP, Biophys. J. 85 (2003) 599–611.
[5] A.F. Fradkov, V.V. Verkhusha, D.B. Staroverov, M.E. Bulina, Y.G. Yanushevich, V.I. Martynov, S. Lukyanov, K.A. Lukyanov, Far-red fluorescent tag for protein labelling, Biochem. J. 368 (2002) 17–21.
[6] S. Shrestha, S.K. Deo, Anthozoa red fluorescent protein in biosensing, Anal. Bioanal. Chem. 386 (2006) 515–524.
[7] R. Bizzarri, C. Arcangeli, D. Arosio, F. Ricci, P. Faraci, F. Cardarelli, F. Beltram, Development of a novel GFP-based ratiometric excitation and emission pH indicator for intracellular studies, Biophys. J. 90 (2006) 3300–3314.
[8] L.J. Galietta, P.M. Haggie, A.S. Verkman, Green fluorescent protein-based halide indicators with improved chloride and iodide affinities, FEBS Lett. 499 (2001) 220–224.
[9] G.S. Baird, D.A. Zacharias, R.Y. Tsien, Circular permutation and receptor insertion within green fluorescent proteins, Proc. Natl. Acad. Sci. USA 96 (1999) 11241–11246.
[10] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin, Nature 388 (1997) 882–887.
[11] S. Jayaraman, P. Haggie, R.M. Wachter, S.J. Remington, A.S. Verkman, Mechanism and cellular applications of a green fluorescent protein-based halide sensor, J. Biol. Chem. 275 (2000) 6047–6050.
[12] R.M. Wachter, D. Yarbrough, K. Kallio, S.J. Remington, Crystallographic and energetic analysis of binding of selected anions to the yellow variants of green fluorescent protein, J. Mol. Biol. 301 (2000) 157–171.
[13] P. Eli, A. Chakrabartty, Variants of DsRed fluorescent protein: development of a copper sensor, Protein Sci. 15 (2006) 2442–2447.
[14] Y. Rahimi, S. Shrestha, T. Banerjee, S.K. Deo, Study of Metal Binding to DsRed-Monomer, Bioluminescence and Chemiluminescence: Chemistry, Biology and Applications, World Scientific, 2006.
[15] Y. Rahimi, Metal affinity-based purification of a red fluorescent protein, Chromatographia 65 (2007) 429–433.
[16] J.P. Sumner, N.M. Westerberg, A.K. Stoddard, T.K. Hurst, M. Cramer, R.B. Thompson, C.A. Fierke, R. Kopelman, DsRed as a highly sensitive, selective, and reversible fluorescence-based biosensor for both Cu(+) and Cu(2+) ions, Biosens. Bioelectron. 21 (2006) 1302–1308.
[17] Y. Rahimi, S. Shrestha, S.K. Deo, Study of metal binding to monomeric red fluorescent protein, DsRed-Monomer, in: L.J. Kricka, Stanley, P.E. (Eds.), Proceedings of the 14th International Symposium on Bioluminescence and Chemiluminescence, San Diego, CA, Part 5, 2006, pp. 235–238.
[18] J.H. Fox, J.A. Kama, G. Lieberman, R. Chopra, K. Dorsey, V. Chopra, I. Volitakis, R.A. Cherny, A.I. Bush, S. Hersch, Mechanisms of copper ion mediated Huntington's disease progression, PLoS ONE 2 (2007) e334.
[19] I.G. Macreadie, Copper transport and Alzheimer's disease, Eur. Biophys. J. (2007).
[20] D.R. Richardson, Y. Suryo Rahmanto, Differential regulation of the Menkes and Wilson disease copper transporters by hormones: an integrated model of metal transport in the placenta, Biochem. J. 402 (2007) e1–e3.
[21] M.V. Matz, A.F. Fradkov, Y.A. Labas, A.P. Savitsky, A.G. Zaraisky, M.L. Markelov, S.A. Lukyanov, Fluorescent proteins from nonbioluminescent Anthozoa species, Nat. Biotechnol. 17 (1999) 969–973.
[22] Living ColorsTM DsRed Monomer Fluorescent Protein, CLONTECHniques, 2005.
[23] N.M.W. James, P. Sumner, Andrea K. Stoddard, Carol A. Fierke, Raoul Kopelman, Cu^{+} and Cu^{2+} sensitive PEBBLE fluorescent nanosensors using DsRed as the recognition element, Sens. Actuat. B: Chem. 113 (2005) 760–767.
[24] Y. Rahimi, S. Surestha, S.K. Deo, Metal affinity-based purification of a red fluorescent protein, Chromatographia 65 (2007) 429–433.
[25] E.D. Walter, M. Chattopadhyay, G.L. Millhauser, The affinity of copper binding to the prion protein octarepeat domain: evidence for negative cooperativity, Biochemistry 45 (2006) 13083–13092.
[26] L. Regan, The design of metal-binding sites in proteins, Annu. Rev. Biophys. Biomol. Struct. 22 (1993) 257–287.
[27] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, third ed., Springer, New York, 2006.
[28] B. Bowen, N. Woodbury, Single-molecule fluorescence lifetime and anisotropy measurements of the red fluorescent protein, DsRed, in solution, Photochem. Photobiol. 77 (2003) 362–369.
[29] C.Q. Jiang, T. Wang, Study of the interactions between tetracycline analogues and lysozyme, Bioorg. Med. Chem. 12 (2004) 2043–2047.
[30] M.A. Andrade, P. Chacon, J.J. Merelo, F. Moran, Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network, Protein Eng. 6 (1993) 383–390.