Structural Characterization of a Blue Chromoprotein and Its Yellow Mutant from the Sea Anemone *Cnidopus Jacoiicus*

Received for publication, July 20, 2006, and in revised form, October 2, 2006. Published, JBC Papers in Press, October 2, 2006. DOI 10.1074/jbc.M606021200

Mitchell C. Y. Chan†, Satoshi Karasawa§, Hideaki Mizuno†, Ivan Bosanac‡, Dona Ho†, Gilbert G. Privé§, Atsushi Miyawaki‡, and Mitsuhiko Ikura§

From the †Division of Signaling Biology, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5G 1L7, Canada, the Laboratory for Cell Function and Dynamics, Advanced Technology Development Center, Brain Science Institute, RIKEN, Wako City, Saitama 351-0198, Japan, §Amalgaam Co. Ltd., 2-9-3 Itabashi, Itabashi-ku, Tokyo 173-0004, Japan, and the Medical & Biological Laboratories Co. Ltd., 3-5-10 Marunouchi, Naka-ku, Nagoya City 460-0002, Japan

Green fluorescent protein (GFP) and its relatives (GFP protein family) have been isolated from marine organisms such as jellyfish and corals that belong to the phylum Cnidaria (stinging aquatic invertebrates). They are intrinsically fluorescent proteins. In search of new members of the family of green fluorescent protein family, we identified a non-fluorescent chromoprotein from the Cnidopus japonicus species of sea anemone that possesses 45% sequence identity to dsRed (a red fluorescent protein). This newly identified blue color protein has an absorbance maximum of 610 nm and is hereafter referred to as cjBlue. Determination of the cjBlue 1.8 Å crystal structure revealed a chromophore comprised of Gln63-Tyr64-Gly65. The ring stacking between Tyr64 and His197 stabilized the cjBlue trans chromophore conformation along the Ca2–Cβ2 bond of 5-[(4-hydroxyphenyl)methylene]-imidazolinone, which closely resembled that of the "kindling fluorescent Protein" and Rtm5. Replacement of Tyr64 with Leu in wild-type cjBlue produced a visible color change from blue to yellow with a new absorbance maximum of 417 nm. Interestingly, the crystal structure of the yellow mutant Y64L revealed two His197 imidazole ring orientations, suggesting a flip-flop interconversion between the two conformations in solution. We conclude that the dynamics and structure of the chromophore are both essential for the optical appearance of these color proteins.

The green fluorescent protein (GFP) from Aequorea victoria has gained widespread interest as a biological reporter in living cells (1). Since its discovery, considerable efforts have been devoted to protein engineering, in conjunction with isolation of new GFP homologs, to expand the visible spectrum and properties of GFP protein family (1, 2). Characterized GFP protein family can be divided into two groups, the fluorescent proteins (FPs) and the non-fluorescent chromoproteins (CPs) (3, 4). The GFP chromophore arises through a unique autocatalytic post-translational modification of a tripeptide, usually X-Tyr-Gly, in the primary sequence. The conformation and interaction of the chromophore with its local environment determines the spectral properties of the protein. X-ray crystallographic studies (5–7) have revealed the general relationship between the trans non-co-planarity of chromophores found in CPs and the cis co-planarity of chromophores found in FPs, with the exception of eqFP611, which has a trans co-planar chromophore.

To date, four CPs from the Anthozoan species have been characterized: Rtm5 from Montipora efflorescens (8), gtCP from Gonioptora tenuidens (9), aeCP957 from Actinia equine (10), and asFP595 from Anemone sulcata (KFP) (5). Three-dimensional structures of Rtm5 (6) and KFP (5) have been solved previously, both of which show the same fold as GFP and contain an internal chromophore. Studied CPs have exhibited absorbance maxima in a confined range of 560–597 nm (11–14); no CP has been thus far found to absorb at absorbance maxima greater than 600 nm. Here we present a new CP from the Cnidopus japonicus sea anemone species, which absorbs at 610 nm. We report the molecular cloning, characterization and structure determination of this blue CP, hereafter termed cjBlue. We have also generated a yellow mutant variant from cjBlue with a single mutation at the 64 position (Tyr to Leu) using semi-random mutagenesis. We discuss the structural basis for the blue chromophore formation of wild-type cjBlue and for the blue-to-yellow shift in cjBlue(Y64L) mutant, which lacks an aromatic amino acid in the tripeptide chromophore. Elucidation of the structural details of cjBlue and cjBlue(Y64L) helps to answer not only why cjBlue and cjBlue(Y64L) absorb different colors, but also contributes to our better understanding of why FPs can fluoresce having a structural architecture similar to that of CPs.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

1. Both authors contributed equally to this work.
2. To whom correspondence may be addressed. E-mail: matsushi@brain.riken.go.jp.
3. Holds Canada Research Chair in Cancer Structural Biology. To whom correspondence may be addressed: Division of Signaling Biology, Ontario Cancer Institute and Dept. of Medical Biophysics, University of Toronto, 101 College St., TMDT Rm. 4-804, Toronto, Ontario M5G 1L7, Canada. Tel.: 416-581-7550; Fax: 416-581-7564; E-mail: mikura@uhnres.utoronto.ca.
4. The abbreviations used are: GFP, green fluorescent protein; KFP, kindling fluorescent protein; FP, fluorescent protein; CP, chromoprotein; TCEP, Tris(2-carboxyethyl)phosphine.
Crystal Structures of Chromoproteins

EXPERIMENTAL PROCEDURES

cDNA Cloning and Gene Construction—A sample of the C. japonicus was acquired from the ocean near the Uozu city in Toyama. Total RNA was isolated from the sea anemone by guanidine thiocyanate extraction. Synthesis, amplification using degenerate primers, and generation of full-length cDNA were performed as described previously (23) using the following degenerate primers: 5′-GAAGGRTGYGTCAAYGGRCCAY-3′ and 5′-ACVGGDCCATYDGVAAGAAARTT-3′ (R = Arg or Gly; Y = Cys or Thr; V = Arg, Cys, or Gly; D = Arg, Gly, or Thr). The cDNA encoding the protein-coding region was amplified using primers containing 5′ BamHI and 3′ EcoRI sites. The digested product was then cloned in-frame into the BamHI/EcoRI sites of pRSETB (Invitrogen) for bacterial expression. Site-directed and semi-random mutations were introduced as described (19).

Protein Expression and Purification—CjBlue and cjBlue(Y64L) were subcloned into a pET28a expression vector. Seleno-1-methionine-labeled protein was produced using minimal M9 media and expressed with N-terminal His6 tag using B834 (DE3) Escherichia coli strain (Novagen). Cells were grown in a shaker incubator at 37 °C until an A490 of 1.20 was reached. Upon induction with 1 mM isopropyl 1-thio-galactopyranoside, the temperature was lowered to 20 °C, and the protein was allowed to express for 48 h. Protein containing the N-terminal polyhistidine tag was purified using nickel-nitrilotriacetic acid resin (Qiagen). The N-terminal His6 tag was subsequently removed using thrombin followed by size exclusion chromatography (Superdex 75, Amersham Biosciences) to achieve satisfactory levels of purity. Incorporation seleno-1-methionine was confirmed via electrospray mass spectrometry. The purified protein was concentrated to 25 mg/ml in a crystallization buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM Tris(2-carboxyethyl)phosphine (TCEP)).

Structure Determination—The protein solution was concentrated to 25 mg/ml in Tris-HCl (pH 7.5) with 150 mM NaCl and 2 mM Tris(2-carboxyethyl)phosphine (TCEP). Blue plated crystals (P21), with approximate dimensions of 0.4 × 0.3 × 0.08 mm, were grown in hanging drops containing 2 μl of mother liquor at 22 °C for 2 days. The mother liquor contained 0.2 mM NaH2PO4, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20% polyethylene 3350, 20% glycerol, and 2 mM TCEP. The crystals were micro-seeded for two rounds, cryo-protected in 20% glycerol, and flushed cooled to 100 K in a stream of nitrogen gas. The cjBlue SAD (Single Anomalous Dispersion) data were collected at 19-ID beamline at the Advanced Photon Source synchrotron facility and were processed with HKL2000. Data were collected at 0.5° oscillation in 30 s exposures and to 99.3% completeness at 1.8 Å. The crystal belongs to the P21 space group with cell dimensions a = 73.86 Å, b = 126.85 Å, c = 100.51 Å, β = 102.10, and with 8 molecules/asymmetric unit. The cjBlue(Y64L) native data were collected in-house on the Bruker X8 PROTEUM. 0.5° oscillations were collected in 45 s exposures. Data were collected to 100% completeness at 2.0 Å. Unit cell dimensions were: a = 74.08 Å, b = 126.93 Å, c = 100.08 Å, and β = 101.97. The cjBlue structure served as a search model for cjBlue(Y64L) in molecular replacement with the chromophore removed to minimize bias. Both structure refinements were performed using crystallography NMR software 1.1.

Spectroscopy—The cjBlue, cjBlue(Y64L), cjBlue(H197S), and cjBlue(Y64L/H197S) samples from their respective stock solutions were exchanged into buffer containing 20 mM NaHPO4 and 150 mM NaCl to measure each individual absorbance spectrum. Concentration of each sample was diluted from the stock 25 mg/ml solution to 1 mg/ml. 0.5 ml of diluted sample was gently transferred into a 1-cm path length quartz cuvette. Absorbance was measured using an Ultrospec 2000 UV-visible spectrometer (GE Healthcare). An absorbance scan was initiated from 250 to 750 nm at 1-nm increments.

RESULTS

cDNA Isolation and Protein Purification—Degenerate primers were employed to amplify cDNAs isolated from the sea anemone, C. japonicus. The primers covered several conserved amino acid sequences identified from among GFP-like fluorescent proteins found in other Anthozoa species. The missing 5′ and 3′ ends of the cDNA fragment were amplified using the rapid amplification of cDNA ends strategy. The resultant open reading frame coded 225 amino acids with high sequence homologies to KFP (5), hcCP (15), eqFP611 (7), and Rtm55 (6) (Fig. 1). The full-length protein was expressed in E. coli with a His6 tag at the N terminus and purified using metal affinity chromatography. The protein referred as cjBlue is a chromoprotein, which does not fluoresce but is dark green in color. CjBlue at pH 7.4 displayed a major absorption wavelength maximum at 610 nm (ε = 66,700 M−1 cm−1) (Fig. 2) showing the longest wavelength absorption of all known CPs. The absorption spectrum of cjBlue showed high resistance to acidity (up to pH 4) (data not shown).

Structure of cjBlue—The crystal structure of cjBlue, solved by SAD, was determined to 1.8 Å. The cjBlue crystal belongs to the P21 space group. A final Rfactor and Rfree of 19.5 and 22.5%, respectively, were reached (Table 1). The final cjBlue model contained eight subunits (residues 5–232), 16 PO4, and 1330 water molecules in an asymmetric unit. Further, the tertiary structure of cjBlue shares a similar fold to GFP (16), dsRed (17), KFP (5), and Rtm55 (6). Each subunit consists of β-strands forming a β-barrel with a central helix running co-axially with the axes of the β-barrel (Fig. 3). The central helix of cjBlue connects the chromophore to the rest of the protein (Fig. 3).

The crystal structure of cjBlue revealed a pair of four chemically identical subunits that were intimately in contact with one another (Fig. 3A), in a manner similar to that observed for eqFP611 (7), Rtm55 (6), and dsRed (17). This molecular association is stabilized by 1) inter-barrel interface interactions and 2) association of the C terminus with β-strands of adjacent subunits (Fig. 3). Asn232, Thr231, Glu232, and Arg251 are residues located at the inter-barrel interface forming hydrogen bonds between subunits. The C-terminal tail interaction involves hydrogen bonding between Ser227, His231, and Asn232 at one tail and Asp196, Arg198, Glu200, and Thr224 within β-strands of an adjacent pair of subunit.

The cjBlue chromophore consists of a 5′-[4-(4-hydroxyphenyl)methylene]-imidazoline group with an acylimine bond found between the Ca and nitrogen atom positioned at Gln63 (Figs. 3

Downloaded from www.jbc.org at TORONTO WESTERN HOSPITAL on January 12, 2007
imidaazole ring of His\(^{197}\), located near the chromophore, shows a stacking orientation with the \(p\)-hydroxyphenyl ring of \(-3.9\) Å.

The cjBlue chromophore adopts a \(trans\) conformation between the \(Ca_2\)--\(C_β_2\) bond with noticeable bond angle distortion. Because of internal steric hindrance between O2 and C82–H, an increase in the \(C_2–Ca_2–C_β_2\) bond angle (141°) and the \(Ca_2–C_β_2–Cγ_2\) bond angle (117°) was observed. Cys\(^{145}\) also appears to contribute to the \(trans\) conformation by sterically hindering the \(p\)-hydroxyphenyl ring from adopting a \(cis\) conformation.

Chromophores adopting a \(trans\) conformation, with the exception of eqFP611, lack planarity between the imidazolinone and \(p\)-hydroxyphenyl ring because of internal collision of O2 and C82–H (supplemental Fig. 1). For example, structures such as Rtm5 (6) and KFP (5) adopt a \(trans\) non-co-planar conformation, whereas eqFP611 (7) has a \(trans\) co-planar conformation. Chromophore planarity can be evaluated through the dihedral angles between the imidazolinone and \(p\)-hydroxyphenyl rings (Fig. 4). Chromophores with a \(cis\) conformation, such as dsRed, have a nearly perfect co-planarity (dihedral angle <1°). In contrast, Rtm5 (6) and KFP (5) have a \(trans\) non-co-planar conformation with a dihedral angle of 35° and 20°, respectively. Similarly the dihedral angle of cjBlue is 40°. A second assessment of chromophore planarity is the measurement of torsion angles between \(\chi_1\) (\(C_2–Ca_2–C_β_2–Cγ_2\)) and \(\chi_2\) (\(Ca_2–C_β_2–Cγ_2–C82\)). Rtm5 (6) and KFP (5) have a \(\chi_1\) and \(\chi_2\) of 169°, -136° and 171°, -163°, respectively, whereas the torsion angles of cjBlue are 178° and -141°.

**Structural Comparison of cjBlue(Y64L) with Wild-type cjBlue**

To examine site-specific roles of the chromophore-forming tripeptide, Gln–Tyr–Gly, we employed semi-random site-specific mutagenesis on cjBlue (19). Random substitutions of these and other amino acids surrounding the chromophore were simultaneously introduced into the protein. We found that Tyr\(^{64}\) could be replaced by some other amino acid without losing any light absorbing ability. For example, substitution of Leu only resulted in a blue shift of the absorption peak. This variant protein, cjBlue(Y64L), was yellowish in color and absorbed light maximally at 418 nm (Fig. 2). Most FPs/CPs have Tyr at this position, whereas blue- and cyan-emitting FPs derived from *Aequorea* GFP have His and Trp, respectively.

To investigate the structural properties of this aromaticless chromophore, we determined the crystal structure of cjBlue(Y64L) to 2.0 Å using molecular replacement and cjBlue as the search model. A final \(R_{factor}\) and \(R_{free}\) of 19.8 and 24.4%,
respectively, were reached. The overall quaternary and tertiary structure of cjBlue(Y64L) was found to be similar to cjBlue, but significant differences were observed in the chromophore structure and environment (Fig. 3).

In comparison with the wild-type, the overall size of the cjBlue(Y64L) chromophore is smaller than that of the cjBlue chromophore. The cjBlue(Y64L) chromophore consists of a single imidazolinone ring and has the wild-type tyrosine ring

### Table 1

<table>
<thead>
<tr>
<th>Sample/Data set</th>
<th>Wavelength</th>
<th>Resolution</th>
<th>Reflections (total/unique)</th>
<th>Completeness</th>
<th>$R_{merge}$</th>
<th>$(I/I_{o})$ (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cjBlue (Peak)</td>
<td>0.9793</td>
<td>50.0–1.6</td>
<td>8,835,277/335,346</td>
<td>99.3 (100)</td>
<td>7.7 (26.1)</td>
<td>12.0 (9.0)</td>
</tr>
<tr>
<td>cjBlue(Y64L) (Native)</td>
<td>1.5419</td>
<td>50.0–1.8</td>
<td>1,054,020/121,926</td>
<td>100 (100)</td>
<td>6.2 (38.1)</td>
<td>39.65 (3.0)</td>
</tr>
</tbody>
</table>

### Refinement statistics

<table>
<thead>
<tr>
<th>Sample/Data set</th>
<th>Resolution</th>
<th>Reflections (working/test)</th>
<th>Total number of atoms</th>
<th>$R_{crys}$</th>
<th>$R_{free}$</th>
<th>(\text{B}) Value</th>
<th>r.m.s. (\sigma) deviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>cjBlue (Peak)</td>
<td>50.0–1.8</td>
<td>318,579/16,767</td>
<td>15,730</td>
<td>19.5/22.5</td>
<td>19.5</td>
<td>0.006</td>
<td>1.673</td>
</tr>
<tr>
<td>cjBlue(Y64L) (Native)</td>
<td>50.0–2.0</td>
<td>107,910/5,761</td>
<td>15,706</td>
<td>19.8/24.4</td>
<td>16.2</td>
<td>0.006</td>
<td>1.624</td>
</tr>
</tbody>
</table>

\(R_{merge} = \frac{\sum_{i} \sum_{h} |I_h - \langle I_h \rangle|}{\sum_{i} \sum_{h} I_h} \times 100\), where \(I\) is the intensity of \(i\) observations for reflection \(h\) and \(\langle I\rangle\) is the mean intensity of the reflection.

\((I/I_{o})\), mean intensity/mean standard deviation.

\(R_{crys} = 100 \times \frac{\sum |F_{o} - F_{c}|}{\sum |F_{o}|}, \) where \(F_{o}\) and \(F_{c}\) are the observed and calculated structure factor magnitudes, respectively.

\(R_{free}\) as for \(R_{crys}\) except calculated for 4.7% of the reflections not used for model refinement.

\(\text{r.m.s.}^{\sigma}\), root mean square.

### Figure 3

The four subunit association, monomer, and electron density map of the chromophore environment. The representative top view of the cjBlue four subunit arrangements is shown in A. The monomeric cjBlue subunit is shown in B. CjBlue 2\(F_o - F_c\) density map contoured at 1.0 \(\sigma\) is shown in C. CjBlue(Y64L) electron density 2\(F_o - F_c\) map contoured at 1.0 \(\sigma\) is shown in D.

Crystal Structures of Chromoproteins
replaced with an isobutyl moiety from leucine. This generates a new 5-isobutyl-imidazolinone group, which forms the cjBlue(Y64L) chromophore (Fig. 3). As compared with the cjBlue chromophore, the overall electron system conjugation is reduced despite the presence of an acylimine bond between the Cα and nitrogen atom of Gln63.

Similar to cjBlue, the deeply buried chromophore in cjBlue(Y64L) forms extensive contacts with neighboring residues and internal water molecules (Fig. 4). The isobutyl moiety creates a new hydrogen bond network between the cjBlue(Y64L) chromophore and water molecules. Three water molecules are found in this network for chromophore stabilization within the β-barrel (Fig. 4). Water molecule W4 forms a hydrogen bond with O2 (3.2 Å) and N2 (3.5 Å) of the chromophore and a third hydrogen bond with W3 (2.4 Å), which in turn hydrogen bonds with W2 (2.8 Å) (Fig. 4). Completion of the bridging coordination between the chromophore and β-barrel is achieved by W2 hydrogen bonding to Thr158 (2.9 Å). The cjBlue(Y64L) water molecule (W1) is shifted from an equivalent water molecule (W1) position in cjBlue forming a new hydrogen bond with Glu145 (3.0 Å), Thr158 (3.0 Å), and Thr176 (2.7 Å). This point mutation also brings the chromophore into closer proximity with His197 (Fig. 5). Such proximity between His197 and the chromophore, not previously observed in cjBlue, appears to contribute to cjBlue(Y64L) absorbance. A striking difference between the cjBlue and cjBlue(Y64L) structures is that His197 assumes two different conformations at a nearly identical population within the cjBlue crystal structure. In conformation A, His197 χ1 is 117° and χ2 of 51°. Although His197 in conformation B has dihedral angles χ1 of −64° and χ2 of −65°.

To assess the importance of His197 in the optic properties of cjBlue and cjBlue(Y64L), we generated cjBlue(H197S) and cjBlue(Y64L/H197S) mutants. Not surprisingly, both cjBlue(H197S) and cjBlue(Y64L/H197S) yielded a colorless protein with previous absorbance peaks abolished (Fig. 2). Proper protein folding of cjBlue(H197S) and cjBlue(Y64L/H197S) was confirmed through circular dichroism analysis (data not shown).
DISCUSSION

In this study, we determined the crystal structure of a blue CP, cjBlue, to 1.8 and 2.0 Å, respectively, and compared it with the crystal structures of Rtms5 (6) and dsRed (18). We suggest that the cjBlue chromophore follows a similar formation mechanism as suggested for Rtms5 (6, 8) and dsRed (18, 20) (supplemental Fig. 1). However, noticeable differences are seen in the planarity of the hydroxyphenyl ring among the non-fluorescent cjBlue, the weakly fluorescent Rtms5 (6), and the highly fluorescent dsRed (18). The cjBlue and Rtms5 chromophores adopt a trans non-co-planar conformation with the hydroxyphenyl ring rotated 40° and 35° out of plane, respectively (supplemental Fig. 2). In comparison, the dsRed chromophore adopts a cis co-planar conformation with a nearly perfect planarity (<1°).

Another piece of information is that the chromophore of a highly fluorescent protein, eqFP611, has a trans co-planar conformation (Fig. 5). In Rtms5, Arg^{197} is found in the same position as His^{197} and favors the trans chromophore orientation through hydrogen bonding with the phenoxy group (6). Mutagenesis of His^{197} to Ser^{197} in cjBlue results in the loss of absorbance at 610 nm (Fig. 2), which suggests that His^{197} is required for catalysis of the chromophore or that the imidazole ring sustains the trans non-co-planar conformation of the chromophore. Second, interactions between the phenoxy group of the chromophore with Glu^{145} of β-strand 7 and Thr^{158} of β-strand 8 stabilize the orientation of the chromophore (Figs. 1 and 4). Of note, Rtms5 has Glu^{148} in β-strand 7 and Asn^{161} in β-strand 8 found at the equivalent position (6). The interaction of these residues with the chromophore contributes to the chromophore conformational stability.

Through semi-random mutagenesis studies, we have identified a chromophore mutant, cjBlue(Y64L) in which the hydroxyphenol group of tyrosine is replaced with the isobutyl moiety of the leucine, resulting in a significant blue shift in the absorbance spectrum (610–417 nm). Visually this mutant appears yellow in color (Fig. 2). The same blue shift was achieved by the mutagenesis of Tyr^{64} to Met. To investigate the structural basis for this color shift, the crystal structure of cjBlue(Y64L) was determined to 2.0 Å. The cjBlue(Y64L) chromophore structure comprises a single cyclical imidazolinone ring and forms a unique hydrogen bond network with its surrounding residues (Fig. 4); Glu^{145} of β-strand seven hydrogen bonds with Arg^{147} of β-strand 7 instead of the chromophore. Thr^{158} of β-strand 8 no longer interacts with the chromophore but rather forms new hydrogen bonds with other neighboring residues.

Strikingly, the crystal structure of the cjBlue(Y64L) mutant revealed two alternative side-chain conformations of His^{197} (Fig. 5, B and C), both of which increased proximity to the chromophore relative to that in the wild-type structure. In conformation A, the distance between the side chain of His^{197} and the chromophore is 3.1–5.5 Å, whereas in conformation B the distance increases to 4.2–4.5 Å (Fig. 5). It is possible that these two conformations coexist in solution, thereby contributing to the blue-shifted absorbance of cjBlue(Y64L). Interestingly, the isobutyl moiety of the cjBlue(Y64L) chromophore appears to have missing electron density in our structure, suggesting that the conformational flexibility of His^{197} is intimately coupled with the chromophore conformation. It remains to be deter-

FIGURE 5. The importance of His^{197} in cjBlue and cjBlue(Y64L). The ring-stacking effect of His^{197} in the p-hydroxyphenyl ring of the cjBlue chromophore is shown in A. The proximal effect of His^{197} conformation A and conformation B on the cjBlue(Y64L) imidazolinone ring is depicted in B and C, respectively. Distances between the atoms are represented with dashed red lines and numbers corresponding to the distances in angstroms.
mined what exact contributions each conformer has to generate the yellow appearance.

cjBlue and the cjBlue(Y64L) mutant set a new absorbance range (417–610 nm) for CPs. The structures of cjBlue and cjBlue(Y64L) provide valuable insights into the understanding of the non-fluorescent properties of this sea anemone protein. The cjBlue crystal structure provided a better understanding for the importance of the ring stacking effect between Tyr64 and His197. The cjBlue(Y64L) crystal structure revealed the interconversion between two His197 imidazole ring conformations on the CP optical behavior. We conclude that the dynamics and structure of the chromophore are both essential for the optical appearance of these color proteins. Future studies on the chromophore formation mechanism will further increase our knowledge of CPs and strengthen our understanding of their spectral properties.

Acknowledgments—We thank the staff of 19-ID beamline at Advanced Photo Source for help on data collection and Dr. Emil Pai of the University of Toronto for his encouragement. We also thank lab members of Drs. M. Ikura and E. Pai for helpful discussions.

REFERENCES

22. Deleted in proof.