Structural Characterization of a Blue Chromoprotein and Its Yellow Mutant from the Sea Anemone *Cnidopus Japonicus*[*][S]

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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 Gln[63]-Tyr[64]-Gly[65]. The ring stacking between Tyr[64] and His[197] stabilized the cjBlue *trans* chromophore conformation along the Cα2–Cβ2 bond of 5-[(4-hydroxyphenyl)methylene]-imidazolinone, which closely resembled that of the "Kindling Fluorescent Protein" and Rtms5. Replacement of Tyr[64] 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 His[197] 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)[4] 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: Rtms5 from *Montipora efflorescens* (8), gtCP from *Goniopora tenuidens* (9), aeCP597 from *Actinia equine* (10), and asFP595 from *Anemonia sulcata* (KFP) (5). Three-dimensional structures of Rtms5 (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.

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′-GAAGGRTGYGTCAAYGGRCAY-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-L-methionine-labeled protein was produced using minimal M9 media and expressed with N-terminal His$_6$ tag using B834 (DE3) *Escherichia coli* strain (Novagen). Cells were grown in a shaker incubator at 37 °C until an A_{600} of 1.20 was reached. Upon induction with 1 mM isopropyl 1-thio-β-D-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 His$_6$ tag was subsequently removed using thrombin followed by size exclusion chromatography (Superdex 75, Amersham Biosciences) to achieve satisfactory levels of purity. Incorporation seleno-L-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-carboxethyl)phosphine (TCEP). Blue plated crystals (P2$_1$), 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 M NaH$_2$PO$_4$, 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 flashed 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 P2$_1$ 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 NaHPO$_4$ 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 Rtms5 (6) (Fig. 1). The full-length protein was expressed in *E. coli* with a His$_6$ 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 P2$_1$ space group. A final R_{factor} and R_{free} of 19.5 and 22.5%, respectively, were reached (Table 1). The final cjBlue model contained eight subunits (residues 5–232), 16 PO$_4$, 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 Rtms5 (6). Each subunit consists of β-strands 11 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. 3*A*), in a manner similar to that observed for eqFP611 (7), Rtms5 (6), and dsRed (17). This molecular association is stabilized by 1) inter-barrel interface interactions and 2) association of the C terminus tails with β-strands 11 of adjacent pair of subunits (Fig. 3). Asn[20], Asn[21], Thr[103], Gln[105], and Arg[179] are residues located at the inter-barrel interface forming hydrogen bonds between subunits. The C-terminal tail interaction involves hydrogen bonding between Ser[227], His[231], and Asn[232] at one tail and Asp[196], Arg[198], Glu[200], and Thr[224] within β-strands 11 of an adjacent pair of subunit.

The cjBlue chromophore consists of a 5-[(4-hydroxyphenyl)-methylene]-imidazolinone group with an acylimine bond found between the Cα and nitrogen atom positioned at Gln[63] (Figs. 3

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                              ┌──────────β1──────────┐        ┌────β2────┐    ┌────β3────┐
cjBlue    100.0%   -----------MASKISDNVRIKLYMEGTVNNHHFMCEAEGEGKPYEGTQMENIKVTKGGPL  51
KFP        61.0%   MRGSHHHHHHGSASFLKKTMPFKTTIEGTVNGHYFKCTGKGEGNPFEGTQEMKIEVIEGGPL  62
hcCP       59.0%   -----------MAGLLKESMRIKIYMEGTVNGYHFKCEGEDGNPFEGTQNMRIRVTEGAPL  51
eqFP611    52.0%   ---------IHMNSLIKENMRMMVVMEGSVNGYQFKCTGEGDGNPYMGTQMTRIKVVEGGPL  51
Rtms5      51.0%   ------------MSVIATQMTYKVYMSGTVNGHYFEVEGDGKGRPYEGEQTVKLTVTKGGPL  50
dsRed      45.0%   --------MRSSKNVIKEFMRFKVRMEGTVNGHEFEIEGEGEGRPYEGHNTVKLKVTKGGPL  54
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       ┌────────α1────────┐         ┌─α2─┐      ┌───β4───┐   ┌───β5───┐
cjBlue    PFSFDILTPNCQYGSVAITKYTSGIPDYFKQSFPEGFTWERTTIYEDGAYLTTQQETKLDGN  113
KFP       PFAFHILSTSCMYGSKTFIKYVSGIPDYFKQSFPEGFTWERTTTYEDGGFLTAHQDTSLDGD  122
hcCP      PFAFDILSPCCAYGSKTFIKHTSGIPDYFKQSFPEGFTWERTTIYEDGGVLTAHQDTSLEGN  113
eqFP611   PFAFDILATSFMYGSKTFIKHTKGIPDFFKQSFPEGFTWERVTRYEDGGVFTVMQDTSLEDG  113
Rtms5     PFAWDILSPQCQYGSIPFTKPED-IPDYVKQSFPEGFTWERIMNFEDGAVCTVSNDSSIQGN  111
dsRed     PFAWDILSPQFQYGSKVYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDG  114
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    ┌───β6───┐      ┌─α3─┐    ┌───β7───┐    ┌───β8───┐        ┌────β9────┐
cjBlue    CLVYNIKILGCNFPPNGPVMQKKTQGWEPCCEMRYTRDGVLCGQTLMALKCADGNHLTCHLR  175
KFP       CLVYKVKILGNNFPADGPVMQNKAGRWEPGTEIVYEVDGVLRGQSLMALKCPGGRHLTCHLH  184
hcCP      CLIYKVKVLGTNFPADGPVMKKISGGWEPCTEIVYQDNGVLGRNVMALKVSGRPPLICHLH  175
eqFP611   CLVYHAKVTGVNFPSNGAVMQKKTKGWEPNTEMLYPADGGLRGYSQMALNVDGGGYLSCSFE  175
Rtms5     CFTYHVKFSGLNFPPNGPVMQKKTQGWEPHSERLFARGGMLIGNNFMALKLEGGGHYLCEFK  173
dsRed     CFIYKVKFIGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLKGEIHKALKLKDGGHYLVEFK  176
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    ┌────β9────┐         ┌────────β10────────┐    ┌──────────β11──────────┐
cjBlue    TTYRSKKAAKALQMPPFHFSDHRPEIVKVS-ENGTLFEQHESSVARYCQTCPSKLGHN----  232
KFP       TTYRSKKPASALKMPGFHFEDHRIEIMEEV-EKGKCYKQYEAAVGRYCDAAPSKLGHN----  241
hcCP      STYRSKK-ACALTMPGFHFADLRIQMPKKKKDEY--FELYEASVARYSDVPEKAT-------  227
eqFP611   TTYRSKKTVENFKMPGFHFVDHRLERLEES-DKEMFVVQHEHAVAKFCD-LPSKLGRL----  231
Rtms5     TTYKAKKP---VKMPGYHYVDRKLDVTNHN-KDYTSVEQCEISIARKPVVA----------  220
dsRed     SIYMAKKP---VQLPGYYYVDSKLDITSHN-EDYTIVEQYERTEGRHHLFL----------  223
```
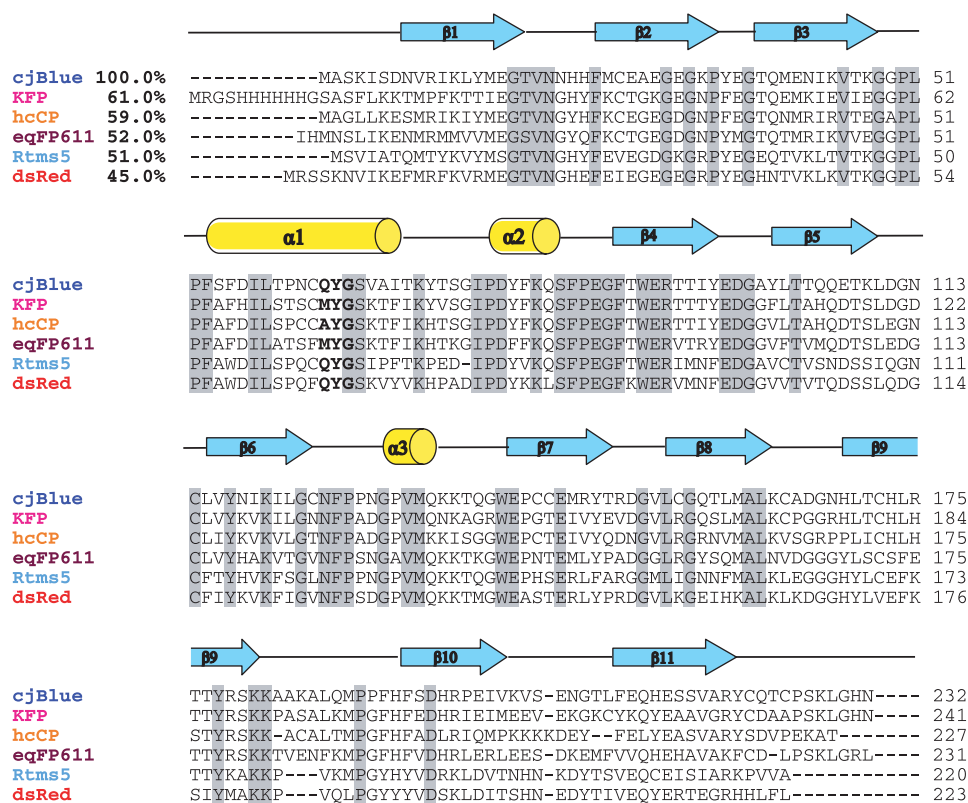
FIGURE 1. **Sequence alignment of cjBlue with other GFP-like family members.** The conserve residues are highlighted in *gray*. The chromophore region is in *bold*. The *blue arrow* above the sequence alignment indicates the β-strand regions. The *yellow cylinder* above the sequence alignment indicates the α-helices region. The percent identity to cjBlue is noted beside each sequence. National Center for Biotechnology Information (NCBI) accession numbers for sequences used in the alignment are: KFP (5) (1XMZA), (15) (Q95W85); eqFP611 (7) (1UISA); Rtms5 (6) (1MOU); and dsRed (17) (1GGXA).
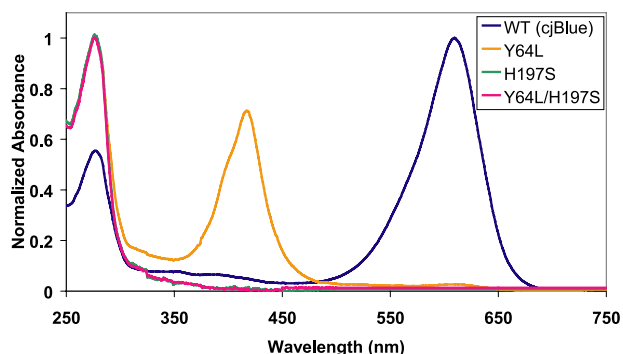

FIGURE 2. **Absorbance spectra of cjBlue and mutant variants.** A pH of 7.0, a temperature of 22 °C, and the mature form of CPs were used to standardize the absorbance profile. Wild-type (*WT*) cjBlue is shown in *blue*, cjBlue(Y64L) in *yellow*, cjBlue(H197S) in *green*, and cjBlue(Y64L/H197S) in *purple*.

and 4) (6). The additional double bond at Gln[63] is comparable with the double bond found at Gln[66] of dsRed (17, 18). Like other FPs and CPs, the cjBlue chromophore is buried deeply within the β-barrel (Fig. 3) with substituents hydrogen bonded to neighboring residues and internal water molecules. These residues include Thr[158] (2.5 Å), Glu[145] (3.1 Å), and water molecule W1 (3.3 Å), which are hydrogen bonded to the oxygen of the *p*-hydroxyphenyl ring (Fig. 4). Remarkably, water molecules near the cjBlue chromophore were all found in the same positions as those seen in the crystal structure of Rtms5 (6). The

imidazole 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 Cα2–Cβ2 bond with noticeable bond angle distortion. Because of internal steric hindrance between O2 and Cδ2–H, an increase in the C2–Cα2–Cβ2 bond angle (141°) and the Cα2–Cβ2–Cγ2 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 Cδ2–H (supplemental Fig. 1). For example, structures such as Rtms5 (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, Rtms5 (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 χ_1 (C2–Cα2–Cβ2–Cγ2) and χ_2 (Cα2–Cβ2–Cγ2–Cδ2). Rtms5 (6) and KFP (5) have a χ_1 and χ_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 aromatic-less 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%,

TABLE 1
Data collection and refinement statistics for cjBlue and cjBlue(Y64L)

			Data collection			
Sample/Data set	Wavelength	Resolution	Reflections (total/unique)	Completeness	R_{merge}[a]	$\langle I \rangle / \langle \sigma(I) \rangle$[b]
	Å	Å		%	%	
cjBlue (Peak)	0.9793	50.0–1.6	8,835,277/335,346	99.3 (100)	7.7 (26.1)	12.0 (9.0)
cjBlue(Y64L) (Native)	1.5419	50.0–1.8	1,054,020/121,926	100 (100)	6.2 (38.1)	39.65
						(3.0)

			Refinement statistics				
						r.m.s.[e] deviations	
Sample/Data set	Resolution	Reflections (working/test)	Total number of atoms	R_{cryst}[c]$/R_{free}$[d]	$\langle B \rangle$ Value	Bond	Angle (°)
	Å				Å^2	Å	
cjBlue (Peak)	50.0–1.8	318,579/16,767	15,730	19.5/22.5	19.5	0.006	1.673
cjBlue(Y64L) (Native)	50.0–2.0	107,910/5,761	15,706	19.8/24.4	16.2	0.006	1.624

[a] $R_{merge} = \Sigma_h\Sigma_i|I_{hi} - \langle I_h \rangle|/\Sigma_h\Sigma_i|\langle I_h \rangle| \times 100$, where I is the intensity of i observations for reflection h and I_h is the mean intensity of the reflection.
[b] $\langle I \rangle/\langle \sigma I \rangle$, mean intensity/mean standard deviation.
[c] $R_{cryst} = 100 \times \Sigma|F_{obs} - F_{calc}|/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor magnitudes, respectively.
[d] R_{free} as for R_{cryst} except calculated for 4.7% of the reflections not used for model refinement.
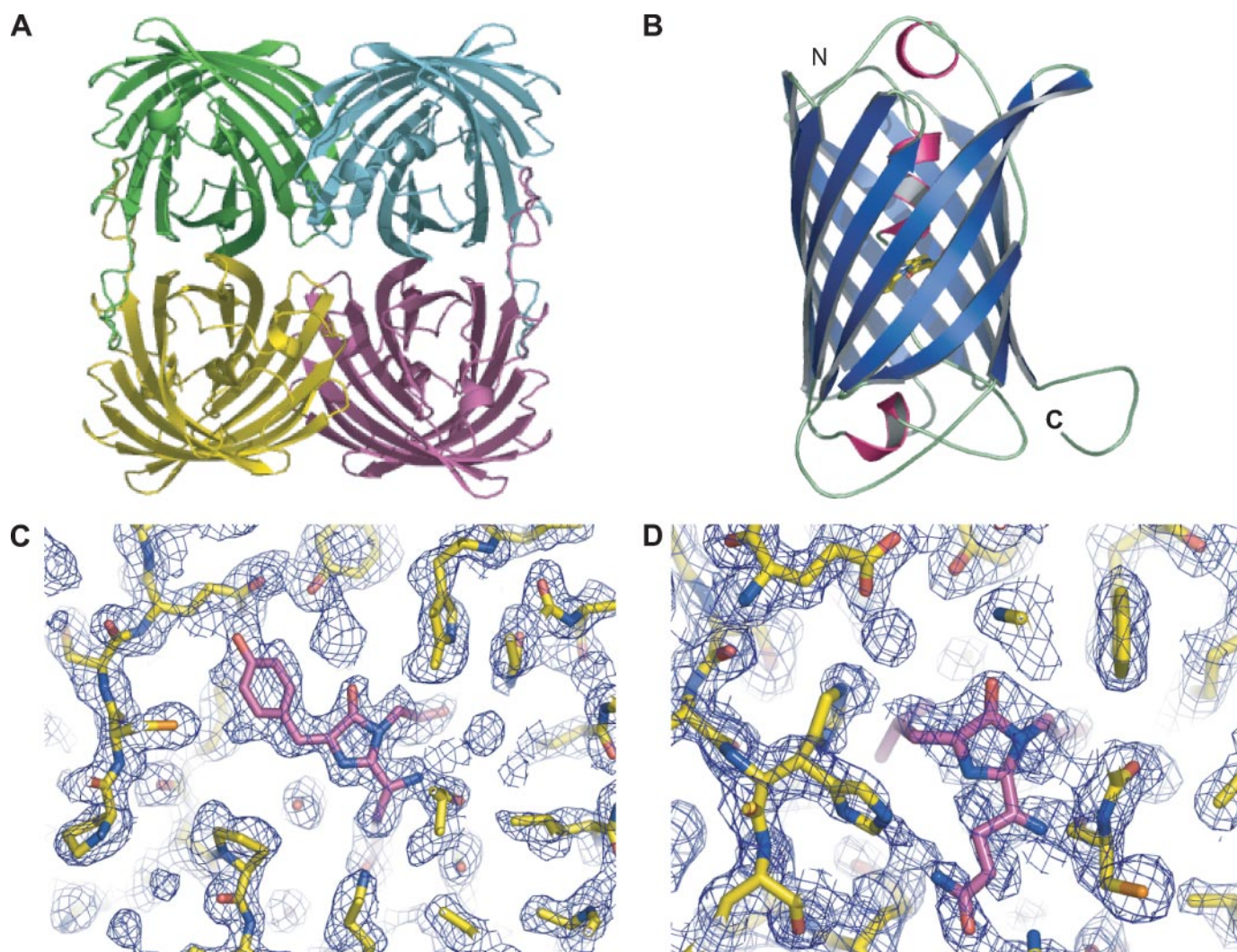[e] r.m.s., root mean square.

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 $2F_o - F_c$ density map contoured at 1.0 σ is shown in *C*. cjBlue(Y64L) electron density $2F_o - F_c$ map contoured at 1.0 σ is shown in *D*.

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
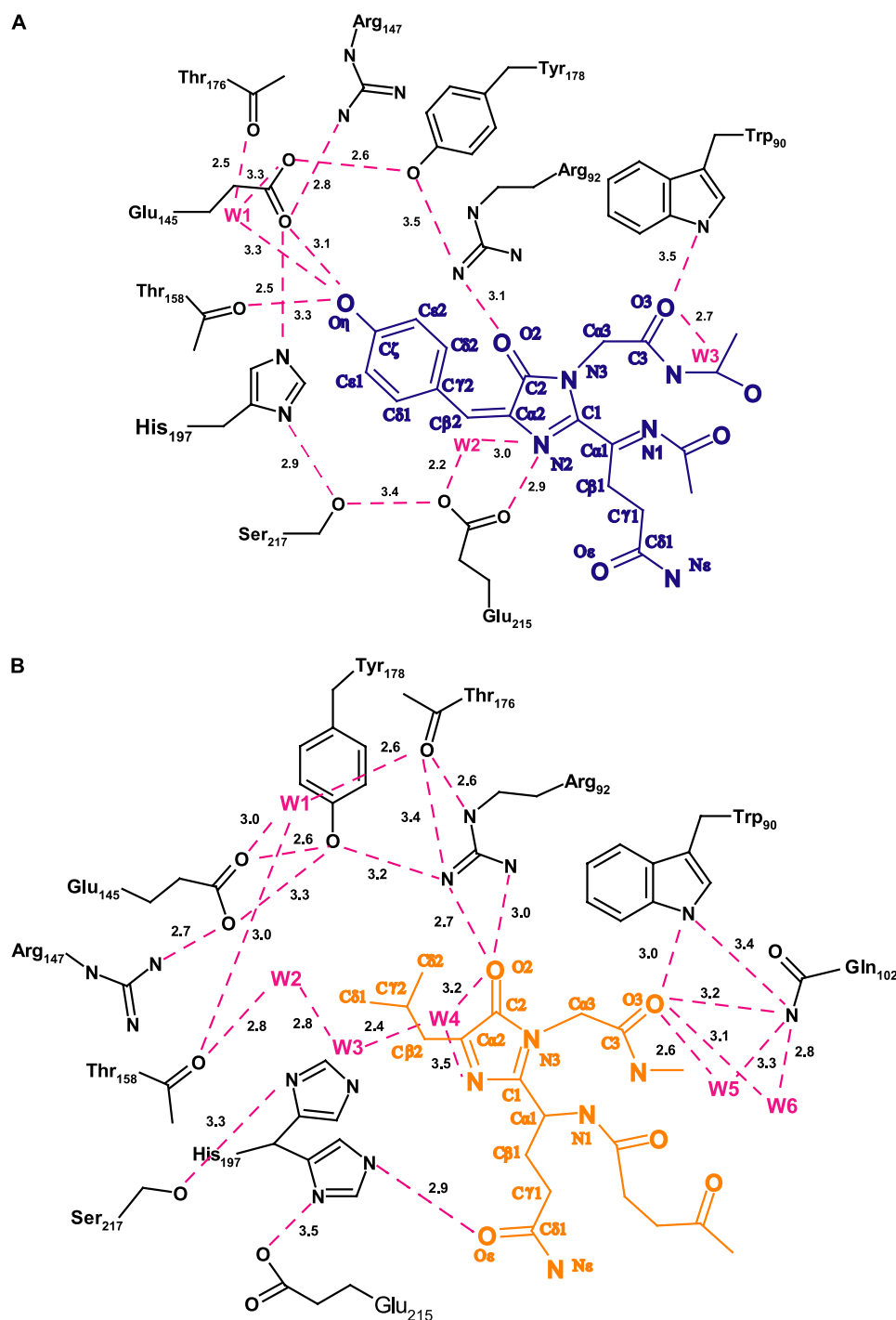
FIGURE 4. **Coordination of the cjBlue and cjBlue(Y64L) chromophore environment.** A two-dimensional schematic representation of the cjBlue chromophore (*blue*) coordination is shown in *A*, and the cjBlue(Y64L) (*orange*) chromophore coordination is shown in *B* with the two conformations of His[197]. The *purple letter W* represents water molecules. Hydrogen bonds are represented with *dashed purple lines* with numbers indicating the distance in angstroms. A cut-off distance for hydrogen bonding is 3.50 Å. The atoms forming the chromophore of cjBlue and cjBlue(Y64L) are represented in *A* and *B*, respectively.

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 Thr[158] (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 Glu[145] (3.0 Å), Thr[158] (3.0 Å), and Thr[176] (2.7 Å). This point mutation also brings the chromophore into closer proximity with His[197] (Fig. 5). Such proximity between His[197] 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 His[197] assumes two different conformations at a nearly identical population within the cjBlue crystal structure. In conformation A, His[197] Nϵ is 3.1 and 5.5 Å away from Cβ2 and N2 of the chromophore, respectively. In conformation B, the distance of His[197] Nϵ is 4.2 and 4.6 Å away from Cβ2 and N2 of the chromophore, respectively. Conformation A, His[197] has corresponding dihedral angles χ_1 of 117° and χ_2 of 51°. Although His[197] in conformation B has dihedral angles χ_1 of −64° and χ_2 of −65°.

To assess the importance of His[197] in the optic properties of cjBlue and cjBlue(Y64L), we generated cjBlue(H197S) and cjBlue-

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 π-system conjugation is reduced despite the presence of an acylimine bond between the Cα and nitrogen atom of Gln[63].

(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).
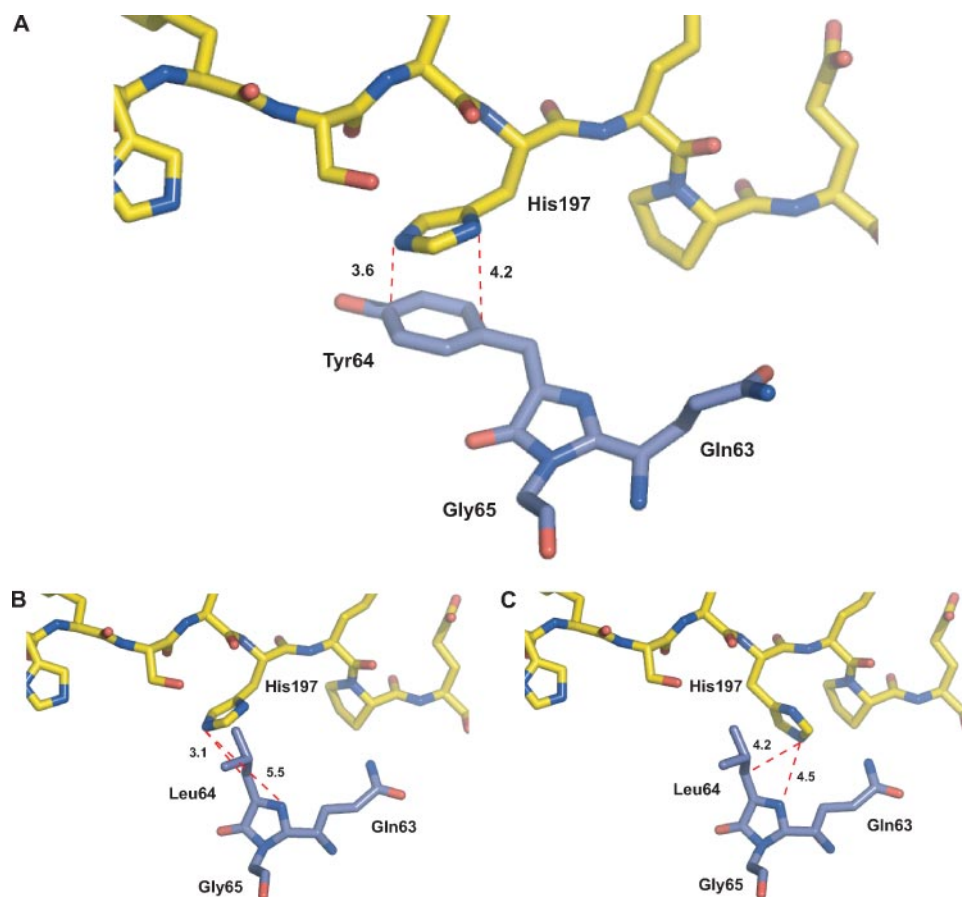
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FIGURE 5. **The importance of His[197] in cjBlue and cjBlue(Y64L).** The ring-stacking effect of His[197] in the *p*-hydroxyphenyl ring of the cjBlue chromophore is shown in *A*. The proximal effect of His[197] conformation A and conformation B on 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.

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. Taken altogether, there is a correlation between the chromophore co-planarity and high fluorescence quantum yield.

Further inspection of the cjBlue crystal structure revealed a number of residues, which contributed to the *trans* non-co-planar conformation of the cjBlue chromophore. First, His[197] appears to stabilize chromophore conformation through parallel ring-stacking interaction with the hydroxyphenyl ring (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 conformation 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-

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 Tyr[64] and His[197]. The cjBlue(Y64L) crystal structure revealed the interconversion between two His[197] 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.

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