Structure and Reactivity of the Chromophore of a GFP-like Chromoprotein from *Condylactis gigantea*†

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ABSTRACT: Here we present the study of the chromophore structure of the purple chromoprotein from *Condylactis gigantea*. Tandem mass spectrometry and ^{1}H and ^{13}C NMR of the chromopeptide reveal that the protein contains a chromophore with a chemical structure identical to that of the red fluorescent protein from *Discosoma* sp. A single A63G substitution demonstrates that the nature of the first amino acid of the XYG chromophore-forming sequence is dispensable for the chromoprotein red shift development. It has been recently proposed that post-translational reactions at the acylimine, a chemical group that accounts for the red fluorescence, might be an additional source of spectral diversity of proteins homologous to the *Aequorea victoria* green fluorescent protein (GFP). We have examined the reactivity of the chromophore acylimine group within the *C. gigantea* purple chromoprotein. Like other proteins with the acylimine-modified chromophore, the purple chromoprotein suffers a hypsochromic spectral shift to the GFP-like absorbance (386 nm) upon mild denaturation. NMR analysis of the chromopeptide suggests this hypsochromic spectral shift is due to H_2O addition across the C═N bond of the acylimine. However, unlike the red fluorescent protein from *Discosoma* sp., denatured under harsh conditions, the wild-type chromoprotein exhibits only slight fragmentation, which is induced by complete hydrolysis of the acylimine. A model suggesting the influence of the amino acid X side chain on protein fragmentation is presented.

During the past decade, the green fluorescent protein from *Aequorea victoria* (GFP)[1] has become a valuable tool that enabled direct visualization of intracellular processes in live cells (*1*). The recent discovery of GFP-like proteins from *Anthozoa* species offers new perspectives on their applications in molecular and cellular biology (*2*−*8*). According to optical properties, GFP-like proteins can be conventionally classified into two general groups: proteins with inherent fluorescence (FPs) and naturally nonfluorescent (or weakly fluorescent) chromoproteins (CPs) (*3*, *8*). Noteworthy, all known CPs can be converted to fluorescent analogues by mutagenesis (*4*, *9*).

According to the type of chromophore structure, GFP-like proteins can be classified into at least three subfamilies: GFP, DsRed, and Kaede. Chromophore assembly within the proteins of the GFP-subfamily is a two-stage process, which includes an autocatalytic cyclization reaction followed by dehydrogenation. All of the green-emitting proteins apparently contain this type of chromophore, *p*-hydroxybenzylideneimidazolinone (Figure 1, structure **a**, highlighted) (*3*). In the DsRed subfamily, the first two reactions are the same as in GFP, and the third reaction is additional dehydrogenation (presumably autocatalytic oxidation that requires O_2) (Figure 1, structure **b**), which extends the π-electron-conjugated structure of the green chromophore (*10*−*12*). Several FPs with red and far-red emission, and CPs as well, were found to possess a DsRed-related structure (*13*−*15*). The green form of the proteins of the Kaede subfamily is produced in a manner similar to that of GFP, but the conversion to the red state is a light-dependent reaction (*16*). A number of FPs of this subfamily have been recently identified, all of them containing the HYG consensus sequence in the chromophore-forming region (*16*−*20*).

The acylimine C═N bond (Figure 1, structure **b**, highlighted), which is the result of additional dehydrogenation in the DsRed-like chromophore, accounts for the red-shifted absorption and emission spectra and, due to its reactivity, is a source of potential modifications of the DsRed-like structure. It has been recently proposed that nucleophilic reactions at the acylimine C═N bond might take place upon maturation of naturally occurring GFP-like proteins (Figure 1, structures c and g). These studies suggest that the

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[1] Abbreviations: FP, fluorescent protein; CP, chromoprotein; GFP, green fluorescent protein from *A. victoria*; DsRed, red fluorescent protein from *Discosoma* sp.; asFP595, purple chromoprotein from *Anemonia sulcata*; zFP538, yellow fluorescent protein from *Zoanthus* sp.; gtCP, chromoprotein from *G. tenuidens*; cgCP, purple chromoprotein from *C. gigantea*; hcCP, chromoprotein from *H. crispa*; aeCP, blue chromoprotein from *Actinia equina*; Kaede, fluorescent protein from *Trachyphyllia geoffroyi*; Rtms5, blue chromoprotein from *Montipora efflorescens*; Rtms1, chromoprotein from *Montipora efflorescens*; ESI, electrospray ionization; HMBC, heteronuclear multiple-bond correlation spectroscopy; HMQC, heteronuclear multiple-quantum correlation spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; ROESY, two-dimensional rotating-frame Overhauser effect spectroscopy.

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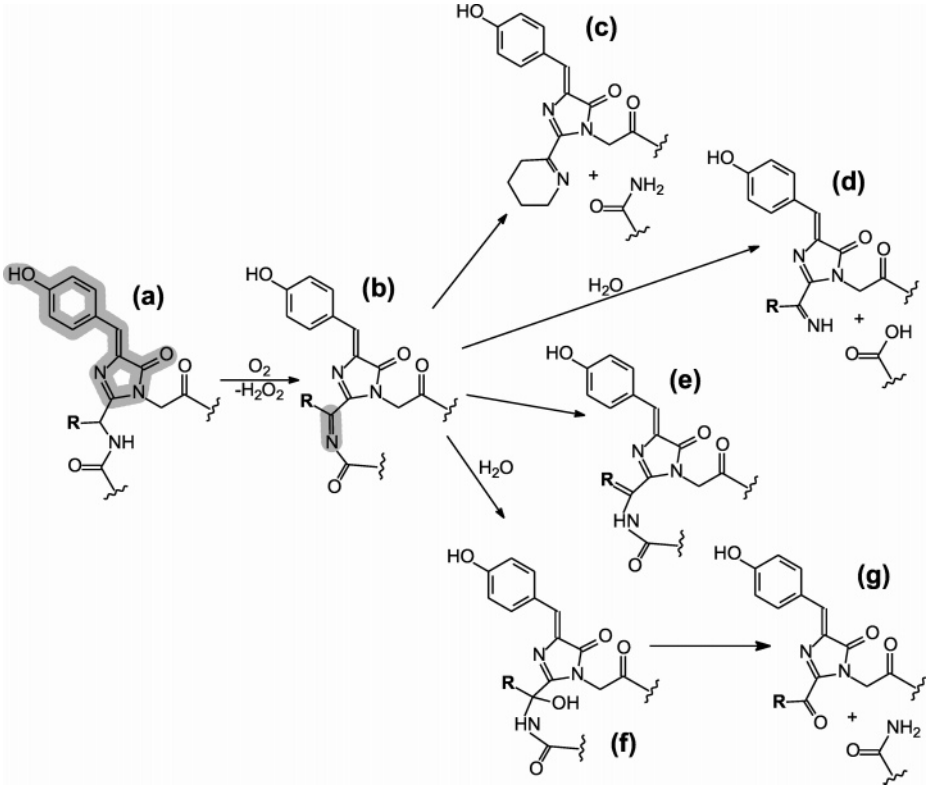

FIGURE 1: DsRed chromophore synthesis and proposed acylimine reaction pathways. The DsRed chromophore (structure **b**) results from autocatalytic oxidation (O_2-mediated dehydrogenation) of a GFP-like structure (structure **a**, *p*-hydroxybenzylideneimidazolinone, highlighted). Due to acylimine reactivity, the C═N bond (structure **b**, highlighted) of the DsRed chromophore is a potential source of subsequent reactions. Upon zFP538 maturation, the lysine side chain amino group (R- in structure **b** corresponds to the side chain of Lys-66 in this particular case) is proposed to react with the carbon of the C═N bond of the acylimine, resulting in structure **c** (*21*). Structure **g** is a proposed structure of the asFP595 chromophore (*22*, *23*). Structure **d** is an alternative imino-substituted chromophore structure of zFP538 and asFP595, which was proposed to result from spontaneous polypeptide cleavage between the nitrogen and carbonyl carbon of the acylimine (*24*, *25*). Structures **d**−**g** might be expected as a result of protein denaturation as well.

transiently appearing DsRed-like acylimine is an intermediate step in the maturation process of zFP538 and asFP595 (*21*−*23*). In contrast, an unusual chemistry of acylimine within zFP538 and asFP595 chromophores has recently been reported (Figure 1, structure **d**) (*24*, *25*). Due to intramolecular reactions at the acylimine, the polypeptide chains of zFP538 and asFP595 suffer a break upon protein maturation. The mechanisms of these reactions, especially for asFP595, have not been unambiguously determined (*21*−*25*). Unlike zFP538 and asFP595, the proteins of the DsRed subfamily originally represent single-polypeptide proteins. However, upon denaturation, both DsRed and gtCP undergo quantitative fragmentation (*10*, *15*).

The purple chromoprotein from the sea anemone *Condylactis gigantea* (cgCP) belongs to a group of so-called far-red proteins, since its mutant variants display emission in the far-red range (622 nm) (*4*). In contrast to DsRed and a chromoprotein from *Goniopora tenuidens* (gtCP) (*10*, *15*), cgCP exhibits substantially reduced fragmentation efficiency upon denaturation, which in the case of DsRed and gtCP was proposed to result from acylimine hydrolysis (Figure 1, structure **g**). These observations prompted us to investigate the chemical nature of the cgCP chromophore. We report here that cgCP contains a chromophore of the DsRed-like structure, with the acylimine substituent extending the overall conjugated π-system. Our results provide evidence that the side chain of the first chromophore-forming amino acid is dispensable for cgCP red shift development. We further examine acylimine reaction pathways upon cgCP denaturation and explore the reasons for the suppressed acylimine reactivity. Our findings demonstrate that upon denaturation the cgCP chromophore acylimine readily reacts with H_2O, yielding a sufficiently stable carbinolamide derivative in this case (Figure 1, structure **f**). The results also suggest that the efficiency of protein fragmentation and, accordingly, the completeness of acylimine hydrolysis are dependent on the nature of the first chromophore-forming amino acid.

MATERIALS AND METHODS

Protein Expression, Purification, and Mutagenesis. Plasmids pQE30-cgCP, pQE30-hcCP, and pQE30-aeCP were a generous gift from K. A. Lukyanov (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry). The recombinant proteins with the N-terminal six-His tag were expressed in *Escherichia coli* (*4*) and purified from the cell lysates by metal affinity chromatography on Ni−NTA resin (Qiagen). Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene). The resulting plasmids were sequenced to confirm mutations.

Spectroscopy and SDS−PAGE Analysis. Absorbance spectra were recorded on a Cary 50 Bio UV−vis spectrophotometer (Varian). For the time course observations of the protein hypsochromic spectral transition upon denaturation, the protein solution was adjusted to pH 1.8 via addition of 0.1 M HCl (mild denaturing conditions). Protein samples were analyzed by SDS−PAGE with 15% polyacrylamide

gels. For acid-induced fragmentation of the proteins, 5 μL of 1.0 M HCl was added to 45 μL of the protein solution, and the samples were boiled for 5 min (harsh denaturing conditions) and immediately cooled to 4 °C. Fifty microliters of cooled methanol and an equal volume of chloroform were added to the acidified protein solution. After being shaken vigorously, the samples were centrifuged, and the upper phase was accurately discarded, preventing disturbance of the interphase. One hundred microliters of cooled methanol was added to the lower phase, and the samples were centrifuged again. The resulting protein pellets were washed twice by centrifugation in cool methanol, dried in vacuo, and dissolved in electrophoresis buffer.

Chromopeptide Isolation and Mass Spectrometry. Purified His-tagged cgCP at 6 mg/mL was denatured by addition of a 0.1 M HCl solution to a final pH of 2.8. Pepsin (Sigma) was further added in a 1:30 (w/w) ratio, and the digest was carried out at room temperature for 24 h. The chromopeptide was isolated by a reverse-phase HPLC procedure described previously (*15*). Peptides absorbing at 380 nm were collected, and amino acid sequencing was performed with an Applied Biosystems 491 sequenator. Peptide masses were analyzed with a Bruker Daltonics Esquire 3000 Plus mass spectrometer equipped with an electrospray source of ionization and an ion trap analyzer. Peptides were injected at a flow rate 1.5 μL/min of 0.1% formic acid, 50% methanol solution.

Acquisition of NMR Data. NMR spectra were acquired on a Bruker Avance DRX 500 spectrometer. The spectra were recorded at 30 °C and pH 3.2. The cgCP chromopeptide (800 μg) was dissolved in 0.6 mL of D_2O or 10% D_2O (99.95% D_2O, Stohler Isotope Chemicals). The two-dimensional (2D) TOCSY (*26*) (τ_m = 80 ms) and ROESY (*27*) (τ_m = 400 ms) spectra were recorded in a phase-sensitive mode (*28*). The WATERGATE technique was used for suppression of the strong solvent resonance (*29*). Natural abundance two-dimensional heteronuclear $^1H-^{13}C$ HMQC (*30*, *31*) and $^1H-^{13}C$ HMBC (*32*) spectroscopy were used for the same chromopeptide sample. Chemical shifts of individual protons were referenced to the H_2O signal [chosen as 4.75 ppm at 30 °C with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate)]. The ^{13}C chemical shifts were referenced indirectly (*33*). NMR spectra were processed and quantified using XWINNMR (Bruker).

RESULTS

Hypsochromic Spectral Transition and Fragmentation. Upon mild denaturation, DsRed-like proteins were shown to undergo a hypsochromic shift to the GFP-like absorbance, and more drastic treatments (short time boiling in 0.1 M HCl) caused the cleavage into two polypeptide fragments, detected by SDS-PAGE (*10*, *15*). We have employed these previous observations in testing GFP-like proteins to examine the nature of their red-shifting modification. Initially, we selected two homologous chromoproteins from the sea anemones *C. gigantea* (cgCP) and *Heteractis crispa* (hcCP) with the red-shifted compared to DsRed absorbance (*4*). Upon denaturation at pH 1.8 and room temperature (herein called "mild denaturing conditions"), the absorbance peaks of native proteins, at 571 nm for cgCP and 578 nm for hcCP (Figure 2A), instantly shifted to a 436 nm peak (Figure 2B). The 436 nm form of both proteins further gradually converted

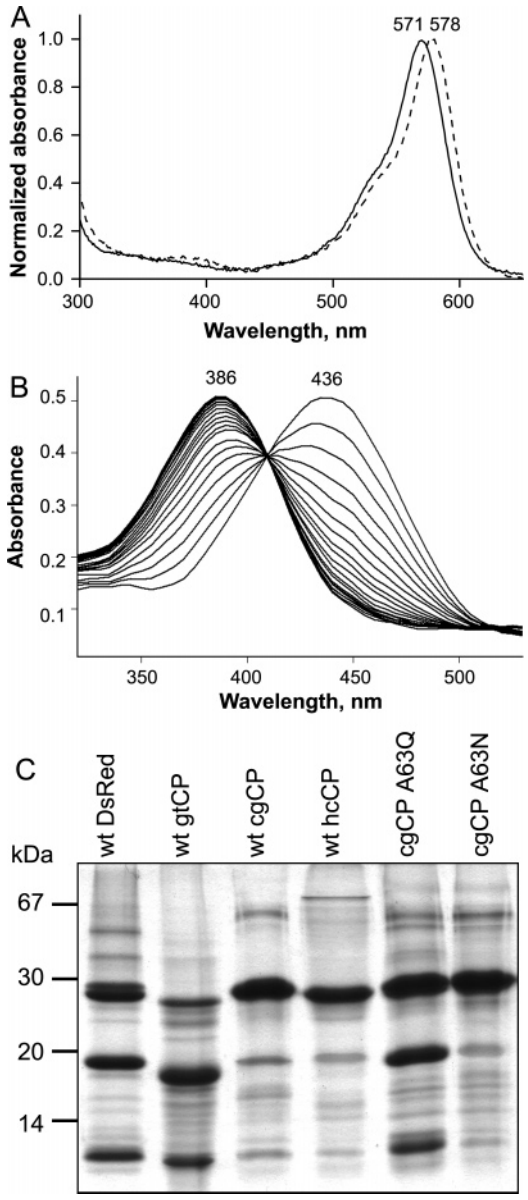

FIGURE 2: Hypsochromic spectral transition and fragmentation. (A) Absorbance spectra of the mature wild-type cgCP (—) and hcCP (- - -) at pH 8.0 (0.65 mg/mL protein). (B) Hypsochromic spectral transition of cgCP upon denaturation at pH 1.8 at room temperature. The solution of mature protein (1.18 mg/mL protein) was adjusted to pH 1.8, and the spectra were recorded in 2 min intervals (the first spectrum with maximal absorbance at 436 nm was recorded 5 s after the pH adjustment). (C) Fragmentation of the wild-type and variant forms after preboiling in 0.1 M HCl for 5 min. The labels above the individual lanes indicate wild-type or variant proteins. Multiple minor bands often observed in SDS−PAGE after boiling the protein in 0.1 M HCl are apparently the result of partial hydrolysis of peptide bonds and polypeptide oligomerization under these conditions.

to the GFP-like absorbance, peaked at 386 nm (Figure 2B, shown for cgCP). A quite similar hypsochromic spectral transition with the same maximum of the chromophore red form (436 nm) has recently been reported for two other GFP-like proteins, DsRed and gtCP (*15*).

Both wild-type cgCP and hcCP, boiled in electrophoresis buffer, exhibited the major full-length protein band in SDS−PAGE, with a molecular mass of ~28 kDa (not shown). Consistent with previous observations (*10*, *15*), DsRed and gtCP preboiled in 0.1 M HCl for 5 min (herein termed "harsh

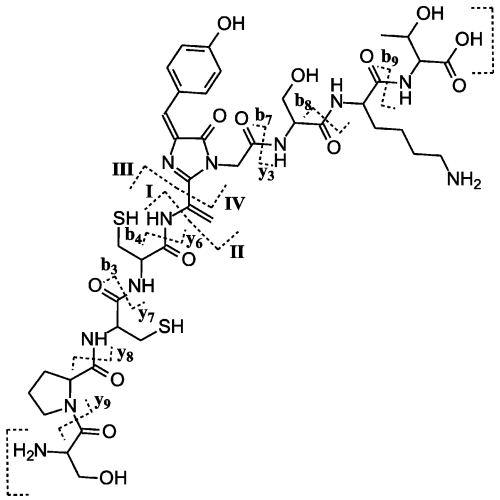

FIGURE 3: Structure of the pepsin-derived cgCP chromopeptide deduced from MS/MS spectra. The identified MS/MS fragments are enclosed in brackets (the *m*/*z* values and assignments of the fragments are listed in Table 1).

denaturing conditions") both exhibited three bands in SDS−PAGE, one of them with the mass of ∼28 kDa, corresponding to the intact protein, and two intense bands of ∼10 and ∼18 kDa, indicative of acylimine hydrolysis (Figure 2C, lanes 1 and 2). However, preboiled under the same conditions (0.1 M HCl), cgCP and hcCP exhibited only faint bands corresponding to 10 and 18 kDa fragments (Figure 2C, lanes 3 and 4). Incomplete fragmentation made us doubtful about the nature of cgCP and hcCP chromophore bathochromic modifications. In the experiments described herein, we have chosen cgCP as a specimen to further investigate the chromophore structure and chemical background underlying the above-mentioned phenomena.

Structure of the cgCP Chromophore Determined by Mass Spectra. Wild-type cgCP was digested by pepsin, and the chromophore-bearing peptide absorbing at 386 nm was isolated by HPLC. Peptide sequencing revealed that Ser-Pro- is the N-terminal amino acid sequence of the cgCP chromopeptide. ESI mass spectra of the chromopeptide contained a +1 charged peak at *m*/*z* 994.4. This value corresponds to the mass of 993.4 Da of the chromopeptide. The Ser-Pro-Cys-Cys-Ala-Tyr-Gly-Ser-Lys-Thr sequence (*4*) was consistent with these mass spectral results, taking into account that the mass of 993.4 Da is 22 Da lower than that calculated (1015.4 Da) for the unmodified peptide with the same sequence. These results suggested that the cgCP chromophore is derived from a cyclization reaction (H_2O elimination) and two sites of dehydrogenation (the loss of $2H_2$), just as reported for the DsRed chromophore (*10*). The structure of the cgCP chromopeptide was further investigated in tandem mass spectrometry experiments. Collision-induced cgCP chromopeptide ion fragmentation yielded a number of daughter ions consistent with fragment masses after splitting of the polypeptide backbone of the sequence given above (Figure 3 and Table 1). Analysis of the MS/MS data showed that both fragment III at *m*/*z* 432.1 and fragment II (*m*/*z* 587.2) contain an additional dehydrogenation site (Figure 3). In contrast, fragments IV (*m*/*z* 563.2) and I (*m*/*z* 408.1) contained only one site of dehydrogenation. In the DsRed MS/MS spectrum (*10*), the fragment similar to cgCP fragment II has been interpreted as the result of intramolecular

Table 1: Assignment of the Peaks Detected in the MS/MS Spectrum of the cgCP Chromopeptide Ion[a]

m/*z* (observed)	*m*/*z* (calculated)	assignment	relative amplitude
288.1	288.1	b_3[b]	6
335.2	335.2	y_3	8
391.1	391.1	b_4	29
408.1	408.1	c_4 (**I**)	8
432.1	432.1	a_5 (**III**)	3
563.2	563.2	$x_5 + 2H$ (**IV**)	17
587.2	587.2	z_6 (**II**)	21
604.2	604.3	y_6	6
660.1	660.2	b_7	2
707.2	707.3	y_7	16
747.2	747.2	b_8	2
810.2	810.3	y_8	32
875.3	875.3	b_9	61

[a] Experimental and calculated *m*/*z* values of the fragment peaks of the parent cgCP chromopeptide ion are listed. Amino acid sequence of cgCP (*4*) and post-translational modifications reported for DsRed (*10*) were used to obtain the calculated *m*/*z* values. The observed mass loss of 22 Da, as compared with that of the unmodified peptide with the same sequence, was considered to be due to a cyclization reaction (H_2O elimination, −18 Da) and two sites of dehydrogenation (−4 Da). A mass loss of 20 Da of the chromophore-bearing fragments is consistent with the cyclization reaction (H_2O elimination, −18 Da) and one site of dehydrogenation (−2 Da). [b] The structures of the assigned ion types are shown in Figure 3.

attack of the side chain amido nitrogen of Gln-66 on the reactive acylimine carbon. However, Ala-63 of cgCP occupying a position equivalent to that of DsRed Gln-66 is not expected to participate in this reaction. Therefore, a possible interpretation of the cgCP chromopeptide MS/MS data is a double bond located between the α- and *β*-carbons of the Ala-63 side chain (Figure 3). This tentative location was further examined in the experiments described below.

The Side Chain of Amino Acid 63 Is Not Involved in Dehydrogenation. To test a potential role of the side chain in the dehydrogenation reaction, a mutant of cgCP lacking the side chain at position 63 was constructed. Assuming that green-to-red maturation of cgCP is a result of additional dehydrogenation at the side chain, the A63G mutant was not expected to produce the "red" chromophore. Conversely, the absorbance peak around 571 nm (absorbance maximum of wild-type cgCP) would indicate the formation of the red chromophore within the A63G mutant in the only one possible acylimine form. At the early stages of maturation, the A63G mutant exhibited a major absorbance peak at 513 nm, obviously corresponding to the immature GFP-like chromophore, and a smaller peak at 582 nm. The peak intensity at 582 nm increased with time and reached its maximal value within 48 h (Figure 4). The completely mature A63G variant still contained a substantial amount of the immature form as judged by absorbance spectra. Nevertheless, the maximum at 582 nm suggests that cgCP originally acquires a red-shifting modification in the form of acylimine.

The Hypsochromic Spectral Transition Is a Result of H_2O Addition across the C═N Bond of Acylimine. The pepsin-derived cgCP chromopeptide, which corresponds to a GFP-like form of the protein after mild denaturation (absorbance maximum at 386 nm, Figure 2B), was investigated by ^1H and ^{13}C NMR spectroscopy. Initially, exchangeable chromopeptide amide protons were identified by spectral comparison of samples dissolved in H_2O or D_2O. Afterward, 2D homonuclear TOCSY and ROESY and heteronuclear $^1H-$

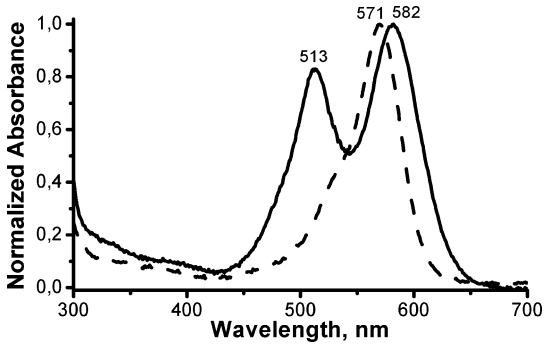

FIGURE 4: Absorbance spectra of wild-type cgCP (- - -) and the mature A63G variant (—).

Table 2: ^{1}H and ^{13}C NMR Chemical Shifts (parts per million) for the cgCP Chromopeptide[a]

residue (spin system)	^{13}C	^{1}H	group	residue (spin system)	^{13}C	^{1}H	group
Ser (1)		—	NH_2	Gly (7)		no	NH
	55.81	4.42	$C^{\alpha}H$		45.36	4.72, 4.86	$C^{\alpha}H$
	167.11		C′		169.61		C′
	59.42	3.90, 3.99	$C^{\beta}H_2$	Ser (8)		8.59	NH
Pro (2)		—	N		56.09	4.52	$C^{\alpha}H$
	61.16	4.37	$C^{\alpha}H$		172.14		C′
	174.20		C′		61.75	3.89	$C^{\beta}H_2$
	25.68	1.96, 2.22	$C^{\beta}H_2$	Lys (9)		8.34	NH
	25.28	1.81, 1.96	$C^{\gamma}H_2$		54.27	4.44	$C^{\alpha}H$
	48.50	3.55, 3.72	$C^{\delta}H_2$		173.76		C′
					31.10	1.79, 1.90	$C^{\beta}H_2$
Cys (3)		8.35	NH		22.53	1.45	$C^{\gamma}H_2$
	54.40	4.43	$C^{\alpha}H$		26.84	1.69	$C^{\delta}H_2$
	172.13		C′		39.86	3.00	$C^{\epsilon}H_2$
	25.93	2.89	$C^{\beta}H_2$			no	$N^{\zeta}H_2$
				Thr (10)		7.91	NH
Cys (4)		8.02	NH		60.67	4.22	$C^{\alpha}H$
	50.28	4.75	$C^{\alpha}H$		176.73		C′
	173.30		C′		68.38	4.27	$C^{\beta}H_2$
	26.55	2.71, 2.92	$C^{\beta}H_2$		19.75	1.16	$C^{\gamma}H_3$
Ala (5)		8.53	NH				
	61.29	no	$C^{\alpha}H$				
	162.02		C′				
	24.88	1.87	$C^{\beta}H_3$				
Tyr (6)		no	N				
	—	no	C^{α}				
	173.57		C′				
	133.95	7.38	$C^{\beta}H$				
	126.51		C^{γ}				
	136.48	8.18	$C^{\delta}H$				
	116.95	7.04	$C^{\epsilon}H$				
	160.26		C^{Z}				

[a] The spectra were recorded in H_2O and D_2O at 30 °C and pH 3.2.

^{13}C HMQC and $^{1}H-^{13}C$ HMBC spectra were used for identification of the amino acid $^{1}H-^{13}C$ spin systems. Altogether, 10 heteronuclear spin systems were identified, and each was assigned to a specific amino acid residue of the chromopeptide (Table 2). Heteronuclear spin systems 1−4 and 8−10 were attributed to the corresponding unmodified amino acids, whereas spin systems 5−7 differed from typical ones of unmodified Ala, Tyr, and Gly, respectively. Ala, Tyr, and Gly residues form cyclic imidazolinone structure. The cross-peak in the $^{1}H-^{13}C$ HMBC spectrum is due to the coupling of α-protons of Gly-65 and C′ of Ala-63, which is located at 162.02 ppm. The latter also exhibits a cross-peak with the CH_3 protons of Ala-63 (the singlet at 1.87 ppm). This suggests covalent bonding between the nitrogen of Gly-65 and C′ of Ala-63. Moreover, the signal of an amide proton of Gly is absent from the NMR spectra of the chromopeptide, since the Gly nitrogen is connected with two carbon atoms of the imidazolinone heterocycle of the chromophore center. The singlet signal of one proton unit from the modified Tyr CβH vinyl proton shows strong nuclear Overhauser effect connectivity with the aromatic protons (8.18 ppm) of this amino acid. The signals of amide and CαH protons of Tyr-64 are absent from the NMR spectra of the chromopeptide, which is indicative of cyclization reaction at Nα and dehydrogenation at Cα of Tyr-64. A singlet at 8.53 ppm corresponds to the amide proton of Ala-63, which excludes the presence of the C═N acylimine bond in the "green" form of cgCP chromopeptide. A singlet (1.87 ppm) of three proton units, which is due to the protons of the CH_3 group of Ala-63, shows nuclear Overhauser effect connectivity with the NH proton of Ala-63. These data strongly argue against hypothetical isomerization of acylimine to enamide upon denaturation (Figure 1, structure **e**). The signal of the CαH proton of Ala-63 was not observed in ^{1}H NMR spectra. The signal of CH_3 protons of Ala-63 (singlet at 1.87 ppm) is shifted downfield from the normal position of Ala CH_3 protons (doublet at 1.37 ppm). Moreover, ^{13}C NMR spectra showed the peak at 61.29 ppm, which corresponds to Cα of Ala-63. This Cα chemical shift value also differs from that usually observed for unmodified Cα of Ala (52.39 ppm). Therefore, the obtained data are consistent with the presence of a hydroxy group at Cα of Ala-63, as a result of H_2O addition across the acylimine C═N bond (Figure 1, structure **f**).

If this proposed reaction pathway is correct, then the complete hydrolysis of the acylimine would lead to cleavage of the modified Ala-63 Nα−Cα bond and formation of the O═Cα derivative of Ala-63 would be expected. The peptic digest of cgCP, preboiled in 0.1 M HCl, was subjected to HPLC fractionation. Together with 386 nm-absorbing, species a chromopeptide with a 410 nm absorbance maximum was isolated. ESI mass spectra of this chromopeptide showed a +1 charged peak at 605.3 *m*/*z*. This value exactly corresponds to the calculated mass (604.3 Da) of the keto derivative of the cgCP chromopeptide with the Ala-Tyr-Gly-Ser-Lys-Thr parent sequence (Figure 1, structure **g**).

Effect of Substitution at Position 63 on cgCP Fragmentation. Although the results given above indicated that the acylimine is indeed a bathochromic modification of the cgCP chromophore, we have further explored the reasons for suppressed hydrolysis of the acylimine observed in SDS−PAGE experiments. To this end, the influence of amino acid 63, the first chromophore-forming residue, on protein fragmentation upon denaturation has been examined. We have constructed and characterized mutants at this position. Except for zFP538 and asFP595, in which fragmentation occurs upon maturation and seems to be a result of protein autocatalysis (*21*, *22*), comparative sequence analysis of the previously characterized DsRed-like proteins suggested that glutamine at position 63 in DsRed and gtCP (*10*, *15*) favors protein fragmentation. The A63Q variant of cgCP exhibited an absorbance maximum at 576 nm characteristic of the mature protein and, like A63G, the maximum at 520 nm apparently corresponding to the immature form. A63Q indeed exhibited enhanced fragmentation (Figure 2C, lane 5) as compared with wild-type cgCP (Figure 2C, lane 3). These results suggested that the side chain of glutamine 63 promotes protein fragmentation. A possible explanation of this phe-

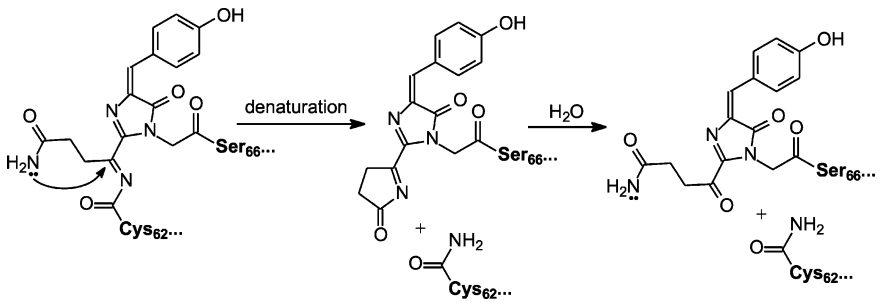

FIGURE 5: Proposed mechanism for A63Q mutant fragmentation.

nomenon is a mechanism in which the amido group of the side chain of Gln-63 attacks the acylimine carbon to yield an intermediate cyclic structure facilitating C═N bond cleavage (Figure 5). If this proposed mechanism is correct, the amino acids, which are unable to form intermediate cyclic structures, would not favor fragmentation. To check this assumption, the A63N point mutant was generated. The asparagine side chain lacks one CH_2 group compared to glutamine and therefore would not be expected to form intermediate cyclic structure, just as shown in Figure 5. The mature red form of A63N absorbed maximally at 584 nm and, like other mutants of cgCP, contained a noticeable amount of an immature form (523 nm). In contrast to A63Q, the A63N mutant proved to be resistant to fragmentation (Figure 2C, lane 6). Highly homologous to cgCP, hcCP (Figure 2C, lane 4) and aeCP (aeCP597, not shown) (*34*), which contain glutamic acid and methionine at this position, respectively, and the A63G mutant of cgCP also exhibited increased resistance to fragmentation.

DISCUSSION

Early studies of the red fluorescent protein from *Discosoma* sp. (DsRed) revealed that the protein loses the red-shifted absorbance upon mild denaturation and reverts back to the GFP-like absorbance spectrum. Harsher treatments were shown to cleave the protein quantitatively into two fragments (*10*). These properties were proposed to originate from reactions of the acylimine group, a constituent part of the red chromophore. It was also assumed that these reactions proceed by a pathway common for simple acylimines (Figure 1, structures f and g) (*10*). However, uncommon chemical reactions have been recently suggested for the formation of zFP538 and asFP595 chromophores (*24*, *25*, *35*). It was proposed that both zFP538 and asFP595 share the same imino-substituted chromophores resulting from spontaneous polypeptide cleavage between the nitrogen and carbonyl carbon of the acylimine (Figure 1, structure **d**) (*24*, *25*). In contrast, crystallographic data of both proteins and direct synthesis of a model chromophore of asFP595 suggested that zFP538 and asFP595 contain different chromophore structures (Figure 1, structures c and g) (*21*−*23*). The more conceivable mechanism was proposed therein, implying nucleophilic reactions at the C═N bond of acylimine. Moreover, crystallographic data showed unambiguously that both zFP538 and asFP595 naturally exist in the fragmented form, suggesting these nucleophilic reactions are protein-mediated. However, it appears that acylimine properties of the DsRed-like chromophore deserve more comprehensive studies, since this reactive group is a potential source of modifications, which might consequently lead to the fluorescence color change.

Initially, we relied on the previously reported properties of a DsRed-like acylimine (*10*) and carried out screening of several GFP-like proteins to assess the nature of their red-shifting modification. Upon mild denaturation, both cgCP and hcCP underwent hypsochromic spectral transitions, which could be described by a one-stage conversion from the red state to the GFP-like absorbance (Figure 2B), as previously observed for DsRed and gtCP (*15*). As expected, hasher treatments of DsRed and gtCP led to quantitative cleavage at the acylimine, which was reflected by the appearance of two intense fragment bands (Figure 2C, lanes 1 and 2). However, incubated under the same conditions, cgCP and hcCP exhibited only faint fragment bands (Figure 2C, lanes 3 and 4), implying that the red-shifting modification in this case displays considerably enhanced resistance to the cleavage. The last observation seemed to cast doubt on the possible implication of the acylimine in π-system extension of the cgCP and hcCP chromophores. Herein, we have chosen cgCP to further investigate the chromophore structure and chemical basis underlying these phenomena.

ESI mass spectra of the chromophore-bearing peptide suggested that the cgCP chromophore is derived from a cyclization reaction, which is accompanied by dehydration, and two sites of dehydrogenation, as reported previously for DsRed (*10*). MS/MS spectra of the cgCP chromopeptide allowed tentative localization of an additional dehydrogenation between the α- and β-carbons of the Ala-63 side chain (Figure 3). This location of an additional double bond differed from that determined for DsRed (Figure 1, structure **b**). Therefore, MS/MS experiments increased the likelihood that dehydrogenation might occur at the side chain of Ala-63 upon cgCP maturation.

To test this possibility, we have constructed the A63G mutant of cgCP. Absorbance spectra demonstrated that this point mutant indeed develops the red-shifted absorbance upon maturation of the protein (Figure 4). Since A63G lacks the side chain at position 63, these results suggested that the red shift of cgCP is due to dehydrogenation between the α-carbon and nitrogen of Ala-63 (Figure 6, structure **I**). These results also exclude the possibility that at the early stages of cgCP maturation the side chain of Ala-63 is implicated in the dehydrogenation reaction, which is followed by isomerization to the acylimine later upon protein maturation. Exhaustive substitutions at the equivalent position have been introduced recently into zFP538 (*21*). The yellow emission of this protein was proposed to result from an intramolecular reaction of a transiently appearing DsRed-like acylimine and the terminal amino group of the Lys-66 side chain (Figure

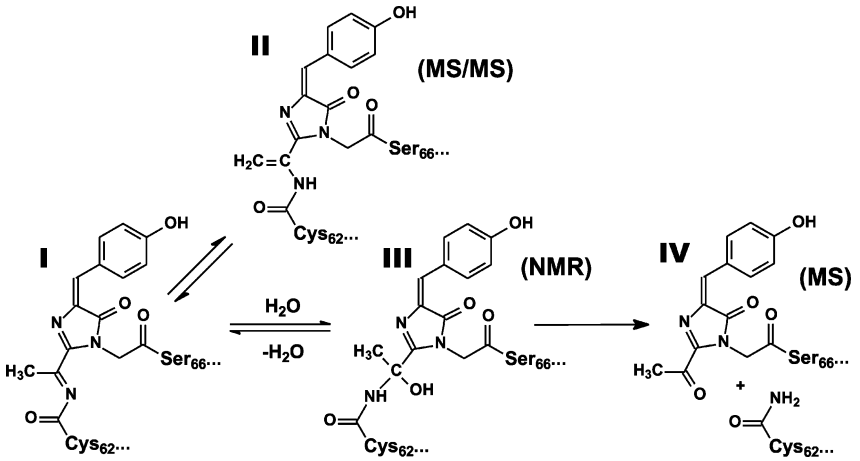

FIGURE 6: Summary scheme of the cgCP chromophore conversions upon protein denaturation. The proposed structures were deduced from the experiments shown in brackets.

1, structure **c**), which is, like Ala-63 of cgCP, the first amino acid of the chromophore-forming triplet of zFP538. Substitutions of Lys-66 with glutamate or aspartate led to partial formation of the red species, which were proposed to be due to a trapped DsRed-like acylimine intermediate. Interestingly, K66G and many other variants of zFP538 developed green fluorescence and did not produce intermediate red species. These results suggest that the side chain of amino acid 66 of zFP538 is crucial for formation of the red species, and it seems likely that compared to cgCP some complementary intermediate reactions are responsible for a transiently appearing DsRed-like acylimine in *Zoanthus* species.

Since the chromopeptide we used in MS experiments corresponds to the protein, which has completed the hypsochromic transition to the GFP-like form (Figure 2B, the 386 nm-absorbing form), MS/MS experiments also increased the likelihood that the acylimine C═N bond might undergo isomerization to the side chain upon denaturation of the protein (Figure 1, structure **e**), and this isomerization would account for the hypsochromic spectral transition. Furthermore, isomerization of acylimine to the more stable enamide derivative would explain an increased resistance of cgCP to fragmentation under harsh conditions. On the other hand, hypsochromic spectral transition might be a result of reversible hydration of the acylimine C═N bond upon denaturation (Figure 1, structure **f**), and as proposed for DsRed (*10*), under strongly dehydrating conditions of electrospray MS experiments, H_2O could be eliminated. To decide between these two possibilities, the pepsin-derived chromopeptide was analyzed by ^1H and ^{13}C NMR spectroscopy. Importantly, NMR analysis was carried out on the same chromopeptide (absorbance at 386 nm), which was used in MS experiments. ^1H NMR spectra revealed the signals of NH (8.53 ppm) and CH_3 protons (1.87 ppm) of Ala-63 altered from usually observed CαH spin properties (both signals corresponding to NH and CH_3 protons are the singlets). Moreover, the signal of the CαH proton of Ala-63 was absent from ^1H NMR spectra. The lack of a Cα proton and a characteristic paramagnetic shift of CH_3 signals (1.87 and 24.88 ppm, Table 2), and of ^{13}Cα (61.29 ppm) of Ala-63 as well, are consistent with the presence of a hydroxy group at Cα of Ala-63. Hence, NMR data argue against hypothetical isomerization of acylimine upon protein denaturation and evidence in favor of a hypsochromic spectral transition (Figure 2B) to be due to the reaction of water addition across the acylimine C═N bond (Figure 6, structure **III**). The acylimine−enamide tautomerism has recently been studied by theoretical calculations of the DsRed chromophore structure. Consistent with our NMR data, ground-state optimizations predicted the acylimine derivative of a DsRed chromophore to be energetically more favorable than the corresponding enamide (*10*). Therefore, the equilibrium between structures **I** and **II** in Figure 6 must be shifted to **I**. A possible interpretation of our MS/MS data is that the C═N bond of the acylimine, which is restored upon MS dehydrating conditions (Figure 6, structure **I**), is resistant to collision-induced splitting, and the double bond between α- and β-carbons of Ala-63 detected in MS/MS spectra of the cgCP chromopeptide corresponds to the enamide tautomer of the acylimine (Figure 6, structure **II**).

Unlike simple carbinolamides, the carbinolamide derivative of the cgCP chromophore (Figure 6, structure **III**) proved to be a sufficiently stable compound, which explains partial fragmentation of the protein under harsh conditions. When the peptic digest of cgCP, preboiled in 0.1 M HCl, is subjected to HPLC separation, the major 386 nm-absorbing fraction, which corresponds to carbinolamide species, is detected. However, a minor chromopeptide with an absorbance maximum of 410 nm has also been isolated. ESI mass spectra of the minor chromopeptide showed the mass exactly corresponding to the keto derivative of the cgCP chromopeptide, the terminal product of acylimine hydrolysis (Figure 6, structure **IV**). These MS data also support the nucleophilic H_2O addition to be a correct mechanism for the acylimine reactivity upon protein denaturation. Hence, our data argue against the possibility of a spontaneous cleavage between the nitrogen and carbonyl carbon of the acylimine, which was proposed for zFP538 and asFP595 (Figure 1, structure **d**) (*24*, *25*).

To elucidate the reasons for the variable reactivity of the acylimine within DsRed-like proteins, the mutagenesis at position 63 of cgCP has been performed. Incomplete fragmentation of HcRed, a fluorescent variant of hcCP, has been recently proposed to be due to the alternative chemistry of the acylimine within a trans non-coplanar chromophore (*36*). More recently, an impact of the chromophore surroundings in Rtms5 and Rtms1 on protein fragmentation upon harsh denaturation has been investigated. It was proposed

that the amino acid immediately preceding the first chromophore-forming residue X and substitution at position 146 facilitating chromophore trans to cis isomerization significantly influence the level of cleavage (*37*). In our experiments, we proceeded on the assumption that protein unfolding upon harsh denaturation would reduce the influence of chromophore surroundings on protein fragmentation. Therefore, we have explored the role of the first amino acid of the XYG chromophore-forming sequence. Substitutions at this position of cgCP suggested that glutamine promotes fragmentation, probably due to an intermediate cyclic structure (Figure 5), which is the result of a reaction of the side chain amido group and the carbon of the acylimine C═N bond. We cannot exclude the role of the chromophore surroundings in protein fragmentation at the early stages of unfolding, but as far as both Rtms5 and Rtms1 also contain glutamine at this position (*37*), the nature of the side chain of X seems to be important.

In summary, we have investigated the chromophore structure of cgCP, examined acylimine reaction pathways upon protein denaturation, and explored the role of the first amino acid of the chromophore-forming sequence in protein maturation and fragmentation. We anticipate that further understanding of the mechanisms modulating acylimine reactivity in DsRed-like proteins should be useful in the rational design of fluorescent probes with new optical properties.

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