Traditional GFP-Type Cyclization and Unexpected Fragmentation Site in a Purple Chromoprotein from *Anemonia sulcata*, asFP595†

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ABSTRACT: The purple chromoprotein (asFP595) from *Anemonia sulcata* belongs to the family of green fluorescent protein (GFP). Absorption and emission spectra of asFP595 are similar to those of a number of recently cloned GFP-like red proteins of the DsRed subfamily. The earlier proposed asFP595 chromophore structure [Martynov, V. I.; et al. (2001) *J. Biol. Chem. 276*, 21012−21016] was postulated to result from an "alternative cyclization" giving rise to a pyrazine-type six-membered heterocycle. Here we report that the asFP595 chromophore is actually very close in chemical structure to that of zFP538, a yellow fluorescent protein [Zagranichny, V. E.; et al. (2004) *Biochemistry 43*, 4764−4772]. NMR spectroscopic studies of four chromophore-containing peptides (chromopeptides) isolated under mild conditions from enzymatic digests of asFP595 and one chromopeptide obtained from DsRed revealed that all of them contain a *p*-hydroxybenzylideneimidazolinone moiety formed by Met-65/Gln-66, Tyr-66/67, and Gly-67/68 of asFP595/DsRed, respectively. Two asFP595 chromopeptides are proteolysis products of an isolated full-length polypeptide containing a GFP-type chromophore already formed and arrested at an earlier stage of maturation. The two other asFP595 chromopeptides were isolated as proteolysis products of the purified chromophore-containing C-terminal fragment. One of these has an oxo group at Met-65 C^{α} and is a hydrolysis product of another one, with the imino group at Met-65 C^{α}. The *N*-unsubstituted imino moiety of the latter is generated by spontaneous polypeptide chain cleavage at a very unexpected site, the former peptide bond between Cys-64 C′ and Met-65 N^{α}. Our data strongly suggest that both zFP538 and asFP595 could be attributed to the DsRed subfamily of GFP-like proteins.

Green fluorescent protein (GFP)[1] from jellyfish *Aequorea victoria* (*1*) and its homologues, colored proteins from *Anthozoa* species (*2*−*6*), belong to a unique group of chromoproteins whose chromophores result from spontaneous posttranslational modifications of the folded polypeptides. GFP-like proteins with fluorescence spectra covering the entire visible wavelength range are most promising for visualizing the expression of a number of genes simultaneously. The overlapping absorption and fluorescence spectra of GFP-like proteins open up opportunities for studies of protein−protein interactions in growth and differentiation using fluorescence resonance energy transfer (FRET). Besides the biotechnology and cellular biology applications (for a recent review see ref *7*), GFP-like proteins are of significant interest as the subjects of basic protein chemistry and enzymology because their chromophores are composed only of modified amino acid residues of precursor polypeptides and do not involve any prosthetic group.

Currently, about 30 members of the GFP-like protein family have been described (*8*) that could be divided into 2 groups, fluorescent proteins (FPs) and naturally nonfluorescent chromoproteins (CPs). Remarkably, all known CPs can be converted into fluorescent analogues with the help of mutagenesis (*6*) or by irradiation with light of fixed wavelength (*9*, *10*). Despite the great diversity of the colors of *Anthozoa*'s GFP-like proteins, this variability seems to result from fairly conservative transformations. At present, few reactions have been shown to be sufficient for generating the chromophore in different FPs and CPs. All known fluorescent proteins contain an invariant Tyr-Gly consensus sequence in the chromophore-forming region and, upon maturation, possess the chromophore core built from *p*-hydroxybenzylideneimidazolinone as a structural unit. Nevertheless, different groups of colors of FPs and CPs are currently considered to stem from chemically distinct types of imidazolinone derivatives (*11*).

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[1] Abbreviations: asFP595 (also known as asCP or asulCP), purple chromoprotein from *Anemonia sulcata*; CP, nonfluorescent chromoprotein; DsRed (also known as drFP583), red fluorescent protein from *Discosoma* sp.; eqFP611, far-red fluorescent protein from *Entacmaea quadricolor*; FP, fluorescent protein; GFP, green fluorescent protein from *Aequorea victoria*; gtCP, purple-blue chromoprotein from *Goniopora tenuidens*; Kaede, fluorescent protein from a stony coral, *Trachyphyllia geoffroyi*; zFP538 (also known as zoanYFP), yellow fluorescent protein from *Zoanthus* sp.

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According to the chromophore structure, GFP-like proteins can be divided into several subfamilies. The X-ray structures of the central members of two subfamilies, GFP and DsRed, have been determined (*12*−*14*). The DsRed subfamily was joined recently by two GFP homologues, pocilloporin (*4*) and eqFP611 (*5*), on the basis of their X-ray crystal structure studies. More recently, another GFP-like protein, gtCP (*15*), was postulated to be a member of the DsRed subfamily on the basis of spectroscopic studies of the denatured protein. We have shown previously (*16*) that a yellow fluorescent protein, zFP538 (*2*), could also be attributed to the DsRed subfamily on the basis of the zFP538 chromophore-containing peptide (chromopeptide) structures studied by NMR. We have also mapped an unexpected site of zFP538 polypeptide spontaneous fragmentation, which could be, in general, characteristic of the DsRed subfamily since the GFP subfamily has no such feature. Another type of polypeptide cleavage, the formal β-elimination promoted by UV irradiation, was described recently for the GFP-like protein called Kaede (*17*).

On the basis of this background, the unusual "alternative" cyclization (*18*) proposed for asFP595 (*3*) (recently renamed as asCP (*6*, *9*) or asulCP (*8*)) effectively places this protein in a novel CP class of its own (*8*). In the course of our studies of zFP538 chromopeptides (*16*) we used both DsRed and asFP595 chromopeptides as spectral controls. The data obtained strongly suggest that asFP595 chromopeptides have a chromophore structure different from the one proposed earlier (*18*). This finding prompted us to study the asFP595 chromopeptide structures by means of NMR using the DsRed chromopeptide as a control. The data presented in this paper allow us to conclude that asFP595 has a chromophore structure very similar, if not identical, to that of zFP538 (including the potential to fragment at the unusual site) and thus should also belong to the DsRed subfamily of GFP-like proteins.

EXPERIMENTAL PROCEDURES

Expression and Isolation of DsRed and asFP595. The proteins were purified as fusions with the N-terminal sequence MRGS[H]$_6$GSAQ preceding the DsRed and asFP595 polypeptides. The plasmids harboring DsRed (also known as drFP583) and asFP595 cDNAs were kindly provided by Dr. S. A. Lukyanov (*2*). We used the asFP595 double point mutant T70A/A148S (*3*) with enhanced fluorescence. Due to that property of the mutant, we prefer the old "asFP595" name (*3*) instead of the recently suggested "asCP" (*6*, *9*) or "asulCP" (*8*). This asFP595 mutant has the same spectral properties as wild-type CP except for the higher fluorescence quantum yield (*3*). Thus, the mutant asFP595 used in this study is considered to have the same chromophore structure as wild-type CP (*3*). The proteins were expressed and isolated by affinity chromatography on Ni−NTA (Qiagen, Germany) as described previously (*16*).

Denaturation and Proteolysis of DsRed and asFP595. Isolation of Chromopeptides. The preparation of DsRed was denatured with acid (0.1 M HCl, pH 2.0), and then dry pepsin at an enzyme:substrate ratio of 1:30 (w/w) was added. Proteolysis at 37 °C was monitored by HPLC. After several hours of incubation, the hydrolysate was loaded onto a Jupiter C4, 300 Å, 5 μm (Phenomenex) HPLC column. The column was eluted with acetonitrile gradient in 13 mM trifluoroacetic acid with absorbance monitoring at 420 nm. The lyophilized chromophore-containing fraction was dissolved in 50 mM Tris−HCl, pH 7.5, and dry trypsin (1:25, w/w) was added. Proteolysis at 37 °C was monitored by HPLC and was followed by HPLC separation under the same conditions. The fraction with an absorbance at 420 nm was treated with carboxypeptidase B (under the conditions described in ref *16*), which resulted in peptide DsRed-II after additional HPLC purification under the same conditions.

asFP595 was denatured with 13 mM trifluoroacetic acid and then immediately applied to the HPLC column to separate (under the conditions described above) the full-length polypeptide from its chromophore-containing C-terminal fragment as we previously described for zFP538 (*16*). The C-terminal fragment of asFP595 was treated with pepsin at pH 2.0. Proteolysis at 37 °C was monitored by HPLC. The proteolytic mixture was subjected to HPLC purification (under the same conditions), which resulted in chromopeptides asFP595-II and asFP595-III. These two peptides were never exposed to an alkali in the course of their isolation because peptide asFP595-III is very unstable and spontaneously converts into peptide asFP595-II upon storage in water at room temperature even at neutral pH. The chromophore-containing asFP595 full-length polypeptide was hydrolyzed with trypsin and gave, after several rounds of HPLC purification under the same conditions, chromopeptides asFP595-Ia and asFP595-Ib.

Absorption, Fluorescence, and NMR Spectroscopy. All the experiments were performed as described previously (*16*).

RESULTS

Chromopeptide Properties and Structures. Peptides asFP595-Ia and asFP595-Ib showed spectral properties very similar to those of chromopeptides isolated from GFP (*19*−*22*) and those of zFP538 peptides I and IV (*16*). Two spectral forms, with $\lambda_{max} = 385$ and 450 nm for acidic and alkaline conditions, respectively, were observed (data not shown). ^{1}H NMR spectroscopy (resonance assignments are presented in the Supporting Information) revealed the signals from two Ser residues, Met, and Cys (in both peptides asFP595-Ia and asFP595-Ib) and, additionally, from Lys and Ala in peptide asFP595-Ia. In both peptides asFP595-Ia and asFP595-Ib, there were no signals from the C$^\alpha$H proton of Tyr while the singlet signal of one proton unit from the modified Tyr C$^\beta$H vinyl proton showed strong nuclear Overhauser effect (NOE) connectivity with the δ-protons of the aromatic ring. Taken together, these data lead to the formulas of peptides asFP595-Ia and asFP595-Ib shown in Figure 2. These are, respectively, SC*MYG*SKA and SC*MYG*S derivatives containing the GFP-type chromophore built from the italic residues Met-65, Tyr-66, and Gly-67 (amino acid numbering accords with ref *18*).

The spectral properties of peptides DsRed-II (Figure 1A) and asFP595-II (Figure 1B) are very similar to those of the zFP538 peptide II (*16*) (hereinafter referred to as "peptide zFP538-II", Figure 3, structure d) and those of the earlier described chromopeptide (*18*) isolated from the asFP595 tryptic hydrolysate (reproduced from ref *18* as peptide asFP595-IV in Figure 2). On the basis of spectrophotometric and spectrofluorimetric titrations which yielded the same pK_a values (data not shown), we can conclude that peptide

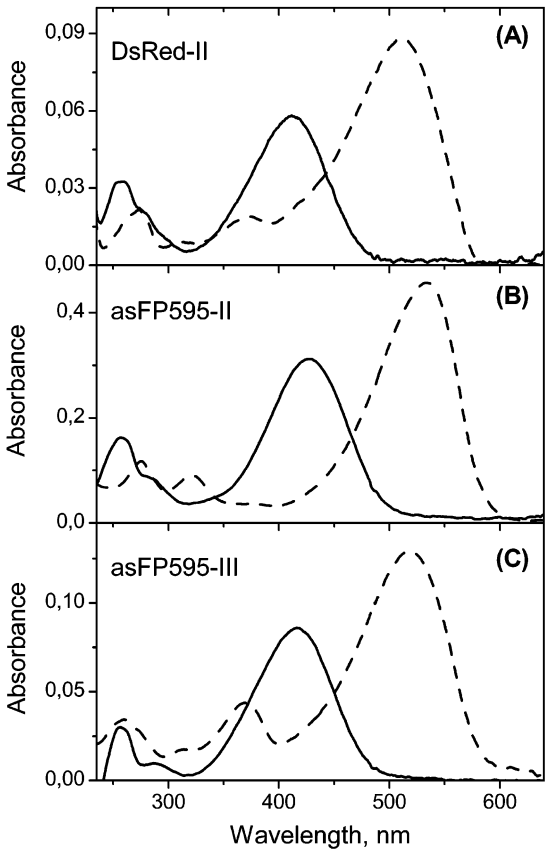

FIGURE 1: Absorption spectra of peptides DsRed-II (A), asFP595-II (B), and asFP595-III (C) at pH 3.0 (solid lines) and at pH 9.0 (dashed lines). The concentrations were the same for every pair of curves in each panel, but not determined.

asFP595-II contains the chromophore identical to that in peptide asFP595-IV described in ref *18*. According to ^{1}H NMR data (resonance assignments are presented in the Supporting Information) the peptides DsRed-II and asFP595-II (Figure 2) are derived from the tetra- and hexapeptides QYGS and MYGSKA, respectively. Both peptides contain a core chromophore nearly identical to that in peptide zFP538-II (*16*), with an oxo group at the Gln-66 (DsRed-II) or Met-65 (asFP595-II) C^α carbon.

The chromophore structure previously suggested in ref *18* for the tryptic chromopeptide from asFP595 (reproduced as peptide asFP595-IV in Figure 2) cannot be attributed to peptide asFP595-II because there is no signal from the $C^\alpha H$ proton of Met-65 in its ^{1}H NMR spectra. Moreover, the signals from the $C^\beta H_2$ protons of Met-65 in peptide asFP595-II (as well as of Gln-66 in peptide DsRed-II) are shifted far downfield from the normal positions of the unmodified amino acid residues. The same was observed for peptide zFP538-II (*16*), indicating the sp^2 hybridization state of C^α carbons at the DsRed Gln-66 (in peptide DsRed-II) and at the asFP595 Met-65 (in peptide asFP595-II). The sp^2 hybridization state of Met-65 C^α in peptide asFP595-II is also inconsistent with the previously suggested pyrazine-type six-membered heterocycle structure (*18*) for the asFP595 chromophore (reproduced as asFP595-IV in Figure 2). Finally, the peptide asFP595-II is a hydrolysis product of asFP595-III (see below). This precursor lacks the amide proton at Gly-67, the nitrogen of which is linked to three carbon atoms. Therefore, peptide asFP595-II should also lack the amide proton at the Gly-67 residue. This is also inconsistent with the structure of asFP595-IV.

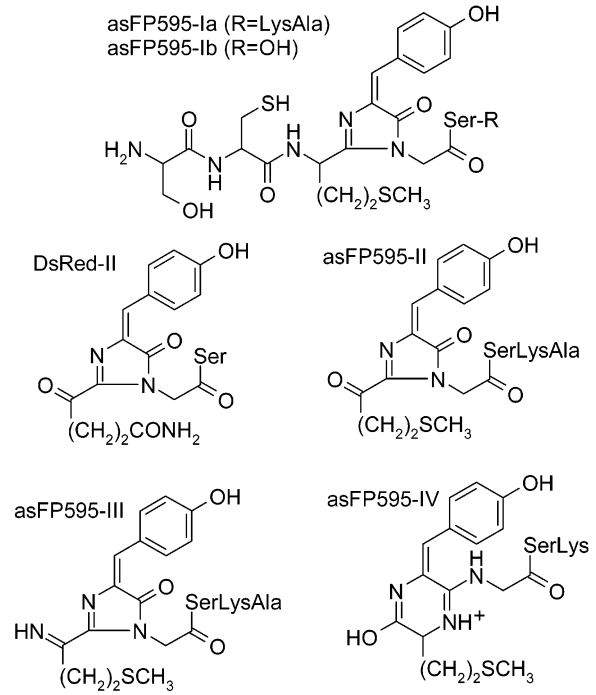

FIGURE 2: Structures of peptides asFP595-Ia and asFP595-Ib (top), DsRed-II (middle left), asFP595-II (middle right), and asFP595-III (bottom left) determined in the present study. The structure asFP595-IV (bottom right), earlier suggested for the acidic form of the chromopeptide isolated from the asFP595 tryptic hydrolysate, is reprinted with permission from ref *18*. Copyright 2001 *Journal of Biological Chemistry*.

The spectral properties of peptide asFP595-III (Figure 1C) resemble those of peptide III from a yellow fluorescent protein, zFP538 (*16*) (hereinafter referred to as "peptide zFP538-III", Figure 3, structure a). The only difference is that peptide asFP595-III has only one alkaline form ($\lambda_{max} \approx$ 520 nm, Figure 1C), which is similar to the "intermediate alkaline form" of the peptide zFP538-III (*16*). No conversion into the other alkaline form (like the "final alkaline form" of the peptide zFP538-III, $\lambda_{max} = 470$ nm, ref *16*) was observed for peptide asFP595-III. On the basis of the spectral and chemical (i.e., conversion into peptide asFP595-II) properties of peptide asFP595-III and ^{1}H NMR data (resonance assignments are presented in the Supporting Information), we propose that the structure of peptide asFP595-III is analogous to that of peptide zFP538-III (Figure 3, structure a). Peptide asFP595-III (Figure 2) is an MYGSKA hexapeptide derivative with a GFP-type *p*-hydroxybenzylideneimidazolinone moiety (formed by the Met-65, Tyr-66, and Gly-67 amino acid residues) and with an imino group at Met-65 C^α.

DISCUSSION

We have isolated four chromopeptides from asFP595, a purple CP from *Anemonia sulcata*. Two of them, asFP595-Ia and asFP595-Ib, were obtained after proteolytic degradation of the isolated asFP595 full-length polypeptide containing the immature GFP-type chromophore. Two other peptides, asFP595-II and asFP595-III, were the products of enzymatic proteolysis of the isolated asFP595 C-terminal fragment,

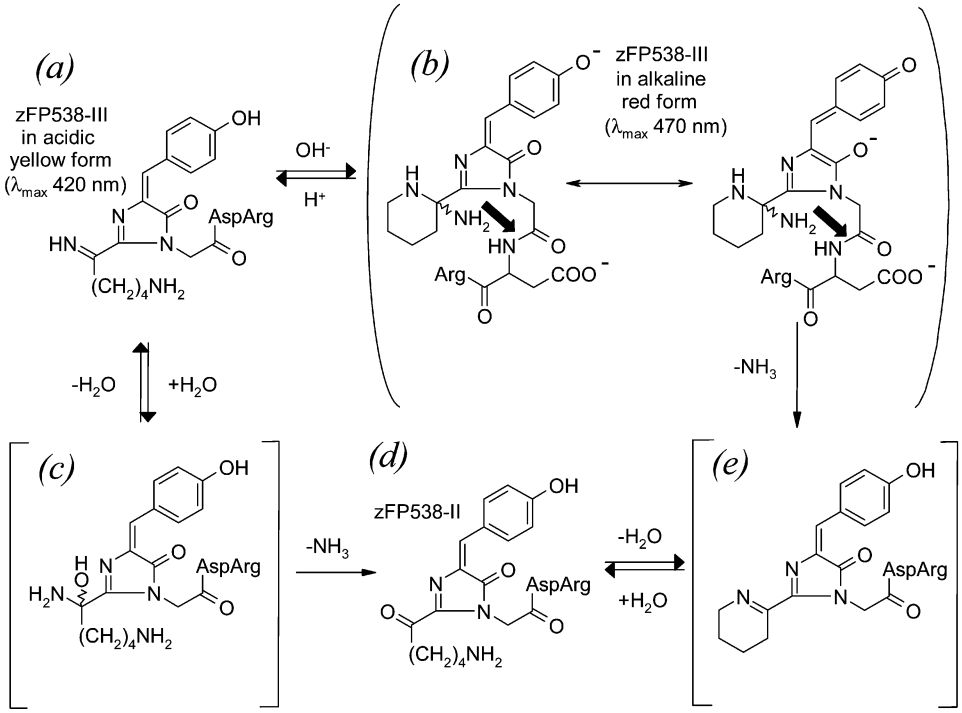

FIGURE 3: Scheme outlining the proposed interconversions of the chromopeptides isolated from zFP538 (*16*). Peptide zFP538-III in its acidic yellow form with $\lambda_{max} = 420$ nm (structure a) transforms into the proposed asymmetric *gem*-diamine structure of this peptide in its "final alkaline" red form (with $\lambda_{max} = 470$ nm) observed at equilibrium at alkaline pH (presented as the resonance structure of a phenolate anion (*21*), structure b); labile Gly-68−Asp-69 peptide bonds sensitive to boiling for 5 min in 0.7 M 2-mercaptoethanol, 62 mM Tris−HCl, pH 6.8 (*16*), are indicated by arrows. Deamination of this *gem*-diamine will give rise to a cyclic Schiff base (structure e) whose hydrolysis, in turn, could result in the keto peptide zFP538-II (structure d). The latter could also appear according to the alternative mechanism (which is the only possibility for the peptide asFP595-III hydrolysis resulting in peptide asFP595-II): water molecule addition across the C═N bond gives carbinolamine (structure c), whose subsequent deamination also results in peptide zFP538-II (structure d).

which appeared as a result of the protein spontaneous fragmentation which occurs in the course of the chromophore maturation or upon asFP595 denaturation. Peptide asFP595-II has an oxo group at the N-terminal Met-65 C^{α} and is similar in its chromophore structure to peptide zFP538-II (*16*) and to peptide DsRed-II, whose chromophore structure was theoretically predicted earlier (*23*). The "keto" peptide asFP595-II is the product of spontaneous hydrolysis of "imino" peptide asFP595-III (HN═C< + H_2O → O═C< + NH_3). Similar peptides, imino peptide zFP538-III and keto peptide zFP538-II (Figure 3, structures a and d, respectively), were previously isolated from a yellow fluorescent protein, zFP538 (*16*).

We conclude that peptide asFP595-II is identical in its structure to asFP595 chromopeptide previously isolated by Martynov and co-workers (*18*) with the exception of the additional Ala residue at the asFP595-II C-terminus. This residue appeared instead of the wild-type Thr-70 as we were working with asFP595 mutant T70A/A148S (*3*). In other words, we argue that the authors of ref *18* did propose an incorrect structure for the asFP595 chromophore formed as a result of "alternative cyclization". Actually, the traditional GFP-type cyclization giving rise to a five-membered imidazolinone cycle occurs in the course of asFP595 chromophore maturation. Additional support for this conclusion is the isolation from the asFP595 full-length polypeptide of peptides asFP595-Ia and asFP595-Ib (Figure 2) with an immature chromophore identical to that in GFP.

It should be noted that at acidic pH both peptides asFP595-Ia and asFP595-Ib have absorption maxima at 385 nm as do chromopeptides isolated from GFP (*19*−*22*) and peptides I and IV from zFP538 (*16*). Their absorbance at 430 nm under HPLC conditions (pH 4.0) described in ref *18* is very low. In addition, the yield of these peptides from the enzymatic digest of the total asFP595 preparation is also very low because, after expression in *Escherichia coli*, the content of asFP595 with an immature chromophore is not as high as in the case of DsRed (cf. Figure 1A in ref *18* and Figure 6, lane C, in ref *23*). Apparently, because of low absorbance at 430 nm and low yields, chromopeptides with GFP-type chromophores (similar to one in our peptides asFP595-Ia and asFP595-Ib) were not isolated in ref *18* from the tryptic hydrolysate of asFP595. It should be stressed that peptide asFP595-III is very unstable even at neutral pH. This could be the reason the imino peptide was also not found in the asFP595 tryptic hydrolysate (*18*): upon trypsinolysis at pH 7.8 and 37 °C for 4 h, this imino peptide, asFP595-III, will be stoichiometrically converted into the keto peptide asFP595-II. In our isolation procedure the exposure to an alkali was excluded.

Taken together, the data obtained allow us to conclude that the asFP595 chromophore is very similar, if not identical, in its structure, maturation pathway, and some chemical properties to the zFP538 chromophore (*16*). Therefore, both asFP595 and zFP538 could be attributed to the DsRed subfamily of GFP-like proteins. Moreover, asFP595 possesses the same unusual site of spontaneous polypeptide chain splitting in the vicinity of its chromophore, between Cys-64 C′ and Met-65 N^{α}. As in the case of zFP538 (*16*), it

still remains unclear whether this polypeptide fragmentation is a direct result of chromophore maturation or only a denaturation artifact as it is in the case of DsRed (*13*, *14*, *23*).

Taking into account the spectral properties of the studied chromopeptides (more exactly, the existence of only one alkaline form of peptide asFP595-II), it could be suggested that conversion of peptide zFP538-III from the "intermediate" (λ_{max} = 529 nm) to the "final" (λ_{max} = 470 nm) alkaline form described in ref *16* involves interaction of the Lys-66 ϵ-amino group with the imino group, giving rise to an asymmetric *gem*-diamine structure (Figure 3, structure b). The shorter conjugated system in the latter can account for the blue-shifted absorption and emission properties of this final alkaline form compared to the intermediate one as was shown for synthetic model DsRed chromophores (*24*). This is also consistent with the known chemical properties of *gem*-diamines (which are relatively stable under alkaline, but not acidic, conditions, ref *25*) and could explain the peptide zFP538-III imino group protection against prolonged incubations under alkaline conditions at pH 8.0 and 37 °C for 24−48 h (*16*). The imino and primary amino moieties can be regenerated (Figure 3, b → a) upon acidification of the alkaline solution of peptide zFP538-III.

It is known (*25*) that *gem*-diamines are the intermediates of imino exchange (transalkylidenation) reaction, $R_2C{=}NH + H_2N{-}R' \rightarrow [R_2C(NH_2){-}NH{-}R'] \rightarrow R_2C{=}N{-}R' + NH_3$. This reaction was used to interpret the mass spectrum of peptide zFP538-III (*16*). The final product of this conversion, a cyclic Schiff base (Figure 3, structure e), could serve as a precursor for the keto peptide zFP538-II (Figure 3, structure d). However, since peptide asFP595-III lacks the appropriate primary amino group, the conversion of imino peptide asFP595-III into keto peptide asFP595-II should follow another hydrolysis mechanism, $R_2C{=}NH + H_2O \rightarrow [R_2C(OH){-}NH_2] \rightarrow R_2C{=}O + NH_3$. In this case, direct addition of the water molecule across the C=N double bond gives rise to the intermediate carbinolamine (similar to structure c in Figure 3). Finally, it could be speculated that the proposed *gem*-diamine structure of the peptide zFP538-III final alkaline form is a step toward the hydrolysis of the Gly-68−Asp-69 peptide bond under boiling in the presence of 0.7 M 2-mercaptoethanol at pH 6.8 for 5 min (*16*). This *gem*-diamine fragment could also be a part of the yellow chromophore of native zFP538 (with an already cleaved polypeptide chain). This additional chemistry is consistent with the blue shift of zFP538 absorption and emission maxima in comparison with those in the spectra of other DsRed-like proteins. The emission maximum of peptide zFP538-III fluorescence in the final alkaline form matches that of the native zFP538 yellow emission at 538 nm (*16*).

Nevertheless, it could be concluded that participation of the ϵ-amino group at Lys-66 must be excluded from the mechanism of zFP538 spontaneous fragmentation between Phe-65 C′ and Lys-66 N^{α} (*16*) because the corresponding position in asFP595 is occupied by a methionine residue. This is not surprising because the primary amino group (which is usually protonated under physiologic conditions) is not nucleophilic. Another amino acid side chain might interact with the proposed DsRed-type *N*-acylimine (*26*) in the course of the chromophore maturation or upon denaturation of the protein with a mature chromophore prior to its exposure to the water-containing solvent. To the best of our knowledge, the results described here and in ref *16* are the first reports of spontaneous polypeptide chain cleavage giving rise to an *N*-unsubstituted imine. If asFP595 and zFP538 chromophores do proceed to the stage of *N*-acylimine, this could be the second (after ref *26*) report of *N*-acylimine decomposition into an *N*-unsubstituted imine, which, this time, was detected directly.

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SUPPORTING INFORMATION AVAILABLE

Proton chemical shift values for peptides asFP595-Ia, asFP595-Ib, asFP595-II, asFP595-III, and DsRed-II (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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