zFP538, a Yellow Fluorescent Protein from Coral, Belongs to the DsRed Subfamily of GFP-Like Proteins but Possesses the Unexpected Site of Fragmentation†

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ABSTRACT: The yellow fluorescent protein (zFP538) from coral *Zoanthus sp.* belongs to a family of green fluorescent protein (GFP). Absorption and emission spectra of zFP538 show an intermediate bathochromic shift as compared with a number of recently cloned GFP-like red fluorescent and nonfluorescent chromoproteins of the DsRed subfamily. Here we report that the zFP538 chromophore is very close, if not identical, in chemical structure to that of DsRed. To gain insight into the mechanism of zFP538 fluorescence and chromophore structure and chemistry, we studied three chromophore-containing peptides isolated from enzymatic digests of zFP538. Like GFP and DsRed chromophores, these contain a *p*-hydroxybenzylideneimidazolinone moiety formed by Lys-66, Tyr-67, and Gly-68 of zFP538. One of the peptides studied, the hexapeptide FKYGDR derivative, is a proteolysis product of the zFP538 full-length polypeptide containing a GFP-type chromophore already formed and arrested at an earlier stage of maturation. The two other peptides are the derivatives of the pentapeptide KYGDR resulted from the protein in which the chromophore maturation process had been completed. One of these has an oxogroup at Lys-66 C^{α} and is a hydrolysis product of another one, with the imino group at Lys-66 C^{α}. The *N*-unsubstituted imino moiety of the latter is generated by spontaneous polypeptide chain fragmentation at a very unexpected site, the former peptide bond between Phe-65 C′ and Lys-66 N^{α}. Also observed in the entire protein under mild denaturing conditions, this fragmentation is likely the feature of native zFP538 chromophore that distinguishes it chemically from the DsRed chromophore.

Green fluorescent protein (GFP)[1] from jellyfish *Aequorea victoria* (*1*) and its homologues, colored proteins from *Anthozoa* species (*2*, *3*), belong to a unique group of chromoproteins whose chromophores result from autocatalytic 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. Mutually overlapping absorption and fluorescence spectra of GFP-like proteins open up fresh opportunities for studies of protein−protein interactions in growth and differentiation using fluorescence resonance energy transfer (FRET). Besides the biotechnology and cellular biology applications, GFP-like proteins are of significant interest as the subjects of basic protein chemistry and enzymology because their chromophores are composed of modified amino acid residues of precursor polypeptides lacking any prostheic group.

In GFP, the chromophore is formed by three amino acid residues, Ser-65, Tyr-66, and Gly-67 (*4*), that make the *p*-hydroxybenzylideneimidazolinone moiety, which is covalently linked to the protein polypeptide chain and hidden inside a specific β-can tertiary structure (*5*). A chromophore structure of red fluorescent protein, DsRed, suggested in ref *6* differs from that of GFP by an additional dehydrogenation of the C^{α}-N bond at Gln-66 that corresponds to GFP Ser-65. The DsRed X-ray crystal structure had been determined independently by two groups (*7*, *8*). Recently, the crystal structures of two other GFP homologues, pocilloporin (*9*) and eqFP611 (*10*), were published, both showing a chromophore chemically identical to that of DsRed but with different configurations of the *p*-hydroxybenzylidene group. SDS/PAGE reveals a spontaneous fragmentation of the DsRed polypeptide chain promoted by its boiling in diluted acid or alkali (*6*). The same was reported for three other red GFP homologues, asFP595 (*11*), gtCP (*12*) (after boiling in

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[1] Abbreviations: asFP595 (also known as asCP or asulCP), purple chromoprotein from *Anemonia sulcata*; DsRed (also known as drFP583), red fluorescent protein from *Discosoma sp.*; eqFP611, far-red fluorescent protein from *Entacmaea quadricolor*; ESI-MS, electrospray ionization mass spectrometry; GFP, green fluorescent protein from *Aequorea victoria*; gtCP, purple-blue chromoprotein from *Goniopora tenuidens*; Kaede, fluorescent protein from a stony coral *Trachyphyllia geoffroyi*; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; zFP538, yellow fluorescent protein from coral *Zoanthus sp*.

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acid), and Kaede (*13*) (after UV-irradiation of intact protein).

The chromophore structure of yellow fluorescent protein is of special interest because zFP538 could be either a FRET donor or acceptor in interactions with other fluorescent proteins. Excitation and emission maxima of zFP538 fluorescence appear right between these of GFP and of red GFP homologues (DsRed subfamily). Thus, the exact structure of zFP538 chromophore is of fundamental interest as well. The goal of the current study was isolation of zFP538 chromophore-containing peptides (chromopeptides), investigation of their physicochemical and spectral properties, and elucidation of their chromophore structures. The reported results are crucial for solving the native zFP538 chromophore structure, for the study of its chemistry, and for the precise mapping of the zFP538 polypeptide spontaneous fragmentation site. The latter has been found at the very unexpected point. This fragmentation is likely the additional feature of native zFP538 chromophore which is identical to that of DsRed, at least at the latest (if not final) maturation step.

EXPERIMENTAL PROCEDURES

Expression and Isolation of zFP538. The protein was purified as a fusion with the N-terminal sequence MRGS-$[H]_6$GSAQ preceding the wild-type zFP538 (residues 3−231, see Figure 4A). The plasmid harboring zFP538 cDNA under the control of *lac* promoter was a kind gift of Dr. S. A. Lukyanov (*2*). Overnight culture of freshly transformed cells (*E. coli* strain JM109) was diluted (1:50) with a fresh LB medium containing ampicillin and grown at 37 °C. Expression was induced by IPTG, and the cells were grown for 6−12 h more. After cell harvesting and sonication, insoluble material was spun out and the supernatant was loaded onto a Ni−NTA (Qiagen, Germany) column handled according to the manufacturer's instructions. As judged from SDS/PAGE and HPLC, zFP538 in the eluate was nearly homogeneous.

Denaturation and Proteolysis of zFP538. A solution of zFP538 in 6 M urea, 20 mM Tris-HCl, pH 7.4, was heated in boiling water bath until its fluorescence disappeared, cooled to room temperature, and supplemented with 5 mM $CaCl_2$ and thermolysin. For acidic denaturation, 0.1 M HCl was added dropwise to the zFP538 solution to adjust its pH to 2.0. Then dry pepsin was added. Trypsin, chymotrypsin, or carboxypeptidase B was dissolved in 50 mM Tris-HCl, pH 7.5, together with dried peptide pellets. Proteolyses were performed at a 1:25 (w/w) enzyme/substrate ratio at 37 °C and monitored by HPLC.

Isolation of Chromopeptides. The products of zFP538 peptic digest were first separated on a MonoS column (Pharmacia, Sweden) with 2.0−7.2 pH gradient. Prior to trypsin addition, pH of the collected fractions was adjusted to 7.8. The peptides were separated by HPLC on a Jupiter C4, 300 Å, 5 μm (Phenomenex, USA) column with acetonitrile gradient in 13 mM trifluoroacetic acid with absorbance monitoring at 420 nm. Finally, an Ultrasphere ODS, 5 μm (Beckman, USA) column was used, first under the same conditions, then with methanol gradient in 7 mM NaH_2PO_4, pH 4.9.

Amino Acid Analysis and Sequencing. Amino acid analysis was performed according to the standard technique using an LC-5000 (Biotronik, Germany) amino acid analyzer. N-terminal amino acid sequences were determined using a 477A protein sequencer (Applied Biosystems, USA).

Mass Spectrometry. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained using a Vision 2000 time-of-flight (TOF) mass spectrometer (ThermoBioAnalyses, England). Electrospray ionization (ESI) mass spectra were recorded using a MAT 900S mass spectrometer (Finnigan, USA). To establish the exact molecular mass in high-resolution ESI-MS experiments, propylenglycol was used as an internal standard.

Absorption and Fluorescence Spectroscopy. Absorption spectra were recorded on an S1000−10 spectrometer (Ocean Optics Inc., USA). Fluorescence spectra were obtained using a RF5301 spectrofluorimeter (Shimadzu, Japan).

NMR Spectroscopy. NMR experiments were performed on Varian Unity 600 or Bruker Avance DRX 500 spectrometers. The peptides were dissolved in 0.6 mL of H_2O (10% D_2O) or D_2O (99.95% deuterium, Stohler Isotope Chemicals). Two-dimensional (2D) double quantum filtered correlation spectroscopy (DQF−COSY) (*14*), 2D total correlation spectroscopy (TOCSY) (*15*) with mixing time (τ_m) of 70 ms, and 2D rotating-frame Overhauser effect spectroscopy (ROESY) (*16*) with τ_m of 400 ms were performed in the pure phase-absorption mode by collecting hypercomplex data (*17*). The WATERGATE (*18*) technique was used for the suppression of strong solvent resonance. Natural abundance heteronuclear 2D correlation experiments (multiple quantum correlation spectroscopy (^{1}H-^{13}C-HMQC) (*19*, *20*) and multiple bond correlation spectroscopy (^{1}H-^{13}C-HMBC) (*21*)) were made on the same set of samples in the absolute value mode. A relaxation delay of 2.0−3.0 s was used. The ^{1}H chemical shifts were measured relative to the H_2O signal (arbitrary chosen as 4.75 ppm at 30 °C). The ^{13}C chemical shifts were referenced indirectly (*22*). NMR spectra were processed and quantified using VNMR (Varian) or XWIN-NMR (Bruker) programs. Complete proton and carbon resonance assignment was obtained by combination of standard procedures (*21*, *23*).

RESULTS

Proteolytic Degradation of zFP538 and Isolation of Chromopeptides. Like GFP and its homologues, the native zFP538 is resistant to a wide variety of proteases. Prolonged incubation with trypsin, chymotrypsin, thermolysin, elastase, staphylococcal protease V8, or thermolysin induced no change in spectral properties of zFP538 or its chromatographic or electrophoretic mobility. To provoke proteolytic degradation of zFP538, heating in 6 M urea and subsequent incubation with thermolysin were used. Cleavage of zFP538 with thermolysin allowed a low-yield isolation of minor peptide I, while the major chromophore-containing fractions were combined and subsequently cleaved by trypsin yielding fraction II+III. HPLC separation of fraction II+III with methanol gradient in phosphate buffer resulted in two individual compounds designated as "peptide II" and "peptide III". Alternative acidic denaturation followed by pepsin hydrolysis was applied as well. The pepsin digest of zFP538 gave two chromophore-containing fractions; of these, the minor remained unanalyzed, while the major one was converted into fraction II+III by trypsin hydrolysis. The amount of peptide II in fraction II+III strongly depended on the denaturation method used. It accounted for several percent after heating in 6 M urea and for 50−70% after acidic treatment without heating.

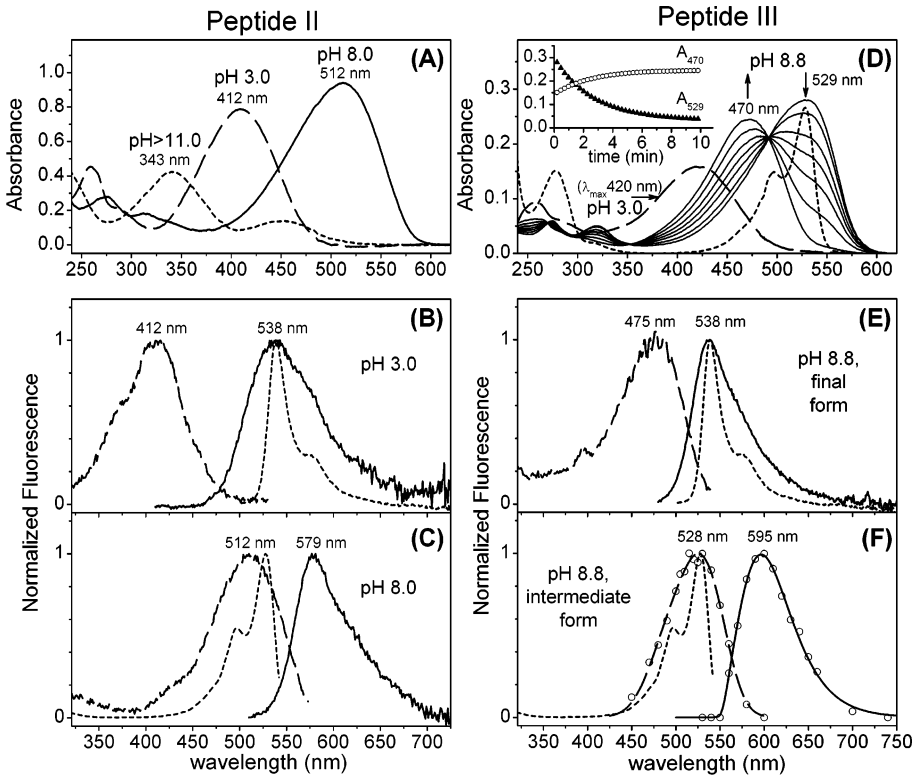

FIGURE 1: Absorption (A, D) and fluorescence (B, C, E, F) spectra of peptides II (A−C) and III (D−F) at various pH superimposed on the spectra of native zFP538 (B−F, dotted lines). (A) Peptide II absorption spectra. The chromopeptide exists in three pH-dependent forms: yellow (λ_{max} 412 nm) at pH 3.0 (dashed line), red (λ_{max} 512 nm) at pH 8.0 (solid line), and colorless (λ_{max} 343 nm) at pH > 11.0 (dotted line). Concentrations were the same for all the three curves, but not determined. The conversion of the chromopeptide from yellow to red is reversible. The chromopeptide conversion from red to colorless is only partly reversible and reflects chromophore chemical destruction. (B) Excitation (dashed line) and emission (solid line) spectra of peptide II in its acidic yellow form (with absorption λ_{max} 412 nm) in 150 mM NaCl, pH 3.0, superimposed on the emission spectrum of native zFP538 in 20 mM Tris-HCl, pH 8.8 (dotted line). Excitation scanning with emission wavelength fixed at 538 nm (dashed line). Emission scanning with excitation wavelength fixed at 410 nm (solid line). Emission scanning with excitation wavelength fixed at 490 nm (dotted line). (C) Excitation (dashed line) and emission (solid line) spectra of peptide II in its alkaline red form (with absorption λ_{max} 512 nm) in 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, superimposed on the excitation spectrum of native zFP538 in 20 mM Tris-HCl, pH 8.8 (dotted line). Excitation scanning with emission wavelength fixed at 595 nm (dashed line). Emission scanning with excitation wavelength fixed at 510 nm (solid line). Excitation scanning with emission wavelength fixed at 540 nm (dotted line). (D) Peptide III absorption spectra. pH of peptide III solution, pH 3.0 (dashed line), was adjusted to 8.8 with dilute NaOH, and then absorption spectra were recorded at various times (solid lines). Concentrations were the same for all the curves, but not determined. The intermediate form (λ_{max} 529 nm) first appears and then spontaneously converts into the final form (λ_{max} 470 nm). The absorption spectrum of native zFP538 in 20 mM Tris-HCl, pH 8.8, is also presented (dotted line). Time course of peptide III solution absorbance at 470 (open circles) and 529 (black triangles) nm after pH adjustment to 8.8 (inset). (E) Excitation (dashed line) and emission (solid line) spectra of peptide III in its alkaline final form (with absorption λ_{max} 470 nm) existing in the equilibrium state at pH 8.8 superimposed on the emission spectrum of native zFP538 in 20 mM Tris-HCl, pH 8.8 (dotted line). Excitation scanning with emission wavelength fixed at 540 nm (dashed line). Emission scanning with excitation wavelength fixed at 470 nm (solid line). Emission scanning with excitation wavelength fixed at 490 nm (dotted line). (F) Reconstructed (see text) excitation (dashed line) and emission (solid line) spectra of peptide III in its alkaline intermediate form (with absorption λ_{max} 529 nm) at pH 8.8 superimposed on the excitation spectrum of native zFP538 in 20 mM Tris-HCl, pH 8.8 (dotted line). Excitation scanning with emission wavelength fixed at 600 nm (dashed line). Emission scanning with excitation wavelength fixed at 520 nm (solid line). Excitation scanning with emission wavelength fixed at 540 nm (dotted line).

Characterization of the Properties of Chromopeptides by Amino Acid Analysis, MS, and UV−Vis Spectroscopy. Peptide I demonstrates a single peak of 765.8 Da in MALDI-TOF MS. Its amino acid analysis reveals Phe, Asp, and Arg at a ratio of 1:1:1. Chymotrypsin cleavage of peptide I produces Phe, while carboxypeptidase B releases C-terminal Arg. Amino acid analyses of peptides II and III reveal Asp and Arg in equimolecular amounts. Treatment of peptides II and III with carboxypeptidase B also releases C-terminal Arg.

According to high-resolution ESI-MS, the major peak of peptide II is detected at $m/z = 617.2681$ corresponding to a singly charged cation with the brutto formula $C_{27}H_{37}N_8O_9$ (calculated monoisotopic M_r 617.2682 Da). The same method (but at a lower resolution) gave two peaks for peptide III, a major ($m/z = 599.27$) and a minor one ($m/z \sim 617.27$). The latter could be attributed to traces of peptide II, a product of peptide III degradation. Indeed, upon incubation at 20 °C in water, peptide III produces chromatographically detectable amounts of peptide II. The treatment of peptide III with $NaBH_4$ in water results in a stable product, peptide IV, with a molecular mass of 618.7 Da according to MALDI-TOF MS.

Absorption spectra of peptides I and IV at various pH were very similar to those of chromopeptides isolated from GFP (*4*, *24*−*26*) and showed only two forms, with λ_{max} 385 and 450 nm for acidic and alkaline conditions, respectively (data not shown). Absorption spectra of peptide II (Figure 1A) closely resemble those of the chromopeptide isolated from the purple chromoprotein asFP595 (*11*). Both forms of peptide II occurring in acidic or mild alkaline solutions are fluorescent compounds (Figure 1B,C).

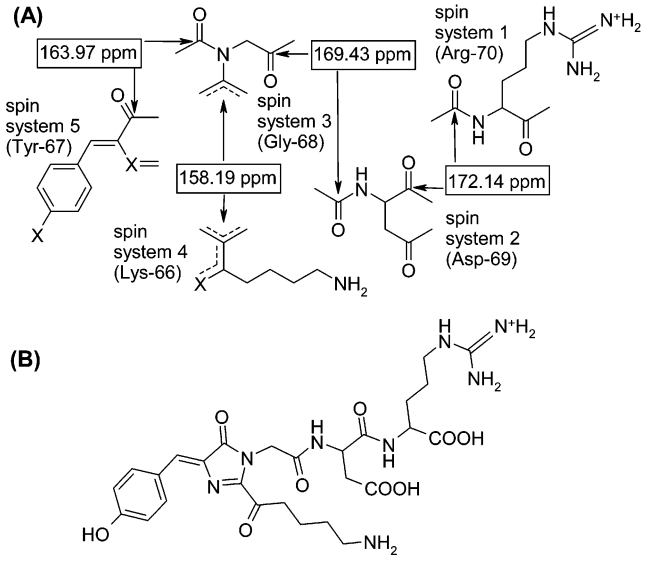

FIGURE 2: Five ^{1}H-^{13}C heteronuclear spin systems of peptide II (A) identified in NMR experiments (see text) and the deduced peptide II structure (B). X represents heteroatom, N or O. Chemical shift values of Lys-66, Tyr-67, Gly-68, and Asp-69 $^{13}C'$ nuclei are shown in rectangles.

Absorption spectra of peptide III (Figure 1D) possess some amazing features. At low pH, a form with λ_{max} 420 nm is observed. A pH shift toward the alkaline region gives an intermediate form with λ_{max} 529 nm. This one is unstable and spontaneously converts into the final form with λ_{max} 470 nm. The conversion rate is pH-dependent: the higher pH, the faster equilibrium is approached. The conversion is reversible: acidification gives rise to the yellow form (λ_{max} 420 nm), and alkalization turns it back into the alkaline final form (λ_{max} 470 nm) via the intermediate one (λ_{max} 529 nm).

Both alkaline forms of peptide III are fluorescent compounds. The emission maximum of the alkaline final form matches that of the native zFP538 (Figure 1E). Kinetic analysis of fluorescence intensities at various wavelengths upon transition from an alkaline intermediate to the final form allowed reconstruction of fluorescence spectra of the peptide III alkaline intermediate form (Figure 1F). The excitation maximum at 528 nm matches that of the native zFP538, the emission maximum at 595 nm matches that of asFP595 chromopeptide fluorescence (*11*). The spectral properties of isolated zFP538 chromopeptides are somewhat similar to those of the synthetic model DsRed chromophores (*27*).

Characterization of Chromopeptides by NMR Spectroscopy. Peptides II and III were characterized by ^{1}H- and ^{13}C NMR spectroscopy. First, the labile (exchangeable with the solvent) amide protons were identified by spectral comparison of samples dissolved in H_2O or in D_2O. After that, 2D homonuclear (TOCSY, DQF-COSY, and ROESY) and heteronuclear (^{1}H-^{13}C-HMQC and ^{1}H-^{13}C-HMBC) spectra were used for the identification of the amino acid ^{1}H-^{13}C spin systems (heteronuclear spectra for peptide II and ^{1}H and ^{13}C resonance assignments for peptides II and III are presented as Supporting Information). Five heteronuclear spin systems were identified for peptide II and as many for peptide III. These showed only a slight difference between the two peptides (the spin systems for peptide II are schematically presented in Figure 2A, the deduced structure of peptide II is shown in Figure 2B).

The spin systems 1, -CO-NH-CH[$(CH_2)_3$-NH-C(=N^+H_2, $-NH_2$)]-CO-, and 2, -CO-NH-CH(CH_2-CO-)-CO-, were attributed to unmodified Arg and Asp, respectively. We can connect these spin systems to each other by the intervening Asp C′=O group (underlined above), since Asp $^{13}C'$ is observed in the both spin systems 1 and 2. The spin system 3, -CO-N(C=)-CH_2−CO-, represents modified Gly and could be connected via Gly $^{13}C'$ to downstream Asp. The signal of amide proton at Gly is absent from the NMR spectra of peptides II and III, and Gly nitrogen is connected to three carbon atoms. The peptide II spin system 4, =C-C(=X)-$(CH_2)_4$-NH_2 (X represents heteroatom, N or O), could be attributed to modified Lys with anomalous chemical shifts of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ carbons and $C^{\beta}H_2$ protons. These peaks at 156.51, 32.50, and 2.84 ppm, respectively, are shifted far out of the normal position in lysine indicating the sp^2 hybridization state of Lys C^{α}. We could connect the peptide II spin system 4 to the Gly spin system 3 via Lys $^{13}C'$. The spin system 5, =X-C(=CH-C_6H_4-X)-CO-, represents modified Tyr connected to the Gly spin system 3 through Tyr $^{13}C'$. The singlet signal of one proton unit from the modified Tyr $C^{\beta}H$ vinyl proton shows strong nuclear Overhauser effect (NOE) connectivity with the aromatic δ-protons of the Tyr phenyl ring. The signals of amide and $C^{\alpha}H$ protons at Tyr are absent from the NMR spectra of peptides II and III. We could not detect the signals from Lys $^{13}C^{\alpha}$ and $^{13}C'$ in the peptide III heteronuclear correlation spectra due to the instability of peptide III and low intensity of the corresponding signals.

The product of the peptide III reduction with $NaBH_4$, peptide IV, has been studied by means of ^{1}H NMR spectroscopy only (proton chemical shifts are also presented as Supporting Information). Peptide IV is a 1:1 mixture of two compounds with similar spin systems and minor (0.003−0.02 ppm) differences in the proton chemical shifts. The ^{1}H NMR spectra of both compounds in the mixture preserve the signals of all the peptide III spin systems, except for those of the spin system 4 corresponding to modified Lys. One proton unit signal corresponding to the $C^{\alpha}H$ proton appeared at ∼4.73 ppm in the peptide IV ^{1}H NMR spectra. At the same time, the multiplet corresponding to modified Lys $C^{\beta}H_2$ protons at 3.25 ppm disappeared, and instead, two new signals, at 1.91 and 1.94 ppm, were observed. These chemical shifts are close to those specific for $C^{\beta}H_2$ protons of unmodified lysine. Therefore, under $NaBH_4$ action on peptide III, the double bond at modified Lys C^{α} is reduced, and this C^{α} carbon changes its hybridization state from sp^2 to sp^3 and becomes chiral. Then, peptide IV is a diastereomeric mixture of two compounds with the opposite configuration of the newly acquired asymmetric center, C^{α} of Lys.

Chromopeptide Structures. The data obtained permit us to propose the structures for peptides I−IV (Figure 3). All of them contain a *p*-hydroxybenzylideneimidazolinone moiety analogous to that described for GFP (*4*) and DsRed (*7*, *8*) chromophores and formed by Lys-66, Tyr-67, and Gly-68 of zFP538 (amino acid numbering accords with wild-type zFP538 (*2*)). Peptide I, FKYGDR hexapeptide derivative, contains the same chromophore as reported for GFP chromopeptides (*4*, *25*). Peptides II−IV are KYGDR pentapeptide derivatives. Racemic Lys-66 C^{α} of peptide IV is bound to the primary amino group resulting from reduction of the peptide III imino group with $NaBH_4$, which, under

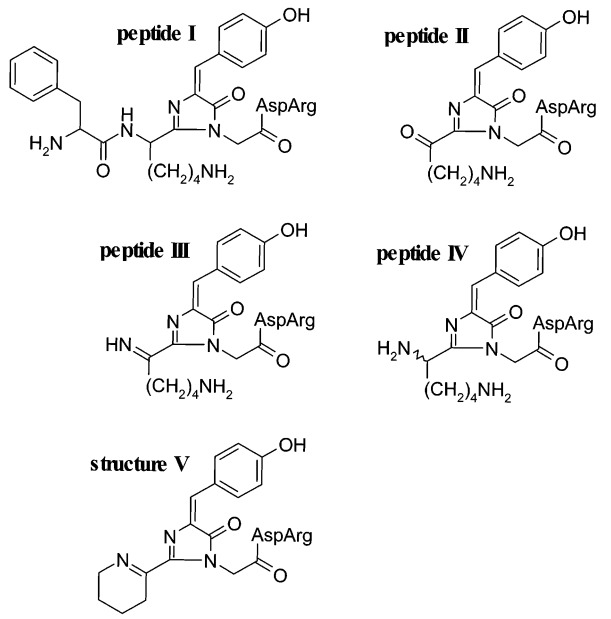

FIGURE 3: Proposed structures of peptides I−IV and cyclic Schiff base (structure V), a possible MS collision product of peptide III.

the conditions applied, may act as a selective reducing agent (*28*). In fact, we were unable to detect any reduction of peptide II (containing an oxogroup at Lys-66 C^{α}) under the same conditions. The upfield position of the peptide II Lys-66 $^{13}C^{\alpha}$ chemical shift (156.51 ppm) comparing to the normal value (~200 ppm) for the carbon of an ordinary keto group could be explained by enolization and/or by the involvement into the conjugated system. The major peak in the peptide III mass spectrum at $m/z = 599.27$ could be interpreted as a product of the intramolecular transalkylidenation (imino exchange) (*29*) reaction

$$R_2C{=}NH + H_2N\text{-}R' \rightarrow R_2C{=}N\text{-}R' + NH_3$$

under ionization conditions between an imino group at C^{α} and the ϵ-amino group of Lys-66. This reaction will give rise to cyclic Schiff base (structure V in Figure 3). Structure V must be excluded for peptide III in water solution at pH 3.2 because the signals from the ϵ-amino group protons of Lys-66 are clearly seen in NMR spectra. Hydrolysis of V should give peptide II, and this conversion could be reversible. Peptide III spontaneous conversion into peptide II observed even in water at 20 °C represents a standard irreversible hydrolysis of unsubstituted ketimine (III + H_2O → II + $NH_3\uparrow$) resulting in ketone and ammonia.

It is interesting to compare our NMR data with these of Mizuno et al. (*13*) on Kaede chromopeptide pep_Ra. This one is a HYGNR pentapeptide derivative containing the GFP-type *p*-hydroxybenzylideneimidazolinone moiety and unsubstituted at C^{α} α,β-dehydrogenated His-62 corresponding to zFP538 Lys-66. Signals of the side chains and C^{α}s of zFP538 Lys-66 and Kaede His-62 N-terminal residues are different as expected. But C′ carbons of the N-terminal residues and the rest C-terminal parts of Kaede pep_Ra and zFP538 peptides II−IV are very similar in either chemical shift values or homo- and heteronuclear correlations. The upfield (~5 ppm) shift of the Tyr-67 $^{13}C'$ signal in peptide II and the downfield shifts of the Tyr-67 $C^{\beta}H$ proton (~0.7 ppm) and $^{13}C^{\beta}$ (~13 and ~8 ppm in peptides II and III, respectively) signals in peptides II and III compared to the corresponding signal positions in Kaede pep_Ra might be ascribed to the differences in the conjugated double bond systems of these peptides.

Fragmentation of the zFP538 Polypeptide Chain. As seen from structures of peptides I−III, their C-termini have resulted from proteolysis (both trypsin and thermolysin can hydrolyze the Arg-70−Ile-71 peptide bond). The same holds true for the N-terminus of peptide I since the Gly-64−Phe-65 peptide bond is sensitive to thermolysin. The cleavage of the zFP538 polypeptide between Phe-65 and Lys-66 in the course of formation of peptides II and III could not be explained by thermolysin, pepsin, or trypsin proteolysis. Spontaneous fragmentation of the zFP538 polypeptide chain in the vicinity of its chromophore was also shown by two other methods, SDS/PAGE and HPLC.

SDS/PAGE of zFP538 reveals three polypeptides with M_r of 9, 19, and 28 kDa (data not shown). The same was reported for DsRed (*6*), asFP595 (*11*), gtCP (*12*), and Kaede (*13*). Similarly to DsRed (*6*), unboiled zFP538 migrates in SDS/PAGE as a diffuse band with $M_r > 100$ kDa and is brightly yellow fluorescent before Coomassie staining (*30*). Excised from the gel after SDS/PAGE, the 19-kDa fragment of zFP538 showed the N-terminal sequence starting from Asp-69 (A. P. Savitsky and P. A. Savitsky, personal communication).

zFP538 could also be separated into three components (designated as peaks A, B, and C) by HPLC under denaturing acidic conditions: isolated polypeptides are virtually identical to those detected by SDS/PAGE (with M_r of 9, 19, and 28 kDa, respectively). Determined by MALDI-TOF MS, the sum of molecular masses of polypeptides at peaks A and B is always equal (within experimental error) to the molecular mass of full-length polypeptide at peak C, plus the mass of two water molecules: A + B = C + $2H_2O$. However, probably due to irreversible oxidation of numerous cysteine side chains, peak C polypeptide M_r varies from one zFP538 preparation to another and often exceeds significantly (up to 200−300 Da) the calculated value. This precluded the direct determination of the exact fragmentation site of the full-length zFP538 on the basis of MS data only.

As judged from N-terminal amino acid sequence, the 9-kDa peak A polypeptide corresponds to the N-terminal fragment of zFP538, whereas the 19-kDa peak B polypeptide is the C-terminal portion bound to the chromophore which blocks its sequencing (Figure 4A). Boiling of the peak B polypeptide for 5 min in 0.7 M 2-mercaptoethanol, 62 mM Tris-HCl, pH 6.8, partially removes the chromophore from its N-terminus and allows determining the N-terminal sequence starting from Asp-69. Peptide III under the same conditions releases dipeptide AspArg, while peptide II remains completely unchanged. We conclude that both peptide III and a part of the 19-kDa peak B polypeptide preparation contain the same chromophore. It is tempting to speculate that the "peptide III type" structure also appears in zFP538 in the course of SDS/PAGE sample processing which releases the 19-kDa fragment N-terminal Asp-69 for Edman degradation. Taking into account the presence of intact Gly-68−Asp-69 peptide bond in peptides II and III, it could be concluded that the zFP538 fragmentation between Gly-68 and Asp-69 observed in SDS/PAGE is the artifact of sample processing which also includes boiling with

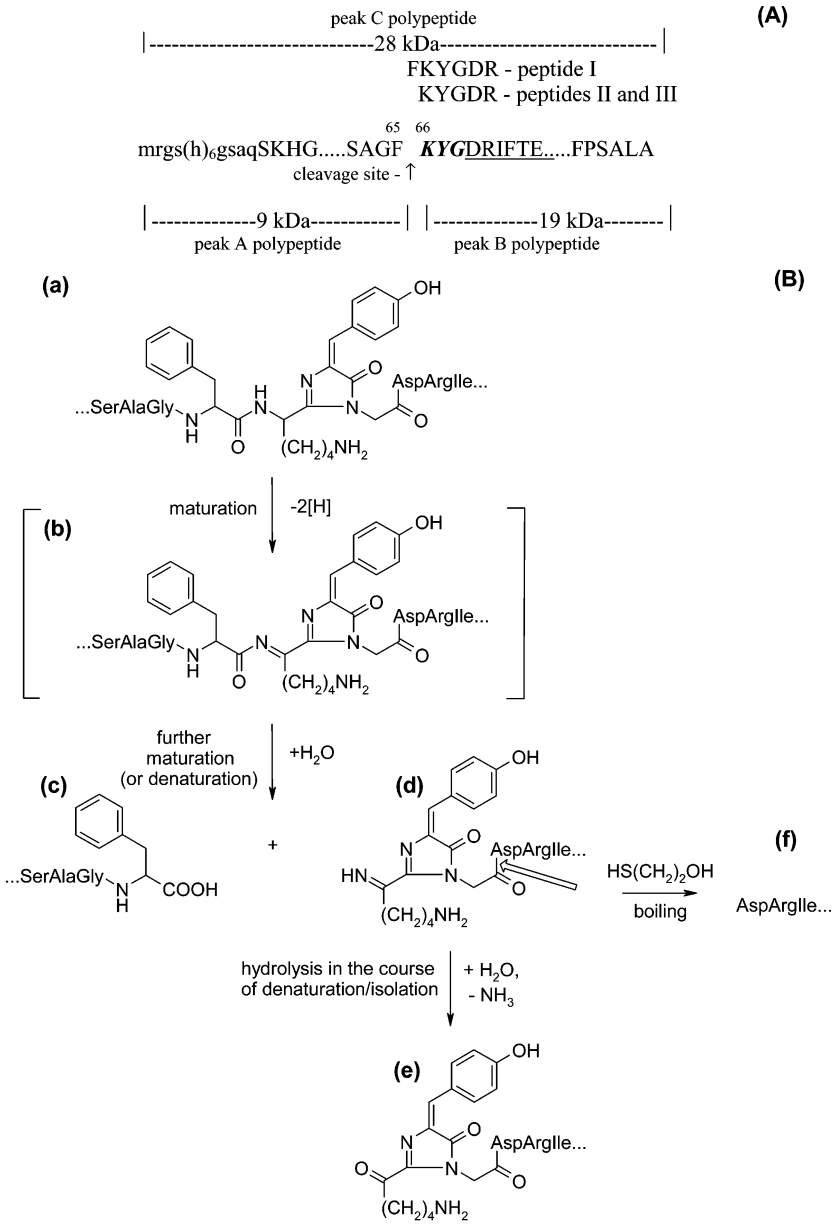

FIGURE 4: Fragmentation of zFP538. (A) Scheme outlining how the zFP538 polypeptide chain splits during maturation or denaturation. The position of the cleavage is indicated by an arrow. Residues corresponding to the expression vector backbone are in lowercase, ones forming the chromophore are in bold italic. The amino acid sequence determined after peak B polypeptide boiling with 2-mercaptoethanol is underlined. Sequences of isolated chromopeptides I−III are also shown and aligned with zFP538 structure. (B) A complete scheme of the zFP538 chromophore formation and degradation upon the protein denaturation. The first step is the GFP-type chromophore formation (structure a) including cyclization by linking of Lys-66 C′ to Gly-68 nitrogen and the subsequent Tyr-67 dehydrogenation. Then, the DsRed-type chromophore (structure b) appears as a result of Lys-66 $C^{\alpha}-N$ bond dehydrogenation. Cleavage of the former peptide bond between Phe-65 and Lys-66 in the course of the final maturation step or upon zFP538 denaturation under mild conditions results in splitting of the protein into 9- and 19-kDa fragments (structures c and d, respectively) as also shown in panel A. Spontaneous hydrolysis of the imine-containing chromophore (structure d) leads to the C-terminal fragment with the "peptide II type" group at its N-terminus (structure e). Boiling of the initial splitting product (structure d, labile Gly-68−Asp-69 peptide bond sensitive to SDS/PAGE sample processing is indicated by an arrow) in the presence of 2-mercaptoethanol removes the chromophore (giving rise to structure f) and allows determining of the N-terminal sequence starting from Asp-69 (underlined in panel A).

2-mercaptoethanol. Lability of the Gly-68−Asp-69 peptide bond in the "peptide III type" chromophore (structure d in Figure 4B) under the action of 2-mercaptoethanol remains to be explained.

Therefore, peptides II and III are proteolysis products of the 19-kDa peak B polypeptide, the preparation of which evidently contains "keto"- and "imino"-chromophores and thus represents a mixture of two compounds (structures d and e in Figure 4B). Additional support for this statement is the fact that the 19-kDa fragment is frequently found in SDS/PAGE as unresolved doublet (data not shown) probably presenting a mixture of the polypeptide with "peptide II type" chromophore (structure e in Figure 4B) and one without chromophore (structure f in Figure 4B) originated from imine-containing fragment (structure d in Figure 4B) after SDS/PAGE sample processing. Peptide II is not a direct (and a major) product of the zFP538 native chromophore degradation upon the protein denaturation. Instead, peptide III, the earlier product of the zFP538 native chromophore destruction, is the apparent peptide II precursor. Isolated with

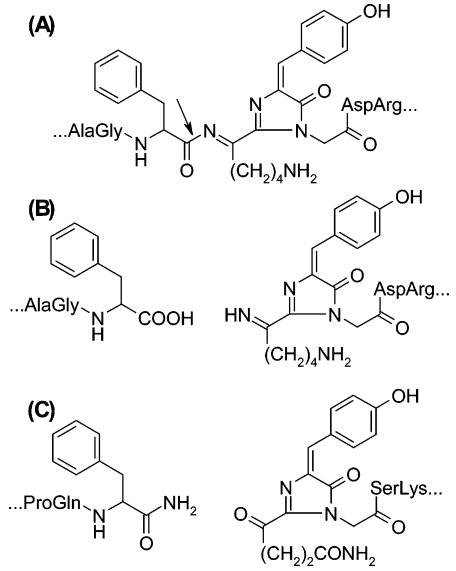

FIGURE 5: Proposed structures of zFP538 chromophore (A and B) and predicted products of DsRed fragmentation (C). (A) DsRed-type chromophore with the unusual potential of *N*-acylimine hydrolysis and polypeptide splitting. Cleavage site, the former peptide bond (between Phe-65 and Lys-66) is indicated by an arrow. More likely, this structure represents only the intermediate step in the zFP538 chromophore maturation. (B) "Peptide III type" structure with the *N*-unsubstituted imino group at Lys-66 C^{α} resulted from DsRed-type chromophore (presented in panel A) in the course of unexpected fragmentation of the zFP538 polypeptide chain at the final step of chromophore maturation. This scheme is also presenting the early splitting products upon zFP538 denaturation if its chromophore is identical to that of DsRed. (C) The products of DsRed chromophore hydrolysis predicted by Gross and co-workers (*6*).

high yields independently of the denaturation method applied, peptide III definitely does contain the Lys-66 N^{α} atom in its structure. This means that the major pathway of spontaneous fragmentation of zFP538 implies cleavage of the bond between Phe-65 C′ and Lys-66 N^{α}. This fragmentation was observed even under mild denaturing conditions of the affinity chromatography on Ni−NTA in 6 M guanidine hydrochloride which was added to zFP538 solution without heating. Hexahistidine-tagged full-length polypeptide and its N-terminal 9-kDa fragment remained bound to the metal-chelating resin while the 19-kDa chromophore-containing C-terminal fragment was eluted from the column in a void volume (data not shown).

Lys-66 N^{α} goes first with the C-terminal chromophore-containing peak B fragment (structure d in Figure 4B) upon zFP538 polypeptide fragmentation observed under mild denaturing conditions, only later leaving it as NH_3 similarly to the peptide III conversion into peptide II: $HN{=}C< + H_2O \rightarrow O{=}C< + NH_3$. Therefore, it should specially be noted that the N-terminal peak A fragment (structure c in Figure 4B) has no C-terminal carbamoyl ($-CONH_2$) group as might be expected according to the fragmentation model, *N*-acylimine hydrolysis, earlier (*6*) suggested for DsRed: $-CO-N{=}C< + H_2O \rightarrow -CO-NH_2 + O{=}C<$ (see also Figure 5*C*). High-yield isolation of peptide III with the *N*-unsubstituted imino group at Lys-66 C^{α} excludes this mechanism (at least as a major pathway) of the polypeptide chain splitting in case of zFP538.

Peptide I is a proteolysis product of the full-length zFP538 polypeptide (structure a in Figure 4B) containing a GFP-type chromophore already formed and arrested at an earlier stage of maturation. Spontaneous fragmentation of zFP538 can be detected only after complete maturation of its yellow chromophore because no splitting has been observed in the zFP538 "evergreen" point mutant Lys66Met (data not shown). This mutant demonstrates wild-type GFP phenotype, characteristic green fluorescence (*31*). The same was reported for the "permanently green" point mutant (Lys83Arg) of DsRed (*6*) and several mutants of Kaede (*13*). In the case of Kaede (*13*), the photoinduced splitting site was precisely mapped between N^{α} and C^{α} of His-62 that corresponds to zFP538 Lys-66. Thus, the Kaede N-terminal fragment has a carbamoyl ($-CONH_2$), not a carboxyl (-COOH) group at the C-terminal end.

It still remains unclear whether the zFP538 polypeptide fragmentation is a direct result of chromophore maturation or only a denaturation artifact as it is in the case of DsRed (*6*−*8*). It seems likely that only the zFP538 X-ray crystal structure determination at high resolution could finally answer this question. But the additional consideration in favor of the polypeptide chain splitting between Phe-65 C′ and Lys-66 N^{α} in the mature zFP538 chromophore is a recently published (*12*) report on the chromopeptide isolation from gtCP which was declared to have the DsRed-type chromophore on the basis of the absorption spectra studied upon the protein denaturation. Similarly to DsRed (*6*), this GFP homologue demonstrated its polypeptide chain splitting seen in SDS/PAGE after preboiling in 0.1 M HCl for 5 min (*12*). On the other hand, isolation from a gtCP chymotrypsin digest of the chromopeptide SPQSQYGSIPF (chromophore-forming residues are underlined) has been described (*12*). This peptide was postulated to contain the intact *N*-acylimine group (see below) but lacks any fragmentation in the vicinity of its chromophore.

DISCUSSION

The Structure of zFP538 Chromophore. Gross L. A. and co-workers (*6*) proposed a DsRed chromophore structure analogous to that of GFP with the additional dehydrogenation of the $C^{\alpha}-N$ bond at Gln-66 that corresponds to zFP538 Lys-66. The planarity of Gln-66 C^{α} had been supported by DsRed X-ray crystal structures (*7*, *8*). Fragmentation of the DsRed polypeptide chain was not observed in the crystal structures, and the Phe-65 C′=O bond is not coplanar to the chromophore plane containing also the Gln-66 $C^{\alpha}{=}N$ double bond. This is consistent with the *N*-acylimine structure and the sp^2 hybridization state (*32*) of Gln-66 N^{α} whose unshared electron pair could thus not participate in the amide stabilization of the former Phe-65−Gln-66 peptide bond, which, in addition, exists in a rare cis configuration (*8*). Chromophores chemically identical to that in DsRed but differing in their configurations were recently discovered in two other GFP homologues, pocilloporin (*9*) and eqFP611 (*10*), on the basis of X-ray crystal structure determination.

It is known (*33*−*36*) that *N*-acylimines having the hydrogen atom in the β-position to the azomethine nitrogen are unstable compounds and practically always rearrange into *N*-acylenamines (enamides): $-CO-N{=}CR-CH_2- \rightarrow -CO-NH-CR{=}CH-$. Several supported exceptions to this rule are the very special cases of *N*-acylimines with endocyclic (*37*, *38*) azomethine bond or when this one participates in quinone-

like conjugation (*39*). DsRed, pocilloporin, and eqFP611 present the only examples of stable *N*-acylimine of this type known to date. The stability of *N*-acylimine moiety discovered in these red GFP homologues could be explained by the very specific environment existing inside the native globular protein. Nevertheless, *N*-acylimine conversion into enamide might be expected in case of the protein denaturation. As a result, the α,*β*-dehydrogenated residue of modified Gln-66 will necessarily appear in the full-length DsRed polypeptide chain under denaturing conditions. The same should be predicted for the above-mentioned chymotryptic chromopeptide isolated from gtCP (*12*), although either native gtCP or its chromopeptide were declared to contain the same intact DsRed-type chromophore with the acylimine-substituted imidazolone group. Alternatively, this gtCP chromopeptide presents an example of stable *N*-acylimine with *β*-hydrogen, existing in water solution and thus surprisingly resistant toward hydrolysis. This theoretical possibility seems to be very improbable.

It is known (*40*) that a polypeptide chain with the α,*β*-unsaturated residue could be easily cleaved at the $C^{\alpha}-N$ bond under the action of various reagents including acid and alkali. This mechanism could explain the DsRed fragmentation observed in SDS/PAGE and promoted by the protein boiling in 0.1 M KOH or 0.1 M HCl (*6*) due to hydration of the proposed Gln-66 $C^{\alpha}=C^{\beta}$ double bond followed by polypeptide chain cleavage (*40*). Gross L. A. and co-workers (*6*) gave another explanation: *N*-acylimine does not tautomerize into enamide, and water molecule addition across the Gln-66 $C^{\alpha}=N$ double bond gives rise to *N*-acylcarbinolamine, which is then decomposed to form a $C^{\alpha}=O$ group. Whatever the mechanism is, the result of the polypeptide chain fragmentation is the same, namely, C-terminal amide and N-terminal ketone (Figure 5C):

$$\text{-CO-N=CR-} + H_2O \rightarrow .?. \rightarrow \text{-CO-}NH_2 + \text{O=CR-} \quad (1)$$

As a first approach to the zFP538 chromophore, it seems reasonable to look at the *N*-acylimine structure suggested for DsRed (*6*). We failed to find an example of enamide hydrolysis (-CO-NH-CR=CH- + $H_2O \rightarrow$ −COOH + H_2N-CR=CH-) into the carboxylic compound and enamine (see, for example, (*32*−*39*, *41*−*43* and refs therein); enamine, in its turn, could then isomerize into a more stable imine tautomer, H_2N-CR=CH- → HN=CR-CH_2- (*29*)). Hydrolysis of *N*-acylketimine practically always gives rise to a carbonyl compound and amide (see eq 1). We have found only several examples of alternative decomposition of *N*-acylimine under anhydrous conditions (*36*, *41*, *42*). Among them, of special interest is the interaction of zinc-containing Reformatsky reagents with *N*-benzoyl diphenylketimine, $Ph_2\underline{C}$=N-CO-Ph (*36*). In this case, the nucleophile attacks the carbonyl carbon, which is usually less electrophilic center of *N*-acylimine than the azomethine carbon (underlined above). The subsequent acidic hydrolysis of the adduct gives rise to benzophenone ($Ph_2C=O$), which is evidently the product of diphenylketimine ($Ph_2C=NH$) hydrolysis as judged from the analysis of the other reaction products. To the best of our knowledge, this is the only example of *N*-acylimine conversion into *N*-unsubstituted imine which was detected indirectly.

If the zFP538 chromophore is identical to that of DsRed, the isolation of peptide III with the *N*-unsubstituted imino group is significant, in that, to our knowledge, this is the first example of *N*-acylimine hydrolysis resulting in carboxylic compound and *N*-unsubstituted imine:

$$\text{-CO-N=CR-} + H_2O \rightarrow .?. \rightarrow \text{-COOH} + \text{HN=CR-} \quad (2)$$

It seems likely that some amino acid side chain function catalyzes this process by nucleophilic attack on Phe-65 C′ atom similarly to described in ref *36*. Additional consideration in favor of this type of cleavage is that sp^2 hybridized imine nitrogen represents a much better leaving group than standard nitrogen of a normal peptide bond.

In conclusion, it could be proposed that the native zFP538 chromophore does contain a five-membered imidazolinone moiety, analogous to GFP, appearing at an earlier stage of maturation (structure a in Figure 4B). Then, some additional process (absent from GFP) takes place leading to oxidation of Lys-66 C^{α} (evident in some zFP538 chromopeptides studied) and zFP538 polypeptide fragmentation (seen at least under denaturing conditions). There are two possibilities as to the nature of additional chemistry relevant to zFP538 chromophore. First, the zFP538 chromophore could be identical to that of DsRed (Figure 5A). In this case, fragmentation of the protein polypeptide chain is a denaturation/isolation artifact. The unusual way of *N*-acylimine hydrolysis (cf.: eqs 1 and 2; Figure 5B and Figure 5C) upon zFP538 denaturation is subject to further investigation. Next, fragmentation of the zFP538 polypeptide chain could be a result of the chromophore maturation in the course of which the bond between Phe-65 C′ and Lys-66 N^{α} has been cleaved, and the *N*-unsubstituted imino group appears at Lys-66 C^{α} to form a "peptide III type" chromophore structure (Figure 5B). The unusual fragmentation should be expected directly inside the protein globule rather than in water solvent upon denaturation because *N*-acylimines, in general, exist only under anhydrous conditions and, as a rule, are quickly hydrolyzed into amides and ketones in the presence of traces of moisture (*32*−*34*, *36*). Additional strong support for the "peptide III type" (2-imino-derivative of imidazolinone) structure of zFP538 chromophore is the similarity of the peptide III absorption and fluorescence spectra to these of the native protein (Figure 1D−F). Therefore, the structure presented in Figure 5B seems to be the most likely for the native chromophore of zFP538.

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SUPPORTING INFORMATION AVAILABLE

Heteronuclear ^{1}H-^{13}C-HMQC and ^{1}H-^{13}C-HMBC spectra for peptide II are presented in Figure 1. ^{1}H (for peptides II−IV) and ^{13}C (for peptides II and III) resonance assignments are listed in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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