Synthesis and Properties of the Chromophore of the asFP595 Chromoprotein from Anemonia sulcata †

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Synthesis and Properties of the Chromophore of the asFP595 Chromoprotein from *Anemonia sulcata*†

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ABSTRACT: A model compound for the chromophore within the purple nonfluorescent GFP-like chromoprotein asFP595 was synthesized. The postulated structure of the chromophore, 2-acetyl-4-(*p*-hydroxybenzylidene)-1-methyl-5-imidazolone, was taken from the high-resolution crystal structure analysis of intact asFP595 [Quillin, M. L., Anstrom, D., Shu, X., O'Leary, S., Kallio, K., Lukyanov, K. A., and Remington, S. J. (2005) Kindling Fluorescent Protein from *Anemonia sulcata*: Dark-State Structure at 1.38 Å Resolution, *Biochemistry 44*, 5774−5787]. Erlenmeyer lactonization and oxidation of the methylene group attached to the heteroaromatic moiety with selenium dioxide were used at the key stages of the synthesis. The spectral properties of the model chromophore in solution and their dependence on the pH and polarity of the solvent were investigated. In water, the chromophore was found to exist in two forms, neutral and anionic, with a pK_a of 7.1. In a dimethylformamide solution, the spectral properties of the anionic form closely match those of the native protein, demonstrating that under these conditions, the compound is an excellent model for the chromophore within native asFP595.

The green fluorescent protein (GFP)[1] from the hydroid jellyfish *Aequorea victoria* is known to a broad readership because of its extensive use in molecular and cell biology as a noninvasive genetically encoded fluorescent label (*1*). GFP possesses a unique ability to form its chromophore within the protein globule without any external enzymatic activities or cofactors except for molecular oxygen (*2*). Chromophore formation in GFP is an autocatalytic process consisting of two main steps: cyclization of the protein backbone at positions 65−67 (Ser-Tyr-Gly) followed by dehydrogenation of the Tyr66 side chain (*3*−*6*). In its final form [4-(*p*-hydroxybenzylidene)-5-imidazolone], the GFP chromophore comprises two rings. One cycle originates from Tyr66; the other, a five-membered heterocycle, forms when the nitrogen of Gly67 bonds with the carbonyl carbon of Ser65. The resulting structure is a system of conjugated double bonds which is capable of absorbing and emitting light in the visible region.

Recently, great spectral diversity was revealed for a family of GFP-like proteins found in *Anthozoa* species (*7*, *8*). Studies have demonstrated that this color diversity is based primarily on the diversity of chemical structures within the different proteins; however, the protein can influence absorption and emission maxima to some extent. For example, the chromophore within the red fluorescent protein DsRed includes a GFP-like core but carries an additional double bond in the peptide linkage preceding the chromophore (between the Cα atom and the amide N of Gln66), which extends the conjugated π-electron system (*9*−*11*). The additional double bond apparently results from reaction of molecular oxygen with an intermediate GFP-like chromophore in a separate step (*9*). The acylimine linkage extends the conjugation of the chromophore through the polypeptide backbone, causing the red shift. The resulting chemical structure, which can exist in either the trans or cis configuration, determines the spectral class for many natural red fluorescent proteins as well as for some nonfluorescent purple-blue chromoproteins (*12*−*15*). However, other modifications have been reported. In the photoconvertible fluorescent protein Kaede (*16*), a red-emitting chromophore is formed upon UV irradiation. The process results in cleavage of the protein backbone between the Cα atom and the amide N of His62 (corresponding to Ser65 in GFP) and, subsequently, formation of a double bond between the Cα and Cβ atoms of His62 (*17*). Crystallographic studies showed that the yellow fluorescent protein zFP538 from button polyp *Zoanthus* contains a three-ring chromophore derived from that of GFP. The third ring was proposed to result from a transimination reaction in which a transiently appearing DsRed-like acylimine is attacked by

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[1] Abbreviations: asFP595, purple chromoprotein from *A. sulcata*; DMF, dimethylformamide; DsRed, red fluorescent protein from *Discosoma*; GFP, green fluorescent protein from *Ae. victoria*; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; zFP538, yellow fluorescent protein from *Zoanthus*.

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the terminal amino group of lysine 66, cleaving the polypeptide backbone (*18*).

The chromophore within the chromoprotein asFP595 from the sea anemone *Anemonia sulcata* (*19*) has been studied. Martynov et al. (*20*) proposed an unusual structure that included a six-membered heterocycle formed after cleavage of the protein backbone. However, later studies showed the proposed chemical structure to be incorrect (*21*, *22*).

Chemical synthesis of natural compounds makes it possible to obtain large amounts of a pure substance with a known structure, to characterize it in detail, and to support the results of prior investigation into the chemical structure. Investigation of a synthetic GFP chromophore clarified details of its spectral behavior (*23*). However, structural studies of the chromophores found within red-shifted GFP-like proteins are complicated by the instability of the isolated compounds in solution (*9*, *14*, *20*, *24*). Chemical modifications can also occur during mass spectral analysis or intense X-ray irradiation. Ideally, one should use a combination of independent approaches to arrive at a reliable conclusion about the chromophore structure within the native protein. Chemical synthesis appeared to be a useful method for studying the chromophores of GFP-like proteins that allow to check results obtained by other methods.

Except for a model GFP chromophore (*23*), no other natural chromophores from GFP-like proteins have been synthesized to date. The only work on this matter described synthesis of a compound similar to but not identical to the chromophore within DsRed (*25*). Here, we present the synthesis of a model chromophore for the purple nonfluorescent chromoprotein asFP595, based on the proposed chromophore structure resulting from high-resolution crystallographic studies of the protein (*22*).

EXPERIMENTAL PROCEDURES

NMR spectra were recorded on Bruker DRX-500 and Bruker AC200-P instruments. UV−vis spectra were recorded with a Beckman DU520 spectrophotometer. Fluorescence excitation and emission spectra were measured with a Varian Cary Eclipse fluorescence spectrophotometer.

*2-Ethyl-4-(p-propionyloxybenzylidene)oxazolone (**1**).* *N*-Propionylglycine (1.31 g, 0.01 mol), sodium propionate (0.97 g, 0.01 mol), *p*-hydroxybenzaldehyde (1.22 g, 0.01 mol), and propionic anhydride (10 mL) were refluxed for 2 h. Propionic anhydride was partially removed on a rotary evaporator, and then cold water (30 mL) was added. The resulting crystalline solid was washed with cold 70% ethanol and dried in vacuo: yield 1.96 g (72%); ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.10 (t, 3H, CH_3), 1.25 (t, 3H, CH_3), 2.65 (m, 4H, $2CH_2$), 7.20 (m, 3H, vinylic CH and aromatic), 8.20 (d, 2H, aromatic); ^{13}C NMR (DMSO-d_6) δ 8.8, 9.1, 22.4, 27.1, 122.5, 129.2, 130.8, 132.4, 133.4, 152.6, 167.4, 170.4, 172.4.

*3-(p-Hydroxyphenyl)-N-methyl-2-(propionylamino)-2-propenamide (**2**).* Ethanol (5 mL) and a 40% aqueous methylamine solution (1 mL) were added to oxazolone **2** (1.37 g, 0.05 mol). The reaction mixture was stirred for 20 min, and then the solvents were removed. The resulting crystalline solid is practically pure **2**. Synthesis of **3** does not require isolation of **2**, and it may start directly from **1** as a one-pot procedure: ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.27 (t, 3H, CH_3), 2.31 (q, 2H, CH_2), 2.65 (d, 3H, N-CH_3), 6.72 (d, 2H, aromatic), 7.01 (s, 1H, vinylic), 7.36 (d, 2H, aromatic), 7.73 (br m, 1H, NH-Me), 9.10 (s, 1H, NH), 9.72 (s br, 1H, OH).

*2-Ethyl-4-(p-hydroxybenzylidene)-1-methyl-5-imidazolone (**3**).* Compound **2** (1.24 g, 0.05 mol) was refluxed with 5 mL of ethanol, 1 mL of 40% aqueous methylamine, and 100 mg of potassium carbonate for 4 h. The reaction mixture was cooled to room temperature, diluted with 50 mL of water, acidified with concentrated HCl to pH 3, and left overnight at 4 °C. The yellow needles that formed were filtered, washed with a small amount of cold ethanol, and dried: yield 94%; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.25 (t, 3H, CH_3), 2.61 (q, 2H, CH_2), 3.09 (s, 3H, N-CH_3), 6.82 (d, 2H, aromatic), 6.90 (s, 1H, CH vinylic), 8.10 (d, 2H, aromatic); ^{13}C NMR (DMSO-d_6) δ 9.2, 21.3, 25.9, 115.8, 125.3, 125.7, 134.2, 136.2, 159.7, 165.7, 170.1.

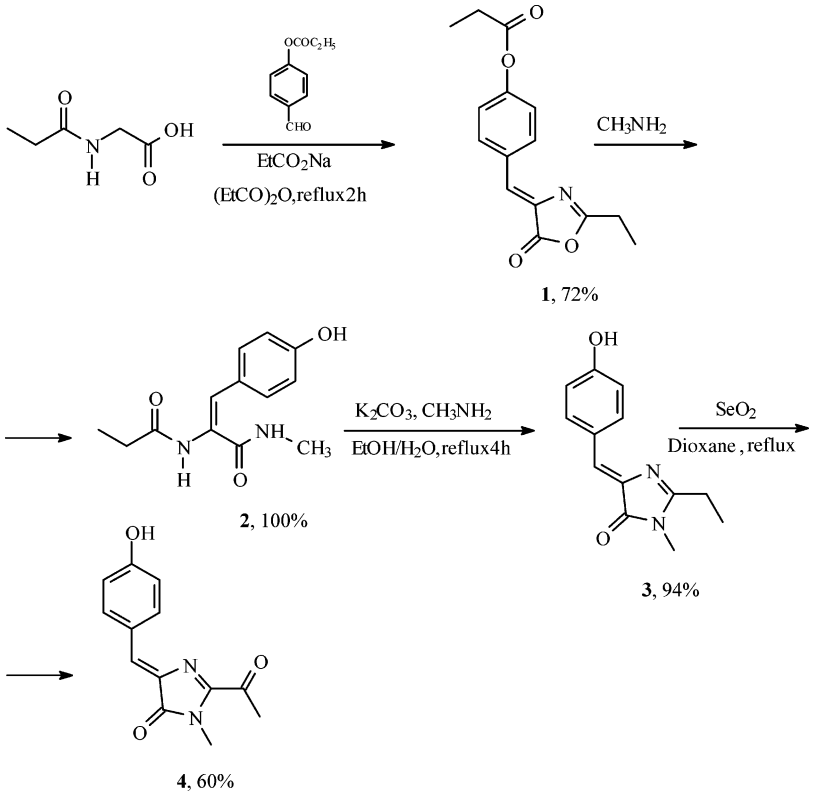
*2-Acetyl-4-(p-hydroxybenzylidene)-1-methyl-5-imidazolone (**4**).* Compound **3** (1.0 g, 4.35 mmol) and selenium dioxide (0.48 g, 4.35 mmol) were suspended in 10 mL of absolute dioxane and refluxed with stirring for ∼40 min, the reaction course being monitored by TLC on silica gel analytical plates in a 95:5 chloroform/ethanol mixture. The R_f of the product is 0.55, while that of the starting material is 0.47. Chromatograms are revealed whether in NH_3 vapors (the stain of the residual starting compound becomes yellow and that of the product becomes bright red) or in a saturated $KMnO_4$ solution with subsequent washing with excess water. Unidentified byproducts with an R_f of ≈0.3 having similar coloration with the product are also formed. After the reaction is complete, dioxane is removed at the rotary evaporator, and the solid residue is recrystallized from 96% EtOH (elementary Se is removed by careful decantation or hot filtration), giving 640 mg (60%) of the crude **4**. Further recrystallization from 96% EtOH affords pure **4** as a fine crystalline red solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.65 (s, 3H, $COCH_3$), 3.32 (s, 3H, N-CH_3), 6.95 (d, 2H, aromatic), 7.42 (s, 1H, CH vinylic), 8.25 (d, 2H, aromatic); ^{13}C NMR (DMSO-d_6) δ 26.2, 28.2, 116.2, 124.9, 134.2, 135.4, 135.8, 153.6, 161.4, 169.6, 192.8.

RESULTS

Synthesis. A general approach to the 4-arylidene-5-imidazolones includes Erlenmeyer azlactone formation followed by aminolysis with the primary amine of choice and subsequent cyclization under alkaline conditions (*25*). We used this approach to synthesize 2-ethyl-4-(*p*-hydroxybenzylidene)-1-methyl-5-imidazolone (**3**) (Scheme 1). Oxidation of **3** with 1 equiv of selenium dioxide in dioxane gave 2-acetyl-4-(*p*-hydroxybenzylidene)-1-methyl-5-imidazolone (**4**) in 60% yield. ^{1}H and ^{13}C NMR spectra of **4** are fully consistent with the proposed structure. In particular, the peak at 192.8 ppm in the ^{13}C NMR spectrum refers to a key carbonyl group in the acetyl substituent.

Spectral Properties of the Model asFP595 Chromophore. The absorption spectrum of compound **4**, the proposed model chromophore, demonstrated a clear dependence on pH. In a water solution at acidic pH, the absorption maximum was at 418 nm, while at neutral and slightly basic pH, the maximum was at 520 nm. Interconversions of these forms by pH titration were found to be completely reversible with a pK_a of 7.1 (Figure 1A). We attribute this to the equilibrium between the neutral and anionic forms (structures **4a** and

Scheme 1: Synthesis of the asFP595 Chromophore



4b, respectively; Figure 1A) of the chromophore, similar to the well-characterized behavior of the GFP chromophore (*23*, *26*).

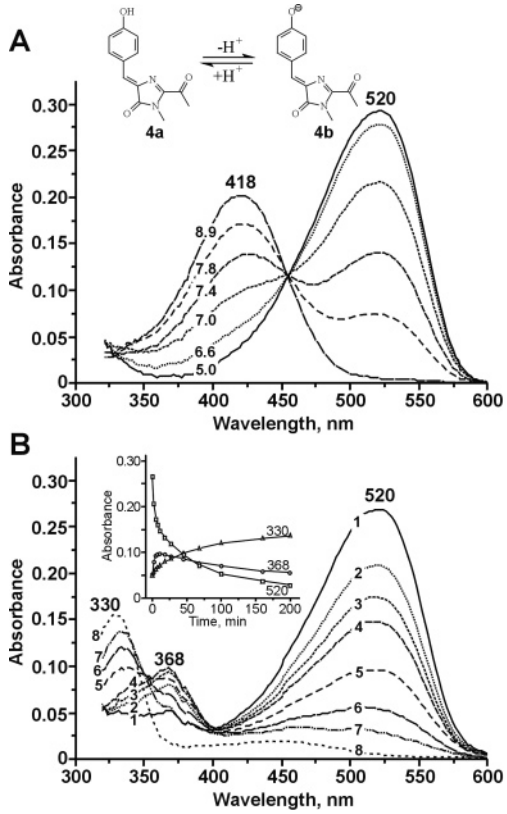


FIGURE 1: (A) Spectrophotometric titration of a chromophore **4** water solution. (B) Degradation of the chromophore in 10 mM aqueous NaOH. Curves 1−8 correspond to incubation for 0, 2, 5, 11, 45, 100, 200, and 1200 min, respectively. The inset shows absorption changes at 334, 368, and 520 nm during the first 200 min.

Table 1: Absorption Characteristics of the Chromophore in Different Solvents[a]

	absorption maximum (nm) [extinction coefficient (M^{-1} cm^{-1})]	
solvent	neutral form	anionic form
water	418 (35 000)	520 (47 000)
ethanol	425 (40 000)	542 (72 000)
2-propanol	428 (39 000)	552 (73 000)
DMF	422 (38 000)	572 (87 000)

[a] Spectra for neutral and anionic forms were measured in 10 mM HCl and 10 mM NaOH, respectively.

At pH ≥11.0, the model chromophore underwent slow degradation (half-life of ∼20 min at pH 12) into unidentified products with absorption at 368 and 330 nm (Figure 1B). Acidification at different time points during this process resulted in the appearance of different amounts of neutral form **4a** corresponding to the current amount of anionic form **4b** (not shown). Further studies are required to solve the degradation pathway, which is in fact irrelevant to the structure and spectral properties of the model asFP595 chromophore.

The nature of the solvent had a strong impact on the absorption maximum of the anionic form of the chromophore, **4b** (Table 1). The most red-shifted absorption maximum at 572 nm was found in DMF, which is very close to the absorption maximum of the asFP595 protein (568 nm) (Figure 2A). A DMF solution of chromophore form **4b** (pH 8.3) showed weak red fluorescence at 603 nm (Figure 2B). We estimated the chromophore fluorescence quantum yield at room temperature to be 2.1×10^{-3}. The wild-type asFP595 protein demonstrated fluorescence at the same wavelength, but remarkably, the quantum yield was 10-fold lower (2.2×10^{-4}). The similarity of absorbance and fluorescence

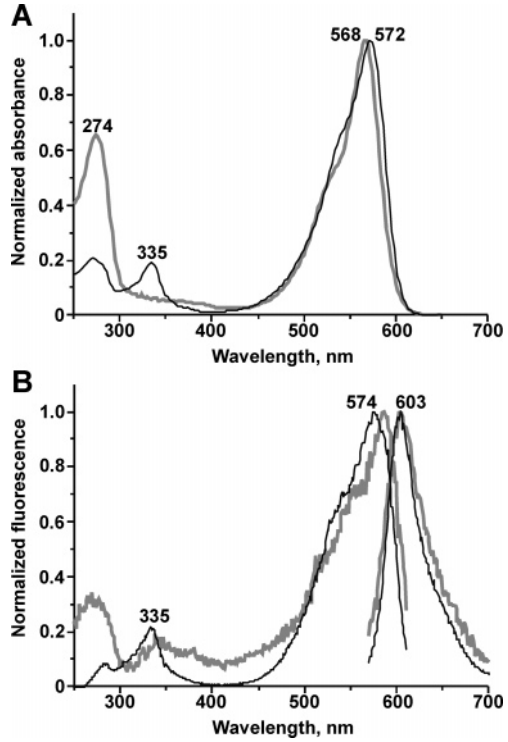


FIGURE 2: Comparison of absorption (A) and excitation−emission (B) spectra of chromophore **4b** in DMF (thin black lines) and native asFP595 (thick gray lines). Within each pair of lines, the emission spectrum is the one at longer wavelengths.

spectra of the synthetic chromophore and the native protein strongly suggests that form **4b** represents the chromophore state within asFP595.

Knowledge of the absorption characteristics of the model chromophore provides an opportunity to estimate the true extinction coefficient of the native proteins carrying this chromophore. Often, extinction coefficients for GFP-like proteins are calculated on the basis of the absorbance of a known amount of the protein (*7*, *19*, *27*, *28*). This method often gives underestimated and poorly reproducible values due to incomplete maturation of the protein, the extent of which depends on the conditions of its expression [for instance, the extinction coefficient for DsRed was variously determined to be 22 500 (*7*), 75 000 (*27*), and 57 000 M^{-1} cm^{-1} (*28*)]. To overcome this problem, one can obtain reliable estimates of the concentration of the mature chromophore in acid- or base-denatured protein samples where chromophore absorption is practically independent of the specific protein environment. Acid-denatured asFP595 possesses an absorbance peak at 430 nm (*20*) which closely resembles that for the model chromophore in acidic 2-propanol. On the basis of the extinction coefficient of this chromophore form (39 000 M^{-1} cm^{-1}; see Table 1), one can estimate the extinction coefficient for native asFP595 to be 150 000 M^{-1} cm^{-1}. This value is much higher than that determined previously (56 000 M^{-1} cm^{-1}) on the basis of the protein concentration (*19*). This underestimate may have resulted from contamination by large amounts of immature asFP595 in the protein sample.

DISCUSSION

Here we present the first synthetic chromophore for a red-shifted protein of the GFP superfamily. The pathway developed is highly efficient, giving a 41% overall yield of final product **4**. The key stage of this synthesis is oxidation of the methylene group at the 2 position of the imidazolone ring with selenium dioxide.

In an earlier publication, the asFP595 chromophore was proposed to possess a six-membered heterocycle (*20*). The structure was elucidated on the basis of a combination of indirect methods. Crystallographic studies of asFP595-A143G have clearly demonstrated this structure to be incorrect (*22*). Apparently, the peak at *m*/*z* 564.6 of the asFP595 chromopeptide detected via MALDI-TOF (*20*) was erroneously attributed to cation radical $[M^{\bullet}]^{+}$. Considering this *m*/*z* value to be a protonated molecular ion, $[M + H]^{+}$, the mass of 563.6 Da of the chromopeptide would exactly correspond to the chromophore structure presented here.

Very recently, another attempt to determine the structure of the chromophore within asFP595 was published (*21*). It was stated that a protein backbone break in asFP595 between the carbonyl C of Cys64 and the amide N of Met65 leads to the presence of a free imino group at the 2 position of the imidazolone core. This conclusion was based primarily on the similarity of the spectrophotometric behavior of these peptides and that of chromopeptides derived from zFP538 (*24*). However, no direct evidence (NMR or MS) for the presence of the proposed unsubstituted ketimine group was presented (*21*, *24*). It is known that N-unsubstituted ketimimes are readily hydrolyzed in aqueous solutions (*29*) and thus would be difficult or impossible to purify under the conditions described in refs *21* and *24*. Further evidence against the proposal for a chromophore with an N-unsubstituted ketimime follows from the chemistry of the acylimine moiety which is the most probable precursor of the mature zFP538 and asFP595 chromophores. Indeed, *N*-acylimines undergo nucleophilic attack (e.g., solvolysis by methanol) at the C═N bond (*30*), which in the case of asFP595 should result in the "oxo" form of the chromophore. Finally, recent crystallographic studies (*18*) gave evidence that the chromophore within native zFP538 contains a GFP core conjugated with a six-membered cyclic imine formed by the ϵ-amino group of Lys66 and the Cα atom of Lys66. The corresponding chromopeptide was detected by Zagranichny et al. but was considered to be an artifact (*24*). Thus, we suggest that the "imino" chromophore proposed in refs *21* and *24* is highly unlikely to be present in either zFP538 or asFP595.

All known chromophore structures within GFP-like proteins include the 4-(*p*-hydroxybenzylidene)-5-imidazolone heterocyclic core. The nature of a substituent at the 2 position of this core plays a dramatic role in protein spectral properties. The asFP595 chromophore carries an additional carbonyl group at this position. As we demonstrate here, a single C═O group conjugated with the GFP core is sufficient to provide a very strong bathochromic shift (compared to the GFP chromophore) of ∼50 and ∼100 nm in absorption spectra for the neutral and anionic forms, respectively. Other known substituents appear to have a smaller impact on spectral properties. For example, the C═N bond in zFP538 provides a modest red shift (*18*). In DsRed-like chromophores, the more substantial bathochromic shift is determined by up two additional double bonds (C═N−C═O group). However, in this case, the available crystal structures (*10*−*13*) indicate that the C═O bond is out of the chro-

mophore plane so that its conjugation with the chromophore and hence the bathochromic shift are reduced from what they might otherwise be. As follows from the spectral properties of the Kaede-like chromophore (*16*, *17*, *31*) and model synthetic compounds (*25*), conjugated C═C bonds have the smallest impact on the red shift.

Compound **4** was found to be unstable at high pHs, in agreement with observations of chromophore instability in alkali-denatured asFP595 (*20*). During base-catalyzed degradation of chromophore **4**, a compound absorbing at 368 nm is formed first (Figure 1B). Apparently, this absorption peak corresponds to the peak at 380 nm in the spectrum for alkali-denatured asFP595 that was earlier erroneously attributed to a reversibly deprotonated chromophore (*20*). We believe that the 368 nm spectral form can be assigned to a product of hydrolysis of the five-membered heterocycle in compound **4**. The peak at 330 nm (Figure 1B) corresponds to a product of further degradation. This compound probably contains a conjugated π-system formed by the phenolic ring plus an additional double bond like *p*-hydroxybenzaldehyde, as this compound has the same absorption maximum (330 nm) at basic pH. Notably, a spectral form absorbing at ~450 nm accumulates during the degradation process. This form becomes clearly distinguishable at the last stages of the process (see curve 8 in Figure 1B) and also can be detected due to a permanent increase in the ratio between absorbance at 450 and 520 nm. Most probably, the 450 nm form represents a GFP-like chromophore structure. However, further studies will be required to determine the exact pathways of asFP595 chromophore degradation.

The positions of the absorption maxima for GFP (*23*) and asFP595 chromophores depend strongly on the nature of the solvent. In particular, the absorption maximum wavelength of anionic form **4b** increased in the following order: water > ethanol > 2-propanol > dimethylformamide (Table 1). We attribute this behavior to solvent protonic acidity rather than solvent polarity as stated by Niwa and co-workers (*23*). We propose that hydrogen bonds between the negatively charged chromophore phenolic oxygen and its surroundings (protein or solvent shell) substantially increase the energy required for excitation, causing the behavior of the anionic chromophore to more closely resemble that of neutral protonated form **4a**.

In DMF, anionic form **4b** demonstrated spectral properties that are similar to those of native asFP595 in terms of shape and peak positions in absorption, excitation, and emission spectra. Thus, we believe this form represents the chromophore state within asFP595. Unexpectedly, we found the fluorescence quantum yield of the free chromophore to be much higher than that of asFP595. The reverse is normally true for fluorescent proteins. For example, the GFP chromophore is essentially nonfluorescent in solution at room temperature but possesses a very high quantum yield within the native protein (*23*). For the free chromophore, it is widely held that photoisomerization is responsible for nonradiative energy dissipation, whereas in GFP, the protein shell restricts chromophore motion. The structural basis for the lack of fluorescence in asFP595 and other GFP-like chromoproteins is at present unclear. However, our data suggest that the protein shell in asFP595 actively favors the nonradiative processes, making the chromophore even less fluorescent than in solution. In turn, this suggests that light absorption, rather than fluorescence emission, is central to the biological function of asFP595.

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REFERENCES

1. Lippincott-Schwartz, J., and Patterson, G. H. (2003) Development and use of fluorescent protein markers in living cells, *Science 300*, 87−91.
2. Heim, R., Prasher, D. C., and Tsien, R. Y. (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein, *Proc. Natl. Acad. Sci. U.S.A. 91*, 12501−12504.
3. Shimomura, O. (1979) Structure of the chromophore of *Aequorea* green fluorescent protein, *FEBS Lett. 104*, 220−222.
4. Cody, C. W., Prasher, D. C., Wastler, W. M., Prendergast, F. G., and Ward, W. W. (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein, *Biochemistry 32*, 1212−1218.
5. Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Crystal structure of the *Aequorea victoria* green fluorescent protein, *Science 273*, 1392−1395.
6. Yang, F., Moss, L. G., and Phillips, G. N., Jr. (1996) The molecular structure of green fluorescent protein, *Nat. Biotechnol. 14*, 1246−1251.
7. Matz, M. V., Fradkov, A. F., Labas, Y. A., Savitsky, A. P., Zaraisky, A. G., Markelov, M. L., and Lukyanov, S. A. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species, *Nat. Biotechnol. 17*, 969−973.
8. Labas, Y. A., Gurskaya, N. G., Yanushevich, Y. G., Fradkov, A. F., Lukyanov, K. A., Lukyanov, S. A., and Matz, M. V. (2002) Diversity and evolution of the green fluorescent protein family, *Proc. Natl. Acad. Sci. U.S.A. 99*, 4256−4261.
9. Gross, L. A., Baird, G. S., Hoffman, R. C., Baldridge, K. K., and Tsien, R. Y. (2000) The structure of the chromophore within DsRed, a red fluorescent protein from coral, *Proc. Natl. Acad. Sci. U.S.A. 97*, 11990−11995.
10. Wall, M. A., Socolich, M., and Ranganathan, R. (2000) The structural basis for red fluorescence in the tetrameric GFP homolog DsRed, *Nat. Struct. Biol. 7*, 1133−1138.
11. Yarbrough, D., Wachter, R. M., Kallio, K., Matz, M. V., and Remington, S. J. (2001) Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-Å resolution, *Proc. Natl. Acad. Sci. U.S.A. 98*, 462−467.
12. Prescott, M., Ling, M., Beddoe, T., Oakley, A. J., Dove, S., Hoegh-Guldberg, O., Devenish, R. J., and Rossjohn, J. (2003) The 2.2-Å crystal structure of a pocilloporin pigment reveals a nonplanar chromophore conformation, *Structure 11*, 275−284.
13. Petersen, J., Wilmann, P. G., Beddoe, T., Oakley, A. J., Devenish, R. J., Prescott, M., and Rossjohn, J. (2003) The 2.0-Å crystal structure of eqFP611, a far red fluorescent protein from the sea anemone *Entacmaea quadricolor*, *J. Biol. Chem. 278*, 44626−44631.
14. Martynov, V. I., Maksimov, B. I., Martynova, N. Y., Pakhomov, A. A., Gurskaya, N. G., and Lukyanov, S. A. (2003) A purple-blue chromoprotein from *Goniopora tenuidens* belongs to the DsRed subfamily of GFP-like proteins, *J. Biol. Chem. 278*, 46288−46292.
15. Shagin, D. A., Barsova, E. V., Yanushevich, Y. G., Fradkov, A. F., Lukyanov, K. A., Labas, Y. A., Semenova, T. N., Ugalde, J. A., Meyers, A., Nunez, J. M., Widder, E. A., Lukyanov, S. A., and Matz, M. V. (2004) GFP-like proteins as ubiquitous metazoan superfamily: Evolution of functional features and structural complexity, *Mol. Biol. Evol. 21*, 841−850.
16. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein, *Proc. Natl. Acad. Sci. U.S.A. 99*, 12651−12656.
17. Mizuno, H., Mal, T. K., Tong, K. I., Ando, R., Furuta, T., Ikura, M., and Miyawaki, A. (2003) Photo-induced peptide cleavage in the green-to-red conversion of a fluorescent protein, *Mol. Cell 12*, 1051−1058.
18. Remington, S. J., Wachter, R. M., Yarbrough, D. K., Branchaud, B., Anderson, D. C., Kallio, K., and Lukyanov, K. A. (2005)

zFP538, a yellow fluorescent protein from *Zoanthus* contains a novel three-ring chromophore, *Biochemistry 44*, 202−212.
19. Lukyanov, K. A., Fradkov, A. F., Gurskaya, N. G., Matz, M. V., Labas, Y. A., Savitsky, A. P., Markelov, M. L., Zaraisky, A. G., Zhao, X., Fang, Y., Tan, W., and Lukyanov, S. A. (2000) Natural animal coloration can be determined by a nonfluorescent green fluorescent protein homolog, *J. Biol. Chem. 275*, 25879−25882.
20. Martynov, V. I., Savitsky, A. P., Martynova, N. Y., Savitsky, P. A., Lukyanov, K. A., and Lukyanov, S. A. (2001) Alternative cyclization in GFP-like proteins family. The formation and structure of the chromophore of a purple chromoprotein from *Anemonia sulcata*, *J. Biol. Chem. 276*, 21012−21016.
21. Zagranichny, V. E., Rudenko, N. V., Gorokhovatsky, A. Yu., Zakharov, M. V., Balashova, T. A., and Arseniev, A. S. (2004) Traditional GFP-type cyclization and unexpected fragmentation site in a purple chromoprotein from *Anemonia sulcata*, asFP595, *Biochemistry 43*, 13598−13603.
22. Quillin, M. L., Anstrom, D., Shu, X., O'Leary, S., Kallio, K., Lukyanov, K. A., and Remington, S. J. (2005) Kindling Fluorescent Protein from *Anemonia sulcata*: Dark-State Structure at 1.38 Å Resolution, *Biochemistry 44*, 5774−5787.
23. Niwa, H., Inouye, S., Hirano, T., Matsuno, T., Kojima, S., Kubota, M., Ohashi, M., and Tsuji, F. I. (1996) Chemical nature of the light emitter of the *Aequorea* green fluorescent protein, *Proc. Natl. Acad. Sci. U.S.A. 93*, 13617−13622.
24. Zagranichny, V. E., Rudenko, N. V., Gorokhovatsky, A. Y., Zakharov, M. V., Shenkarev, Z. O., Balashova, T. A., and Arseniev, A. S. (2004) zFP538, a yellow fluorescent protein from coral, belongs to the DsRed subfamily of GFP-like proteins but possesses the unexpected site of fragmentation, *Biochemistry 43*, 4764−4772.
25. He, X., Bell, A. F., and Tonge, P. J. (2002) Synthesis and spectroscopic studies of model red fluorescent protein chromophores, *Org. Lett. 4*, 1523−1526.
26. Brejc, K., Sixma, T. K., Kitts, P. A., Kain, S. R., Tsien, R. Y., Ormo, M., and Remington, S. J. (1997) Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein, *Proc. Natl. Acad. Sci. U.S.A. 94*, 2306−2311.
27. Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2000) Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral, *Proc. Natl. Acad. Sci. U.S.A. 97*, 11984−11989.
28. Campbell, R. E., Tour, O., Palmer, A. E., Steibach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) A monomeric red fluorescent protein, *Proc. Natl. Acad. Sci. U.S.A. 99*, 7877−7882.
29. Layer, R. W. (1963) The chemistry of imines, *Chem. Rev. 63*, 489−510.
30. Heng Suen, Y., and Kagan, H. B. (1965) Iminomagne'siens. III. Proprie'te's d'acylimines et acyle'namines, *Bull. Soc. Chim. Fr. 5*, 1460−1463.
31. Pakhomov, A. A., Martynova, N. Y., Gurskaya, N. G., Balashova, T. A., Martynov, V. I. (2004) Photoconversion of the chromophore of a fluorescent protein from *Dendronephthya* sp., *Biochemistry (Moscow) 69*, 901−908.

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