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Chromophore Structure of the Kindling Fluorescent Protein asFP595 from *Anemonia sulcata*

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Green fluorescent protein (GFP) and its red-emitting homologues are well-known tools for visualizing intracellular processes in live cells.[1] The red fluorescent protein from *Discosoma* sp. (DsRed) and some of its nonfluorescent homologues (chromoproteins, CPs) contain an acylimine-substituted version of the GFP chromophore, 2-(*N*-acylimino)-4-(*p*-hydroxybenzylidene)-5-imidazolinone (Scheme 1, structure **a**).[2,3] In contrast to DsRed, the fluorescence of photoactivatable proteins can be switched on and off by illumination with light of the appropriate wavelength, allowing selective and direct tracking of tagged objects.[4] The photoactivatable protein asFP595 from *Anemonia sulcata*, often referred to as a "kindling fluorescent protein," has extremely weak fluorescence at 595 nm with a quantum yield $\Phi_{fluor} < 0.001$.[5] However, upon illumination with green light (~570 nm), the protein is activated into a transiently fluorescent state that can be rapidly quenched by illumination with blue light (~450 nm).[6] Unlike proteins of the DsRed subfamily, autocatalytic synthesis of the asFP595 chromophore is accompanied by a polypeptide chain break near the chromophore center (Scheme 1).[7] To fully exploit the potential of photoactivatable proteins, a detailed understanding of the molecular events underlying this phenomenon is essential. However, the mechanism of the chromophore-forming reaction as well as the exact structure of the asFP595 chromophore presently remains obscure.

It has been recently proposed that the asFP595 chain break is the result of an unusual polypeptide cleavage between the nitrogen and carbonyl carbon of the acylimine, which produces an imino-substituted variant of the GFP-like chromophore (Scheme 1, structure **b**).[8] X-ray analysis also revealed a GFP-like structure; however, even at 1.38 Å resolution, it failed to unambiguously distinguish between the nitrogen and oxygen atoms of the chromophore substituent (Scheme 1, structures **b** and **c**, highlighted).[9] Absorbance and fluorescence spectra of the synthesized acetyl-substituted model chromophore in dimethylformamide closely matched those of asFP595.[10]

To solve the controversy regarding the chromophore structure, we have isolated terminal proteolytic peptides adjacent to the asFP595 cleavage site and analyzed them by tandem mass spectrometry. We first tested the stability of the asFP595 chromophore moiety under conditions that were employed in the chromopeptide preparations prior to MS analysis. It was earlier demonstrated that DsRed-like proteins undergo a hypsochromic shift to the GFP-like absorbance (~380 nm) upon denaturation at mild conditions, and this spectral shift is due to H_2O addition across the C═N bond of the chromophore acylimine.[2,3c] Harsher treatments (short time boiling in 0.1 M HCl) result in a complete hydrolysis of the acylimine, splitting DsRed into two fragments and shifting the absorbance maximum to 425 nm.[2,3b] Indeed, upon denaturation at mild conditions (pH 2.8), the absorbance peak at 558 nm of native DsRed instantly shifted to 455 nm and then gradually converted to the GFP-like absorbance peak at 383 nm (Figure 1A). Similar spectral behavior would be expected for the supposed imino-substituted version (Scheme 1, structure **b**) of the asFP595 chromophore. However, under acidic conditions (pH 2.8), the absorbance peak of native asFP595 at 568 nm shifted to 430 nm and did not further change (Figure 1B). These results suggested that, in contrast to DsRed, the asFP595 chromophore does not undergo hydration under acidic conditions.

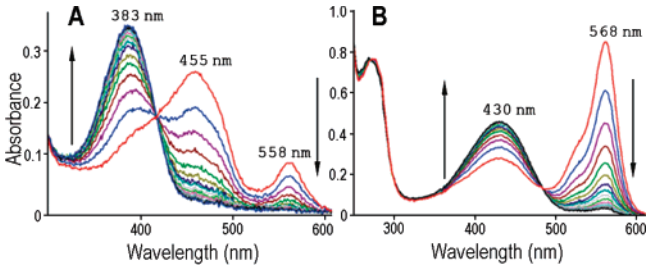
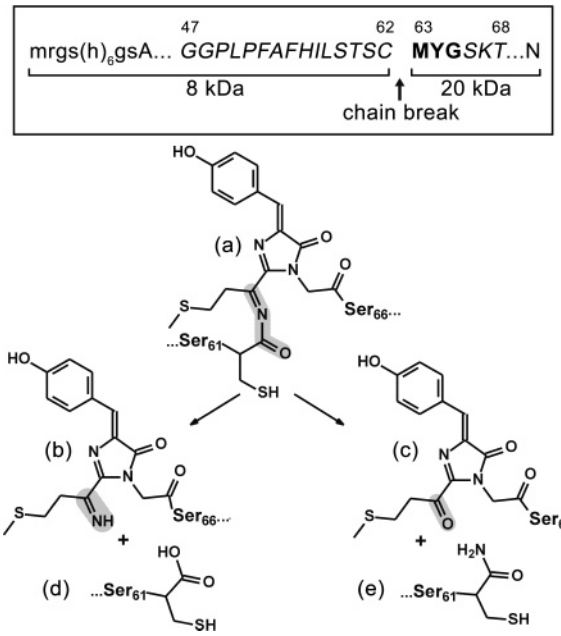


Figure 1. (A) Spectral transitions of DsRed upon denaturation at pH 2.8. Absorbance spectra upon incubation of DsRed at pH 2.8 were recorded in 1 min intervals. (B) Spectral transitions of asFP595 upon denaturation at pH 2.8. Absorbance spectra upon incubation of asFP595 at pH 2.8 were recorded in 2 min intervals.

Scheme 1. asFP595 Polypeptide Chain Break and Proposed Reactions



After denaturation at pH 2.8, wild-type asFP595 was subjected to pepsin digestion, and the digest was separated by reverse-phase HPLC (10 mM sodium phosphate buffer, pH 4.0). The chromophore-bearing peptide fraction that absorbed at 430 nm was collected and analyzed by MALDI-TOF mass spectrometry. The mass spectrum of the chromopeptide contained a major +1 charged peak at 665.2 *m/z*. Further fragmentation of the 665.2 *m/z* ion with

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Table 1. Fragment Ions Detected in Secondary Mass Spectra of the Synthetic and Isolated Wild-Type Peptides *GGPLPFAFHILSTSC*

C-Terminal Ions				N-Terminal Ions			
ion type	*m*/*z* (calcd)[a]	*m*/*z* (synth)[b]	*m*/*z* (w-t)[c]	ion type	*m*/*z* (calcd)[a]	*m*/*z* (synth)[b]	*m*/*z* (w-t)[c]
y3	310.1	310.0	309.0	**a4**	297.1	297.1	297.0
y7	760.4	760.1	759.1	**b6**	569.1	569.1	569.0
y8	907.5	907.3	906.2	**a7**	612.2	612.2	612.0
y9	978.5	978.4	977.3	**b7**	640.2	640.1	640.1
y10	1125.6	1125.4	1124.5	**b9**	924.4	924.3	924.2
y11	1222.6	1222.5	1221.4	**a11**	1122.6	1122.5	1122.5
y13	1432.8	1432.7	1431.6	**b12**	1237.6	1237.6	1237.5
y14	1489.8	1489.6	1488.5	**b13**	1338.7	1338.7	1338.7
y15	1546.8	1546.8	1545.6	**b14**	1425.8	1425.8	1425.7

[a] Fragment *m*/*z* values calculated for the COOH-terminating peptide. [b] The *m*/*z* values observed in mass spectra of the synthetic peptide. [c] The *m*/*z* values observed in mass spectra of the wild-type peptide.

tandem mass spectrometry gave rise to peaks at 546.2, 418.3, and 331.1 *m*/*z* assigned to b_5, b_4, and b_3 ions, which correspond to the loss of the carboxy terminal Thr, Lys-Thr, and Ser-Lys-Thr residues, respectively. Thus the parent 665.2 *m*/*z* ion is consistent with the fragment [chromophore]-Ser-Lys-Thr, in which the chromophore is assumed to derive from the Met-Tyr-Gly sequence and to have a keto-substituted GFP-like structure (Scheme 1, at the top and structure **c**). It has recently been demonstrated that the *N*-acylimino-substituted chromophore of DsRed-like proteins is resistant to hydrolysis at acidic MS conditions.[3b] It is therefore unlikely that the keto derivative detected in mass spectra is the result of a complete hydrolysis of the supposed imino-substituted chromophore upon matrix-assisted laser desorption.

In contrast to the chromophore moiety, the presumptive carboxy or carboxamido terminal groups of the 8 kDa fragment (Scheme 1, at the top and structures **d** and **e**) are expected to be stable products, whatever MS conditions are used. By gel-filtration chromatography on Sephadex G-100, we have isolated the 8 kDa fragment and further digested it by a protease from *S. aureus* strain V8. Purified by HPLC, a C-terminal peptide of the 8 kDa fragment was analyzed by MALDI-TOF. The major peak at 1545.6 *m*/*z* was consistent with a V8 protease cleavage after Glu-46 and a peptide sequence of GGPLPFAFHILSTSC. We have synthesized a peptide with the same sequence containing a COOH group at the C-terminus. Both wild-type and synthetic peptides were subjected to fragmentation in tandem mass spectrometry. Fragment comparison shows that N-terminal ions of the synthetic and wild-type peptides are similar in their *m*/*z* values (Table 1). Conversely, C-terminal ions of the wild-type peptide are 1 *m*/*z* unit lighter than corresponding ions of the synthetic peptide. These results suggest that the carboxamide is a C-terminal group of the 8 kDa fragment. Thus, mass spectral analysis of terminal peptides adjacent to the asFP595 chain break is consistent with the reaction shown in Scheme 1 (right), which results in a carbonyl derivative of a GFP-like chromophore (Scheme 1, structure **c**).

Altogether, the obtained results suggest that the asFP595 chromophore derives from an intermediate DsRed-like structure, and the protein chain break results from hydrolysis of an acylimine substituent of a DsRed-like chromophore. Consequently, this reaction yields a carboxamide terminus on the 8 kDa fragment and a carbonyl-substituted chromophore within the 20 kDa fragment of the protein.

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Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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