Alternative Cyclization in GFP-like Proteins Family.
The Formation and Structure of the Chromophore of a Purple Chromoprotein from
Anemonia sulcata

Vladimir I. Martynov¶, Alexander P. Savitsky§, Natalya Yu. Martynova¶, Pavel A. Savitsky§,
Konstantin A. Lukyanov¶, and Sergey A. Lukyanov¶#

From the ¶Shemiakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, Miklukho-
Maklaya 16/10, 117871 Moscow, Russia; and §Institute of Biochemistry RAS, Leninsky pr. 33,
117071 Moscow, Russia;

#To whom correspondence should be addressed: Shemiakin and Ovchinnikov Institute of
Bioorganic Chemistry RAS, Miklukho-Maklaya 16/10, 117997 Moscow, Russia. Tel./fax: 7
(095) 330-7056; E-mail: luk@ibch.ru

Running title: asFP595 Chromophore Structure

The abbreviations used are: GFP, green fluorescent protein; FP, fluorescent protein; asFP595,
purple chromoprotein from Anemonia sulcata; drFP583 (DsRed), red fluorescent protein from
Discosoma sp.; DNS-Cl, dansyl chloride.
SUMMARY

*Anemonia sulcata* purple protein (asFP595) belongs to a family of GFP-like proteins from *Anthozoa* species. Like GFP, asFP595 apparently forms its chromophore by modifying amino acids within its polypeptide chain. Until now, the GFP-like proteins from *Anthozoa* were thought to contain chromophores with the same imidazolidinone core as GFP. Mass spectral analysis of a chromophore-containing tryptic pentapeptide from asFP595 demonstrates that chromophore formation in asFP595 is stoichiometrically the same as that in GFP: One H₂O and two H⁺ are released as a Schiff base and dehydrotyrosine are formed. However, structural studies of this asFP595 chromopeptide show that, in contrast to GFP, the other peptide bond nitrogen and carbonyl carbon are required for chromophore cyclization—a reaction that yields the six-membered heterocycle 2-(4-hydroxybenzylidene)-6-hydroxy-2,5-dihydropyrazine.

Spectrophotometric titration reveals three pH-dependent forms of the asFP595 chromopeptide: yellow (Abs. max = 430 nm) at pH 3.0, red (Abs. max = 535 nm) at pH 8.0, and colorless (Abs. max = 380 nm) at pH 14.0. The pKₐ’s for these spectral transitions (6.8 and 10.9) are consistent with the ionization of the phenolic group of dehydrotyrosine and deprotonation of the amidinium cation in the chromophore heterocycle, respectively. The amidinium group in asFP595 accounts for the protein’s unique absorption spectrum, which is substantially red-shifted relative to that of GFP. When the asFP595 chromophore cyclizes, the Cys-Met bond adjacent to the chromophore hydrolyzes, splitting the chromoprotein into 8 kDa and 20 kDa fragments. HPLC analysis of a tryptic digest of denatured asFP595 shows that a pentapeptide with the cleaved Cys-Met bond is the only fragment associated with the red-shifted absorbance. These results imply that fragmentation of asFP595 is a critical step in protein maturation.
INTRODUCTION

The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* is a well-known light-emitting protein, which is widely used for visualizing gene expression in living cells and organisms (1-6). The most outstanding feature of GFP is the creation of its chromophore, a self-catalyzed reaction consisting of two main steps: cyclization of the protein backbone at positions 65–67 (Ser-Tyr-Gly) followed by dehydrogenation of the Tyr-66 side chain (7-10). In its final form, the GFP chromophore comprises two cyclic structures. One cycle originates from Tyr-66; the other, a 5-membered heterocycle, forms when the nitrogen of Gly-67 bonds with the carbonyl carbon of Ser-65.

Recently, a number of GFP homologues were cloned from *Anthozoa* species (11-13). As a group, these GFP-like proteins display colors spanning the visible spectrum. Sequence comparisons of all known fluorescent proteins (FPs) reveal that Tyr-66 and Gly-67 (numbering in accordance with GFP) are invariant. The most impressive feature of some of these proteins is a dramatic difference in their absorption maxima. Presumably, there are three plausible explanations for the structural basis of color differences. First, spectral variability could arise from different noncovalent interactions of the chromophore with its environment. Second, GFP-like imidazolidinone structure might be extended by an additional reaction. Third, chromophore formation could occur via an alternative pathway yielding a different heterocyclic structure. Apparently, all three possibilities might be embodied among diverse FPs. Among a number of GFP mutants, for example, spectral diversity results from differences in the way a chromophore interacts with its protein environment (for review see ref. 14). Gross and coworkers have demonstrated that the red emitter drFP583 (DsRed) derives its spectral quality from an additional
autocatalytic dehydrogenation, which extends the chromophore’s system of conjugated \( \pi \)-bonds (15).

Recently, we reported the discovery of a GFP-like purple chromoprotein, asFP595, from the sea anemone *Anemonia sulcata* (13). This protein is naturally nonfluorescent and is responsible for the purple coloration of the anemone tentacles. *In vitro*, asFP595 absorbs light maximally at 572 nm and displays extremely weak red fluorescence at 595 nm (quantum yield < 0.001). We have found that the spectral and biophysical properties of both the native and denatured forms of asFP595 differ markedly from GFP and drFP583. These observations prompted us to investigate the chemical nature of the asFP595 chromophore in order to identify the structural basis for the outlined differences. Our studies show that the asFP595 chromophore forms via an alternative pathway—one that requires protein cleavage in addition to amino acid modification.
EXPERIMENTAL PROCEDURES

Trypsin digestion of asFP595 and purification of the chromopeptide—After dialysis against 10 mM tris-HCl buffer pH 8.0 containing 1 mM EDTA, asFP595 was denatured by acidification to pH 2.3 with dilute HCl. The solution with denatured protein was immediately adjusted to pH 7.8 with dilute NaOH. After addition of trypsin (20% by weight) the suspension was continuously stirred at room temperature for 4 hours. Finally, the clarified digest was adjusted to pH 4.0 and applied to a HPLC column (Beckman Ultrasphere ODS, 4.5 x 250 mm). The column was equilibrated with a starting buffer of 10 mM sodium phosphate pH 4.0, at a flow rate 0.4 ml/min. Protein was eluted with linear gradient of 10 mM sodium phosphate pH 4.0, containing 60% of acetonitrile. The eluent was monitored at 210 nm and 430 nm.

Protein concentration was determined with a Bio-Rad protein assay using bovine serum albumin as a standard. SDS-PAGE analyses routinely employed 15% polacrylamide gels (18) calibrated with low molecular-weight standards from Amersham Pharmacia Biotech Inc. Proteins were transferred to Immobilon P membrane, using a semi-dry Hoefer transfer unit following the protocol provided by PE Biosystems (17). Automated Edman degradation was performed with Applied Biosystems 491 sequenator. Amino acid analyses were done with a Biotronik amino acid analyser LC 3000.

Mass spectra—Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired using a Vision 2000 (Thermo Bio Analyses) mass spectrometer operating in the linear mode. Samples of peptides (10–20 pmole each) prepared by HPLC were directly deposited in the sample well of the MALDI plate using 2,5-dihydroxybenzoic acid as a matrix.
Spectroscopy—Absorption spectra were recorded on a Beckman DU520 UV/VIS Spectrophotometer. A Perkin-Elmer LS50B Fluorescence Spectrophotometer was used for registration of the excitation-emission spectra.

**Carboxypeptidase A treatment**—Reaction was carried out in 0.2 M N-ethylmorpholine acetate buffer pH 8.5 for 4 hours at 37°C. The blotted fragments of asFP595 (30 µg of protein) were excised from Immobilone membrane and incubated with carboxypeptidase A. Finally, the liberated amino acids were identified as dansyl derivatives on thin-layer silica gel plates or on polyamide sheets.
RESULTS

Fragmentation of asFP595—Based on its cDNA sequence (13), monomeric asFP595 should have a mass of 27.2 kDa. However, when we analyzed recombinant asFP595 (expressed by E. coli and purified using metal affinity chromatography) by SDS-PAGE, we detected very little protein at 28 kDa. Instead, most of the protein distributed between two major bands at 20 kDa and 8 kDa (Fig. 1A). After blotting onto PVDF membrane, these two bands were analyzed directly by Edman degradation and by carboxypeptidase digestion. Amino acid sequencing of the 8 kDa band revealed the Met-Arg-Gly-Ser-His(6)-Gly-Ser-Ala sequence, which corresponds to the N-terminal part of the expressed protein (13). Edman degradation of the 20 kDa band provided no sequence information. The 20 kDa band was further analyzed by reaction with dansyl chloride (DNS-Cl). We reasoned that, although the amino acid participating in cyclization of the chromophore may contain a free \( \alpha \)-NH\(_2\) group, the adjoining peptide bond might be somehow modified and therefore resistant to Edman degradation. However, dansylation of the 20 kDa fragment followed by acid hydrolysis failed to reveal an accessible \( \alpha \)-NH\(_2\) group, though intrachain \( \varepsilon \)-Lys and \( \alpha \)-Tyr dansyl derivatives were observed.

Carboxypeptidase A treatment of the blotted 20 kDa band liberated Asn and His as expected for the C-terminus of the protein. Similar analysis of the 8 kDa band showed the presence of Cys, Ser and Thr. Taking into account the molecular mass of 8 kDa, we have found the characteristic Thr-Ser-Cys sequence at positions 62-64 just before the predicted chromophore motive Met-Tyr-Gly.

The above results indicate that the mature asFP595 undergoes fragmentation by splitting at the Cys-Met bond, yielding two of the three bands observed on SDS-PAGE. The minor 28 kDa
band corresponds to the full-length protein, whereas the major 8 kDa and 20 kDa bands correspond to the N- and C-terminal fragments of the protein respectively (Fig. 1B).

Spectral characteristics of denatured asFP595—Under acidic conditions (pH 2.5), asFP595’s absorption peak occurs at 430 nm (not shown). Under alkaline conditions (pH 14.0), the protein’s absorption maximum shifts to an even shorter wavelength, the peak in this case occurring at 380 nm. The pH-dependent spectral shift for asFP595 is the opposite of that observed for GFP. Under acidic conditions, GFP absorbs maximally at 380 nm; under basic conditions, at 445 nm. Prolonged incubation of asFP595 at pH 14.0 led to a decrease in the absorbance at 380 nm, implying that the asFP595 chromophore is unstable and undergoes degradation under alkaline conditions. In contrast, incubation of asFP595 at pH 3.0 at room temperature for several hours did not alter the characteristic 430 nm absorbance.

Spectral behavior of asFP595 trypsin-derived chromopeptide—The absorption spectra (Fig. 2A) of an HPLC-purified chromopeptide (a peptide produced by digesting asFP595 with trypsin) were similar to those for the denatured protein, asFP595. For example, at pH 3.0 the peptide showed an absorption maximum at 430 nm. This peak shifted to 380 nm when the pH was adjusted to 14.0. As the pH increased from pH 3.0 to pH 9.0, the peak, formerly at 430 nm, shifted to 535 nm (Fig. 2A) and the color of the peptide solution changed from yellow to red. This pH-dependent transition showed an isobestic point at 466 nm with a pKₐ of 6.8 (Fig. 3). The yellow to red color shift is reversible; titrating back to pH 3.0 restored the absorbance at 430 nm. For GFP, the pH-dependent shift from 380 to 445 nm is known to be due to ionization of a phenolic group (19), which has a pKₐ of 7.9–8.1 (7, 29). Presumably, the pH-dependent shift from 430 to 535 nm for the asFP595 chromopeptide also corresponds to ionization of a phenolic group. But the relatively lower pKₐ of the asFP595 implies the presence of a stronger electron-
 withdrawing group in the asFP595 chromophore. As the chromopeptide-containing solution was titrated to pH 14.0, the 535 nm absorbance peak shifted to 380 nm as was seen for the denatured asFP595. The pKₐ of this transition was 10.9 (Fig. 3), suggesting that a protonated base is crucial for the chromophore’s structure. This transition is only partially reversible because at pH 14.0 the chromophore gradually falls apart as discussed above.

The red form of the chromopeptide exhibits emission spectra similar to that of asFP595 (Fig. 2B) with the emission peak at 595 nm and excitation maximum at 535 nm. Since the 430 nm (yellow) and 380 nm (colorless) forms of the chromopeptide do not show an appreciable emission at 595 nm, it is reasonable to assume that the structure of the chromophore in the 535nm-absorbing peptide represents the structure of the chromophore in mature asFP595.

**Structural studies of the chromopeptide**—Acid hydrolysis of the chromopeptide yielded 1.8 mol glycine, 1 mol lysine, and 0.6 mol serine. Edman degradation, or reaction with DNS-Cl failed to reveal any accessible N-terminal NH₂-group of the chromopeptide. Carboxypeptidase A treatment of the purified peptide liberated Ser and Lys. Collectively, these results lead us to conclude that the isolated chromopeptide corresponds to the N-terminal pentapeptide from the 20 kDa fragment - Met-Tyr-Gly-Ser-Lys. (Fig. 1B).

With MALDI-time-of-flight mass spectrometry (MALDI/TOF MS), the chromopeptide was detected as a molecular ion at \( m/z = 564.6 \) (The \( M_r \) for the precursor peptide Met-Tyr-Gly-Ser-Lys was calculated to be 584.6 Da.). To confirm that the mass of 564.6 corresponded to the cation radical and not to the protonated molecular ion \([M+H]^+\), MALDI/TOF MS experiments were carried out in both positive and negative ion detection modes and included the synthetic hexapeptide Thr-Gly-Glu-Asn-His-Lys as an internal standard (Fig. 4A & B). The masses of the peptide standard, detected by MALDI/TOF MS in positive and negative ion modes, differed by
2u, a value that corresponds to the difference between the [M+H]^+ and [M-H]^− molecular ions. In contrast, the masses of the chromopeptide differed by only 1u, corresponding to the difference between the M^{++} and [M-H]^− ions. Because the chromophore contains an extended system of conjugated \( \pi \)-electrons, it is reasonable to conclude that the molecule stabilizes the cation radical by delocalizing the charge over the entire \( \pi \) framework. Resonance effects such as these have also been used to explain the stability of charged radicals formed from other polyunsaturated compounds including carotenoids (20), glucuronide metabolites of certain aromatic drug molecules such as indomethacine (21), and highly conjugated textile dyes (22). Comparing the mass of the molecular ion at \( m/z = 564.6 \) with the calculated mass of the corresponding unmodified peptide (584.6 Da), one can conclude that the cyclization reaction of the asFP595 chromophore is stoichiometrically the same as that for the GFP chromophore: One H\(_2\)O and two H^+ are released as a Schiff base and dehydrotyrosine are formed. Since the N-terminal methionine of the chromopeptide does not contain an accessible \( \alpha \)-NH\(_2\) group, a covalent bond to this nitrogen is probably formed during cyclization. Based on the results of the carboxypeptidase A treatment of the chromopeptide, the carbonyl carbons of Ser and Gly remain unmodified. Therefore, the tyrosine carbonyl is the only reasonable candidate for the nucleophilic attack by the methionine NH\(_2\) group (Fig. 5a, b).

The results presented above can be summarized as follows (Fig. 5). After the Schiff base forms, the N-acylamidine bond undergoes hydrolysis (c) cleaving the Cys-Met peptide bond and fragmenting the protein. Cyclization is followed by oxidation of the tyrosine side chain by molecular oxygen (similar to the reaction in GFP [25, 26]), yielding dehydrotyrosine. N,N'-disubstituted amidines usually exist in the two resonance forms shown. Protonation of the imino nitrogen of form c leads to resonance form d and a C=N double bond within the heterocycle.
Amidines are strong bases (23). The amidinium cation is a strong electron withdrawing substituent, which shifts the absorbance of asFP595 to longer wavelengths as compared to GFP. Ionization of the phenolic group of dehydrotyrosine shown in e accounts for the yellow ($\lambda_{\text{max}}$ 430 nm) to red ($\lambda_{\text{max}}$ 535 nm) transition of the chromopeptide. Deprotonation of amidinium cation abolishes the long-wavelength (red) form of the chromophore, yielding the product shown in f, which absorbs at 380 nm.

**Base-catalyzed opening of the chromophore heterocycle**—As discussed above, under alkaline conditions, asFP595 and the chromopeptide each display a 380 nm absorption, which declines over time as the chromophore degrades. We realized that if we could identify the degradation products we could reconstruct the chromophore structure. To this end, the purified chromopeptide was incubated in 1 N NaOH in the dark at room temperature for 24 hours, conditions that normally do not hydrolyze ordinary peptide bonds. Dansylation of the hydrolysis products revealed the presence of methionine, apparently the only amino acid liberated upon decomposition of the chromopeptide (Fig. 5g). Dansylation followed by hydrolysis in 6 N HCl for 8 hours at 105°C showed that tyrosine is the N-terminal amino acid of a newly formed tetrapeptide (Fig. 5g), though a much lower amount of glycine was also detected. Extensive hydrolysis in 6 N HCl for 24 hours followed by dansylation yielded dansyl-derivatives of Gly, Ser, and Lys. It should be mentioned, that extensive acid-catalyzed hydrolysis of dehydrotyrosine yields p-hydroxybenzaldehyde and glycine (7, 8). The conditions we routinely employ for liberating N-terminal residues labeled with DNS-Cl (hydrolysis for 8 hours) might explain why tyrosine, though the predominant, is not the only hydrolysis product: the treatment also liberates a small amount of glycine. Also, it should be noted that we were unable to distinguish dehydro-Tyr from Tyr-dansyl derivatives using thin-layer chromatography.
DISCUSSION

Chromophore formation in asFP595 is unlike that in GFP or any other GFP-like protein studied to date. asFP595 is unique because, after the autocatalyzed cyclization of its chromogenic amino acids, the polypeptide chain splits into two fragments with $M_r$'s of 8 kDa and 20 kDa. Our results suggest that this fragmentation is a critical step in the maturation of asFP595.

The purple protein splits when the Cys64-Met65 bond adjacent to the chromophore is cleaved in an autocatalyzed reaction—the final step in protein maturation. Since all GFP-like proteins, including asFP595, possess an extraordinarily rigid structure, harsh denaturating conditions must be applied to study this phenomenon. Therefore, it was initially unclear to us if the splitting is an inevitable consequence of inadequate treatment of the protein or if fragmentation of asFP595 is a necessary step in protein maturation. To resolve these uncertainties, we optimized the conditions for denaturation and analyzed the chromophore-bearing peptides after proteolytic digestion. We found that alkaline conditions, sufficient for denaturation, lead to irreversible degradation of the asFP595 chromophore. In a mildly acidic solution, the protein denatures but the chromophore is chemically stable. Therefore, acid-induced denaturation followed by a short digestion with trypsin (at a high trypsin to protein ratio) at room temperature was selected as the optimal method for isolating asFP595’s chromophore-bearing peptide.

Recently, Gross et al. (15) showed that the long-wavelength fluorescence of drFP583 can be explained by an additional autocatalytic dehydrogenation, a reaction that extends the system of overlapping $p$ orbitals in the GFP-like chromophore. The dehydrogenation forms an acylimine at the 2$^{\text{nd}}$ position of the 4-(p-hydroxybenzylidene)-5-imidazolone. The acylimine bond exists only in the undenatured protein: Harsh denaturation of drFP583 leads to hydrolysis of the acylamine,
which is located at the Phe64-Gln65 junction (numbering based on GFP)—the same position as the Cys64-Met65 cleavage in asFP595. The hydrolysis splits drFP583 into two fragments and causes the red chromophore to revert to its nascent green form. If the red shifted absorbance of asFP595 had been a property of the full-length protein (as in drFP583), then the characteristic 430 nm absorbance must have been fully associated with the peptide with uncleaved Cys-Met bond. However, fractionation of a trypsin digest of asFP595 by HPLC showed that the 430 nm absorbance distributes with the pentapeptide Met-Tyr-Gly-Ser-Lys. Our structural studies show that this sequence is located at the N-terminus of a 20 kDa fragment produced when the Cys64-Met65 peptide bond breaks in the full-length protein. Thus, these results favor a model in which full-length asFP595 represents an immature form of the purple protein. In this model, fragmentation of asFP595 is a critical step in protein maturation.

The chromophore structure presented here illustrates why fragmentation is crucial for the development of the mature asFP595 chromophore. The pKₐ’s of the N-acyl-N,N’-disubstituted amidines, like b in Fig. 5, are considerably decreased in comparison with unacylated amidines (24). This means the protonated amidinium cation is much more stable in mature (fragmented) asFP595 in which the N-acylamidine bond has been cleaved (Fig. 5c), that explains bathochromic shift upon fragmentation. Noteworthy, since the amidines have resonance forms, both nitrogens of the amidinium cation should lie in the plane of the conjugated system.

According to our data, the chromopeptide from asFP595 does not contain an acylimine bond. (An acylimine bond is responsible for the red-shifted fluorescence of drFP583 [15, 30, 31]). Hydrolysis of acylimine bond would yield an amide of cysteine at the carboxyl terminus of the 8 kDa fragment. Carboxypeptidase A treatment of the 8 kDa fragment liberated cysteine, not cysteine amide. Carboxypeptidase A does not display amidase activity (27) and the absolute
requirement for this enzyme is the presence of an amino acid in the terminal position with a free \(\alpha\)-carboxyl group (28). Base-catalyzed opening of the chromophore heterocycle confirms the above data, showing that methionine \(\alpha\)-nitrogen does not exist in the form of a cysteine amide leaving group, but it is indeed located within chromophore heterocycle.

We interpret these discrepancies to mean that drFP583 and asFP595 mature by different pathways. Indeed, even after prolonged maturation, drFP583 still contains a substantial amount of GFP-like green chromophore (15, 16). asFP595, on the other hand, exhibits no green fluorescence (or GFP-like absorbance) at any time during its maturation. Thus, it is likely that the green GFP-like chromophore in drFP583 is an intermediate in the maturation of red fluorescent protein. And, although this intermediate is a prerequisite for forming the red chromophore, it does not appear to be a part of the pathway that leads to formation of the purple (asFP595) chromophore. This idea is supported by the spectra of the drFP583 and asFP595 denaturation products. When drFP583 is denatured at alkaline pH, it displays a UV-visible absorbance spectrum similar to that of denatured GFP, demonstrating that the drFP583 chromophore is a derivative of imidazolidinone (15). Under the same denaturing conditions, asFP595 yields a product absorbing at 380 nm.

Shortly before this report was published, an unusually small GFP-like chromoprotein asCP562 from *Anemonia sulcata* was described (32). In comparing the predicted amino acid sequence of asCP562 with the known sequence of asFP595 (13), we find that these proteins have an identical stretch of 131 amino acids at their N-termini. Their cDNAs differ by four bases and one frame shift at the position corresponding to the 132-nd amino acid residue (CCCC in asFP595 cDNA versus CCCCC in asCP562 cDNA).

Armed with this information, we decided to study the maturation of truncated asFP595. We generated a pQE30-based clone expressing cDNA identical to the cDNA sequence reported for
asCP562 (32). The shortened protein, however, was completely colorless. In light of this result, we believe that Wiedenmann et al. (32) have misinterpreted the asCP562 length because of a sequencing mistake. Consequently, the observed 19.1 kDa band for asCP562 does not comprise the N-terminal sequence of asFP595, but presumably corresponds to the C-terminal 20 kDa fragment of mature asFP595 (see Fig. 1). Interpreted this way, the results reported by Wiedenmann et al. support our finding that fragmentation plays a critical role in the development of asFP595 red shift. They have shown that only the 19 kDa band exhibits red-shifted fluorescence associated with the native protein.

Acknowledgments—We thank Natalya I. Khoroshilova for the perfect experiments with dansyl derivatives of amino acids, Arkady F. Fradkov for cloning of the truncated asFP595 cDNA, and Alexander S. Arseniev for fruitful discussion. We are grateful to Louis Wollenberger (CLONTECH) and Maria E. Bulina for the help in the manuscript preparation.
REFERENCES


17. User Bulletin (1993) No. 58, PE Biosystems, Foster City CA


FIGURE LEGENDS

**Fig. 1. Fragmentation of asFP595.** A, after expression in *E. coli*, purified asFP595 was analyzed on a 15% SDS-polyacrylamide gel. The 28 kDa band corresponds to full-length protein. The 8 kDa and 20 kDa bands are the N- and C-terminal fragments of the protein, respectively. Protein bands were visualized by staining the gel with Coomassie Blue. Molecular weight standards are shown on the left of the gel. B, Scheme outlining how the asFP595 polypeptide chain splits during maturation. The position of the cleavage is indicated by an arrow. (Residues corresponding to the expression vector backbone are in lowercase.) The amino acid sequence of the chromopeptide is shaded.

**Fig. 2. Absorption and emission spectra of the purified asFP595 tryptic chromopeptide.** A, chromopeptide exists in three pH-dependant forms: yellow (\(\lambda_{\text{max}}\) 430 nm) at pH 3.0 (dashed line), red (\(\lambda_{\text{max}}\) 535 nm) at pH 8.0 (solid line) and colorless (\(\lambda_{\text{max}}\) 380 nm) at pH 14.0 (dotted line). The yellow and colorless forms exhibit the same maxima as the acid- and alkali-denatured asFP595. B, excitation (dotted line) and emission (solid line) spectra of the HPLC-purified chromopeptide of asFP595 in 20 mM tris-HCl buffer containing 150 mM NaCl, pH 8.5.

**Fig. 3. Spectrophotometric titration of the purified asFP595 chromopeptide.** Purified chromopeptide in 10 mM sodium phosphate buffer pH 3.0 was titrated by addition of microliter quantities of dilute NaOH solution. Absorbance of the chromopeptide was monitored at 430 nm (circles), at 535 nm (squares) and at 380 nm (triangles). The measured pK\(_{a}\) of 6.8 corresponds to conversion of the chromopeptide from yellow to red; and pK\(_{a}\) 10.9 corresponds to the conversion from red to colorless.
Fig. 4. MALDI/TOF mass spectra of the chromopeptide. Mass spectra of the purified chromopeptide were acquired in positive (A) and negative (B) ion detection modes with the inclusion of a synthetic hexapeptide Thr-Gly-Glu-Asn-His-Lys (calculated Mr 684.7) as an internal standard. Mass of 587.4 in (A) corresponds to the Na-form of the chromopeptide.

Fig. 5. A complete scheme of the asFP595 chromophore formation, pH-dependant conversions and degradation. The first step in asFP595 chromophore formation is cyclization which is the result of a nucleophilic reaction between the α–nitrogen of Met 65 and carbonyl carbon of Tyr 66 catalyzed by the protein. After cyclization, the N-acylamidine bond (the former peptide bond between Cys 64 and Met 65) hydrolyses resulting in splitting of the protein into 8 kDa and 20 kDa fragments as shown in Fig. 1. The chromopeptide of asFP595 exists in the three pH-dependant forms (absorption maxima are indicated). The determined pKa’s of 6.8 and 10.9 are consistent with ionization of phenolic group of dehydrotyrosine and deprotonation of amidinium cation respectively. The red form of the chromopeptide exhibits the same emission maximum as the native protein (595 nm) suggesting, that this form is a major structure of the chromophore in mature asFP595. The colorless form of the chromophore is unstable and undergoes degradation by hydrolysis at two C=N bonds yielding free methionine and the shortened polypeptide.
Figure 1

A

B

mrgs(h)_{gsASF}.....STSC \text{MYGSKT.....KLGHN}

8 kDa cleavage site 20 kDa
Figure 2
Figure 3
Figure 4
Figure 5