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# Photoconversion of the Chromophore of a Fluorescent Protein from *Dendronephthya* sp.

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**Abstract**—A green fluorescent protein from the coral *Dendronephthya* sp. (Dend FP) is characterized by an irreversible light-dependent conversion to a red-emitting form. The molecular basis of this phenomenon was studied in the present work. Upon UV-irradiation at 366 nm, the absorption maximum of the protein shifted from 494 nm (the green form) to 557 nm (the red form). Concurrently, in the fluorescence spectra the emission maximum shifted from 508 to 575 nm. The green form of native Dend FP was shown to be a dimer, and the oligomerization state of the protein did not change during its conversion to the red form. By contrast, UV-irradiation caused significant intramolecular changes. Unlike the green form, which migrates in SDS-polyacrylamide gels as a single band corresponding to a full-length 28-kD protein, the red form of Dend FP migrated as two fragments of 18- and 10-kD. To determine the chemical basis of these events, the denatured red form of Dend FP was subjected to proteolysis with trypsin. From the resulting hydrolyzate, a chromophore-containing peptide was isolated by HPLC. The structure of the chromophore from the Dend FP red form was established by methods of ESI, tandem mass spectrometry (ESI/MS/MS), and NMR-spectroscopy. The findings suggest that the light-dependent conversion of Dend FP is caused by generation of an additional double bond in the side chain of His65 and a resulting extension of the conjugated system of the green form chromophore. Thus, classified by the chromophore structure, Dend FP should be referred to the Kaede subfamily of GFP-like proteins.

*Key words*: GFP, GFP-like proteins, DsRed, Kaede, fluorescent protein

During the last decade, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been widely used in molecular and cell biology as a transcription reporter, biomarker, or biosensor [1-8]. Such exclusive popularity GFP was acquired due to its unique features: the chromophore is completely encoded as standard DNA triplets of the GFP gene; during the translation, a two-stage autocatalytic assemblage of the chromophore occurs inside the polypeptide chain of the protein [9, 10]. The first stage of this reaction is an autocatalytic cyclization of the polypeptide chain at positions 65-67 (Ser-Tyr-Gly) resulting in an intermediate imidazoline-5-one. During the second stage, the side chain of Tyr66 is dehydrated, which results in a conjugated system of the imidazolidinone heterocycle and phenolic ring of Tyr66. These chemical reactions produce a chromophore center inside the protein and are mainly responsible for its optical properties, although the interaction of the chromophore with the protein environment also causes various spectral features of GFP-like proteins.

About 30 new GFP-like proteins have been recently cloned from different of *Anthozoa* species [11-15]. Some of these proteins are characterized by emission in the red and far-red regions of the spectrum [11, 14]. Studies of this red shift have shown that in some of the proteins the chromophore generation is preceded by three successive reactions. The first two reactions are the same as in GFP (the "green" chromophore is synthesized). The third reaction (an additional autocatalytic dehydrogenation) results in an additional C=N bond in the main chain of the protein that causes a significant shift in the spectra [16, 17]. Note that the last stage of the chromophore synthesis in these proteins is light-independent and seems to additionally need the presence of $O_2$ [16]. Various proteins emitting in the red and far-red regions are found to contain similar chromophores [18-20]. Based on this fea-

*Abbreviations*: GFP) green fluorescent protein from *Aequorea victoria*; DsRed) red fluorescent protein from *Discosoma* sp.; Kaede) fluorescent protein from *Trachyphyllia geoffroyi*; Dend FP) fluorescent protein from *Dendronephthya* sp.; ESI) electrospray ionization; MS/MS) secondary mass-spectra; ESI/MS/MS) tandem mass spectrometry.

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ture, proteins of the GFP family were combined in the DsRed subfamily [20].

The "red" chromophore in the recently described protein from the coral *Trachyphyllia geoffroyi* (Kaede) is generated by quite another mechanism [21, 22]. The green form of this protein is produced similarly to synthesis of the "green" chromophore of GFP, but the conversion to the red form is light-dependent. In the present work, this conversion was studied with a Kaede homologous protein from *Dendronephthya* sp.

## MATERIALS AND METHODS

**Isolation of the recombinant protein.** To provide the expression of Dend FP, the full-length cDNA encoding the amino acid sequence of the protein was cloned in the vector pQE30 (Qiagen, USA). The recombinant protein containing the insertion 6 His in the N-terminal region was expressed in *E. coli* and isolated from the cell lysate by affinity chromatography on Ni-NTA agarose (Qiagen).

**Absorption spectra** were determined with a Cary 50 Bio spectrophotometer (Varian, USA); fluorescence spectra were determined with a Cary Eclipse spectrofluorimeter (Varian).

**SDS electrophoresis** was performed in 15% polyacrylamide gels, which were then stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA).

**Dend FP was photolyzed** using a UV-transilluminator (model CL-215, Ultra-Violet Products, Inc., USA), the source power not indicated.

**Oligomeric structure of the protein was determined by gel-filtration** on a column (0.7 × 60 cm) with Sephadex G-100 (Amersham Pharmacia Biotech., England) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. The column was calibrated with GFP and its homologs with known molecular weights (see "Results").

**Trypsinolysis and isolation of the chromophore-containing peptide.** The purified Dend FP was denatured by addition to the protein solution of NaOH to the final concentration of 0.1 M. The resulting solution of the denatured protein was supplemented with Tris (Sigma, USA) to the concentration of 10 mM and titrated with HCl to the final pH 7.8. To solution of the denatured Dend FP, trypsin (Fluka, USA) was added at the protein/enzyme ratio of 30 : 1 (w/w). The protein was trypsinized at 37°C for 4 h, then the hydrolysis was stopped by titration with acetic acid to pH 4.0. The resulting hydrolyzate was applied onto an HPLC column with reverse phase (Ultrasphere ODS, BD Biosciences, USA) equilibrated with 10 mM sodium phosphate buffer (pH 4.0). Peptides were eluted with a linear gradient of acetonitrile (Merck, Germany) in the same buffer. Peptide fractions were detected simultaneously at 210 and 430 nm with a Variable Wavelength 165 detector (BD Biosciences).

**Mass-spectrometry.** Mass-spectrometry of the chromopeptide was performed with a Thermo Finnigan LCQ deca XP mass-spectrometer equipped with an electrospray source of ionization (ESI) and an "ion trap" analyzer. The peptide was introduced into the source of ionization in the quantity of 5 pM in 0.1% formic acid in water–methanol mixture (50 : 50 v/v) at the rate of 3 μl/min. The working temperature of the capillary was 210°C, the gaseous envelope of the capillary was fixed at 7 units, and the needle potential was 3.2 kV. In MS/MS experiments, the relative energy of collision was 29-35% and the isolation width was 1.2 daltons.

**$^1$H-NMR spectroscopy.** NMR was performed with a Bruker Avance DRX 500 spectrometer. The spectra were recorded at 30°C and pH 5.0. The chromopeptide under study (350 μg) was dissolved in 0.6 ml of 10% $D_2O$. The two-dimensional TOCSY (total correlation spectroscopy) spectrum [23] ($\tau_m$ = 80 msec) was recorded in a phase-selective regime [24]. The strong signal of the solvent was suppressed by the WATERGATE technique [25]. Chemical shifts of $^1$H were determined relatively to that of $H_2O$ (chosen as 4.75 ppm at 30°C with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate). Constants of the spin–spin interaction of protons (H–CC–H) were determined in one-dimensional spectra with numerical resolution of 0.25 Hz per point. NMR spectra were processed using the XWINNMR software (Bruker, Germany).

## RESULTS

**Spectral changes caused by UV-irradiation of the protein.** The recombinant Dend FP purified on Ni-NTA agarose had an absorption spectrum specific for many GFP-like proteins (Fig. 1a) with the maximum at 494 nm and a shoulder at 470 nm. After the protein denaturation caused by 0.1 M HCl (pH 1.8) or by 0.1 M NaOH (pH 14.0), the maximums in the visible spectrum were recorded at 380 and 450 nm, respectively (not shown). Depending on pH, these two forms (380 and 450 nm) of the denatured protein interconverted in the manner characteristic for conversion of the GFP-chromophore caused by dissociation of the phenolic group of Tyr66. On UV-irradiation at 366 nm of the green form of Dend FP, significant changes were recorded in the absorption spectra of the native protein. The absorption of the green form at 494 nm fell, along with appearance of an absorption band at 557 nm with a shoulder at 521 nm (Fig. 1b). This conversion was associated with the visual change in the protein color from green-yellow to red. Changes were also recorded in the absorption spectra of the denatured protein (not shown). Unlike the green form of Dend FP, the red form of the denatured protein was characterized by new pH-dependent forms with maximums at 430 nm (0.1 M HCl) and 500 nm (0.1 M NaOH). These parame-

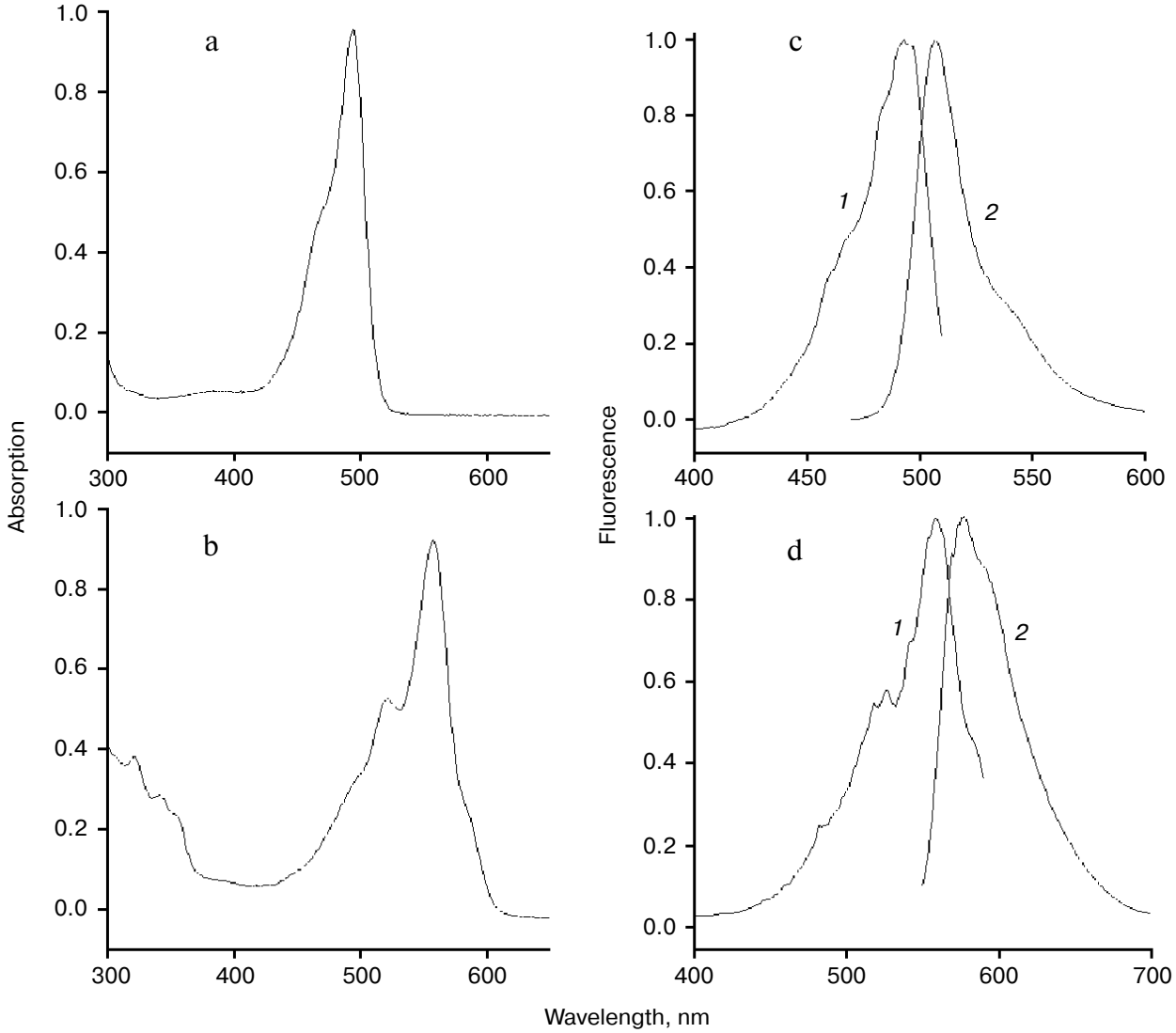


**Fig. 1.** Spectral changes during the photoinduced conversion of Dend FP. Absorption spectra of the green (a) and red (b) forms of the native Dend FP. Normalized spectra of excitation (*1*) and emission (*2*) of the green (c) and red (d) forms of native Dend FP.

ters were used by us later during the isolation to detect the chromophore-containing peptide of the red form of Dend FP. The UV-irradiation also caused significant changes in fluorescence spectra of the native protein. Initially, Dend FP had the emission maximum at 508 nm (Fig. 1c) that correlated with emission in the green region. As a result of the photoinduced conversion, the protein emitted a bright red light with the maximum at 575 nm (Fig. 1d).

**Light-dependent fragmentation of the polypeptide chain of Dend FP.** The green form of Dend FP migrated in SDS-polyacrylamide gels as a single band, which corresponded to the apparent mass of 28 kD characteristic for full-length GFP-like proteins (Fig. 2, lane *1*). However, during UV-irradiation of the protein, the intensity of the 28-kD band decreased. Concurrently, two protein fragments corresponding to the apparent masses of 18 and 10 kD appeared (Fig. 2, lane *2*). Considering the masses of these fragments and also the location of invariant X-Tyr-Gly chromophore-forming amino acids in the polypeptide chain of the protein, it was suggested that the fragmentation of Dend FP should occur due to a break in the polypeptide chain not far from the chromophore center.

**Determination of oligomeric structure of the native protein.** Proteins of the GFP family have a very tightly packed polypeptide chain. And this seems to explain why on calibration of gel-filtration columns with the standard set of proteins bovine serum albumin (67 kD) was eluted in approximately the same volumes of the eluting buffer as the 112-kD protein DsRed of the GFP family. Therefore, to calibrate the columns for determination of oligomerization state of Dend FP, we used homologs of

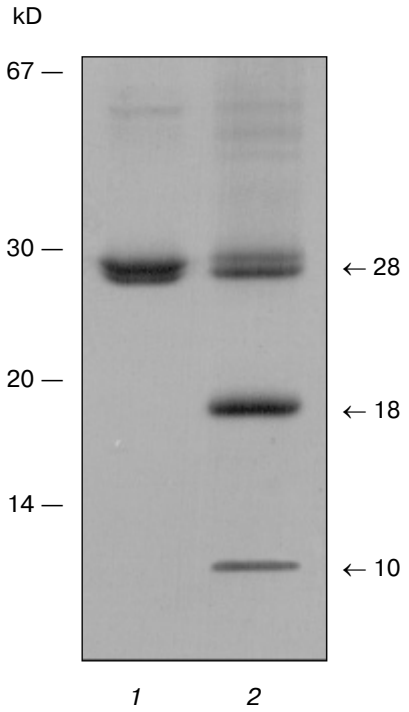


**Fig. 2.** Light-dependent fragmentation of Dend FP detected by SDS electrophoresis in 15% polyacrylamide gels: *1*) green form of Dend FP; *2*) red form of the protein.

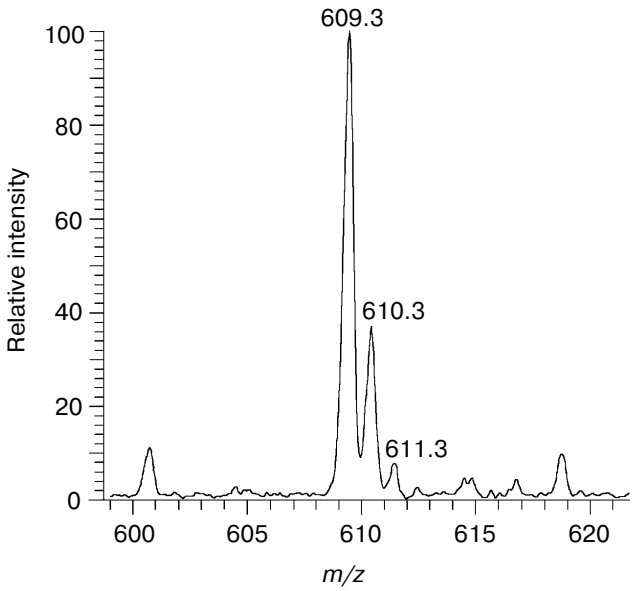


**Fig. 3.** ESI mass-spectrum of the Dend FP chromopeptide. The mass scale (m/z) corresponds to positive single-charged ions $(M + H)^+$.

this protein from the GFP family with the known oligomeric structure: DsRed and GFP (DsRed is a tetramer and GFP from *Aequorea victoria* is a monomer). The weight of the native protein determined by gel filtration was 60 kD for both the green and red form of Dend FP. Thus, the native protein is a dimer, and its oligomerization state does not change during its photoconversion to the red form.

**Isolation of the chromophore-containing peptide of the red form of Dend FP.** The denatured red form of Dend FP was treated with trypsin, and the resulting hydrolyzate of the protein was applied onto a reversed-phase column and separated by HPLC. The fractions were detected concurrently at two wavelengths: 210 and 430 nm. The resulting chromopeptide fraction with absorption at 430 nm was used in studies on the structure.

**Mass spectrometry.** All attempts to determine the N-terminal amino acid sequence by automated amino acid sequencing were unsuccessful. Therefore, it was suggested that the light-dependent conversion of the protein to the red form should result in a break of the polypeptide chain immediately in front of the chromophore center with the accompanying loss of $\alpha$-$NH_2$ by His65. Thus, it seemed that the amino acid sequence of the chromopeptide might be determined only by mass spectrometry. The fraction of the Dend FP chromopeptide isolated by HPLC was analyzed further by ESI mass spectrometry. In ESI mass-spectra a single-charged molecular ion $MH^+$ = 609.3 was found which correlated with the chromopeptide weight of 608.3 daltons (Fig. 3). The calculated weight of the original, unmodified pentapeptide was 645.3 daltons. On comparing these two values (with the difference of 37 daltons), it was suggested that the Dend FP chromopeptide should be a result of an intramolecular cyclization (with the loss of one molecule of $H_2O$) and dehydrogenation (with the loss of $H_2$), just similarly to events in the GFP chromophore. Moreover, the additional difference of 17 daltons seemed to correlate with the loss of $\alpha$-amino group by His65 and the concurrent formation of an additional double bond in the side chain of this amino acid (–1H). Later, all these hypotheses were tested by tandem mass spectrometry and NMR techniques. Secondary (MS/MS) spectra resulting due to fragmentation of the ion of the Dend FP chromopeptide displayed a set of peaks (Fig. 4). There was found among them a peak with m/z of 609.3 which corresponded to the initial molecular ion of the peptide and a number of positive ions with weights corresponding to those of the fragments produced by cleavage of His-Tyr-Gly-Asn-Arg of the polypeptide chain. The assignment of MS/MS peaks resulting from the collision-induced fragmentation of the Dend FP chromopeptide main chain (according to the accepted classification, depending on the break point of the main chain, $a^+$, $b^+$, $c^+$ and $x^+$, $y^+$, $z^+$ are ions, respec-

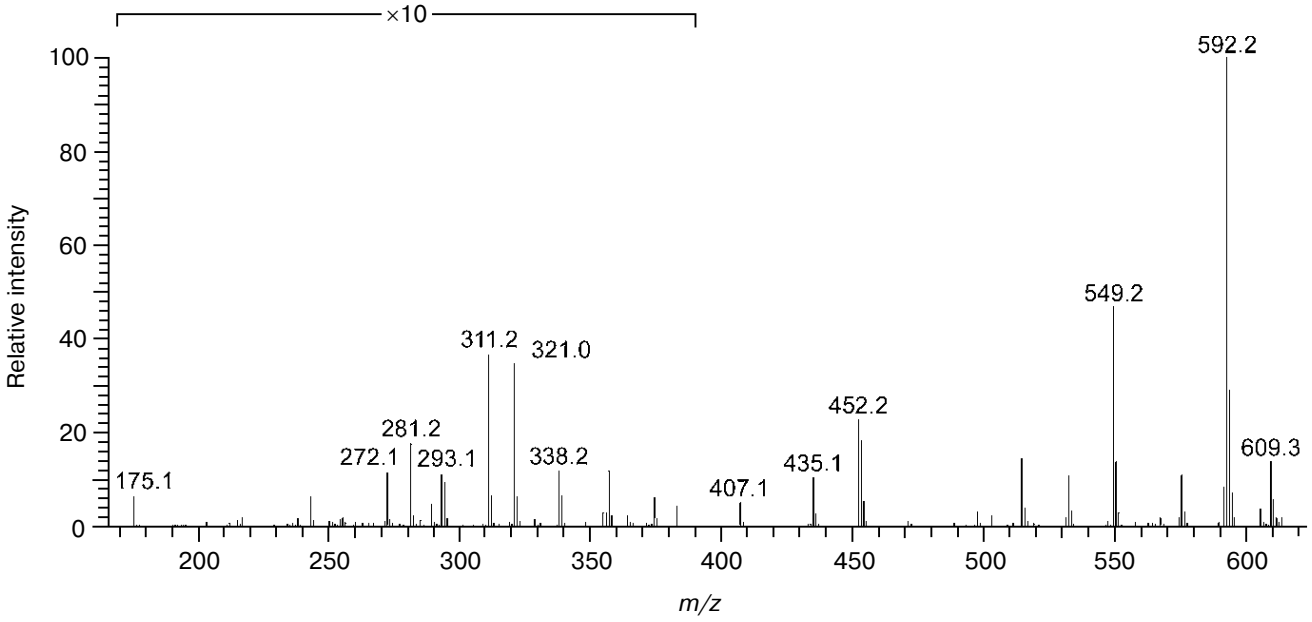


**Fig. 4.** ESI/MS/MS spectrum of the Dend FP chromopeptide.

tively) is given in the table. These findings confirmed the origin of the chromopeptide as a result of intramolecular photolytic cleavage of the bond between the amino group and the α-carbon of His65 and also to tryptic proteolysis of the bond between Arg69 and Val70 of the polypeptide chain of the protein (Fig. 5). The fine structure of the chromophore group was more accurately determined by NMR.

**Study on structure of the Dend FP chromophore by $^1$H-NMR spectroscopy.** In studies on the structure of the Dend FP chromopeptide, proton spin systems were identified with homonuclear one- and two-dimensional TOCSY spectra. Based on analysis of NMR spectra (Fig. 5a), protons of the specimen under study were assigned. In the spectrum five spin systems were identified: two of them were, respectively, -NH-$C_\alpha$H(X)-$C_\beta H_2$-$C_\gamma H_2$-$C_\delta H_2$-NH- (1) and -NH-$C_\alpha$H(X)-$C_\beta H_2$-CO- (2), and three spin systems which included aromatic residues and/or unsaturated bonds, such as **(-**HC=CH**-)**$_2$ (3), -CH=CH- (4), and -CH=X-CH- (5). Spin systems 1 and 2 were Arg residues (NH 8.03 ppm, $^3J_{NH\text{-}C_\alpha H}$ 7.6 Hz; $C_\alpha$H 4.18 ppm; $C_\beta H_2$ 1.65 and 1.80 ppm; $C_\gamma H_2$ 1.46 ppm; $C_\delta H_2$ 3.03 ppm and $N_\varepsilon$H 6.97 ppm; at 6.50 ppm a signal of protons of the guanidine group was recorded) and Asn residues (NH 8.92 ppm, $^3J_{NH\text{-}C_\alpha H}$ 6.6 Hz; $C_\alpha$H 4.7 ppm; $C_\beta H_2$ 2.79 and 2.90 ppm; signals of amide protons of the side chain were recorded at 6.93 and 7.63 ppm). Two doublets of two proton units each (6.99 and 8.12 ppm, $^3$J 8.6 Hz, spin system 3) corresponded to signals of the aromatic ring of the modified Tyr66, a singlet at 7.24 ppm corresponded to its $C_\beta$H at the double bond -$C_\alpha$=$C_\beta$-. Spin system 4 was a fragment -$C_\alpha$H=$C_\beta$H- (6.85 and 7.86 ppm, $^3$J 16.2 Hz) of the modified His65 with *trans*-oriented protons. Two doublets (with J ~ 0.4 Hz) of one proton unit each at 7.71 and 8.47 ppm were signals of C4H and C2H, respectively, of the modified histidine. At 20°C, a sixth spin system was recorded in the spectrum: two signals, each of one proton unit, interacting with each other with J 17.7 Hz. This spin system corresponded to $C_\alpha H_2$ protons of Gly. Based on these findings, the structure of the chromophore-containing peptide Dend FP was proposed (Fig. 5b, compound III).

The main peaks in MS/MS spectra resulting from the collision-induced fragmentation of the Dend FP chromopeptide (relative amplitudes were calculated on the assumption of the peak amplitude of m/z 592.2 $(M\text{-}NH_2)^+$ as 100%)

| *m/z* (observed) | *m/z* (calculated) | Assignment | Relative amplitude |
|---|---|---|---|
| 175.1 | 175.1 | $y_1$ | 0.6 |
| 272.1 | 272.1 | $z_2$ | 1.2 |
| 281.2 | 281.1 | $c_2$ | 1.8 |
| 293.1 | 293.1 | $a_3$ | 1.1 |
| 321.0 | 321.1 | $b_3$ | 3.5 |
| 338.2 | 338.1 | $c_3$ | 1.2 |
| 407.1 | 407.1 | $a_4$ | 5 |
| 435.1 | 435.1 | $b_4$ | 10 |
| 452.2 | 452.2 | $c_4$ | 23 |

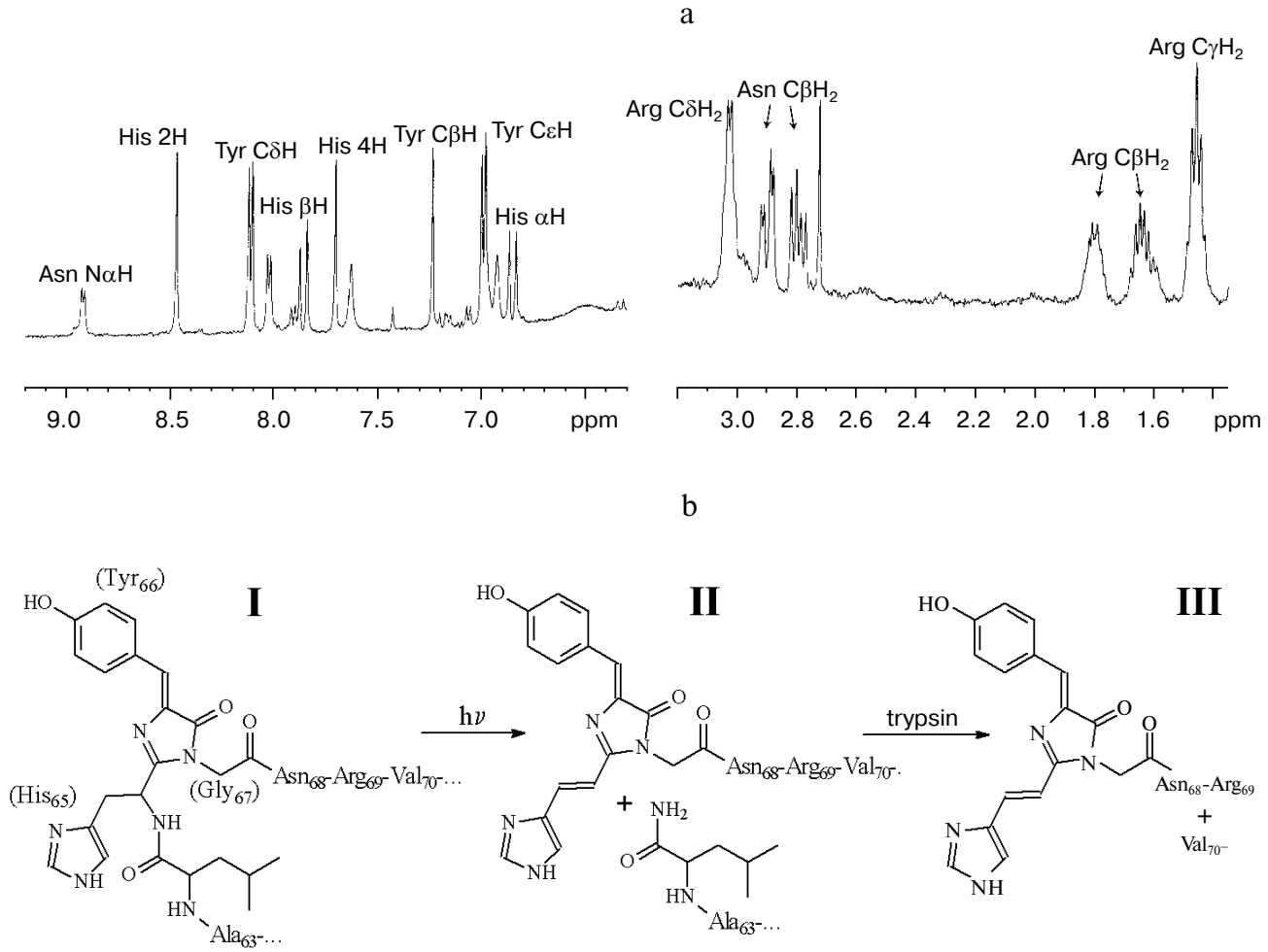


**Fig. 5.** Structure of the chromophore of the red form of Dend FP. a) $^1$H-NMR spectrum of the Dend FP chromopeptide in 10% $D_2O$ at 30°C, pH 4.0. Regions of NH and protons of aromatic/unsaturated bonds (to the left); to the right the region of aliphatic protons is shown. b) General scheme of conversions resulting in formation of the Dend FP chromopeptide.

## DISCUSSION

During recent years, both GFP and GFP-like proteins have been actively used as probes to directly follow processes in the living cell [26]. Optimization of these probes significantly depends on establishment of their structure, which predetermines their optical features. Thus, studies on formation mechanisms and structure of chromophores of GFP-like proteins seem to be of invaluable help for construction of new probes with desirable features. This problem is also of fundamental importance. According to present concepts, all chromoproteins are produced upon combination of the chromophore group (prosthetic group) with the synthesized polypeptide chain of the protein (apoprotein). GFP-like proteins contradict these routine concepts: they are self-sufficing enzymatic machines because they synthesize the chromophore center from amino acids of the polypeptide chain. Therefore, these proteins are extremely interesting as objects of basic research in protein chemistry and chemical enzymology.

A paper [21] published in 2002 presented a description of a protein of the GFP family from the coral *Trachyphyllia geoffroyi* (Kaede), which changed the color of the emitted light after UV-irradiation. In the same work, the unique features of this protein were used for introduction of local intracellular optical labels, in particular, to visualize processes in individual neurons when these neurons were in a primary cell culture of high density. However, the protein from *Trachyphyllia geoffroyi* has significant disadvantages, because, unlike the monomeric GFP, it is a tetramer. In the present work under study were the nature and features of the light-dependent conversion of a Kaede homolog, a protein from the coral *Dendronephthya* sp., which was recently cloned at the Institute of Bioorganic Chemistry, Russian Academy of Sciences [15].

Spectral features of Dend FP are slightly unlike those of the protein Kaede. The absorption spectrum of the green form of Dend FP has the maximum at 494 nm, whereas the maximal absorption of the green form of Kaede is at 508 nm. The emission maximums in the fluorescence spectra are also different: 508 nm for Dend FP and 518 nm for Kaede. The absorption and fluorescence spectra of the red forms of these proteins are also different. The red form of Dend FP has the absorption maximum at 557 nm (572 nm for Kaede) and the emission maximum at 575 nm (582 nm for Kaede). As in all GFP-like proteins, denaturation of Dend FP is accompanied by a hypsochromic shift in the absorption spectra. This effect is caused by disorders in the noncovalent interactions of the chromophore with the side chains of the surrounding amino acids in the native protein. As a result, the contribution of these interactions to the total spectrum in the denatured protein is negligibly small, and the absorption spectrum of the denatured protein rather adequately characterizes the chemical structure of the chromophore itself. The green form of the denatured Dend FP had absorption maximums at 380 nm (pH 1.8) and 450 nm (pH 14.0). On titration, these two forms of the denatured protein with the maximums at 380 and 450 nm interconverted. Based on the specific absorption maximums of these two forms and their pH-dependent conversion, it was suggested that the green form of native Dend FP should contain the chromophore with just the same chemical structure as GFP (*para*-hydroxybenzyliden-5-on-imidazoline). Unlike the green form, the red form of denatured Dend FP had quite other pH-dependent spectral forms with maximums at 430 nm (pH 1.8) and 500 nm (pH 14.0) that indicated significant changes in the chromophore structure during the photoinduced conversion of the protein. Moreover, this feature (the absorption in the visible region at 430 nm) was afterwards used by us for selection of peptides when isolating the chromopeptide by HPLC.

The spectral shift to the red region (compared to the spectra of GFP) in proteins of the DsRed subfamily is known to be due to additional autocatalytic dehydrogenation of the peptide bond immediately in the vicinity of the chromophore [16-20]. Thus, the resulting additional C=N bond extends the conjugated π-electron system of the original GFP-like chromophore. Concurrently, the labile C=N bond is responsible for the partial fragmentation of this subfamily proteins after denaturation, although in the native state they are full-length. A GFP-like protein is also known which is fragmented during maturation, and this fragmentation seems to be important for production of the "red" chromophore [27]. Dend FP was fragmented quite similarly (the same apparent masses of the fragments in SDS/PAGE), and this suggested splitting at the same (or close) bond C=N (in proteins of the DsRed subfamily). However, the light-dependence of the Dend FP fragmentation suggested quite another mechanism of this phenomenon.

Unlike Kaede, which is a tetrameric complex [21], Dend FP is a dimer. All known wild forms of GFP family proteins with emission in the red region of the spectrum are tetramers, and this is disadvantageous for their utilization as optical probes. Note that the light-dependent conversion of Dend FP to the red form is not accompanied by changes in its dimeric structure. Thus, these features of the wild protein are promising for preparation of Dend FP mutants with monomeric structure.

On isolation of the chromophore-containing peptide Dend FP, we considered that many chromophore-bearing structures of GFP family proteins include labile bonds, which can by hydrolyzed during isolation of the chromopeptide. The Dend FP chromopeptide purified by HPLC exhibited the same pH-dependent spectral forms as the protein after denaturation (430 and 500 nm). Thus, it was concluded that the chromopeptide used by us for structural studies retained the intact chemical structure of the chromophore, which was inherent in the native protein.

The secondary MS/MS mass-spectra of the chromopeptide displayed a positive ion specific for the initial chromopeptide, as well as daughter ions, which were fragments resulting from detachment of amino acids Arg and Asn of the C-terminal sequence of the peptide (table). These findings indicated that the chromopeptide was produced due to proteolytic cleavage of the protein by trypsin by the bond of Arg69-Val70 (Fig. 5b). It was reasonable to suggest that photolytic fragmentation of the protein, detected by SDS/PAGE, occurs as a result of a break of the bond between the amino group and α-carbon of His65. In this case, a double bond was suggested to be generated in the side chain of this amino acid with subsequent involvement of the imidazoline nucleus into the total conjugated system of the chromophore (Fig. 5b). The chromopeptide weight of 608.3 daltons determined by ESI mass-spectrometry strictly corresponded to the weight calculated for such a structure of the chromophore. This hypothetic structure was further confirmed by $^1$H-NMR.

When this article was in preparation, data on structure of the chromophore of protein Kaede were published [22]. According to these data, the chromophore centers of Dend FP and Kaede have as a whole similar structure, although chemical shifts of the protons recorded in NMR spectra of the chromopeptides are slightly different. Thus, our findings and the data published recently on the chemical structure of the chromophore allowed us to assign Dend FP and Kaede to the same protein subfamily of the GFP family.

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