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14. ABSTRACT

This report results from a contract tasking Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS as follows: During the past decade, Green Fluorescent Protein (GFP) has become one of the most widely used fluorescent probes that enable direct visualization of the intracellular processes in the living cell. The discovery of the red-emitting GFP-like proteins from Anthozoa species promises considerable broadening of the scope of biochemical applications of these probes. Further rational designing of new red-emitting probes with desired optical properties is tightly associated with understanding of the chemical background of the red shift phenomenon. Recently, three highly homologous green (zFP506), yellow (zFP538) and red (zFP576) fluorescent proteins from Zoanthus sp. were cloned. More recently it was shown, that in contrast to GFP-like chromophore structure of zFP506, the chromophore of the yellow fluorescent protein zFP538 undergoes additional modification of a transiently appearing DsRed-like structure. Our preliminary studies indicate that the red fluorescent protein from Zoanthus sp. zFP576 apparently contains the chromophore differing from both zFP538 and DsRed. The proposed research is aimed at the study of zFP576 chromophore structure.

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**Final
Technical Report of
ISTC 3223**

**THE STRUCTURE OF THE CHROMOPHORE WITHIN A RED
FLUORESCENT PROTEIN FROM ZOANTHUS sp.**

(From 1 June 2005 to 31 May 2006 for 12 months)

**Vladimir Ivanovich Martynov
(Project Manager)
Shemiakin and Ovchinnikov Institute of Bioorganic Chemistry RAS**

May 2006

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**THE STRUCTURE OF THE CHROMOPHORE WITHIN A RED FLUORESCENT
PROTEIN FROM ZOANTHUS sp.****(From 1 June 2005 to 31 May 2006 for 12 months)****Vladimir Ivanovich Martynov
(Project Manager)****Shemiakin and Ovchinnikov Institute of Bioorganic Chemistry RAS****Summary of the project**

The overall objective of this one-year Project was to determine the structure of the chromophore of a red fluorescent protein from *Zoanthus sp.* (zFP576). The chromophore structure of zFP576 was determined by ESI-mass-spectrometry, tandem mass-spectrometry and NMR analysis of the zFP576 chromopeptide. These studies revealed that the red fluorescent protein zFP576 from *Zoanthus sp.* contains a novel modification of the chromophore, which is the result of decarboxylation of Asp 66, the first of the three chromophore-forming amino acids. There were doubts, whether decarboxylation proceeds upon maturation of the wild-type protein, or if it is a result of protein denaturation, which is the necessary step in chromopeptide isolation. To resolve these doubts, the crystal structure of the wild-type zFP576 protein was determined at a 2.4 Å resolution. The crystallographic study supported the MS and MS/MS data obtained for the chromopeptide and showed that decarboxylation of Asp 66 is indeed the post-translational modification of the mature zFP576. Due to Asp 66 decarboxylation zFP576 contains a modified Ala residue at the 66th position. Another GFP-like protein from *Condylactis gigantea* (cgCP) originally contains modified Ala at this position. By tandem mass-spectrometry, ¹H- and ¹³C-NMR of the cgCP chromopeptide we have found that the cgCP protein contains the chromophore of the structure identical to that of zFP576, however, the absorbance of the former protein is shifted far to the red as compared to the latter. Importantly, single A63G (equivalent to the position 66th in zFP576) substitution demonstrated that the nature of the first amino acid of the XYG chromophore-forming sequence is dispensable for the cgCP red shift development. Therefore, unlike zFP576, decarboxylation of the first chromophore-forming amino acid is not necessary for the cgCP red shift acquisition.

Keywords: Anthozoa; *Zoanthus sp.*; coral proteins; fluorescence; fluorescent proteins; GFP-like proteins; DsRed; chromophore; acylimine; decarboxylation.

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Introduction

According to the Work Plan, the first stages of our study were aimed at characterization of the zFP576 chromophore type by spectrophotometric and SDS/PAGE experiments. In the second part, we planned isolating the zFP576 chromophore-bearing peptide and elucidating the chromophore structure by ESI-mass-spectrometry, tandem mass-spectrometry and NMR. Therefore, the overall objective of this one-year Project was to determine the structure of the chromophore of a red fluorescent protein from *Zoanthus sp.*

Results

Task 1. Spectral transition of zFP576 upon denaturation.

The spectral diversity of GFP-like proteins is considered to derive from different types of chromophores involved in a protein structure and the way by which a chromophore interacts with a protein environment. In the denatured state chromophore interactions with aminoacid side chains do not contribute significantly to the overall absorption spectra, so that upon denaturation, the protein visible spectrum reflects the type of implicated chromophore structure. Moreover, some of GFP-like proteins undergo hypsochromic spectral transition to a GFP-like absorbance, which is indicative of reactive “red-shifting” groups, which extend a GFP-like π -electron chromophore structure. Upon heat-induced (100°C) or alkaline denaturation (0.15 M NaOH) the chromophore of zFP576 converted instantly to a “green” form, i.e. absorption spectra of zFP576 were very similar to the denatured GFP with the maxima at 378 nm (pH 3.0) and 451 nm (pH 14.0). Spectrophotometric titration of the “green” form of zFP576 (not shown) indicated the presence of an ionizable group with pKa 8.0, which correlated with the value previously reported for the GFP chromophore phenolic group. Altogether, these data suggested that the zFP576 chromophore shares common features with GFP and contains p-hydroxybenzylideneimidazolinone as a core structure. However, denaturation at acidic conditions (0.1 M HCl) resulted in more complex spectral changes. In this case, absorption peak at 552 nm of the native zFP576 instantly shifted to a 424 nm peak (Fig. 1B), which is proposed to correspond purely to the absorbance of the “red” form of a chromophore of zFP576. Upon incubation, the 424 nm-form further gradually converted to a GFP-like absorption, peaked at 378 nm. Upon denaturation at the same conditions (0.1 M HCl) cgCP also showed hypsochromic spectral transition (Fig. 1A). In the case of cgCP spectral transition upon denaturation was shown recently to be due to reactive acylimine group. However, comparison of spectral behavior of cgCP (Fig. 1A) and zFP576 (Fig. 1B) upon acid-induced denaturation reveals different absorption maxima of the “red” chromophores of cgCP (436 nm) and zFP576 (424 nm). Moreover, there is a pronounced difference in the extinction of the “red” chromophores of cgCP and zFP576.

A highly homologous to zFP576 fluorescent protein zFP506, which emits green light, does not show spectral transition at the same conditions. Upon denaturation in 0.1 M HCl an absorption peak at 493 nm of the native zFP506 immediately shifted to 382 nm and did not further changed upon incubation (Fig. 1C). These results support the assumption that hypsochromic spectral transition of zFP576 is due to a labile chemical group, characteristic for the red chromophore.

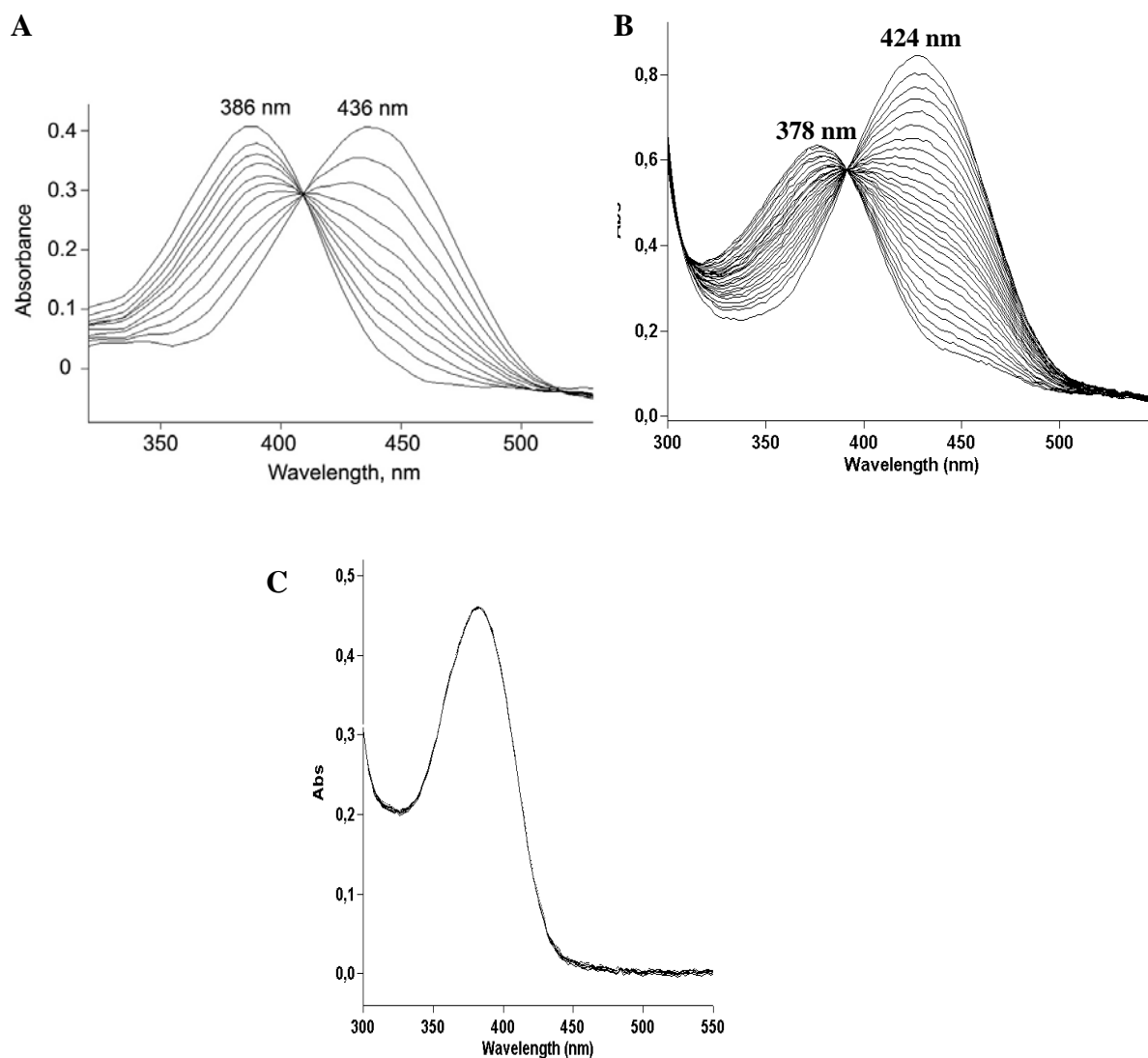


Fig. 1. Spectral behavior of GFP-like proteins upon denaturation in 0.1 M HCl. Hypsochromic spectral transition of cgCP (A) and zFP576 (B). zFP506 does not show spectral transition (C)

Task 2. Fragmentation of zFP576 upon denaturation as determined by SDS/PAGE.

It was shown earlier that GFP migrates as a single band in SDS/PAGE with Mr of 28 kDa. It was demonstrated later, that DsRed partially splits into the fragments, the extent of fragmentation depending on the conditions of denaturation. Later on, asFP595 was shown to exist predominantly in a fragmented form regardless of denaturing conditions. These data suggest that the conditions and the extent of fragmentation are characteristic features for a chromophore type of GFP-like proteins. Fragmentation of DsRed was proposed to occur after the complete hydrolysis of an acylimine, the red-shifting modification of the DsRed chromophore, and does not exceed 50% of the total protein, since mature DsRed contains red and green species in approximately equal ratio.

As expected, DsRed preboiled in 0.1 M HCL (Fig. 2, lane 1) showed appreciable amount of cleaved protein, as detected by SDS/PAGE. The green fluorescent protein zFP506 from *Zoanthus sp.* migrated as a single band either after boiling in the sample buffer or after preboiling in 0.1 M HCL (Fig. 2, lanes 2, 3) suggesting that zFP506 chromophore does not

contain labile chemical groups. The red fluorescent protein zFP576 boiled in the sample buffer migrated predominantly as a single band, indicating that the mature protein originally exists as a single-chain polypeptide (Fig. 2, lane 4). The situation changed when the sample of zFP576 was pretreated with 0.1 M HCL (Fig. 2, lane 5). In this case, zFP576 showed fragment bands of Mr 18 kDa and 10 kDa. However, comparison of fragmentation of DsRed (Fig. 2, lane 1) and zFP576 (Fig. 2, lane 5) revealed the lesser extent of cleavage of zFP576.

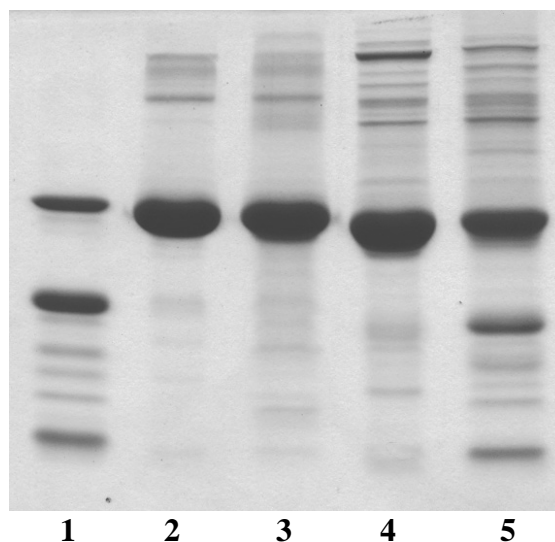


Fig. 2. Fragmentation of the proteins upon denaturation. Lane 1 – DsRed preboiled in 0.1 M HCL followed by boiling in the electrophoresis sample buffer; Lane 2 - zFP506 boiled in the electrophoresis sample buffer; Lane 3 - zFP506 boiled in 0.1 M HCL and electrophoresis buffer; Lane 4 - zFP576 boiled in the electrophoresis buffer; Lane 5 - zFP576 boiled in 0.1 M HCL and electrophoresis buffer.

Altogether, both spectral and SDS/PAGE results are consistent with the presence of a labile chemical group in the zFP576 chromophore structure. However, zFP576 and DsRed-like proteins according to our results differ in absorption maxima and extinction of their chromophore “red” forms and the extent of fragmentation as well.

Task 3. Optimization of the denaturing conditions.

We have shown previously that the zFP576 chromophore contains a chemically labile group and converts to a GFP-like form upon denaturation in 0.1 M HCl (see Report 1, Task 1). Therefore, the present part of work was aimed at the selection of denaturing conditions, if any, upon which the chromophore of zFP576 would remain intact. The characteristic absorption at 424 nm (see Report 1, Task 1) was used as the criterion for the chromophore intactness. zFP576 proved to be extremely resistant to different denaturing agents. The protein retained its native absorption maximum at 552 nm upon prolonged incubation at room temperature in 8M urea, 6M guanidine HCL, 1% sodium dodecyl sulfate or in the pH range between 3 and 12. Other conditions commonly used for protein denaturation also did not change the native absorption spectra of zFP576. For example, organic solvents, such as 50% methanol did not cause denaturation at room temperature. Treatment with pronase did not affect either native absorption properties or mobility of protein in PAAG electrophoresis. On the other hand, either heat-induced, alkaline denaturation or other harsh denaturing conditions resulted in irreversible loss of a red-shifted absorption of zFP576 chromophore.

Since below pH 2, in 0.1 M HCl, the zFP576 chromophore also loses the red-shifting modification and readily converts to a GFP-like form, we further tested the behavior of the protein in the pH range between 2 and 3. In contrast to other GFP-like proteins, within the pH range between 2.5 and 3.0 denaturation of zFP576 was slow and incomplete, as judged by predominant absorbance at 552 nm and a small peak at 424 nm. Below pH 2.5 the peak at 424 nm dominated over 552 nm absorbance, however neutralization of this protein solution caused complete renaturation of the protein as judged by disappearance of 424 nm absorbance and reappearance of 552 nm peak (Fig. 3). This behavior of the protein has limited the proteases choice to those ones that display proteolytic activity at acidic conditions.

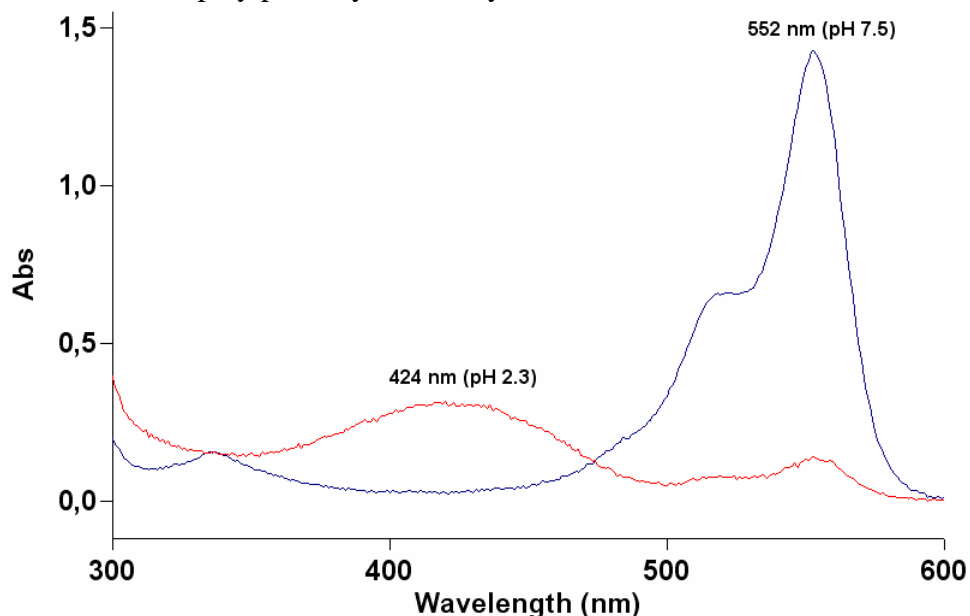


Fig. 3. Denaturation of zFP576 at pH 2.3 and renaturation at pH 7.5

Task 4. Digestion of denatured zFP576 with proteolytic enzymes.

Since the range of choice of enzymes suitable for the generation of 424 nm absorbing peptides of zFP576 was restricted to those ones that exhibit optimum activity at acidic pHs, initially we have chosen pepsin for proteolytic digestion of the denatured protein. Overnight digestion of denatured zFP576 at pH 2.4 (in a 1/20 w/w enzyme to protein ratio) with pepsin did not significantly alter the overall absorbance spectrum of the denatured protein, except for the appearance of a small peak at 380 nm characteristic for the GFP-like chromophore. Generation of the chromophore-bearing peptides and protein digestion was tested by HPLC and SDS/PAGE. SDS/PAGE analysis showed several fragments of masses above 15 kDa, which are probably the result of limited proteolysis of the protein. Several peptides absorbing at 380 nm were obtained after HPLC separation. However, no chromophore-bearing fragments absorbing at 424 nm were detected during HPLC fractionation of the digest. The above results suggested that the 424 nm-form of zFP576 is resistant to pepsin digestion. These results also suggested that only the GFP-like 380 nm-form, which derives from a completely denatured protein, is susceptible to proteolytic digestion.

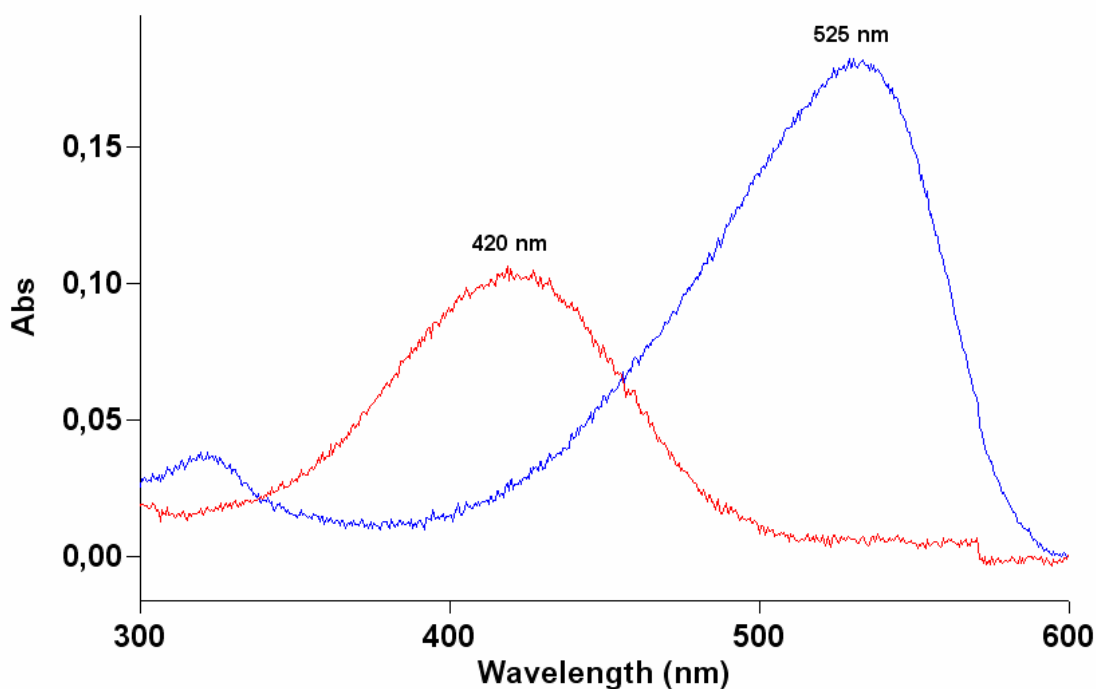


Fig. 4. Hydrolysis of the 380 nm-form of zFP576 after overnight incubation at room temperature in 0.1 M HCl.

We were anxious that below pH 2 (0.1 M HCl), the conditions that lead to complete protein denaturation and conversion to the GFP-like 380 nm-form, zFP576 chromophore labile group(s) might undergo hydrolysis, as is the case for the DsRed-like chromophore. Indeed, overnight incubation of zFP576 in 0.1 M HCl at room temperature gave rise to a product absorbing at 420 nm, which shifted to 525 nm at pH 8.0 (Fig. 4). This behavior was very similar to the absorbance spectral shift of the 2-keto derivative of the DsRed-like chromophore after hydrolysis of the acylimine. Therefore, acidic conditions turned out to be inappropriate for zFP576 denaturation and for generation of chromophore-bearing peptides by pepsin digestion. Eventually, we have chosen basic conditions to minimize hydrolysis. Denaturation of zFP576 was performed at pH 14.0 (0.1 M NaCl). After denaturation (few seconds), protein solution was adjusted to pH 7.8 and chymotrypsin was finally added (enzyme to protein ratio 1/30 w/w). Digestion was performed at room temperature for 24 hours. SDS/PAGE of the chymotryptic digest showed a wide band below 6 kDa. HPLC fractionation of the digest showed one major peak absorbing at 380 nm.

Task 5. Isolation of a chromophore-bearing peptide.

The chymotryptic digest of alkali-denatured zFP576 was adjusted to pH 3.0 by addition of 0.1% acetic acid and subjected to fractionation on Bio-Gel P-2 (0.7x40cm column) in 10 mM sodium phosphate buffer (pH 3.0). Fraction absorbing at 380 nm was further separated by HPLC on a reverse-phase Ultrasphere ODS column (4.5x250 mm) equilibrated with 10 mM sodium phosphate buffer pH 4.0. Peptides were eluted with a linear gradient of acetonitrile. Figure 5 shows a routine HPLC profile of zFP576 digest separation. The chromopeptide fractions were monitored at 380nm. A major 380nm-absorbing chromopeptide was eluted at 24% of acetonitrile. Alternatively, HPLC separation was carried out in 0.05% TFA solution, but this conditions led to multiple peaks and poor yield of the chromopeptide, probably due to chromophore decomposition.

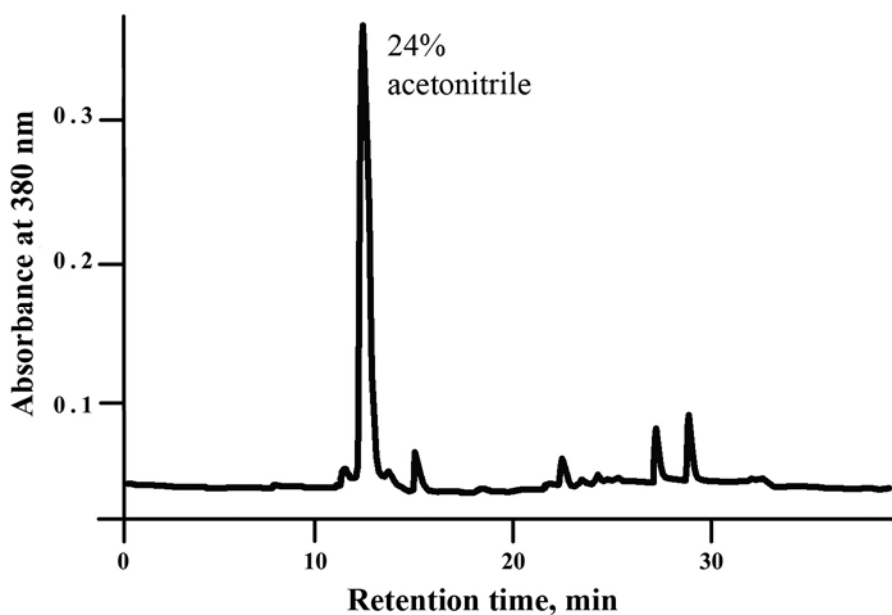


Fig. 5. HPLC separation of 380nm-absorbing peptide species, obtained after zFP576 chymotryptic digest fractionation on Bio-Gel P-2. The chromopeptide fractions were detected at 380nm

Titration of the isolated chromopeptide gave rise to a characteristic 450 nm peak in absorbance spectra at pH 9.0, which shifted back to 378 nm when pH was adjusted to 3.0 (not shown). This pH-dependent transition was very similar to the behavior of a 378 nm-form of denatured zFP576 itself, suggesting that the terminal product of denaturation of zFP576 and the isolated chromopeptide include structurally identical chromophores. By now we have obtained 200 μ g of the HPLC-purified zFP576 chromopeptide for MS, MS/MS and NMR experiments, which are to be carried out in the next quarter according to the Work Plan of the Project.

Task 6. Determination of aminoacid sequence of the chromopeptide

The zFP576 chromophore-bearing peptide derived from extensive chymotryptic digestion was subjected to Edman degradation using Applied Biosystems 491 sequencer. Analysis of the degradation data revealed that Ser-Ala-Ala-Phe - is the N-terminal amino acid sequence of the zFP576 chromopeptide indicating that chymotrypsin cleaves the peptide bond of zFP576 between L 60 and S 61. Acid hydrolysis of the zFP576 chromopeptide followed by amino acid analysis showed the amino acid content of 0.8 mol of serine, 2.5 mol of alanine, 1.8 mol of phenylalanine, 0.3 mol of tyrosine, 1.0 mol of aspartic acid, 0.5 mol of glycine, 0.8 mol of arginine, and 1,1 mol of leucine. Carboxypeptidase treatment revealed that phenylalanine is the C-terminal amino acid of the purified zFP576 chromopeptide. Altogether, the above data suggested that the zFP576 chromopeptide derives from an additional cleavage of the peptide bond between F 71 and T 72, yielding the fragment Ser-Ala-Ala-Phe-Asp-Tyr-Gly-Asn-Arg-Leu-Phe (Fig. 6).

Notably, the isolated chromopeptide contains -Asp-Tyr-Gly- sequence. The sequence -X-Tyr-Gly- has been shown to be involved in the autocatalytic synthesis of the chromophore in GFP-like proteins. The first amino acid X of the chromophore-forming triplet was shown to be variable and the two others are invariant in all known GFP-like proteins.

MAHSKHGLTDDMTMHFRMEGCVDPGHKVFIEGNGNGNPFKKGQFINLVCVIE
 GGPLPFSEDILSAAFDYGNRLFTEYPEGIVDYFKNSCPAGYTWHRSF^{RF}FE
 DGAVCICSADITVNVRENCIYHESTFYGVNFPADGPVMMKMTTNWEPSCE
 KIIPINSQKILKGDVSMYLLLLKDGGRYRCQFDTIYKAKTEPKEMPDWHFI
 QHKLNREDRSDAKNQKWQLIEHAIASRSALP

Fig. 6. The complete amino acid sequence of zFP576 (Yanushevich, Y.G., et al., (2003) *Bioorg. Khim. (Moscow)* 29, 356-360) and the amino acid sequence of the isolated chromopeptide (shaded in grey).

Task 7. ESI mass-spectrometry of the zFP576 chromopeptide.

The zFP576 chromopeptide-containing fraction after HPLC separation was further analyzed by ESI mass-spectrometry. ESI mass-spectra of zFP576 chromopeptide (Fig. 7) contained a +1 charged peak at $m/z = 1194.6$. This value corresponds to the mass of 1193.6 Da of the chromopeptide.

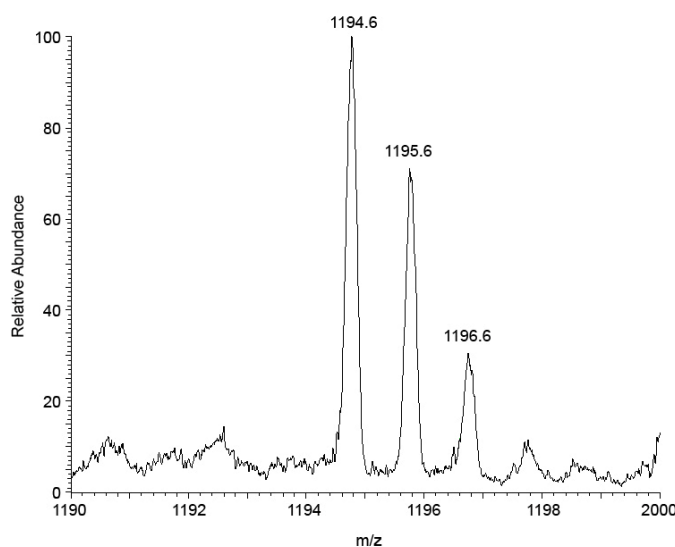


Fig. 7. ESI mass-spectrum of the zFP576 chromopeptide.

The mass of the original unmodified peptide with the sequence Ser-Ala-Ala-Phe-Asp-Tyr-Gly-Asn-Arg-Leu-Phe was calculated to be 1259.6 Da. The comparison of the calculated and experimental masses (the difference is 66 Da) suggested that the zFP576 chromopeptide derives from intramolecular cyclization reaction (the loss of H₂O molecule) and two sites of dehydrogenation (the loss of 2H₂), similar to the chromophore-forming reactions of DsRed. Moreover, an additional difference of 44 Da was tentatively attributed to the loss of CO₂ as a result of proposed Asp-66 decarboxylation. These assumptions were further checked in tandem mass-spectrometry and NMR experiments.

Task 8. Tandem mass-spectrometry and NMR of the isolated zFP576 chromopeptide

Tandem mass-spectra of the zFP576 chromopeptide showed a number of daughter ions. Among them was a peak of m/z 1194.6, which corresponds to the original monocharged chromopeptide ion. Several +1 positively charged ions corresponding to the fragmentation of

Ser-Ala-Ala-Phe-Asp-Tyr-Gly-Asn-Arg-Leu-Phe peptide backbone were detected as well. Table 1 lists the assignments of MS/MS peaks, which are the result of collision-induced fragmentation of the zFP576 chromopeptide main chain (according to the accepted nomenclature and due to the main chain definite cleavage sites, the peaks were assigned to the a⁺, b⁺, c⁺ and x⁺, y⁺, z⁺ ions respectively).

Table 1. The MS/MS major peaks obtained after the collisional-induced fragmentation of zFP576 chromopeptide.

m/z (observed)	m/z (calculated)	Assignment	Relative amplitude
418.1	418.2	a ₅	2
532.1	532.3	z ₄	3
549.1	549.3	y ₅	2
777.2	777.4	x ₆ +2H	9
801.1	801.4	y ₆	100
818.2	818.4	y ₇	24
888.1	888.4	a ₉	2
916.1	916.4	b ₉	20
965.2	965.4	y ₈	3
1001.2	1001.5	a ₁₀	5
1029.2	1029.5	b ₁₀	23
1036.2	1036.5	y ₉	4
1046.3	1046.5	c ₁₀	7
1176.3	1176.6	b ₁₁	23
1177.3	1177.6	y ₁₁	33

The obtained MS/MS data indicated that the zFP576 chromopeptide indeed derives from cyclization and oxidation reactions, just as in the case of GFP, one additional dehydrogenation site between nitrogen and α -carbon of Asp 66, as in DsRed chromophore, and a novel modification of the chromophore, - the decarboxylated side chain of Asp 66 (Fig. 8).

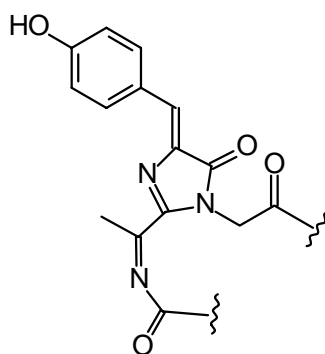


Fig. 8. Proposed structure of the zFP576 chromophore as deduced from MS and MS/MS experiments.

The chymotrypsin-derived zFP576 chromopeptide was further investigated by $^1\text{H-NMR}$ spectroscopy. For proton spin systems identification of the zFP576 chromopeptide we used homonuclear one-dimensional TOCSY (total correlation) spectroscopy. The chromopeptide (350 μg) was dissolved in 0.6 ml of H_2O or D_2O . Initially, the exchangeable with the solvent amide protons were identified by spectral comparison of samples dissolved in $\text{H}_2\text{O}/\text{D}_2\text{O}$. Afterwards, 1D homonuclear TOCSY spectra were used for identification of the amino acid ^1H - spin systems.

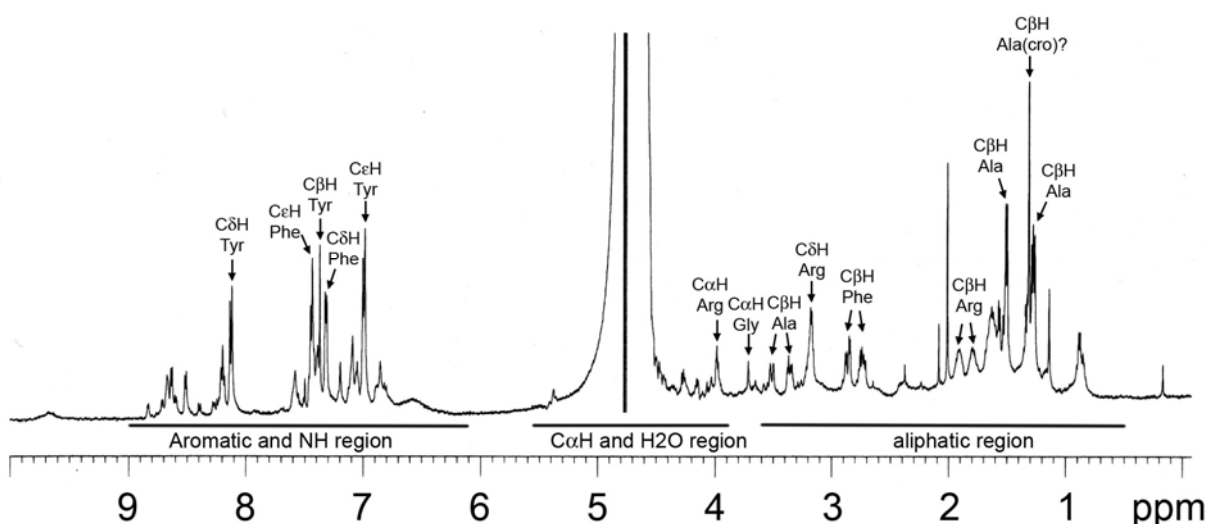


Fig. 9. ^1H NMR spectrum of the zFP576 chromopeptide.

The aromatic region of the $^1\text{H-NMR}$ spectrum revealed the signals of the chromophore Tyr-67 δ - and ϵ -protons at 8.14 and 7.0 ppm respectively. The peak at 7.4 ppm was attributed to the single β -proton of Tyr-67 vinyl group. The aliphatic region of the $^1\text{H-NMR}$ spectrum was not of a good quality, therefore, we could draw only tentative assignments shown in Fig. 9. A singlet of three proton units at 1.3 ppm was attributed to the CH_3 -group, the decarboxylation product of Asp-66 side chain.

The crystal structure of zFP576.

By structural analysis of the zFP576 chromopeptide we have determined, that the side chain of Asp 66 is decarboxylated. However, there were doubts, whether decarboxylation proceeds within a native protein upon maturation, or if it is a result of protein denaturation, which is a necessary step before chromopeptide isolation. To resolve these doubts, the crystal structure of

the wild-type zFP576 protein was determined at a 2.4 Å resolution. The crystal structure, refined to a crystallographic R factor of 0.203 (R_{free} = 0.249), adopted the β-barrel fold composed of 11 strands similar to that of all GFP-like proteins. The zFP576 chromophore, originating from the protein sequence Asp66-Tyr67-Gly68, exhibited a two-ring structure typical of GFP-like proteins. The bond geometry of the residue 66 showed the strong tendency of the corresponding C_α atom to *sp*² hybridization as a consequence of N-acylimine bond formation. The zFP576 chromophore contained the 65–66 *cis*-peptide bond characteristic of the red fluorescent proteins. The chromophore phenolic ring adopted a *cis*-conformation coplanar with the imidazolinone ring. The crystallographic study supported MS and MS/MS data and also showed that the side chain of the chromophore-forming residue Asp66 is decarboxylated (Fig. 10).

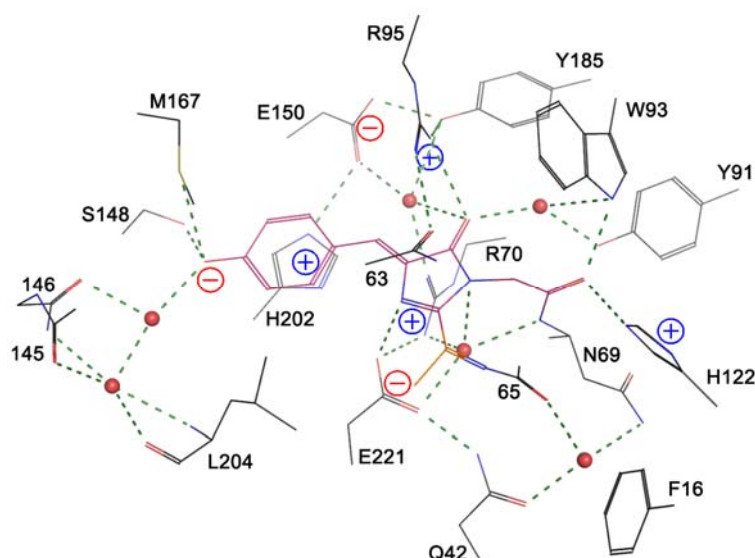


Fig. 10. The chromophore and its environments in the wild-type red fluorescent protein zFP576. The zFP576 chromophore (in the center) environments include a number of charges that form a network of salt-bridges and hydrogen-bond interactions (dashed lines). The residues located above and below the chromophore plane are shown in black and grey respectively. The red balls represent the water molecules. The remnant (the result of decarboxylation) of the Asp 66 side chain is shown in orange. Adapted from Pletneva N., Pletnev S., Tikhonova T., Popov V., Martynov V., and Pletnev V., (2006) *Crystal structure of a red fluorescent protein from Zoanthus, zRFP574, reveals a novel chromophore, Acta Crystallogr D Biol Crystallogr. D62, 527-532.*

Determination of the structure and reactivity of the red chromophore within a GFP-like chromoprotein from *Condylactis gigantea*

The GFP-like chromoprotein from *Condylactis gigantea* (cgCP) contains Ala at the first position of the XYG sequence, which includes the chromophore-forming amino acids. zFP576 also contains Ala at this position, as the result of Asp 66 decarboxylation (Fig. 8). By tandem mass-spectrometry, ¹H- and ¹³C-NMR of the cgCP chromopeptide we have found that the cgCP protein contains the chromophore of the structure identical to zFP576, however the absorbance maximum of the former wild type protein is shifted far to the red as compared to the latter. Interestingly, single A63G (amino acid X) substitution in cgCP demonstrated that the nature of the first amino acid of the XYG chromophore-forming sequence is dispensable for the red shift development. Therefore, unlike zFP576, decarboxylation of the first chromophore-forming amino acid is not necessary for the cgCP red shift acquisition. We have shown that, similar to zFP576, cgCP suffers a hypsochromic spectral shift to the GFP-like absorbance (386 nm) upon mild denaturation. NMR analysis of the cgCP chromopeptide suggested this hypsochromic spectral shift to be due to H₂O addition across the C=N bond of the acylimine. However, unlike DsRed, denatured at harsh conditions wild type cgCP showed only slight fragmentation, which is

induced by complete hydrolysis of the acylimine. We have explored the influence of the amino acid X side chain on cgCP fragmentation. Substitutions at this position of cgCP suggested that glutamine promotes fragmentation, probably due to an intermediate cyclic structure, which is the result of a reaction of the side chain amido group and the acylimine C=N carbon. Altogether, our findings suggested that the acylimine group within the cgCP chromophore conforms to the chemistry of nucleophilic addition to the C=N bond.

Concluding remarks

According to the Work Plan of the Project, the chromophore structure of a red fluorescent protein from *Zoanthus sp.* was determined by ESI-mass-spectrometry, tandem mass-spectrometry and NMR of the isolated chromopeptide. This structure was supported by crystallographic studies of the protein. Combined, these experiments revealed that the red fluorescent protein from *Zoanthus sp.* contains a novel modification of the chromophore, which is the result of post-translational decarboxylation of Asp 66, the first of the three chromophore-forming amino acids. This additional post-translational modification is likely to play a key role in the bathochromic shift of zFP576. Presently, the mechanism of decarboxylation is obscure, however we anticipate that the mechanistic pathways of red shift acquisition by DsRed and zFP576 are different.

List of published papers and reports with abstracts

1. Pakhomov A.A., Pletneva N.V., Balashova T.A., and Martynov V.I. (2006) Structure and reactivity of the chromophore of a GFP-like chromoprotein from *Condylactis gigantea*, *Biochemistry* **45**, 7256-7264

Biochemistry, ASAP Article 10.1021/bi060207q S0006-2960(06)00207-8

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Structure and Reactivity of the Chromophore of a GFP-like Chromoprotein from *Condylactis gigantea*

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Abstract:

Here we present the study of the chromophore structure of the purple chromoprotein from *Condylactis gigantea*. Tandem mass spectrometry and ^1H and ^{13}C NMR of the chromopeptide reveal that the protein contains a chromophore with a chemical structure identical to that of the red fluorescent protein from *Discosoma* sp. A single A63G substitution demonstrates that the nature of the first amino acid of the XYG chromophore-forming sequence is dispensable for the chromoprotein red shift development. It has been recently proposed that post-translational reactions at the acylimine, a chemical group that accounts for the red fluorescence, might be an additional source of spectral diversity of proteins homologous to the *Aequorea victoria* green fluorescent protein (GFP). We have examined the reactivity of the chromophore acylimine group within the *C. gigantea* purple chromoprotein. Like other proteins with the acylimine-modified chromophore, the purple chromoprotein suffers a hypsochromic spectral shift to the GFP-like absorbance (386 nm) upon mild denaturation. NMR analysis of the chromopeptide suggests this hypsochromic spectral shift is due to H_2O addition across the $\text{C}=\text{N}$ bond of the acylimine. However, unlike the red fluorescent protein from *Discosoma* sp., denatured under harsh conditions, the wild-type chromoprotein exhibits only slight fragmentation, which is induced by complete hydrolysis of the acylimine. A model suggesting the influence of the amino acid X side chain on protein fragmentation is presented.

2. Pletneva N., Pletnev S., Tikhonova T., Popov V., Martynov V., and Pletnev V., (2006) Crystal structure of a red fluorescent protein from *Zoanthus*, zRFP574, reveals a novel chromophore, *Acta Crystallogr D Biol Crystallogr.* **D62**, 527-532.

Structure of a red fluorescent protein from *Zoanthus*, zRFP574, reveals a novel chromophore

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The three-dimensional structure of the red fluorescent protein (RFP) zRFP574 from the button polyp *Zoanthus* sp. (two dimers per asymmetric unit, 231 ± 4 amino acids) has been determined at 2.4 Å resolution in space group C2221. The crystal structure, refined to a crystallographic R factor of 0.203 (R_{free} = 0.249), adopts the α -barrel fold composed of 11 strands similar to that of the yellow fluorescent protein zYFP538. The zRFP574 chromophore, originating from the protein sequence Asp66-Tyr67-Gly68, has a two-ring structure typical of GFP-like proteins. The bond geometry of residue 66 shows the strong tendency of the corresponding C α atom to sp² hybridization as a consequence of N-acylimine bond formation. The zRFP574 chromophore contains the 65–66 cis peptide bond characteristic of red fluorescent proteins. The chromophore phenolic ring adopts a cis conformation coplanar with the imidazolinone ring. The crystallographic study has revealed an unexpected chemical feature of the internal chromophore. A decarboxylated side chain of the chromophore-forming residue Asp66 has been observed in the structure. This additional post-translational modification is likely to play a key role in the bathochromic shift of the zRFP574 spectrum.

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List of presentations at conferences and meetings with abstracts

1. Pakhomov A.A., Pletneva N.V., Martynov V.I. The molecular basis for the green to red conversion of the fluorescent protein from *Dendronephthya* sp. The 30th FEBS Congress and 9th IUBMB Conference "The Protein World", Budapest, Hungary 2-7 July 2005, The abstract is published in *FEBS Journal* (2005) **272**, Supplement 1, p. 384.

The molecular basis for the green to red conversion of the fluorescent protein from *Dendronephthya* sp.

Abstract number: G2-089P

Pakhomov A. A., Pletneva N. V., Martynov V. I.

The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* and its homologs from corals have a broad application in biotechnology as visual reporters for events in living cells. Their applicability in cellular biology is due to autocatalytic synthesis of the chromophore from amino acids inside the protein shell. Thus, these reporters do not require any external agents for the fluorescence appearance. In general the chromophore structure defines spectral properties of the protein whereas alterations in the chromophore environment provides the shift of absorption/emission maxima of no more than some tens of nanometers. In this work we determined the molecular basis of green (emission maximum at 504 nm) to red (emission maximum at 575 nm) conversion of the fluorescent protein from the coral *Dendronephthya* sp. (DendFP) under UV-irradiation. UV-illumination causes fragmentation of the polypeptide backbone of DendFP, as seen in SDS-PAGE gels. To clarify the chemical nature of this phenomenon DendFP was subjected to proteolytic digestion with trypsin. The chromophore-containing peptide was isolated from the tryptic digest by HPLC. The structure of the chromopeptide containing the "red" chromophore was determined by ESI, ESI/MS/MS mass-spectrometry and NMR. The data obtained suggest that the photoinduced green-to-red conversion results in the cleavage of Dend FP polypeptide chain between Leu 64 and His 65 and double bond formation in the side chain of His 65. Consequently, the red shift is explained by the extended p-electron system, which involves the newly formed C=C double bond and imidazole group of His 65. Recently, the analogous photoconversion was found in a fluorescent protein from *Trachyphyllia geoffroyi* (Kaede). Therefore, DendFP can be attributed to the Kaede subfamily of GFP-like proteins in the classification based on the chromophore structure.

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