ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 3, pp. 250-259. © Pleiades Publishing, Ltd., 2009.
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MINI-REVIEW

Posttranslational Chemistry of Proteins of the GFP Family

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Received September 12, 2008
Revision received October 28, 2008

Abstract—This review focuses on the current knowledge about posttranslational chemistry underlying the diverse optical properties of GFP-like proteins.

DOI: 10.1134/S000629790903002X

Key words: posttranslational modifications, fluorescence, chromophore, GFP, DsRed, KFP, Kaede

Posttranslational modifications (PTM) are one of the most important ways of regulating the biological activity of proteins and enzymes. The following three main PTM categories can be distinguished by the type of reactions: proteolytic cleavage of the polypeptide chain (removal of signal sequences, protein sequences determining its intracellular localization, autocatalytic cleavage of inteins), attachment of a non-protein chemical group (phosphorylation, glycosylation, prenylation, etc.), and formation of inter- and intramolecular bonds (disulfide bonds, ligation of exteins, etc.). PTM occur with the involvement of specialized enzymes performing polypeptide processing and due to autocatalysis. All known protein modifications are present in the RESID database (http://www.ncifcrf.gov/RESID/) [1]. Some enzymes do not need exogenous cofactors and use cofactors that are derivatives of protein structures obtained as a result of PTM [2]. The latter group includes proteins of the GFP family, the chromophore of which is synthesized autocatalytically due to posttranslational reactions that do not require exogenous cofactors except for molecular oxygen [3]. Thus, autocatalytic PTM of GFP-like proteins underlie their unique ability for self-tuning to a certain spectral range [4]. This property is used in the modern molecular and cell biology for the labeling of cell structures [5].

The first representative of the GFP family, green fluorescent protein, was found in 1961 in photocytes of the bioluminescent jellyfish *Aequorea victoria* [6] belonging to the class Hydrozoa. In 1999, the genes of GFP-homologous proteins with fluorescence in the longer-wavelength spectral region were cloned from non-luminescent corals of the class Anthozoa [7]. In spite of a rather distant homology of primary structures (~20%), fluorescent proteins from the corals have a considerable homology of tertiary structures. Like GFP, the molecules of the homologous proteins are shaped as a cylinder with walls formed of 11 segments of β-folded structures. The α-helix containing the chromogenic sequence of -Xxx-Tyr-Gly- amino acids passes through the central axis of this cylinder. After the cylinder structure has been formed, the protein enters the phase of maturation characterized by autocatalytic reactions within the -Xxx-Tyr-Gly- sequence, which lead to chromophore synthesis and determine the fluorescent properties of the protein. These reactions result in the formation in GFP of a chromophore, i.e. 4-(*p*-hydroxybenzylidene)imidazolid-5-one (*p*-HBI), which is spatially located in the center of the cylinder (Scheme 1, structure VI). The *p*-HBI is also the initial compound at further synthesis of chromophores of homologous proteins from the corals. In the latter case, *p*-HBI undergoes additional PTM, which introduce changes into its conjugated π-electron system.

In the recent years much experimental material has accumulated promoting deeper insight into the biochem-

Abbreviations: asFP595, photoactivatable protein from *Anemonia sulcata*; avGFP, green fluorescent protein from *Aequorea victoria*; DendFP, fluorescent protein from *Dendronephthya* sp.; DsRed, red fluorescent protein from *Discosoma* sp.; EosFP, fluorescent protein from *Lobophyllia hemprichii*; *p*-HBI, 4-(*p*-hydroxybenzylidene)imidazolid-5-one; Kaede, fluorescent protein from *Trachyphyllia geoffroyi*; KikG, fluorescent protein from *Favia favus*; PTM, posttranslational modifications; zFP538, yellow fluorescent protein form *Zoanthus* sp.; z2FP574, red fluorescent protein from *Zoanthus* sp. 2.

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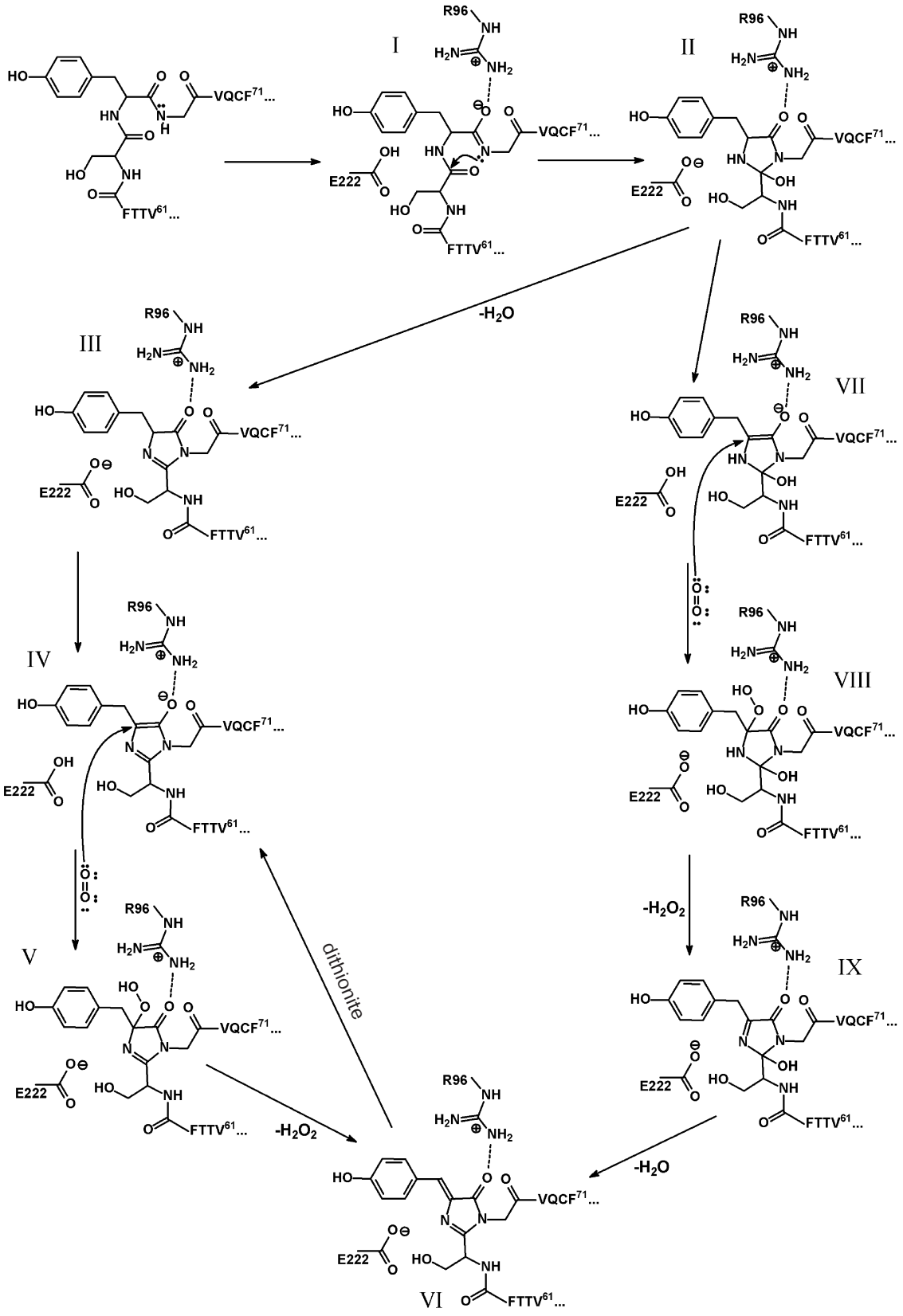


Mechanism of biosynthesis of the chromophore of green fluorescent protein. Two alternative schemes of *p*-HBI biosynthesis are presented, on the right and on the left

Scheme 1

ical mechanisms of chromophore synthesis in GFP-like proteins. Understanding of the biochemical mechanisms is necessary for creation of a molecular model for directed action on the photophysical properties of these proteins. This review generalizes modern concepts of posttranslational reactions determining the diversity of the optical properties of GFP-like proteins.

FORMATION OF GFP CHROMOPHORE

Polypeptide chain cyclization. It was originally supposed that PTM of the polypeptide chain with the formation of imidazolid-5-one structure is unique and typical only of the GFP-like proteins. However, recent studies have shown that histidine ammonia-lyase (HAL) [8], phenylalanine ammonia-lyase (PAL) [9], and tyrosine aminomutase (TAM) [10] use such reaction for the synthesis of electrophilic residue methylidene-imidazolid-5-one in the active center of enzymes [11]. In GFP-like proteins, *p*-HBI synthesis has three major stages: cyclization with the formation of a covalent bond between the nitrogen atom of Gly67 and the carbonyl carbon atom of amino acid Xxx of the tripeptide -Xxx65-Tyr66-Gly67-, dehydration, and oxidation followed by formation of an exocyclic C=C bond (Scheme 1) [12].

Posttranslational modification of GFP-like proteins begins from the moment when the polypeptide acquires cylinder-shaped β-folded spatial structure. In this structure, the central α-helical segment has significant distortions as a bend in the region of the chromophore-forming tripeptide -Xxx-Tyr-Gly-. These distortions are supposed to initiate autocatalytic PTM of GFP [13]. The X-ray structure analysis of GFP mutants before and after cyclization reaction has shown that the formed bend results in a close contact of the nitrogen atom of Gly67 and the carbonyl carbon atom of amino acid Xxx65, which later participate in this nucleophilic reaction. Besides, hydrogen bonds typical of undistorted α-helix are not formed in the region of the bend, significantly decreasing the energy barrier of the reaction [13].

The chromophore of GFP-like proteins is in a cavity that consists of amino acids influencing the electron density and charge distribution within its conjugated π-system [14, 15]. These amino acids mostly determine the optical properties of a particular protein. Obviously, this cavity contains amino acids catalyzing the reactions of chromophore formation. The overwhelming majority of GFP-like proteins contain invariant Arg96 and Glu222 in immediate proximity to the chromophore (Scheme 1). The Arg96 residue is supposed to participate in formation of a bend in the region of the chromophore-forming sequence and contribute to deprotonation of the amide nitrogen atom of Gly67 increasing its nucleophilic strength (Scheme 1, structure I). Crystallographic studies of the mutant containing the chromophore-forming sequence -Gly-Gly-Gly- in the state preceding cyclization show that the side chain of Arg96 prevents the formation of hydrogen bonds between the amino acids in positions 62 and 66 as well as 65 and 68 of the α-helix [13]. The substitution of alanine or methionine for Arg96 by site-directed mutagenesis results in significant retardation of the chromophore formation [16, 17]. It has been established that the positively charged guanidine group of Arg96 in all GFP proteins forms a hydrogen bond with the carbonyl oxygen of Tyr66, considerably decreasing the energy of activation of the cyclization reaction [3, 13, 17]. The amino group of Lys96 of mutant R96K does not form a hydrogen bond with the carbonyl group of Tyr66 [17]. In this case, the activation role of Arg96 is only partially compensated by the lysine residue in this position [18]. Thus, it is considered that Arg96 induces structural reconstructions necessary for the coincidence of electron orbitals before cyclization, provides deprotonation of the Gly67 nitrogen atom augmenting its nucleophilicity, and stabilizes the iminol intermediate during cyclization (Scheme 1, structure I) [19]. It has also been shown that Arg96 can promote the formation of cyclic enolate (Scheme 1, structure IV) at other stages of chromophore synthesis that will be described in detail below.

Amino acid residue Glu222 also plays a significant role in the catalysis of polypeptide chain cyclization. The E222Q substitution noticeably decreases the reaction rate at physiological pH values. However, the reaction rate for this mutant significantly increases at pH 9-10. In accordance with the proposed kinetic model, the reaction rate is largely determined by the chemical group with basic properties and pK_a of 9.2. In this work, it has been suggested that Glu222 in the native protein avGFP functions as a general base promoting the formation of intermediate enolate [16].

Dehydration and oxidation. Since polypeptide chain cyclization is a thermodynamically unfavorable process [13], its driving force is supposed to be subsequent reactions. The reaction of dehydration results in formation of a compound with a resonance-stabilized π-electron system (Scheme 1, structure IV), which probably causes the shift of equilibrium towards the cyclic product [13]. It is also supposed that such "conjugation-trapping" mechanism is facilitated by further oxidation of the Cα–Cβ-bond of Tyr66 with the formation of an aromatic system of imidazolid-5-one heterocycle and its conjugation with the Tyr phenol ring (Scheme 1, structure VI). Indeed, the X-ray diffraction analysis of the mutant containing -Gly-Gly-Gly- as a chromophore-forming sequence shows that cyclization is possible only in the presence of oxygen. Neither heterocycle formation nor subsequent attachment of an oxygen molecule occurs under anaerobic conditions [13].

Authors of work [20], who have studied the kinetics of GFP chromophore formation, consider that oxidation precedes the reaction of dehydration (Scheme 1, struc-

tures VII-IX). This work has shown that oxidation is the rate-limiting stage of chromophore formation (rate constant ($k_r = 4.8 \cdot 10^{-4}$ sec^{-1}) was determined for the variant of protein avGFP-trix) and is accompanied by the release of an equimolar amount of peroxide in relation to the initial protein. At the same time, according to the mass spectra, the protein loses 2 Da, which corresponds to the formation of an additional double bond (Scheme 1, structure IX). The same work has revealed the existence of a certain lag period between the reaction of oxidation and acquirement of the fluorescence by the protein. This is supposedly associated with the reaction of dehydration resulting in the formation of a final conjugated system of π-electrons of the mature protein chromophore ($k_r = 1.57 \cdot 10^{-3}$ sec^{-1} for avGFP-trix). Thus, these data are evidence in favor of the cyclization–oxidation–dehydration scheme (Scheme 1, on the right); in this model, primarily oxidation is the motive force of the cyclization reaction by the "conjugation-trapping" mechanism [20]. The conjugated system of double bonds formed after oxidation is, in this case, the supposed cause of the shift of equilibrium towards the cyclic product. In the protein obtained in work [21], hydrogen atoms at the β-carbon of the Tyr66 chromophore were replaced by two deuterium atoms. The study of the effect of this isotope substitution on the kinetics of chromophore synthesis led to a conclusion that the Cβ protons of Tyr66 do not participate in reduction of molecular oxygen. On the contrary, authors of paper [21] suggest that oxidation affects first of all the hydrogen atoms of the imidazolone cycle (Scheme 1, structures VIII and IX). It has also been suggested that at the last stage of chromophore synthesis Arg96 functions as a base accepting the Cβ proton of tyrosine, thus contributing to the formation of the final *p*-HBI structure (Scheme 1, structure VI) [21].

It should be mentioned that the substitutions of nonaromatic amino acid residues for Tyr66 in the chromophore-forming tripeptide -Xxx-Tyr-Gly- have practically no effect on the cyclization reaction [3, 22]. It is interesting that Tyr66 substitution, having no effect on the first reaction, can significantly change the direction of further PTM [23]. The performed experiments suggest that the degree of unsaturation of the side chain of residue 66 is directly associated with the yield of dehydration product. In the native GFP-like proteins, the Tyr66 phenol group apparently shifts the equilibrium towards completely dehydrated product [12, 22]. It has also been shown that the Y66L substitution results in the formation of a cross-link between Leu66 and His148 from the surroundings of GFP chromophore [22]. Analogous cross-links with the imidazole cycle of substrate histidine are typical of 4-methylidene-imidazolid-5-one structure in the active center of HAL [11]. Besides, Tyr66 substitutions in GFP-like proteins can cause the hydrolysis of polypeptide chain of the protein and reaction of decarboxylation [23]. The substitution of phenylalanine for Tyr66 in the chromophore-forming sequence also leads to posttranslational reactions untypical of GFP, which seem to be associated directly with the chemical mechanism of chromophore synthesis in the native protein [24]. The X-ray structure analysis has shown that chromophore synthesis in the mutant GFPsol Y66F is accompanied both by oxidation with the formation of a double bond between the Cα–Cβ atoms of amino acid 66 typical of native protein and by splitting of the Cα–Cβ-bond followed by the cleavage of the benzyl residue from Phe66. As a result of the latter reaction, a product with an oxygen molecule attached to the Cα atom of amino acid 66 has been identified. This work suggests that further direction of PTM (oxidation or homolytic cleavage) is controlled by residue Glu222 [24].

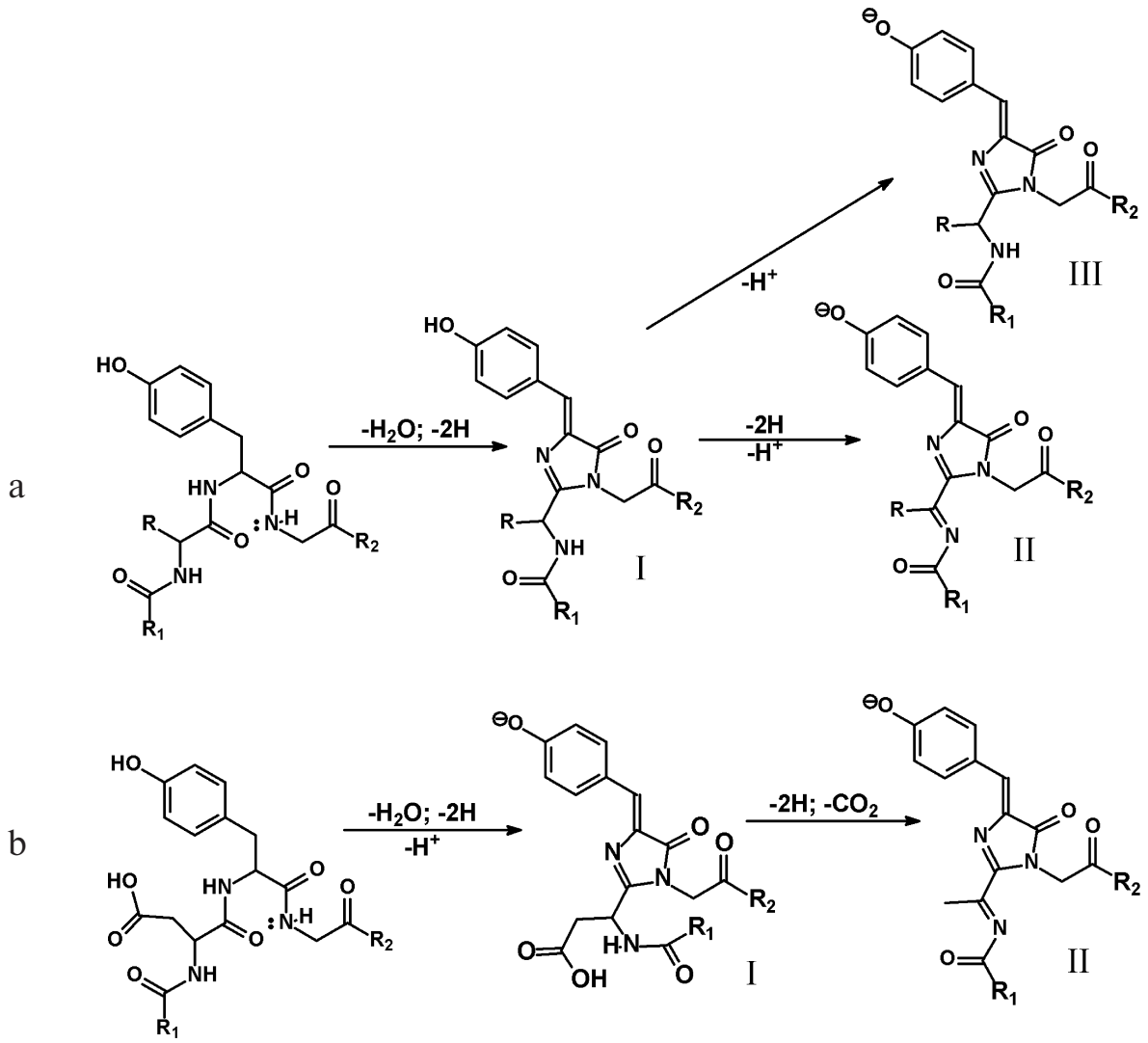
Reduction of mature GFP chromophore (GFPsol-variant) by dithionite (Scheme 1) was also used in the study of the oxidation reaction [25]. After the removal of the reductant, the protein was once again oxidized under the action of air oxygen and became fluorescent. Spatial structure (resolution 1.25 Å) of dithionite-treated GFP obtained under anaerobic conditions shows that the reduced chromophore maintains the dehydrated cyclic structure of imidazolone but loses coplanar configuration. The resulting structure clearly points to sp^3-hybridization of the Cβ atom of Tyr66 as a result of reduction of the double bond between Cα–Cβ of tyrosine. However, the Cα atom is characterized by sp^2-hybridization, which is evidenced by the planar structure of the imidazolone cycle. These structural data suggest the involvement of cyclic enolate (Scheme 1, structure IV) as the intermediate in chromophore oxidation [25]. This structure is supposedly stabilized by the positively charged guanidine group of Arg96 located at a distance of a hydrogen bond from the enol oxygen. The carboxylic group of Glu222 as a general base probably also participates in cyclic enolate formation, polarizing the H_2O molecule in close proximity to the Cα-carbon of Tyr66 and thus facilitating deprotonation of the Cα-carbon [16].

FURTHER MODIFICATIONS OF *p*-HBI RESULTING IN BATHOCHROMIC SHIFT

Additional oxidation of the chromophore of red fluorescent protein. After the cloning of the GFP gene (protein has emission maximum at 508 nm), attempts were made for some time to obtain mutants of this protein fluorescing in the longer wavelength region of the spectrum. These efforts resulted in the YFP variant (yellow fluorescent protein with emission maximum at 529 nm) after T203Y substitution in the protein with the same chromophore as that of GFP [26]. Further attempts to obtain a GFP mutant with emission in the red region of the spectrum failed for a long time. Authors of the work [27] used the method of directed GFP evolution to obtain a

mutant protein fluorescing in the red spectral region. However, this variant was supposed to contain an additionally modified *p*-HBI chromophore [27]. Not long ago, GFP homologs were found in coral polyps of the class Anthozoa [7]. Blue, green, yellow, and red fluorescent and colored non-fluorescent proteins (chromoproteins) were obtained by cloning [28, 29]. Heterochromatic homologous proteins were found in other marine organisms as well [30].

DsRed, a GFP homolog obtained from the coral *Discosoma* sp., has bright-red fluorescence with emission maximum at 583 nm. High-resolution mass spectrometry showed that the mass of the chromophore-containing peptide isolated from DsRed proteolytic hydrolyzate was by 2 Da lower than the mass of an analogous chromopeptide isolated from the "green" mutant of the protein. Tandem mass spectrometry showed that this difference in masses corresponded to additional oxidation and formation of a double bond Cα=N in the amino acid in position 65 of the chromophore (Scheme 2a, structure II; for simplicity, the numeration of amino acids of homologous protein here and further corresponds to the GFP sequence) [31]. Quantum-mechanical calculations performed in the same work showed that extension of the π-electron system of the initial GFP chromophore due to the formed acylimine (O=C–N=C) corresponded to the observed 75-nm shift in the DsRed fluorescence spectra compared with the GFP spectra. Later on, these data on the chromophore structure were completely confirmed by the crystal structures of DsRed [32, 33]. At present, the mechanism of additional oxidation of DsRed chromophore and the amino acids responsible for the cataly-



Supposed mechanisms of synthesis of chromophores of red fluorescent proteins DsRed and z2FP574: a) scheme of biosynthesis of DsRed chromophore; b) reaction of coupled oxidation–decarboxylation of Asp66 during the synthesis of the z2FP574 chromophore

Scheme 2

sis of this reaction have not been established. The nature of the oxidant forming the DsRed-type chromophore has not been determined exactly enough either, because the removal of oxygen leads, in particular, to inhibition of the synthesis of intermediate *p*-HBI [31].

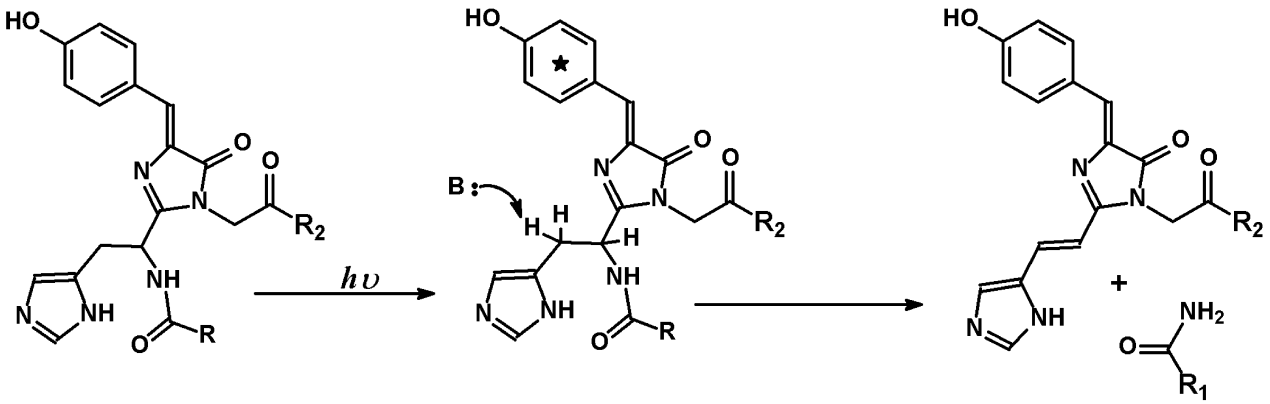
It has been established that most fluorescent proteins from corals with emission in the far-red spectral region (610-650 nm) and their non-fluorescent analogs contain a DsRed-type chromophore [34-38]. However, in contrast to DsRed, the chromophore of non-fluorescent proteins, as shown by their crystal structures, has non-planar *trans*-conformation [34]. Mutations in the surroundings of the chromophore promoting *trans-cis*-isomerization of the latter convert non-fluorescent analogs into proteins with emission in the far-red spectral region [29, 39]. Based on the crystal structures of mutant proteins (DsRed derivatives), it has been assumed recently that the quantum efficiency of fluorescence and, consequently, the fluorescence ability of proteins from corals significantly depend on the chromophore planarity [40].

Originally it was supposed that the synthesis of a DsRed-type chromophore results in formation of an intermediate compound with a structure analogous to the structure of *p*-HBI and that the chromophore at the intermediate stage contains phenolate anion corresponding to fluorescence in the green spectral range. However, the kinetic studies of maturation of DsRed-like proteins show that the green fluorescent form is "dead-end" (Scheme 2a, structure III) and does not result in the formation of a "red" chromophore [41]. In this work, it was supposed that the intermediate form is the one that fluoresces in the blue spectral region and contains a non-dissociated phenol (Scheme 2a, structure I). This supposition seems to be applicable also to most chromoproteins with the DsRed-type chromophore [41].

The studies of posttranslational chemistry of protein z2FP574 from *Zoanthus* sp. 2 have shown that, in contrast to DsRed, the red fluorescence of z2FP574 is a result of oxidative decarboxylation of Asp66, the first of the three chromophore-forming amino acids -Asp-Tyr-Gly- (Scheme 2b) [42, 43]. Thus, although the final acylimine-substituted chromophore is an equivalent of the DsRed chromophore, it is apparently the result of different post-translational reactions. Besides, in contrast to DsRed, the kinetics of maturation of native and mutant proteins from *Zoanthus* indicates the presence of an intermediate "green" ionized form of *p*-HBI during the formation of the "red" chromophore of z2FP574 [42].

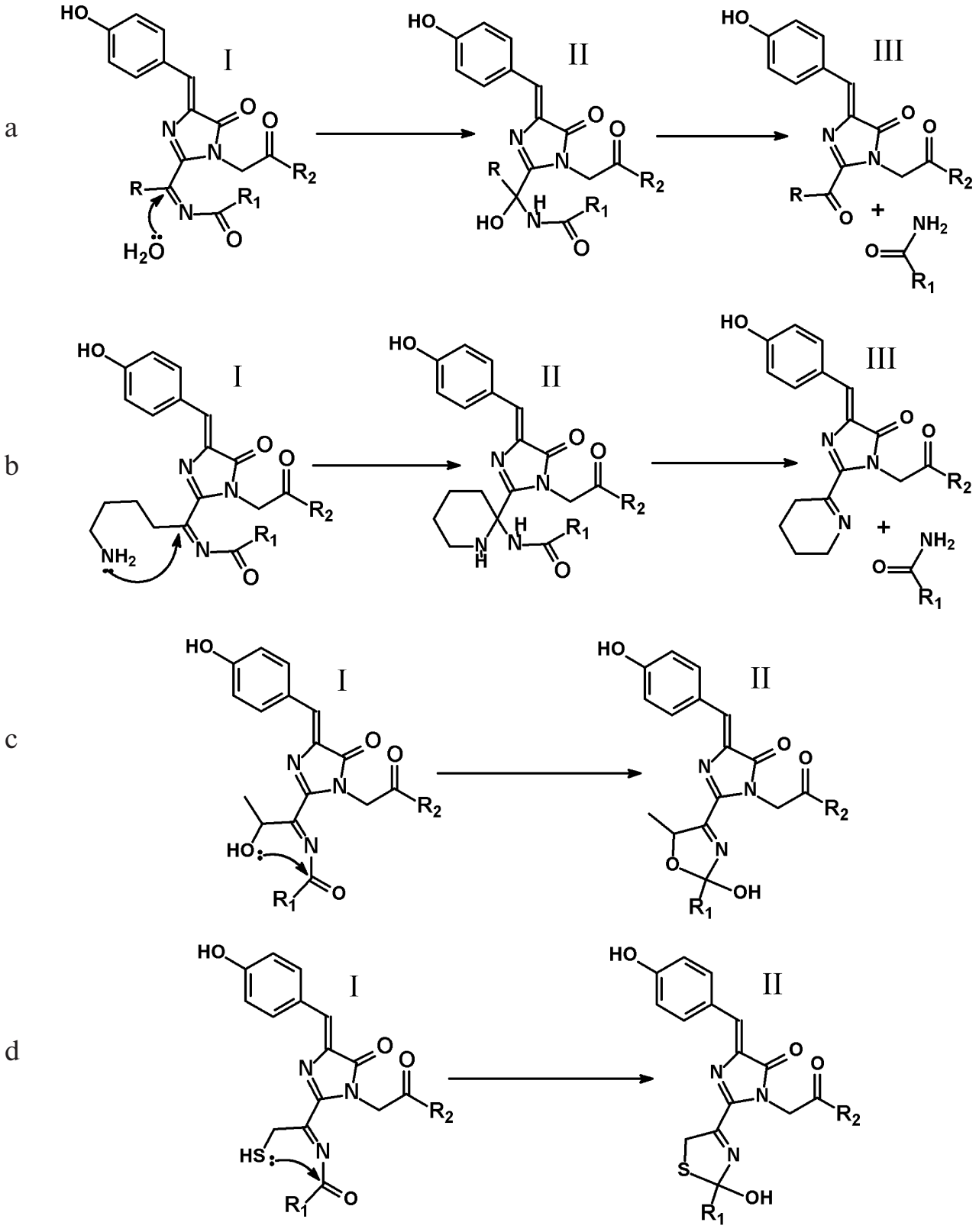
Photolytic conversion of Kaede chromophore. Some GFP proteins from Anthozoa possess a unique property of changing the emission maximum on exposure to intensive light of a certain wavelength (the phenomenon of photoconversion). This phenomenon was revealed for the first time in the study of fluorescent protein Kaede from the coral *Trachyphyllia geoffroyi* [44]. On exposure to light of ~400 nm, this protein is able to change the color of emitted light irreversibly from green to red. Later on it was shown that at least two other proteins have the same properties: DendFP and EosFP [45, 46]. All of them contain the sequence of -His-Tyr-Gly- amino acids forming the chromophore. Mass spectrometry, NMR, and X-ray structure analysis have shown that, in contrast to additional oxidation and formation of an acylimine substituent in DsRed, the Kaede-type proteins undergo photolysis with the polypeptide chain break immediately before the chromophore and formation of an additional double bond in the side chain of His65 with extension of the π-system of the *p*-HBI chromophore (Scheme 3) [45, 47, 48].

It appears that the reaction involves the protonated form of a *p*-HBI-chromophore, because the photoconversion rate increases at lower pH values. The photoconversion action spectrum also corresponds to the absorption spectrum of the protonated form of the *p*-HBI-chromophore [44]. The reaction is supposed to proceed by the mechanism of E-2-type β-elimination. It is necessary



Mechanism of photolytic conversion of Kaede protein

Scheme 3



Posttranslational reactions of acylimine-substituted derivative of *p*-HBI: a) proposed scheme of chromophore synthesis of asFP595 from *Anemonia sulcata*; b) biosynthesis of the chromophore of yellow fluorescent protein zFP538 from the coral polyp *Zoanthus* sp.; c) synthesis of the chromophore of orange fluorescent protein mOrange; d) biosynthesis of the chromophore of orange fluorescent protein mKO

Scheme 4

that glutamate should be present in the chromophore surroundings in the position analogous to that of Glu222 of avGFP, which plays the role of a general base, accepting the Cβ-proton of histidine [48]. Unfortunately, the proposed mechanism does not explain the main point, how light initiates this conversion. The recently obtained crystal structure of Kaede supposes the involvement of a water molecule located in the immediate proximity to the imidazole ring of the chromophore-forming His65 [49]. The photoconvertible mutant was obtained from an ordinary GFP-like protein (KikG from the coral *Favia favus*) by means of amino acid substitutions in the chromophore surroundings [50]. The results obtained in this work show that the presence of histidine in the first position of the chromophore-forming tripeptide is an obligatory but insufficient condition for the reaction of β-elimination. Besides this substitution, seven more mutations were performed in the chromophore surroundings, which made it possible to obtain a KikGR variant capable of photoconversion [50].

POSTTRANSLATIONAL REACTIONS OF DsRed CHROMOPHORE

The acylimine substituent formed after additional *p*-HBI oxidation (Scheme 2a, structure II) is a compound with rather high reactivity that can enter into further posttranslational reactions. The reactivity of acylimine of the DsRed-type chromophore can be exemplified by the reactions proceeding on denaturing of these proteins. Upon denaturation, the water molecule is attached to the C=N bond of acylimine (Scheme 4a, structure II), which partially disturbs the extension of the DsRed chromophore π-electron system and results in the formation of a usual *p*-HBI chromophore with absorption spectrum typical of GFP [31, 36]. The hydrolysis reaction leading to the polypeptide chain cleavage at the C=N bond of acylimine is possible under more stringent denaturing conditions (Scheme 4a, structure III). A number of proteins can be distinguished where chromophores are supposed to be the derivatives of the DsRed-type chromophore, and which are formed as a result of additional reactions at the acylimine group. However, both the amino acids catalyzing posttranslational reactions at the acylimine and the intermediate acylimine residue itself have not been identified directly in the course of maturation of these proteins. As opposed to the reaction of additional oxidation of the DsRed chromophore and photolytic conversion of Kaede, further PTM at the acylimine group usually do not result in chromophore π-electron system extension and do not cause additional bathochromic shift in protein spectra.

In a recent work, the reaction of acylimine group was proposed to result in yellow fluorescence of protein zFP538 from the coral polyp *Zoanthus* sp. The crystal structure and mass-spectrometric analyses have shown that the zFP538 chromophore is a tricyclic structure, the additional six-membered cycle being formed as a result of nucleophilic transimination reaction (Scheme 4b). According to the proposed mechanism, the amino group of Lys66 of the chromophore-forming tripeptide attacks the α-carbon of acylimine (Scheme 4b, structures I-III) followed by formation of an additional tetrahydropyridine heterocycle [51, 52]. The spectrum of another protein, mOrange (emission maximum at 562 nm), obtained as a result of mutations in the monomeric DsRed [53], is also shifted to the blue region as compared with the emission maximum of the initial protein (584 nm). It is supposed that this shift is a consequence of a cyclization reaction, in which the hydroxyl of chromophore-forming Thr66 attacks the carbonyl carbon atom of acylimine yielding an unusual 2-hydroxy-dihydrooxazolic heterocycle (Scheme 4c) [40]. The synthesis of the chromophore of orange fluorescent protein (emission maximum at 559 nm) from the coral *Fungia concinna* occurs by an analogous mechanism. In accordance with the crystal structure and mass spectrometry data of the monomeric variant of this protein (mKO), PTM within the tripeptide -Cys65-Tyr66-Glu67- result in the synthesis of a tricyclic compound. It is supposed that in this case the sulfhydryl group of Cys65 enters into the nucleophilic reaction with the carbonyl carbon of acylimine, forming an additional 2-hydroxy-3-thiazoline heterocycle (Scheme 4d) [54].

Photoactivatable protein asFP595 from *Anemonia sulcata* initially has extremely weak fluorescence at 595 nm. However, on exposure to green light (540-560 nm) it is activated into a transiently fluorescent state characterized by slow relaxation into the original non-fluorescent state [55, 56]. A number of structural studies have shown that, in contrast to DsRed, the autocatalytic synthesis of asFP595 chromophore is accompanied by a polypeptide chain breakage [57-59]. The exact structure of asFP595 chromophore was determined by the tandem mass spectrometry of the terminal proteolytic peptides adjacent to the polypeptide chain breakage (Scheme 4a, structure III). The data obtained in this work suggest that, as in the case of DsRed denaturation under harsh conditions, the posttranslational synthesis of the asFP595 chromophore occurs via autocatalytic hydrolysis of the intermediate acylimine-substituted chromophore [60]. The authors of work [61] synthesized a compound similar in structure to the supposed asFP595 chromophore. The resultant acetyl-substituted *p*-HBI in dimethylformamide had absorption and fluorescence spectra similar to those of the native protein [61].

This work was supported by the program "Molecular and Cell Biology" of the Russian Academy of Sciences, the Russian Foundation for Basic Research (project No. 09-04-00212), and by the Russian Federal Agency for Science and Innovations (State contract 02.513.12.3013).

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