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Photo-Induced Peptide Cleavage in the Green-to-Red Conversion of a Fluorescent Protein

Short Article

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Summary

Green fluorescent protein from the jellyfish (*Aequorea* GFP) and GFP-like proteins from coral species encode light-absorbing chromophores within their protein sequences. A coral fluorescent protein, Kaede, contains a tripeptide, His^{62}-Tyr^{63}-Gly^{64}, which acts as a green chromophore that is photoconverted to red. Here, we present the structural basis for the green-to-red photoconversion. As in *Aequorea* GFP, a chromophore, 4-(*p*-hydroxybenzylidene)-5-imidazolinone, derived from the tripeptide mediates green fluorescence in Kaede. UV irradiation causes an unconventional cleavage within Kaede protein between the amide nitrogen and the α carbon (Cα) at His^{62} via a formal β-elimination reaction, which requires the whole, intact protein for its catalysis. The subsequent formation of a double bond between His^{62}-Cα and -Cβ extends the π-conjugation to the imidazole ring of His^{62}, creating a new red-emitting chromophore, 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone. The present study not only reveals diversity in the chemical structure of fluorescent proteins but also adds a new dimension to posttranslational modification mechanisms.

Introduction

To understand better the physicochemistry of absorption and fluorescence emission of green fluorescent protein from the jellyfish *Aequorea victoria* (*Aequorea* GFP) (Shimomura, 1979; Prasher et al., 1992; Cody et al., 1993; Ormö et al., 1996; Yang et al. 1996) and GFP-like proteins from *Anthozoa* species (Matz et al., 1999; Gross et al., 2000; Wall et al., 2000; Yarbrough et al., 2001), it is imperative to have structural information regarding their chromophores. The currently accepted mechanism for fluorescence development of *Aequorea* GFP is shown in Figure 1A. An internal tripeptide, Ser^{65}-Tyr^{66}-Gly^{67}, autocatalytically forms a chromophore, 4-(*p*-hydroxybenzylidene)-5-imidazolinone, by nucleophilic attack of Gly^{67}-Nα on the carbonyl of Ser^{65}, dehydration, and oxidation of the α-β bond in Tyr^{66} (Heim et al., 1994; Tsien, 1998; Reid and Flynn, 1997). One example of GFP-like protein is a red-emitting fluorescent protein, DsRed (Matz et al., 1999). DsRed fluoresces first green and then red, implying the existence of some modification of chromophore structure during its maturation (Baird et al., 2000; Mizuno et al., 2001). Recent structural studies have shown that a tripeptide in DsRed (Gln^{66}-Tyr^{67}-Gly^{68}) analogous to the chromophore-forming sequence in *Aequorea* GFP forms the same structure, 4-(*p*-hydroxybenzylidene)-5-imidazolinone, and that the Cα-Nα bond of Gln^{66} then oxidizes as the protein matures (Figure 1B) (Gross et al., 2000; Wall et al., 2000; Yarbrough et al., 2001).

Kaede is a recently cloned fluorescent protein from a stony coral, *Trachyphyllia geoffroyi* (Ando et al., 2002). It contains a tripeptide, His^{62}-Tyr^{63}-Gly^{64}, which acts as a green chromophore that can be photoconverted to red, and thus provides a simple and powerful technique for regional optical marking. Supplemental Figure S1 (http://www.molecule.org/cgi/content/full/12/4/1051/DC1) shows high-contrast delineation of a single neuron in a dense culture using 405 nm light from a violet laser diode. The green state of Kaede shows two absorption peaks at 380 and 508 nm, corresponding to neutral and ionized forms, respectively. The photoconversion is highly sensitive to irradiation with UV or violet light (350–410 nm), which excites the neutral form. Interestingly, it has been observed that the photoconverted Kaede dissociates into 19- and 10 kDa fragments on SDS/PAGE (Ando et al., 2002). This finding prompted us to explore an attractive possibility that the irradiation results in cleavage of the peptide chain, thus effecting the color change from green to red.

Results and Discussion

A Peptide Cleavage Found in the Red Kaede Protein

Tandem mass spectrometry combined with liquid chromatography electrospray ionization (LC-ESI/MS/MS) after trypsinolysis revealed that the 19 kDa fragment contained sequences consistent with the C terminus of Kaede (underlined in Figure 2A), suggesting that the cleavage occurred near the chromophore-forming tripeptide, His^{62}-Tyr^{63}-Gly^{64}. The tryptic peptide containing the cleavage site was expected to appear as a single species in the green Kaede sample and to be split into two in the red one. Tryptic peptide fragments from the green and red Kaede was subjected to reverse phase chromatography for separation. Comparison of the elution profiles indicated that there were one peptide specific to green Kaede (pep_G) and two to red (pep_Ra

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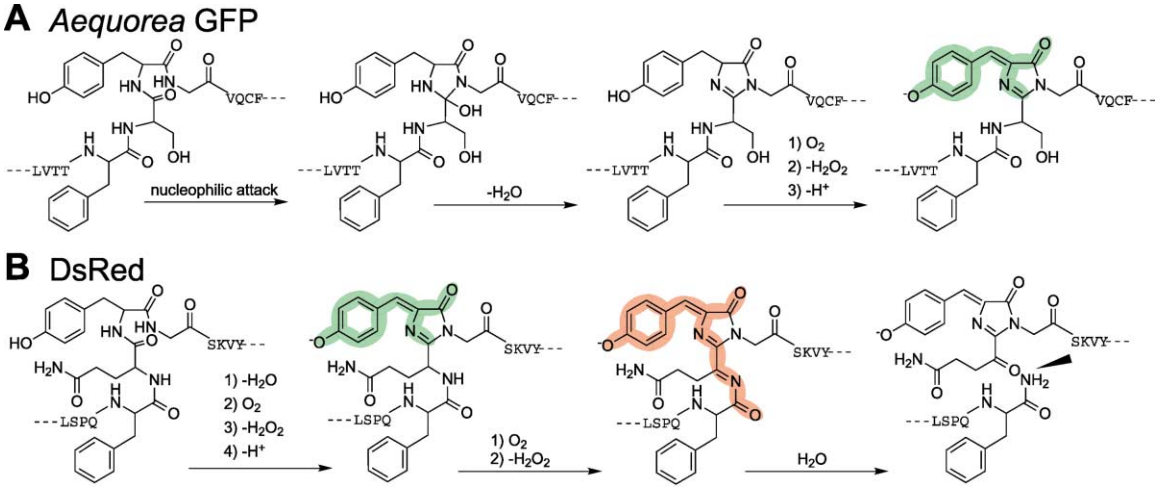

Figure 1. A Comparison of Reported Schemes for the Autocatalytic Formation and Maturation of Chromophores in Fluorescent Proteins

(A) *Aequorea* GFP. (B) DsRed. The cleavage site is indicated by an arrowhead. π-conjugation for visible-light absorption is indicated in green or red. Neighboring amino acids (single-letter code) have been added.

and pep_Rb) (Figure 2B). We believed that pep_G was split into pep_Ra and pep_Rb.

pep_G: The Tryptic Peptide Containing the Green Chromophore

Based on the amino acid sequence of Kaede, the tryptic peptide bearing His^{62}-Tyr^{63}-Gly^{64} is predicted to contain residues from Glu^{46} to Arg^{66} (a green bar in Figure 2A). The ESI/MS/MS spectrum of collision-induced fragments of pep_G identified amino acid sequences corresponding to Pro^{51}-Phe^{61} and Asn^{65}-Arg^{66} (Figure 2C), indicating that pep_G is the tryptic peptide containing the green chromophore. Because the three amino acid residues—His^{62}, Tyr^{63}, and Gly^{64}—were not identified in the spectrum, and the fragment ion containing His^{62}-Gly^{64} (y_5^+) exhibited a molecular mass (626.21 Da) that was 20.09 Da lower than the theoretical value (646.30 Da), we hypothesized that modification of the tripeptide had occurred. The reduction of 20.09 Da suggests cyclization (loss of H_2O) and oxidation (O_2-mediated loss of H_2) of the peptide to form a 4-(*p*-hydroxybenzylidene)-5-imidazolinone, as occurs in *Aequorea* GFP.

Site of the Photo-Induced Cleavage of Kaede Protein

Next, we examined the site of cleavage between pep_Ra and pep_Rb. Pep_Rb did not show any absorption above 300 nm (results not shown), indicating the absence of a complete chromophore structure in this peptide. The ESI/MS/MS spectrum of pep_Rb clearly identified the amino acid residues from Glu^{46} to Phe^{61} (Figure 2D, a black bar in Figure 2A). It was concluded that the Phe^{61} located at the C terminus must therefore border on the cleavage site. With respect to the C-terminal end structure, two points must be considered. First, pep_Rb was detected as a divalent cation by ESI/MS (Figure 2E), although it contains no basic amino acids, suggesting the presence of additional amino group in this peptide. Second, the y_1^+, y_2^+, y_3^+, and y_4^+ ions all showed a loss of 1 Da relative to their respective theoretical values (Figure 2D). These results strongly suggested that the C-terminal end was not a carboxyl group (-COOH) generated by hydrolysis but a carbamoyl group (-$CONH_2$). This structure explains both the creation of the divalent cation on the ESI/MS through protonation of both the C-terminal and N-terminal amino groups, as well as the 1-Da mass reduction observed in the y^+ ions. It was also concluded that the cleavage site was between His^{62}-Cα and -Nα (Figure 3A).

The Structural Basis for the Green-to-Red Photoconversion of Kaede

The other cleavage product was pep_Ra (a red bar in Figure 2A), which should contain the red chromophore. Figure 3A illustrates the events proposed to be involved in chromophore modification. The photocleavage involves elimination of a carboxamide (species **4b**) at His^{62}-Cα and removal of a proton at His^{62}-Cβ. The resulting structure, 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone (species **5a**), features extended π-conjugation, accounting for the green-to-red conversion of Kaede. The structure is consistent with our mass spectral data on pep_Ra. The ESI/MS spectrum showed a peak for a monovalent ion at m/z = 609.09 (Figure 2F), from which the molecular weight of pep_Ra was calculated to be 608.08 Da. This value is 37.22 Da lighter than the value of 645.30 Da expected for the pentapeptide His-Tyr-Gly-Asn-Arg. Since the region corresponding to His^{62}-Tyr^{63}-Gly^{64} lost 20 Da upon formation of the green chromophore, an additional reduction by 17 Da can be attributable to the photocleavage reaction. The mass reduction can be explained by an extraction of an amino group from His^{62} (−16 Da) and liberation of a proton at His^{62}-Cβ (−1 Da).

To our knowledge, what we present here is the first report of a peptide cleavage in an intact protein at an Nα-Cα bond via a formal β-elimination reaction (Figure 3B, right), while it is well accepted that peptide cleavage occurs at the peptide bond, the bond between the car-

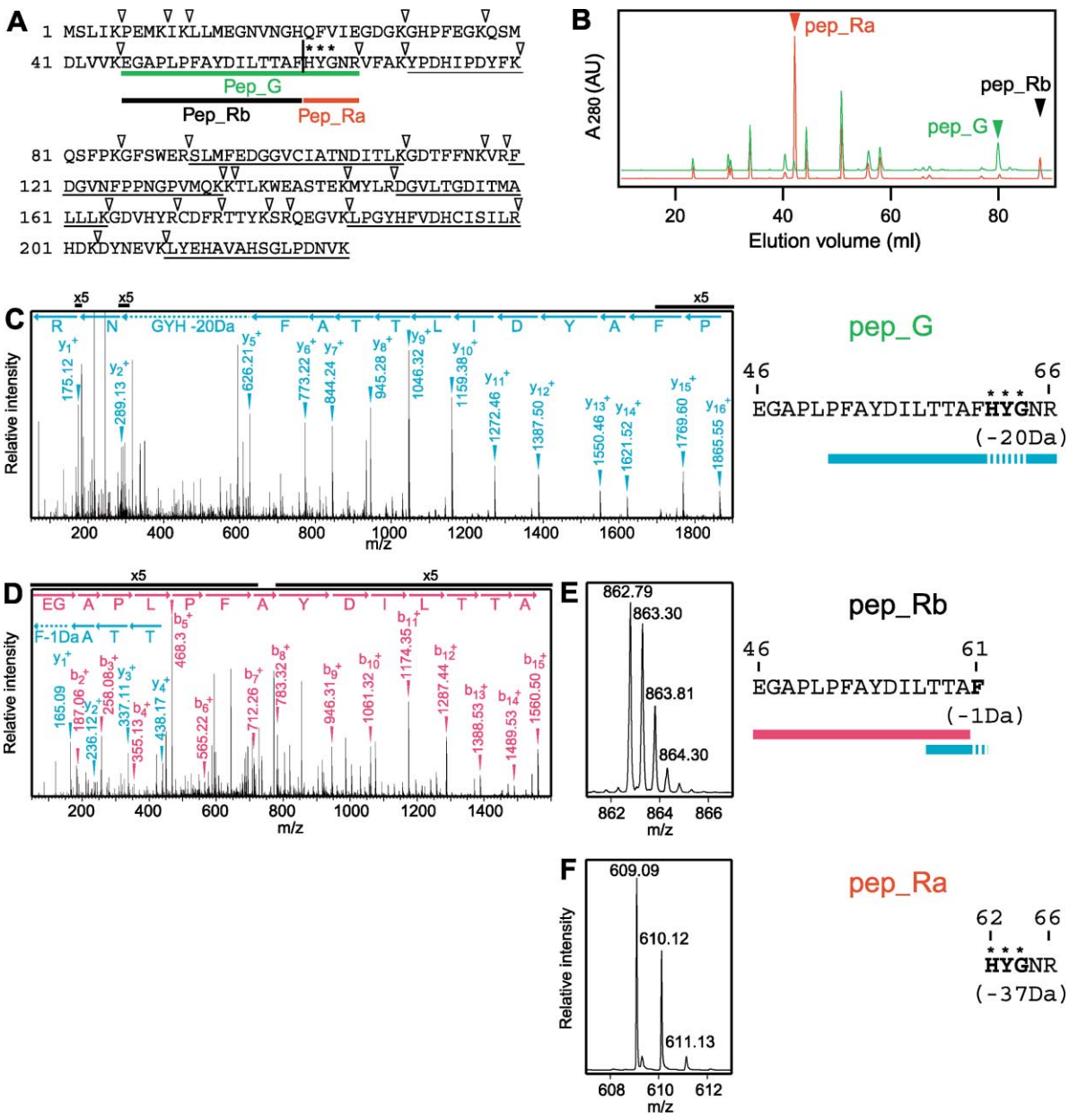

Figure 2. Mass Spectroscopic Analysis of Tryptic Peptide Fragments from the Green and Red Kaede

(A) Amino acid sequence of Kaede. The observed sequences obtained by ESI-MS analysis using the tryptic digest from the 19 kDa fragment are underlined. The chromophore-forming amino acids are indicated by asterisks. Open arrowheads indicate putative trypsin digestion sites. Tryptic peptide fragments around the chromophore are indicated by thick bars. They are pep_G (green), pep_Rb (black), and pep_Ra (red), which are written in the same colors in (B)–(F). A vertical line indicates the photocleavage site.

(B) Comparison of tryptic digests between green- and red-state Kaede by reverse phase chromatography. Tryptic digests of the green- (green trace) and red- (red trace) fluorescing isoforms were applied onto an Inartsil ODS-3 column and eluted with a 0%–90% acetonitrile linear gradient. Elution of peptides was monitored by absorbance at 280 nm. The unique peptides in each sample (pep_G, green trace; pep_Ra and pep_Rb, red trace) are indicated by arrowheads.

(C) ESI/MS/MS spectrum of pep_G. Cyan arrowheads indicate peaks corresponding to C-terminal fragment ions (y^+) produced through peptide bond breakage by collision-induced dissociation.

(D) ESI/MS/MS spectrum of pep_Rb. Magenta and cyan arrowheads indicate peaks corresponding to N- and C-terminal fragment ions (b^+ and y^+, respectively). In (C) and (D), horizontal arrows with single-letter codes indicate amino acid residues deduced from the fragment ions. At positions denoted as dotted arrows, reductions in molecular mass were observed. Intensity is multiplied by 5 times in some regions denoted as "x5."

(E) ESI/MS spectrum of pep_Rb.

(F) ESI/MS spectrum of pep_Ra.

Predicted amino acid sequences of the analyzed peptides are shown at the right sides of the respective spectra. The chromophore-forming amino acids are indicated by asterisks. Magenta and cyan bars under sequences indicate deduced amino acid residues by analyses of b^+ and y^+ ion series, respectively. The residues that showed reductions in molecular mass are indicated by boldface.

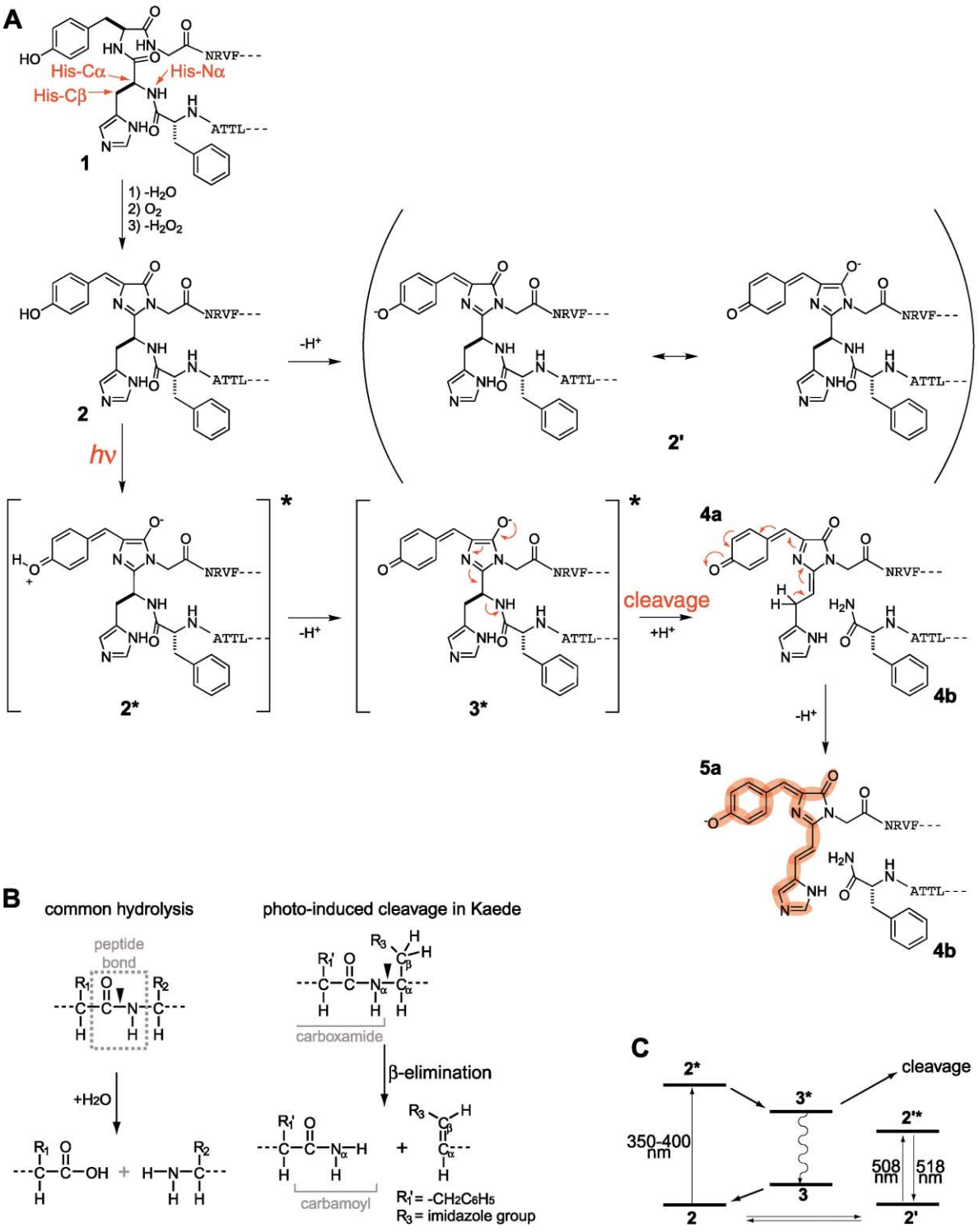

Figure 3. Molecular Mechanisms Proposed for the Green-to-Red Photoconversion

(A) Scheme for the formation and photo-induced extension of the chromophore of Kaede. Structures derived from Phe[61], His[62], Tyr[63], and Gly[64] are drawn, and the neighboring amino acids (single-letter code) are added. The crucial atoms for the photocleavage reaction are indicated in red in the structure of the precursor peptide (**1**). 4-(*p*-hydroxybenzylidene)-5-imidazolinone (**2**) is formed from **1** with the same mechanism for the chromophore formation of *Aequorea* GFP (Figure 1A). The chromophore in species **2** is a neutral form and nonfluorescent. Deprotonation at the hydroxyl group of Tyr[63] results in a green-emitting species (**2′**). The resonance structures of species **2′** are in parentheses. After excitation of the neutral form (**2**) by UV or violet light ($h\nu$), the excited state (**2***) releases proton to form the excited intermediate (**3***) (Chattoraj et al., 1996; McAnaney et al., 2002). The *p*-quinone methide-type charge density distribution has been supported by calculation for the LUMO of the neutral chromophore (Tozzini and Nifosì, 2001). Then cleavage occurs at the Nα-Cα bond of His[62] to eliminate a carboxamide group-containing peptide (**4b**). The subsequent loss of a proton from His[62]-Cβ gives a *trans* double bond between His[62]-Cα and -Cβ, leading to the extension of the conjugated π system (**5a**).

bonyl carbon and amide nitrogen atoms, through acid or protease hydrolysis (Figure 3B, left).

Determination of the Detailed Structure of the Red Chromophore by NMR

To validate our proposed structure of the red chromophore, we then carried out extensive NMR analysis on pep_Ra. A chemically synthesized pentapeptide, His-Tyr-Gly-Asn-Arg (pep_C) was used as a control. Comparison of the 1D ^{1}H-NMR spectrum of pep_C (Figure 4A) with that of pep_Ra (Figure 4B) revealed drastic changes in the chemical shifts and *J*-coupling pattern for Tyr^{63} and His^{62} resonances. The multiplet peak corresponding to Tyr^{63}-Hβ of pep_C (3.01 ppm) (Figure 4A) was shifted into the aromatic/vinyl region in the pep_Ra spectrum (7.07 ppm) (Figure 4B). This downfield shift was accompanied by a characteristic change in NMR spin properties; the peak changed from a multiplet to a singlet. Furthermore, the peak corresponding to Tyr^{63}-Hα of pep_C at 4.61 ppm in Figure 4A disappeared in the spectrum of pep_Ra (Figure 4B). These observations convincingly support the formation of a double bond between Cα and Cβ of Tyr^{63} in pep_Ra. The presence of a covalent bond between amide nitrogen of Gly^{64} and the carbonyl carbon of His^{62} was verified by the ^{13}C-edited heteronuclear multiple bond correlation (^{13}C-^{1}H HMBC) spectrum (Bax and Summer, 1986) of pep_Ra (Figure 4C), which showed a correlation between Gly^{64}-Hα and 2C of the imidazolinone ring (Figure 4G, cyan lines). All these results confirm the presence of a 4-(*p*-hydroxybenzylidene)-5-imidazolinone chromophore structure in the red chromopeptide.

The double bond formation between His^{62}-Cα and -Cβ was evidenced by exceptionally large downfield shifts of 2.4 and 4.2 ppm for the His^{62}-Hα and -Hβ resonances, respectively (Figures 4A and 4B). In the pep_Ra spectrum (Figure 4B), both the His^{62}-Hα and -Hβ resonances appeared as doublets with a *J*-coupling constant of 16.0 Hz, indicating that the two protons existed in a *trans* position (Figure 4G). Furthermore, the downfield shift of the His^{62}-4H peak (Figures 4A and 4B) is consistent with the expansion of π-conjugation to the imidazole ring through the Cα-Cβ double bond.

To establish the preferred conformation of the red chromophore, we carried out ^{1}H-^{1}H rotational nuclear Overhauser effect spectroscopy (ROESY) at three bonds within the structure of 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone (Figure 4G). First, a rotational nuclear Overhauser effect (ROE) was observed between His^{62}-Hα and Gly^{64}-Hα but not between His^{62}-Hβ and Gly^{64}-Hα (Figure 4D), confirming that His^{62}-Hα and 1N of imidazolinone ring are in an s-*cis* conformation. Second, ROEs between His^{62}-Hβ and Tyr^{63}-2,6H, as well as between His^{62}-Hβ and Tyr^{63}-3,5H (Figures 4E and 4F) demonstrate that Tyr^{63}-Hβ and 3N of imidazolinone exist in a *trans* position, as occurs for the chromophore of *Aequorea* GFP (Ormö et al., 1996; Yang et al., 1996). Finally, an ROE between His^{62}-4H and Tyr^{63}-3,5H (Figure 4F) supports an s-*trans* conformation between His^{62}-Hβ and His^{62}-1N.

Diversity of Chromophore Modification Mechanisms in the GFP-like Protein Family

Both DsRed and Kaede have π-conjugation structures similar to that of *Aequorea* GFP, feature 4-(*p*-hydroxybenzylidene)-5-imidazolinone within their chromophores, and emit green fluorescence in their immature and unphotoconverted states, respectively (Gross et al., 2000; Wall et al., 2000; Yarbrough et al., 2001). The processes involved in the development of red fluorescence, however, are different. While DsRed generates an acylimine (-C=N-C=O) by an oxidation reaction to expand the π-conjugation structure at the 2-position of the imidazolinone (Gross et al., 2000), Kaede becomes red specifically upon UV irradiation, which causes a peptide cleavage as a result of an elimination reaction. Its cleavage site is between Nα and Cα of the His residue, which subsequently becomes involved in the red chromophore.

It has been reported that a fraction of DsRed that was boiled and then subjected to SDS/PAGE showed two fragment bands of apparent masses 15 and 22 kDa (Gross et al., 2000). After the denaturation of the protective protein shell, the C=N bond in the acylimine was thought to hydrolyze under harsh conditions. It appears that peptide cleavage does not occur in the intact mature DsRed. In contrast, Kaede cleavage should occur in the intact protein upon irradiation with UV or violet light, leading to formation of the structure 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone, which accounts for the green-to-red conversion of Kaede.

Another GFP-like protein absorbing at a long wavelength is asFP595 (Lukyanov et al., 2000), whose proposed chromophore structure is different from that of DsRed or Kaede. According to a study by Martynov et al. (2001), the tripeptide Met^{65}-Tyr^{66}-Gly^{67} of asFP595 undergoes a cyclization reaction involving nucleophilic attack by the Met^{65}-Nα onto the Tyr^{66} carbonyl, producing a six-membered heterocycle ring structure. Following dehydration and oxidation of the α-β bond of Tyr^{66}, the peptide cleaves at the peptide bond between Cys^{64} and Met^{65}, splitting the protein into 8- and 20 kDa fragments. Although this peptide cleavage completes the chromophore, a hydrolysis, rather than elimination, reaction actually cleaves the peptide bond. Furthermore, the hydrolysis does not depend on illumination.

(B) Peptide cleavage reactions. Conventional cleavage at the peptide bond, the bond between the carbonyl carbon and amide nitrogen atoms through acid or protease hydrolysis (left). Photo-induced peptide cleavage at an Nα-Cα bond in intact Kaede protein (right).

(C) Provisional model for the photophysics of the green Kaede. Direct excitation of the anionic chromophore (**2′**) results in green fluorescence. Upon excitation of the neutral chromophore, **2*** rapidly converts to **3*** through the excited-state proton transfer (Chattoraj et al., 1996). **3*** is an intermediate anionic chromophore in a nonequilibrium protein environment, and may correspond to I* in the three-state model for the photophysics of wild-type *Aequorea* GFP (Chattoraj et al., 1996; McAnaney et al., 2002; Brejc et al., 1997; Weber et al., 1999). The nonfluorescence of the neutral form of Kaede is explained by (1) no direct fluorescence (**2***→ **2**), (2) radiationless decay of **3*** to **3**, and (3) no conversion from **3*** to **2′***.

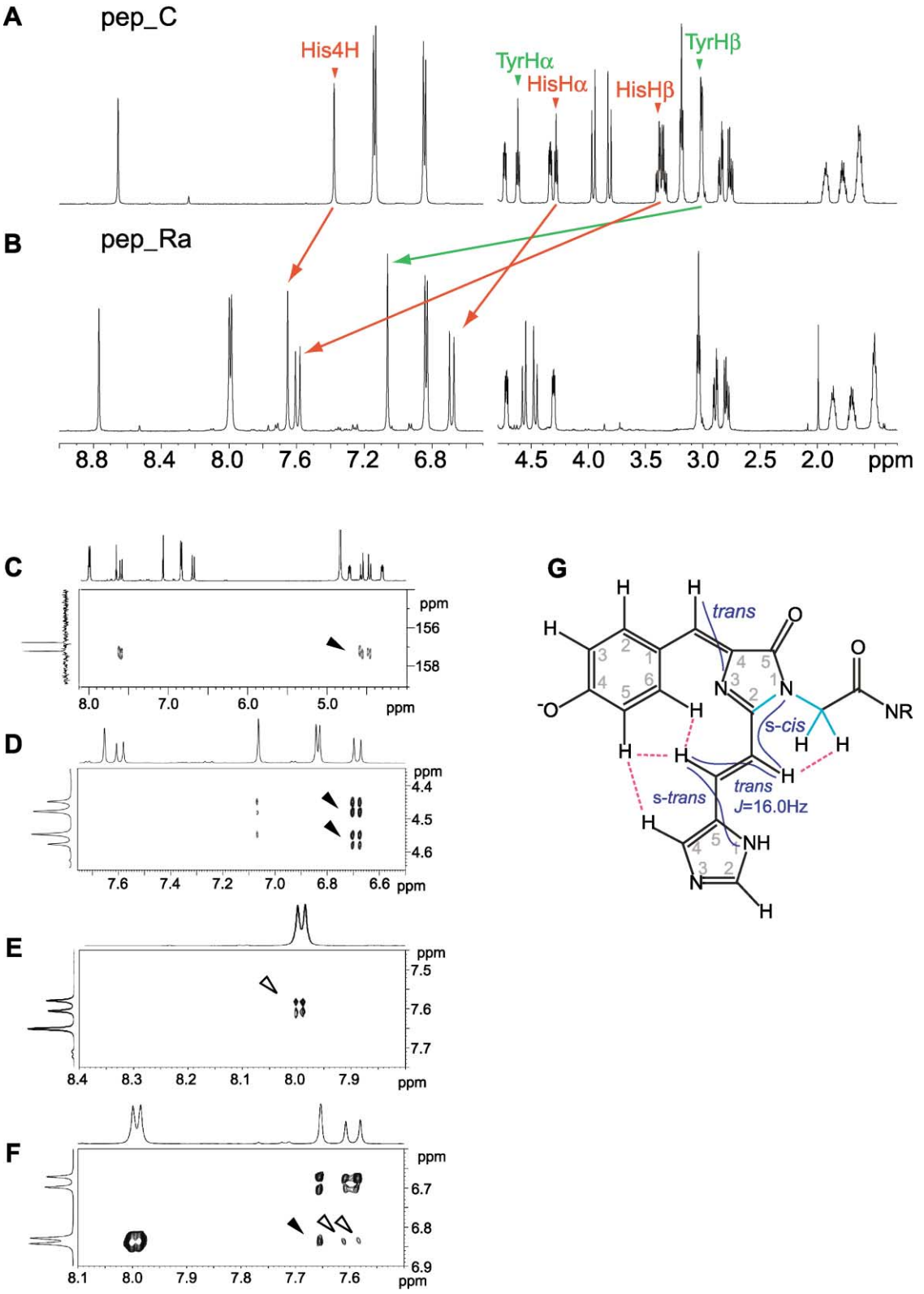

Figure 4. Determination of the Detailed Structure of the Red Chromophore by NMR

(A and B) ^{1}H NMR spectra of pep_C and pep_Ra, respectively. Downfield (9.0-6.5 ppm) and upfield (4.8-1.3 ppm) regions of the spectra are displayed in the left and right, respectively. Arrowheads indicate peaks assigned to His-4H, His-Hα, and Hβ (red arrowhead), and Tyr-Hα and Hβ (green arrowhead). Arrows indicate shifts of some peaks in pep_Ra.

(C) Expanded ^{13}C-^{1}H HMBC spectrum with a mixing time of 80 msec. Arrowhead indicates spots corresponding to a correlation between Gly-Hα and 2C of imidazolinone.

Photophysics of the Photo-Induced Peptide Cleavage

It is interesting to know if His^{62} is replaceable with other amino acids. Substitution of tyrosine, tryptophan, aspartate, or arginine dimmed or abolished the fluorescence. Substitution of all other amino acids yielded green-emitting mutants that did not exhibit photo-induced cleavage as well as photoconversion (Supplemental Table S1 [http://www.molecule.org/cgi/content/full/12/4/1051/DC1]). Thus, His^{62} is requisite for initiating the β-elimination reaction. It is possible that the imidazole of His^{62} gets protonated on its 3N and participates in the reaction by supplying a proton from its 1N to the carboxamide leaving group in **4b** (Figure 3A). In fact, 1N of the imidazole is positioned near the cleavage site as revealed by the determination of the red chromophore conformation (Figure 4G). On the other hand, the green chromopeptide, pep_G, was not cleaved with UV irradiation (data not shown). Also, a mutation outside the chromophore-forming region, Ala69Ser, generated a mutant that fluoresced green but did not photoconvert (data not shown). Therefore, the photo-induced peptide cleavage and resulting expansion of π-conjugation appear to require a strict three-dimensional structure for the catalysis. The crystal structures of the green and red Kaede will provide complementary information to further our understanding of the conversion mechanism.

Also, ultra-fast time-resolved spectroscopy of Kaede will reveal excited state dynamics in the protein (Figure 3C). These forthcoming studies will provide an answer to the fundamental question of why excitation of the neutral form of the green chromophore causes the chemical reaction, while excitation of the anionic form gives fluorescence. At present, species **3*** and **2′*** in Figure 3A are indistinguishable. We have only a speculation that the hydrogen-bond network around the chromophore is different between the two species, analogous to the three-state model for the photophysics of wild-type *Aequorea* GFP (Chattoraj et al., 1996; McAnaney et al., 2002; Brejc et al., 1997; Weber et al., 1999). Only in species **3*** may the network connect the phenolic hydroxyl of Tyr^{63} to the imidazole ring of His^{62} so that the proton on 1N of His^{62} imidazole is efficiently released for the cleavage reaction.

Experimental Procedures

Production and Purification of Kaede Protein

Recombinant Kaede protein was produced in *E. coli* and purified as described previously (Ando et al., 2002), except that all procedures were done in the dark. Photoconversion was performed on green Kaede by illumination at 365 nm using a UV illuminator in 150 mM NaCl and 10 mM MOPS (pH 7.0).

Purification of Chromopeptides after Trypsinization

Protein was denatured and trypsinized with sequence-grade modified trypsin (Promega, USA) in accordance with the supplier's protocol. Peptides were purified by reverse phase chromatography (Inertosil ODS-3, GL Science, Japan) and eluted with a 0 to 90% acetonitrile linear gradient in the presence of 0.1% trifluoroacetate. Pep_Ra was further purified by size exclusion chromatography (Superdex Peptide, Pharmacia) using 30% acetonitrile:0.1% trifluoroacetate as eluent.

ESI-MS/MS Analysis of Chromopeptides

Peptides were applied onto a Cadenza C18 column (Michrom BioResources, USA) installed on a MAGIC 2002 HPLC system (Michrom BioResources) and eluted at a flow rate of 1 μl/min with a 2 to 90% acetonitrile linear gradient in the presence of 0.1% formic acid. Peptides in the eluate were ionized by positive-mode nanoflow-LC ESI (MDS Proteomics, Denmark) at 2.4 kV of capillary voltage and introduced into a QSTAR quadrupole-TOF mass spectrometer (AB/MDS Sciex, Canada).

NMR Analyses of Chromopeptides

Peptides were lyophilized from D_2O and redissolved in 99.999% D_2O (Isotec Inc., USA). Sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (Isotec Inc.) was used as a standard for calibration of NMR spectra. ^{13}C NMR spectra were acquired using a Bruker Advance 500 MHz spectrometer equipped with a ^{13}C cryoprobe. Other spectra were acquired using a Bruker Advance 600 MHz spectrometer equipped with a QXI probe.

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(D–F) Expanded ^1H-^1H ROESY spectra of 500 msec mixing time. Both positive (solid line) and negative (dotted line) signals are indicated. Closed arrowheads indicate spots corresponding to correlations between His-Hα and Gly-Hα (D), and between His-4H and Tyr-3,5H (F). Open arrowheads indicate spots corresponding to correlations between His-Hβ and Tyr-2,6H (E), and between His-Hβ and Tyr-3,5H (F).
(G) Summary of NMR analyses regarding *cis-trans* isomers of the structure, 2-[(1*E*)-2-(5-imidazolyl)ethenyl]-4-(*p*-hydroxybenzylidene)-5-imidazolinone. Cyan lines indicate a correlation between Gly-Hα and 2C of imidazolinone observed on the HMBC spectrum (C). ROEs are indicated by magenta dotted lines (D–F). Assignments and correlations observed in NMR analyses are shown in Supplemental Data (http://www.molecule.org/cgi/content/full/12/4/1051/DC1).

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