

# Ca<sup>2+</sup>-Regulated Photoproteins: Structural Insight into the Bioluminescence Mechanism

EUGENE S. VYSOTSKI\*,†,‡ AND JOHN LEE‡

*Institute of Biophysics, Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia, and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602*

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## ABSTRACT

The bioluminescent jellyfish has contributed two famous proteins to modern science: green fluorescent protein or GFP, which finds wide use as a probe in cell biology studies, and aequorin, which has been used for intracellular calcium measurement for more than 30 years. More recently, obelin, a protein from the bioluminescent hydroid and also in the family of what are called “Ca<sup>2+</sup>-regulated photoproteins”, has been shown to have very attractive properties both in general applications and for basic structural biology investigations. This review will survey the new information into their molecular mechanism of bioluminescence action.

In their seminal investigation of the bioluminescence of the jellyfish *Aequorea* in the early 1960s, Shimomura and co-workers<sup>1</sup> isolated a small protein that required only the addition of calcium ions to generate the bioluminescence emission. They aptly named this new protein “aequorin” and also found that the presence or absence of oxygen had no effect on the bioluminescence intensity, a property contrasting with that of other bioluminescence reactions that were under close study at that time, such as those from the firefly and the bioluminescent bacteria. In fact, the majority of the great variety of bioluminescence mechanisms that have received study have the properties of classical enzyme–substrate reactions, the enzyme being generically termed “luciferase” and the substrate “luciferin”. It was therefore proposed<sup>2</sup> that aequorin and some other bioluminescent proteins be classified as “photoproteins”, distinguishing them from the luciferases that required external substrates including

molecular oxygen for bioluminescence activity. Though other kinds of photoproteins have been described,<sup>2</sup> the great majority of photoproteins now known to exist are stimulated to luminescence by calcium or certain other inorganic ions. The term “calcium-regulated photoproteins” was suggested by Blinks<sup>3</sup> to refer to this group to be consistent with the terminology used for the larger family of calcium-binding proteins and also because calcium regulates but is not essential for bioluminescence emission. The terms “Ca<sup>2+</sup>-activated” and “Ca<sup>2+</sup>-binding” photoproteins, have been preferred by some authors.

Ca<sup>2+</sup>-regulated photoproteins are found in and are responsible for the light emission of a variety of bioluminescent marine organisms, mostly coelenterates. The best known of these is aequorin mentioned above from *Aequorea*.<sup>1</sup> Although Ca<sup>2+</sup>-regulated photoproteins have apparently been detected in a great many (more than 25) different coelenterates<sup>4</sup> and probably there are many other organisms yet containing photoproteins, only the five shown in Figure 1 have yielded sequence information via cDNAs: aequorin,<sup>5,6</sup> halistaurin (mitrocomin),<sup>7</sup> and phialidin (clytin)<sup>8</sup> from *Aequorea*, *Halistaura (Mitrocoma)*, and *Phialidium (Clytia)* and obelins from the marine hydroids *Obelia longissima*<sup>9</sup> and *O. geniculata*.<sup>10</sup> All Ca<sup>2+</sup>-regulated photoproteins show high sequence homology and contain three “EF-hand” calcium-binding consensus sequences<sup>10–12</sup> (Figure 1, cyan). Apoproteins expressed by *Escherichia coli* can be charged to active photoproteins by incubating them with synthetic coelenterazine under calcium-free conditions in the presence of O<sub>2</sub> and reducing reagents.<sup>13</sup> The mechanism of the charging is unknown.

The chemical mechanism of aequorin bioluminescence was extensively reviewed by Ohmiya and Hirano in 1996.<sup>14</sup> More recently with the advances in knowledge of the spatial structure of some photoproteins, there is the potential for understanding how the protein structure itself and, in particular, the key residues in the active site determine the different properties among the photoproteins. The possibility arises that based on structural information, rational mutations can be planned to engineer photoprotein properties, modulation of bioluminescence color, flash kinetics, etc., to suit applications to analytical methodologies.

## Nature of the Bioluminescent Reaction

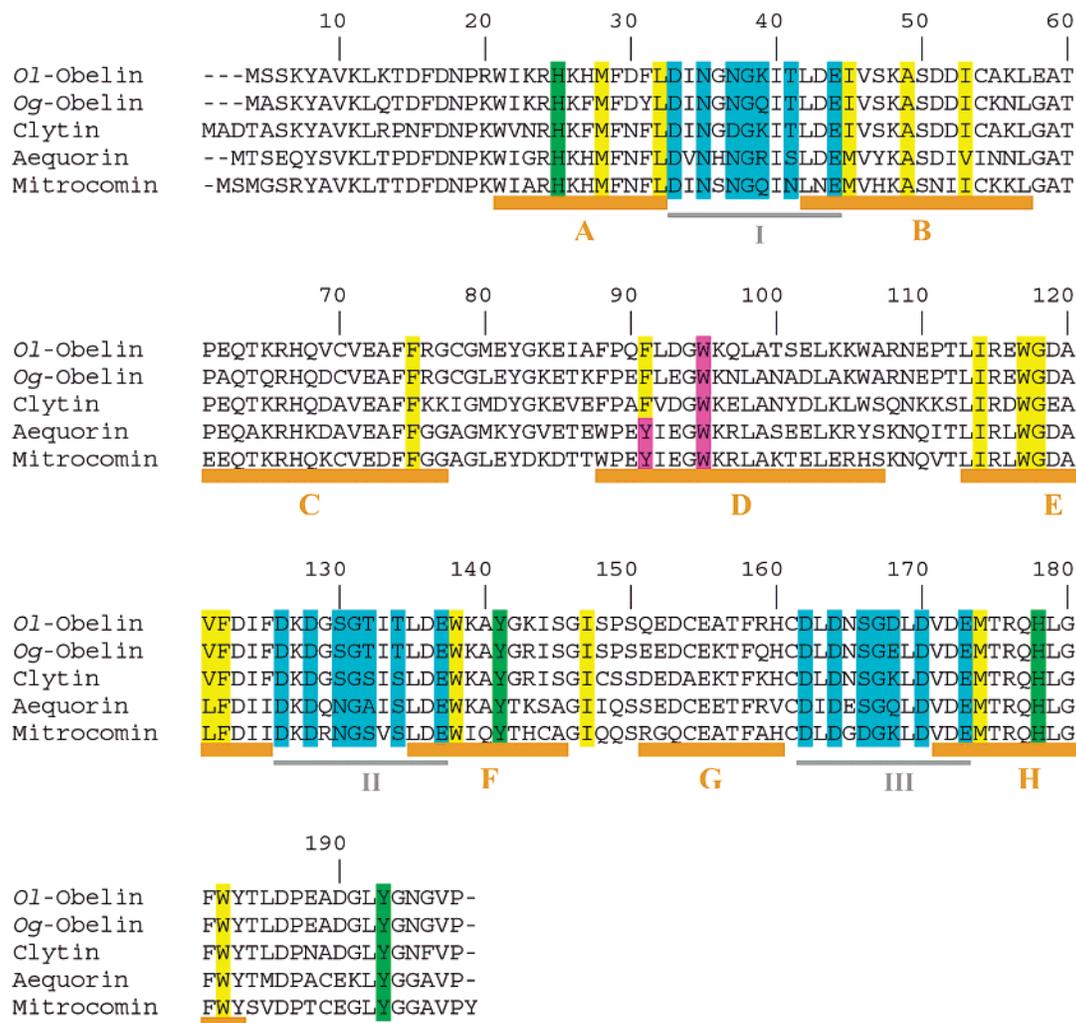
A distinguishing property of photoproteins already mentioned is that there is no requirement for an added substrate, a luciferin such as that in other bioluminescent systems. However, to yield blue light emission ( $\lambda_{\max} = 465\text{--}495\text{ nm}$ ), an electronic excited state of a molecule must be populated by a chemical reaction with at least 70 kcal/mol of exergonicity. Such an amount of energy cannot be produced just by the binding of Ca<sup>2+</sup> to the photoprotein but clearly must be the result of an intra-

Eugene Vysotski was born in Krasnoyarsk, USSR, in 1952 and obtained his B.S. and M.S. degrees in Biophysics at Krasnoyarsk State University in 1975 and Ph.D. in Biophysics from the Institute for Biophysics, Russian Academy of Sciences, Siberian Branch, in 1987. He was appointed to the Institute of Biophysics there and became Head of the Photobiology Laboratory in 1991. He has made numerous research visits at the Friday Harbor Laboratories, University of Washington, and to the University of Georgia, since 1993. His research interest has been in mechanisms and various applications of bioluminescence, and he has published on bacterial bioluminescence and the photoproteins.

John Lee was born in Sydney, Australia, in 1935 and received his B.Sc. (Hons) degree in Physical Chemistry in 1956 and Ph.D. in Nuclear Chemistry in 1960 at the University of New South Wales, Australia. In 1961–1963, he had a postdoctoral fellowship in the Biology Department at Johns Hopkins University, Baltimore, in the laboratory of Howard Seliger. Since 1969, he has been at the University of Georgia where he is Professor of Biochemistry and Molecular Biology. Early research concentration was on the chemistry of electrons and positrons and in the last 30 years on the biophysics of bioluminescence systems, laser spectroscopy, fluorescence dynamics, and structural biology of bioluminescent proteins.

† Russian Academy of Sciences, Siberian Branch.

‡ University of Georgia.



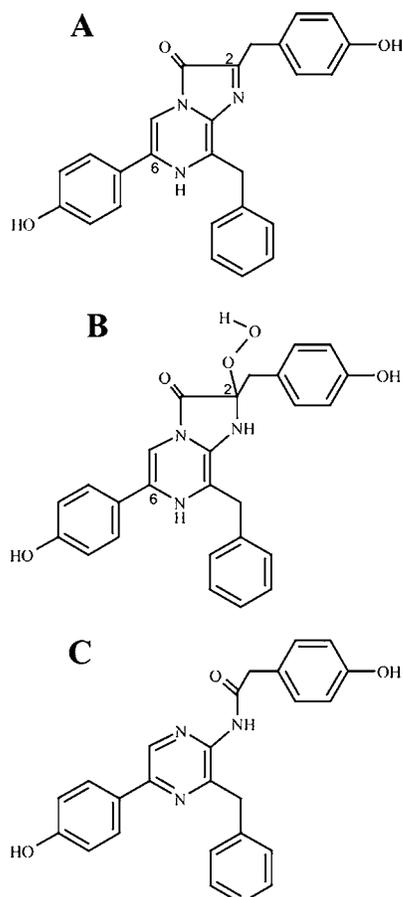
**FIGURE 1.** Sequence alignment among the Ca<sup>2+</sup>-regulated photoproteins: aequorin,<sup>5</sup> mitrocomin,<sup>7</sup> clytin,<sup>8</sup> and obelins from *O. longissima*<sup>9</sup> and *O. geniculata*.<sup>10</sup> The residues found from spatial structures of aequorin and obelins to be around the active site are shown in yellow, green, and pink. The residues participating presumably in bioluminescent reaction are marked in green. The residues that probably take part in formation of light emitter through their hydrogen bonds are shown in pink. Helices A–H are shown in brown underbars according to the *O1*-obelin 3D structure. Calcium-binding loops I–III are shown as gray underbars. The calcium-binding consensus sequences within the loops are marked in cyan.

molecular chemical reaction within the photoprotein itself. The binding of calcium ions serves to regulate or trigger that reaction.

The Ca<sup>2+</sup>-regulated photoproteins consist of a single polypeptide chain of about 22 kDa to which the substrate, 2-hydroperoxy-coelenterazine, an imidazolopyrazinone derivative (Figure 2), is tightly though not covalently bound. Coelenterazine figures prominently in the field of bioluminescence, being referred to by Cormier and co-workers<sup>15</sup> as “coelenterate-type luciferin” because it serves as a conventional luciferin in many coelenterate luciferase systems. However, it is now becoming apparent that coelenterazine is the luciferin for luciferases from many other marine species, as well as being found in nonbioluminescent animals.<sup>16–18</sup> Moreover, it is probably not synthesized in coelenterates; most likely coelenterazine is obtained by these animals in their diet as demonstrated for the hydromedusa *Aequorea victoria*.<sup>19</sup> The function of coelenterazine in nonbioluminescent animals is not known.

The energy-yielding reaction in photoprotein bioluminescence is an oxidative decarboxylation of the coelenterazine with the evolution of CO<sub>2</sub>, and the energy is deposited into the excited state of the product, followed by the emission of a photon.<sup>20,21</sup> The oxidized end-product is called coelenteramide (Figure 2C). The oxygen required for the reaction is derived from the peroxy substitution on the coelenterazine itself and so explains why the reaction kinetics is not influenced directly by the availability of free molecular oxygen.<sup>1</sup>

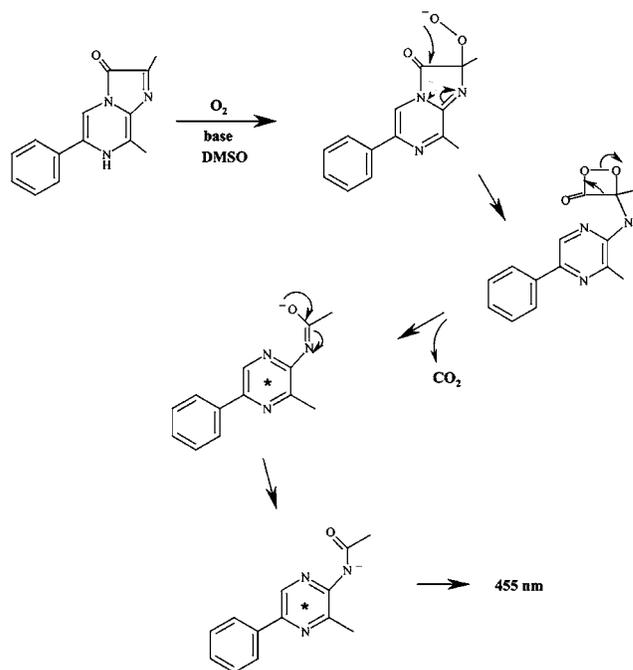
The photoproteins do not exhibit visible fluorescence in their unreacted state, but at least three of them become highly fluorescent once they have reacted. After the aequorin bioluminescence reaction, the protein displays an intense blue fluorescence ( $\lambda_{\max} = 469$  nm) and for this reason in the literature has been called “blue fluorescent protein”.<sup>22</sup> The fluorescence spectral distribution of blue fluorescent protein is almost identical to the bioluminescence spectrum of aequorin. This is in sharp contrast to the obelins,<sup>10</sup> which also have a blue bioluminescence but



**FIGURE 2.** Coelenterazine (A), 2-hydroperoxy-coelenterazine derivative (B), and coelenteramide (C).

at a longer wavelength ( $\lambda_{\text{max}} = 485$  or  $495$  nm, depending on the type), and the product fluorescence is not the same but even further shifted into the green ( $\lambda_{\text{max}} = 510$  or  $520$  nm). Because the name “green fluorescent protein” has already been conferred on another well-known protein also originally found in *Aequorea*, we prefer to use the names “Ca<sup>2+</sup>-discharged aequorin”, “Ca<sup>2+</sup>-discharged obelin”, etc. for these product proteins. The bright fluorescence of the Ca<sup>2+</sup>-discharged photoproteins indicates that the coelenteramide remains bound within the spent protein, because coelenteramide is not fluorescent in aqueous solution. Fluorescence anisotropy measurements also indicate that the motion of coelenteramide in the apo-obelin binding pocket is highly restricted.<sup>23</sup> For aequorin but not obelin, the fluorescence of the Ca<sup>2+</sup>-discharged photoprotein is diminished if the calcium is removed attributed to dissociation of the coelenteramide from the apoaequorin. For Ca<sup>2+</sup>-discharged obelin, the coelenteramide remains tightly bound even in the calcium-free state.

McCapra and Chang<sup>24</sup> suggested a mechanism for the bioluminescent reaction from a model study of the chemiluminescence of a coelenterazine analogue in an aprotic solvent (Figure 3). They showed that the oxidative decarboxylation of coelenterazine occurs through several intermediates. The reaction of coelenterazine with oxygen produces a primary oxygenation product, the C2-hydro-



**FIGURE 3.** The McCapra/Chang mechanism of the chemiluminescence of a coelenterazine analogue.<sup>24</sup>

peroxide, and this is deprotonated and closed to a dioxetane, a strained four-member  $\alpha$ -peroxylactone ring (Figure 3). The hydroperoxide intermediate in both the chemiluminescence and bioluminescence reactions is now verified by more recent studies,<sup>25–29</sup> but the experimental verification of the dioxetane is more elusive. However from isotope labeling experiments showing that one oxygen atom in the CO<sub>2</sub> originates from molecular oxygen, there is general agreement that the pathway in Figure 3 represents the bioluminescence chemistry.

In DMSO and a strong base such as potassium *tert*-butoxide, the chemiluminescence reaction in Figure 3 has a spectral emission maximum at 455 nm. The amide is produced in a high yield, and under the same basic solution conditions, the fluorescence spectrum of the authentic amide matches the chemiluminescence spectrum. The fluorescing species was identified as the excited amide anion because without added base the fluorescence maximum is at 380 nm, assigned to the neutral species. Hori et al.<sup>30</sup> extended this model with a study of the chemiluminescence properties of a number of other coelenterazine analogues. On the basis of the spectral similarity of this amide anion fluorescence to the bioluminescence from aequorin, it has been generally agreed until recently that the excited amide anion was the origin of the aequorin bioluminescence.

Shimomura and Teranishi<sup>31</sup> carried out fluorescence studies of coelenteramide and five analogues in solvents of different polarities and also determined the effect of inclusion of strong and weak bases. They proposed that coelenteramide can form five kinds of excited states, shown in Figure 4, with fluorescence maxima listed depending on solvent polarity. In neutral solvents of increasing polarity, the fluorescence spectral maxima were in the range 387–409 nm, clearly assignable as being from

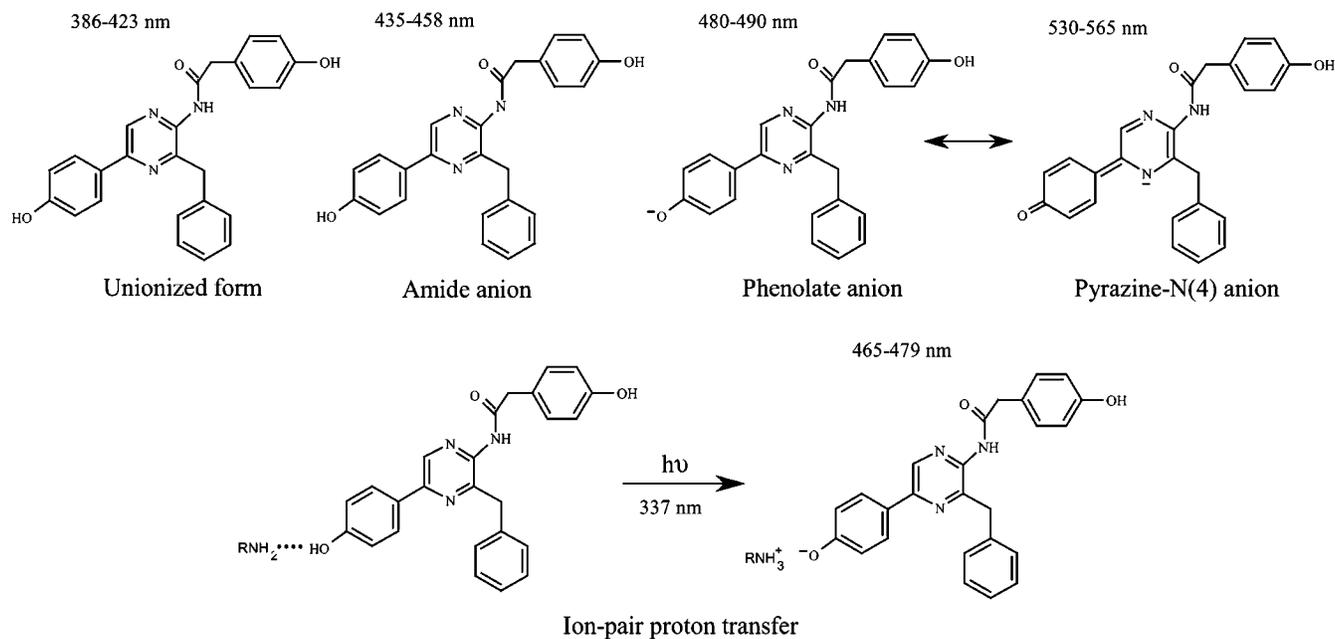


FIGURE 4. Fluorescence forms of coelenteramide.<sup>31</sup> The range of fluorescence maxima is indicated.

the excited state of un-ionized coelenteramide. In benzene with the addition of the strong base *t*-BuOK/*t*-BuOH, the fluorescence maximum shifted to 480 nm and was assigned as being from the excited coelenteramide phenolate. In the more polar solvents acetonitrile or DMSO, basic conditions produced a yellow fluorescence, a maximum in the 535–550 nm range, and Shimomura and Teranishi attributed this to the predominance of the pyrazine-N(4) anion resonance form of the phenolate anion (Figure 4). Coelenteramide analogues having the 6-(*p*-hydroxy)phenyl position blocked show fluorescence at 435–458 nm in basic solvents, assignable again as being from the excited amide anion species.

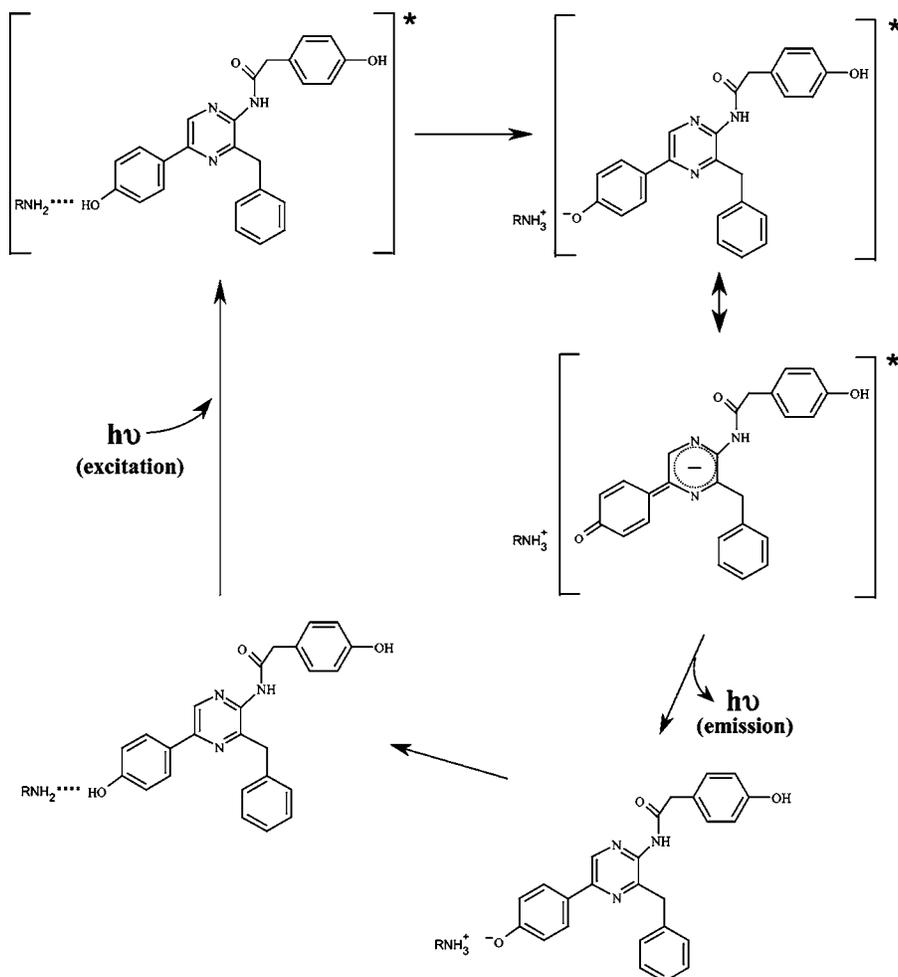
A very relevant observation was that the addition of *n*-butylamine to a benzene solution of coelenteramide produced a bimodal fluorescence spectrum with maxima at 397 and 467 nm. The fluorescence at 467 nm was assigned as being from an ion-pair excited state. In the ground state, the phenolic OH is hydrogen-bonded to the amine without ionic dissociation (Figure 4); thus, the excitation peaks (302 and 337 nm) closely correspond to those of the neutral coelenteramide.<sup>31</sup> On excitation, however, the phenolic OH proton transfers to the amine and the resulting fluorescence emission (465–479 nm) of the ion pair becomes close to, but not identical with, that of the phenolate anion species (480–490 nm). The excited-state proton transfer is feasible because, as is well-known, the phenolic OH in the excited state is a much stronger acid than that in its ground state. Hirano and co-workers<sup>32</sup> made a systematic study of the fluorescence of coelenteramide and came to similar conclusions about ion pairing with amines. They suggested that because the fluorescence of the coelenteramide phenolate anion was very sensitive to solvent polarity (fluorescence ranged from 592 nm in polar solvents such as DMSO to 466 nm in benzene), the singlet-excited state of the phenolate ion has an intramolecular charge-transfer character. In the

excited state of the coelenteramide phenolate anion, the negative charge migrates from the electron-donating 4-oxidophenyl group to the electron-accepting pyrazine ring to give a charge-transfer state in a manner similar to other reported electron donor–acceptor compounds (Figure 5).<sup>32</sup> They concluded that this configuration of the phenolate anion, rather than the amide anion, is the light emitter in aequorin bioluminescence.

It is now generally agreed that in the bioluminescence systems involving coelenterazine, that is, the photoproteins and *Renilla* luciferase, or the related *Vargula* luciferin–luciferase reaction, the bioluminescence occurs according to the mechanism suggested by McCapra and Chang.<sup>14,24,33</sup> Variation in spectral properties in each case can be fully accounted for by differences in the ionic state of the excited product and by the influence of the environment of the binding site cavity that can perturb the energy level of the product excited state.

## The Function of Active Site Residues in Formation of the Excited State

The crystal structures of several photoproteins have been solved: aequorin with resolution of 2.3 Å,<sup>28</sup> obelin from *O. longissima* at 1.7<sup>34</sup> and 1.1 Å,<sup>29,35</sup> and some other obelins.<sup>23,36–38</sup> As expected from the homology of their primary sequences, all photoproteins have the same compact globular structure (Figure 6A). The tertiary structure contains two sets of four helices comprising helix–turn–helix (HTH) motifs, I and II in the N-terminal region and III and IV in the C-terminal region (Figures 1 and 6A). The peroxy-coelenterazine binding pocket is highly hydrophobic and is formed by residues originating from each of the helices (Figure 6B). In addition, several hydrophilic side chains are also directed internally. These are His22 and Tyr138 and His175 from HTH I and IV,



**FIGURE 5.** The scheme for formation of the singlet-excited state of the phenolate anion of coelenteramide with an intramolecular charge-transfer character.

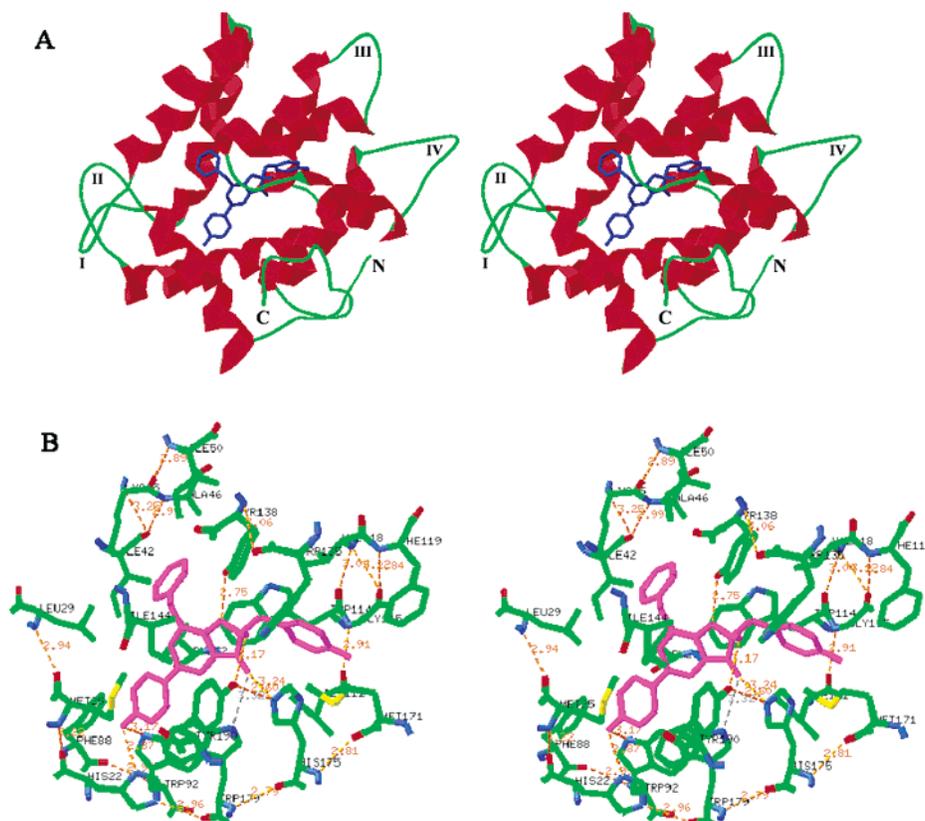
respectively, and Tyr190, which is near the C-terminus of the protein. Most of the contacts between the protein moiety and the peroxy-coelenterazine are observed for residues in HTH III.

It can be seen in Figure 1 that almost all the residues forming the peroxy-coelenterazine binding pocket are conserved in the sequence of each type of photoprotein. Therefore, it would be predicted that the spatial structure of the peroxy-coelenterazine binding pocket should also be conserved in each. The superposition of the peroxy-coelenterazine and some active site residues of two photoproteins, aequorin and obelin, clearly supports this expectation (Figure 7).

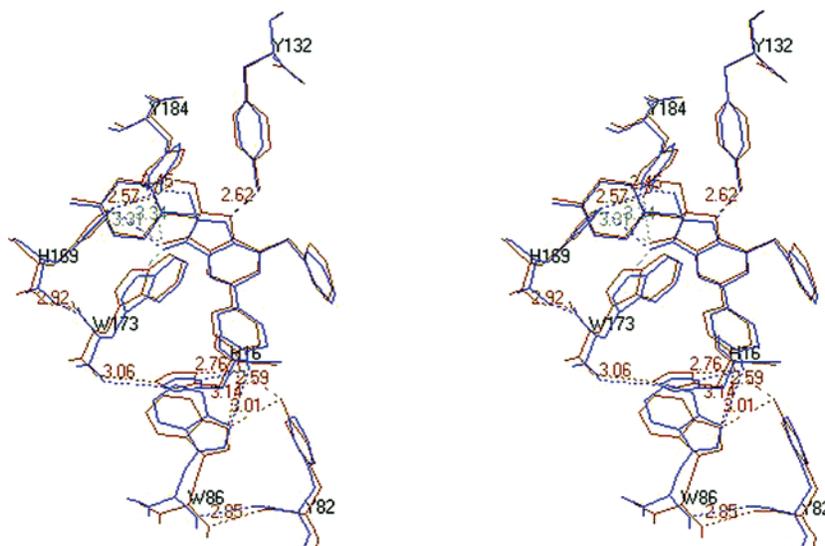
The importance of certain amino acid residues for the bioluminescence of these photoproteins was apparent before the three-dimensional structure was solved. Site-directed mutagenesis and direct chemical modifications implicated some tryptophan, histidine, and cysteine residues and the C-terminal proline. Aequorin mutants with replacement of tryptophan residues for phenylalanine exhibited various bioluminescence activities and spectra including a W86F mutant that displayed a bimodal emission with maxima at 455 and 400 nm.<sup>39</sup> Ohmiya and co-workers concluded that Trp86 could be involved in the generation of the excited state. The solved photoprotein

structures<sup>28,29,34</sup> support this suggestion because Trp92, Trp114, Trp135, and Trp179 (numbered according to the obelin sequence; Figures 7 and 8) are among the residues that make close contact with the peroxy-coelenterazine. For instance, the side chains of Trp92 and Trp179 “sandwich” the 6-(*p*-hydroxy)phenyl ring of coelenterazine, rendering the planes of the phenyl ring and the Trp92 indole (Trp86 for aequorin) almost parallel (Figures 6B and 7). The Trp114 and Trp135 side chains would appear to restrict any motion of the *p*-hydroxybenzyl group. Assuming that this sandwich is preserved in the Ca<sup>2+</sup>-discharged structure, this would account for the rigid binding of the coelenteramide, as concluded from the observation of a high fluorescence anisotropy,<sup>23</sup> a feature with direct biological function in that a rigid environment would favor a high fluorescence quantum yield for the coelenteramide.

To understand why the substitution of Trp86 in aequorin enhances bioluminescence emission from the neutral excited state of coelenteramide, we produced the corresponding obelin mutant, W92F, and determined its spatial structure.<sup>23,36</sup> The bioluminescence of W92F obelin was a violet color as a result of the addition of a new band with  $\lambda_{\max} = 405$  nm having an intensity similar to that of the band at the longer wavelength. This substitution of Trp92 in obelin results in a larger relative intensity of the



**FIGURE 6.** Stereoview of (A) the overall three-dimensional structure of obelin (PDB 1EL4). The peroxy-coelenterazine is in blue. Roman numbers I–IV designate the loops of HTH motifs; N and C letters indicate N- and C-terminus of the photoprotein, respectively. Panel B shows the stereoview of the peroxy-coelenterazine binding site region with the hydrogen bond network within 4 Å. Peroxy-coelenterazine is colored in pink.



**FIGURE 7.** Stereoview of superposition of some active site residues and peroxy-coelenterazine molecules of aequorin (PDB 1EJ3; brown) and obelin (PDB 1EL4; blue). The residues are numbered according to the aequorin sequence.<sup>28</sup> Hydrogen bond distances are shown for the aequorin structure.

shorter wavelength emission than in the case of W86F aequorin. The crystal structure of W92F obelin solved at 1.72 Å resolution revealed no significant differences between the dimensions of the active sites of WT and W92F obelins (Figures 8 and 9).

In particular, the proximity of His22 to the OH in both obelin and W92F obelin immediately suggests an explana-

tion for the formation of the different bioluminescent excited states (Figure 9). The primary excited product, the amide anion, would be expected to be rapidly protonated before it has a chance for radiative emission. An amide has a very high  $pK$  and even though the  $pK^*$  might be lowered a few units, there are a number of proton sources in the binding cavity. One candidate is the Tyr138, which



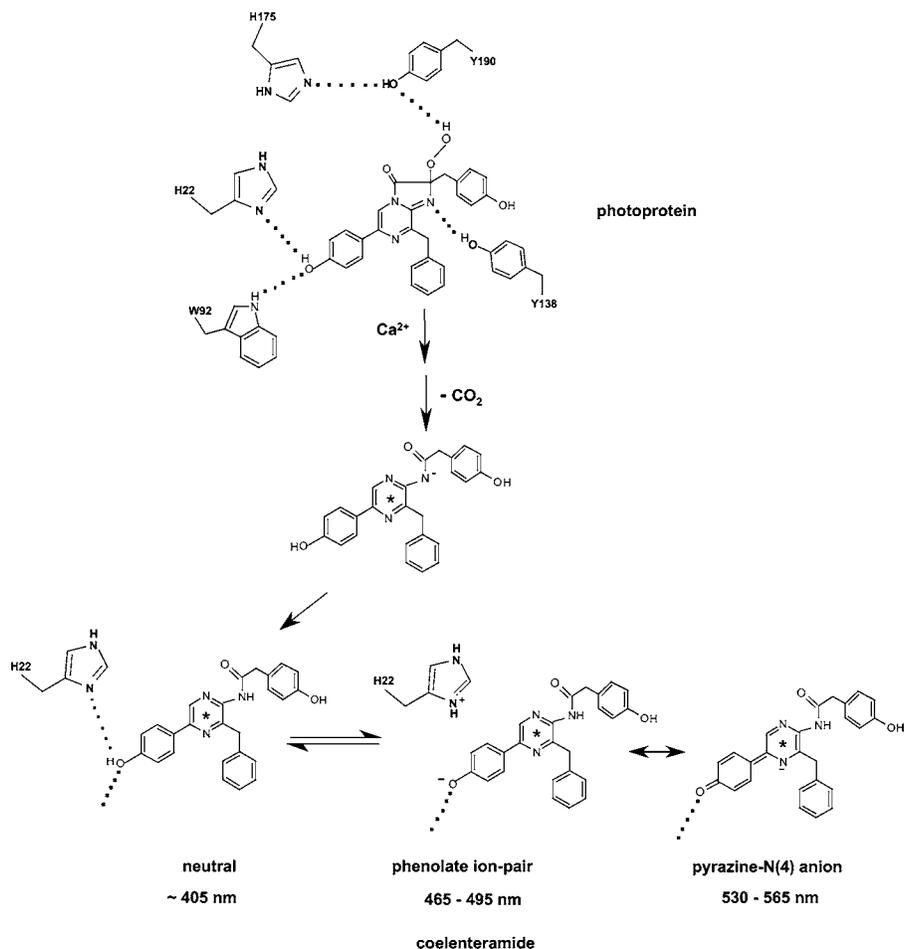


FIGURE 10. Formation of bioluminescent excited states within the binding cavity of obelin.

of the Ca<sup>2+</sup>-discharged obelin is in the green, knowledge of the spatial structure of the mutants should provide a rational basis for the design of a mutant with green bioluminescence.

### Proton-Relay Mechanism for the Ca<sup>2+</sup> Trigger

In the binding site of aequorin and obelin, the H-bond network around the coelenterazine is very similar. It is on this basis that a proton-relay hypothesis has been formulated as to how Ca<sup>2+</sup> binding could trigger the bioluminescence reaction<sup>23</sup> (Figure 11). In any protein crystal structure, hydrogen bonds are inferred if the separation of a putative H-donor and acceptor is less than about 3 Å, and in Figures 11, 12, and 13, these inferred H-bonds are indicated by the dash lines between the donor H atoms and the acceptor. The H atom itself is too light for its electron density to be detectable by the X-ray crystallography method, although some recent atomic resolution (below 1 Å) structures are challenging that assertion. Coelenterazine hydroperoxide is not stable in free solution, but in the hydrophobic environment of the binding site, the hydroperoxide group appears to be stabilized by the H-bond to Tyr190. In turn there is an H-bond from Tyr190 to His175. This same arrangement is seen in all other obelins and in aequorin.<sup>28,29,37,38</sup>

The first step in the model chemiluminescence reaction of McCapra and Chang is the formation via the strong

base of the peroxyanion, which then cyclizes to the dioxetane. Figure 11 incorporates this part of the McCapra/Chang mechanism in steps II–IV. In the protein active site, it is suggested that His175 plays the role of the base via its H-bond to Tyr190.

The H-bond distance between Tyr190 and His175 is 2.64 Å (Figure 13), which is indicative of an H-bond with moderately strong electrostatic character.<sup>41</sup> The hypothetical mechanism is that as a direct result of Ca<sup>2+</sup> binding, the Tyr190–His175 H-bond becomes stronger, increasing the electrostatic contribution, being equivalent to saying that the His175 is partially protonated (step I). Because the tyrosine and hydroperoxide have similar pKs around 10, there will be a probability that the hydroperoxide will protonate the tyrosinate and the peroxy anion (step II) then has another probability of irreversible nucleophilic addition to the C3-carbon of coelenterazine to form the committed dioxetanone intermediate (step III). The exergonicity of this last step provides the thermodynamic feasibility of the overall process.

How should the Ca<sup>2+</sup> binding initiate this shift? In the superfamily of EF-hand calcium-binding proteins, the bound calcium ion is found associated specifically in a consensus sequence in the loop region of the HTH motifs. In practice, the identification of the bound Ca<sup>2+</sup> is by its strong electron density and, as well, in almost all cases, by a bipyramidal pentagonal coordination with a bond

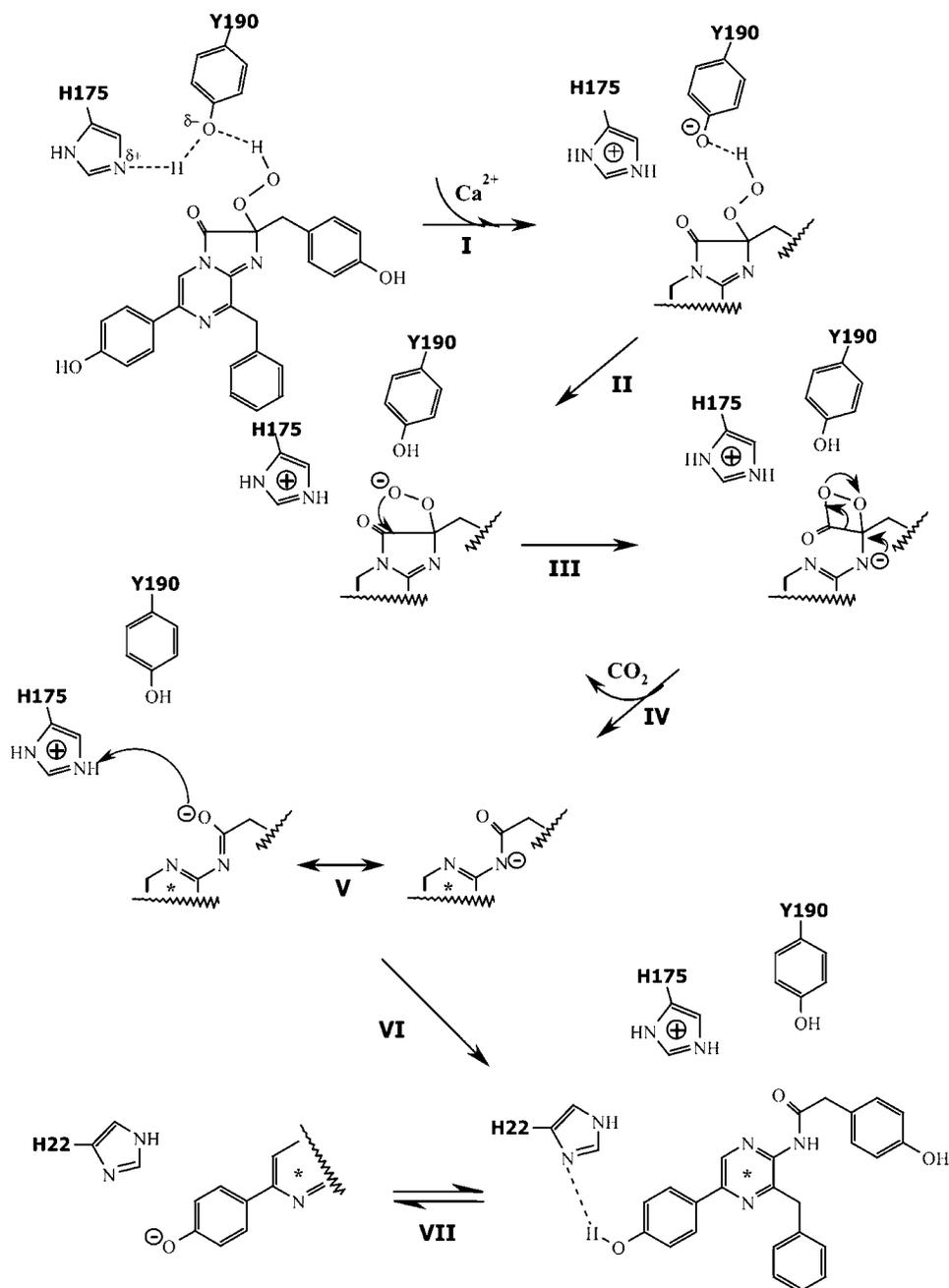


FIGURE 11. Proton-relay mechanism of the Ca<sup>2+</sup> trigger and formation of the bioluminescent emitters.

length close to 2.4 Å between the central atom and the coordination partner.<sup>42</sup> The recent structure of an obelin crystal with Ca<sup>2+</sup> bound in loop I indicates adherence to these average bond length specifications.

In the published spatial structures of photoproteins, the loop structures are not prepositioned for calcium binding, that is, especially in the two C-terminal loops, some movement of the residues must occur on Ca<sup>2+</sup> binding to happily accommodate the coordinating atoms to the required 2.4 Å separation. In photoproteins, the first step in the generation of high-intensity bioluminescence must be the binding of Ca<sup>2+</sup> to the loops within the EF-hands. It is observed in Figures 12 and 13 that certain residues within the exiting helices of loops III and IV, namely, Tyr138, His175, and Trp179, as well as Tyr190,

have critical proximity to the substrate in the reactive center. Therefore any conformational adjustment in the binding loops accompanying Ca<sup>2+</sup> binding can be expected to propagate into shifts of the hydrogen bond donor–acceptor separations around the coelenterazine, the ones apparently essential for the hydroperoxide stability, the networks O34, Tyr190, His175, O18, and N1 to Tyr138. Because the pKs of the tyrosine hydroxyl and the hydroperoxide are very close, as already noted, and the position of His175 is poised to act as a general base, the destabilization of the substrate is thereby triggered.

To initiate the shift of hydrogen donor–acceptor separations, the small spatial shift of the exiting α-helix of loop IV probably will be enough since the most of the residues mentioned above, including His175, which very

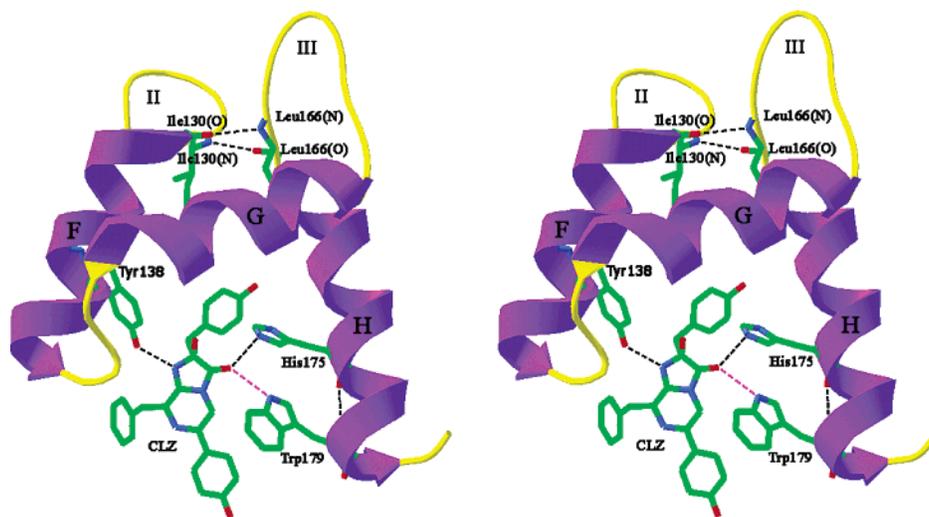


FIGURE 12. Stereoview of loop–loop connectivity and relationship to the bound coelenterazine (PDB 1EL4).

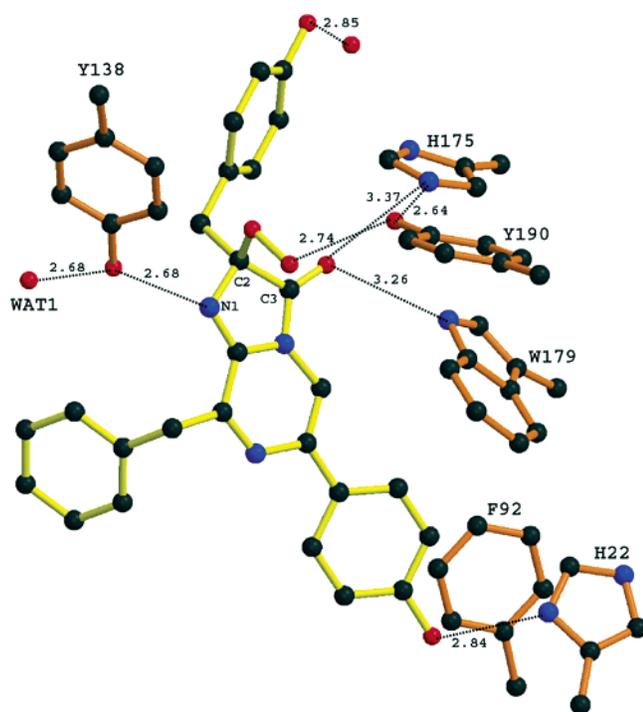


FIGURE 13. The interaction of peroxy-coelenterazine with the residues at the binding pocket.

likely is the key residue, are found in this  $\alpha$ -helix. The notion that a histidine residue in this position is very important for photoprotein activity is supported by observations on aequorin. Site-directed mutagenesis of the five histidine residues in aequorin has shown that substitution of His169 (corresponding to His175 for obelin) to alanine, phenylalanine, or tryptophan leads to complete loss of activity, whereas modification of the remaining four histidine residues yielded mutant aequorins with varying bioluminescence activities.<sup>43</sup> The process of hydrogen donor–acceptor separation may be fast and will be irreversible because it initiates the chemical reaction of coelenterazine decarboxylation, and its rate would then be independent of calcium concentration.

Evidence for a conformational change on Ca<sup>2+</sup> binding has been shown by NMR.<sup>44</sup> There is a clear difference in

the <sup>15</sup>N-heteronuclear single quantum coherence (HSQC) spectra between obelin and its Ca-loaded state. A structural change also occurs in aequorin following calcium addition. This was monitored by an increase in tryptophan residue fluorescence, but the rate of this change is much slower than the bioluminescence emission rate.<sup>45</sup>

## Conclusions

The foregoing ideas about the mechanism of Ca<sup>2+</sup> triggering of photoprotein bioluminescence and production of the different ionic excited states have to be based on spatial structure information, which is only available for the photoprotein itself (obelin or aequorin). The relevant structure, however, is that of the Ca<sup>2+</sup>-loaded, Ca<sup>2+</sup>-discharged photoprotein or, even more desirable but hardly attainable, the structure at the instant of bioluminescence emission. NMR evidence shows that the protein attains a variety of conformations depending on bound ligands, Ca<sup>2+</sup>-loading, etc., and for aequorin, there may be a slow conformation change following the completion of the bioluminescence reaction. In studies of the large family of Ca<sup>2+</sup>-binding proteins, the goal is to obtain the structures of both Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-loaded states so that the pathway of conformation change, which is the basis of the Ca<sup>2+</sup>-signaling function of the protein, might be understood. For the photoproteins, we predict from the hypothesis that the Ca<sup>2+</sup>-bound state will show a shift in the structure of the C-terminal helix resulting in change in separation of the two key residues in the active site, His175 and Tyr190, with a preservation of H-bonding between His22 and the hydroxy group shown in Figure 11. Three-dimensional structural information required to test the hypothesis is being actively pursued.

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