Crystal Structure of a Ca\(^{2+}\)-discharged Photoprotein

**IMPLICATIONS FOR MECHANISMS OF THE CALCIUM TRIGGER AND BIOLUMINESCENCE**

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Ca\(^{2+}\)-regulated photoproteins are members of the EF-hand calcium-binding protein family. The addition of Ca\(^{2+}\) produces a blue bioluminescence by triggering a decarboxylation reaction of protein-bound hydroperoxycoelenterazine to form the product, coelenteramide, in an excited state. Based on the spatial structures of aequorin and several obelins, we have postulated mechanisms for the Ca\(^{2+}\) trigger and for generation of the different excited states that are the origin of the different colors of bioluminescence. Here we report the crystal structure of the Ca\(^{2+}\)-discharged photoprotein obelin at 1.96-Å resolution. The results lend support to the proposed mechanisms and provide new structural insight into details of these processes. Global conformational changes caused by Ca\(^{2+}\) association are typical of the class of calcium signal modulators within the EF-hand protein superfamily. Accommodation of the Ca\(^{2+}\) ions into the loops of the EF-hands is seen to propagate into the active site of the protein now occupied by the coelenteramide where there is a significant repositioning and flipping of the His-175 imidazole ring as crucially required in the trigger hypothesis. Also the H-bonding between His-22 and the coelenterazined found in the active photoprotein is preserved at the equivalent position of coelenteramide, confirming the proposed rapid excited state proton transfer that would lead to the excited state of the phenolate ion pair, which is responsible for the blue emission of bioluminescence.

Calcium-binding proteins are probably one of the most extensively studied protein families. The main reason for such attention is because these proteins regulate numerous vital intracellular events in living cells and organisms. The members of this family are related not necessarily by any similarity in function but by the fact that most of them selectively bind calcium through a homologous structural unit known as the EF-hand (1, 2). The three-dimensional structures of more than fifty EF-hand proteins have been determined and these fall into about twelve functional types (3) that can in part be combined into two functional classes, Ca\(^{2+}\) sensor proteins and Ca\(^{2+}\) signal modulators. Upon binding Ca\(^{2+}\), the Ca\(^{2+}\) sensor proteins undergo large conformational changes, which may be functionally related. The well known representatives of this group, calmodulin and troponin C, appear to function by exposing a large hydrophobic area upon binding Ca\(^{2+}\), which enables them to target certain receptor proteins. In contrast, the Ca\(^{2+}\) signal modulators show a much smaller conformational transition upon loading with Ca\(^{2+}\). A well known Ca\(^{2+}\) signal modulator is parvalbumin, the first structure determined for an EF-hand protein. It is not known how the configurations of the EF-hand structural motifs determine whether Ca\(^{2+}\) binding will induce a conformational transition or not, and for many of these proteins even the cellular function is debated.

There is however, one subfamily of proteins in this broad Ca\(^{2+}\)-binding protein family, the Ca\(^{2+}\)-regulated photoprotein subfamily that has a well established function. It is to emit blue photons upon the appearance of Ca\(^{2+}\), a function evidently with some survival benefit for the host, although the real nature of the benefit is not known. Aequorin, named for the jellyfish _Aequorea_ from which it was isolated, was the first Ca\(^{2+}\)-regulated photoprotein to be discovered (4) and has been extensively investigated (5). Aequorin emits blue light upon the addition of Ca\(^{2+}\), but the bioluminescence from the jellyfish itself is green, corresponding to the fluorescence of the famous green fluorescent protein, which is excited to fluoresce secondarily by the energy generated from the biochemical reaction in the aequorin. Ca\(^{2+}\)-regulated photoproteins have now been detected in a variety of bioluminescent marine organisms, mostly coelenterates (6). The primary sequence of aequorin determined after almost 25 years revealed the canonical Ca\(^{2+}\)-binding EF-hands (7, 8), and this is now known to be common in the sequences of photoproteins from other genera, such as obelin from _Obelia_ (9, 10), and all show high homology to aequorin.

The bioluminescence activity of these photoproteins arises from the chemical breakdown of coelenterazine, an imidazolopyrazine derivative substituted by a hydroperoxy at the C-2 position and tightly but noncovalently bound within the photoprotein (Scheme 1A, _R_\(^1_1 = OH, R_2 = benzyl, R_3 = p\)-hydroxybenzyl). Early in the history of this subject McCapra and Chang (11) investigated the chemiluminescence of a certain imidazolopyrazine derivative (Scheme 1A, _R_\(^1_1 = H, R_2 = R_3 = methyl) and established the reaction mechanism in Scheme 1. In the photoprotein the otherwise highly labile hydroperoxycoelenterazine (Scheme 1B) is stabilized within an active site cavity. A photoprotein, in other words, can be regarded as an enzyme having a stabilized reaction intermediate. The binding of Ca\(^{2+}\) initiates the decarboxylation resulting in the excited
state of the product, coelenteramide (Scheme 1C), which remains bound within the protein. Calcium is not essential for the bioluminescence of photoproteins because alone they give off a very low level of light emission known as calcium-independent luminescence (12); however, the light intensity is increased up to 1 million-fold or more upon the addition of calcium.

The bioluminescence spectral distributions of photoproteins are broad with maxima in the range of 465 to 495 nm depending on the type. Photoproteins themselves are hardly fluorescent but the Ca\(^{2+}\)-discharged photoproteins have strong fluorescence with type-dependent maxima in the range of 465 to 520 nm. Fluorescence studies of coelenteramide and its analogues have shown that the differences of the bioluminescence spectra among photoproteins are attributable to the activation of different ion excited states of coelenteramide under the influence of the environment of the active site. These states include the neutral coelenteramide with a fluorescence spectral maximum around 400 nm, the amide monoanion with a fluorescence spectral maximum around 450 nm, a phenolate ion pair with a fluorescence spectral maximum in the range of 465 to 495 nm, and a pyrazine-N(4) anion with a fluorescence spectral maximum in the range of 530 to 565 nm (13, 14).

Recently the crystal structures of several photoproteins have been solved. These are aequorin (15), obelin from Obelia longissima (16, 17), and some other obelins (18, 19). As expected from the homology of the primary sequences, all the photoproteins have the same compact globular structure. The tertiary structure contains two sets of four helices composed of helix-turn-helix motifs I and II in the N-terminal end and III and IV in the C-terminal end. The hydroperoxycelenterazine binding cavity is highly hydrophobic and is formed by residues originating from each of the helices. In addition, several hydrophilic side chains are also directed internally. These are His-22, Tyr-138, and His-175 from helix-turn-helix motifs I and IV, respectively, and Tyr-190, which is near the C terminus of the protein (the residues are numbered according to the obelin sequence). The side chains of these hydrophilic residues form a network of hydrogen bonds that apparently stabilizes the highly labile hydroperoxycelenterazine.

It has been generally considered that, as with other calcium-binding proteins, a structural change induced by Ca\(^{2+}\) is responsible for initiating the full bioluminescence activity. Indeed, heteronuclear single quantum coherence-NMR experiments indicate five distinct conformation states controlled by the binding of the various ligands, Ca\(^{2+}\), coelenterazine, and coelenteramide (Fig. 1) (20). The structure of conformation state II has been determined for aequorin and obelin. Based on these spatial structures and the detection of the hydrogen-bond network in the binding cavity we have proposed mechanisms for how the binding of Ca\(^{2+}\) might trigger the bioluminescence reaction and for how different excited states could result. Clearly however, for testing these hypotheses and for revealing intimate details of the processes, the structures of several of these conformation states would be highly desirable to have in hand.

In this paper we report the crystal structure of a Ca\(^{2+}\)-discharged photoprotein bound with the product of the bioluminescent reaction, coelenteramide (conformation state IV in Fig. 1) solved at 1.96-Å resolution. The results lend support to the proposed mechanisms for the Ca\(^{2+}\) trigger and for the formation of the different excited states of photoprotein bioluminescence, providing new structural insight into the details of these processes.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—High purity recombinant W92F obelin\(^{1}\) was prepared as described elsewhere (19). Apo-W92F obelin was converted to a photoprotein with synthetic coelenterazine (Prolume Ltd., Pittsburgh, PA). The procedure for discharging W92F obelin was to slowly add CaCl\(_2\) to a 1 mg/ml solution of the protein in 10 mM bis-Tris, pH 7.0, until bioluminescence emission ceased and the solution was colorless. This procedure and the crystallization of the Ca\(^{2+}\)-discharged W92F obelin are described in detail elsewhere (21). The crystallization condition is 1.5 x tri-sodium citrate in 0.1 x Na-Hepes buffer, pH 7.5, at 4 °C. Crystals took 1 month to grow to a size suitable for diffraction with dimensions of 0.15 x 0.1 x 0.05 mm. The crystal fluorescence of the Ca\(^{2+}\)-discharged W92F obelin was examined. A single crystal was transferred to a droplet containing only the crystallization mother liquor. The green fluorescence from the crystal that was observed under a stereomicroscope with excitation at 350 nm (Fig. 5) clearly indicated that coelenteramide is bound within the protein.

**Data Collection and Data Processing**—A crystal was directly mounted to a fiber loop and flash-frozen into liquid nitrogen before the data collection process. A 360° data set of Ca\(^{2+}\)-discharged W92F obelin was collected at the in-house copper x-ray source with a Bruker Smart 6000 charge-coupled device detector. An 8° of 2θ was used for the purpose of extending the data resolution to 1.9 Å. The data were processed and scaled by the Bruker program Proteum. The crystal of Ca\(^{2+}\)-discharged W92F obelin belongs to a P4\(_1\)2\(_1\)2 space group with unit cell dimensions of a = b = 53.4 Å, and c = 144.0 Å. There is one molecule in the asymmetric unit. Data collection statistics are collected in Table I.

**Phasing and Refinement**—Rigid body refinement for Ca\(^{2+}\)-discharged W92F obelin was directly carried out with the structure of W92F obelin (Protein Data Bank accession code 1JF2) as the starting model using Refmac5 (22), resulting in an R factor of 30.3% and an R-free factor of 30.7%. Further refinement of Ca\(^{2+}\)-discharged W92F obelin was extended to 1.96 Å using Refmac5. The electron density of coelenteramide was clearly shown in the center of the protein molecule. Each cycle of refinement was followed by manual model adjustment using XTALVIEW (23). The final refinement statistics are shown in Table I. The final stereochemical parameters of the structure were evaluated with the programs PROCHECK (24) and MolProbity (25). Atomic coordinates have been deposited to the Protein Data Bank with accession code 1S36.

**RESULTS**

**Overall Structure of Ca\(^{2+}\)-discharged W92F Obelin**—Although study of several types of Ca\(^{2+}\)-discharged photoproteins was attempted, only the Ca\(^{2+}\)-discharged W92F obelin produced crystals of sufficient quality to enable a structure solution at 1.96-Å resolution (Table I) (21). This W92F mutant of obelin emits two bands of bioluminescence, one blue and the other in the violet range, but the fluorescence of the Ca\(^{2+}\)-

\(^{1}\)The abbreviations used are: W92F obelin, Obelia longissima obelin mutant with substitution of Trp to Phe; bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-hydroxyethyl)propane-1,3-diol; r.m.s.d., root mean square deviation.
removal of calcium ions from the product; V, the apoprotein; by heteronuclear single quantum coherence-NMR spectroscopy the addition of Ca\(^{2+}\) to cause the bioluminescence reaction; IV, upon removal of calcium ions from the product; V, Ca\(^{2+}\) bound to the apoprotein. The structure reported here represents state IV.

### Table I

**X-ray crystallographic statistics**

<table>
<thead>
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<th>Data collection and processing statistics</th>
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<tbody>
<tr>
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<td>Resolution (Å)</td>
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<tr>
<td>Completeness (last shell) (%)</td>
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<td>Rmerge (5) (last shell) (%)</td>
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</table>

<table>
<thead>
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<td>Number of solvent atoms</td>
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<tr>
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<tr>
<td>(R_{merge}) value ((Å))</td>
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</tr>
<tr>
<td>r.m.s.d. bond angles (degree)</td>
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</tr>
</tbody>
</table>

\[ R_{merge} = \frac{\Sigma_{hkI} Σ_{I}(|I_{hkI}|-|I_{hkI}|)\sqrt{\Sigma_{hkI}}} {\Sigma_{hkI} I_{hkI}}. \]

The discharged protein is green like the wild type obelin, and other biochemical properties are also the same. The three-dimensional structures of W92F and the wild type are also practically identical (18, 19), so it is valid to assume that the two structures of W92F before and after the bioluminescence generally represents the conformation change occurring in a photoprotein reaction.

Fig. 2 shows that the crystal structure of the Ca\(^{2+}\)-discharged photoprotein retains the same overall scaffold as the undischarged photoprotein (18, 19). The r.m.s.d. from the 185 C-α atomic positions of Ca\(^{2+}\)-discharged W92F obelin versus W92F obelin is only 0.306 Å. Fig. 2b shows the well conserved structural features between these two protein states, the one primed with the hydroperoxycoelenterazine and the other with the bioluminescence reaction product, coelenteramide. This observation suggests that Ca\(^{2+}\)-regulated photoproteins belong to the category of Ca\(^{2+}\) signal modulators rather than to the category of Ca\(^{2+}\) sensors in the EF-hand protein family (3). The first six residues at the N terminus and the four residues at the loop region of the third helix-turn-helix motif were not observed in the electron density maps and were assumed to be disordered.

**Structure of the Active Site Cavity**—The coelenteramide fits precisely into the electron density (Fig. 3a), and it is buried in a highly hydrophobic cavity situated at the center of the protein structure (Fig. 2a) in the same place as the precursor hydroperoxycoelenterazine (18, 19), surrounded by residues from each of the eight helices of the protein. This solvent-inaccessible cavity apparently provides the necessary environment for efficient excited state product generation and fluorescence. Going from the hydroperoxycoelenterazine to coelenteramide, the biggest structural change of the molecule (Fig. 3b) is in the reaction center around the C-2 position (C-2, O-33, and C-10, Fig. 4), resulting in an obvious orientational deviation of the phenol group at the C-10 position. The other parts of the coelenterazine molecule also adjust positions a little but not dramatically.

The key residues facing into the binding cavity and interacting with the hydroperoxycoelenterazine do not shift positions after the reaction except for His-175 and Tyr-138 (Figs. 3b and 4). The Tyr-138 was in an H-bonding interaction with the N-1 of hydroperoxycoelenterazine but has now moved away with a slight rotation (Fig. 4, 4.33 Å, 13°). The His-175 has flipped the imidazole ring perpendicular (63°) to the original conformation toward the hydroxyl group of Tyr-190. A water molecule (blue ball) originally hydrogen-bonded to Tyr-138 is no longer there after reaction, and two new water molecules (brown balls) are introduced into the binding cavity.

The published crystal structures of photoproteins all have typical EF-hand spatial structure characteristics, but the loop structures III and IV are not prepositioned for Ca\(^{2+}\) binding. Some movements of the ligating residues must have to occur upon Ca\(^{2+}\) binding to accommodate the coordinating atoms to the required 2.4-Å separation commonly observed in the Ca\(^{2+}\)-loaded structures of other EF-hand proteins (26), including the Ca\(^{2+}\) in loop I of obelin (17). The hypothesis that we formulated (19) for the way in which calcium binding triggers the photoprotein bioluminescence is that the loop residue displacement to accommodate the Ca\(^{2+}\) is transmitted to the helices of EF-hand IV where the critical His residue (His-175 in obelin) is located. The spatial displacement of His-175 is crucial for Ca\(^{2+}\) triggering according to the proposed mechanism because it disrupts the hydrogen-bond network by which the hydroperoxycoelenterazine had been stabilized. That this His-175 plays an essential role for bioluminescence activity is supported by observations of aequorin. Site-directed mutagenesis of the five histidine residues in aequorin has shown that substitution of His-169 (corresponding to His-175 for obelin) to Ala, Phe, or Trp leads to complete loss of activity, whereas modification of the remaining four histidine residues yields mutant aequorins with varying bioluminescence activities (27).

In Scheme 2 we show how this Ca\(^{2+}\)-triggered displacement of the imidazole ring of His-175 could lead to the intermediate steps resulting in generation of the product excited states. With the caution that a change of the His-175 to Tyr-190 with a hydrogen-bond distance of 2.64 to 2.54 Å is hardly significant at this resolution, reducing this separation would make the H-bond stronger (28) by an increased electrostatic character equivalent to a partial positive charge on the His-175 and then...
leave Tyr-190 partially in the negatively charged phenolate form. We represent this in Scheme 2 (step 1) by a transient proton transfer from Tyr-190 to His-175. Because the Tyr-190 phenol and the hydroperoxy group have similar pK values, proton transfer from the hydroperoxy is feasible (step 2) leaving the peroxy anion to immediately undertake a nucleophilic attack on the C-3 position of the coelenterazine to form the dioxetanone intermediate (step 3). The uphill reaction sequence would be favored by the exergonicity from the irreversible formation of the dioxetanone. In the product structure (Fig. 4) it is also seen that a new water molecule has moved into position bridging Tyr-190 and the carbonyl oxygen through hydrogen bonds.

The primary excited state in the reaction in Scheme 1 is the coelenteramide amide anion but within the protein cavity this would be rapidly protonated to the excited state of the neutral coelenteramide. In the photoprotein (18) Tyr-138 makes an H-bond to the N-1 atom of hydroperoxycoelenterazine (2.68 Å) and is an obvious first choice as a proton donor. However, as Tyr-138 moves away to 4.33 Å after the reaction (Fig. 4), a more likely candidate proton would be for the one that protonated His-175 (Scheme 2) to return to Tyr-190 with the bridging water molecule now conveniently located to relay a proton to the carbonyl oxygen of the coelenteramide (step 6). Thus the proton relay cycle is completed, and the photoprotein is essentially turned over like an enzyme. Turnover has in fact been demonstrated in the case of aequorin (29).

The present structure does not reveal the loop residue displacements required in the trigger mechanism because, although the crystal was grown from the protein solution in the presence of calcium, the precipitant used for successfully obtaining a good crystal was sodium citrate, which is a strong cation chelator (21). Consequently the crystal contains no bound Ca²⁺ and therefore corresponds to conformation state IV in Fig. 1. However the crystals do give a green fluorescence similar to that of a solution of Ca²⁺-discharged W92F obelin with or without the presence of calcium ions (Fig. 5) (19). Calcium binding shifts the fluorescence maximum to a longer wavelength by only 10 nm, so it is reasonable to assume that the coelenteramide binding environment in the current crystal structure (the IV conformation state of Fig. 1) is practically the same as in the Ca²⁺-loaded state (conformation state III of Fig. 1). It is probable that the difference in the NMR spectra observed for these two conformation states arises from the displacement of residues involved in the Ca²⁺ binding.

The suggested mechanism (Scheme 2) also provides a reasonable explanation for calcium-independent luminescence. It is conceivable that proton transfer from Tyr-190 to His-175 could also occur in the absence of Ca²⁺ through the spontaneous motion of these residues resulting in a decrease in H-bond distance. The extremely small probability of this occurrence would probably account for this phenomenon.
Fluorescence and Bioluminescence Spectra—The bioluminescence emission from W92F obelin is bimodal with one band maximum at 405 nm and the other similar to the band maximum from wild type obelin at 485 nm (18). Other position 92 mutants also produce bimodal emission spectra with W92R obelin almost completely monomodal at the shorter wavelength (30). The 405-nm band originates from the excited state of the neutral coelenteramide bound to the protein. On the basis of the photoprotein structure and fluorescence model studies it has been suggested that the 485-nm band is from the excited state of the 5-phenolate ion of coelenteramide in an ion pair interaction with His-22 (19, 30) (Scheme 2). In the first electronic excited state, the $pK_a$ of the 5-hydroxyphenyl group should be sufficiently near that of the His-22 to make rapid proton transfer feasible, particularly as the His-22 H-bond interaction in W92F obelin (2.84 Å) is practically unchanged (2.82 Å) in the Ca$^{2+}$-discharged photoprotein (Fig. 4).

The fluorescence spectra of wild type and W92F obelin have practically the same maximum values (18). As the bioluminescence and fluorescence are believed to originate from the same excited state species, a 25-nm redshift of the fluorescence from the bioluminescence would result if the final structure of the binding cavity were more polar than that at the instant of bioluminescence emission. Model studies do show that the fluorescence spectra of coelenteramide anions shift to a longer wavelength in more polar solvents. It is noted that in comparison with hydroperoxycelenterazine in the photoprotein cavity, two new water molecules are now in H-bonding interactions with the coelenteramide (Fig. 4). If the water molecule interacting with N-4 were to arrive there only in some relaxation period following the bioluminescence emission, then plausibly this would render the environment more polar for the fluorescence measurement than during the bioluminescence.

DISCUSSION
Evidence from the structure of the active site cavity in the Ca$^{2+}$-discharged photoprotein lends support to the ideas concerning the mechanism through which the Ca$^{2+}$ triggers the reaction and the pathway leading to generation of the bioluminescent excited states. The His-22 that was proposed to have
an excited state ion pair interaction to explain the population of alternative ionic excited states can clearly fulfill this role, as it is seen to remain unchanged in its H-bonding to the hydroxy oxygen of the 5-hydroxyphenyl substituent of coelenteramide. The binding site residue His-175, already implicated as having a key role in triggering the bioluminescence reaction, shifts position to provide a pathway for a proton relay process to destabilize the hydroperoxycelenterazine. Additionally, two water molecules move into interaction with the reaction product coelenteramide, one of which would provide a means for the proton relay to cycle. To complete the photoprotein story with more direct support for the trigger mechanism, it will be necessary to solve the structure of the Ca$^{2+}$-loaded conformation (III), which should reveal the effect of residue shifts in the Ca$^{2+}$-binding loops propagating into the shift of the His-175 position. Perhaps this shift would then be more substantial. Other questions yet to be addressed include the origin of spectral differences between the types of photoprotein bioluminescence and the reason for the shift of fluorescence from the bioluminescence of discharged obelins, a phenomenon that has not been observed in the case of aequorin. Probably all of these spectral differences can be attributed to variations in effective dielectric constants in the different binding cavities, and it will be important to have the Ca$^{2+}$-buffered aequorin structure to provide evidence in this regard.

If very little structural change occurs in Ca$^{2+}$-regulated photoproteins following interaction with Ca$^{2+}$, then these proteins would appear to fall in the Ca$^{2+}$ signal modulator class of EF-hand proteins and potentially implement similar functions in living cells peculiar to this class of EF-hand proteins, i.e. modulation of calcium influx. The photoproteins are located in special light-emitting cells (photocytes), and in the case of the hydroid Obelia for example (31), the bioluminescence response from these cells is subject to complex regulation. The influx of calcium into nonluminescent endodermal cells through conventional voltage-dependent calcium channels is required for the excitation-luminescence coupling. Then the subsequent diffusion of this calcium, via gap junctions, into the neighboring photocytes triggers a localized luminescence response. Following intense stimulation, the local rise in calcium elicits a second wave of luminescence that is supported by a voltage-independent calcium permeability mechanism in the photocyte plasma membrane. This suggests that photoproteins might also modulate calcium signals in this organism. Tantalizing questions for future illumination remain, however, including how a protein that modulates calcium signals has evolved to produce bioluminescence in coelenterates but not in related organisms and in which other organisms this same protein might be found.

**REFERENCES**

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