All three Ca^{2+} -binding loops of photoproteins bind calcium ions: The crystal structures of calcium-loaded apo-aequorin and apo-obelin

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Abstract

The crystal structures of calcium-loaded apo-aequorin and apo-obelin have been determined at resolutions 1.7 Å and 2.2 Å, respectively. A calcium ion is observed in each of the three EF-hand loops that have the canonical calcium-binding sequence, and each is coordinated in the characteristic pentagonal bipyramidal configuration. The calcium-loaded apo-proteins retain the same compact scaffold and overall fold as the unreacted photoproteins containing the bound substrate, 2-hydroperoxycoelenterazine, and also the same as the Ca^{2+} -discharged obelin bound with the product, coelenteramide. Nevertheless, there are easily discerned shifts in both helix and loop regions, and the shifts are not the same between the two proteins. It is suggested that these subtle shifts are the basis of the ability of these photoproteins to sense Ca^{2+} concentration transients and to produce their bioluminescence response on the millisecond timescale. A mechanism of intrastructural transmission of the calcium signal is proposed.

Keywords: bioluminescence; EF-hand; fluorescent protein; proton relay; calcium-binding loops; aequorin; obelin; diffraction

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Aequorin and obelin are Ca^{2+} -regulated photoproteins obtained from certain bioluminescent coelenterates, the jellyfish *Aequorea* and the hydroid *Obelia*. Aequorin, discovered in the early 1960s (Shimomura et al. 1962), was found to require only the addition of calcium ions to produce a blue bioluminescence emission. The primary sequence of aequorin, determined more recently, revealed that it has three canonical Ca^{2+} -binding EF-hands (Charbonneau et al. 1985; Inouye et al. 1985). Subsequently, the same was also

found for the obelin sequence (Illarionov et al. 1995), and this is now known to be common for photoproteins from other genera (Fagan et al. 1993; Inouye and Tsuji 1993; Markova et al. 2002; Fig. S1), indicating that they are members of the EF-hand calcium-binding protein family (Moncrief et al. 1990; Kawasaki et al. 1998), one of the most extensively studied protein families. The EF-hand proteins are distinguished from other Ca^{2+} -binding proteins in that they have common calcium-binding helix-loop-helix (HLH) motifs consisting of two helices that flank a “canonical” sequence loop region of 12 contiguous residues from which the oxygen ligands for the calcium ion coordination are derived. The calcium ion is coordinated in a pentagonal bipyramidal array with an average 2.4 Å separation to oxygen atoms (Strynadka and James 1989). Frequently one or two water molecules are also involved in ligating to the calcium.

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Abbreviations: HLH, helix-loop-helix; HSQC, heteronuclear single quantum coherence; RMSD, root mean square deviation; SAD, single wavelength anomalous dispersion.

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Proteins of the EF-hand family are grouped on the basis of their EF-hand content, not by any similarity in function that they might perform in living organisms, and in fact, for many of these proteins even their cellular function is debated. However, the Ca^{2+} -regulated photoproteins have a well-established function. It is to emit blue photons on the appearance of Ca^{2+} , a bioluminescence function evidently having some survival benefit for the host, although the real nature of the benefit is not known. The bioluminescence activity of these photoproteins arises from a Ca^{2+} -triggered chemical breakdown of “coelenterazine,” an imidazolopyrazine derivative substituted by a hydroperoxy, this derivative being tightly but noncovalently bound within the photoprotein (for reviews, see Ohmiya and Hirano 1996; Vysotski and Lee 2004). The binding of Ca^{2+} initiates an oxidative decarboxylation of the coelenterazine resulting in the excited state of the product, coelenteramide. Calcium is not essential for the bioluminescence of photoproteins because alone they give off a very low level of light emission called the “calcium-independent luminescence” (Allen et al. 1977); however, the light intensity is increased up to 1 million-fold or more on the addition of calcium.

It has been generally considered that, as with other calcium-binding proteins, a structural change induced by Ca^{2+} binding is responsible for initiating the full bioluminescence activity. Indeed, from ^{15}N -HSQC NMR experiments on obelin, it was concluded that there were five distinct conformation states controlled by the binding of various ligands: apo-protein (state **I**), apo-protein with Ca^{2+} (state **V**), the photoprotein itself with coelenterazine (state **II**), and the product with coelenteramide with (state **III**) or without (state **IV**) Ca^{2+} (Fig. 1; Lee et al. 2001). The three-dimensional structures of only two of these states are known, state **II**, the “charged” photoproteins aequorin and obelin (Head

et al. 2000; Liu et al. 2000, 2003), and state **IV**, the Ca^{2+} -discharged obelin containing the bound product coelenteramide without Ca^{2+} (Deng et al. 2004a,b). However, for revealing details of photoprotein bioluminescence and the formation of an enzyme–substrate complex consisting of coelenterazine, oxygen, and protein, it would be highly desirable to get the spatial structures of all of these conformation states. Here we report the structures of the two Ca^{2+} -loaded apo-photoproteins, Ca^{2+} -loaded apo-aequorin and apo-obelin, in conformational state **V**. In both apo-proteins a Ca^{2+} is bound in its characteristic coordination at each of the three canonical Ca^{2+} -binding loops with retention of the same compact structure as the unreacted photoproteins. Subtle shifts in structure, which may be the basis for the Ca^{2+} triggering, are described, and a mechanism of intrastuctural transmission of the calcium signal is proposed.

Results and Discussion

If we are to attempt to account for the intricacies of function of these photoproteins on the basis of their spatial structure, it is necessary to make a very detailed structural analysis and so a brief overview might be first useful to consider. There is very little gross structural difference among the different conformation states of the photoproteins, but closer examination reveals that there are local changes among the different structures as well as different intraprotein interactions between the aequorin and obelin. Probably most significant are the properties of the three calcium-binding loops. It is known that loop residue positions have to move in order to bind a Ca^{2+} in its typical coordination. It has been proposed that a loop should have a higher affinity for Ca^{2+} if the coordinating residues do not have to move much for accommodation of the Ca^{2+} . It would follow that loop I belonging to EF-hand motif I should have a higher affinity than the loops in EF-hands III and IV, and these required position shifts and therefore the affinities differ between aequorin and obelin. However, the situation is quite complex because there are loop–loop H-bonds as well as EF-hand helix–helix interactions, so that the binding to one loop will influence the binding to the others. On the basis of a previously formulated hypothesis that a displacement of His175 in EF-hand IV of obelin was responsible for triggering the bioluminescence, a mechanism of transmission of the calcium signal is proposed.

Overall structure

Figure 2 shows that the structures of the Ca^{2+} -loaded apo-photoproteins (Fig. 1, state **V**), retain the same compact scaffold and characteristic two-domain fold as the undischarged photoproteins (state **II**) (Head et al. 2000; Liu et al. 2000, 2003). Table 1 shows that the same conclusion can be made for comparison with the Ca^{2+} -discharged obelin (Fig. 1, state **IV**), which retains coelenteramide (Deng et al. 2004b).

Figure 1. Photoprotein conformation states. Apo-protein (state **I**), photoprotein (with 2-hydroperoxycoelenterazine in the absence of Ca^{2+}) (state **II**), Ca^{2+} -discharged photoprotein (protein with the reaction product, coelenteramide, and bound Ca^{2+}) (state **III**), Ca^{2+} -discharged photoprotein without Ca^{2+} (protein with coelenteramide without Ca^{2+}) (state **IV**), Ca^{2+} -loaded apo-protein (state **V**). The photoprotein conformational states were revealed by HSQC-NMR spectroscopy study of obelin (Lee et al. 2001).

Figure 2. Crystal structures of Ca^{2+} -loaded apo-aequorin and apo-obelin and their comparison. (A) Cartoon drawing of the crystal structures of Ca^{2+} -loaded apo-aequorin (state V). (B) The crystal structure of aequorin (state II, monomer B of the dimer; PDB code 1EJ3) is presented with the same orientation as Ca^{2+} -loaded apo-aequorin. A–H indicate the helices and I–IV the loops of the E–F hands. (C) Stereoview of a superimposition of Ca^{2+} -loaded apo-aequorin (state V; magenta) and aequorin (state II; gray). (D) Ribbon diagram of Ca^{2+} -loaded apo-obelin (state V). Part of the C terminus is disordered. (E) Superimposition of Ca^{2+} -loaded apo-obelin (state V; cyan) and Ca^{2+} -loaded apo-aequorin (state V; magenta). (A,D) Ca^{2+} ions are shown as red balls. The figures were drawn with MOLSCRIPT (Kraulis 1991). The photoprotein conformational states II and V are from Figure 1.

A calcium ion is found at each of the expected Ca^{2+} -binding sites, EF-hand loops I, III, and IV, in both proteins (Fig. 2A,D), and the N termini of both proteins are disordered. The C terminus of Ca^{2+} -loaded apo-obelin is not observed in the electron density map and is assumed to be disordered as well.

Although Ca^{2+} -loaded apo-aequorin and apo-obelin share a similar overall fold, easily discerned local structure differences are found for almost every part of the structures, both in the helical and the loop regions (Fig. 2E). The RMSD of the C_α atoms of the two Ca^{2+} -loaded apo-protein structures (from residue 15 to 181 of Ca^{2+} -loaded apo-obelin) is 2.27 Å. But the RMSD of the same residue range of aequorin and obelin, for example, is only 1.48 Å. This means that the similarity in structure between the photoproteins

is greater than between the Ca^{2+} -loaded apo-proteins. The magnitude of this difference leads us to suggest that the different conformational transients following Ca^{2+} binding might be responsible for the differences in properties such as bioluminescence kinetics.

Structural comparison of different states of photoproteins

Table 1 is a collection of RMSDs of different conformation states. The overall RMSD for the aequorin states V versus II is 2.15 Å and for the same obelin states, 2.91 Å. It should be noted that for both proteins, the displacement of the C-terminal domain (containing EF-hand loops III, IV) is much bigger than that of the N-terminal domain (containing EF-hand loops I, II). Another finding is that there is hardly any difference in RMSD whether the Ca^{2+} -loaded apo-obelin is compared to either obelin (Fig. 1, state II) or the Ca^{2+} -discharged obelin (Fig. 1, state IV).

In both the aequorin and obelin structures (Fig. 1, state II) the C terminus caps the substrate cavity containing the hydroperoxycoelenterazine, resulting in a solvent-inaccessible and nonpolar environment (Fig. 2B). This condition apparently optimizes the efficient population of the first electronic excited state of coelenteramide and favors a high quantum yield for its fluorescence (Watkins and Campbell 1993). In the obelin case for state IV, the C terminus cap remains over the coelenteramide (Deng et al. 2004b), but its position cannot be detected in the Ca^{2+} -loaded apo-obelin. In the Ca^{2+} -loaded apo-aequorin structure, however, the C terminus is opened up. This was proposed by Kendall et al. (1996) as the reason apo-aequorin can have a luciferase

Table 1. RMS deviation values of different states of Ca^{2+} -regulated photoproteins

RMSD of C_α atoms (Å)	Ca^{2+} -loaded apo-aequorin vs. aequorin (state V vs. state II)	Ca^{2+} -loaded apo-obelin vs. obelin (state V vs. state II)	Ca^{2+} -loaded apo-obelin vs. Ca^{2+} -discharged obelin (state V vs. state IV)
Overall ^a	2.15	2.91	2.94
N-terminal domain	1.42	1.28	1.27
C-terminal domain	2.27	3.36	3.37
Loop I	1.34	1.19	0.83
Loop II	0.40	0.51	0.41
Loop III	1.39	1.56	— ^b
Loop IV	2.6	1.92	1.93

Photoprotein conformational states II, IV, and V are from Figure 1. Values are based on structures in the following PDB entries: aequorin, 1EJ3 (Head et al. 2000); obelin, 1EL4 (Liu et al. 2000); Ca^{2+} -discharged W92F obelin, 1S36 (Deng et al. 2004b).

^a For residues 11 to 177; the 14 residues from the C terminus are also not included in the calculation due to their obvious deviation.

^b Loop III is disordered (Deng et al. 2004b).

function. Assuming that uncapping occurs in both photoproteins going from state **III** to state **IV**, this would explain why the coelenteramide dissociates away in the presence of the organic solvent used for the crystallization, or in the case of Ca^{2+} -discharged aequorin in solution, by removal of bound Ca^{2+} (Shimomura and Johnson 1970, 1975).

Another noticeable difference is that particularly in the case of aequorin going to the Ca^{2+} -loaded apo-aequorin (state **V**), the structure becomes more elongated, especially the EF-hand motifs III and IV move closer toward each other, leaving no space in the cavity for coelenteramide.

Figure 3. Structure of calcium-binding loop I in different photoprotein states. (A) Obelin (PDB code 1EL4; state **II**). (B) Ca^{2+} -soaked obelin crystal (PDB code 1QV1). (C) Ca^{2+} -loaded apo-obelin (state **V**). (D) Ca^{2+} -discharged W92F obelin (PDB code 1S36; state **IV**). (E) Aequorin monomer B of the dimer (monomers A and B are very similar) (PDB code 1EJ3; state **II**). (F) Ca^{2+} -loaded apo-aequorin (state **V**). Water molecules are represented by blue balls. Ca^{2+} ions are the green balls. The residues capable of binding Ca^{2+} are marked in red. The figures were drawn with MOLSCRIPT (Kraulis 1991). The photoprotein conformational states **II**, **IV**, and **V** are from Figure 1.

Figure 4. Structure of calcium-binding loop III in different photoprotein states. (A) Obelin (PDB code 1EL4; state **II**). (B) Ca^{2+} -loaded apo-obelin (state **V**). (C) Aequorin monomer B of the dimer (monomers A and B are very similar) (PDB code 1EJ3; state **II**). (D) Ca^{2+} -loaded apo-aequorin (state **V**). Loop III of Ca^{2+} -discharged W92F obelin is not shown because it is disordered. Water molecules are represented by blue balls. Ca^{2+} ions are green balls. The residues capable of binding Ca^{2+} are marked in red. The figures were drawn with MOLSCRIPT (Kraulis 1991). The photoprotein conformational states **II** and **V** are from Figure 1.

Calcium-binding loops

Figures 3–5 show how the 12 residues of the EF-hand calcium-binding loops I, III, and IV shift their positions when going to the Ca^{2+} -loaded states (Fig. 1, state **V**). The typical geometrical arrangement of oxygen atoms in a pentagonal bipyramid is observed with the Ca^{2+} ion occupying the center of the pyramid. All three Ca^{2+} -binding sites of both apo-proteins (Figs. 3C,E; 4B,D; 5B,D) contribute six oxygen ligands derived from the carboxylic side-chain groups of aspartate and glutamate residues, carbonyl groups of the peptide backbone or side chain of asparagine, and hydroxyl group of serine to the metal ion, with a separation of ~ 2.4 Å (Table S1). The seventh ligand comes from the oxygen atom of a water molecule. Based on studies of EF-hand calcium-binding proteins from different sources, it has been observed that high-affinity Ca^{2+} -binding sites have either no water or at most one water ligand (Strynadka and James 1994). The three Ca^{2+} -binding sites of the present Ca^{2+} -loaded apo-photoprotein structures each contains only one water molecule as a ligand, indicating that they all can have high affinity for calcium.

Liu et al. (2003) reported that an obelin crystal that had been soaked with a very low concentration of Ca^{2+} such as

Figure 5. Structure of calcium-binding loop IV in different photoprotein states. (A) Obelin (PDB code 1EL4; state II). (B) Ca^{2+} -loaded apo-obelin (state V). (C) Ca^{2+} -discharged W92F obelin (PDB code 1S36; state IV). (D) Aequorin monomer A of the dimer (PDB code 1EJ3; state II). (E) Aequorin monomer B of the dimer (PDB code 1EJ3; state II). (F) Ca^{2+} -loaded apo-aequorin (state V). Water molecules are represented by blue balls. Ca^{2+} ions are the green balls. The residues capable of binding Ca^{2+} are marked in red. The figures were drawn with MOLSCRIPT (Kraulis 1991). The photoprotein conformation states II, IV, and V are from Figure 1.

not to trigger the bioluminescence, had a Ca^{2+} bound in loop I only. They concluded that loop I must have a higher affinity for Ca^{2+} than the other loops. In fact, the ligands in loop I more than in loops III and IV of obelin appear to be pre-positioned for the binding, requiring only small shifts to properly accommodate Ca^{2+} (Table 1; Fig. 3A–C; Strynadka et al. 1997). It was also noted that the coordination was not in the usual pentagonal bipyramidal configuration as the invariant ligand Glu41 (Fig. 3B) was absent, hardly shifted from its position in obelin. This Ca^{2+} -soaked state in the crystal might represent an intermediate in calcium binding in solution (Fig. 3B), going from the calcium-free state (Fig. 3A) to the properly coordinated calcium-loaded state (Fig. 3C). It can be expected that in solution there could be a dynamic equilibrium set up with the Glu41 able to move in to ligate Ca^{2+} . Adding higher concentration of Ca^{2+} to the soaked crystal or prolongation of soaking with the same calcium concentration caused it to crack accompanied by bioluminescence emission.

Strynadka and James (1994) proposed that conformational “pre-forming” of the ligands in the binding loop would decrease an energetic (presumably entropic) cost in ordering this site for optimal calcium binding. As a conse-

quence, the Ca^{2+} -binding loop I should have higher affinity for calcium than Ca^{2+} -binding loops III and IV. In other words, calcium ion will first go to the Ca^{2+} -binding loop I. The residues in Ca^{2+} -binding loop III are at close orientations but not quite the right positions for calcium binding (Fig. 4) and have to adjust a little to accommodate a calcium ion. The RMSD between Ca^{2+} -loaded and Ca^{2+} -free states (state V vs. state II) of the residues of this loop is higher than that of loop I (Table 1). Most displacement is also observed for the Glu at loop residue position 12. Therefore, on the assumption of the inverse relation of residue displacement to the affinity to calcium, loop III should bind calcium with less affinity than loop I.

Among the three Ca^{2+} -binding loops of obelin, loop IV has to undergo the largest change in terms of residue reorientation and repositioning (Fig. 5; Table 1), and therefore the affinity of this Ca^{2+} -binding loop may be less than that of the others. According to the hypothesis for the mechanism of triggering photoprotein bioluminescence suggested by us, the crucial step of bioluminescence initiation is a spatial displacement of His175 (Deng et al. 2004b; Vysotski and Lee 2004). The significant conformational change of Ca^{2+} -binding loop IV to accommodate calcium supports this

requirement because His175 is found in the exiting α -helix of this loop.

In the Ca^{2+} -binding loop structure comparison of aequorin and Ca^{2+} -loaded aequorin (Figs. 3–5), it is easily seen that the loop regions of the EF-hand motifs I (Fig. 3E) and III (Fig. 4C) of aequorin do not have a geometry as optimized as has obelin for accommodating Ca^{2+} . In aequorin a couple of residue side-chain ligands have to reorient, whereas in obelin, only the bidentate ligand Glu needs to move significantly. For the loop region of the EF-hand motif IV (Fig. 5), aequorin also needs to change a lot more than does obelin for Ca^{2+} -binding. Aequorin was crystallized as a dimer (Head et al. 2000). The loop residue conformations of monomer A are more removed from the final Ca^{2+} -occupied conformation than that of monomer B. It appears that structures of the Ca^{2+} -binding loop residues of obelin and aequorin are different. The loop regions of obelin need less conformational changes than do those of aequorin for Ca^{2+} attachment; in other words, obelin is pre-positioned for Ca^{2+} binding more than aequorin. As a consequence, this could be the reason that obelin has a faster response to Ca^{2+} concentration change than does aequorin (Campbell 1974; Moisesescu et al. 1975; Illarionov et al. 2000a).

Interactions between EF-hand motifs in N- and C-terminal domains

In the family of EF-hand proteins, the EF-hand motif almost always occurs in pairs. This double motif appears to be important for correct structural folding, and is thought to increase the affinity of each EF-hand for calcium (Kretsinger 1980; Kawasaki et al. 1998; Nelson and Chazin 1998). The paired EF-hands display extensive hydrophobic interactions between the two EF-hand motifs, and a short β -type interaction between the two binding loops.

The EF-hand motif I of photoproteins is paired with EF-hand motif II, which has the characteristic structural features of an EF-hand motif but not the canonical sequence in the loop for calcium binding. The two loops of EF-hand motifs I and II show typical interactions in both obelin and aequorin (Fig. 1, state II). In obelin, the loop I is bound with the loop II by means of hydrogen bonds between main-chain nitrogen and carbonyl oxygen atoms of Ile37 and Ile83, the side-chain $\text{O}^{\delta 1}$ of Asp40, and the main-chain nitrogen atom of Gly80, and between N^{ϵ} of Lys36 and the side-chain oxygen atom of Glu82 (Fig. S2). Aequorin displays exactly the same hydrogen-bond interaction pattern between these loops, even though a threonine in aequorin is found in a position corresponding to Ile83 in obelin without affecting the hydrogen-bond network.

In Ca^{2+} -loaded apo-obelin and Ca^{2+} -loaded apo-aequorin (Fig. 1, state V), the hydrogen-bond pattern of loop–loop interaction is different. The binding of calcium ion in loop I abolishes the hydrogen bond between Lys36 and Glu82 of

obelin and the same is observed for Ca^{2+} -loaded apo-aequorin versus aequorin. In obelin the hydrogen bond distances between the main-chain nitrogen atom of Ile37 and the main-chain carbonyl oxygen atom of Ile83, and the main-chain carbonyl oxygen atom of Ile37 and the main-chain nitrogen atom of Ile83 are 3.03 Å and 2.80 Å, respectively; in the Ca^{2+} -loaded state of apo-obelin, the distances are switched to 2.91 Å and 3.03 Å. The accommodation of calcium in the loop makes both distances of the corresponding interactions in Ca^{2+} -loaded apo-aequorin shorter. The hydrogen bond distances between $\text{O}^{\delta 1}$ of Asp40 and the main-chain nitrogen atom of Gly80 that bind helices B and C of obelin, are practically unchanged, whereas an increase in distance on calcium binding occurs in the case of aequorin.

The loops of EF-hand motifs III and IV interact with each other as well both in Ca^{2+} -free (Fig. 1, state II) and in Ca^{2+} -loaded states (Fig. 1, state V) of obelin and aequorin. The interaction occurs mainly through hydrogen bonds between main-chain atoms of Ile and Leu in both photoproteins (Fig. S3). However, in the case of aequorin there is an additional hydrogen bond between the main-chain carbonyl oxygen atom of Gly122 and the main-chain nitrogen atom of Val162 (Fig. S3b). The accommodation of a calcium ion in these loops produces changes in hydrogen bonding between them with similar trends as observed in the N-terminal domain. In the Ca^{2+} -free state (state II) of obelin the hydrogen bond distances between the main-chain nitrogen atom of Ile130 and the main-chain carbonyl oxygen atom of Leu166, and the main-chain carbonyl oxygen atom of Ile130 and the main-chain nitrogen atom of Leu166, are 2.89 Å and 3.01 Å, respectively (Fig. S3a). In the Ca^{2+} -loaded state (state V) the distances are switched to 3.09 Å and 2.84 Å (Fig. S3c). The corresponding distances in aequorin become shorter in the Ca^{2+} -loaded state (state V) of apo-aequorin (Fig. S3b,d). The hydrogen bonding between Gly128 and Val162 in aequorin is abolished after Ca^{2+} binding (Fig. S3b,d).

These observations allow the reasonable assumption that these two photoproteins may undergo different conformational transients of the Ca^{2+} -binding loops following the binding of calcium, an additional possible origin for the observed differences in bioluminescence kinetics.

Interhelical angles and interaction of the α -helices

The conformational changes that result from calcium binding to loops of the EF-hand motifs are usually described by the interhelical angles that represent more complex rearrangements of the interfaces between the α -helices of the domains. Although such a description of Ca^{2+} -induced conformational transients is available only for a few Ca^{2+} -binding proteins, it provides a useful framework within which different types of conformational changes can be examined.

For instance, the EF-hand motifs of calmodulin and troponin C are approximately antiparallel in the Ca^{2+} -free state, but they become nearly perpendicular in the Ca^{2+} -loaded state. This reorientation of α -helices exposes a hydrophobic patch, which is the basis of the biological function of these two proteins in living cells.

Unlike calmodulin or troponin C, the interhelical angles in both obelin and aequorin are almost perpendicular already in their Ca^{2+} -free state (Fig. 1, state **II**), and the Ca^{2+} binding does not change these angles significantly (Table 2; Fig. S4). In comparison to the effects in calmodulin and troponin C, we can conclude that the α -helices of Ca^{2+} -regulated photoproteins in state **II** (Fig. 1) are optimally oriented for calcium binding. This also is in agreement with our conclusion derived from analysis of the orientation of the residues of the Ca^{2+} -binding loops. As a consequence of the only small conformational shifts that are required to trigger the bioluminescence, these photoproteins are able to respond to the appearance of Ca^{2+} on the millisecond time-scale.

Although these changes are observed for the Ca^{2+} -loaded apo-states (Fig. 1, state **V**) of photoproteins, we suppose that similar displacements of the α -helices will occur when the coelenteramide is bound (Fig. 1, state **III**) because, for instance, the presence of coelenteramide in Ca^{2+} -discharged obelin (state **IV**) does not drastically affect the orientation of α -helices as compared with obelin itself (Table 2).

A much-debated question regarding the bioluminescence of Ca^{2+} -regulated photoproteins is the precise series of molecular events produced by calcium binding to the protein that results in triggering the bioluminescence. As just mentioned, an obelin crystal soaked with a low concentration of Ca^{2+} produced a spatial structure in which one calcium ion occupied the Ca^{2+} -binding loop I (Liu et al. 2003). Therefore, the first molecular event in solution is the calcium binding with the Ca^{2+} -binding loop I, and any rearrangement of α -helices A and B caused by this binding.

The α -helices in these photoproteins exhibit many interactions, van der Waals, ion pairing, and hydrogen bonds.

Especially there are many hydrogen bonds between helix A and helix H, and it should be again noted that His175, crucial for the bioluminescence triggering (Deng et al. 2004b; Vysotski and Lee 2004), is found in the exiting helix H before the C terminus of the protein. This hydrogen-bond connectivity is strictly conserved between obelin and aequorin. Figure 6 shows the hydrogen-bond interhelical network for the Ca^{2+} -free (state **II**) and Ca^{2+} -loaded states (state **V**) of obelin and aequorin. In the Ca^{2+} -free state, hydrogen bonds from the Arg to His are found in both proteins (Fig. 6A,B). The Arg hydrogen bond binds together helix A and the C terminus that caps the substrate cavity containing the hydroperoxycoelenterazine, resulting in a solvent-inaccessible and nonpolar environment. The hydrogen bonds His-Trp and Arg-Phe probably also serve this goal pulling together helix A and helix H. In Ca^{2+} -free aequorin (state **II**) there are also numerous additional hydrogen bonds that bind these two α -helices and also helix A to the C terminus but are lacking in obelin and probably produce a more stable interaction in the case of aequorin.

In the Ca^{2+} -loaded states (state **V**) of apo-obelin (Fig. 6C) and apo-aequorin (Fig. 6D), the hydrogen-bond network is completely different compared to the Ca^{2+} -free states (state **II**). In the case of Ca^{2+} -loaded apo-obelin (state **V**), the hydrogen bonds binding together helices A and H are gone and there are only two hydrogen bonds remaining between Arg21 and Arg17 and the residues from the C terminus. This clearly shows that helix A and helix H move away from each other upon calcium binding. Different changes are observed for Ca^{2+} -loaded apo-aequorin. In this case, despite the fact that the hydrogen-bond pattern is also different for the Ca^{2+} -loaded state (state **V**) from in the Ca^{2+} -free state (state **II**), helix A and helix H remain connected by numerous hydrogen bonds. The different character of changes in hydrogen-bond interactions between these two α -helices and between helix A and the C terminus in aequorin and obelin clearly implies that these photoproteins undergo different conformational transients on calcium binding.

Table 2. Interhelical angles in EF-hand motifs of different states of Ca^{2+} -regulated photoproteins (degrees)

EF-hand	Aequorin (state II)	Ca^{2+} -loaded apo-aequorin (state V)	Obelin (state II)	Soaked obelin	Ca^{2+} -loaded apo-obelin (state V)	Ca^{2+} -discharged obelin (state IV)
I	70.5	80.6	71.4	73.2	77.4	73.2
II	111.6	106.7	107.3	108.8	108.2	110.9
III	112.2	116.3	111.1	110.5	122.4	111.3
IV	104.3	105.6	106.5	106.1	99.2	106.4

Interhelical angles were calculated using the program interhlx (K. Yap, University of Toronto). Values are based on structures in the following PDB entries: aequorin, 1EJ3 (Head et al. 2000); obelin, 1EL4 (Liu et al. 2000); soaked-obelin, 1QV1 (Liu et al. 2003); Ca^{2+} -discharged W92F obelin mutant, 1S36 (Deng et al. 2004b). Photoprotein conformational states **II**, **IV**, and **V** are from Figure 1.

Figure 6. Hydrogen-bond interactions between helix A and helix H, and helix A and the C terminus in conformation states **II** and **V**. (A) Obelin (PDB code 1EL4; state **II**). (B) Aequorin (PDB code 1EJ3, monomer B; state **II**). (C) Ca^{2+} -loaded apo-obelin (state **V**). (D) Ca^{2+} -loaded apo-aequorin (state **V**). The photoprotein conformation states **II** and **V** are from Figure 1. The residues for the Ca^{2+} -loaded apo-aequorin are numbered according to that used for the aequorin structure (PDB code 1EJ3).

In contrast to helix A, helix B forms only one hydrogen-bond interaction with helix C in both aequorin and obelin. Therefore, the probability of calcium signal transmission from the EF-hand motif I to other parts of the photoprotein molecule through changes of helix A is higher than through changes in helix B. As already mentioned, although calcium-induced conformational changes are represented by big changes in interhelical angles on calcium binding in some proteins, this is not the case for the Ca^{2+} -regulated photoproteins (Table 2; Fig. S4).

Figure 7 shows the hydrogen-bond structure of helix A in obelin (state **II**), Ca^{2+} -loaded apo-obelin (state **V**), aequorin (state **II**), and Ca^{2+} -loaded aequorin (state **V**). For clarity, only the main-chain atoms are shown. The intrahelical hydrogen-bond connectivity of helix A in the Ca^{2+} -free state (state **II**) of obelin is typical for an α -helical structure with the hydrogen bond distances indicated for each carbonyl to the fourth following residue. Although in general the intrahelical hydrogen bonds of helix A are maintained in the Ca^{2+} -loaded state (state **V**) of apo-obelin, there is a small increase of 0.1–0.3 Å in each bond length (Fig. 7B), which

accumulates to a significant stretching of the helix length. In other words, the accommodation of calcium ion in the Ca^{2+} -binding loop of the EF-hand motif I makes the incoming helix A less stable, and this can also be imagined as a pulling of helix A in the direction of the N terminus.

The intrahelical hydrogen-bond patterns of helix A for aequorin (Fig. 7C) and obelin (Fig. 7A) are almost the same. However, on going to the Ca^{2+} -bound state (state **V**), the changes are completely different in that for aequorin, there appears to be some compaction of the incoming helix A.

Despite the difference in character of these changes in the two photoproteins, in both cases a helix A displacement will be transmitted to helix H via their hydrogen-bond interactions.

Intrastructural transmission of the calcium signal

According to our hypothesis (Deng et al. 2004b; Vysotski and Lee 2004), displacement of His175 that lies in helix H is a crucial step for triggering the bioluminescence. We first considered that binding of one Ca^{2+} to the loop IV preced-

The likelihood that each binding event will trigger a bioluminescence response depends on three factors: the on-rate

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for Ca^{2+} binding, which is probably proportional to the binding affinity; the degree of residue shift for Ca^{2+} ligation and the rate of propagation of this change to helix H; and the amount of any position shift that His175 undergoes as a result. There are also hydrogen-bond interactions between loops III and IV, so that binding to the one should no doubt alter the positions of the key binding residues in the other.

Therefore, at least for obelin the sequence of molecular events on calcium binding leading to the triggering of bioluminescence could be as follows:

- (1) A calcium ion is preferentially bound to loop I as evident from the observations of the soaked obelin structure and expected from the “pre-formed” nature of this loop itself.
- (2) The binding of calcium ion to this loop and optimization of the pentagonal, bipyramidal geometry produces a “twist” of the EF-hand motif I around a pivot point by means of changes in hydrogen bond distances between the main-chain atoms of Ile37 and Ile83. The accommodation of calcium ion also changes slightly the interhelical angle between helices A and B.
- (3) All these changes produce a pulling of helix A in the direction of the N terminus of the protein.
- (4) Since helix A is tightly bound with helix H and the C terminus through numerous hydrogen bonds, the changes in helix A will result in a displacement of helix H and the C terminus. In other words, the binding of only one calcium ion into the Ca^{2+} -binding loop of the EF-hand motif I could be sufficient to trigger the bioluminescence.
- (5) The binding of a calcium ion to loop IV and the optimization of the pentagonal, bipyramidal geometry produces a “twist” of the EF-hand motif IV around a pivot point by means of changes in hydrogen bond distances between the main-chain atoms of Ile130 and Leu166. At the same time, the accommodation of calcium ion induces a small change of interhelical angle between helices H and G.
- (6) The displacement of helix G produces a rearrangement of helix F, which is hydrogen-bonded with helix G. The displacement of helix F can therefore adjust the Ca^{2+} -binding loop of the EF-hand motif III increasing its affinity for calcium and facilitating its binding. The accommodation of calcium into this Ca^{2+} -binding loop completes the rearrangements of α -helices F and E of the EF-hand motif III and again leads to an additional stimulation of the bioluminescence. In fact, the accommodation of the third calcium ion and the rearrangements in the EF-hand motif III complete all the structural rearrangements in the photoprotein molecule, pro-

ducing a final conformation that is optimal for effective bioluminescence.

Although we have considered in detail only the sequence of molecular events for obelin, we assume that in the case of aequorin, the “calcium signal transmission” through the photoprotein structure should be in general the same. The minor distinctions could probably serve as a molecular basis for the reported differences in bioluminescence properties. For instance, if the energy cost for the helicity increase of aequorin helix A is higher than for pulling obelin helix A, then it might result in a slower rearrangement of helix H in aequorin. Therefore, a slower bioluminescence response on calcium addition will occur for aequorin than for obelin as is observed (Illarionov et al. 2000a).

Based on aequorin bioluminescence titration with Ca^{2+} it has been proposed (Shimomura 1995; Shimomura and Inouye 1996) that only two Ca^{2+} ions are required for light emission and that the third Ca^{2+} is unrelated. Other authors using various experimental approaches with both obelin and aequorin have concluded that three Ca^{2+} ions were necessary (Moisesescu et al. 1975; Allen et al. 1977; Campbell et al. 1979; Illarionov et al. 2000a; Markova et al. 2002). The sequence of molecular events occurring in a photoprotein molecule suggested by us based on structural data and the mechanism for triggering the bioluminescence (Deng et al. 2004b; Vysotski and Lee 2004) implies that binding of even one calcium ion into Ca^{2+} -binding loop I will be enough to set off bioluminescence. The binding of the other two calcium ions is a cooperative event leading to a greater stimulation of bioluminescence, especially the binding of the second calcium ion to the Ca^{2+} -binding loop of EF-hand motif IV. Our recommendation is that in view of the different affinities of the binding sites and probably cooperativity among them, one must interpret bioluminescence kinetic results with due caution.

Another study relevant to this debate was an investigation of obelin mutants with substitutions of key residues in each of the loops participating in calcium coordination in the Ca^{2+} -binding loops (Illarionov et al. 2000b). The authors consecutively “switched off” the Ca^{2+} -binding loops of obelin and studied some properties of these mutants. As a result of this investigation they concluded that all calcium-binding loops are necessary for effective bioluminescence. The structural data in the present work lead to the same conclusion but also allow us to account for the results obtained for obelin mutants from a structural point of view. The interhelical angles of both obelin and aequorin are almost perpendicular already in the Ca^{2+} -free state (Fig. 1, state II), and therefore we concluded that all EF-hand motifs of Ca^{2+} -regulated photoproteins are already pre-formed to bind calcium ion. The same conclusion derives from analyses of orientation of the key residues participating in calcium binding of the Ca^{2+} -binding loops. Therefore, if even some

Ca²⁺-binding loop(s) were mutated and did not bind calcium ion, the nonmutated loop(s) would probably do it even with the same affinity. Since the α -helices are all interacting with each other by hydrogen bonds, even the binding of one calcium into one binding loop could produce a displacement of helix H, but the path of calcium signal transmission in each mutant would be different in comparison with wild-type photoproteins, and therefore there will be a different effect on some bioluminescent properties of the mutated photoproteins such as bioluminescence response kinetics or the calcium concentration–effect curve, as examples, and, indeed, this is what is observed (Illarionov et al. 2000b).

Materials and methods

Protein preparation and crystallization

The N terminus of photoproteins is very flexible allowing different conformations (Head et al. 2000; Liu et al. 2000, 2003) that could impede the ability to crystallize the protein and also lead to reduced crystal quality. This could be especially important for aequorin, which produced crystals diffracting to only 2.3 Å resolution (Head et al. 2000). Therefore, to improve the chance of successful crystallization here, six residues from aequorin's N terminus (GenBank accession no. AAA27716) were cut off by using PCR. The truncated gene was cloned in expression vector pET22b⁺ (Novagen). The resulting plasmid was named pET22⁺-A7. After verification of the nucleotide sequence, the pET22⁺-A7 was introduced into *Escherichia coli* strain BL21(DE3)-Gold (Stratagene) for expression of apo-aequorin. The apo-obelin from *Obelia longissima* was expressed as previously described (Markova et al. 2000).

The *E. coli* BL21(DE3)-Gold cells were grown in media containing 200 µg/mL ampicillin or 50 µg/mL carbenicillin. For protein production, the transformed *E. coli* BL21-Gold was cultivated with vigorous shaking at 37°C in LB medium containing ampicillin and induced with 1 mM IPTG when the culture reached an OD₆₀₀ of 0.5–0.6. After addition of IPTG, the cultivation was continued for 3 h.

State V (Fig. 1) can be produced in two ways either by adding Ca²⁺ to apo-photoprotein or by removing coelenteramide from Ca²⁺-discharged photoprotein (Fig. 1, state III) with some appropriate solvent. Our initial plan in this study was to determine the crystal structures of the Ca²⁺-discharged photoproteins (Fig. 1, state III), that is, the ones containing all three Ca²⁺ and the product coelenteramide. The Ca²⁺-discharged photoproteins in aqueous solution before crystallization display strong fluorescence, bright blue for the case of aequorin and green for the obelin. This property verifies that the coelenteramide is bound within the protein because the fluorescence of coelenteramide is quenched in aqueous solution. On determining the structures, however, the coelenteramide molecule was absent. Evidently the organic precipitants that were included for successful crystal formation were sufficiently nonpolar as to dissolve the coelenteramide out of the protein. Indeed, this was verified by fluorescence studies that showed that in the presence of the crystallization precipitants, either 2-methyl-2,4-pentanediol or polyethylene glycol 10,000, the fluorescence of the Ca²⁺-discharged aequorin and obelin solutions is completely quenched. This indicates release of the bound coelenteramide, and, therefore, in fact, the crystals of Ca²⁺-loaded apo-

aequorin and Ca²⁺-loaded apo-obelin (Fig. 1, state IV) were produced.

High-purity recombinant aequorin (7.97 mg/mL) and obelin (7.93 mg/mL) were produced as previously described (Illarionov et al. 2000a; Markova et al. 2002). The proteins were homogeneous according to LC-Electrospray Ionization Mass Spectrometry. Apo-aequorin was converted to aequorin with synthetic coelenterazine (Prolume Ltd) and obelin—with coelenterazine-h, a coelenterazine analog having a 2-benzyl substitution instead of 2-(p-hydroxy) benzyl. Both Ca²⁺-discharged photoproteins were obtained according to a procedure described elsewhere (Deng et al. 2004a,b). The Ca²⁺-discharged aequorin and h-obelin were concentrated to 40 mg/mL and 24 mg/mL, respectively. The protein concentrations were measured by the Bradford method with chicken albumin in 1 mM CaCl₂, 10 mM bis-Tris (pH 7.0) as a standard. The modified microbatch method was used for crystallization (Chayen et al. 1990; D'Arcy et al. 1996). The crystals of Ca²⁺-loaded apo-aequorin were grown from 0.02 M calcium chloride, 30% (v/v) 2-methyl-2,4-pentanediol, and 0.1 M sodium acetate (pH 4.6) during less than 1 wk of incubation at 4°C. The maximum size of crystals was 0.35 × 0.3 × 0.25 mm. The crystals of Ca²⁺-loaded apo-obelin were grown from 20% (w/v) polyethylene glycol 10,000 and 0.1 M HEPES (pH 7.5) within 4 wk of incubation at 18°C. The maximum size of crystals was 0.05 × 0.1 × 0.15 mm.

Data collection and processing

All crystals were directly mounted to a fiber loop and flash-frozen in liquid nitrogen before data collection. The methodology based on anomalous scattering could be used based on the sulfur atom content in the protein and the calcium ions in the Ca²⁺-loaded apo-aequorin crystals, and a complete data set was collected for ab initio single-wavelength anomalous dispersion (SAD) phasing using an in-house chromium X-ray source with a longer wavelength (2.2909 Å) and Rigaku R-Axis IV detector. The maximum resolution was 2.5 Å because of instrumental limitation. One high-resolution data set of Ca²⁺-loaded apo-aequorin and one data set of Ca²⁺-loaded apo-obelin were collected to 1.7 Å resolution and 2.2 Å, respectively, at Beamline 22ID in the facilities of the South East Regional Collaborative Access team (SER-CAT) at the Advanced Photon Source with a wavelength of 0.97 Å. The data were processed separately using the program HKL2000 (Otwinowski and Minor 1997). The crystal of Ca²⁺-loaded apo-aequorin belongs to the P₄₃2₁2 space group with unit cell dimensions of $a = b = 54.4$ Å, $c = 135.1$ Å. The crystal of Ca²⁺-loaded apo-obelin belongs to the P₄₁2₁2 space group with unit cell dimensions of $a = b = 58.6$ Å, $c = 110.4$ Å. There is one molecule per asymmetric unit for all protein crystals. Data collection statistics are presented in Table 3.

Phasing

Phases of Ca²⁺-loaded apo-aequorin were determined from the SAD data collected from the chromium X-ray source using the program SOLVE (Terwilliger and Berendzen 1999). Low-resolution data (3.0 Å) were used to search for the eight sulfur sites and the three calcium sites. Three calcium ions and four of the sulfur sites were found and used to estimate the protein phases. The phased chromium X-ray data were then subjected to density modification with the program RESOLVE (Terwilliger 2000). The electron density map obtained after SOLVE and RESOLVE showed clear electron density for most of the molecule including side-chain density. On the basis of the electron density map and the information provided from the automated model-building by

Table 3. X-ray crystallographic statistics

	Ca ²⁺ -loaded apo-aequorin		Ca ²⁺ -loaded apo-obelin
Data collection statistics			
Wavelength (Å)	2.2909	0.97	0.97
Space group	P4 ₃ P ₁ 2	P4 ₃ 2 ₁ 2	P4 ₁ 2 ₁ 2
Resolution overall/outer (Å)	50–2.5/2.59–2.5	50–1.7/1.76–1.7	50–2.2/2.28–2.2
Completeness overall/outer (%)	99.5/–95.2	98.8/91.7	99.8/99.4
<i>R</i> _{merge} overall/outer (%) ^a	5.3/11.2	3.8/27.0	7.4/25.1
<i>I</i> / <i>σ</i> ₁ overall/outer	40.5/13.7	45.9/4.7	20.4/4.7
Refinement statistics			
Resolution range (Å)		50.0–1.7	50.0–2.2
<i>R</i> _{work}		0.21	0.20
<i>R</i> _{free}		0.23	0.24
Free <i>R</i> -value test set (%)		5	5
Mean <i>B</i> -factor (Å ²)		30.4	27.2
RMSDs from ideality			
Bond lengths (Å)		0.019	0.015
Bond angles (°)		1.6	1.3

^a $R_{\text{merge}} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl,i} I_{hkl,i}$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with the Miller indices h , k , and l , and $\langle I_{hkl} \rangle$ is the mean intensity of that reflection.

RESOLVE, the chain was readily traced from residues 11 to 191 with the program XTALVIEW (McRee 1999).

The orientational and positional parameters of the Ca²⁺-loaded apo-obelin molecules in the unit cell were determined by the molecular replacement method with CNS1.0 (Brünger et al. 1998). The search model used in the calculation was W92F obelin (PDB code 1JF2) with some truncations. The cross-rotation function and translation function search gave a less than satisfactory result. Rigid-body refinement was with the use of RefMac 5.0 (Murshudov et al. 1997) but gave a solution only slightly better than alternatives, an *R* factor of 53.4%, and an *R*-free factor of 56.9%. The graphics program XTALVIEW was used to observe the crystal packing and the initial electron density map. The $2F_o - F_c$ map showed that part of the protein density was continuous with some side-chain electron density. However, the model did not match the electron density well.

Structure refinement

The initial model of Ca²⁺-loaded apo-aequorin was refined against the chromium X-ray data using CNS 1.0. Each cycle of refinement such as minimization, simulated annealing, and temperature factor refinement was followed by a manual rebuilding. The resulting model was subsequently refined against the high-resolution data collected at the synchrotron with phases gradually extended to 1.7 Å using the maximum likelihood refinement with the program RefMac 5.0. SIGMAA weighted phases were used to calculate $2F_o - F_c$ and $F_o - F_c$ maps with the program XTALVIEW to aid the model building. The first three residues at the N terminus were not observed in the electron density maps and were assumed to be disordered.

The refinement of Ca²⁺-loaded apo-obelin was carried out using RefMac 5.0. The model was gradually built up through the phase improvement by manual model adjustment and missing atom rebuilding after each cycle of refinement with the use of XTALVIEW. The first 10 residues at the N terminus and the last 12 residues at the C terminus were not observed in the electron

density maps. The final refinement statistics of the three molecules is shown in Table 3. The final stereochemical parameters of the structures were evaluated with the programs PROCHECK (Laskowski et al. 1993) and MolProbity (Lovell et al. 2003).

Accession numbers

The coordinates and structure factors of Ca²⁺-loaded apo-aequorin and Ca²⁺-loaded apo-obelin have been deposited into the Brookhaven Data Base under accession codes 1SL8 and 1SL7, respectively.

Electronic supplemental material

Table S1 collects the calcium-ligand distances for the two Ca²⁺-loaded apo-photoproteins. Figure S1 compares the sequences of the aequorin studied by Head et al. (2000), the aequorin-A7 used in this work, obelin, and the Ca-loaded forms. Figure S2 shows the loop I to loop II interactions of EF-hand motifs AB and CD. Figure S3 shows the loop III to loop IV interactions of EF-hand motifs EF and GH. Figure S4 shows the repositioning of the EF-hand motifs of obelin on calcium binding.

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