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Preparation and X-ray crystallographic analysis of the Ca^{2+}-discharged photoprotein obelin

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Ca^{2+}-regulated photoproteins belong to the EF-hand Ca^{2+}-binding protein family. The addition of calcium ions initiates bright blue bioluminescence of the photoproteins, a result of the oxidative breakdown of coelenterazine peroxide to coelenteramide. Crystals of the Ca^{2+}-discharged W92F mutant of obelin from *Obelia longissima* have been grown, representing the first crystallization of a photoprotein after the Ca^{2+}-triggered bioluminescence. A green fluorescence observed from the crystals clearly demonstrates that coelenteramide, the bioluminescence product of coelenterazine peroxide, is bound within the protein. The diffraction pattern exhibits tetragonal Laue symmetry. Systematic absences indicate that the space group is either $P4_32_12$ or $P4_12_12$. The unit-cell parameters are $a = b = 53.4$, $c = 144.0$ Å. The crystals diffract to 1.9 Å resolution.

1. Introduction

The Ca^{2+}-regulated photoproteins consist of a single polypeptide chain (~22 kDa) to which an imidazolopyrazinone derivative (called coelenterazine) is tightly but non-covalently bound (Shimomura & Johnson, 1972). The light-yielding reaction of Ca^{2+}-regulated photoproteins is an oxidative decarboxylation of coelenterazine, with the elimination of a mole of carbon dioxide and the generation of the protein-bound product (called coelenteramide) in an excited state (Shimomura & Johnson, 1972).

Two features of the calcium-regulated photoproteins distinguish them from the luciferin–luciferase bioluminescence systems that operate through this same chemical mechanism but have classical enzyme–substrate kinetics. The first is that the bioluminescence kinetics of photoproteins is not influenced by oxygen concentration (Shimomura *et al.*, 1962; Morin & Hastings, 1971). It was suggested therefore that in aequorin the coelenterazine was bound as the stabilized peroxycoelenterazine intermediate (Shimomura & Johnson, 1978) and this proposal has been confirmed in a recent structural study (Head *et al.*, 2000). The second feature is that although calcium is not essential for the luminescence (photoproteins alone give off a very low level of light emission called 'calcium-independent luminescence'), the light intensity is increased up to 10^6-fold or more on the addition of calcium. It is supposed therefore that the binding of Ca^{2+} induces conformational changes in the photoprotein which destabilize the peroxycoelenterazine and allow it to decompose to the excited state of the protein-bound coelenteramide, followed by the emission of the blue bioluminescence.

The three-dimensional structures of obelin from *Obelia longissima* (Liu *et al.*, 2000) and aequorin from *Aequorea* (Head *et al.*, 2000) revealed that the photoprotein molecule is almost entirely helical, highly compact and globular. The overall folds of obelin and aequorin are very similar and are characterized by four sets of helix–loop–helix (HLH) structural motifs wrapping around to form a hydrophobic core cavity that accommodates the coelenterazine peroxide. Similar to other Ca^{2+}-binding proteins, the four HLH motifs are arranged in pairs. The loops of HTH motifs I, III and IV form typical calcium ion-binding sites and these motifs are therefore referred as EF-hand motifs. The loop of HTH motif II is not functional for calcium binding because it does not have the canonical sequence for calcium binding. The structures of obelin and aequorin confirmed the prediction made earlier that Ca^{2+}-regulated photoproteins belong to the EF-hand Ca^{2+}-binding protein family (Charbonneau *et al.*, 1985; Inouye *et al.*, 1985; Illarionov *et al.*, 1995).

Although aequorin and obelin are very similar to each other in primary and spatial structures, there are some obvious differences. Both photoproteins emit blue light, but the light emission of obelin (λ_{max} = 485 nm) (Markova *et al.*, 2002) is shifted to longer wavelength in comparison with aequorin bioluminescence (λ_{max} = 465 nm). Also, a shoulder at 400 nm that is clearly revealed in the obelin bioluminescence spectrum is not observed in the aequorin spectrum. Another difference is in the fluorescence of calcium-discharged photoproteins (after bioluminescence

cence has been triggered by the addition of Ca^{2+} ions to the solution) on excitation by near-UV. Although both proteins have approximately the same excitation maximum (~350 nm), the Ca^{2+}-discharged aequorin displays a blue fluorescence with λ_{max} = 465 nm that matches its bioluminescence spectrum, whereas the Ca^{2+}-discharged obelin has a green fluorescence with λ_{max} = 510 nm that is shifted from its bioluminescence maximum by 25 nm. It is assumed that the coelenteramide-binding pockets in these Ca^{2+}-discharged photoproteins differ from each other. Recently, we have produced a W92F obelin mutant that displays a bimodal light-emission spectrum with violet and blue emission bands and determined its three-dimensional structure (Deng *et al.*, 2001; Vysotski *et al.*, 2003). In spite of the difference in bioluminescence spectra between this mutant and wild-type obelin, the fluorescence spectrum of Ca^{2+}-discharged W92F obelin is green (λ_{max} = 510 nm), similar to that of Ca^{2+}-discharged wild-type obelin, suggesting that the environment of coelenteramide bound in these two Ca^{2+}-discharged proteins is the same.

An HSQC-NMR spectroscopy study of obelin showed that the protein undergoes clear conformational changes through five states: the first is the apoprotein, the second is when the Ca^{2+} is bound with the apoprotein, the third is on charging the apoprotein with coelenterazine in the absence of Ca^{2+}, the fourth on the addition of Ca^{2+} to cause the bioluminescence reaction and the fifth on removal of calcium ions from the product (Lee *et al.*, 2001). In order to completely elucidate the mechanism of this bioluminescent reaction and the structural transients accompanying each step of the mechanism, the three-dimensional structures of each conformational state need to be solved.

This paper reports the successful production and characterization of crystals of the Ca^{2+}-discharged form of W92F obelin that diffract to 1.96 Å resolution. These crystals represent the first crystallization of a photoprotein after the bioluminescence reaction triggered by calcium ions. The three-dimensional structure of the protein bound with coelenteramide, the product of bioluminescent reaction of Ca^{2+}-regulated photoproteins, will provide more insight into the mechanism of coelenterazine-dependent bioluminescence and the role of the protein moiety in that bioluminescence.

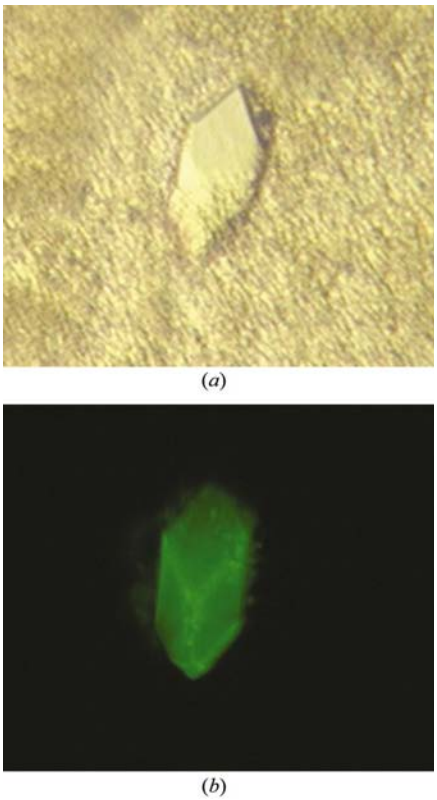

Figure 1
(*a*) Crystal of Ca^{2+}-discharged W92F obelin (0.05 × 0.1 × 0.25 mm); (*b*) fluorescence of the crystal on excitation by near-UV.

2. Materials and methods

2.1. Protein production and purification

Site-directed mutagenesis was performed on the template pET19-OL8 *Escherichia coli* expression plasmid carrying the *O. longissima* wild-type apo-obelin (Markova *et al.*, 2001). A mutation resulting in the amino-acid change W92F was introduced using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) as described elsewhere (Deng *et al.*, 2001; Vysotski *et al.*, 2003).

For protein production, *E. coli* BL21-Gold cells with the W92F obelin plasmid were cultivated with vigorous shaking at 310 K in LB medium containing ampicillin and induced with 1 m*M* isopropyl β-D-thiogalactopyranoside when the culture reached an OD_{600} of 0.5–0.6. After induction, cultivation was continued for 3 h.

The W92F obelin was purified as previously reported (Deng *et al.*, 2001; Vysotski *et al.*, 2003). The purified protein was concentrated to approximately 8–10 mg ml^{-1}, desalted on a BioGel P2 column equilibrated with 1 m*M* EDTA, 10 m*M* sodium/potassium phosphate buffer pH 7.3 and concentrated again to approximately the same concentration. Protein was homogeneous according to LC-Electrospray Ionization Mass Spectrometry and the mass was in excellent agreement with that calculated from the sequence. The apo W92F obelin was converted to photoprotein with synthetic coelenterazine (Prolume Ltd, Pittsburgh, USA).

To prepare Ca^{2+}-discharged W92F obelin, a solution of W92F obelin was diluted tenfold with 10 m*M* bis-Tris buffer pH 7.0. $CaCl_2$ solution in water was then gradually added into the diluted protein solution with stirring at room temperature. The final concentration of calcium was 1 m*M*. During this procedure, a bright violet bioluminescence was observed. After the bioluminescence emission ceased, the yellow protein solution had turned colorless, indicating that coelenterazine had been converted into coelenteramide. To test for the presence of bound coelenteramide, the final product was excited with near-UV. The Ca^{2+}-discharged W92F obelin displayed green fluorescence. The Ca^{2+}-discharged protein was then concentrated to 28 mg ml^{-1} with the use of Millipore (Bedford, MA, USA) centrifugal tubes. The protein concentration was measured by the Bradford method with chicken albumin in 1 m*M* $CaCl_2$, 10 m*M* bis-Tris pH 7.0 as a standard.

2.2. Crystallization

Crystals (Fig. 1*a*) were grown by the modified microbatch method (Chayen *et al.*, 1990; D'Arcy *et al.*, 1996) using an ORYX 1–6 protein crystallization robot from Douglas Instruments Ltd (East Garston, UK) for screening initial crystallization conditions. Optimization was performed manually. Equal volumes (0.5 µl) of the protein and the crystallization solutions were mixed in the wells of a Nunc HLA plate. The mixed solutions were covered with 10 µl of paraffin oil. After all the crystallization droplets had been set up, the HLA plate was sealed using 4 ml of a combination of silicon and paraffin oil (7:3) and was incubated at 277 K for over two weeks. The best conditions for crystallization of Ca^{2+}-discharged W92F obelin were 1.5 *M* trisodium citrate in 0.1 *M* Na HEPES buffer pH 7.5.

3. Results and discussion

It took one month for the initial crystals to appear and they were relatively small (maximum dimensions of ~0.05 × 0.05 × 0.1 mm) in a precipitation droplet with a protein concentration of 14 mg ml^{-1}. Success in obtaining crystals was also variable and by the time the crystals appeared

Table 1
Data-processing statistics.

Values in parentheses are for the outer shell.

Resolution range (Å)	20–1.96 (2.05–1.96)
Unique reflections	15775 (1982)
Mosaicity	0.4
Completeness (%)	99.5 (99.2)
Redundancy	14.9 (14.1)
$I/\sigma(I)$	9.37 (2.86)
$R_{\rm merge}$† (%)	6.0 (18.8)

† $R_{\rm merge} = \sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}\langle I_{hkl}\rangle$, 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.

the droplet was almost dried out, indicating possible high solubility of the protein. This observation and the length of time it took to form crystals in the incubator partially explains our earlier failure using lower protein concentration (8 mg ml^{-1}). To be sure that coelenteramide is still bound in the protein crystal, the fluorescence of the Ca^{2+}-discharged W92F obelin crystals was examined. A single crystal was transferred to a droplet containing only the crystallization mother liquor. The green fluorescence from the crystal observed under a stereomicroscope with excitation at 350 nm (Fig. 1*b*) clearly indicates that coelenteramide is bound within the protein.

For X-ray analysis, the crystal of the Ca^{2+}-discharged W92F obelin was directly mounted on a fiber loop (Teng, 1990) containing a minimal amount of mother liquor and flash-frozen (Hope, 1988) in liquid nitrogen before the data-collection process. No cryoprotectant was used prior to the flash-freezing of the crystals. A 360° data set was collected from the Ca^{2+}-discharged W92F obelin crystal on an in-house copper X-ray source with a Bruker Smart 6000 CCD detector. An offset of 8° in 2θ was used for the purpose of extending the data resolution to 1.96 Å. The Bruker program *Proteum* was used for data processing. The data-processing statistics are given in Table 1. The diffraction pattern of the Ca^{2+}-discharged W92F obelin exhibits tetragonal Laue symmetry. Systematic absences indicate that its space group is either $P4_32_12$ or $P4_12_12$. The unit-cell parameters are $a = b = 53.4$, $c = 144.0$ Å. Assuming one molecule per asymmetric unit, the Matthews coefficient is 2.34 Å^3 Da^{-1}, corresponding to a solvent content of 45%. The determination of the three-dimensional structure of the Ca^{2+}-discharged W92F obelin is currently in progress.

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