Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Preparation and X-ray crystallographic analysis of recombinant obelin crystals diffracting to beyond 1.1 Å

Eugene S. Vysotski,[a,b] Zhi-Jie Liu,[b] John Rose,[b] B. C. Wang[b] and John Lee[b]*

[a]Photobiology Laboratory, Institute of Biophysics, Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia, and [b]Department of Biochemistry and Molecular Biology, University of Georgia, Athens GA 30602, USA

Correspondence e-mail: jlee@arches.uga.edu

Received 26 June 2001
Accepted 4 October 2001

Crystals of recombinant obelin, the Ca^{2+}-regulated photoprotein from the marine hydroid *Obelia longissima*, have been grown from a solution containing PEG 8000 and potassium phosphate. Hexaminecobalt trichloride was used as an additive to increase the chance of crystallization. The crystals grow in a light yellow cubic form ($0.5 \times 0.5 \times 0.45$ mm) which diffracts to beyond 1.1 Å resolution. The crystals belong to the space group $C2$, with unit-cell parameters $a = 83.43$, $b = 54.92$, $c = 52.99$ Å, $\beta = 112.00^\circ$. The asymmetric unit contains one molecule. Crystals exposed to calcium ion before and after X-ray irradiation emit light, confirming that the crystals consist of an active photoprotein.

1. Introduction

Obelin from the hydroid *O. longissima* is a single-subunit protein (22.2 kDa) consisting of 195 amino-acid residues (Bondar *et al.*, 1992; Illarionov *et al.*, 1995). Obelin, together with aequorin and other photoproteins that originate mainly from marine bioluminescent coelenterates (Morin, 1974), belongs to the subfamily of calcium-regulated photoproteins which is a part of the larger calcium-binding protein family (Moncrief *et al.*, 1990). The common property of these proteins that combines them together and distinguishes them from other calcium-binding protein subfamilies is that calcium ion binding triggers a bioluminescent response arising from oxidation of the substrate (called coelenterazine) which is tightly bound within the protein moiety (Shimomura & Johnson, 1978). All photoproteins whose primary structures have been described at present show a high homology and contain three EF-hand calcium-binding sites (Tsuji *et al.*, 1995).

Recently, the tertiary structures of the Ca^{2+}-regulated photoproteins aequorin (Head *et al.*, 2000) and obelin (Liu *et al.*, 2000) were solved at resolutions of 2.3 Å and 1.7 Å, respectively. In general, as expected from the homology of their primary sequences (Illarionov *et al.*, 1995), both aequorin and obelin have the same tertiary structure, a compact globule with four HTH motifs. The coelenterazine-oxygen substrate in both photoproteins lies in a corresponding position proximate to a Tyr-His-Trp triad of probable catalytic relevance (Head *et al.*, 2000; Liu *et al.*, 2000). However, the striking difference between the two photoprotein structures concerns the oxygen substituted at the C2 position on the coelenterazine. In the obelin electron-density map at 1.7 Å resolution, only a single oxygen could be fitted at this position (Liu *et al.*, 2000). Head *et al.* (2000) concluded that in aequorin the electron density near the C2 position is consistent with the coelenterazine bound with peroxide in accordance with the peroxide structure expected.

To obtain more conclusive evidence about the discrepancy in the form of the bound coelenterazine in these two otherwise very closely related photoproteins, we decided to grow the obelin crystals in a different form of better quality. Here, we describe the successful preparation and X-ray analysis of obelin crystals that diffract to beyond 1.1 Å. Using such quality crystals, a three-dimensional structure of the photoprotein can be solved in detail and

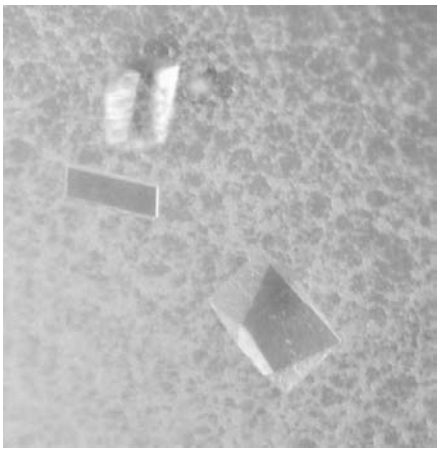

Figure 1
Crystal of the photoprotein obelin grown from 23% PEG 8000, 50 m*M* KH_2PO_4 pH 5.8 with addition of 0.1 *M* hexaminecobalt chloride to the drop. Approximate dimensions are $0.5 \times 0.5 \times 0.45$ mm.

the presence of the single oxygen substitution at the C2 position of coelenterazine in obelin is confirmed.

2. Materials and methods

2.1. Protein purification

High-purity recombinant obelin was obtained according to the previously described procedure (Illarionov *et al.*, 2000) with slight modifications (Markova *et al.*, 2001). The apoobelin obtained after extraction with 6 *M* urea and through the first step of purification on a DEAE-Sepharose Fast Flow column was concentrated by ultrafiltration in an Amicon cell (YM10 membrane) to a final volume of 5–6 ml. To fold the protein, the concentrated protein sample containing 6 *M* urea was diluted with buffer containing 5 m*M* EDTA, 10 m*M* DTT, 20 m*M* Tris–HCl pH 7.0 and coelenterazine (Prolume Ltd) (approximately 1 mol coelenterazine per 1 mol apoobelin) and was incubated overnight at 277 K. The final concentration of urea in the protein sample after dilution was approximately 0.3 *M*. The charged protein was then additionally purified on a Bio-Scale DEAE 10 anion-exchange column equilibrated with 5 m*M* EDTA, 20 m*M* Tris–HCl pH 7.0. The obelin was eluted with a linear salt gradient of NaCl from 0 to 0.35 *M* at a flow rate of 2 ml min^{-1}. This chromatography step allows separation of the obelin from uncharged protein. The final protein was homogeneous according to SDS–PAGE and LC-electrospray ionization mass spectrometry. The N-terminal amino-acid sequence of the obelin showed that the first methionine is digested on producing the recombinant apoobelin in *Escherichia coli* cells.

2.2. Crystallization

For crystallization, the protein was exchanged into a buffer containing 10 m*M* potassium/sodium phosphate, 1 m*M* EDTA pH 7.4 by gel filtration on a BioGel P2 column and was concentrated to approximately 8–10 mg ml^{-1} using Amicon Centricon-10 tubes. Crystals were grown by the hanging-drop vapor-diffusion technique. We were unsuccessful at obtaining any crystals using the Hampton Research Kits (Lagnua Niguel, CA, USA), so we designed experiments taking into account the properties of obelin. It was shown (Vysotski *et al.*, 1989; Bondar *et al.*, 1992) that ammonium sulfate and PEG stabilized natural obelin. Therefore, various concentrations of ammonium sulfate and PEGs of different molecular weights (MW 4000, 6000, 8000 and 10 000) at different pH values were tested. No crystals were formed with ammonium sulfate alone but several high-quality crystals were obtained with PEG 8000 containing KH_2PO_4. However, the conditions were very irreproducible. Therefore, additional optimization was made using the Additive screens from Hampton Research. The additional screening revealed that hexaminecobalt trichloride improved reproducibility. The best precipitant was a solution containing 23% PEG 8000, 50 m*M* KH_2PO_4 pH 5.8, with addition of 0.1 *M* hexaminecobalt trichloride to the drop. The

Table 1
Data-processing statistics.

Resolution (Å)	No. of reflections	Completeness (%)	$I/\sigma(I)$	R_{merge}†
99.00–2.37	9075	99.3	27.5	0.032
2.37–1.88	9056	100.0	33.8	0.038
1.88–1.64	8992	100.0	28.5	0.055
1.64–1.49	8971	99.9	25.2	0.050
1.49–1.39	8946	99.8	22.0	0.051
1.39–1.30	8962	99.7	18.4	0.062
1.30–1.24	8940	99.7	15.0	0.076
1.24–1.18	8727	97.7	11.9	0.095
1.18–1.14	6863	76.8	8.8	0.114
1.14–1.10	5104	56.9	6.4	0.135
99.00–1.10	83636	93.0	26.2	0.042

† $R_{merge} = \sum\sum_j |F - \langle F\rangle| / \sum\langle F\rangle$.

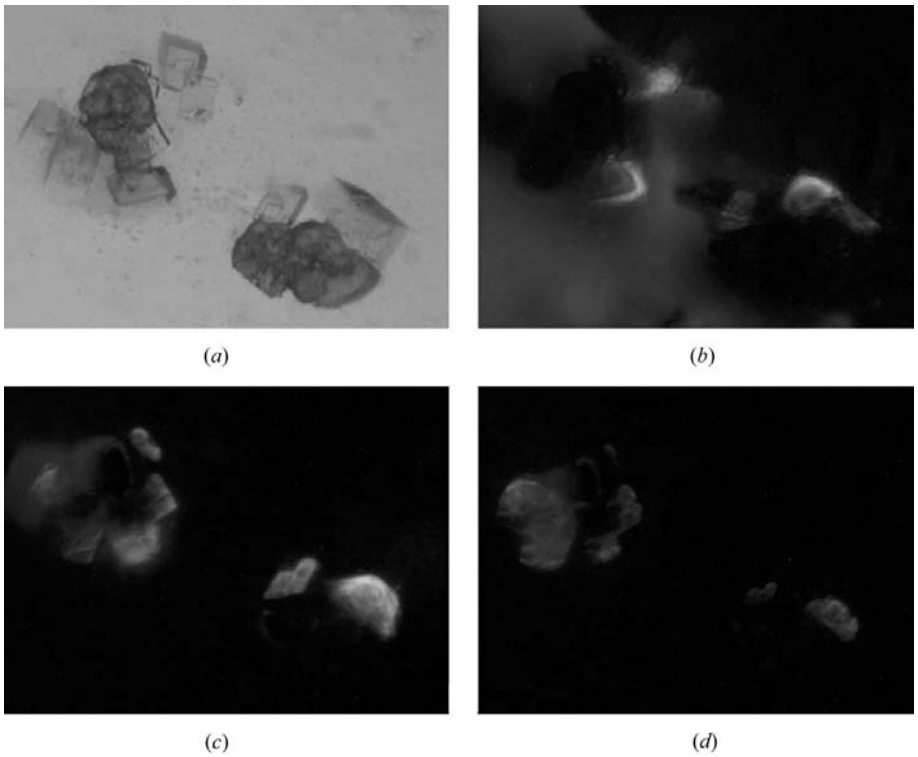

Figure 2
Luminescent images of obelin crystals after the crystals were soaked into solution with $CaCl_2$. (*a*) Before soaking. (*b*) 10 s after soaking. (*c*) 20 s after soaking. (*d*) 30 s after soaking.

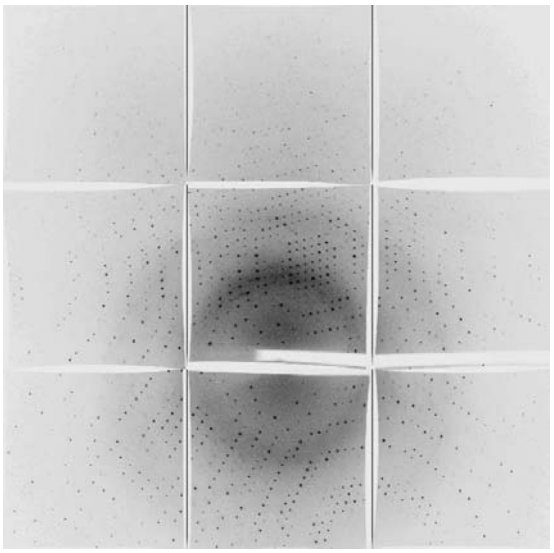

Figure 3
The diffraction pattern for the obelin crystals recorded on the APS1 detector at Structure Biology Center beamline of the Advanced Photon Source (APS), Argonne National Laboratory. The crystals diffract to beyond 1.1 Å.

drop consisted of 9 µl protein, 5 µl precipitant solution and 1 µl additive. Obelin crystals grew as secondary light yellow cubic crystals on the surface of brown salt crystals (Fig. 1) and grew to dimensions of about 0.5 × 0.5 × 0.45 mm after 5–10 d at 277 K.

To expose crystalline obelin to calcium, the single crystal was transferred from the mother drop by loop into a drop containing liquor from the parent well with 10 m*M* $CaCl_2$. Luminescence from the crystal was observed in the dark as bright spots which lasted more than 5 min (Fig. 2). During light emission the crystal deteriorated mechanically as periodically examined with a stereomicroscope.

2.3. X-ray data collection

For the X-ray analysis the crystal was suspended in a fiber loop containing a minimal amount of mother liquor (Teng, 1990) and flash-cooled (Hope, 1988) to 100 K in a mixture of 25% glycerol and 75% mother liquor as cryoprotectant.

3. Results and discussion

The crystal diffracted to beyond 1.1 Å resolution as recorded on the APS1 CCD detector at the Structure Biology Center's beamline in the Advanced Photon Source (APS) (Fig. 3), Argonne National Laboratory using 1.0 Å X-rays. The processing statistics are given in Table 1. The crystal-to-detector distance was 110 mm and three 180° rotations of the crystal in steps of 0.5° oscillation were measured. The first rotation was to measure the low-resolution diffraction with $2\theta = 0.0^\circ$ and $\chi = 0.0^\circ$, the second rotation was to measure high-resolution diffraction with $2\theta = 20.0^\circ$ and $\chi = 0.0^\circ$ and the third rotation was to measure high-resolution diffraction with $2\theta = 20.0^\circ$ and $\chi = 30.0^\circ$. Data processing was carried out using the program *HKL*2000 (Otwinowski & Minor, 1997) and the results indicated that the crystals belong to a monoclinic lattice, with unit-cell parameters $a = 83.43$, $b = 54.92$, $c = 52.99$ Å, $\beta = 112.00^\circ$. Examination of the distribution of intensities indicated the space group to be *C*2. The Matthews coefficient (V_M) (Matthews, 1968) is calculated to be 2.54 $Å^3\ Da^{-1}$ for one molecule per asymmetric unit, which corresponds to a calculated solvent content of 51%.

A preliminary structure determination (Vysotski *et al.*, 2001) confirms the conclusion from our earlier report (Liu *et al.*, 2000) that the substrate coelenterazine is substituted at the C2 position by a single oxygen. Completion of the structural details is currently in progress.

We wish to thank Rongguang Zhang, Gerd Rosenbaum and Andrzej Joachimiak for the support of data collection. This work was supported by ONR grant N 00014-99-1-0414 and in part by grant 99-04-48452 from the Fundamental Research Foundation of the Russian Academy of Sciences.

References

Bondar, V. S., Trofimov, K. P. & Vysotski, E. S. (1992). *Biochemistry (Moscow)*, **57**, 1020–1027. In Russian.
Head, J. F., Inouye, S., Teranishi, K. & Shimomura, O. (2000). *Nature (London)*, **405**, 372–376.
Hope, H. (1988). *Acta Cryst.* B**44**, 22–26.
Illarionov, B. A., Bondar, V. S., Illarionova, V. A. & Vysotski, E. S. (1995). *Gene*, **153**, 273–274.
Illarionov, B. A., Frank, L. A., Illarionova, V. A., Bondar, V. S., Vysotski, E. S. & Blinks, J. R. (2000). *Methods Enzymol.* **305**, 223–249.
Liu, Z. J., Vysotski, E. S., Chen, C. J., Rose, J. P., Lee, J. & Wang, B. C. (2000). *Protein Sci.* **9**, 2085–2093.
Markova, S. V., Vysotski, E. S. & Lee, J. (2001). *Bioluminescence and Chemiluminescence 2000*, edited by J. F. Case, P. J. Herring, B. H. Robison, S. J. Haddock, L. J. Kricka & P. E. Stanley, pp. 115–118. Singapore: World Scientific Publishing Company.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moncrief, N. D., Kretsinger, R. H. & Goodman, M. (1990). *J. Mol. Evol.* **30**, 522–562.
Morin, J. G. (1974). *Coelenterate Biology: Reviews and New Perspectives*, edited by L. Muscatine & H. M. Lenhoff, pp. 397–438. New York: Academic Press.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Shimomura, O. & Johnson, F. H. (1978). *Proc. Natl Acad. Sci. USA*, **75**, 2611–2615.
Teng, T.-Y. (1990). *J. Appl. Cryst.* **23**, 387–391.
Tsuji, F. I., Ohmiya, Y., Fagan, T. F., Toh, H. & Inouye, S. (1995). *Photochem. Photobiol.* **62**, 657–661.
Vysotski, E. S., Bondar, V. S. & Letunov, V. N. (1989). *Biochemistry (Moscow)*, **54**, 781–789. In Russian.
Vysotski, E. S., Liu, Z.-J., Deng, L., Rose, J, Wang, B. C. & Lee, J. (2001). *Bioluminescence and Chemiluminescence 2000*, edited by J. F. Case, P. J. Herring, B. H. Robison, S. J. Haddock, L. J. Kricka & P. E. Stanley, pp. 135–138. Singapore: World Scientific Publishing Company.