REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED
17-10-2002	Final report	12-3-1999 to 31-12-2002

4. TITLE AND SUBTITLE

Crystal and solution structure of the photoprotein Obelin

5a. CONTRACT NUMBER

5b. GRANT NUMBER

N00014-02-1-0230

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Lee, John

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dept. of Biochemistry and Molecular Biology
University of Georgia, Athens, GA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

10. SPONSOR/MONITOR'S ACRONYM(S)

ONR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AILABILITY STATEMENT

Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The three-dimensional structures of several types of recombinant obelin have been determined to atomic resolution. Obelin is a calcium-regulated photoprotein and the origin of the bioluminescence from the marine hydroid polyp Obelia. A W92F mutant obtained showed a violet bioluminescence emission but without change in dimensionality of the substrate binding site. The structures are typical of the super-family of calcium-binding E-F hand proteins. The high quality of the crystals also allowed a novel crystallographic method of anomalous scattering from the protein sulfur, to be demonstrated. The substrate coelenterazine is bound within the protein substituted as a 2-hydroperoxide. The NMR study indicated that the solution secondary structure did not differ substantially from the crystal.

15. SUBJECT TERMS

bioluminescence, calcium, X-ray crystallography, NMR, photoprotein

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			John Lee
Unclass.	Unclass.	Unclass.	UL	4	19b. TELEPHONE NUMBER (Include area code) 706-542-1764

FINAL REPORT

GRANT #: N00014-02-1-0230

PRINCIPAL INVESTIGATOR: John Lee, Ph.D.

INSTITUTION: University of Georgia

GRANT TITLE: Crystal and Solution Structure of the Photoprotein Obelin

AWARD PERIOD: 12 March 1999 - 31 December 2002

OBJECTIVES: To solve the three-dimensional structure of the bioluminescent photoprotein Obelin. The crystal structure will be solved by X-ray crystallography provided that quality crystals can be grown, and the solution structure by high-field NMR.

APPROACH: With the collaboration of Dr. John Blinks at the Friday Harbor Labs, University of Washington, and Dr. Eugene Vysotski, Institute of Biophysics, Krasnoyarsk, Russia, obelin and a number of its mutants have been developed into high expression systems. Preliminary crystallization trials were promising and NMR results together indicated that both crystal and solution structure determinations should be feasible. Screening trials will be done to establish the conditions for obtaining the highest quality crystals. Diffraction will be measured both at-home facilities and with synchrotron beam time available at the APS and elsewhere. For the NMR investigations the proteins will be enriched with ^{13}C and ^{15}N so that the standard barrage of three-dimensional NMR experiments can be carried out for the purpose of resonance assignments. Restraints for structure will be obtained from a series of NOESY experiments. Facilities are available on campus, both 600 MHz and 800 MHz machines.

ACCOMPLISHMENTS: High quality crystals of recombinant obelin from *Obelia longissima* were produced and enabled the development of the technique of sulfur single wavelength anomalous scattering for the *de novo* analysis of protein structure in general, a significant advance in crystallographic techniques. A high-resolution (1.7 Δ) structure was determined. Crystals of a different crystal form allowed an improvement to 1.1 Δ. With access now to the APS at Argonne and application of ShelxD procedures, the structure has been now refined to 0.96 Å, the highest resolution of EF-hand proteins.

Contrary to the expectation that the bound substrate coelenterazine, should be substituted as a peroxide, the structure of obelin from *O. longissima* from two crystal forms at resolutions 1.7 Δ and 1.1 Δ, both showed electron density of only a single oxygen substituted at the 2-position of the coelenterazine. This contrasted with the

finding in the structure of the related photoprotein of a peroxy substitution, although the peroxy electron density at the 2-position was weak, but explained as due to radiation damage. Both photoproteins have overall structures typical of the calcium-regulated protein family, four helix-turn-helix motifs. Less common however, is that only three of these motifs are EF-hands with ability to bind Ca^{2+}. Cloning and expression of recombinant obelin from another species, *O. geniculata*, was carried out. The two obelins have 86% sequence identity and very similar spacial structure. A contrasting finding is the presence in the *O. geniculata* structure of strong electron density at the C2-position of the coelenterazine accounting for the two peroxidic oxygens. The two obelins differ only slightly in respect to bioluminescence sensitivity and response to Ca^{2+} with little interference by $Mg^{2+}$and low Ca^{2+}-independent luminescence. They differ in bioluminescence spectral maximum, 495 and 485 nm respectively, and in the product fluorescence, 520 and 510 nm maxima.

The W92F mutant of obelin was overexpressed, crystallized, and structure solved to 1.7 . This mutant emits a violet bioluminescence, in contrast to the blue of the wild-type obelin. A mechanism for the production of the violet bioluminescence from W92F was proposed. Mutant shows no difference in dimensionality from the wild-type, except for the absence obviously of the Trp-92 residue and again the presence of two-oxygen electron density at the C2-substitution position of the ligand. When the refinement of the wild type structure was completed to 0.96 , a weak electron density at the second oxygen became evident. The explanation for these varying densities of the peroxy oxygens is not apparent.

CONCLUSIONS: The three-dimensional structure of obelin is typical of the super-family of calcium-binding E-F hand proteins. Obelin is an unusually cooperative protein in being able to form high quality crystals for X-ray structure study, recently giving diffraction beyond 1.0 Å so that the structure could be refined to atomic resolution (0.96 Å), the highest for any E-F hand protein. The high quality of the crystals also allowed a novel crystallographic method of anomalous scattering from the protein sulfur, to be demonstrated. The substrate coelenterazine is bound within the protein substituted as a 2-hydroperoxide. The NMR study indicated that the solution structure did not differ substantially from the crystal. In spite of initial promises, technical problems frustrated the goal of solving the spatial structure by the NMR method.

SIGNIFICANCE: The spatial structures of two species of obelin, of the related photoprotein aequorin, and of an obelin mutant that emits a violet color of bioluminescence, give insight into the machinery by which calcium triggers the light emitting reaction, the method the protein uses to stabilize the intermediate hydroperoxide, and the means of

controlling the nature of the product excited state. This information provides the basis for rational mutational engineering to produce novel photoproteins with useful properties, color, temperature stability, etc.

PATENT INFORMATION: None

AWARD INFORMATION: None

PUBLICATIONS AND ABSTRACTS:

1. Vysotski, E.S., Liu, Z-J., Rose, J., Wang, B.C., and Lee, J. Preparation and preliminary study of crystals of the recombinant calcium-regulated photoprotein obelin from the bioluminescent hydroid *Obelia longissima*. Acta Cryst. D55, 1965-1966 (1999).

2. *De novo* Structure Determination of the Photoprotein Obelin at 1.7 Å Resolution Using Single Wavelength Sulfur Anomalous Scattering Data. Zhi-Jie Liu, Eugene S. Vysotski, John Rose, John Lee, and B.C. Wang. Protein Sci.9: 2085-2093 (2000).

3. Lee J, Glushka JN, Markova SV, and Vysotski ES. (2001). Protein conformational changes in obelin shown by ^{15}N-HSQC nuclear magnetic resonance. *Bioluminescence and Chemiluminescence 2000* (Case JF, Herring PJ, Robison BH, Haddock SHD, Kricka LJ, Stanley PE, eds.)pp. 99-102. World Scientific Publishing Company, Singapore.

4.Vysotski ES, Liu Z-J, Deng L, Rose J, Wang BC, and Lee J.(2001).Obelin crystal structure: implications for the bioluminescence mechanism. *Bioluminescence and Chemiluminescence 2000* (Case JF, Herring PJ, Robison BH, Haddock SHD, Kricka LJ, Stanley PE, eds.)pp. 135-138. World Scientific Publishing Company, Singapore.

5.Markova SV, Vysotski ES, and Lee J. (2001). Obelin hyperexpression in *E. coli*, purification and characterization. *Bioluminescence and Chemiluminescence 2000* (Case JF, Herring PJ, Robison BH, Haddock SHD, Kricka LJ, Stanley PE, eds.) pp. 115-118. World Scientific Publishing Company, Singapore.

6. Vysotski ES, Liu Z-J, Rose J, Wang BC, and Lee J. (2001). Preparation and X-ray crystallographic analysis of recombinant obelin crystals diffracting to beyond 1.1 Δ. Acta Cryst. D57 1919-1921.

7.Deng L., Vysotski ES, Liu Z-J, Markova SV, Malikova NP, Lee J, Rose J, Wang B-C. (2001). Structural basis for the emission of violet bioluminescence from a W92F obelin mutant. FEBS Lett.506: 281-285.

8.Svetlana V. Markova, Eugene S.Vysotski, John R. Blinks,

7.Deng L., Vysotski ES, Liu Z-J, Markova SV, Malikova NP, Lee J, Rose J, Wang B-C. (2001). Structural basis for the emission of violet bioluminescence from a W92F obelin mutant. FEBS Lett.506: 281-285.

8.Svetlana V. Markova, Eugene S.Vysotski, John R. Blinks, Ludmila P. Burakova, B-C. Wang, and John Lee. (2002) Obelin from the Bioluminescent Marine Hydroid *Obelia geniculata*: Cloning, Expression, and Comparison of some Properties with those of other Ca^{2+}-Regulated Photoproteins. Biochemistry 41: 2227-2236.

9. Carbon-13, nitrogen-15, and proton NMR assignments and solution structure of the photoprotein obelin from the bioluminescent hydroid, *Obelia longissima*. John Lee, John N. Glushka, and Eugene S. Vysotski. Biophys. J. 78:481A (2000).

RECENT ABSTRACTS (out of a total of 13):

16. Obelin Hyperexepression in *E. coli*, Purification and Characterization. Svetlana V. Markova, Eugene S. Vysotski, and John Lee. Luminescence 15: 214(2000).

18. Preparation and X-ray crystallographic analysis of recombinant obelin crystals diffracting to beyond 1.1 Å. Eugene S. Vysotski, Zhi-Jie Liu, John Rose, B.C.Wang, and John Lee. American Crystallograhic Society Annual Meeting, P66, Los Angeles, CA (July 2001).

19. Atomic resolution structure of obelin: soaking with calcium enhances electron density of a second oxygen substituted at the C2-position of coelenterazine. Zhi-Jie Liu, Eugene Vysotski, Lu Deng, John Lee, John Rose, and Bi-Cheng Wang. Luminescence 17:99(2002).

20. The crystal structure of the calcium-regulated photoprotein obelin from *Obelia geniculata*. Lu Deng, Eugene Vysotski, Zhi-Jie Liu, Svetlana Markova, John Lee, John Rose, and Bi-Cheng Wang. Luminescence 17:87(2002).

21. Substitution of Trp92 by Phe in obelin produces a violet bioluminescence and an increase in the speed of bioluminescence response to calcium. Eugene Vysotski, Lu Deng, Svetlana Markova, John Blinks, Zhi-Jie Liu, Michelle Herko, Natalia Malikova, Ludmila Frank, Bi-Cheng Wang, and John Lee. Luminescence 17:112(2002).

22. High resolution (1.17 Δ) structure of obelin with coelenterazine h. Lu Deng, Eugene Vysotski, Bruce R. Branchini,Zhi-Jie Liu, John Lee, John Rose, and Bi-Cheng Wang. Luminescence 17:87(2002).