Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Spatial structure of the novel light-sensitive photoprotein berovin from the ctenophore *Beroe abyssicola* in the Ca^{2+}-loaded apoprotein conformation state

Galina A. Stepanyuk [a,b,1], Zhi-Jie Liu [a,c,d,1], Ludmila P. Burakova [b,e], John Lee [a], John Rose [a], Eugene S. Vysotski [b,e,*], Bi-Cheng Wang [a]

[a] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA
[b] Photobiology Laboratory, Institute of Biophysics Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia
[c] National Laboratory of Biomacromolecules, Institute of Biophysics Chinese Academy of Sciences, Beijing 100101, China
[d] Institute of Molecular and Clinical Medicine, Kunming Medical University, Kunming 650500, China
[e] Laboratory of Bioluminescence Biotechnology, Institute of Fundamental Biology and Biotechnology, Siberian Federal University, Krasnoyarsk 660041, Russia

ARTICLE INFO

Article history:
Received 1 May 2013
Received in revised form 3 July 2013
Accepted 17 July 2013
Available online xxxx

Keywords:
Coelenterazine
Calcium
Bioluminescence
Luciferase

ABSTRACT

The bright bioluminescence of ctenophores, found in oceans worldwide, is determined by Ca^{2+}-regulated photoproteins, functionally identical to and sharing many properties of hydromedusan photoproteins. In contrast, however, the ctenophore photoproteins are extremely sensitive to UV and visible light over the range of their absorption spectrum. The spatial structure of a novel light-sensitive photoprotein from the ctenophore *Beroe abyssicola* in its apoform bound with three calcium ions is determined at 2.0 Å. We demonstrate that the apoberovin is a slightly asymmetrical compact globular protein formed by two domains with a cavity in the center, which exactly retains the fold architecture characteristic of hydromedusan photoproteins despite their low amino acid sequence identity. However, the structural alignment of these two photoprotein classes clearly shows that despite the high similarity of shape and geometry of their coelenterazine-binding cavities, their interiors differ drastically. The key residues are appearing to be crucial for stabilizing the CBV in berovin by amino acid residues having completely different side chain properties. Evidently, these replacements must be responsible for the distinct properties of ctenophore photoproteins such as sensitivity to light or the fact that the formation of active photoprotein from apophotoprotein, coelenterazine, and oxygen is more effective at alkaline pH.

1. Introduction

Bioluminescence is a widely distributed phenomenon among marine dwellers [1,2]. Many of these luminous organisms generate light by oxidation of coelenterazine, an imidazopyrazinone derivative [3,4]. The chemical mechanism of light emission appears to be common among various organisms utilizing coelenterazine but often differs in the detailed biochemical process, probably as necessitated by the behavioral function of bioluminescence [5].

The Ca^{2+}-regulated photoproteins constitute a unique class of protein biochemistry. They are responsible for the light emission of a variety of marine coelenterates. The best known and studied of these photoproteins are aequorin from the jellyfish *Aequorea* and obelin from the hydroids *Obelia* — both belonging to the class Hydrozoa. Ca^{2+}-regulated photoproteins are "precharged" bioluminescent proteins that are triggered to emit light by binding Ca^{2+} or certain other inorganic ions [6]. The reaction does not require the presence of molecular oxygen or any other cofactor — the photoprotein and the triggering ion are the only components required for light emission. Since the energy emitted as light is derived from the "charged" photoprotein, that molecule can react only once, i.e., it does not "turn over" as an enzyme does. In this respect, as well as in the lack of a requirement for molecular oxygen or any other cofactor, the reaction is strikingly different from that of classical bioluminescent systems in which an enzyme (luciferase) catalyzes the oxidation of a smaller organic substrate molecule (luciferin) with the creation of an excited state and the emission of light. This feature prompted Shimomura and Johnson to coin the term "photoprotein" to describe proteins that contain a reactive organic molecule in its bioluminescent reaction system [7]. Although they described other kinds of photoproteins, the great majority of photoproteins presently known are stimulated to luminescence by calcium ions and the term "Ca^{2+}-activated photoproteins" was applied to them by Hastings and Morin [8]. Later the term "Ca^{2+}-regulated photoproteins" was suggested to refer to this group, first because these proteins are

* Corresponding author at: Photobiology Laboratory, Institute of Biophysics Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia. Tel.: +7 391 249 4430; fax: +7 391 243 3400.
E-mail address: eugene.vysotski@gmail.com (E.S. Vysotski).
[1] These authors contributed equally to this work.

1570-9639/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbapap.2013.07.006

members of the family of calcium-regulated proteins and second, because calcium regulates the function of these proteins but is not essential for it [9]. Ca^{2+}-free photoproteins emit a very low level of light named "Ca^{2+}-independent bioluminescence" but the light intensity increases to 1-million fold or even more after the addition of calcium [10].

All hydromedusan Ca^{2+}-regulated photoproteins known to date consist of a single polypeptide chain with a molecular mass of ~22 kDa [11], and have a hydrophobic cavity in which a peroxy-substituted coelenterazine, 2-hydroperoxycoelenterazine, is tightly but non-covalently bound. The Ca^{2+} binding to the protein induces oxidative decarboxylation of the 2-hydroperoxycoelenterazine with generation of the protein-bound product, coelenteramide, in its excited state [12,13]. Excited coelenteramide reverts to its ground state with the production of blue light, a broad spectrum with maxima in the range 465–495 nm, depending on the photoprotein type [14]. In the past decade the crystal structures of the photoproteins aequorin [15], obelin [16,17], and clytin [18] as well as their ligand-dependent conformation states [19–21] have been determined. Based on these findings a credible mechanism of hydromedusan photoprotein bioluminescence has been suggested [14,22,23].

Ctenophores (comb jellies) are found in oceans worldwide and nearly all species are luminous [24,25]. The bright bioluminescence of these organisms is also determined by Ca^{2+}-regulated photoproteins that are functionally identical to and share many properties of hydromedusan photoproteins [26,27]. Ctenophore photoproteins are also a stable complex of apoprotein and 2-hydroperoxycoelenterazine requiring only Ca^{2+} addition to produce bioluminescence [6]. However, in contrast to hydromedusan photoproteins, the ctenophore photoprotein complex is extremely sensitive to UV and visible light; the bioluminescence capacity deteriorates on exposure to light over its entire absorption spectral range, most likely due to photodestruction of the bound 2-hydroperoxycoelenterazine [28,29].

Recently cDNA genes encoding several ctenophore photoproteins have been cloned. These are berovin from *Beroe abyssicola* [30], bolinopsin from *Bolinopsis infundibulum* [31,32], mnemiopsin from *Mnemiopsis leidyi* [33,34], and the photoprotein from *Bathocyroe fosteri* [35]. The analysis of amino acid sequences revealed that similar to hydromedusan photoproteins [11], the ctenophore photoproteins also contain three canonical sequence loop regions, each of 12 contiguous residues, which supply the oxygen ligands needed for calcium ion coordination. This property brings ctenophore photoproteins into the family of EF-hand Ca^{2+}-binding proteins [36], one of the most numerous and extensively studied protein families. Noteworthy is that although ctenophore photoproteins are functionally identical in many properties to hydromedusan photoproteins, the degree of identity of their amino acid sequences is very low; the highest degree of identity between berovin and hydromedusan photoproteins, for example, is only 29.4% [30]. Thus, these photoproteins might be regarded as a novel type of Ca^{2+}-regulated photoprotein distinct from hydromedusan photoproteins.

In this paper we determine the crystal structure at 2.0 Å of the apoberovin from the ctenophore *B. abyssicola* bound with calcium ions. We demonstrate for the first time that the Ca^{2+}-regulated photoprotein berovin displays structural features characteristic of the Ca^{2+}-binding proteins belonging to the EF-hand family. Despite the low degree of identity between their sequences, berovin retains the same fold architecture as the Ca^{2+}-regulated photoproteins of Hydrozoa.

2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutagenesis was performed on the template pET22b-BA13 *Escherichia coli* expression plasmid carrying the wild-type berovin gene [30]. Mutations resulting in the desired amino acid changes were carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the protocol supplied with the kit. Mutations W192F and Y204F were introduced with the following primers, respectively:

- For1 5′-CATCTCTTTAGAAAGTTT**TTC**ATGGAGCCTTACGAT-3′
- Rev1 5′-ATCGTAAGGCTCCAT**GAA**AAACTTTCTAAAGAGATG-3′
- For2 5′-ACAGTGGGACGGAGTC**TTC**GCTTATAAGTA-3′
- Rev2 5′-TACTTATAAGC**GAA**GACTCCGTCCCACTGT-3′.

The substitutions introducing the mutations are in bold, underlined. The presence of the mutations was verified by DNA sequencing.

2.2. Protein production and purification

The *E. coli* strain B834 (DE3) cells (Novagen) were transformed with plasmid pET22b-BA13 containing the gene encoding apoberovin from *B. abyssicola* (GenBank: JN673815) [30] without any purification tags. To produce apoberovin labeled by Se-Met, the transformed *E. coli* cells were cultivated with vigorous shaking in minimal PASM-5052 auto-inducing media [37] during 16 h at 37 °C. Most of the apoprotein accumulated in inclusion bodies that can be easily isolated by centrifugation. The apoberovin was purified as previously reported for recombinant obelin [38,39] with little modification. After chromatography on DEAE Sepharose Fast Flow in 6 M urea, the protein sample was diluted 10-fold by 1 mM $CaCl_2$, 20 mM Tris–HCl pH 7.1 for refolding and loaded on a DEAE-10 column (Bio-Rad) for additional purification. The protein was eluted with a linear gradient (0–0.5 M) of NaCl, the main peak was collected, and then protein was concentrated on Amicon centrifugal filters (Millipore) with a buffer change for 1 mM calcium acetate, 10 mM Bis-Tris-propane pH 6.5.

The wild type berovin and its mutants for bioluminescence measurements were produced in *E. coli* strain BL21(DE3)-CodonPlus-RIPL (Stratagene) harboring corresponding plasmids and purified as previously described [30]. The wild type apoberovin and its mutants were charged with coelenterazine in 0.5 M NaCl, 5 mM EDTA, 50 mM Bis-Tris-propane pH 9.0 by incubating overnight at 4 °C. Ion-exchange chromatography on a Mono Q column (GE Healthcare) was used to separate the berovin from its apoprotein. Before loading on the Mono Q column, the protein samples were diluted 20-fold with a buffer 5 mM EDTA, Tris–HCl pH 7.2. All manipulations with the charged wild type berovin and its mutants including bioluminescence and spectral measurements were performed under dim red light to avoid photoinactivation of its bioluminescence property. Coelenterazine was obtained from Prolume Ltd. (Pinetop, USA).

Protein concentration was determined with the D_c Bio-Rad protein assay kit.

2.3. Bioluminescent assay and spectral measurements

The specific bioluminescent activities of wild type berovin and its mutants were measured by integrating the light signal over 10 s. Bioluminescence was initiated by rapid injection of 10 μL photoprotein solution at concentration of 40 nM, into a luminometer cell containing 490 μL of 2 mM $CaCl_2$ in 50 mM Bis-Tris-propane–HCl pH 8.5 at room temperature. Only freshly purified proteins were used for measurements.

Bioluminescence spectra were measured with a Varian Cary Eclipse spectrofluorimeter (Agilent Technologies) in 50 mM Bis-Tris-propane pH 7.0 (scanning speed 200 nm/s, slit 5 nm). Bioluminescence was initiated by adding the $CaCl_2$ solution in the same buffer. Emission spectra were corrected using the computer program supplied with the instrument. The concentration of free calcium was ~0.5 μM to provide an approximately constant light level during the spectral scan. If any substantial change of bioluminescence intensity took place during the spectral scan, the data points were corrected for this.

Please cite this article as: G.A. Stepanyuk, et al., Spatial structure of the novel light-sensitive photoprotein berovin from the ctenophore Beroe abyssicola in the Ca^{2+}-loaded apoprotein conformation state, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbapap.2013.07.006