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All Ca^{2+}-binding loops of light-sensitive ctenophore photoprotein berovin bind magnesium ions: The spatial structure of Mg^{2+}-loaded apo-berovin

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ABSTRACT

Light-sensitive photoprotein berovin accounts for a bright bioluminescence of ctenophore *Beroe abyssicola*. Berovin is functionally identical to the well-studied Ca^{2+}-regulated photoproteins of jellyfish, however in contrast to those it is extremely sensitive to the visible light. Berovin contains three EF-hand Ca^{2+}-binding sites and consequently belongs to a large family of the EF-hand Ca^{2+}-binding proteins. Here we report the spatial structure of apo-berovin with bound Mg^{2+} determined at 1.75 Å. The magnesium ion is found in each functional EF-hand loop of a photoprotein and coordinated by oxygen atoms donated by the side-chain groups of aspartate, carbonyl groups of the peptide backbone, or hydroxyl group of serine with characteristic oxygen-Mg^{2+} distances. As oxygen supplied by the side-chain of the twelfth residue of all Ca^{2+}-binding loops participates in the magnesium ion coordination, it was suggested that Ca^{2+}-binding loops of berovin belong to the mixed Ca^{2+}/Mg^{2+} rather than Ca^{2+}-specific type. In addition, we report an effect of physiological concentration of Mg^{2+} on bioluminescence of berovin (sensitivity to Ca^{2+}, rapid-mixed kinetics, light-sensitivity, thermostability, and apo-berovin conversion into active protein). The different impact of physiological concentration of Mg^{2+} on berovin bioluminescence as compared to hydromedusan photoproteins was attributed to different affinities of the Ca^{2+}-binding sites of these photoproteins to Mg^{2+}.

1. Introduction

Ca^{2+}-regulated photoproteins are single-chain proteins which are responsible for the light emission of a variety of marine organisms [1]. The best known and best studied of these are aequorin, first isolated in 1962 by Shimomura et al. [2] from the jellyfish *Aequorea victoria*, and obelin from the hydroid *Obelia longissima* [3]. The Ca^{2+}-regulated photoproteins are "precharged" bioluminescent proteins that are triggered to emit light by binding calcium or certain other inorganic ions. 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, the photoprotein molecule reacts 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) yielding the product in the excited state and following light emission [1]. However the oxygen is involved in a photoprotein bioluminescence — O_2 is needed for the formation of an active photoprotein from apoprotein and coelenterazine at Ca^{2+}-free conditions. In fact, the photoprotein is an enzyme containing the stabilized reaction intermediate, 2-hydroperoxycoelenterazine, which is tightly but non-covalently bound within inner protein cavity. The photoprotein light emission reaction is an oxidative decarboxylation of peroxy-substituted coelenterazine with the elimination of carbon dioxide and generation of the protein-bound product, coelenteramide, in the S_1 excited state [4,5]. The excited product then relaxes to the ground state accompanied by light emission with a maximum within the range 465–495 nm, depending on the photoprotein type [6].

Bioluminescence of ctenophores (comb jellies) ubiquitously distributed in the oceans [7] is also caused by Ca^{2+}-regulated photoproteins [1]. Although ctenophore photoproteins are identical to hydromedusan

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photoproteins in many properties [8,9], in contrast of these, they are extremely sensitive to UV and visible light. Ctenophore photoproteins lose the ability to bioluminescence on exposure to light over its entire absorption spectral range [10,11]. In the past decade, cDNA genes encoding Ca^{2+}-regulated photoproteins from ctenophores *Beroe abyssicola* [12], *Bolinopsis infundibulum* [13,14], *Mnemiopsis leidyi* [15,16], and *Bathocyroe fosteri* [17] have been cloned. The comparison of ctenophore photoprotein amino acid sequences with those of aequorin [18,19], clytin [20,21], and mitrocomin [22] from the jellyfish *A. victoria*, *Clytia gregaria*, and *Mitrocoma cellularia*, respectively, and obelins [23,24] from the hydroids *O. longissima* and *O. geniculata* revealed a very low degree of identity (only 29.4%) [12,17]. This suggests that ctenophore photoproteins represent a novel type of Ca^{2+}-regulated photoproteins which differs from hydromedusan photoproteins. However, despite the differences, both ctenophore and hydromedusan Ca^{2+}-regulated photoproteins contain three Ca^{2+}-binding motifs formed by two α-helices that flank a canonical sequence loop region consisting of 12 contiguous residues which supply the oxygen ligands for Ca^{2+} coordination [25]. The crystal structures of Ca^{2+}-regulated photoproteins from different organisms determined in the past decade [26–29] confirmed the existence of helix–loop–helix structures in photoproteins, three of which can bind calcium ions [29–31]. This structure feature brings photoproteins into the family of EF-hand Ca^{2+}-binding proteins [32]. These proteins are the extensively studied protein family as they are involved in a regulation of numerous cellular functions from fertilization, contraction, cell differentiation and proliferation, to apoptosis and cancer through control of $[Ca^{2+}]_i$ [33].

The Ca^{2+}-regulated photoproteins have attracted great interest owing to their broad applications in analytical assays *in vivo* and *in vitro* [3]. However, the main use of photoproteins derives from their ability to emit light on Ca^{2+} binding, allowing them to be applied to detect calcium ions within living cells [34,35]. Despite the availability of other recombinant photoproteins, only aequorin is widely used as an intracellular Ca^{2+} indicator though there are a number of shortcomings that limit its utility. The most significant drawback is that physiological concentrations of magnesium ions [36] considerably slow the bioluminescence response of aequorin on a sudden change of Ca^{2+} concentration and decrease its sensitivity to calcium [37,38] that is obviously caused by competition of Mg^{2+} with calcium for Ca^{2+}-binding sites [39]. Bioluminescence of other Ca^{2+}-regulated photoproteins is less sensitive to magnesium [40] that is apparently due to the variations in amino acid composition of the Ca^{2+}-binding loops and, consequently, lead to different affinities to Mg^{2+}. For instance, only Ca^{2+}-binding loops I and III of aequorin bind magnesium ions [39]. At that, the affinities of Ca^{2+}-binding loops to Mg^{2+} were found to be different though the degree of identity of amino acid sequences of these loops is 66.7%. It should be pointed out that there is no direct evidence of binding magnesium ions by the Ca^{2+}-binding loops of other Ca^{2+}-regulated photoproteins.

In the present study, we report the crystal structure of apo-berovin from *B. abyssicola* with three magnesium ions bound at its Ca^{2+}-binding sites determined at 1.75 Å resolution, as well as the effect of Mg^{2+} on berovin bioluminescence properties.

2. Materials and Methods

All reagents were used as received without further purification unless otherwise stated and were obtained from Sigma-Aldrich. The coelenterazine was purchased from Prolume Ltd (Pinetop, USA).

2.1. Protein Production and Purification

To produce protein, the plasmid p22-BA containing the gene encoding apo-berovin from *B. abyssicola* without any purification tags [12], was transformed into *E. coli* cells strain BL21(DE3)-CodonPlus-RIPL (Novagen, USA). Then the transformed cells were cultivated with vigorous shaking at 37 °C in LB medium containing ampicillin and induced with 1 mM IPTG when the culture reached an OD_{600} of 0.6. After addition of IPTG, the cultivation was continued for 3 h. Most of the apo-berovin produced was accumulated inside *E. coli* cells in inclusion bodies that can be easily isolated by centrifugation. The apo-berovin was purified as previously described [12,41,42]. For crystallization experiments, the apo-berovin was prepared by dilution of apoprotein sample in 6 M urea obtained after chromatography on DEAE Sepharose Fast Flow in buffer 2 mM EDTA, 10 mM Tris–HCl pH 9.0 and the following concentration on Amicon centrifugal filters (Millipore, USA). For bioluminescence measurements, the berovin was prepared by dilution of apoprotein sample in 6 M urea obtained after chromatography on DEAE Sepharose Fast Flow in buffer 0.5 M NaCl, 5 mM EDTA, 20 mM Tris–HCl pH 9.0, concentration on Amicon centrifugal filters, and incubation with coelenterazine (apoprotein/coelenterazine molar ratio 1/1.1) overnight at 4 °C. The coelenterazine concentration was determined using the absorption coefficient $\varepsilon_{435\ nm} = 9800\ cm^{-1}\ M^{-1}$ [1]. To separate apoprotein from the charged berovin the photoprotein was additionally purified by ion-exchange chromatography on Mono Q column (GE Healthcare) equilibrated with 5 mM EDTA, 20 mM Tris–HCl pH 7.2. Before loading on the Mono Q column, the berovin sample was diluted 20-fold with 5 mM EDTA, 20 mM Tris–HCl pH 7.2. The berovin was eluted with a linear salt gradient (0–0.5 M NaCl in 5 mM EDTA, 20 mM Tris–HCl pH 7.2). The obtained protein samples were of high purity according to SDS PAGE.

Obelin and aequorin were produced and purified as described elsewhere [41,42].

2.2. Crystallization, Data Collection, Structure Solution, and Crystallographic Refinement

The initial crystallization trial was carried out with apo-berovin sample in concentration of 14.5 mg mL^{-1}. A Mosquito crystallization robot (TTP LabTech, UK) and 384 commercially available conditions were used for initial screening. A cluster of translucent rods (about 0.005 × 0.01 × 0.03 mm in size) was grown in 3 weeks at 4 °C in 0.2 M magnesium format, 20% PEG 3350, pH 6.5 (PEG ION, Hampton Research, USA). The apo-berovin crystals suitable for diffraction data collection were grown in 1 week at 16 °C after manual optimization of this condition with different apo-berovin concentrations using the hanging-drop vapor-diffusion technique. The best crystals (0.05 × 0.1 × 0.2 mm) were obtained at apo-berovin concentration of 20 mg mL^{-1}. The crystals were frozen in liquid nitrogen in a cryoprotectant solution of glycerol. The diffraction data set was collected at wavelength of 0.9792 Å at beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (Shanghai, China). Data reduction was carried out with the HKL2000 suite [43]. Phases were determined by molecular replacement with PHASER [44] using apo-berovin structure (PDB entry 4MN0) [29] as search model. The final models were refined with PHENIX [45] and REFMAC5 [46]. Manual adjustments to the model were performed with the program COOT [47]. The final refinement statistics are shown in Table 1. Visualization and superposition of the molecular structures was carried out using PyMol (DeLano Scientific LLC). Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession code 5BPJ.

2.3. Calcium Concentration-Effect Curve

Measurements were performed with EDTA-free solutions of the photoproteins. EDTA was removed from the photoprotein by gel filtration on a DSalt plastic column (Pierce, USA). The column was equilibrated and eluted with 150 mM KCl, 5 mM Pipes, pH 7.0, which had been passed twice through freshly washed beds of Chelex-100 chelating resin (Sigma-Aldrich) to remove the trace