Journal of Photochemistry and Photobiology B: Biology 127 (2013) 133–139

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

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

# Role of key residues of obelin in coelenterazine binding and conversion into 2-hydroperoxy adduct

Elena V. Eremeeva [a,b,c], Svetlana V. Markova [a,c], Willem J.H. van Berkel [b], Eugene S. Vysotski [a,c,*]

[a] Photobiology Laboratory, Institute of Biophysics, Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia
[b] Laboratory of Biochemistry, Wageningen University, 6703 HA Wageningen, The Netherlands
[c] Laboratory of Bioluminescence Biotechnology, Institute of Fundamental Biology and Biotechnology, Siberian Federal University, Krasnoyarsk 660041, Russia

ARTICLE INFO

*Article history:*
Received 2 July 2013
Received in revised form 10 August 2013
Accepted 20 August 2013
Available online 28 August 2013

*Keywords:*
Bioluminescence
Coelenterazine
Obelin
Aequorin
Photoprotein

ABSTRACT

Bioluminescence of a variety of marine organisms is caused by monomeric $Ca^{2+}$-regulated photoproteins, to which a peroxy-substituted coelenterazine, 2-hydroperoxycoelenterazine, is firmly bound. From the spatial structure the side chains of Tyr138, His175, Trp179, and Tyr190 of obelin are situated within the substrate-binding pocket at hydrogen bond distances with different atoms of the 2-hydroperoxycoelenterazine. Here we characterized several obelin mutants with substitutions of these residues regarding their bioluminescence, coelenterazine binding, and kinetics of active obelin formation. We demonstrate that Tyr138, His175, Trp179, and Tyr190 are all important for coelenterazine activation; substitution of any of these residues leads to significant decrease of the apparent reaction rate. The hydrogen bond network formed by Tyr138, Trp179 and Tyr190 participates in the proper positioning of coelenterazine in the active site and subsequent stabilization of the 2-hydroperoxy adduct of coelenterazine. His175 might serve as a proton shuttle during 2-hydroperoxycoelenterazine formation.



## 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 coelenterazine utilizing organisms but often differs in the detailed biochemical process, probably as necessitated by the behavioral function of bioluminescence [5].

The $Ca^{2+}$-regulated photoproteins constitute one specific class of coelenterazine utilizing molecules. They comprise monomeric polypeptides with a molecular mass of ~22 kDa to which a peroxy-substituted coelenterazine, 2-hydroperoxycoelenterazine, is tightly but non-covalently bound. Since $Ca^{2+}$-regulated photoproteins contain a stable oxygenated reaction intermediate, they are capable of emitting light in proportion to the concentration of protein unlike the luciferases where the amount of light emitted is proportional to the concentration of the substrate luciferin [6]. Bioluminescence is triggered upon binding of $Ca^{2+}$, which induces the oxidative decarboxylation of 2-hydroperoxycoelenterazine with generation of protein-bound reaction product, coelenteramide, in its excited state [7,8]. The excited coelenteramide relaxes to its ground state with the production of blue light with maxima around 465–495 nm depending on the type of photoprotein [9].

Although $Ca^{2+}$-regulated photoproteins have been detected in many different marine organisms [3,10], cDNA sequence information is only available for five hydromedusan photoproteins, namely aequorin [11–13], mitrocomin (halistaurin) [14], and clytin (phialidin) [15–17] from the jellyfishes *Aequorea, Mitrocoma* (*Halistaura*), and *Clytia* (*Phialidium*), and obelins from the hydroids *Obelia longissima* [18,19] and *Obelia geniculata* [20], and for the light-sensitive photoproteins from ctenophore *Beroe abyssicola* [21], *Mnemiopsis leidyi* [22,23], and *Bathocyroe fosteri* [24]. All $Ca^{2+}$-regulated photoproteins contain three calcium-binding consensus sequences characteristic of EF-hand $Ca^{2+}$-binding proteins [25,26]. Apophotoproteins expressed in *Escherichia coli* can be converted to active photoproteins by incubating them with coelenterazine under $Ca^{2+}$-free conditions in the presence of $O_2$ and reducing agents [27].

The main application of $Ca^{2+}$-regulated photoproteins originates from their ability to emit light upon $Ca^{2+}$ binding. Photoproteins have been successfully used to estimate the intracellular $Ca^{2+}$ concentration under steady-state conditions and to study the role of calcium transients in the regulation of cellular function in different types of cells [28]. The cloning of cDNAs encoding apophotoproteins has granted a new approach in photoprotein applications. The recombinant apophotoprotein expressed intracellularly forms

* Corresponding author at: Photobiology Laboratory, Institute of Biophysics, Russian Academy of Sciences, Siberian Branch, Krasnoyarsk 660036, Russia. Tel.: +7 3912494430; fax: +7 391433400.
*E-mail address:* eugene.vysotski@gmail.com (E.S. Vysotski).

1011-1344/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jphotobiol.2013.08.012

the active photoprotein upon binding with coelenterazine, which is added externally and diffuses into the cell. Thus, such cells have, in effect, a "built-in" calcium indicator. This approach is highly valuable because it does not require laborious procedures like microinjection or liposome-mediated transfer and now is widely used [29–31]. The success of such photoprotein applications, however, depends on various factors among which are the rate and efficacy of the generation of active photoprotein from apophotoprotein, coelenterazine, and oxygen, as well as any influence of the cellular environment on these processes.

In the past decade the crystal structures of photoproteins aequorin [32], obelin [33,34], and clytin [35] as well as obelin ligand-dependent conformation states [36–38] have been determined. Based on these findings and mutagenesis studies of some substrate-binding cavity residues [39–42] significant insight has been obtained into the bioluminescence mechanism [9,38,42,43]. At the same time much less is known about the molecular mechanism of active photoprotein complex formation from apophotoprotein, coelenterazine and oxygen (Scheme 1). Previous studies focused primarily on the efficiency of regeneration of wild type apo-aequorin with coelenterazine derivatives as well as the effects of temperature, pH, incubation time and some additives on this process [27,44–46]. It was shown, for example, that dithiothreitol (DTT) or β-mercaptoethanol are required to reduce disulfide bonds in the recombinant apo-aequorin [47]. Furthermore, it was determined that the binding of coelenterazine to apophotoprotein occurs within milliseconds [48], in contrast to the formation of active photoprotein complex [27]. This finding evidently showed that the rate-limiting step of active photoprotein formation is the conversion of coelenterazine into its peroxy derivative.

The substrate-binding cavity of obelin is highly hydrophobic with addition of several hydrophilic side chains (His22, Tyr138, His175, and Tyr190) directed internally. The side chains of Tyr138, His175, Trp179, and Tyr190 are situated within the coelenterazine-binding pocket at hydrogen bond distances with certain atoms of the 2-hydroperoxycoelenterazine (Fig. 1). Recently, it was shown that the hydrogen bond network formed by His175–Trp179–Tyr190 triad participates in positioning and stabilizing the 2-hydroperoxy adduct of coelenterazine and that His175 is critical for the bioluminescence function [49]. In active obelin His175, Trp179, and Tyr190 are located near the C2 atom of coelenterazine, which is the exact position for oxygen to react with the substrate. Thus, it looks reasonable to assume that these residues could be involved in the process of active photoprotein complex formation. Tyr138 is hydrogen bonded to the N1 atom of 2-hydroperoxycoelenterazine (Fig. 1) and potentially might effect the charge distribution in the imidazopyrazinone ring of the coelenterazine molecule via polarizing the N1-nitrogen and thus the efficiency of the coelenterazine reaction with oxygen.

In this paper we report for the first time the study of the function of amino acid residues located in the substrate-binding cavity of $Ca^{2+}$-regulated photoprotein obelin in the formation of 2-hydroperoxycoelenterazine and consequently active obelin. Several obelin mutants with substitutions of Tyr138, His175, Trp179 and Tyr190 were characterized regarding their bioluminescence, coelenterazine binding and kinetics of active obelin formation.

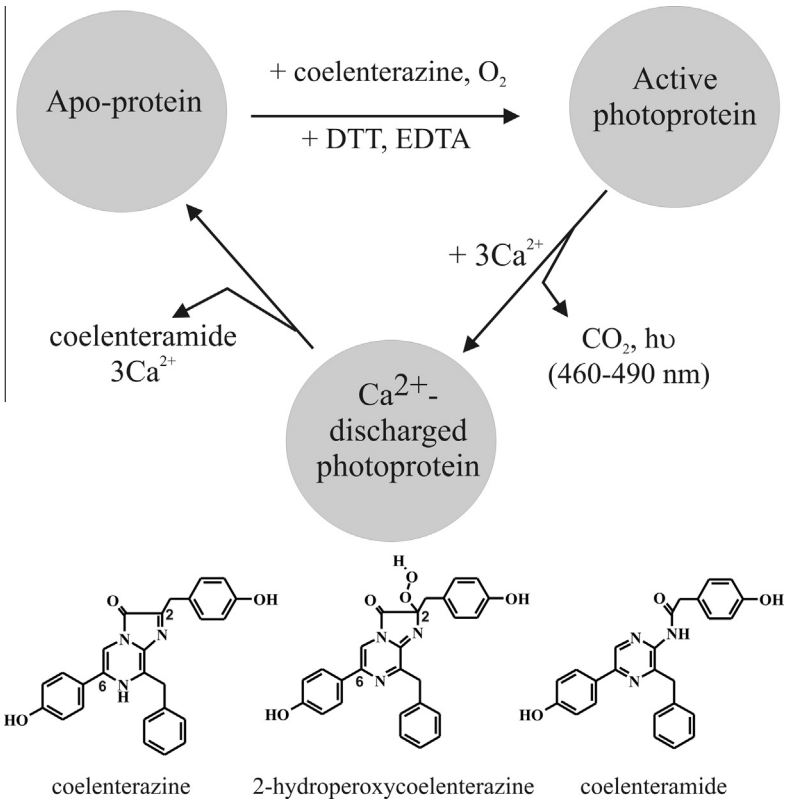
## 2. Experimental methods

### 2.1. Materials

Coelenterazine was obtained from Prolume Ltd. (Pinetop, AZ, USA). Other chemicals, unless otherwise stated, were from Sigma–Aldrich and the purest grade available.

### 2.2. Molecular biology

Site-directed mutagenesis was done on the template pET19-OL8 *E. coli* expression plasmid carrying the *O. longissima* wild type apo-obelin gene [51]. Mutations resulting in the desired amino




**Scheme 1.**