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Bioluminescent and biochemical properties of Cys-free Ca^{2+}-regulated photoproteins obelin and aequorin

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ABSTRACT

Bioluminescence of a variety of marine coelenterates is determined by Ca^{2+}-regulated photoproteins. A strong interest in these proteins is for their wide analytical potential as intracellular calcium indicators and labels for *in vitro* binding assays. The presently known hydromedusan Ca^{2+}-regulated photoproteins contain three (aequorin and clytin) or five (obelin and mitrocomin) cysteine residues with one of them strictly conserved. We have constructed Cys-free aequorin and obelin by substitution of all cysteines to serine residues. Such mutants should be of interest for researchers by the possibility to avoid the incubation with dithiothreitol (or β-mercaptoethanol) required for producing an active photoprotein that is important for some prospective analytical assays in which the photoprotein is genetically fused with a target protein sensitive to the reducing agents. Cys-free mutants were expressed in *Escherichia coli*, purified, and characterized regarding the efficiency of photoprotein complex formation, functional activity, and conformational stability. The replacement of cysteine residues has been demonstrated to affect different properties of aequorin and obelin. Cys-free aequorin displays a two-fold lower specific bioluminescence activity but preserves similar activation properties and light emission kinetics compared to the wild-type aequorin. In contrast, Cys-free obelin retains only ~10% of the bioluminescence activity of wild-type obelin as well as binding coelenterazine and forming active photoprotein much less effectively. In addition, the substitution of Cys residues drastically changes the bioluminescence kinetics of obelin completely eliminating a "fast" component from the light signal decay curve. At the same time, the replacement of Cys residues increases conformational flexibility of both aequorin and obelin molecules, but again, the effect is more prominent in the case of obelin. The values of thermal midpoints of unfolding (T_m) were determined to be 53.3 ± 0.2 and 44.6 ± 0.4 °C for aequorin and Cys-free aequorin, and 49.1 ± 0.1 and 28.8 ± 0.3 °C for obelin and Cys-free obelin, respectively. Thus, so far only Cys-free aequorin is suitable as a partner for fusing with a tag sensitive to reducing agents since the aequorin mutant preserves almost 50% of the bioluminescent activity and can be produced with a substantial yield.

1. Introduction

Bioluminescence of a variety of marine organisms, mostly coelenterates, is caused by Ca^{2+}-regulated photoproteins [1]. The best known of these are aequorin and obelin from the hydrozoans *Aequorea victoria* and *Obelia longissima*, respectively [1,2]. All Ca^{2+}-regulated photoproteins characterized by now are single-chain globular proteins comprising a non-covalently bound oxygenated coelenterazine ligand, 2-hydroperoxycoelenterazine, in its inner cavity [3–7]. Bioluminescence reaction is triggered upon binding of calcium ions to the EF-hand Ca^{2+}-binding loops of a photoprotein. This leads to small conformational changes within a substrate-binding cavity of the photoprotein molecule that initiates an oxidative decarboxylation of the bound 2-hydroperoxy adduct of coelenterazine with the elimination of one mole of CO_2 and generation of the protein-bound product, coelenteramide, in an excited state [8–10]. Its relaxation to the ground state is accompanied by the emission of blue light with λ_{max} at 465–495 nm depending on the photoprotein type [8].

Ca^{2+}-regulated photoproteins draw a strong interest due to their wide analytical potential (mostly as probes of intracellular [Ca^{2+}]) originating from the ability to emit light upon Ca^{2+} binding. The cloning of cDNAs encoding photoproteins from the hydrozoans *Aequorea victoria* [11,12], *Clytia gregaria* [13–15], and *Mitrocoma cellularia* [7,16] as well as *Obelia longissima* [17,18] and *Obelia geniculata* [19] has not only ensured new avenues for applying photoproteins by their expression in cells but has also expanded their availability since Ca^{2+}-regulated photoproteins from various organisms turned out to reveal different properties as intracellular calcium indicators [20,21].

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This approach is highly valuable because such cells have a "built-in" calcium indicator. In addition, Ca^{2+}-regulated photoprotein can be modified to introduce specific targeting sequences providing a selective localization of the photoprotein to a cell region of interest where local calcium transients need to be measured [22,23]. Clearly, this offers an important advantage of photoproteins over calcium fluorescent probes [24].

Ca^{2+}-regulated photoproteins from different organisms reveal a high degree of similarity of amino acid sequences (64–76%) and crystal structures (RMSD 0.50–0.66 Å) [2,7,25]. The most conserved residues are those forming a substrate-binding pocket of photoproteins; only a few residues differ. At the same time, there is just one distinction that significantly affects bioluminescence properties of photoproteins. In aequorin and mitrocomin His, Trp, and Tyr are found near the OH group of the 6-(p-hydroxyphenyl) substituent of 2-hydroperoxycoelenterazine, while in obelins and clytin the corresponding positions are occupied by His, Trp, and Phe [8]. As was demonstrated [26,27], a different arrangement of the hydrogen bond network around p-OH of the phenol attached at C6 of 2-hydroperoxycoelenterazine is responsible for variations in light emission spectra of hydromedusan photoproteins. It was also proposed that a few other residues situated in a substrate-binding pocket may play an important role in photoprotein bioluminescence [28,29]. However much less is known about the function of many other amino acid residues of photoproteins.

Of the hydromedusan Ca^{2+}-regulated photoproteins known to date, aequorin and clytin contain three cysteine residues whereas obelin and mitrocomin contain five cysteine residues (Fig. 1), and one of these residues is strictly conserved (see Cys highlighted in yellow in Fig. 1C). In aequorin, the cysteine residues are located at positions 145, 152, and 180 (Fig. 1A), whereas in obelin they occupy positions 51, 67, 75, 151, and 158 (Fig. 1B). As follows from the structures of these photoproteins (Fig. 1A,B), Cys145 and Cys152 of aequorin are in the same location as Cys151 and Cys158 of obelin, respectively. The other cysteine residues of obelin (i.e., Cys51, Cys67 and Cys75) are situated at the N-terminal domain of the photoprotein molecule (Fig. 1B,C). The sequence alignment (Fig. 1C) shows that Cys51 in obelin corresponds to Cys54 and Cys53 in clytin and mitrocomin, respectively, and Cys67 in obelin matches Cys69 in mitrocomin. In contrast, Cys75, which is located near to the third α–helix of obelin, is unique to this photoprotein. In aequorin, Cys180 corresponds to Cys188 in mitrocomin (Fig. 1C). Earlier it was shown that substitution of any cysteine residue in aequorin leads to a considerable decrease of bioluminescence activity [30]. Despite the results obtained on aequorin mutants with one cysteine substituted, cysteine-free aequorin revealed an increased bioluminescence activity and independence of dithiothreitol (DTT) upon its conversion into active photoprotein as compared to the wild-type photoprotein [30]. This aequorin mutant was proposed as a promising label in binding assays [31,32]. Replacement of the conserved Cys158 in obelin to Ser or Ala resulted in a decrease of bioluminescence activity and stability as well as slowing down the decay kinetics and the rate of apo-obelin conversion into active photoprotein [33]. In addition, the substitutions of cysteine residues also altered the effects of alternative divalent cations such as barium and magnesium on the bioluminescence of both aequorin and obelin [34]. However, the obtained results, especially the gain of bioluminescence activity, are not easily explainable because none of the Cys residues is found in the substrate-binding cavity either before or after the bioluminescent reaction, or in the EF-hand Ca^{2+}-binding loops [4,5,9].

To further investigate the role of Cys residues in photoprotein bioluminescence, we have replaced all cysteine residues in aequorin and obelin with serines which side-chain donor-acceptor properties are very similar to those of cysteines and characterized these Cys-free mutants in terms of efficiency of the photoprotein complex formation and functional activity.

2. Materials and Methods

2.1. Materials

Coelenterazine was obtained from Prolume Ltd. (Pinetop, AZ, USA). All other chemicals, unless otherwise stated, were from Sigma-Aldrich at the purest grade available.

2.2. Molecular Biology

Site-directed mutagenesis was performed on the templates pET19-OL8 [35] and pET19-Aq7 [36] of *Escherichia coli* expression plasmids carrying the wild-type apo-obelin from *O. longissima* and wild-type apo-aequorin genes, respectively. Mutations resulting in the desired amino acid changes were carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol supplied by the manufacturer. Cys-free photoproteins were obtained by successive substitutions of each cysteine residue to serine (three and five cysteines in total in the case of aequorin and obelin, respectively). The plasmids harbouring the mutations were verified by DNA sequencing.

2.3. Apophotoprotein Preparation

Apophotoproteins were produced as previously reported [29,37]. Apophotoproteins from inclusion bodies were extracted by 6 M urea, purified on a DEAE-Sepharose Fast Flow column, and then concentrated with the use of 10 kDa Ultra Centrifugal Filters (Millipore). To fold apophotoproteins, the concentrated samples containing 6 M urea were diluted approximately 20-fold with a solution containing 1 mM EDTA, 20 mM Tris-HCl pH 7.2, concentrated again, and then washed several times with the same buffer to remove any impurities of urea and salts. The apophotoproteins were centrifuged (20,000 g, 10 min) at 4 °C, incubated overnight with 10 mM DTT, centrifuged once again, and then passed through a Superdex 75 column (Amersham Bioscience) equilibrated with freshly prepared 10 mM DTT, 5 mM EDTA, 20 mM Tris-HCl pH 7.2 to produce a monomeric apophotoprotein containing no disulfide bonds or aggregates. The final preparations of apophotoproteins were homogeneous according to SDS-PAGE and gel filtration.

The active wild-type photoproteins and their mutants were produced as described elsewhere [38,39]. Apophotoproteins were activated overnight with a 2-fold molar excess of coelenterazine in a buffer 5 mM EDTA, 10 mM DTT, 20 mM Tris-HCl pH 7.2 at 4 °C. Active photoproteins were separated from apophotoproteins, unbound coelenterazine, and DTT by ion-exchange chromatography on Mono Q column (Amersham Bioscience). Active photoproteins and apophotoproteins were eluted as separate peaks with the linear salt gradient of 1 M NaCl in 5 mM EDTA, 20 mM Tris-HCl pH 7.2. With chromatography elution profiles applied, the yield of active photoprotein was determined as a percentage of apoprotein taken for activation with coelenterazine and loaded on Mono Q column. The concentrations of apophotoproteins and their mutants were determined using the corresponding molar extinction coefficients at 280 nm calculated with the ProtParam tool (http://us.expasy.org/tools/protparam-doc.html) that uses the method of Edelhoch [40].

2.4. Fluorescence Measurements and Determination of Apparent Dissociation Constant of the Apophotoprotein-Coelenterazine Complex

Fluorescence measurements were performed with a Varian Cary Eclipse spectrofluorimeter (Agilent Technologies) in 5 mM EDTA, 10 mM DTT, 20 mM Tris-HCl pH 7.2 at 20 °C. The concentrations of monomeric apophotoproteins were 1.22 μM. Excitation was at 295 nm (slit 5 nm). The fluorescence emission spectra were corrected with the computer program supplied with the instrument. All spectra were taken using a standard quartz cuvette (1 × 1 cm) in a 1-mL initial volume with varying coelenterazine additions in 5- to 10-μL portions up to the