Interchange of aequorin and obelin bioluminescence color is determined by substitution of one active site residue of each photoprotein

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Received 25 November 2004; revised 3 January 2005; accepted 6 January 2005
Available online 13 January 2005

Edited by Peter Brzezinski

Abstract The bioluminescence spectra from the Ca\textsuperscript{2+}-regulated photoproteins aequorin ($\lambda_{\text{max}} = 469$ nm) and obelin ($\lambda_{\text{max}} = 482$ nm) differ because aequorin has an H-bond from its Tyr82 to the bound coelenteramide, not present in obelin at the corresponding Phex. Substitutions of this Phex by Tyr, Trp, or His shifted the obelin bioluminescence to shorter wavelength with F88Y having $\lambda_{\text{max}} = 453$ nm. Removal of the H-bond by the substitution of Y82F in aequorin shifted its bioluminescence to $\lambda_{\text{max}} = 501$ nm. All mutants were stable with good activity and were expressible in mammalian cells, thereby demonstrating potential for monitoring multiple events in cells using multi-color detection.

Keywords: Coelenterazine; Calcium; Reporter protein; Mammalian expression; Fluorescence spectrum

1. Introduction

Aequorin and obelin are members of the family of “calcium-regulated photoproteins”, so-called because it only requires the addition of Ca\textsuperscript{2+} to trigger a light emission. Such photoproteins have been isolated from many types of bioluminescent marine animals, mostly coelenterates [1], the well-known and well-studied representatives being aequorin and obelin derived from the hydromedusa Aequorea [2] and the hydroid Obelia longissima [3], respectively.

During the past 15 years, cloning and sequence analysis has been achieved for cDNAs coding for five different types of photoproteins [4–8] including from two species of Obelia [9–11]. All Ca\textsuperscript{2+}-regulated photoproteins show high sequence homology (identity is $\approx$75–65\%) and contain three “EF-hand” calcium-binding sites [11–13]. Apo-proteins expressed into Escherichia coli can be converted to active photoproteins by incubating them with coelenterazine under calcium-free conditions in the presence of O\textsubscript{2} and a reducing agent.

The bioluminescence reaction involves an intramolecular oxidation of the coelenterazine resulting in the excited state of the product, coelenteramide [14]. This reaction mechanism is apparently common for coelenterazine-dependent luciferases and photoproteins. The bioluminescence has a broad spectral distribution in the range of 465–495 nm depending on the type of photoprotein or luciferase. The bioluminescence spectral maximum of recombinant aequorin is at 462 nm [15], whereas for recombinant obelin from O. longissima it is at a longer wavelength ($\lambda_{\text{max}} = 485$ nm) [11]. Photoproteins themselves are hardly fluorescent but the Ca\textsuperscript{2+}-discharged photoproteins have strong fluorescence. For Ca\textsuperscript{2+}-discharged aequorin, the fluorescence spectral distribution is very similar to the bioluminescence with $\lambda_{\text{max}} = 460$ nm [15]. However, for Ca\textsuperscript{2+}-discharged obelin, the fluorescence is green with $\lambda_{\text{max}} = 510$ nm [11].

Recently, the crystal structures of several photoproteins have been determined [16]. These are aequorin [17], obelin from O. longissima [18,19], and some other obelins [20,21]. Their structures confirm that, in essence, photoproteins can simply be regarded as luciferases containing a stabilized intermediate, which was originally suggested by Hastings and Gibson [22]. Additional structural information is also available for obelin following the Ca\textsuperscript{2+}-triggered bioluminescence reaction [23,24]. As expected from the homology of their primary sequences, all photoproteins have the same compact globular structure. The substrate-binding cavity is highly hydrophobic and is formed by strictly conserved residues. There are only four residues in the cavity of aequorin and obelin that are different but one of them would appear to be especially significant (Fig. 1). In obelin, a Phe is found at sequence position 88, whereas in aequorin the corresponding position is occupied by a Tyr82 (Tyr89 in the full length sequence of Fig. 1A), that is hydrogen bonded to the oxygen atom of the 6-(p-hydroxy) phenyl group of coelenterazine (Fig. 1B).

To elucidate the influence of the residue in this position on the bioluminescent properties of these two photoproteins, several mutants were constructed: obelin with substitution of Phe88 to Tyr, His, Trp, or Arg, and aequorin with substitution of its Tyr82 to Phe. In each case, the bioluminescence and fluorescence spectral properties were affected as would be expected based on the suggested proton-relay mechanism [16]. In addition, we also show that these mutants have good stability and activity, and similar to that reported for aequorin itself [25–27] can be expressed in mammalian cells thereby demonstrating their potential for monitoring multiple events in cells, such as the simultaneous expression of different genes or the measurement of intracellular calcium in different cell compartments, for example.
2. Materials and methods

2.1. Molecular biology

Site-directed mutagenesis was done on the template pET19-OL8 E. coli expression plasmid carrying the O. longissima apo-obelin gene [28] and on the template pET22-A7 expression plasmid carrying the apo-aequorin gene (GenBank Accession No. AAA27716) with its N-terminus truncated by six amino acid residues. Mutations resulting in the amino acid change were carried out using the QuickChange site-directed mutagenesis kit (Stratagene, USA) according to the protocol supplied with the kit. The plasmids harboring mutations were verified by DNA sequencing.

For protein production, the transformed E. coli BL21-Gold was 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 0.5–0.6. After addition of IPTG, the cultivation was continued for 3 h.

2.2. Photoprotein purification

The obelin, truncated aequorin, and their mutants were purified and charged with coelenterazine as previously reported for recombinant obelins [11,29]. It needs to be noted that at the last step of protein purification by ion-exchange chromatography on Mono Q column (Amer sham Bioscience, USA) [11], the photoproteins and their mutants were clearly separated from the uncharged proteins. The final products were homogeneous according to SDS-PAGE.

Since it was found that the truncation of the aequorin had no effect on its stability or bioluminescence properties, and because the naturally occurring aequorin is also “truncated” but for seven amino acid residues from N-terminus [12] in comparison with the sequence deduced from cDNA (Fig. 1A), for simplicity of results’ description we indicated this truncated aequorin as a wild-type photoprotein.

2.3. Ca^{2+}-discharged photoproteins

To prepare samples of Ca^{2+}-discharged proteins, the concentrated solutions of photoproteins and their mutants were diluted 100 times with 50 mM bis-Tris propane, pH 7.0, containing CaCl_2 (final concentration of calcium in a sample = 1 mM). Fluorescence was measured after the bioluminescence reaction ceased.

2.4. Bioluminescence assay

The bioluminescence intensity was measured with a BLM 8801 photometer (SCTB “Nauka”, Russia) by rapid injection of 0.2 ml of 100 mM CaCl_2, 100 mM Tris–HCl, pH 8.8, into the photometer cell containing 0.5 ml of 5 mM EDTA, 100 mM Tris–HCl, pH 8.8, and the photoprotein aliquot.

Fig. 1. (A) The sequence alignment of aequorin [5] and obelin [10]. The pink underbars indicate the helices A–H of the EF-hands. The three known Ca^{2+}-binding loops are marked in gray. Strictly conserved residues between aequorin and obelin sequences, which are found in the coelenterazine-binding pocket, are colored in red and variable residues are in green and blue. The residues mutated in aequorin and obelin are marked in blue. (B, C) The aequorin (PDB 1EJ3) [17] and obelin (PDB 1EL4) [18] spatial structures, respectively, nearby the 6-(p-hydroxy) phenyl group of coelenterazine (CLZ). The hydrogen bonds are shown in brown and their distances are in Å. The residues of aequorin are numbered according to its three-dimensional structure [17].
2.5. Spectral measurements

Absorption spectra were obtained with a UVIKON 943 Double Beam UV/Vis spectrophotometer (Kontron Instruments, Italy). Bioluminescence and fluorescence spectra were measured with an AMINCO spectrophuorometer (Thermo Spectronic, USA). Emission spectra were corrected with the computer program supplied with the instrument. The bioluminescence spectra were measured in 1 mM EDTA, 50 mM bis-Tris propane buffer, pH 7.0, and initiated by injection of CaCl₂ solution in the same buffer. The concentration of free calcium was around 0.5 μM in order to provide an approximately constant light level during the spectral scan. In cases where a substantial change in bioluminescence intensity took place during the spectral scan, the data points were also corrected for bioluminescence decay. The calcium concentration was estimated with the MAXICHELATOR program. All bioluminescence and fluorescence measurements were carried out at room temperature.

2.6. Expression of photoproteins in CHO cells

To express the photoproteins and their mutants in CHO-K1 (Chinese Hamster Ovary) cells (ATCC, USA), the cDNA of the corresponding photoprotein was subcloned in the expression vector pcDNA3.1(+) (Invitrogen, Netherlands) with the use of standard techniques [30,31]. The resulting plasmids were named pcDNA3-Aequorin, pcDNA3-Aequorin Y82F, pcDNA3-obelin, and pcDNA3-Obelin F88Y. All plasmids contained neomycin resistance. The transfection of CHO-K1 cells with recombinant plasmids was performed with FuGENE 6 reagent (Roche Applied Science, Germany) according to the manufacturer’s protocol. The transfected cells were selected in medium with 2 mg/ml geneticin.

2.7. Time course and dose–response experiments

The CHO cells were stably transfected with pcDNA3-Aequorin, pcDNA3-Aequorin Y82F, pcDNA3-obelin, or pcDNA3-Obelin F88Y. The transfected cells were seeded on 384 well plates with 2000 cells per well with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and were grown at 37 °C with 5% CO₂. After 6–8 days, the plates were duplicated and incubated under the same conditions for another 48 h. Then the medium was replaced by 30 μl coelenterazine solution (3 μM coelenterazine in 130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 5 mM NaHCO₃, and 2 mM CaCl₂), the cells were incubated at growth conditions for 4 h, and ATP was added to the cells directly inside the camera system. The measurement was started immediately. The bioluminescence was assayed by integration over a time interval of 1 s with a robotic Bayer HealthCare system based on commercially available CCD camera in combination with dispensing unit.

3. Results

Fig. 2 shows that the bioluminescence spectra of aequorin and obelin can be roughly interchanged by switching the hydrogen bonding to the oxygen of the 6-(p-hydroxy) moiety of the coelenterazine in the binding site. In Fig. 2 (right), it is seen that in obelin the addition of a second hydrogen-bond donor to the hydroxy group by the F88Y substitution shifts the bioluminescence to shorter wavelength, λ_max = 453 nm. Simultaneously, the small shoulder at 400 nm, which is clearly observed in the bioluminescence spectrum of obelin, is absent in the F88Y obelin spectrum. In contrast, in the case of aequorin, the removal of the H-bond donor at the corresponding position by the Y82F substitution shifts its bioluminescence spectrum to the longer wavelength, λ_max = 501 nm. Also, this Y82F aequorin bioluminescence now displays a new shoulder at 400 nm similar to that observed from obelin.

The Ca²⁺-regulated photoproteins themselves are practically non-fluorescent in the visible region until after the addition of Ca²⁺ to produce the bioluminescence reaction, when their products have an efficient fluorescence (Fig. 3). For the case of aequorin, the fluorescence spectral distribution overlaps the bioluminescence [15] but for obelin the fluorescence is shifted 25 nm to the longer wavelength [11]. For F88Y obelin, this longer wavelength shift is preserved but for Y82F aequorin the major fluorescence band (λ_max = 505 nm) practically coincides again with bioluminescence (λ_max = 501 nm). However, there now appears in the aequorin mutant a prominent band with λ_max = 400 nm, which is not seen in any of the other fluorescence spectra.

Table 1 lists some characteristics of a number of obelin mutants with substitution of Phe88 to residues with different donor–acceptor properties of side chains. The shift to shorter wavelength for bioluminescence for F88H obelin is about the same as for F88Y obelin but the shifts for F88W and F88R are not as great. The fluorescence appears at longer wavelength again in all cases by about the same amount, except for F88R where fluorescence is not detected, possibly indicating that the reaction product coelenteramide dissociates from the protein.

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Fig. 2. The bioluminescence spectra of aequorin and obelin are interchanged by a switch of hydrogen bonding by substitution of one active site residue. (A) Bioluminescence of aequorin (dashed line) and its Y82F mutant (solid line). (B) Bioluminescence of obelin (dashed line) and its F88Y mutant (solid line).
Table 1 also shows that all mutants show no changes in bioluminescence yield or kinetics. They are all as stable as the original photoprotein with the exception of F88R obelin and have the same kinetics for charging with coelenterazine (data not shown).

The absorption spectra of mutants are practically unchanged in comparison with the original photoproteins and are therefore not shown. All have an absorption maximum at 460 nm with only small differences in extinction coefficient. Similarly, there are no significant differences in the absorption spectra of Ca\textsuperscript{2+}-discharged photoproteins. All of them have an absorption peak at 280 nm and the distinct absorbance at 350 nm characteristic of bound coelenteramide.

### Table 1

<table>
<thead>
<tr>
<th>Photoprotein</th>
<th>Bioluminescence $\lambda_{\text{max}}$/shoulder (nm)</th>
<th>Fluorescence$^a$ $\lambda_{\text{max}}$/shoulder (nm)</th>
<th>Bioluminescence yield (%)$^b$</th>
<th>Decay rate (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT obelin</td>
<td>482/400</td>
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<td>100</td>
<td>7.2</td>
</tr>
<tr>
<td>F88Y</td>
<td>453</td>
<td>487</td>
<td>100</td>
<td>7.2</td>
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<tr>
<td>F88H</td>
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<td>504</td>
<td>105</td>
<td>7.2</td>
</tr>
<tr>
<td>F88W</td>
<td>477/390</td>
<td>508</td>
<td>100</td>
<td>7.2</td>
</tr>
<tr>
<td>F88R</td>
<td>474/390</td>
<td>ND$^c$</td>
<td>99</td>
<td>7.2</td>
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<tr>
<td>WT aequorin</td>
<td>469</td>
<td>466</td>
<td>100</td>
<td>1.28</td>
</tr>
<tr>
<td>Y82F</td>
<td>500/400</td>
<td>495/400</td>
<td>100</td>
<td>1.28</td>
</tr>
</tbody>
</table>

All measurements are in bis-Tris propane buffer at pH 7.0 and room temperature.

$^a$Following Ca\textsuperscript{2+} addition to discharge bioluminescence.

$^b$Total bioluminescence emission relative to the unmutated photoprotein.

$^c$ND, not detectable.

Fig. 3. The fluorescence spectra of Ca\textsuperscript{2+}-discharged aequorin and obelin are shifted in opposite directions on interchanging the hydrogen bond at the $\beta$-hydroxy position of coelenteramide. (A) Fluorescence of Ca\textsuperscript{2+}-discharged aequorin (dashed line) and its Y82F mutant (solid line). (B) Fluorescence of Ca\textsuperscript{2+}-discharged obelin (dashed line) and its F88Y mutant (solid line).

Fig. 4. The bioluminescence response time course of CHO cells stably expressing WT and mutant photoproteins on ATP addition. (A) Aequorin (solid line) and its Y82F mutant (dashed line). (B) Obelin (solid line) and its F88Y mutant (dashed line). ATP concentration is 1 μM. RLU, relative light units.
Tral coelenteramide excited state, as also seen in obelin bioluminescence. Notice also that the Y82F aequorin bioluminescence also exhibits a shoulder at 400 nm originating from the neutral coelenteramide excited state, as also seen in obelin bioluminescence. The way in which the H-bonding at this position influences these excited states has been discussed in detail in the case of obelin W92F [20,21] and can readily be extended to the case of aequorin. The additional H-bond from Tyr82 in aequorin enhances stabilization of the excited state phenolate lessening the probability of radiation from the neutral state of coelenteramide [16].

Photoproteins are non-toxic when injected into cells and have found wide application for assay of intracellular [Ca^{2+}] [32]. Recently, it has been shown possible to create organisms that express the aequorin gene [25,26,33,34]. Such cells or organisms have, in effect, a “built in” calcium indicator. Since coelenterazine is lipophilic and crosses cell membranes readily, the expressed apophotoprotein can be easily charged to generate calcium-dependent bioluminescence response. Nowadays, this approach has become very popular among researchers.

In addition to direct measurement of [Ca^{2+}], photoproteins can be used in various indirect cell based assays [27]. For instance, if the binding of some agonist with a membrane receptor results in a stimulation of phospholipase C and therefore in activation of the inositol-1,4,5-trisphosphate (InsP3)/Ca^{2+} signaling pathways [35], the “built in” photoprotein can be used for monitoring the activation of this membrane receptor. The P2Y2 receptor is a purinergic G_q-coupled receptor, which is endogenously expressed by CHO cells and which can be activated by ATP or UTP [36,37]. The activation of the P2Y2 receptor with ATP results in activation of phospholipase C through G_q and therefore in a Ca^{2+} influx into the cytosol. These transients in [Ca^{2+}] can be easily determined with photoprotein that is constitutively expressed in cell cytosol.

A number of bioluminescence reporters such as luciferases, Ca^{2+}-regulated photoproteins, or green fluorescent protein (GFP) have now been shown to be useful for non-invasive, real-time imaging of cellular events. A set of probes having signals that can be separated in real-time are of great value for studying the complex mechanisms of cell regulation. One approach to achieve this is construction of bioluminescent and fluorescent reporters emitting light at different wavelengths. Probably, the most impressive progress has been attained in construction of GFP mutants fluorescing at different wavelengths and their application for multi-color imaging of various intracellular events [38-40]. At this time, among the luciferases and photoproteins, only firefly luciferase [41-45] and photoprotein [46] bioluminescence color mutants have been produced and only firefly color mutants have been applied for development of dual imaging systems [47-49].

In this paper, we demonstrate the use of rational mutation of obelin and aequorin to interchange their bioluminescence color. These mutants are practically unchanged in their properties as calcium indicators in comparison with WT photoproteins.

4. Discussion

The present results show how H-bonding to the oxygen atom of the 5-(p-hydroxy) phenyl group of coelenteramide is the prime factor controlling the spectral properties of aequorin and obelin. Removal of the Tyr82 H-bond in the aequorin case changes the spectral properties to resemble obelin and vice versa by adding an H-bond in the obelin case. Notice also that the Y82F aequorin bioluminescence also exhibits a shoulder at 400 nm originating from the neutral coelenteramide excited state, as also seen in obelin bioluminescence. The way in which the H-bonding at this position influences these excited states has been discussed in detail in the case of obelin W92F [20,21] and can readily be extended to the case of aequorin. The additional H-bond from Tyr82 in aequorin enhances stabilization of the excited state phenolate lessening the probability of radiation from the neutral state of coelenteramide [16].

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In this paper, we demonstrate the use of rational mutation of obelin and aequorin to interchange their bioluminescence color. These mutants are practically unchanged in their properties as calcium indicators in comparison with unmutated photoproteins (Figs. 4 and 5). Photoprotein mutants are now available with bioluminescence maxima ranging from 390 to 500 nm [46]. Since the spectral maxima of Y82F aequorin and F88Y obelin are separated by about 50 nm, these mutants have, for example, a potential for development of dual-color reporter systems for simultaneous monitoring of the expression of two different genes, of the activity of two different receptors, or for measurement of calcium concentration in two different compartments of cells.
Acknowledgments: This work was supported by Grant 02-04-49419 of the Russian Foundation for Basic Research and by Grant of “Molecular and Cellular Biology” program of the Russian Academy of Sciences, and Bayer AG (Germany).

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