Calcium Activated Photoproteins, Mechanism of Action and Medical Applications

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Summary

The term photoprotein refers to any protein that generates light when oxidized, normally without the aid of an enzyme catalyst. These proteins do not exhibit a luciferin-luciferase reaction, that is, a normal enzyme-substrate reaction. They display luminescence proportional to the amount of the protein. Such proteins are stable as a luciferin-photoprotein complex, until the addition of Ca^{2+} for the photoprotein aequorin.

The EF-hand motif is the most common calcium-binding motif found in proteins. Several high-resolution structures containing different metal ions bound to EF-hand sites have given new insight into the modulation of their binding affinities. Recently determined structures of members of several newly identified protein families that contain the EF-hand motif in some of their domains, as well as of their complexes with target molecules, are throwing light on the surprising variety of functions that can be served by this simple and ingenious structural motif.

Keywords: Calcium binding; EF-hand protein; photoprotein, bioluminescence; chemiluminescence; coelenterazine; aequorin, obelin, clytin, mnemiopsin.

Introduction

Calcium is involved in many cellular processes including adhesion, gene expression and motility. Therefore concentration of Ca^{2+} within the cell is carefully controlled [1-3]. The cytosolic calcium level of cells is as low as 10–100 nM. However,its concentration may increase to 500-1000 nM in response of stimulation, causing activation of important Ca^{2+} sensors such as calmodulin and troponin C [4]. The cell has several different mechanisms for regulating calcium entry and removal from the cytosol. Opening of voltage-gated, ligand-gated or store-operated calcium channels, or release of calcium from intracellular stores, will result in a rapid increase in its concentration. The calcium activated mechanisms are then balanced by pump mechanisms, such as the plasma membrane ATPase and the sarcoplasmic/endoplasmic ATPase pumps, or the plasma membrane Na^{+}-Ca^{2+} exchange mechanism. All of these mechanisms are based on quickly return calcium ion concentrations to the resting state. The challenge for developing high-throughput (HT) calcium assays, therefore, is to rapidly measure these transient changes in calcium concentration, or convert the transient changes into sustained responses that can be measured at a later time.

Photoproteins

The term photoprotein refers to any protein that generates light when oxidized in proportion to its amount, normally without the aid of an enzyme catalyst. These proteins do not exhibit a luciferin-luciferase reaction, that is, a normal enzyme-substrate reaction. They display luminescence proportional to the amount of the protein. Such proteins are stable as a luciferin-photoprotein complex, until the addition of Ca^{2+} for the photoprotein aequorin. Most of known photoproteins have been reported in marine organisms. Up to present time, about two dozen types of bioluminescent organisms have been identified for which substantial biochemical knowledge is available, and about one third of them involve photoproteins. There are various types of photoproteins: the photoproteins of coelenterates, ctenophores and radiolarians require Ca^{2+} to trigger their luminescence; the photoproteins of the bivalve Pholas and of the scale worm appear to involve superoxide radicals and O_2 in their light-emitting reactions; the photoprotein of euphausiid shrimps emits light only in the presence of a special fluorescent compound; the photoprotein of the millipede Luminodesmus, the only known example of terrestrial origin, requires ATP and Mg^{2+} to emit light. The Ca^{2+}-sensitive photoproteins of coelenterates have been most frequently studied and most widely used. Most of coelenterate photoproteins, including aequorin, halistaurin, obelin and phialidin, emit blue light in aqueous solution when a trace of Ca^{2+} is added, in the presence or absence of molecular oxygen. Their relative molecular masses are about 20 000 and they all contain an identical functional group. Aequorin contains an oxygenated form of coelenterazine in its functional group. When Ca^{2+} is added, aequorin decomposes into three parts, i.e., apo-aequorin, coelenteramide and CO_2, accompanied by the emission of light. Apo-aequorin can be reconstituted into active aequorin indistinguishable from the original sample, by incubation with an excess of coelenterazine in a buffer containing 5 mM-EDTA and a trace of 2-mercaptoethanol. The regeneration process of coelenterate photoproteins in this manner takes place *in vivo*, utilizing stored coelenterazine. The photoproteins of coelenterates, and their chemically modified forms, are useful in measuring and monitoring calcium ions in biological systems, especially in single cells.

Mechanism of photoprotein glowing

Bioluminescence is a type of chemiluminescence based on enzyme-catalyzed reactions. Many of luminous organisms exhibit different bioluminescent reactions depending on enzymatic structure and type of substrates [5]. It is known that bioluminescence of marine coelenterates is the result of a photoprotein reaction [6]. These photoproteins are stable enzyme-substrate complexes which is composed of a single polypeptide chain (Mw ~ 22 kDa), containing three EF-hand Ca^{2+}-binding sequences [7] and a substrate. The substrate is a derivative of coelenterazine, hydroperoxycoelenterazine pre-activated by oxygen and non-covalently bound within a hydrophobic cavity inside the protein [8-10]. All bioluminescent reactions are oxidative decarboxylation of the substrate resulting heterocyclic products characterized by their high fluorescence [11, 12]. Figure 1 illustrates the mechanism of coelenterazine bioluminescence.

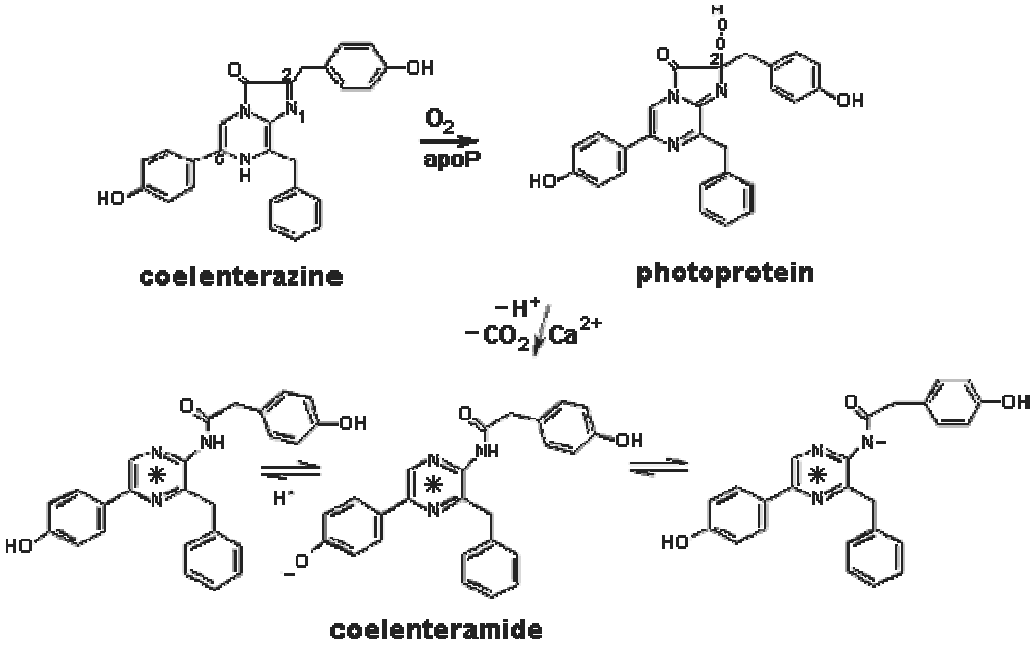

Figure 1. Mechanism of coelenterazine bioluminescence. The bioluminescent reaction is an oxidative decarboxylation of the protein-bound substrate triggered by calcium ions. The excited protein-bound product (*), coelenteramide, is then relaxed to its ground state that results in emission of blue light

The reaction mechanism involves breakdown of a dioxetanone intermediate and formation of carbonyl group (Figure 1). This reaction mechanism is common for all coelenterazine-dependent photoproteins.
As addition of calcium ions to the photoproteins causes a bioluminescent reaction, the term Ca^{2+}-regulated is used for this type of photoproteins [13]. It must be emphasized that bioluminescence intensity of a photoprotein depends on Ca^{2+} concentration. This group of photoproteins can be, therefore, be used as appropriate compounds for measurement of intracellular calcium in some industrial and biomedical applications [14-18].

Bioluminescence spectra of photoproteins
Considering the bioluminescence characteristic of Ca^{2+}-regulated photoproteins, their spectra are complex, broad and asymmetric. The spectra of photoproteins aequorin and obelin from marine coelenterates, jellyfish *Aequorea victoria* [2] and [19] and hydroid *Obelia longissima* [20] have been studied. It has been shown that coelenteramide can exist in several fluorescent forms depending on the solvents. Contribution of each component to bioluminescence of photoprotein could be changed by changing the protonic environment of coelenteramide in the enzyme active center [21]. It has been demonstrated that the protonic environment can be changed by substitution of amino acids using genetic manipulations [22]. Variation in phtoprotein spectra is the basis of constructing bioluminescent markers of different colors. Using modern techniques, various photoproteins could be prepared with bioluminescence colors ranging from violet to green [22].

Different types of Ca^{2+} binding photoproteins

Even though there are many bioluminescent organisms in nature, only a few photoproteins have been isolated and characterized. The only few known coelenterate photoproteins, include aequorin, obelin, clytin, brovin, mitrocumin, phialidin, halistaurin and nmiopsin emit blue light in aqueous solution when a trace of Ca^{2+} is added, in the presence or absence of molecular oxygen.

Two kinds of aequorin-type photoproteins, i.e., halistaurin and phialidin, and four kinds of modified forms of aequorin, i.e., products of acetylation, ethoxycarbonylation, fluorescamine-modification and fluorescein labelling, were prepared. The modified forms of aequorin were more sensitive to Ca^{2+} than was aequorin in their Ca^{2+}-triggered luminescence reactions, whereas halistaurin and phialidin were less sensitive. The emission maxima of luminescence were all within a wavelength range 450-464 nm, except for fluorescein-labelled aequorin, which emitted yellowish light (lambda max. 520 nm). A new technique of measuring Ca^{2+} concentration is suggested.

Aequorin

The calcium-sensitive photoprotein aequorin, isolated from the jellyfish *Aequorea aequorea*, has been used as a probe for detecting calcium ions. The protein is a non-covalent complex of apoaequorin (apoprotein, 21.4 kDa) and a hydroperoxide of coelenterazine [23]. When Ca^{2+} is added, the peroxide of coelenterazine in aequorin decomposes into coelenteramide and CO_2, accompanied by the emission of light with a λ_{max} of about 460 nm [24-26]. Aequorin is then converted into a blue fluorescent protein (BFP-aq), a dissociable complex of Ca^{2+}-bound apoaequorin and coelenteramide. Treatment of coelenterazine with molecular oxygen in the presence of ethylenediaminetetraacetic acid (EDTA) and a reducing reagent can later regenerate aequorin from apoaequorin [25]. This regeneration process is similar to the formation of an enzyme-substrate intermediate, and BFP-aq is analogous to an enzyme-product complex.

Obelin

Obelin is the enzyme-substrate complex of polypeptide with 2-hydroperoxy coelenterazine. The photoprotein causes bioluminescence of marine hydroid *Obelia longissima*. Addition of Ca^{2+} to the photoprotein triggers the bioluminescent reaction with light emission. The product of the bioluminescent reaction, enzyme-bound coelenteramide, is a fluorescent protein that is stable and highly fluorescent. It has been shown that increase in concentration of Ca^{2+} causes an increase in the fluorescence intensity of obelin.

Clytin

Clytin is a member of the aequorin family of photoproteins. It is made up of 189 amino acid residues, contains 3 Ca^{2+}-binding sites, and shows 62% homology in amino acid residues to those in aequorin. The cysteine, tryptophan, and histidine residues, and the C-terminal proline, that are conserved in aequorin and clytin may be involved in the Ca^{2+}-activated bioluminescence of the two proteins. Clytin may also prove useful in the determination of Ca^{2+} [1]. The cDNA for an isotype of clytin, a calcium-binding photoprotein from the luminous jellyfish *Clytia gregarium*, was identified and named

clytin-II. The histidine-tagged apoprotein of clytin-II expressed into the periplasmic space of *Escherichia coli* cells was isolated by nickel chelate affinity chromatography. Recombinant clytin-II regenerated from apoprotein by incubation with coelenterazine was purified. The yield of purified clytin-II was 13 mg from 2 l of cultured cells with purity >95%. The luminescence properties of clytin-II were characterized by comparison with clytin-I and aequorin, and semi-synthetic clytin-II was also prepared. The initial luminescence intensity of clytin-II triggered by Ca^{2+} was 4.5 times higher than that of clytin-I and aequorin, but the luminescence capacity was close to clytin-I and aequorin. Thus, clytin-II is a useful protein, showing high sensitivity in the signal-to-noise ratio of luminescence intensity [27].

A novel histidine-tagged secretion vector in Escherichia coli was constructed and large amounts of highly pure clytin, a calcium-binding photoprotein, was prepared. The histidine-tagged apoclytin expressed into the periplasmic space in E. coli was purified by nickel chelate affinity chromatography. Recombinant clytin was regenerated from apoclytin by incubation with coelenterazine in the presence of dithiothreitol and then purified by anion-exchange chromatography and hydrophobic chromatography. The yield of recombinant clytin was 20mg from 2L of cultured cells with purity greater than 95%. Luminescence properties of recombinant clytin were identical to that of native clytin (phialidin). The Ca^{2+} sensitivity of recombinant clytin is lower than that of aequorin and clytin is suited for measuring higher concentration of Ca^{2+}. Semi-synthetic clytins were also prepared with coelenterazine analogues, and the initial intensity, luminescence capacity and half decay time were characterized [28]

Mnemiopsin

Calcium activated photoprotein, termed mnemiopsin, which emits bioluminescence upon the addition of calcium ion, has been isolated from the Ctenophore, Memiopsis leidyi, and purified by hollow fiber techniques. The system is similar to aequorin, from the jellyfish Aequorea, except that mnemiopsin can be light-inactivated. Mnemiopsin is a photoprotein that was discovered in the ctenophore *Mnemiopsis leidyi* and has been resolved into two distinct isoprotein, namely Mnemiopsin-1 and Mnemiopsin-2, abbreviated m-1 and m-2 [29]. According to our vast literature survey, the sequences of these isoproteins have not been reported up to the beginning of this study. Most of research works preformed on photoproteins have been focused on aequorin and obelin, while there is almost no information about mnemiopsin 3-D structure.

Halistaurin and phialidin

Halistaurin and phialidin are also sensitive to calcium ion concentration. These two types of photoproteins have been identified and reported previously [3, 4, 30], but their calcium sensitivity were not recorded until 1985 that Shimomura & Shimomura [31] prepared four kinds of modified aequorin by acetylation, ethoxycarbonylation, fluorescamine-modification and fluorescein labelling. They found that some of these forms of aequorin were even more sensitive to Ca^{2+}-triggered luminescence reactions. The emission maxima of luminescence for all of these derivatives were within a wavelength 450-464 nm, except for fluorescein-labelled aequorin, which emitted yellowish light (λmax 520 nm).

Photoproteins as diagnostic target for Ca^{2+} concentration in living cells

Calcium is one of the most important second messengers that take participate in regulating many critical processes in cells. Up to present time, many efforts have been made to design appropriate methods for imaging Ca^{2+} in living cells. In this regard, one of the first probes suitable to be used for studying intracellular calcium ions is Ca^{2+}-regulated photoproteins. Malikova et al [28] have constructed 3D photoprotein structure base on the bioluminescence mechanism of two obelin mutants. It was shown that they concurrently showed an altered bioluminescence spectra and calcium affinity. It was demonstrated that how the local changes in [Ca^{2+}] switch exogenous and endogenous stimuli to the corresponding cell response.

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