Methods 46 (2008) 160–166

Contents lists available at ScienceDirect

Methods

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

Calcium-sensitive photoproteins

Marisa Brini *

Department of Biochemistry and Department of Experimental Veterinary Sciences, University of Padova, Viale G. Colombo 3, 35131 Padova, Italy

ARTICLE INFO

Article history:
Accepted 12 September 2008
Available online 10 October 2008

Keywords:
Calcium signaling
Photoproteins
Recombinant probes
Targeted probes
Intracellular organelles

ABSTRACT

The recombinant Ca^{2+} sensitive photoprotein aequorin was the first probe used to measure specifically the Ca^{2+} concentration, $[Ca^{2+}]$, inside the intracellular organelles of intact cells. Aequorin-based methods offer several advantages: (i) targeting of the probe is extremely precise, thus permitting a selective intracellular distribution; (ii) the use of wild-type and low Ca^{2+}-affinity aequorins allows covering a large dynamic range of $[Ca^{2+}]$, from 10^{-7} to 10^{-3} M; (iii) aequorin has a low Ca^{2+} buffering effect and it is nearly insensitive to changes in Mg^{2+} or pH; (iv) it has a high signal-to-noise ratio; (v) calibration of the results in $[Ca^{2+}]$ is made straightforward using a simple algorithm; and (vi) the equipment required for luminescence measurements in cell populations is simple and low-cost. On the negative side, this technique has also some disadvantages: (i) the relatively low amount of emitted light makes difficult performing single-cell imaging studies; (ii) reconstitution of aequorin with coelenterazine is necessary to generate the functional photoprotein and this procedure requires at least 1 h; (iii) in the case of aequorin targeted to high Ca^{2+} compartments, because of the high rate of aequorin consumption at steady-state, only relatively brief experiments can be performed and, because of the steepness of the Ca^{2+}-response curve, the calibrated $[Ca^{2+}]$ values may not reflect the real mean in cells or compartments with dyshomogeneous behavior; and (iv) expression of targeted aequorins requires previous transfection or infection to introduce the appropriate DNA construct, or alternatively the use of stable cell clones.

1. Introduction

The jellyfish *Aequorea victoria* produces a 22 kDa protein that plays a major role in the study of Ca^{2+} signaling. Even if the cloning of aequorin cDNA was reported in 1985 [1] and the expansion of its use occurred in the 1990s, only more recently techniques have been developed to obtain recombinant aequorin of highest purity to permit its structure determination by crystallographic approaches. Aequorin is found to be a globular molecule containing a hydrophobic core cavity that accommodates the ligand coelenterazine [2]. The protein scaffold of aequorin contains four helix loop helix EF hand sites arranged in pairs to form the globular molecule. Each pair of "hands" is arranged back-to-back, forming short stretches of β-sheet, as seen in other EF hand protein.

Aequorin, in its active form, includes a polypeptide moiety covalently bound to the prosthetic group coelenterazine. Upon binding of Ca^{2+} to three high affinity sites (only three EF hand sites can bind Ca^{2+}), an irreversible reaction occurs in which the prosthetic group is released and a photon is emitted (Fig. 1). Due to its Ca^{2+} triggered light emission and the reliable calibration procedures, aequorin has been widely employed for studying intracellular $[Ca^{2+}]$ during the late 1960s and most of the 1970s [3–6]. Since aequorin has been introduced by permeabilization procedures or by microinjection, the technique has been prevalently used in giant cells, i.e., barnacle muscle fibers, oocytes, and cells from the heart, liver and adrenal gland [7]. During the 1980s, the synthetic fluorescent Ca^{2+} indicators [8], that can be easily loaded in numerous cell types, essentially replaced aequorin in the Ca^{2+} measurements. However, the cloning of aequorin cDNA [1] and the wide diffusion of molecular biology techniques, with the possibility of expressing proteins in living cells, has permitted to revaluate aequorin and extensively expand the number of its applications in Ca^{2+} measurements. Indeed, transfecting a cell with a plasmid which allows the recombinant expression of exogenous protein is a simple and effective procedure. By this means, it is possible to introduce aequorin in a wide variety of cell types, different in morphology and in embryological origin [9]. The development of viral transduction to introduce cDNA in the cells has expanded the application to primary cultures, especially of neuronal origin, which are traditionally much more resistant to the canonical transfection procedures [10–12]. Moreover, recently, a couple of papers appeared with the description of aequorin transgenic mice as useful tool to monitor localized Ca^{2+} signals in whole animal in vivo [13,14] The approach appears particularly relevant to obtain large-scale spatio-temporal information on the role of Ca^{2+} signaling, e.g., in the highly coordinated activity of muscle groups in intact animals, or in neuronal tissues, without losing their original function once

* Fax: +39 049 8276125.
E-mail address: marisa.brini@unipd.it

1046-2023/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2008.09.011

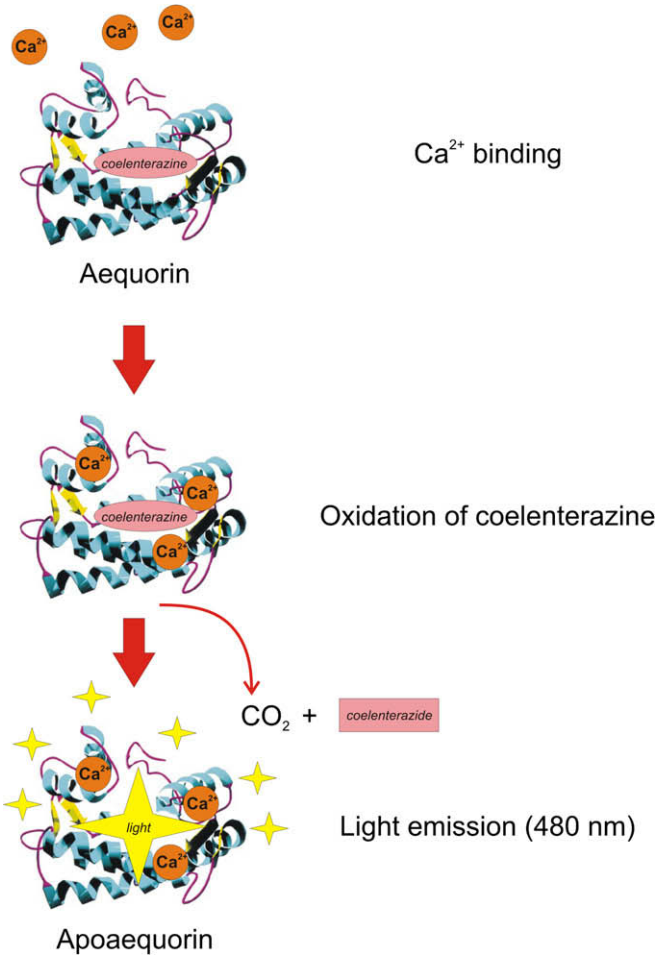

Fig. 1. Schematic model of the irreversible reaction of aequorin. When Ca^{2+} ions bind to the EF-hand binding sites of the reconstituted aequorin a photon is emitted and that molecule of aequorin is irreversibly discharged.

isolated or cultured under an environment different from that of the original tissue.

The great advantage provided by aequorin, being a protein probe, is that its intracellular targeting can be selectively controlled by adding specifically signaling information (i.e., the amino acid sequence which directs the correct sorting of organelle proteins to the proper intracellular location) to the primary sequence of the protein. With this strategy, several aequorin chimeras have been constructed targeted to the mitochondrial matrix, the nucleus, the endoplasmic and sarcoplasmic reticulum, the subplasmalemma region, the Golgi apparatus and the outer face of the inner mitochondrial membrane etc. and thanks to their selective localization they have permitted to monitor Ca^{2+} dynamics in specific cell compartments [15,16]. The same approach can be extended to other compartments and to specific localized domains, i.e., to specific proteins, which are modulators or effectors of the Ca^{2+} signal. The addition of short amino acid sequence encoding strong immunological epitopes ("epitope tagging") has been shown to represent a powerful approach for immunolocalizing transfected protein inside the cells. In particular, the HA1 epitope (nine amino acids of the hemagglutinin protein) induces a low background signal in untransfected cells and thus has been employed as a tag in the developing of aequorin chimeras [17].

Native aequorin can accurately measure [Ca^{2+}] ranging from 0.5 to 10 μM, i.e., reaching concentrations at which most fluorescent indicators are saturated. Thanks to these properties it is useful to estimate large cytosolic [Ca^{2+}] rises, which occur, for example in neurons, but not to accurately measure resting cytosolic [Ca^{2+}] values. Native aequorin is also unsuitable for measurements where [Ca^{2+}] is much higher (e.g., the lumen of the ER and SR, near Ca^{2+} channels, pumps etc.). However, this limitation can be overcome by reducing the affinity between Ca^{2+} and photoprotein. At least three methods can be envisaged, and all three have been used successfully: (i) mutation of one or more of the Ca^{2+} binding sites [18,19], (ii) use of surrogate cations, such as Sr^{2+}, which elicit a slower rate of photoprotein consumption than Ca^{2+} itself [18,20]; (iii) use of modified prosthetic groups, such as coelenterazine n, which decrease the affinity of aequorin for Ca^{2+} [21]. These three approaches can be combined to obtain a clear shift in the Ca^{2+} affinity of the protein. Specifically, the point mutation from Asp to Ala at position 119 in the second EF hand domain of aequorin, because of the cooperativity of the Ca^{2+} binding sites of aequorin, markedly impairs the affinity for the cation, and produces a mutated aequorin which can measure [Ca^{2+}] in the range of 10–100 μM. This range can be further expanded by employing suitable divalent cations as Ca^{2+} surrogate. Sr^{2+} permeates across the Ca^{2+} channels and is actively transported, although with a low affinity, by both the plasma membrane and the sarco-endoplasmic Ca^{2+} ATPases. Altogether, by combining the two approaches, this aequorin probe can measure [cation^{2+}] ranging from the μM to the mM range. Currently, in order to avoid possible discrepancies between Ca^{2+} and Sr^{2+}, and also to specifically monitor [Ca^{2+}] in compartments with high [Ca^{2+}], the use of Sr^{2+} is falling in disuse. This is made possible because of the availability of low affinity coelenterazine analogue (coelenterazine n), which gives rise to an aequorin–coelenterazine functional probe with a very low rate of consumption. Using Ca^{2+}, this synthetic prosthetic group, and mutated aequorin, it is possible to monitor mM concentrations of Ca^{2+} for relatively long periods of time.

The major disadvantage in the use of aequorin is the low amount of light emitted by the photoprotein. Each aequorin molecule emits only one photon, and only a small fraction of the photoprotein pool ($<10^{-3}$) emits light throughout the experiment. This is not a major problem when light output comes from an entire coverslip of transfected cells. However, to detect Ca^{2+} waves or local changes in [Ca^{2+}] it is necessary to carry out single-cell imaging experiments. With aequorin such analysis is still quite difficult and despite special imaging systems have been developed, the approach is technically difficult and yields a low image quality [22]. Thus, aequorin method is essentially recommend for cell population analysis.

2. Description of method

2.1. Available aequorin chimeras

2.1.1. Cytosol (cytAEQ)

Recombinantly expressed wild-type aequorin is exclusively cytosolic and, therefore, it does not require any modification to measure [Ca^{2+}] in this compartment. The only modification made has been at the 5′ end of the coding region to include the HA1 epitope tag in order to verify the correct localisation of the probe [17].

2.1.2. Nucleus (nuAEQ)

The chimeric cDNA encoding an aequorin chimera stable resident in the nucleus was obtained by fusing a portion of the cDNA encoding the glucocorticoid receptor (GR). The GR moiety spans amino acids 407–524, and includes the DNA binding domain and the nuclear localization signal (NLS). NLSs cause nuclear localization because they mediate the translocation through the nuclear pore [23].