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Semisynthetic photoprotein reporters for tracking fast Ca^{2+} transients

Received 00th September 2015,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Changes in the intracellular concentration of free ionized calcium ($[Ca^{2+}]_i$) control a host of cellular processes as varied as vision, muscle contraction, neuronal signal transmission, proliferation, apoptosis etc. The disturbance of Ca^{2+}-signaling causes many severe diseases. To understand the mechanisms underlying the control by calcium and how disorders of this regulation relate to pathological conditions, it is necessary to measure $[Ca^{2+}]_i$. The Ca^{2+}-regulated photoproteins which are responsible for bioluminescence of marine coelenterates are successfully used for this purpose over the years. Here we report the results on comparative characterization of bioluminescence properties of aequorin from *Aequorea victoria*, obelin from *Obelia longissima*, and clytin from *Clytia gregaria* charged by native coelenterazine and coelenterazine analogues *f*, *i*, and *hcp*. The comparison of specific bioluminescence activity, stability, emission spectra, stopped-flow kinetics, sensitivity to calcium, and effect of physiological concentrations of Mg^{2+} establishes obelin-*hcp* as an excellent semisynthetic photoprotein to keep track of fast intracellular Ca^{2+} transients. The rate of rise of its light signal on a sudden change of $[Ca^{2+}]$ is almost 3- and 11-fold higher than those of obelin and aequorin with native coelenterazine, respectively, and 20 times higher than that of the corresponding aequorin-*hcp*. In addition, obelin-*hcp* preserves a high specific bioluminescence activity and displays higher Ca^{2+}-sensitivity as compared to obelin charged by native coelenterazine and sensitivity to Ca^{2+} comparable with those of aequorin-*f* and aequorin-*hcp*.

Introduction

Changes in the intracellular concentration of free ionized calcium ($[Ca^{2+}]_i$) control a host of cellular processes as varied as vision, muscle contraction, neuronal signal transmission, proliferation, apoptosis etc.[1] Hence, disturbances of Ca^{2+}-signaling cause many severe diseases. To study the function of Ca^{2+} in the regulation of intracellular processes, several experimental approaches have been developed. Currently, fluorescent tetracarboxylate calcium indicators developed by Tsien[2] are probably the most widely used substances for measuring $[Ca^{2+}]_i$ transients within living cells due to their availability, stability, non-toxicity and ease of introduction into cells[3]. Although a wide range of chemical fluorescent indicators with different affinities to Ca^{2+} is commercially available and protocols of their use are well established[4], there are shortcomings that restrict their application range. Among the drawbacks of fluorescent dyes is the difficulty to control their targeting to specific subcellular compartments as well as dye extrusion from the cell during long recording experiments.[5-7] In addition, at higher concentrations chemical indicators act as Ca^{2+} buffers, distorting the kinetics of Ca^{2+}-signaling within cells.[4] Another approach to detect $[Ca^{2+}]_i$ in cells is the use of genetically encoded Ca^{2+} indicators which can be broken up into two groups – GFP-based fluorescent sensors[7] (cameleons, camgaroos, pericams etc.) and bioluminescent probes[8] involving Ca^{2+}-regulated photoproteins. A major advantage of genetically encoded Ca^{2+} indicators over fluorescent dyes is that they can be targeted with molecular-scale precision within the cell.[7,9]

Ca^{2+}-regulated photoproteins are small one-subunit globular proteins which are responsible for bioluminescence of marine dwellers, mainly jellyfish and ctenophores.[10,11] Photoprotein represents a stable complex consisting of apoprotein to which a peroxy-substituted coelenterazine, 2-hydroperoxycelenterazine, is tightly but non-covalently bound.[12] The latter has been confirmed by studying the spatial structures of different photoproteins.[13-15] Light emission is initiated upon binding of Ca^{2+} to the EF-hand Ca^{2+}-binding loops of the photoprotein, causing small conformational changes within the internal cavity of the protein. This disturbs the hydrogen bond network stabilizing the peroxy group of 2-hydroperoxycelenterazine[16,17] and triggers an oxidative decarboxylation reaction yielding a mole of CO_2 and the product, coelenteramide, in an excited state. Relaxing of

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[‡]Abbreviation: CTZ, coelenterazine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid; PIPES, 1,4-piperazinebis(ethanesulfonic acid); IPTG, isopropyl β-D-thiogalactopyranoside; RLU, relative light units

Electronic Supplementary Information (ESI) available: Figure S1, Figure S2, Figure S3. See DOI: 10.1039/x0xx00000x

excited coelenteramide to its ground state is accompanied by light emission with λ_{max} in the range of 465-495 nm depending on the photoprotein source.[18]

Although cloning and sequence analysis have been carried out for four hydromedusan Ca^{2+}-regulated photoproteins (aequorins from *Aequorea victoria*[19-21], *Aequorea coerulescens*[22], and *Aequorea macrodactyla*[23], clytins from *Clytia gregaria*[24-26] and *Clytia hemisphaerica*[27], mitrocomin from *Mitrocoma cellularia*,[28-30] obelins from *Obelia longissima*[31,32] and *Obelia geniculata*[33]) and three light-sensitive photoproteins of ctenophores[34-38], only aequorin is widely applied as a genetically encoded intracellular calcium probe. This may be contributed to the fact that the cDNA encoding this photoprotein was the first to be cloned and, more significantly, because natural aequorin is well-characterized[10] and extensively used to measure intracellular $[Ca^{2+}]$ in various types of cells[39].

Naturally, speed and Ca^{2+}-sensitive range of a calcium probe should be in line with the $[Ca^{2+}]_i$ response under investigation. Intracellular compartments, for example, generally display much higher Ca^{2+} concentrations than the cytosol, surpassing the optimal detection range of 0.5-10 μM for wild type aequorin.[39,40] In this perspective, attempts to modify the functional properties of aequorin have been undertaken by both mutagenesis of aequorin[41-43] and the use of coelenterazine analogues[10,44,45]. This has allowed researchers to construct semisynthetic aequorin variants with an altered affinity for calcium[42-43] or a significant shift in their bioluminescence spectrum[46].

There are, as yet, no reported aequorin variants with a speed of rise of the bioluminescent signal which significantly exceeds that of the wild type aequorin. The speed with which luminescence responds to changes in $[Ca^{2+}]_i$ is an important property of Ca^{2+}-regulated photoproteins as the changes occur sometimes at the millisecond timescale, e.g. $[Ca^{2+}]_i$ transients in the post-synaptic compartments of excitatory neurons during neuronal transmission[47,48] or the Ca^{2+}-sparks that initiate cardiac excitation-contraction coupling[49]. Wild type aequorin responds too slowly (k_{rise} characterizing the rate of rise of light signal *in vitro* does not exceed 100-123 s^{-1})[30,50,51] to follow such rapid intracellular calcium transients without distortion. In the presence of physiological concentrations of free ionized Mg^{2+} ($[Mg^{2+}]$) aequorin kinetics are even slower and, in addition, magnesium ions decrease its sensitivity to calcium, further limiting its role as a fast Ca^{2+} reporter[30,39,51].

Other hydromedusan photoproteins reveal a high degree of identity of their amino acid sequences[11] with that of aequorin and have very similar overall spatial structures and structures of coelenterazine-binding cavities[15]. Nevertheless, they differ in many properties. For example, obelins and clytin are considerably "faster" and their bioluminescence kinetics and sensitivity to calcium are practically unaffected by physiological $[Mg^{2+}]$ as compared to aequorin[30,33,51]. At the same time, obelin, clytin, and mitrocomin are less sensitive to calcium as compared to aequorin, even in the presence of 1 mM Mg^{2+}.[30] In addition, semisynthetic Ca^{2+}-regulated photoproteins from different organisms charged by the same coelenterazine analogues reveal different bioluminescence properties.[52,53]

In the present study, we report the bioluminescence properties of semisynthetic aequorins, obelins, and clytins charged by the most frequently used coelenterazine analogues *f*, *i,* and *hcp* (Scheme). The comparison of specific bioluminescence activity, stability, emission spectra, stopped-flow kinetics, sensitivity to calcium, and effect of physiological $[Mg^{2+}]$ reveals obelin-*hcp* as the best photoprotein to keep track of fast intracellular Ca^{2+} transients. This semisynthetic photoprotein responds rapidly to sudden changes in $[Ca^{2+}]$, preserves a high specific bioluminescence activity, and displays an increased Ca^{2+}-sensitivity as compared to the other photoproteins.

Experimental

Materials

Native coelenterazine (CTZ)[‡], NanoFuel®coelenterazine-*f* (CTZ-*f*) and coelenterazine-*i* (CTZ-*i*) were obtained from NanoLight Technology, a division of Prolume Ltd (Pinetop, USA). Coelenterazine-*hcp* (CTZ-*hcp*) was purchased from Biotium (Hayward, USA). Native coelenterazine and its analogues were dissolved and stored as recommended by the manufacturers. All other reagents were from Sigma-Aldrich, unless otherwise stated, and were of analytical reagent grade or better and used as received.

Photoprotein purification

For apophotoprotein production, *Escherichia coli* BL21 Codon plus (RIPL) cells (Stratagene) transformed with pET19-OL8[54], pET22-A7[55], or pET22-CL3[26] were cultivated at 37°C in vigorously shaken ampicillin-containing LB medium till reaching an OD_{590} of 0.6-0.8. After induction with 1 mM IPTG, the cultivation of cells was continued for 3 h. Cells were harvested by centrifugation, the pellet was resuspended in 20 mM Tris-HCl pH 7.2 and disrupted by sonication (20 s × 6) on ice. After centrifugation of the mixture, all apophotoproteins were purified from the inclusion bodies and activated with an excess of native CTZ, CTZ-*f*, CTZ-*i*, or CTZ-*hcp* as reported for the recombinant wild type obelin.[33,51,56] The purification scheme included two steps: i) anion-exchange chromatography of supernatant on DEAE-Sepharose Fast Flow column (Pharmacia, Sweden) in denaturing conditions and ii) anion-exchange chromatography of the charged photoprotein on a Mono Q column (GE Healthcare) to separate the active photoprotein from apophotoprotein that has not been charged with coelenterazines.

Final photoprotein preparations were homogeneous according to SDS-PAGE analysis (data not shown). Protein concentration was determined with D_c Bio-Rad protein assay kit.

Bioluminescence assay

Bioluminescence was measured at 23°C after injection of 50 μl of 60 mM $CaCl_2$, 100 mM Tris-HCl pH 7.5 into a plate well

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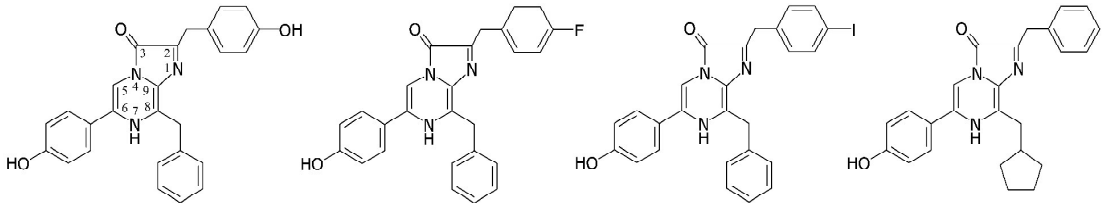

Scheme

containing 100 µl of 2 mM EDTA, 20 mM Tris-HCl pH 7.5, and the photoprotein. The light signal was recorded with a Mithras LB940 plate luminometer (Berthold Technologies) over 0.5-2 min depending on bioluminescence kinetics. Specific activity (RLU/mg) is expressed as the total light emission in relative units (RLU) normalized towards protein concentration.

Spectral measurements

Bioluminescence spectra were obtained at room temperature with a Varian Cary Eclipse spectrofluorimeter (Agilent Technologies) (slit width, 5 nm; scan rate, 200 nm/s). An aliquot of purified protein sample was brought into contact with a Ca^{2+} solution in 20 mM Tris-HCl, pH 7.5 to induce light emission. The free ionized Ca^{2+} concentration was calculated to obtain ~0.5 µM [Ca^{2+}], providing a near-constant light intensity during the spectral scan. The spectra were corrected for spectral sensitivity of the detector with the program supplied with the instrument. If a substantial decay in bioluminescence intensity took place during the measurement, we corrected data points accordingly.

Ca^{2+} concentration-effect curves

Purified samples were buffer-exchanged to zero Ca^{2+} buffer by gel filtration on a 1.5 × 6.5 cm D-Salt Dextran Desalting column (Pierce). The column was equilibrated and eluted with 150 mM KCl, 5 mM PIPES, pH 7.0 previously passed through freshly washed beds of Chelex-100 chelating resin (Sigma) to remove trace amounts of Ca^{2+}. The fractions containing photoprotein were identified by a bioluminescence assay. To exclude possible contamination with EDTA, we used only the first few photoprotein fractions to come off the column to determine Ca^{2+} concentration-effect curves and bioluminescence kinetics.

Ca-EGTA buffers (total [EGTA] = 2 mM) were used to establish Ca^{2+} concentrations ≤ 10 µM while simple dilutions of $CaCl_2$ (in a Chelex-scrubbed solution of 150 mM KCl, 5 mM PIPES, pH 7.0) were used for higher Ca^{2+} concentrations.[57] A 10-µl aliquot of photoprotein sample with a concentration of 1-5 µM was injected by a constant rate syringe CR 700-20 (Hamilton, USA) into 1 ml test solution. Light intensity (L) measurements were converted to units of L/L_{int} by first calculating L/L_{max} and then multiplying L/L_{max} by the maximum peak-to-integral ratio (L_{max}/L_{int}), determined from kinetic measurements carried out under the same conditions with the same photoprotein sample. All measurements were performed at 20°C using a luminometer equipped with a temperature-stabilized cuvette block and neutral-density filters with transmission coefficients to match the intensity of the light signals, which typically vary about 8 log units from low to saturated calcium concentrations.[39]

Ca^{2+}-free luminescence (called "Ca^{2+}-independent luminescence")[58] of photoproteins was measured in the buffer 150 mM KCl, 2 mM EGTA, 5 mM PIPES, pH 7.0.

The apparent dissociation constants (K_d) were estimated from Ca^{2+} concentration-effect curves using a two-state model.[58] The Ca^{2+} concentration detection limit was estimated as described elsewhere.[30] The K_d and $[Ca^{2+}]_{limit}$ values were calculated as the mean of the apparent dissociation constants and the detection limits determined from 3-4 Ca^{2+} concentration-effect curves for each photoprotein. The stated error is the standard deviation.

Rapid-mixing kinetic measurements

The light response kinetics after a sudden step to saturating Ca^{2+} concentration was examined with an SX20 stopped-flow machine (cell volume 20 µl, dead-time 1.1 ms) (Applied Photophysics, UK). Temperature was controlled with a circulating water bath and set at 20°C. The Ca^{2+} syringe contained 40 mM Ca^{2+}, 30 mM KCl, 5 mM PIPES buffer, pH 7.0. The photoprotein was dissolved in a Ca^{2+}-free solution of the same ionic strength: 150 mM KCl, 5 mM PIPES, pH 7.0. The solutions were mixed in equal volumes. Data was fitted in the range from zero to the time when the light signal reaches its

maximum with the equation $L = L_o + a \times (1 - e^{-k\text{rise} \times t})$ and k_{rise} used as an estimate of the rise rate constant. The decay rate constants were calculated by one or two-exponential fitting.[59] The contribution of k_1 and k_2 decay rate constants was estimated as the relative amplitude calculated from the fitted amplitudes a and b with their sum normalized to 1.[59] The rise and decay constants were calculated as a mean of corresponding constants determined from about 10 shots. The stated error is the standard deviation.

When measurements for Ca^{2+} concentration-effect curves and kinetics were made in the presence of Mg^{2+}, the photoprotein samples were pre-equilibrated for 1 h with 1 mM Mg^{2+}. For these measurements, all other solutions (Ca-EGTA buffers, dilutions of $CaCl_2$, and Ca^{2+} solution in a syringe at rapid-mixing kinetic measurements) also contained 1 mM Mg^{2+}.

Results and discussion

Bioluminescence properties of semisynthetic photoproteins

The semisynthetic aequorins, obelins, and clytins were prepared by overnight incubation of the native CTZ and its analogues with the corresponding apophotoproteins. Before measuring their bioluminescence properties, active photoproteins were separated from apophotoproteins and unbound CTZ by chromatography on a Mono Q column.

Specific bioluminescent activities of different photoproteins were determined at saturating [Ca^{2+}]. Specific activities of purified semisynthetic photoproteins are summarized in Table1. For the purpose of comparison, specific activity of the corresponding photoprotein with native coelenterazine is set to 100%. Among semisynthetic photoproteins, only aequorin-*f* reveals specific bioluminescence activity identical to that of the photoprotein charged by native CTZ. In combination with the two other semisynthetic photoproteins, specific bioluminescence activity is somewhat diminished, but still significantly higher as compared to bioluminescence activities of semisynthetic photoproteins charged by other two CTZ analogues[52,53]. Of note is that the photoproteins charged by the same CTZ analogue reveal different decrease of activity, as shown previously for other CTZ analogues[52,53]. For instance, clytin-*hcp* retains only 22% of activity, whereas semisynthetic obelin and aequorin with CTZ-*hcp* respectively preserve 86% and 62% of activity as compared to those of corresponding photoproteins charged by native CTZ (Table 1).

The semisynthetic aequorin, obelin, and clytin charged by CTZ-*f* and CTZ-*i* display bioluminescence maxima shifted for 4-10 nm to longer wavelengths as compared to those of the corresponding photoproteins with native CTZ (Table 1). Aequorin-*f* and aequorin-*i* reveal emission spectra shapes similar to that of aequorin with native CTZ, whereas the replacement of native CTZ in obelin and clytin by these CTZ analogues eliminates a shoulder at 390 nm in their bioluminescence spectra. Obelin-*hcp* and clytin-*hcp* also display a short-wavelength shift of the main bioluminescence maxima for 15 and 20 nm, respectively. In addition, the use of CTZ-*hcp* instead of native CTZ in obelin and clytin also leads to

Table 1 Bioluminescence properties of semisynthetic photoproteins

photoprotein	CTZ	Specific activity (%)	Light emission λ_{max}/shoulder (nm)	FWHM [a] (nm)	without Mg^{2+}		with 1 mM Mg^{2+}		$[Ca^{2+}]_{limit}$, nM	
					K_d, nM	Slope [b]	K_d, nM	Slope	without Mg^{2+}	with 1 mM Mg^{2+}
aequorin	*native*	100	472	89	45 ± 5	2.2	83 ± 5	2.9	24 ± 2	54 ± 2
	f	100	479	93	29 ± 4	2.1	56 ± 5	2.2	21 ± 1	39 ± 2
	i	95	482	90	125 ± 9	2.0	150 ± 10	2.3	110 ± 10	100 ± 13
	hcp	62	452	86	37 ± 5	1.4	100 ± 5	1.9	31 ± 3	90 ± 5
obelin	*native*	100	480/390 (0.15)[c]	88	91 ± 10	2.9	132 ± 11	2.9	81 ± 3	135 ± 5
	f	90	484	90	67 ± 6	2.2	110 ± 10	2.1	67 ± 3	100 ± 5
	i	75	485	90	233 ± 13	2.2	250 ± 15	2.2	200 ± 13	200 ± 10
	hcp	86	464/390 (0.40)	94	18 ± 5	1.6	105 ± 10	2.3	27 ± 1	87 ± 4
clytin	*native*	100	478/390 (0.15)	88	500 ± 35	2.8	500 ± 45	2.8	490 ± 20	490 ± 10
	f	88	482	88	250 ± 15	2.3	270 ± 15	2.3	210 ± 12	210 ± 10
	i	55	483	84	833 ± 45	2.3	1720 ± 50	2.3	690 ± 30	1200 ± 90
	hcp	22	458/390 (0.35)	86	161 ± 11	1.66	357 ± 15	2.0	280 ± 10	280 ± 25

[a] full width at half-maximum
[b] slope of Ca^{2+} concentration-effect curve
[c] contribution of violet light to emission spectrum

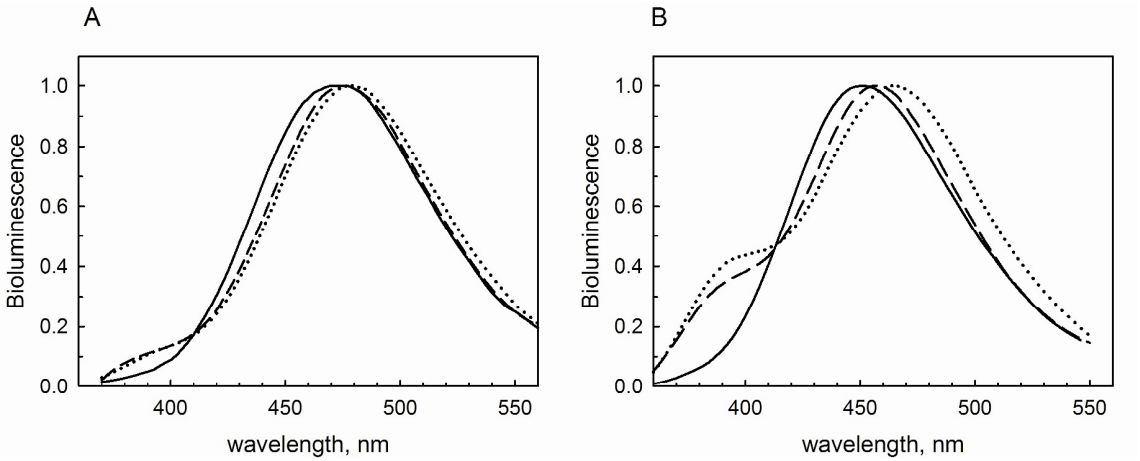

Fig. 1 Bioluminescence spectra of aequorin (solid line), clytin (dashed line), and obelin (dotted line) charged by native CTZ (A) and CTZ-*hcp* (B).

a more than 2-fold increase of the intensity at 390 nm (Fig. 1). The 20-nm blue-shift of the light emission maximum of aequorin-*hcp* does not affect the shape of its spectrum (Fig. 1).

According to the suggested proton-relay mechanism for triggering a bioluminescence reaction by Ca^{2+} and for the formation of different product excited states[16,18], the neutral coelenteramide is the primary excited product in photoprotein bioluminescence (λ_{max} = 390 nm), and coelenteramide excited state emitting light at λ_{max} = 460-490 nm occurs from the excited phenolate anion. Appearance of the phenolate anion owes to rapid transient proton dissociation of the OH group of the 6-(*p*-hydroxyphenyl) substituent of coelenterazine and its 'transient displacement' toward the N atom of His, situated within H-bond distance in all hydromedusan photoproteins.[13-15] The hydrogen bond network near the OH group and the residues participating in its formation significantly affect the yield of phenolate anion and consequently the blue emission.[60] Therefore, we can reasonably suggest that the presence of the cyclopentyl group at the 8 position of coelenterazine instead of the original phenyl group slightly changes the orientation of the OH group of the 6-(*p*-hydroxyphenyl) substituent relatively to His and, consequently, decreases the efficiency of proton dissociation. It accounts for the increase of violet light emission. In addition, in semisynthetic photoproteins with CTZ-*hcp*, the H-bond with a water molecule found close to the OH group of the *p*-hydroxybenzyl group in C2 position in all hydromedusan photoproteins[13-15] should be missing since a benzyl group substitutes the *p*-hydroxybenzyl group (Scheme). It was suggested that the main role of this hydrogen bond is stabilization of 2-hydroperoxycoelenterazine within the internal cavity of photoproteins.[13,61] The absence of this H-bond may also lead to the shift of the OH group of the 6-(*p*-hydroxyphenyl) substituent relatively to His, thereby reducing the efficiency of proton dissociation. Noteworthy is that it does not occur for aequorin; aequorin-*hcp* and aequorin with native CTZ display very similar bioluminescence spectra (Fig. 1). Why substitution of the OH group of the *p*-hydroxybenzyl substituent by F or I results in disappearance of the shoulder found at 390 nm in the spectra of semisynthetic obelin and clytin is obscure.

Sensitivity to calcium

Figure 2 and Table 1 summarize the effect of CTZ analogues on photoprotein sensitivity to Ca^{2+}. The vertical ranges of the Ca^{2+} concentration-effect curves are reduced for all semisynthetic photoproteins as compared to those of photoproteins charged by native CTZ; the curves span vertical ranges of 4.5-6.5 log units for semisynthetic photoproteins and approximately 7 log units for photoproteins with native CTZ. The decrease of vertical span is due to either increase of Ca^{2+}-independent bioluminescence, raising the left end of the curve (semisynthetic photoproteins with *hcp* and *f* coelenterazine analogues) or reduction of L/L_{int} ratio at saturating [Ca^{2+}] (semisynthetic photoproteins charged by CTZ-*i*). The maximum slope of the Ca^{2+} concentration-effect curves for semisynthetic photoproteins with CTZ-*hcp* is 1.4-1.7. The semisynthetic photoproteins charged by CTZ-*f* or CTZ-*i* as well as photoproteins with native CTZ reveal slopes between 2.0 and 2.9. Thus, only two calcium ions are needed to trigger the bioluminescent reaction of aequorin-*hcp*, obelin-*hcp*, and clytin-*hcp*, whereas three calcium ions in this case are required for photoproteins charged by both native CTZ and CTZ analogues *f* and *i*.[33,42]

To estimate the effect of replacing native CTZ to CTZ analogues on the Ca^{2+} sensitivity of aequorin, obelin and clytin, we used two parameters: i) the apparent dissociation constant (K_d) calculated using a two-state model[58] and ii) Ca^{2+} concentration detection limit defined as the [Ca^{2+}] producing a light signal five times over Ca^{2+}-free luminescence level[30] (Table 1). All the photoproteins reconstituted with CTZ-*hcp* and CTZ-*f* display higher sensitivities to Ca^{2+} as compared to those of the corresponding photoproteins charged by native CTZ; accordingly, Ca^{2+} concentration-effect curves for these semisynthetic photoproteins are situated on the left side of the corresponding curves for photoproteins with native CTZ (Fig. 2, left panel). In contrast, the use of CTZ-*i* instead of native CTZ always lowers Ca^{2+}-sensitivity (Fig. 2, left panel). These observations are supported by calculated K_d and [Ca^{2+}] detection limit (Table 1). Free Mg^{2+} is found in cells in concentrations exceeding [Ca^{2+}] by several orders of

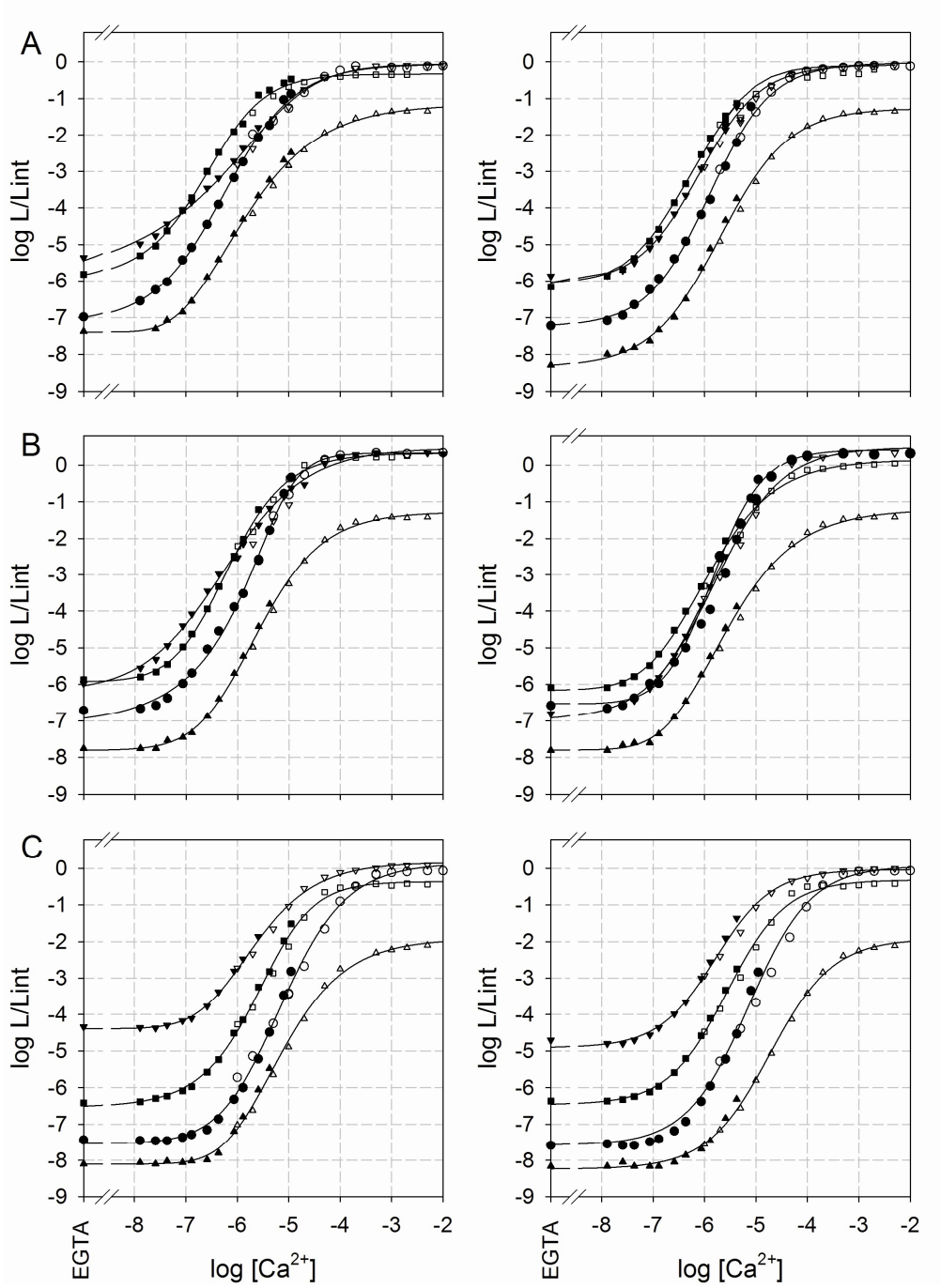

Fig. 2 Ca^{2+} concentration-effect curves for recombinant aequorin (A), obelin from *O. longissima* (B), and clytin (C) reconstituted with native CTZ (circles), CTZ-*f* (squares), CTZ-*i* (triangles up), and CTZ-*hcp* (triangles down) without (left panel) and with 1 mM Mg^{2+} (right panel). *L* and L_{int} are the peak bioluminescence intensity for a given [Ca^{2+}] and the total bioluminescence recorded for a saturating Ca^{2+} dose for the same sample, respectively. Filled symbols, Ca-EGTA buffers; open symbols, dilutions of $CaCl_2$. Before measurements with magnesium, photoproteins were pre-equilibrated with 1 mM Mg^{2+} for 1 h.

magnitude.[62] Mg^{2+} can compete with Ca^{2+} for binding with EF-hand Ca^{2+}-binding loops of a photoprotein and this may lead to a decrease in photoprotein affinity for calcium.[30,39] Therefore, we quantified the Ca^{2+}sensitivity of our semisynthetic photoproteins in the presence of 1 mM Mg^{2+}. The action of Mg^{2+} on Ca^{2+}-sensitivity differs for semisynthetic aequorin, obelin, and clytin (Table 1 and Fig. 2, right panel). The presence of 1 mM Mg^{2+} significantly decreases the Ca^{2+}-sensitivities of aequorin-*hcp* and obelin-*hcp* but is practically without effect for aequorin-*i* and obelin-*i* (Table 1). The impact of magnesium on Ca^{2+}-sensitivities of aequorin and obelin with CTZ-*hcp* is stronger than on these photoproteins with native CTZ. This is especially manifested for obelin-*hcp*, where 1 mM Mg^{2+} almost does not affect sensitivity of obelin charged by native CTZ to

Ca^{2+} (Table 1). At the same time, the reduced Ca^{2+}-sensitivity of aequorin-*i* and obelin-*i* in the presence of 1 mM Mg^{2+} is almost equal to that for corresponding photoproteins with native CTZ. It is evident that the higher sensitivity to Mg^{2+} of photoproteins charged by CTZ-*hcp* causes an increase of Mg^{2+} affinity of their Ca^{2+}-binding sites as compared to the corresponding photoproteins charged by native CTZ. The mechanism behind this is unclear, but it might suggest an impact of CTZ-*hcp* on the conformation of Ca^{2+}-binding loops, since the structure of this CTZ analogue is quite different from native CTZ. In the case of clytin, it is notable that only K_d and $[Ca^{2+}]_{limit}$ of clytin-*i* are significantly influenced by the presence of 1 mM Mg^{2+} (Table 1). For clytin-*hcp*, magnesium ions change the slope of its Ca^{2+} concentration-effect curve and reduce the K_d but have no effect on the $[Ca^{2+}]$ detection limit. Interestingly, similar effects of magnesium on the steepness of Ca^{2+} concentration-effect curves are observed for aequorin-*hcp* and obelin-*hcp*. This is especially evident for obelin-*hcp* with a slope of 1.6 of its Ca^{2+} concentration-effect curve without Mg^{2+}, whereas it is more than 2 in the presence of 1 mM Mg^{2+} (Table 1).

In Ca^{2+}-free solutions, the Ca^{2+}-regulated photoproteins emit a very low level of light, Ca^{2+}-independent luminescence, arising as a result of photoprotein structure fluctuations leading to spontaneous breakdown of 2-hydroperoxycoelenterazine. All semisynthetic photoproteins charged with CTZ-*hcp* and CTZ-*f* show an increased level of Ca^{2+}-independent luminescence as compared to photoproteins charged with native CTZ, whereas the spontaneous luminescence of photoproteins with CTZ-*i* is always notably lower (Fig. 2). This property is in good correlation with the stability of semisynthetic photoproteins at storage. During storage at 4°C, obelin-*f* and clytin-*f* lost half of bioluminescence activity, aequorin-*f* – more than 90% after six weeks, the activity of photoproteins charged by CTZ-*hcp* dropped by half after 1-2 days, whereas bioluminescence activity of photoproteins with CTZ-*i* was virtually unchanged after six weeks. The effect of Mg^{2+} on Ca^{2+}-independent luminescence of semisynthetic photoproteins is very similar to that of corresponding photoproteins with native CTZ, i.e. magnesium ions decrease the level of Ca^{2+}-independent luminescence of aequorins and in a lesser extent clytins, and slightly increase Ca^{2+}-free luminescence of obelins.[30,33,39,42].

Although the increased level of Ca^{2+}-independent luminescence affects the stability of photoproteins and limits their dynamic range as a $[Ca^{2+}]_i$ indicator, this property can be used for the calibration of intracellular photoprotein concentration at $[Ca^{2+}]_i$ resting levels.

Rapid-mixing stopped-flow kinetics

All photoproteins display a rapid increase of light signal after rapid mixing with Ca^{2+}. Within milliseconds a peak in bioluminescence intensity is reached, followed by a slow decay of the light signal lasting seconds. The kinetic characteristics of bioluminescence reactions of semisynthetic photoproteins and the corresponding photoproteins charged by native CTZ show considerable differences in rate of rise of light intensity and bioluminescence decay, as is summarized in Table 2. This is remarkable, as hydromedusan photoproteins reveal a high degree of identity of amino acid sequences and spatial structures.[11] Also, replacement of native coelenterazine by its analogues has different effects on bioluminescence kinetics of different photoproteins.

Among hydromedusan Ca^{2+}-regulated photoproteins tested, aequorin shows the lowest rate of rise of light signal.[30] The k_{rise} values for semisynthetic aequorins reconstituted with CTZ-*f* and CTZ-*hcp* are nearly equal but almost twice less than that of aequorin with native CTZ (Table 2). While aequorin-*f* shows very similar decay kinetics to that of aequorin charged by native CTZ, the decay kinetics of aequorin-*hcp* becomes 2-fold faster (Fig. 3A). Although aequorin-*i* reveals the lowest

Table 2 Rate constants for rise and decay of bioluminescence for semisynthetic photoproteins

Photoprotein	CTZ	k_{rise}, s^{-1}		k_{decay}, s^{-1}			
		without Mg^{2+}	with 1mM Mg^{2+}	without Mg^{2+}		with 1 mM Mg^{2+}	
				k_1	k_2	k_1	k_2
aequorin	*native*	123 ± 1	66 ± 1	0.81 ± 0.01 (1)	-	0.72 ± 0.01 (1)	-
	f	77 ± 4	67 ± 1	0.51 ± 0.01 (1)	-	0.66 ± 0.02 (1)	-
	i	280 ± 4	67 ± 1	0.05 ± 0.01 (1)	-	0.05 ± 0.01 (1)	-
	hcp	68 ± 1	70 ± 1	1.67 ± 0.08 (1)	-	2.2 ± 0.06 (1)	-
obelin	*native*	505 ± 5	490 ± 4	39.71 ± 1.75 (0.66)	4.66 ± 0.05 (0.34)	40.70 ± 1.70 (0.63)	4.08 ± 0.07 (0.37)
	f	552 ± 25	540 ± 31	18.40 ± 1.80 (0.45)	1.10 ± 0.03 (0.55)	16.50 ± 1.00 (0.51)	1.40 ± 0.02 (0.49)
	i	284 ± 5	283 ± 5	12.23 ± 1.25 (0.27)	0.03 ± 0.01 (0.73)	0.03 ± 0.01	-
	hcp	1375 ± 30	1270 ± 38	46.00 ± 0.23 (0.94)	6.06 ± 0.15 (0.06)	46.87 ± 0.08 (0.92)	5.75 ± 0.04 (0.08)
clytin	*native*	413 ± 3	395 ± 5	46.67 ± 2.90 (0.22)	0.86 ± 0.01 (0.78)	0.80 ± 0.01 (1)	-
	f	1450 ± 58	1250 ± 37	53.32 ± 2.00 (0.20)	0.35 ± 0.01 (0.80)	0.35 ± 0.01 (1)	-
	i	665 ± 42	490 ± 46	1.36 ± 0.12 (0.35)	0.01 ± 0.01 (0.65)	0.01 ± 0.01 (1)	-
	hcp	1970 ± 110	1720 ± 122	38.32 ± 1.82 (0.09)	5.32 ± 0.01 (0.91)	5.35 ± 0.02 (1)	-

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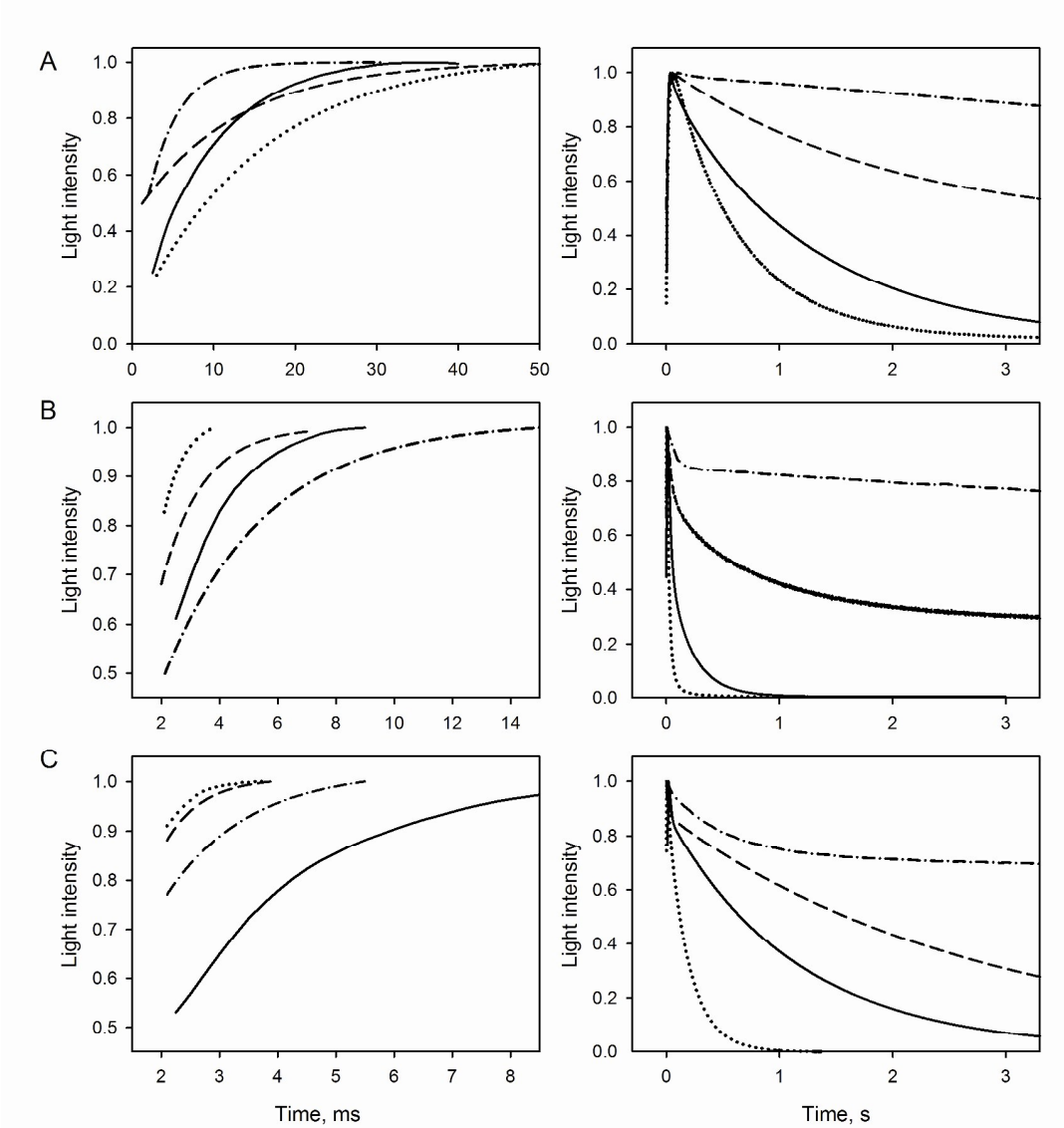

Fig. 3 Stopped-flow records of the rising phase (left panel) and full-time course (right panel) of luminescence signal for aequorin (A), obelin (B), and clytin (C) in pair with native CTZ (solid line), CTZ-*f* (dashed line), CTZ-*i* (dashed and dotted line), and CTZ-*hcp* (dotted line). The display begins at the time the flow was stopped, with each tracing normalized to its own maximum to facilitate comparison of the emission time course.

sensitivity to Ca^{2+} among aequorins (Table 1), the rate of rise of its light signal is more than twice as high as that of aequorin charged by native CTZ and almost 4-fold higher than those of other semisynthetic aequorins (Table 2, Fig 3A, left panel). At the same time, the decay kinetics of aequorin-*i* is much slower as compared to other aequorins (Fig. 3A, right panel), the light signal of aequorin-*i* reaching zero in 100 s instead of less than 10 s found for the other aequorins. These results for aequorin-*i* agree well with those described earlier[10,43].

Bioluminescence of aequorin is most sensitive to the presence of magnesium ions as compared to other hydromedusan photoproteins.[30,42,51] In the presence of 1 mM Mg^{2+} the rate of rise decreases significantly and the decay rate slightly for aequorin charged by native CTZ, while it practically

doesn't affect k_{rise} of aequorin-*f* and aequorin-*hcp* as well as k_{decay} of aequorin-*f*, and slightly increases k_{decay} for aequorin-*hcp* (Fig. S1). The speed of rise of light signal of aequorin-*i* is most sensitive to the magnesium ions; 1 mM Mg^{2+} reduces k_{rise} almost 4 times (Table 2, Fig. S1C). However, Mg^{2+} has no influence on the decay kinetics of the light signal of aequorin-*i*. Noteworthy, despite the initial differences, the values of k_{rise} for different aequorins become approximately equal in the presence of 1 mM Mg^{2+} (Table 2).

The bioluminescence kinetics of obelins from *O. longissima* and *O. geniculata* is faster and less sensitive to physiological [Mg^{2+}] than that of other hydromedusan Ca^{2+}-regulated photoproteins.[30,33,51] The substitution of native CTZ by CTZ-*f* in obelin results in a slight increase in the rising speed of the light signal and a several times decrease of the "fast" and "slow" k_{decay} constant (respectively k_{1decay} and k_{2decay}), thus increasing the duration of light signal up to 5 s unlike 2 s for obelin with native CTZ (Table 2, Fig. 3B). Bioluminescence kinetics of obelin-*f* is unaffected by magnesium ions unlike that of obelin charged by native CTZ, where Mg^{2+} shows a minor effect (Table 2, Fig. S2). In contrast to aequorin, the replacement of native CTZ to CTZ-*i* in obelin yields an approximately 2-fold decrease of k_{rise} (Fig. 3B, left panel) but also, as in the case of aequorin-*i*, a significant decrease of the decay rate (light signal drops to zero in ~100 s) (Fig. 3B, right panel). The presence of Mg^{2+} does not influence k_{rise} but practically eliminates the "fast" decaying component; the decay of light signal can be satisfactorily characterized by a single rate constant, which corresponds to k_{2decay} for obelin-*i* without magnesium (Fig. S2C). Obelin-*hcp* reveals a rate of light signal rise ~3-fold higher than it is for obelin with native CTZ (Table 2; Fig. 3B, left panel). The k_{rise} is even higher than that for the W92F obelin mutant, amounting to 992 ± 37 s^{-1}.[63] In addition, the use of CTZ-*hcp* instead of native CTZ increases the decay rate and contribution of the "fast" component to decay kinetics (Fig. 3B, right panel). The effect of magnesium ions on bioluminescence kinetics of obelin-*hcp* is very similar to that for obelin with native CTZ (Fig. S2D). Of note is that the light signal of obelin-*hcp* is the shortest among the tested photoproteins - it ceases after 0.3 s (Fig. S2D, right panel). This is approximately 6-fold shorter than the duration of light signal of obelin with native CTZ.

In contrast to aequorin and obelin, the semisynthetic clytins paired with each of CTZ analogues increase the speed of rise of their light signals (Fig. 3C, left panel). For instance, the k_{rise} values for clytin-*f* and clytin-*hcp* are increased up to 1450 and 1970 s^{-1}, respectively (Table 2), i.e. the rise rates for these semisynthetic clytins are even higher than those for the corresponding obelins, although k_{rise} of obelin with native CTZ exceeds that of clytin. The 1 mM Mg^{2+} slightly decreases the speed of rise, and the effect of magnesium is approximately equal for both semisynthetic clytins and the clytin charged by native CTZ (Table 2, Fig. S3). Similar to obelin, the decay kinetics of clytin can be satisfactorily described by two rate constants, although the contribution of the "fast" component to decay kinetics is less as compared to that of obelin (Table 2; Fig. 3C, right panel). Whereas the replacement of native CTZ to CTZ-*f* in clytin entails an increase of the rate of the "fast" decaying component and a decrease of rate of the "slow" decaying component, the charging of clytin by CTZ-*hcp* causes the opposite effect (Table 2). The substitution of native CTZ to CTZ-*i* leads to slowdown of the light signal decay, significantly reducing the rates of its "fast" and "slow" components, as is the case for aequorin and obelin (Fig. 3C, right panel). Of note is that for all clytins, the change of shape of the "fast" decaying component in the presence of a physiological Mg^{2+} concentration (Fig. S3, right panel) is such that it cannot be fitted by an exponential anymore. The k_{decay} values of the "slow" component in the presence of 1 mM Mg^{2+} are equal to the values of the decay constants characterizing the "slow" component of the corresponding clytin without Mg^{2+} (Table 2).

Conclusions

Despite the large number of synthesized CTZ analogues[10], only a few display sufficient activity and useful properties when paired with hydromedusan Ca^{2+}-regulated photoproteins. This study reports the results on comparative characterization of bioluminescence properties of aequorin from *A. victoria*, obelin from *O. longissima*, and clytin from *C. gregaria* charged by native coelenterazine and its analogues CTZ-*f*, CTZ-*i*, and CTZ-*hcp*, which are often used instead of native CTZ in measurements of intracellular calcium. In the experiments, we show that, as expected, the semisynthetic photoproteins differ from each other in specific bioluminescence activity, sensitivity to Ca^{2+}, and bioluminescence kinetics as well as sensitivity of their bioluminescence to physiological [Mg^{2+}]. Based on our quantification of these bioluminescence properties, we infer that obelin charged by CTZ-*hcp* is a promising photoprotein to keep track of intracellular Ca^{2+} transients with rise times of only a few milliseconds, e.g. in the post-synaptic compartments of excitatory neurons[47,48] or the Ca^{2+}-sparks at excitation-contraction coupling in heart muscles[49]. The rising rate of its luminescence signal on a sudden change of Ca^{2+} concentration is almost 3- and 11-fold higher than those of obelin and aequorin with native CTZ, respectively, and 20 times higher than that of the corresponding aequorin-*hcp*. In addition, obelin-*hcp* preserves a high specific bioluminescence activity and displays higher Ca^{2+}-sensitivity as compared to obelin charged by native CTZ and Ca^{2+}-sensitivity comparable with those of aequorin-*f* and aequorin-*hcp*. Although k_{rise} values for clytin-*f* and clytin-*hcp* are higher than that for obelin-*hcp*, these semisynthetic photoproteins show a significantly lower Ca^{2+}-sensitivity as compared to that of obelin-*hcp*. This makes them less suitable calcium probes for the 0.1 to 1 μM $[Ca^{2+}]_i$ range. Thus, a suitable pair depending on the task to be addressed can be selected among available hydromedusan recombinant photoproteins and coelenterazine analogues.

Acknowledgments

We would like to thank Bruce Bryan, MD at NanoLight.com, for a gift of coelenterazine analogues *i* and *f*. This work was supported by the grant 14-14-01119 of the Russian Science Foundation.

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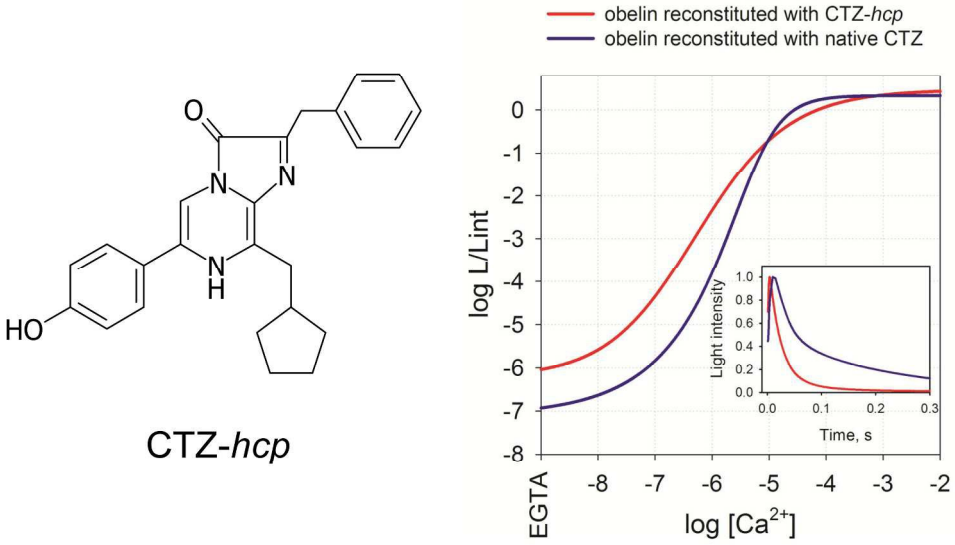

The semisynthetic Ca^{2+}-regulated photoprotein obelin from *Obelia longissima* charged by coelenterazine analogue *hcp* is a promising reporter to keep track of fast intracellular Ca^{2+} transients.
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