ORIGINAL PAPER

Coelenterazine-v ligated to Ca^{2+}-triggered coelenterazine-binding protein is a stable and efficient substrate of the red-shifted mutant of *Renilla muelleri* luciferase

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Abstract It has been shown that the coelenterazine analog, coelenterazine-v, is an efficient substrate for a reaction catalyzed by *Renilla* luciferase. The resulting bioluminescence emission maximum is shifted to a longer wavelength up to 40 nm, which allows the use of some "yellow" *Renilla* luciferase mutants for in vivo imaging. However, the utility of coelenterazine-v in small-animal imaging has been hampered by its instability in solution and in biological tissues. To overcome this drawback, we ligated coelenterazine-v to Ca^{2+}-triggered coelenterazine-binding protein from *Renilla muelleri*, which apparently functions in the organism for stabilizing and protecting coelenterazine from oxidation. The coelenterazine-v bound within coelenterazine-binding protein has revealed a greater long-term stability at both 4 and 37 °C. In addition, the coelenterazine-binding protein ligated by coelenterazine-v yields twice the total light over free coelenterazine-v as a substrate for the red-shifted *R. muelleri* luciferase. These findings suggest the possibility for effective application of coelenterazine-v in various in vitro assays.

Abbreviation

CBP	Ca^{2+}-triggered coelenterazine-binding protein
RM-Luc	Native *Renilla muelleri* luciferase
RM-Y	Long-wavelength shifted *Renilla muelleri* luciferase mutant

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Introduction

The *Renilla* luciferase is an enzyme that is responsible for the bioluminescence of the soft coral *Renilla* [1]. The enzyme catalyzes the oxidative decarboxylation of coelenterazine in the presence of O_2, resulting in the formation of coelenteramide in its excited state and CO_2 as the reaction products [2]. The excited coelenteramide relaxes to its ground state with the production of blue light with a maximum at 480 nm. The bioluminescence in vivo results from the radiationless energy transfer from excited coelenteramide to green-fluorescent protein, which then emits green light with an emission maximum of 509 nm [3]. In addition, the *Renilla* bioluminescence in vivo also involves a third protein called Ca^{2+}-triggered coelenterazine-binding protein (CBP). The CBP is a stable complex of coelenterazine and a single-chain polypeptide with molecular mass about 20 kDa [4]. The primary structure of CBP from *Renilla reniformis*, as determined by direct amino acid

sequencing [5], reveals three canonic Ca^{2+}-binding sites characteristic for the family of EF-hand Ca^{2+}-binding proteins [6, 7]. On addition of calcium, the coelenterazine bound with CBP becomes available for bioluminescence reaction with luciferase, thereby linking the bioluminescence with the nerve net of coral [8]. Recently, a synthetic gene encoding *R. reniformis* CBP was synthesized from oligonucleotides [9] based on the published amino acid sequence of *R. reniformis* CBP [5], and the full-size cDNA encoding the CBP from *R. muelleri* (RM-Luc) has been cloned as well [10]. It was shown that *Renilla* luciferase bioluminescence is more efficient using the recombinant CBP with bound coelenterazine as the substrate than with coelenterazine alone [10]. The determination of crystal structures of CBP with coelenterazine [11] and apo-CBP bound with calcium ions [12] has allowed the reasonable mechanistic suggestion as to why oxygen addition to coelenterazine is inhibited within the CBP cavity, whereas it occurs readily in Ca^{2+}-regulated photoproteins [13, 14], and as to how CBP might interact with luciferase to provide a higher bioluminescent yield of reaction.

Following the cloning of luciferase from the firefly *Photinus pyralis* [15], luciferase genes have become valuable components of biological research. They are used ubiquitously as reporter genes in cell culture experiments, and their use as reporters has been extended into the context of small-animal imaging [16]. The firefly type luciferases, however, are not optimal for employment in imaging. These luciferases are not particularly small (~62 kDa) and are dependent on firefly luciferin, ATP, molecular oxygen, and magnesium for activity. The dependence on ATP especially would hinder the application of these luciferases in vivo, as serum ATP concentrations are generally below 10 nM [17]. Luciferases that use coelenterazine as their substrate (e.g., from *Renilla* [18], *Metridia* [19], and *Gaussia* [20]) are more appropriate for application as bioluminescent reporters, as these enzymes are not ATP dependent and in general require only molecular oxygen in addition to coelenterazine for luminescence. Among them, the *Renilla* luciferase is the most usable [21].

The major limitation for the application of *Renilla* and other coelenterazine-dependent luciferases for bioluminescence imaging in small animals is that they emit blue light (~480 nm), which is preferentially absorbed by biological tissues [22]. In the case of *Renilla* luciferase, which has its spectral maximum at 480 nm, only ~3% of the emitted photons are of wavelength above 600 nm where tissue attenuation is minimized. Consequently, the coelenterazine-dependent luciferases have diminished sensitivity at non-superficial locations.

A few years ago, mutants of *Renilla* luciferase with long-wavelength shifts of bioluminescence spectrum up to 66 nm (peak at 547 nm) have been described [23]. These mutants revealed greater stability and higher light emission than native luciferase. In addition, it is notable that when the coelenterazine-v, an analog of coelenterazine, was used as a substrate, an orange-peaked emission resulted ($\lambda_{max} = 588$ nm) [23]. Coelenterazine-v, which is a significantly more efficient substrate of *Renilla* luciferase than native coelenterazine, only shifts the bioluminescence of native luciferase from blue to green [24]. However, despite very promising results with coelenterazine-v, its application in vivo remains limited. This coelenterazine analog appears to be extremely unstable in solutions and exhibits an order of magnitude increase in a background autochemiluminescence in experiments in vivo. Therefore, the development of alternative coelenterazine analogs, or overcoming this coelenterazine-v instability, might be very important for developing small-animal imaging techniques using red-shifted *Renilla* luciferase mutants.

In the present study, we demonstrate that coelenterazine-v analog stability in vitro can be greatly increased on binding within the Ca^{2+}-triggered coelenterazine-binding protein from *R. muelleri*, and that CBP with this coelenterazine analog is a more efficient substrate of a long-wavelength shifted mutant of *R. muelleri* luciferase than coelenterazine-v alone.

Materials and methods

Materials

Native coelenterazine was purchased from Prolume Ltd. (USA). All chemicals were used as received without further purification.

Molecular biology

To introduce the 5′-terminal TEV-specific protease site and attB1/attB2 flanking recombination sites into RM-Luc coding sequence for subsequent cloning with the Gateway system (Invitrogen), the coding sequence for native *R. muelleri* luciferase (NCBI accession number is EF535511) from pET-19b plasmid [10] was amplified by two-step PCR using "AccuPrime Pfx supermix" (Invitrogen). The first PCR with the gene-specific forward *S-RM-TEV* (5′-GAAAACCTGTATTTTCAGGGCATCATGACGTCAAAAGTTTACGATCC-3′) and reverse *A-RM-attB2* (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTGTTCATT TTTAAGAACACGC-3′) primers was performed for 27 cycles (denaturation, 25 s at 95 °C; annealing, 60 s at 56 °C; extension, 60 s at 68 °C). For the second PCR, the first PCR product was used as a template and the full-size gene was amplified under a similar condition with the second forward *S-attB1-TEV* (5′-GGGGACAAGTTTGTA

C A A A A A A G C A G G C T G A A A A C C T G T A T T T T C A GGGCATC-3′) and the same reverse *A-RM-attB2* primers. To generate an entry clone, pDONR221 (Invitrogen) was used as an entry vector in BP reaction of recombination.

The resulting pDONR221-RM plasmid harboring the native *R. muelleri* luciferase sequence was used as a template for site-directed mutagenesis in order to construct the red-shifted *R. muelleri* luciferase (RM-Y) mutant. The following amino acid substitutions A55T, C124A, C130A, A143M, M253L, S287L, A123T, D154M, E155G, D162E, I163L, and V185L were sequentially introduced into the RM-Luc coding sequence using designed oligonucleotide primers and Quick-Change site-directed mutagenesis kit (Stratagene). These mutations were selected based on the substitutions leading to the red-shifted bioluminescence of *R. reniformis* luciferase (mutant RLuc8.6-535) [23]. The plasmids harboring mutations were verified by DNA sequencing.

To generate the expression plasmid, pDEST-566 vector harboring the N-terminal His$_6$-tag, a carrier protein–maltose-binding protein (MBP), and the *attR1/attR2* sites [25] was used in LR reaction of recombination, which was performed according to the protocol supplied with the kit (Gateway Cloning, Invitrogen).

Synthesis of coelenterazine-v

Coelenterazine-v was synthesized using the described procedures [26, 27]. To produce pure coelenterazine-v, the product was passed over a Silica Gel 60 column (EMD Chemicals) and eluted with a linear gradient of methylene chloride to methanol. The pure coelenterazine-v was characterized by ^{1}H NMR (300 MHz) in CD$_3$OD made acidic with the addition of one drop of DCl. The chemical shifts [δ: 8.62 (d, *J*=9.4, 1 H), 8.60 (d, *J*=9.1, 1 H), 7.56 (d, *J*=9.4, 1 H), 7.53–7.47 (m, 2 H), 7.38–7.11 (m, 6 H), 7.08 (d, *J*=2.4, 1 H), 6.81–6.73 (m, 2 H), 4.55 (s, 2 H), 4.27 (s, 2 H), (phenolic OH is not observed)] of synthesized coelenterazine-v do not exactly correspond to reported values, but the acidic conditions used here likely account for the differences [28]. The calculated mass amounted to 447.48; the determined mass was 448.2 (M+) and 446.4 (M−). The $\varepsilon_{238\ nm}$ in acidic methanol equaled to 23,110 M^{-1} cm^{-1}.

Purification of RM-Y mutant luciferase and CBP

For protein production, *Escherichia coli* BL21-CodonPlus (DE3)-RIPL strain (Stratagene) was transformed with plasmid isolated from LR-RM-Y clones. Transformed *E. coli* cells were cultivated with vigorous shaking at 37 °C in LB medium containing 200 μg/mL of ampicillin and induced with 0.5 mM IPTG when the culture reached an A_{600} of 0.8–1.0. After addition of IPTG, the cultivation was continued for 8 h at 20 °C.

For purification of luciferase, the cell paste was resuspended in Ni-binding buffer (0.2 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 50 mM Na$_2$HPO$_4$/KH$_2$PO$_4$ buffer, pH 7.1) and disrupted with ultrasound (1 min×6) at 0 °C. The mixtures were passed over a 5-mL HisTrap Fast Flow column (Amersham Biosciences) and eluted with an imidazole gradient (0–0.5 M) in 0.3 M NaCl, 50 mM Na$_2$HPO$_4$/KH$_2$PO$_4$ buffer pH 7.1. The luciferase peak was collected and dialyzed against TEV protease cleavage buffer (1 mM DTT, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0). MBP-tag was cleaved by incubation of the fusion protein with TEV protease at ratio 50:1 (*w/w*) overnight at room temperature; then the samples were loaded on a Ni-NTA resin column and the flow-through was collected. The collected sample of RM-Y was dialyzed against 1 mM EDTA, 20 mM Tris–HCl pH 8.0 and loaded on an anion exchange HiTrap Q column (Amersham Biosciences). The active fractions were collected and concentrated on Amicon centrifugal filters (Millipore) in 25 mM NaCl, 0.5 mM EDTA, 20 mM Tris–HCl pH 7.0.

Plasmid pET22b-CBP3 containing the gene encoding CBP from *R. muelleri* (NCBI accession number EF535511) [10] without any purification tags, was used to transform *E. coli* cells, strain BL21-CodonPlus (DE3)-RIPL (Stratagene). For protein production, transformed cells were cultivated with vigorous shaking at 37 °C in LB media containing 200 μg/mL of ampicillin and induced with 1 mM IPTG when the culture reached an A_{600} of 0.8–1.0. After addition of IPTG, the cultivation was continued for 3 h at 37 °C.

Most apo-CBP produced was accumulated inside *E. coli* cells in inclusion bodies that can be easily isolated by centrifugation. The purification was performed as previously reported for recombinant obelin [29] with modification as described in Ref. [10]. The apo-CBP sample in 6 M urea obtained after chromatography on DEAE Sepharose Fast Flow was refolded by desalting on 26/10 HiPrep Desalting column (Amersham Biosciences) in 5 mM EDTA, 20 mM Tris-HCl pH 7.0 buffer, and then mixed with coelenterazine-v (or native coelenterazine) in an equimolar concentration to protein. The mixture was incubated for 3 h at 4 °C, followed by ion-exchange chromatography on UnoQ-10 column (Bio-Rad) to separate CBP from apo-protein and from unbound substrate. The fraction of CBP was collected and concentrated on Amicon centrifugal filters (Millipore) in 25 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl pH 7.0. Protein concentration was measured with DC Bio-Rad protein assay kit. According to SDS-PAGE analysis, the RM-Y luciferase mutant and CBP were of high purity.

Bioluminescence assay

The bioluminescence was measured at room temperature with a Mithras LB940 plate luminometer (Berthold Tech-

nologies) by injecting 10 μL of RM-Y (17.8 ng/mL) into the wells containing 100-μL solution of different concentrations of (a) coelenterazine-v in 25 mM NaCl, 0.5 mM EDTA, 50 mM Tris–HCl pH 7.0; or (b) CBP–coelenterazine-v in 25 mM NaCl, 50 mM CaCl$_2$, 50 mM Tris–HCl pH 7.0. Luminescence data were corrected for a methanol inhibition effect (5–25%) on RM-Y activity. Coelenterazine-v in methanol was prepared by dilution from stock solution of coelenterazine-v in methanol. The concentration was determined using the extinction coefficient for coelenterazine-v ($\varepsilon_{238 \ nm}$=23,110 M^{-1} cm^{-1}) in methanol. The specific bioluminescent activity was determined as integral signal (integration time 20 s).

Thermostability assays

Thermostability was assayed using the purified CBP–coelenterazine-v (0.52 mg/mL in 25 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 7.0) by incubating samples at 37 or 4 °C. Aliquots of 10 μL were removed over periods of time and assayed with RM-Y luciferase. For measurement, the CBP–coelenterazine-v aliquot was mixed in luminometer cuvette with 500 μL of RM-Y luciferase in 25 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 7.0, and then the bioluminescence reaction was initiated by rapid injecting 200 μL of 40 mM CaCl$_2$ in 50 mM Tris–HCl pH 7.0. The coelenterazine-v stability in methanol was tested by storing stock solution (0.24 mM) at −20 °C. Aliquots of 5 μL were removed over periods of time and tested with RM-Y luciferase by rapid injection of 5-μL aliquot into cuvette with 700 μL of RM-Y luciferase in the same buffer as above. In both cases, the RM-Y luciferase concentration in cuvette was 9 ng/mL.

Spectral measurements

Bioluminescence spectra were measured with an AMINCO spectrofluorimeter (Thermo Spectronic, USA) using a slit width of 4 nm and a scan rate of 16 nm/s. All spectra were corrected for intensity change over the scan and for the spectral sensitivity of the instrument with the program supplied by AMINCO. The absorption spectra were obtained with a UVIKON 943 Double Beam UV/VIS spectrophotometer (Kontron Instruments, Italy). The measurements were carried out at room temperature.

Calcium concentration–effect curve

The measurements were carried out as previously described [10, 30] with EDTA-free solutions of CBP and luciferase. EDTA was removed from the CBP and luciferase by gel filtration on a DSalt plastic column (Pierce). The column was equilibrated and eluted with 150 mM KCl, 5 mM PIPES, pH 7.0, which had been previously passed two times through freshly washed beds of Chelex-100 chelating resin. The fractions containing either CBP or luciferase were identified by the bioluminescence assay. To avoid possible contamination with EDTA, only the first few protein fractions to come off the column were used for the determination of Ca^{2+} concentration–effect curves. Ca–EGTA buffers (total [EGTA]=2 mM) were used to establish Ca^{2+} concentrations below 10^{-5} M, and simple dilutions of CaCl$_2$ (in a Chelex-scrubbed solution of 150 mM KCl, 5 mM PIPES, pH 7.0) for the range of Ca^{2+} concentrations from 10^{-6} to 10^{-2} M. The Ca^{2+} buffers were prepared by the two stock-solution method described elsewhere [31]. The peak light intensity (L) was measured after 10 μl of CBP solution (2.32×10^{-8} M) was injected into 1 mL of the test solution that contained a constant amount of luciferase (1.16×10^{-9} M), so that CBP and luciferase in the cuvette were in the ratio of 20:1. All measurements were done at 20 °C maintained with the temperature-stabilized cuvette block of the luminometer.

Results and discussion

The chemical structure of coelenterazine-v differs from that of the native coelenterazine by an additional –CH=CH– group bridging C5 and C20 atoms (Scheme 1). The introduction of an additional conjugated double bond leads to a planar conformation of the 6-(p-hydroxy) phenyl group relative to pyrazine ring and results in an increase of rigidity of the overall structure of the molecule. This steric

Scheme 1 a Native coelenterazine; **b** coelenterazine-v
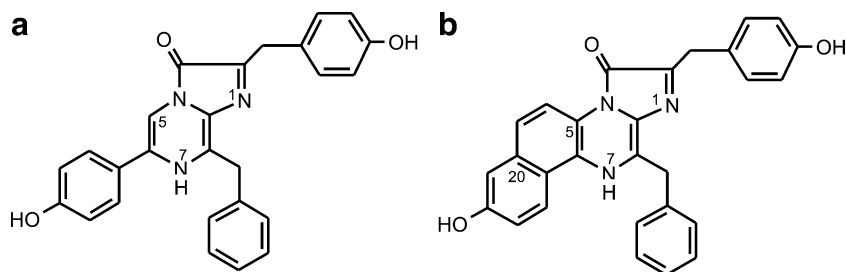
difference of coelenterazine-v when compared with the native coelenterazine drastically decreases the bioluminescence light output of Ca^{2+}-regulated photoproteins and *Oplophorus* luciferase, but significantly increases the efficiency of the reaction catalyzed by *Renilla* luciferase [24]. There was no detectable effect of structure changes on binding coelenterazine-v to apo-CBP. The apo-CBP binds coelenterazine-v very rapidly from Ca^{2+} free solution, similar to that as the native coelenterazine, thus implying there that is sufficient space to accommodate this coelenterazine analog within the CBP cavity despite the presence of the additional –CH=CH– group (Fig. 1). Probably for the same reason, the analog coelenterazine-e, which has –CH$_2$–CH$_2$– linker in place of the –CH=CH–, also effectively ligates to apo-CBP [9]. Consequently, the procedure for preparation of CBP bound with coelenterazine-v required only small modifications from that previously reported for CBP binding with native coelenterazine [10]. In the first step, the purified apo-CBP was refolded and then incubated with coelenterazine-v. To yield a homogeneous holoprotein free of apo-CBP and unbound coelenterazine-v, it was additionally purified on a UnoQ anion exchange column. It should be noted that DTT also was not required for the conversion of apo-CBP to CBP bound with coelenterazine-v. The absorption spectrum of the homogeneous CBP loaded with coelenterazine-v displays two main maxima at 286/462 and two minor maxima at 350/380 nm (Fig. 2a). In addition, there are two apparent shoulders at 310 and 490 nm. The 286 nm/462 nm absorption ratio amounts to 1.6. The absorbance maximum of coelenterazine-v bound with protein is red-shifted from that in methanol (λ_{max}= 455 nm). This effect might be caused by a more hydrophobic environment of coelenterazine-v within the
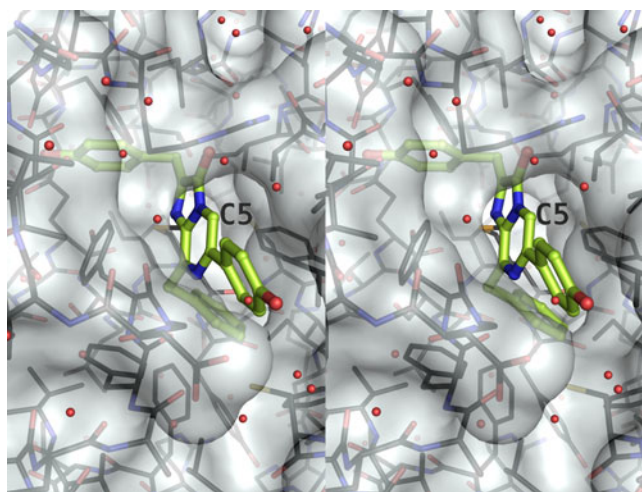

Fig. 1 Stereoview of coelenterazine molecule within CBP cavity (PDB code 2HPS). Coelenterazine molecule is shown as a *stick model in the center*; *red balls* show water molecules

protein. Extinction coefficients of CBP with coelenterazine-v were determined as $\varepsilon_{285\ nm}$=16.2 mM^{-1} cm^{-1} and $\varepsilon_{462\ nm}$= 10.6 mM^{-1} cm^{-1}. Addition of calcium to CBP leads to conformational changes in the protein making coelenterazine-v solvent exposed and available for reaction with *Renilla* luciferase. In the presence of Ca^{2+}, the visible maximum is shifted to 450 nm and the absorbance decreases slowly to zero after less than 3 h of incubation at 4 °C. This effect is attributed to coelenterazine-v dissociation and autooxidation (Fig. 2b).

The luciferases emitting a large percentage of photons in red to near-infrared wavelengths (600–900 nm) have advantages in bioluminescent imaging in intact animals. Hence, the examination of CBP bound with coelenterazine-v as a bioluminescent substrate was carried out using the pure *R. muelleri* luciferase mutant (RM-Y), which has a red-shifted bioluminescence spectrum. To express *Renilla* luciferase mutant as a soluble protein, RM-Y was fused with maltose-binding protein, which greatly enhances solubility of the proteins upon their expression in *E. coli* cells [25]. With this approach, the yield of the pure RM-Y mutant amounts to ~50–60 mg/L of cell culture.

Figure 3 shows the spectra of Ca^{2+}-triggered bioluminescence of RM-Y luciferase mutant with CBP, loaded with either coelenterazine-v or native coelenterazine, as a substrate. As expected, the use of CBP bound with coelenterazine-v produces a longer-wavelength bioluminescence (λ_{max}=574 nm) as compared with CBP loaded with native coelenterazine (λ_{max}=535 nm). Visually, the bioluminescence has the yellow-orange and green-yellowish color, respectively (Fig. 3b). It needs to be noted that the reaction of the RM-Y luciferase mutant with the free coelenterazine-v or native coelenterazine produces identical bioluminescence spectra to those when the substrates are CBP bound (data not shown). It is interesting that the bioluminescence spectrum with coelenterazine-v shows a diminished contribution of a shoulder at 420 nm, which corresponds to the excited electronic state of coelenteramide in neutral form (Fig. 3a). It should also be noted that the RM-Y mutant of *R. muelleri* luciferase has a 25% greater activity and improved thermostability at 37 °C, similar to the RLuc8.6-535 mutant of *R. reniformis* luciferase [23].

Figure 4 shows the concentration dependence of the bioluminescence integrated intensity when RM-Y mutant is assayed with either CBP bound with coelenterazine-v or free coelenterazine-v. The total emitted light with CBP is about 2.2 times higher than that with free coelenterazine-v over most of the range of substrate concentrations, i.e., this difference is practically the same as obtained for CBP loaded by native coelenterazine and wild-type *R. muelleri* luciferase [10].

Generally, the implementation of imaging experiments in vivo requires reporter enzymes and compounds which are
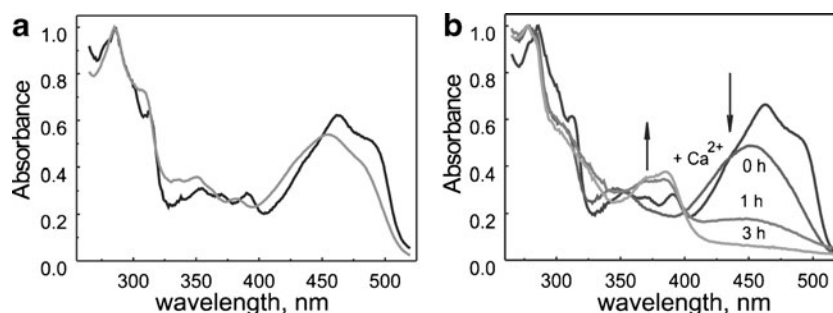
Fig. 2 a Absorption spectra of purified CBP–coelenterazine-v in EDTA-buffer and freshly prepared coelenterazine-v in methanol (*gray lines*). **b** Changes of absorption spectrum of CBP–coelenterazine-v on calcium addition. *Arrows* show increase (at 375 nm) and decrease (at 460 nm) of coelenterazine-v absorbance bands; 0–3 h, time after calcium addition. All spectra are normalized at the short wavelength maximum

sufficiently stable at 37 °C. As mentioned above, one of the limitations of the use of coelenterazine-v is its instability in solutions. Hence, we also tested whether the binding of coelenterazine-v with CBP stabilizes this coelenterazine analog in solution. For that, CBP bound with coelenterazine-v was incubated both at 4 and 37 °C. The coelenterazine-v diluted in methanol and stored in the dark at −20 °C was used as a control. The quantity of the residually active coelenterazine-v was determined in a bioluminescence assay with RM-Y mutant luciferase. A methanol solution of coelenterazine-v possesses poor stability even when kept at low temperature; there was

only ~1% of residual activity detected after 9 days of storage at −20 °C (Fig. 5a). In contrast, the coelenterazine-v bound within CBP (0.52 mg/mL) is stable in 1 mM EDTA, 25 mM NaCl, 50 mM Tris–HCl pH 7.0 over half a year at 4 °C and preserves about 80% activity after 32 h of incubation even at 37 °C (Fig. 5b). It should be noted that there was no effect of room light on the stability of the coelenterazine-v bound in CBP.

It is well-known that free coelenterazine and its analogs are unstable in neutral aqueous solution, undergoing a slow autooxidation over several hours [14]. In an aprotic solvent, coelenterazine is much less stable, reacting with oxygen to generate chemiluminescence. As the "aprotic reaction" is triggered by base, the reaction mechanism is proposed to be by oxygen attack at C2 on the coelenterazine-N7 anion [32, 33]. It has been proposed that in the CBP coelenterazine-binding pocket, hydrogen bonding should result in a net dipole across the ring system, as the C3-oxygen is an acceptor resulting in its obtaining a partial positive charge and at the other end of the molecule, the 6-(*p*-hydroxy) phenyl group and the N7, are H-bond donors, leaving them with partial negative charge [11]. Thus, the partial negative
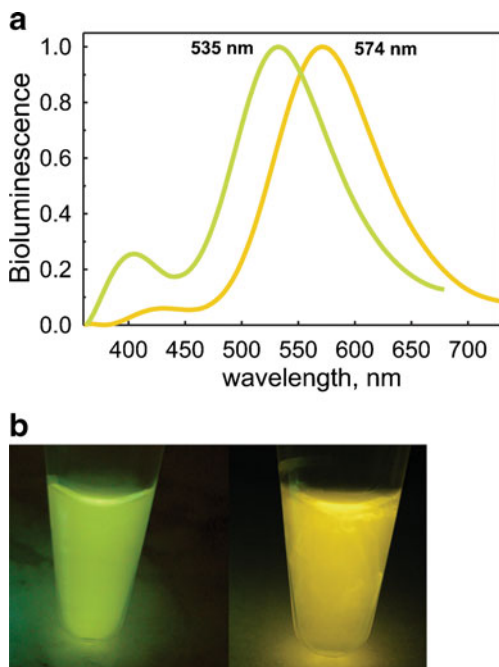

Fig. 3 a Normalized bioluminescence spectra of Ca^{2+}-triggered RM-Y luciferase mutant with CBP bound with native coelenterazine (*greenish*) and CBP bound with coelenterazine-v (*orange*). **b** Photo of calcium-triggered (40 mM) luminescence of RM-Y luciferase mutant (0.4 µg/mL) mixed with CBP bound with either native coelenterazine (*left*) or coelenterazine-v (*right*)
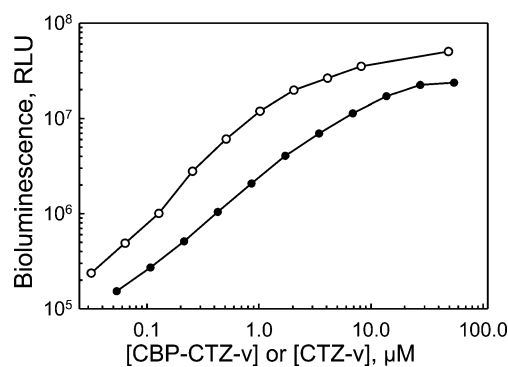

Fig. 4 Log–log plots of RM-Y luciferase bioluminescence assay with CBP–coelenterazine-v (*empty circles*) or free coelenterazine-v (*filled circles*). RLU is integral bioluminescence intensity in relative light units. The luciferase concentration is 4.9 nM. The data on the plots are average of three measurements

Fig. 5 Thermostability of CBP–coelenterazine-v complex (*empty circles*) at 4 °C (**a**), 37 °C (**b**), and free coelenterazine-v in methanol (0.24 mM) at −20 °C (**a**, *filled circles*). L/L_{max}, the residual bioluminescent capacity expressed as a percentage of initial activity
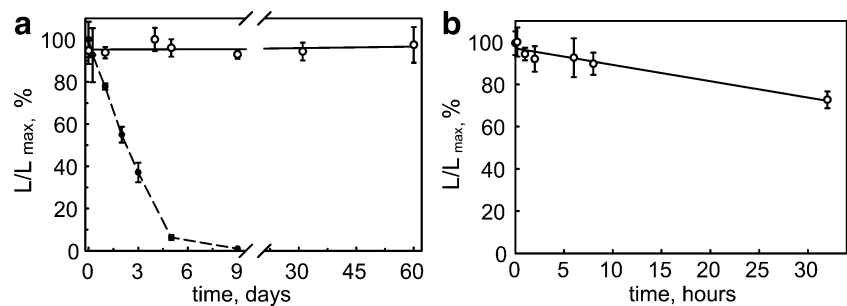
charge on the coelenterazine-N7 bound in CBP should render this position much less acidic than in free solution. Apparently, the same mechanism of stabilization has been implemented in CBP bound with coelenterazine-v. Interactions within the binding site shift the pK and thus reduce the probability of the coelenterazine-v anion formation, the target of oxygen attack.

Figure 6 shows two representations of Ca^{2+} concentration–effect curve for the mixture of CBP–coelenterazine-v and RM-Y mutant luciferase. The curves are log–log plots with light intensities expressed in terms of the ratio either L/L_{max} or L/L_{int}. The utility of representation of Ca^{2+} concentration–effect curves in terms of the ratio L/L_{int} has already been discussed elsewhere [34]. The peak light intensity (L) is measured from a sample when mixed with Ca^{2+}-containing solution under a particular set of circumstances. L_{max} and L_{int} are the peak light intensity and total light, respectively, recorded from an identical sample when mixed with saturating [Ca^{2+}] under the same conditions. The curves are sigmoid. The mixture of
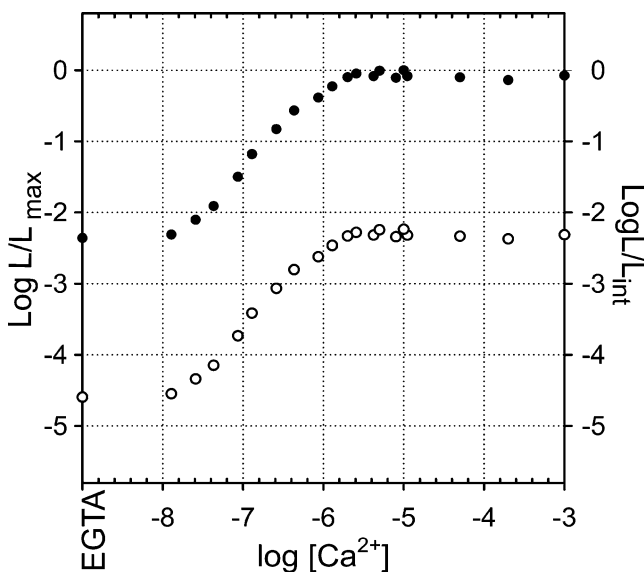
CBP–coelenterazine-v with RM-Y mutant luciferase begins to respond to changes of [Ca^{2+}] starting from 10^{-8} M and saturates at approximately 10$^{-5.5}$ M. The mixture of CBP bound with native coelenterazine and wild-type *Renilla* luciferase responds to [Ca^{2+}] changes from 10^{-7} M to approximately 10^{-5} M under the same conditions [10]. Thus, CBP–coelenterazine-v with RM-Y mutant luciferase reveals a slightly higher affinity to calcium than CBP bound with native coelenterazine and wild-type *R. muelleri* luciferase. The curve spans have a vertical range approximately of 2.5 log units. This is higher by approximately one order as compared with the span of the Ca^{2+} concentration–effect curve for the mixture of CBP–coelenterazine and wild-type *R. muelleri* luciferase. It should be noted, however, that the CBP–coelenterazine-v and RM-Y mutant luciferase mixture exhibits a high level of Ca^{2+}-independent luminescence, which is approximately the same as for CBP loaded by native coelenterazine and wild-type *R. muelleri* luciferase [10], but exceeding the level of Ca^{2+} free luminescence of Ca^{2+}-regulated photoproteins by almost two orders of magnitude [34]. The slope of the Ca^{2+} concentration–effect curve amounts to 1.2, indicating that binding of only two Ca^{2+} to CBP–coelenterazine-v is enough to trigger the RM-Y bioluminescence, whereas for Ca^{2+}-regulated photoproteins, three calcium ions are necessary [13, 34].

Conclusion

A shift of the bioluminescence emission from *Renilla* luciferase is very desirable for in vivo imaging and development of in vitro dual-color bioluminescence assays [35, 36], and this is achieved by using a coelenterazine analog, coelenterazine-v, as a substrate. In the present study, we show a potential utility of CBP from *R. muelleri* as a coelenterazine-v protector against oxidation and as a carrier protein, which allows ready turnover of substrate with *Renilla* luciferase reaction upon Ca^{2+} binding. An important finding is that the reaction of the *R. muelleri* luciferase mutant RM-Y with CBP–coelenterazine-v produces more than twice the total light over that with the free substrate. Thus, at low concentrations of the reaction components, the

Fig. 6 Ca^{2+} concentration–effect curve for CBP bound with coelenterazine-v expressed in terms of the ratio of either L/L_{max} (*filled circles*) or L/L_{int} (*empty circles*)

coelenterazine-v bound within CBP would generate a brighter bioluminescence signal than would free coelenterazine, thereby increasing the assay sensitivity. In addition, this approach for coelenterazine-v protection might be applied in vivo by expressing apo-CBP within mammalian cells by using the same technology developed for the intracellular use of Ca^{2+}-regulated photoproteins [37–39].

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