Cation-driven Optical Properties of Artificial Luciferases

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The present study demonstrates cation-driven optical properties of artificial luciferases (ALucs) from copepod luciferases, as an optical readout for bioanalysis. An assignment of the supersecondary structure code (SSC) of ALucs revealed that ALucs carry a helix-loop-helix structure, which appears at the same sites of the EF-hands of typical Ca²⁺-binding proteins. A mutagenesis study shows that the EF-hand-like structure is a pivotal site for enzymatic activity. The effects of 20 kinds of mono- and multivalent cations on ALuc activities were estimated with column-purified ALuc16. High pH values boost the ALuc activities with both the native coelenterazine and an analog called 6-pi-OH-CTZ. Multivalent cations, Ca(II), Mg(II), and Cr(VI), elevate and prolong the ALuc activities, whereas Co(II), Cu(II) and Pb(II) greatly hamper the ALuc activities. Ca(II) greatly prolongs the optical intensities, suggesting a contribution to the structural robustness of ALucs. The inhibitory effect of multivalent cations on the ALuc activities was utilized for creating dose-response curves. The intrinsic cation-driven selectivity and optical intensity of ALucs enable researchers to constitute de novo biosensors for multivalent cations.

Keywords Bioluminescence, metal cation, artificial luciferase, supersecondary structure code

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Introduction

Bioluminescence provides distinctive bioanalytical benefits of the low-background and high signal-to-noise (S/N) ratio, a wide dynamic range of the signals, and suitability in living-subject imaging.¹ Bioluminescence is generated by luciferases and “photoproteins”, such as Ca²⁺-sensitive aequorins from luminescent organisms. Many researchers have been devoted to create de novo luciferases with excellent optical properties and functionalities for facilitating their applications to bioanalysis as an optical readout.²,³

We recently established a series of artificial luciferases (ALucs) through extracting frequently occurring amino acids from the multiple sequence alignment of copepod luciferases from zooplankton samples collected at the southern deep-sea of Hokkaido, Japan (13 kinds) and other existing luciferases (2 kinds).³,⁴ Copepod luciferases generally share a high homology with each other, and are phylogenetically close to Oplophorus luciferases (OLucs) from deep-sea shrimp (Fig. S1, Supporting Information). Unique supersecondary structure codes (SSCs) of all amino acids in ALuc30 revealed a helix-loop-helix structure,⁵ which resembles a typical “EF-hand” of many Ca²⁺-binding proteins including calmodulin and aequorins (Figs. 1 and S2 (Supporting Information)). Cation-driven bioluminescence of some luciferases was previously reported: e.g., beetle luciferases require Mg²⁺ as a cofactor, which can be substituted for various divalent cations.⁶ An inhibitory feature of several multivalent cations on the OLuc was previously reported, but the mechanism is unclear.⁷ All of the background knowledge inspired us to estimate the potential cation-driven optical properties of ALucs for constructing multivalent cation sensors.

We first examined a structure-activity relationship (SAR) with site-directed mutagenesis, and found that the EF-hand-like structure is a pivotal site for the enzymatic activity of ALucs. An affinity column-purified ALuc16 exerted unique selectivity and optical stability to 20 kinds of mono- and divalent metal cations besides protons, but whose optical spectra were almost invariant due to the multivalent cations. The present study provides new insight on how the optical properties of copepod luciferases link up to multivalent cations.

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Experimental

Reagents

The standard metal cations in Fig. 2 were purchased from Wako Pure Chemicals. The counter anion is chloride. The pcDNA3.1(+) plasmids encoding ALuc16, 23, 25, 30 and 34 were from our previous study.3,6 The plasmids encoding Renilla reniformis luciferase 8.6-535 (RLuc8.6-535) and Gaussia princeps luciferase (GLuc) were custom-synthesized by Eurofins Genomics and subcloned into the pcDNA3.1(+) vector (Invitrogen). A lipofection reagent, TransIT-LT1, was purchased from Mirus. The native coelenterazine (nCTZ) was obtained from the commercial RLuc assay kit (E2820, Promega), whereas the nCTZ analog, named 6-pi-OH-CTZ, was obtained from the authors’ previous study.9 6-pi-OH-CTZ was one of the most efficient CTZ analogs in the precedent study. The Trizma base salt was purchased from Sigma-Aldrich and used for preparing a metal cation-free Tris–HCl buffer, whereas the ingredients for the universal buffer (citric acid, boric acid, KH2PO4) were from Wako Pure Chemicals. Possible metal cation contamination in our reagents was determined with inductively coupled plasma–mass spectrometry (ICP-MS) beforehand, the results of which found that the concentration of multivalent metal cations were less than 0.1 μg/mL (Table S1, Supporting Information).

EF-hand-like region of ALucs

We recently created a series of ALuc by a linkage of frequently occurring amino acids in the alignment of copepod luciferase sequences from zooplankton samples.3,6 The molecular structure of ALuc30 in Fig. 1(A) was previously predicted by a template-based modeling of the amino acid sequence.6,10 The detailed procedure of the present template-based modeling (TBM) approach was previously demonstrated.

In brief, we reviewed the known crystallographic information of existing marine luciferases available from public databases. We found that ALucs share the highest sequence homology with the coelenterazine-binding protein (CBP) derived from Renilla Muelleri (PDB id: 2hps and 2hq8) (16.7%) among structure-available luciferases in databases. Thus, we chose CBP for the molecular-structural template of ALucs. The sequence of CBP was aligned with that of ALuc30 in the view of the supersecondary structure code (SSC). In the alignment, all consensus amino acids in CBP were first substituted with ones of ALuc30. Finally, the molecular structure of ALuc30 was optimized by a molecular mechanics (MM) calculation based on a Polak–Ribiére algorithm.

We performed multiple sequence alignments of ALucs with typical Ca2+-binding proteins using ClustalW 2.1 (SFI). The alignment reveals the putative EF-like motifs, a unique, repeated SSC pattern showing a helix-loop-helix structure (Figs. 1 and S2).

Point-mutation of ALuc25 for ensuring the key amino acids

The pcDNA3.1 plasmids encoding ALuc25 mutants were generated by a site-directed mutagenesis technique, called “Quikchange”, via PCR and the appropriate primers (Fig. 1(B)).11 The mutants were named ALuc25m1 (mutation sites: E150Y, A182Y), m2 (mutation sites: E150W, A182W), m3 (mutation site: E150Y) and m4 (mutation site: E150W). As per the references, the same pcDNA3.1 plasmids encoding GLuc and RLuc8.6-535 were prepared. Aliquots of the plasmids
were transiently transfected into African green monkey kidney-derived COS-7 cells with a lipofection reagent, TransIT-LT1 (Mirus). Sixteen hours after transfection, the cells were lysed with a lysis buffer (Promega) and an aliquot of the lysates (10 μL) was transferred to an optical bottom 96-well plate. Immediately after simultaneous injection of the specific substrate (native coelenterazine; nCTZ) into the plate, the corresponding substrate solution carrying nCTZ (Promega), was diluted 100-fold with a cation-free Tris–HCl buffer (0.05 M, pH 8.2) over 80 mL. The eluted sample was dialyzed to a metal-cation-free Tris–HCl buffer (0.05 M, pH 8.2) at 4°C for 24 h, and finally adjusted to a concentration of 1 mg/mL by dilution.

The purified ALuc16 stock was further diluted 500-fold to 2 μg/mL with the metal-cation-free Tris–HCl buffer (0.05 M, pH 8.2) before experiments. Then, 45 μL of the mixture was then mixed with 5 μL of varying concentrations of a metal cation (Ca(II), Mg(II), Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Pb(II), Al(III), Fe(III), Mo(IV), or Cr(VI)) in a 96-well optical bottom plate (Nunc) for the experiment of Fig. 2(A) (Solution A). On the other hand, the corresponding substrate solution carrying nCTZ (Promega), was diluted 100-fold with the cation-free Tris–HCl buffer (Solution B); 50 μL of Solution B was simultaneously injected into Solution A in the 96-well plate with a multichannel micropipette (Gilson). The microplate was immediately moved to an image analyzer (LAS-4000, FujiFilm) equipped with a cooled charge-coupled device (CCD) camera, and the optical intensities were simultaneously estimated in a precision mode. The measurements were conducted in triplicate (n = 3). The optical images were analyzed with Multi Gauge v3.2 (FujiFilm).

The corresponding bioluminescence spectra (Fig. 2(B)) were also determined with a similar protocol to that of Fig. 2(A). In a 200 μL PCR tube, 50 μL of the mixture of ALuc16 and a metal cation (Ca(II), Fe(III), Ni(II), Zn(II), Mg(II), or Cr(VI);
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conc. = 100 μg/mL) in the metal cation-free Tris–HCl buffer (Solution A) was further mixed with 10 μL of an nCTZ solution (Solution B). The tube was immediately moved into the chamber of a precision spectrophotometer (AB-1850, ATTO) (Fig. 2(B)) and the consequent spectra were taken in an integration of 30 s.

Detailed dose-response curves of ALuc activities were determined with varying concentrations of Pb(II) or vehicle alone for a negative control (Fig. S4, Supporting Information). The experiment was conducted with the same method as that of Fig. 2. The inset shows the optical image taken by LAS-4000, FujiFilm.

The long-term stability of the optical intensities was estimated with the same protocol as that of Fig. 2(A) (Fig. 3(A)). The microplate was placed in the chamber of LAS-4000 and the optical intensities were monitored every 5 min, after substrate injection, for a duration of 60 min.

Results and Discussion

The EF-hand-like structure of ALucs is a pivotal site for ALuc activity

We performed multiple sequence alignments of ALucs with typical Ca2+-binding proteins using ClustalW 2.1 (SFI) (Fig. S2). Although ALucs had poor sequence homology with natural luciferases and Ca2+-binding proteins, the alignment of the SSC sequences reveals a unique, repeated SSC pattern showing a helix-loop-helix structure (Figs. 1(A) and S2), which appears at the corresponding sites of the known EF-hand motif of Ca2+-binding proteins like coelenterazine-binding protein (CBP).12 The structure is rare among non-Ca2+-binding proteins.

Point mutations in the amino acids (E150Y, A182Y, E150W, and A182W) in the proposed EF-hand-like structure completely destroyed the optical intensities of ALuc25 (Fig. 1(B)). These results suggested that the EF-hand-like structure is a structural core of ALuc activities.

Although we discuss results from different ALucs in this study, we expected they are the common optical features of ALucs, considering the sequential similarity between them (Figs. 1(A) and S1). For instance, the only difference between ALuc30 and 34 is the epitope sequence at amino acids 20 – 27 (underlined).

Low proton concentrations elevate the optical intensities of ALucs

The optical intensities according to the proton concentrations (pH) were determined so as to elucidate the smallest cation (H+)-driven feature of ALuc activity (Fig. S3). In the lower pH region, such as pH 4 and 5 (acidic condition), the optical intensities were suppressed to the background level. In contrast, the optical intensities of ALuc23 and ALuc34 were dramatically elevated at pH 7 up to ca. 5 fold, compared to pH 6 (Fig. S3(C)). The ALucs sustained 51 – 53% of the initial optical intensities, even after 20 min at pH 9, whereas GLuc and RLuc8.6-535 showed poor optical stability and retained less than 30% of the initial intensities after 20 min (Fig. S3(D)).

The corresponding feature of ALucs was observed even with a coelenterazine analog, 6-pi-OH-CTZ (Fig. S3(A)). The maximal optical intensities were obtained at pH 9 and the intensities were prolonged up to 41% (ALuc23) and 60% (ALuc34) of the initial intensities, even 20 min after the substrate injection (Fig. S3(A), Supporting Information).

This pH-driven elevation of ALuc activities is highly distinctive from those of other marine luciferases, such as OLuc, GLuc, Cypridina luciferase (CLuc) and Periphylla luciferase (PLuc), whose maximal optical intensities are generally found at ca. pH 7.5, quickly decrease at a pH higher than 8, and lose much of the intensity at ca. pH 9.8,13,14

Since the pKα value of the hydroxyl groups of nCTZ is ca. 7.6 in an aqueous phase,15 nCTZ is considered to be in a deprotonated, anionic form at ca. pH 9 (Fig. S3). The highest optical intensities of ALucs at pH 9 strongly suggest that ALucs provide an optical platform for accommodating anionic nCTZ.

Fig. 3 Cation-driven long-term stability of the optical intensities by ALuc16. (A) Time-course of the bioluminescence intensities with varying concentrations of Ca(II). The intensity variance was monitored every 10 min until 60 min after the substrate injection. Inset shows the relative optical stability between 100 μg/mL of Ca(II) (red) and Mg(II) (green). The optical image shows the prolonged optical intensities with varying concentrations of Ca(II). The overall intensity range was adjusted by time to highlight the relative intensity variance. The scale of the relative luminescence unit (RLU) was shown in the indicators. (B) Time-course of the absolute optical intensities with varying concentrations of Mg(II) (n = 3; standard deviation). The initial optical intensities at 0 min are elevated in a concentration-dependent manner.
and its analogs, compared to other luciferases. Because 6-pi-OH-CTZ has a double bond at position 6 (Fig. S3(B)), the anionic 6-pi-OH-CTZ is much more stable than the anionic nCTZ owing to the unique double bond, and optimally interacts with ALucs at the higher pH region. Actually, 6-pi-OH-CTZ-added ALucs emitted the maximal intensities only at pH 9, whereas nCTZ-added ALucs showed a similar optical intensity at both pH 8 and 9 (Fig. S3(A)).

**Metal cations dominate the optical intensities of ALucs**

The metal cation-driven optical intensities of ALuc16 were examined (Fig. 2). Monovalent cations, such as Li⁺, Na⁺, K⁺ and NH₄⁺ had little effect on the optical intensities of ALuc16 (Fig. S5(B)). The same conclusion as the monocation-driven feature was previously reported with OLuc.⁸ In contrast, multivalent cations were found to greatly dominate the optical intensities of ALuc16 (Fig. 2). Ca(II) and Mg(II) among divalent cations elevate the ALuc16 activities up to 1.5 times, whereas the other divergent cations, such as Mn(II), Co(II), Cu(II), Zn(II) and Pb(II), greatly suppress the ALuc16 activities in the metal cation-free Tris–HCl buffer (0.05 M, pH 8.2). The corresponding repressive effect of Cu(II) and Zn(II) on OLuc was previously reported by Inouye et al., who estimated the influence of several multivalent cations (Ca(II), Mg(II), Cu(II), Zn(II) and Cd(II)) to OLuc activities,⁸ where monovalent cations had little effect on the optical intensity of OLuc; in contrast, Cu(II), Zn(II) and Cd(II) inhibited it.

To date, the correlation between multivalent cations and luciferase activity has been poorly investigated. Few researches have dealt with this issue, and the working mechanism remains unclear. Rodionova et al. reported that Ca(II), Mn(II), and Mg(II) elevate the activities of Fridericia luciferase, which is from the Siberian luminous earthworm Fridericia heliota.²² However, the boosting effect with Mn(II) was not observed with the present ALuc16. A corresponding result with OLuc was previously reported by Inouye et al., who estimated the influence of several multivalent cations (Ca(II), Mg(II), Cu(II), Zn(II) and Cd(II)) to OLuc activities, where monovalent cations had little effect on the optical intensity of OLuc; in contrast, Cu(II), Zn(II) and Cd(II) inhibited it.

The inhibitory effect of Pb(II) was further examined over a long concentration range (Fig. S4). The dose-response curves show that the ALuc16 activities are quickly inhibited by raising concentrations of Pb(II), but not by the vehicle (Tris–HCl, pH 8.2) as a negative control. The linear range is between 1 to 100 μg/mL. The optical intensity is quickly decreased, even by as low as 1 μg/mL of Pb(II) (Fig. S4).

**Metal cations have little effect on the bioluminescence spectra**

We further examined the contribution of metal cations on the bioluminescence spectra. We examined the metal cation-driven variance of the bioluminescence spectra (Fig. 2(B)), where the effective metal cations in Fig 2 were selected, i.e., Ca(II), Fe(III), Ni(II), Zn(II), Mg(II) or Cr(VI). However, in contrast to our speculation, almost no shift of the spectra was found with the metal cations; e.g., Ca(II) and Ni(II) induced only 2 nm of
blue shift and 4 nm of red shifts of the spectrum. The negligible effect of metal cations on the spectra suggests that the multivalent cations do not modulate the electric states of the intermediates of nCTZ in the chemical reaction of the light emission of ALucs.

**Ca(II) contributes to the long-term stability of ALuc activities**

The long-term stability of luciferases is a key determinant for bioassays. Because of the boosting effect on ALuc activities, we chose Ca(II), Mg(II) and Cr(VI) for the cation-driven long-term stability (Fig. 3). A contrasting effect was found in a comparison of the effects of Ca(II) and Mg(II). Although both Ca(II) and Mg(II) boosted the optical intensities, only Ca(II) prolonged the bioluminescence intensities in a concentration-dependent manner (Fig. 3(A), Inset). Ca(II) sustained 60% of the initial optical intensity for 13 min after nCTZ injection, and retained 4% of the optical intensity even after 60 min (Fig. 3(A), optical image). In contrast, the Mg(II) concentration-dependently elevates the ALuc activities, but seldom influences the long-term stability (Figs. 3(A) inset and 4(B)).

The long-term stability suggests that Ca(II) modifies the structural integrity of ALuc16. We thus speculate that Ca(II) binds the EF-hand-like structure of ALuc16 and supports the prolonged optical intensities of ALucs.

**Circular dichroism (CD) measurements of metal cation-mixed ALuc16**

A circular dichroism (CD) measurement was further conducted for reasoning the cation-driven degradation of ALuc16 activities (Fig. 4). The samples were prepared by mixing varying concentrations of Pb(II) or Al(III) with the column-purified and dialyzed ALuc16, and were immediately measured with a CD spectrometer (JASCO, Japan) after preparation.

The variation of the molar ellipticity in the CD spectra at 222 nm reveals that the α-helical portion is gradually decreased by elevating the Al(III) concentrations. The same feature was observed with Pb(II). Pb(II) levels higher than 1 μg/mL showed basal noise spectra. The overall results strongly suggest that metal cations, such as Pb(II) and Al(III), destroy the tertiary structure of ALuc16, leading to decomposition.

Taken together, we assigned four EF-hand-like structures in ALuc sequences, resembling the EF-hands of Ca²⁺-binding proteins. The structure is found to be a key site for the enzymatic activities in a mutagenesis study. Hydrogen ions (pH) and divalent cations dramatically vary the optical intensities of ALucs. The overall optical intensity ranking is listed as follows in descending order: Cr(VI), Mg(II), Ca(II) > Ni(II) > Mo(IV), Cd(II), Fe(III), Zn(II) > Mn(II) and the others (Co(II), Cu(II) and Pb(II)). Although both Ca(II) and Mg(II) boost the ALuc activities, Ca(II) also increases the duration of upregulation, perhaps by stabilizing the structural integrity of the proteins. We further demonstrated that the unique inhibitory effect of metal cations, such as Pb(II), on the ALuc activities can be utilized for creating a novel cation sensor. Little variance of the optical spectra by multivalent cations indicates that they do not modulate the electric states of the intermediates of nCTZ in the chemical reaction of the light emission of ALucs.

The evidence above suggests that multivalent cations may bind the EF-hand-like structure of ALucs and modulate the metal cation-driven optical intensity and stability of ALucs. The unique cation selectivity of ALucs invites researchers to future applications for multivalent cation sensors.

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**References**