Mutant Firefly Luciferase Enzymes Resistant to the Inhibition by Sodium Chloride

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Abstract

Objectives

Firefly luciferase, one of the most extensively studied enzymes, has numerous applications. However, luciferase activity is inhibited by sodium chloride. This study aims to expand the applications of firefly luciferase in the presence of sodium chloride.

Results

We first obtained two mutant luciferase enzymes whose inhibition were alleviated and identified these mutations as Val288Ile and Glu488Val. Under dialysis condition (140 mM sodium chloride), the wild type was inhibited to 44% of its original activity level. In contrast, the single mutants, Val288Ile and Glu488Val, retained 67% and 79% of their original activity, respectively. Next, we introduced Val288Ile and Glu488Val mutations into the wild-type luciferase to create a double mutant using site-directed mutagenesis. Notably, the double mutant retained its activity more than 95% of that in the absence of sodium chloride.

Conclusions

The mutant luciferase, named luciferase CR, was found to retain its activity in various concentrations of sodium chloride. The inhibition of luciferase CR under dialysis condition was more alleviated than either Val288Ile or Glu488Val alone, suggesting that the effect of the double mutation was cumulative. We discussed the effect of mutations on the alleviation of the inhibition by sodium chloride.

Introduction

Firefly luciferase, which generates bioluminescence during the oxidative decarboxylation of D-luciferin in the presence of ATP, has been used for numerous applications such as measuring biomass and cellular conditions (Lundin 2000) and protein-protein interaction (Christopoulos and Chiu 1995; Ohmuro-Matsuyama and Ueda 2016), assaying ATP-related enzymes (Clarke 2005; Lundin 2000), examining D-luciferin-generating enzymes using peptide-modified luciferin or luciferin derivatives (Cali et al. 2006; Liu et al. 2005; Noda et al. 2010; O'Brien et al. 2008), and performing real-time ATP imaging (Grygorczyk et al. 2019). The firefly luciferase gene, *luc*, has been used as a reporter gene (Wood and Gruber 1996) as well as for bioimaging (Kaskova et al. 2016; Yan et al. 2019). In the food industry, the bioluminescence-based ATP assay has received considerable attention, mainly because as a rapid monitoring system, it can ensure the safety of food products and contact surfaces at critical control points during food processing (Champiat et al. 2001; Darchuk et al. 2015; Davidson et al. 1999; Siragusa et al. 1995).

Although the bioluminescence-based methods have numerous applications, their sensitivity is reduced by the presence of various salts. For example, sodium chloride, a commonly used reagent, is known to inhibit luciferase activity (Aledort et al. 1966). The considerable loss in the sensitivity of the bioluminescence-based method limits the applications in the presence of salts.

Mutations in the *luc* gene have been known to produce mutant luciferase enzymes with properties significantly different from those of the wild-type gene. For example, some mutant luciferase enzymes have different bioluminescence spectra (Branchini et al. 2001; Branchini et al. 2005; Branchini et al. 2003; Kajiyama and Nakano 1991; Ugarova et al. 2005), increased enzyme stability (Baggett et al. 2004; Hattori et al. 2002; Kajiyama and Nakano 1993; Koksharov and Ugarova 2012; Law et al. 2006; Pozzo et al. 2018), or different catalytic activity (Aswendt et al. 2019; Fujii et al. 2007; Hirokawa et al. 2002). Previously, we developed the mutant luciferase FM (Ile423Leu, Asp436Gly, Leu530Arg) (Fujii et al. 2007), later named LGR by another group (Pozzo et al. 2018), whose higher catalytic activity resulted in a luminescence intensity 10-fold higher than that of the wild type.

However, mutant luciferase that alleviates inhibition by salt has not been reported. From a random mutant library, this study identified two novel *Photinus pyralis* mutant luciferase enzymes that were found to retain their activities under dialysis condition. Site-directed mutagenesis of the *luc* gene was conducted in these mutations to investigate the effect of substitution with other amino acids. Eventually, by combining the optimized mutations in the *luc* gene, we generated a mutant luciferase enzyme that retains its activity in the presence of various concentrations of sodium chloride.

Methods

Measurement of luciferase activity

Luminescence intensity, expressed as the generated light count per second, was measured in 96-well plates (Thermo Fisher, Massachusetts, USA) using a Microplate reader SH-9000 (Corona Electric, Ibaraki, Japan). The reaction was initiated by the addition of 50 µl of 1 µM ATP and 0.1 µM D-luciferin in Tris–HCl buffer (50 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$) to 50 µl of 0.1 mg/ml luciferase. Luminescence was measured for 5 s after injection at 25 ℃. Luciferase protein concentrations were determined by measuring the absorbance at 280 nm.

The measurement of the inhibition of luciferase activity in the presence of sodium chloride and other salts

Luciferase reactions were performed in the absence or presence of various concentrations of sodium chloride, potassium chloride, calcium chloride, ammonium chloride, and sodium acetate. To test whether the inhibition is reversible or irreversible, luciferase was dissolved in the Tris–HCl buffer in 140 mM sodium chloride. After 120 min, luciferase was diluted 10-fold with the Tris–HCl buffer, mixed with 50 µl of 10 µM ATP and 1 µM D-luciferin in the Tris–HCl buffer to initiate the reaction, and measured. Each experiment was performed with three replicates.

Random mutagenesis and screening

The PCR Random Mutagenesis Kit (Takara, Shiga, Japan) was used to generate a random mutant library of the *Photinus pyralis luc* genes. Random mutations were introduced at the rate of one mutation per

gene. PCR was conducted using the primers 5'-GACTCCATGGAAGACGCCAAAAAC-3' and 5'-GACACTCGAGCAATTTGGACTTTCCGCC-3' to generate mutant insert containing restriction sites *Nco*I and *Xho*I. All mutant genes were cloned into the pET-28a vector (Merck, Darmstadt, Germany) to generate recombinant luciferases containing a C-terminal His-tag.

The vectors containing the mutant *luc* genes were introduced into *Escherichia coli* HMS174 (DE3) (Merck), spread onto Luria–Bertani agar plates containing kanamycin, and incubated at 37 ℃. The colonies were inoculated into a 2YT liquid medium containing kanamycin and induced with 0.1 mM isopropyl-ß-D-thiogalactoside (IPTG) in deep-well plates (Watson, Tokyo, Japan) to produce the luciferase protein.

Crude extracts containing mutant luciferase proteins were prepared by freezing and thawing the recombinant bacteria. Each extract was divided into two portions to measure the luminescence intensities in the absence or presence of 140 mM sodium chloride in a reaction mixture containing 50 µl each of 1 µM ATP and 0.1 µM D-luciferin in the Tris–HCl buffer.

Site-directed mutagenesis

The mutagenesis of the wild-type *luc* was conducted with primers, designed using the manufacturer's specification, and the KOD-plus mutagenesis kit (Toyobo, Osaka, Japan) to generate 19 substitutions of Val288 and Glu488.

Expression and purification of luciferase

The recombinant *luc* was expressed in *E. coli* HMS174 (DE3). The cells were grown at 37 ℃ in 2YT medium containing 1 µg/ml kanamycin. IPTG was added to the medium for incubation at 25 ℃ for 65 h. The induced cells were harvested by centrifugation and stored at − 20 ℃. The cell pellets were resuspended in a bacterial cell lysis reagent (B-PER, Thermo Fisher, Massachusetts, USA). After incubation at 25 ℃ for 15 min, the whole-cell extracts were isolated by centrifugation at 16,000 *g* for 10 min at 4 ℃. The His-tagged recombinant luciferase was purified using the Ni Sepharose 6 Fast Flow column (GE Healthcare, Illinois, USA) according to the manufacturer's instructions. Whole-cell extracts were applied to the columns and washed with a binding buffer of 20 mM NaH_2PO_4, pH 7.4, 500 mM sodium chloride, and 20 mM imidazole. The recombinant luciferase was eluted with an elution buffer (20 mM NaH_2PO_4, pH 7.4, 500 mM sodium chloride, and 500 mM imidazole). The elution buffer was exchanged for the Tris–HCl buffer using a PD-10 Desalting Column (GE Healthcare).

Bioluminescence emission spectra

The bioluminescence emission spectra of the luciferase were measured using a Microplate reader SH-9000. The data were collected between 450 and 700 nm. The reactions were initiated by adding 1.8 ml of substrate including 100 µM ATP and 10 µM D-luciferin into 200 µl of 0.1 mg/ml luciferase in the Tris–HCl buffer.

Results

Inhibition of luciferase activity by sodium chloride

Luciferase is known to be inhibited by sodium chloride (Aledort et al. 1966). Indeed, in the presence of 200 mM sodium chloride, *P. pyralis* luciferase activity was decreased to 28% of the control (Fig. 1). Under dialysis condition (140 mM sodium chloride), luciferase activity was reduced to 44% of the control. Since luciferase was inhibited by potassium chloride, calcium chloride, and ammonium chloride, but not sodium acetate (Fig. 1), the inhibition was likely due to the chloride ion.

Next, we tested whether the inhibition of luciferase activity by sodium chloride was reversible. The luciferase was incubated in the 140 mM sodium chloride for 120 min and then diluted 10-fold; its final luciferase activity was compared with that of the luciferase directly diluted in 14 mM sodium chloride. Since there was no significant difference (within 1% difference in triplicated experiments) between the condition with or without sodium chloride, the inhibition of luciferase activity by sodium chloride was found to be reversible.

Isolation of mutant luciferase enzymes retaining their activities under dialysis condition

Mutations were randomly introduced into the *P. pyralis luc* gene and then mutant luciferase enzymes that retained more than 60% of their activities under dialysis condition were selected. Hence, two mutant luciferase enzymes were obtained from approximately twenty thousand transformants. DNA sequencing revealed that the mutations were localized at Val288, changing to Ile (Val288Ile) and at Glu488, changing to Val (Glu488Val), respectively. Luciferase activities of Val288Ile and Glu488Val mutants under dialysis condition were 67% and 79% of those in the absence of sodium chloride, respectively.

Site-directed mutagenesis was conducted to investigate the effect of substitution with other amino acids at positions 288 and 488 of luciferase (Tables 1 and 2). The substitution of Val288 with nonpolar amino acids, especially Ile, Leu, Met, and Phe, alleviated the inhibition under dialysis condition (Table 1). In contrast, the replacement with polar, positively charged, or negatively charged amino acids, except cysteine, failed to lessen the inhibition under dialysis condition (Table 1).

Table 1
The effect of Vla288 substitutions on luciferase activity in the presence of 140 mM sodium chloride

Luciferase	Substituted amino acid	Molecular weight (g/mol)	Polarity and charge	Relative luminescence intensity (%) [a]
Wild type	Val	117.15	Nonpolar	44.0 ± 0.6
Mutant	Gly	75.07	Nonpolar	16.3 ± 0.1
	Ala	89.09	Nonpolar	21.9 ± 1.3
	Pro	115.13	Nonpolar	19.6 ± 0.4
	Ile	131.17	Nonpolar	67.2 ± 2.9
	Leu	131.17	Nonpolar	46.8 ± 0.6
	Met	149.21	Nonpolar	47.3 ± 0.7
	Phe	165.19	Nonpolar	46.2 ± 0.8
	Trp	204.23	Nonpolar	28.9 ± 0.9
	Ser	105.09	Polar	16.3 ± 0.1
	Thr	119.12	Polar	25.6 ± 0.5
	Cys	121.16	Polar	46.5 ± 1.3
	Asn	132.12	Polar	29.3 ± 0.5
	Gln	146.15	Polar	32.8 ± 0.3
	Tyr	181.19	Polar	38.3 ± 0.9
	Lys	146.19	Positive	23.8 ± 1.8
	His	155.15	Positive	31.1 ± 1.4
	Arg	174.20	Positive	40.8 ± 0.8
	Asp	133.10	Negative	16.1 ± 0.4
	Glu	147.13	Negative	25.1 ± 1.0

[a] The relative luminescence intensity (%) was calculated by dividing the intensity in the presence of 140 mM sodium chloride by that in the absence of sodium chloride. The values are represented as means ± SD (n = 3).

Table 2
The effect of Glu488 substitutions on luciferase activity in the presence of 140 mM sodium chloride

Luciferase	Substituted amino acid	Molecular weight (g/mol)	Polarity and charge	Relative luminescence intensity (%) [a]
wild type	Glu	147.13	Negative	44.0 ± 0.6
Mutant	Gly	75.07	Nonpolar	50.2 ± 0.2
	Ala	89.09	Nonpolar	43.4 ± 1.1
	Pro	115.13	Nonpolar	54.5 ± 1.2
	Val	117.15	Nonpolar	78.7 ± 1.3
	Ile	131.17	Nonpolar	58.0 ± 0.5
	Leu	131.17	Nonpolar	62.0 ± 0.5
	Met	149.21	Nonpolar	73.1 ± 0.7
	Phe	165.19	Nonpolar	56.6 ± 1.1
	Trp	204.23	Nonpolar	40.0 ± 1.2
	Ser	105.09	Polar	50.9 ± 1.0
	Thr	119.12	Polar	66.8 ± 1.7
	Cys	121.16	Polar	69.5 ± 1.1
	Asn	132.12	Polar	30.9 ± 1.1
	Gln	146.15	Polar	34.8 ± 1.4
	Tyr	181.19	Polar	40.2 ± 0.7
	Lys	146.19	Positive	22.9 ± 1.3
	His	155.15	Positive	32.8 ± 0.5
	Arg	174.20	Positive	43.1 ± 1.0
	Asp	133.10	Negative	68.1 ± 1.2

[a] Relative luminescence intensity (%) was calculated by dividing the intensity in the presence of 140 mM sodium chloride by that in the absence of sodium chloride. The values are represented as means ± SD (n = 3).

Alternatively, the substitution of Glu488 with nonpolar amino acids, except Ala and Trp, mostly improved the inhibition (Table 2). Also, the replacement of Glu488 with Ser, Thr, Cys, and Asp also alleviated the

inhibition. By contrast, the substitution with positively charged amino acids failed to ease the inhibition (Table 2). Finally, the best substitution at Val288 and Glu488 was found to be Ile and Val, respectively.

The characterization of a Val288Ile and Glu488Val double mutant luciferase enzyme

A *luc* gene encoding the Val288Ile and Glu488Val double mutant luciferase, tentatively named CR for chloride ion resistance, was constructed with site-directed mutagenesis. Bioluminescence activities of the wild-type, single mutants Val288Ile and Glu488Val, the double mutant CR, and the FM mutant that we previously developed, were tested under dialysis condition and in the absence of sodium chloride (Fig. 2).

The luminescence intensity of the CR double mutant luciferase under dialysis condition retained more than 95% of its activity compared to that in the absence of sodium chloride. The inhibition of CR under dialysis condition was more alleviated than either Val288Ile or Glu488Val alone, suggesting that the effect of the double mutation was cumulative. Unexpectedly, the luminescence intensity of the double mutant luciferase was found to be 460% higher than that of the wild type (Fig. 2). Meanwhile, the luminescent intensities of Val288Ile and Glu488Val were 310% and 280% higher than that of the wild type, respectively, indicating that these mutations not only alleviated the inhibition by sodium chloride but also increased the luciferase activity. Alternatively, the luminescence intensity of the FM mutant (Fujii et al. 2007) increased 500% higher than that of the wild type in the absence of sodium chloride but failed to alleviate the inhibition by sodium chloride (Fig. 2), indicating that mutations increasing the luminescence intensity do not always lessen the inhibition by sodium chloride.

Next, we evaluated the inhibition of wild type, the double mutant CR, and the FM mutant by various concentrations of sodium chloride (Fig. 3). Compared to the wild type, the double mutant CR retained its activity in various concentrations of sodium chloride. In contrast, the FM mutant was more susceptible to sodium chloride inhibition than the wild type. We cocluded the double mutant CR as a new type of mutant luciferase resistant to the inibition by sodium chloride.

Next, we evaluated the wavelength of bioluminescence emission by the wild type and CR. The patterns of bioluminescence wavelength were not significantly different between the enzymes (Fig. 4). The maximum wavelength emitted by luciferase CR was 556 nm.

ATP assay using luciferase CR

We previously developed the higher catalytic mutant luciferase FM (Fujii et al. 2007) and applied this enzyme for efficient assays for ATP as well as D-luciferin-generating enzymes using peptide-modified luciferin (Noda et al. 2010; Noda et al. 2008; Urata et al. 2009). Here we found that luciferase CR showed higher luminescence than FM within various ATP concentrations in the presence of sodium chloride (Fig. 5). Therefore, the mutant luciferase CR would expand the applications of luciferase in the presence of sodium chloride.

Discussion

In this report, two mutant luciferase enzymes that retained more than 60% of their original activity under dialysis condition, were obtained from approximately 20,000 candidates; the mutations were located at Val288 and Glu488. The double mutant luciferase CR (Val288Ile and Glu488Val) retained more than 95% of its original activity under dialysis condition. Our study is the first report of mutant luciferases that lessen the inhibition by salt. Luciferase CR would be useful for the application of firefly luciferase in the presence of sodium chloride.

Electrostatic interaction is a significant force in mediating intramolecular and intermolecular interactions for determining the structure, dynamics, and function of biomolecules (Neves-Petersen and Petersen 2003). In an aqueous solution, ions and small molecules of opposite charges accumulate around a highly charged biomolecule, resulting in electrical neutralization of the biomolecule, or the shielding effect. In the screening or shielding effect, salt molecules shield the long-range electrostatic repulsive forces between the intramolecular charges, decrease the repulsive interactions, and enhance the hydrophobic effects, thus increasing enzyme stability (Curtis et al. 1998; Valente et al. 2005). On the other hand, certain types of salt ions could also shield enzyme-substrate electrostatic interactions, thus inhibiting enzyme activities. The salt inhibition of luciferase might indicate that the positive effect of salt ions could not overcome the negative effect of chloride ions.

The firefly luciferase enzyme, consisting of a large ~ 440 amino acid N-terminal domain and a small ~ 110 amino acid C-terminal domain connected by a short hinge (Fig. 6), catalyzes a sequence of reactions, i.e., adenylation and oxidation, that convert luciferin into an electronically excited state oxyluciferin that emits light (Conti et al. 1996). The interface between the two domains contains the active site, with the N-terminal domain contributing most of the residues responsible for substrate binding, while the C-terminal domain introduces Lys529 and Lys443 that participate in adenylation and oxidation reactions, respectively (Sundlov et al. 2012). The Val288 residue is localized within 5 Å apart from Thr527 that interacts with the active site Lys529 (Fig. 6). Therefore, the mutation of Val288 to Ile might have changed the localization of Thr527 and strengthened the enzyme-substrate electrostatic interactions, resulting in the alleviation of the inhibition.

On the contrary, the Glu488 residue is localized in the flexible region away from the active sites in the N-terminal domain (Fig. 6). It was recently reported that the substitution of histidine in the same flexible region by aspartate (His461Asp) decreased ATP binding affinity and shifted its optimum temperature of activity (Rahban et al. 2017). It was also reported that the substitution of His489 by aspartate (His489Asp) increased protein rigidity but only slightly improved its thermal stability (Rahban et al. 2017). Therefore, the finding that Glu488 in the flexible region contributes in alleviating the inhibition by chloride ion is novel.

Declarations

Acknowledgments

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Compliance with Ethical Standards

Funding

This study was funded by DKK-TOA Corporation.

Conflict of interest

S.Y., K.N. and A.S. are employees of DKK-TOA Corporation. A.K. has no conflicts of interest.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

S.Y. and K.N. developed the original concept of this study. S.Y., K.N. and A.S. performed the experiments. A.K. discussed the experimental data and supervised writing of the manuscript. All authors and contributed to manuscript writing and approve of its contents.

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Figures

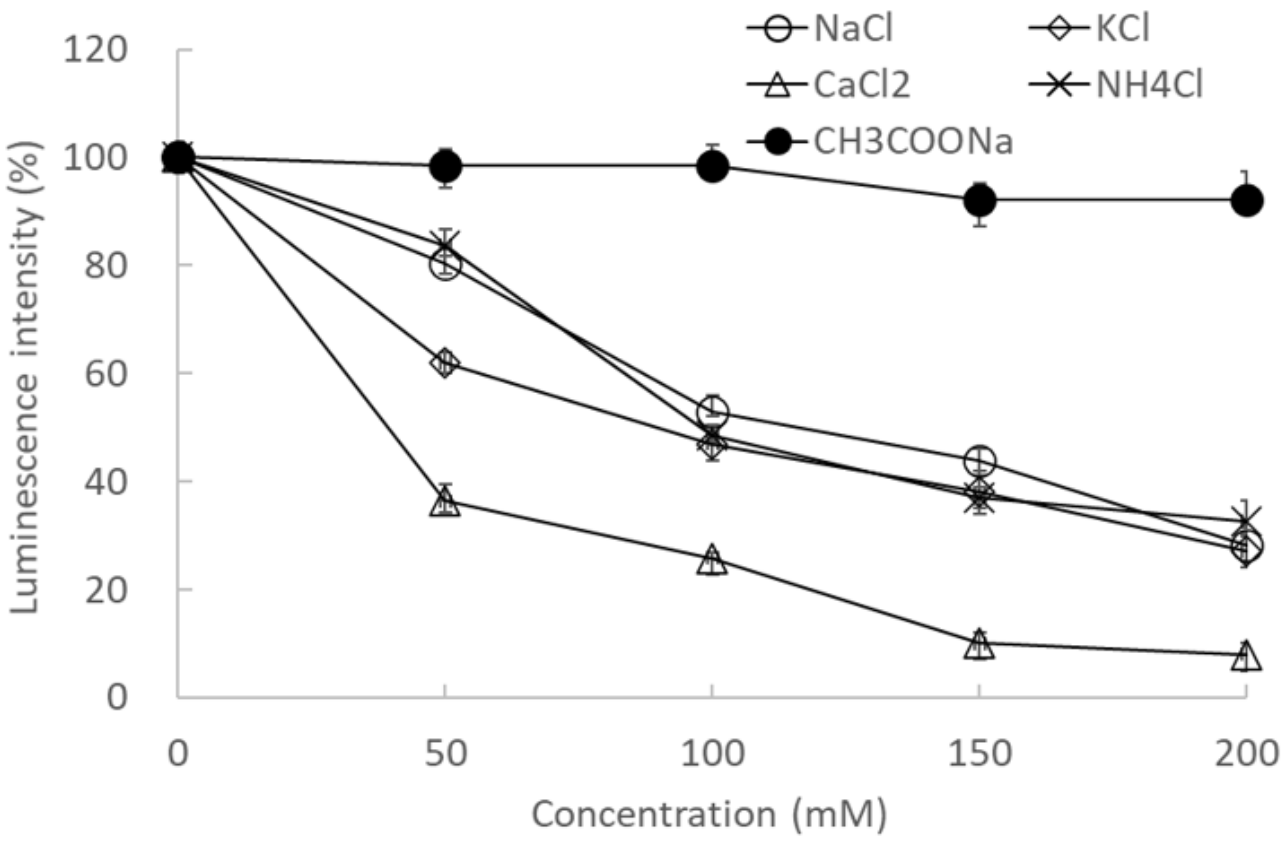

Figure 1

The effects of various concentrations of sodium chloride, potassium chloride, calcium chloride, ammonium chloride, and sodium acetate on the activity of the wild-type luciferase The values are represented as means ± SD (n = 3).

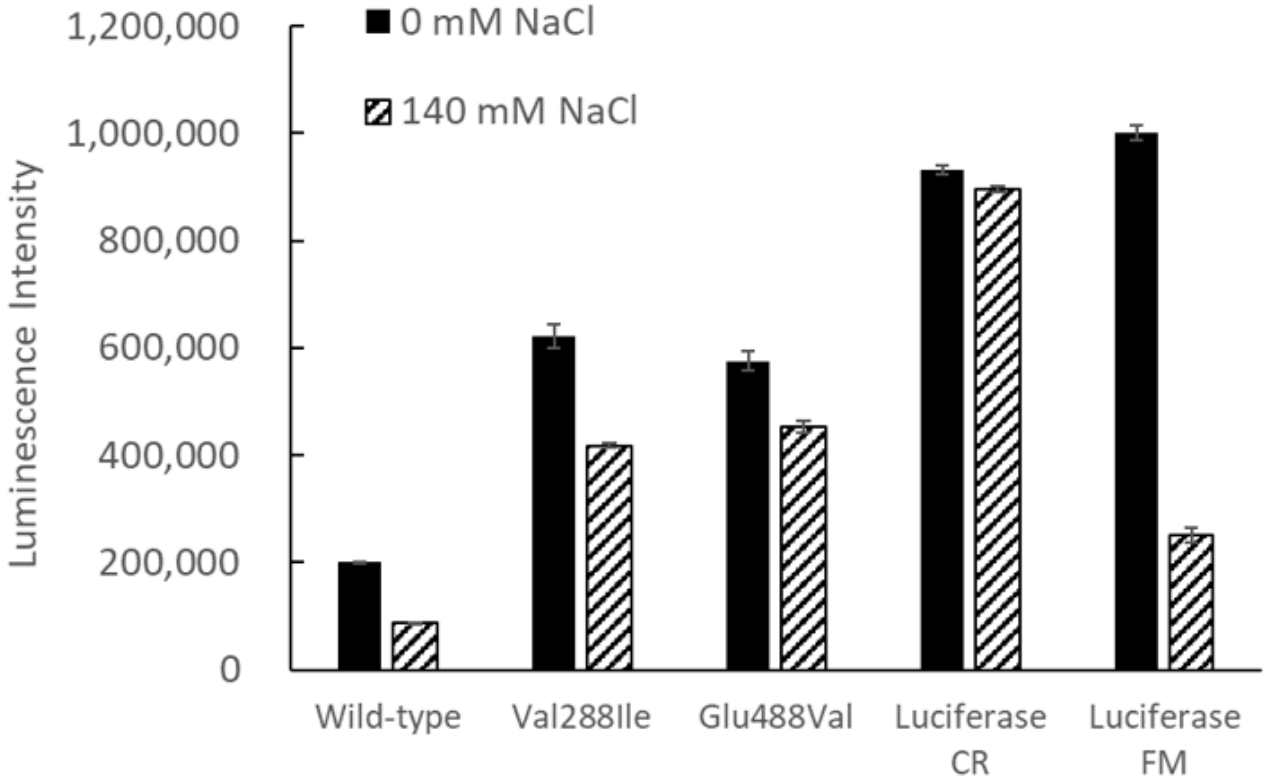

Figure 2

The inhibition of the activity of the wild-type and mutant luciferases by sodium chloride The luminescence intensity of the luciferase reactions in the absence or presence of 140 mM sodium chloride was measured. The values are represented as means ± SD (n = 3).

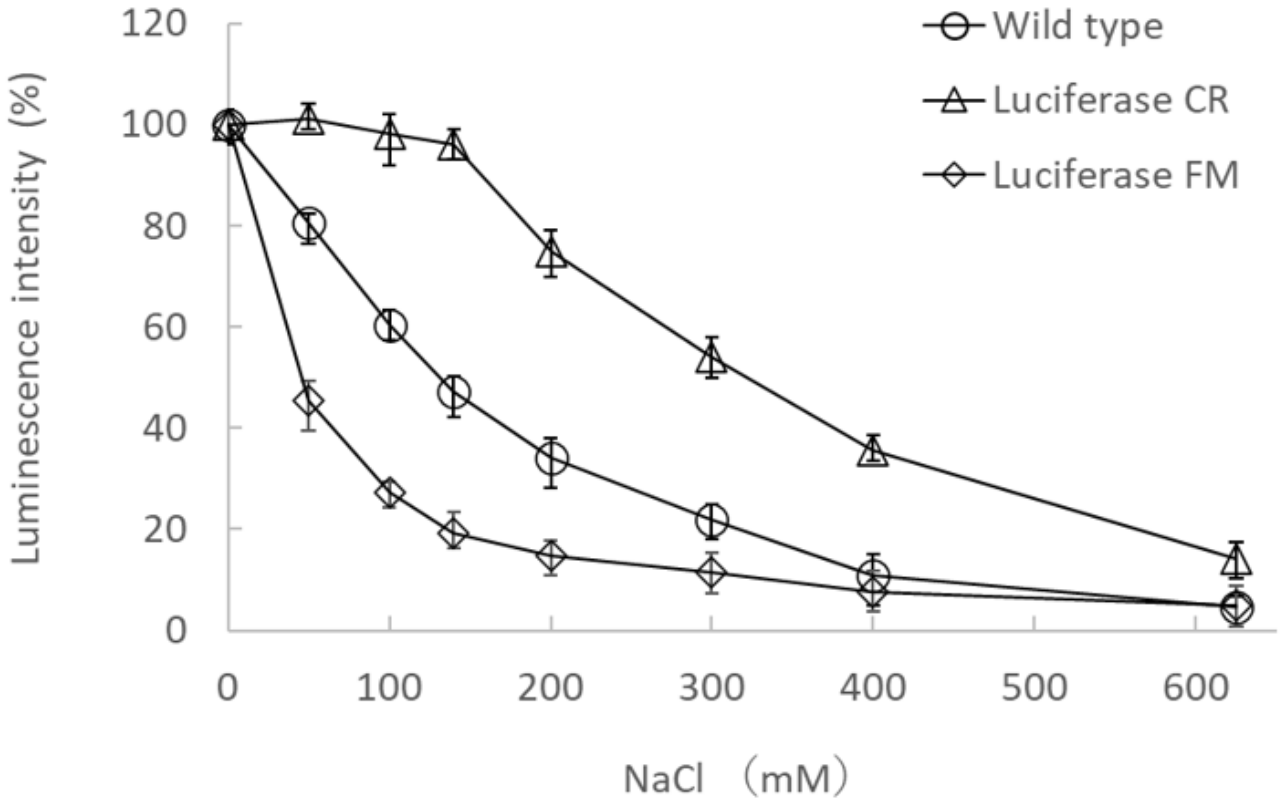

Figure 3

The effect of sodium chloride concentration on the activity of the wild-type, luciferase CR, and FM The luminescence intensity of the luciferase reactions in various concentrations of sodium chloride was measured. The values are represented as means ± SD (n = 3).

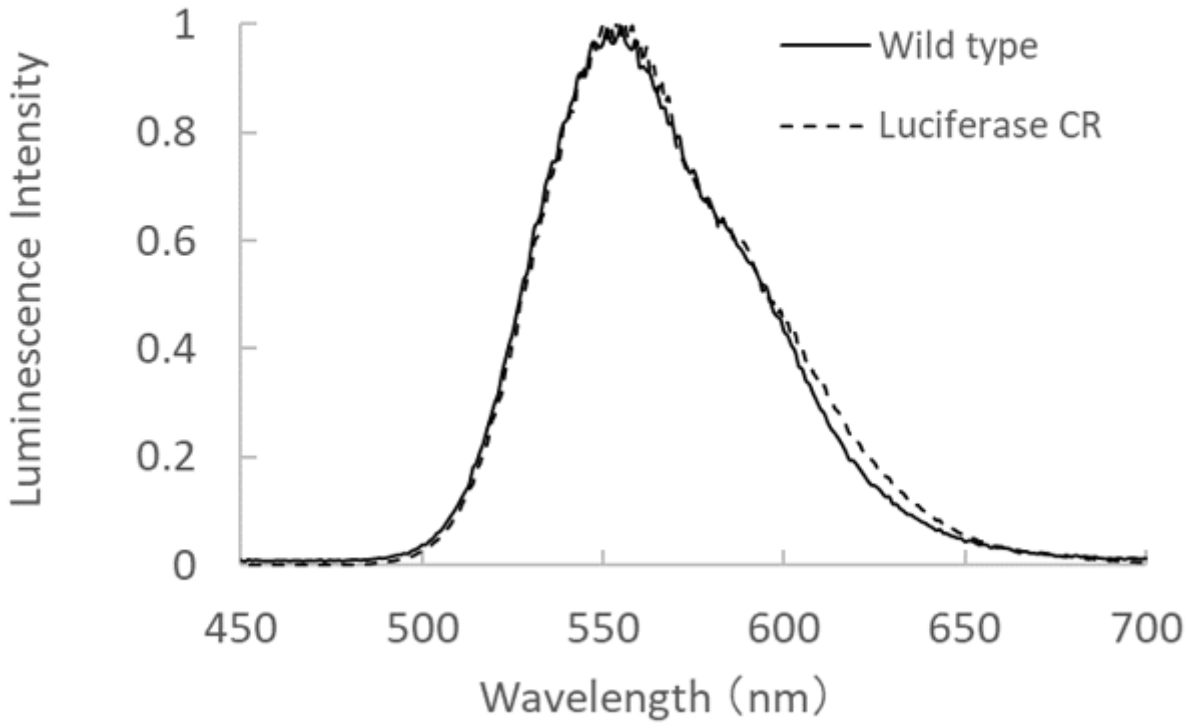

Figure 4

The bioluminescence emission spectra of the wild-type and luciferase CR

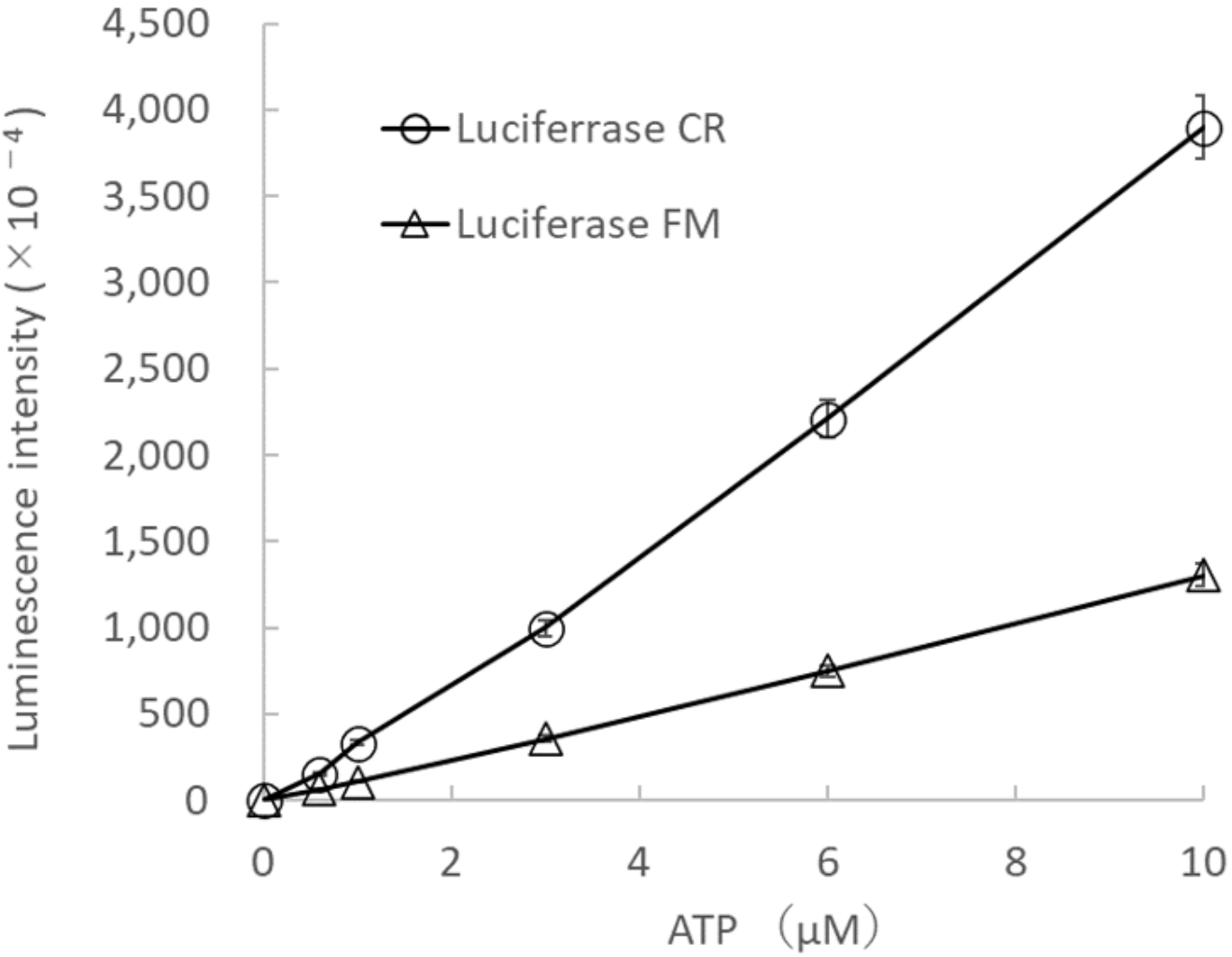

Figure 5

The luminescence intensity of the luciferase reactions in various concentrations of ATP The luminescence intensity of the luciferase reactions in various concentrations of ATP was measured. The values are represented as means ± SD (n = 3).

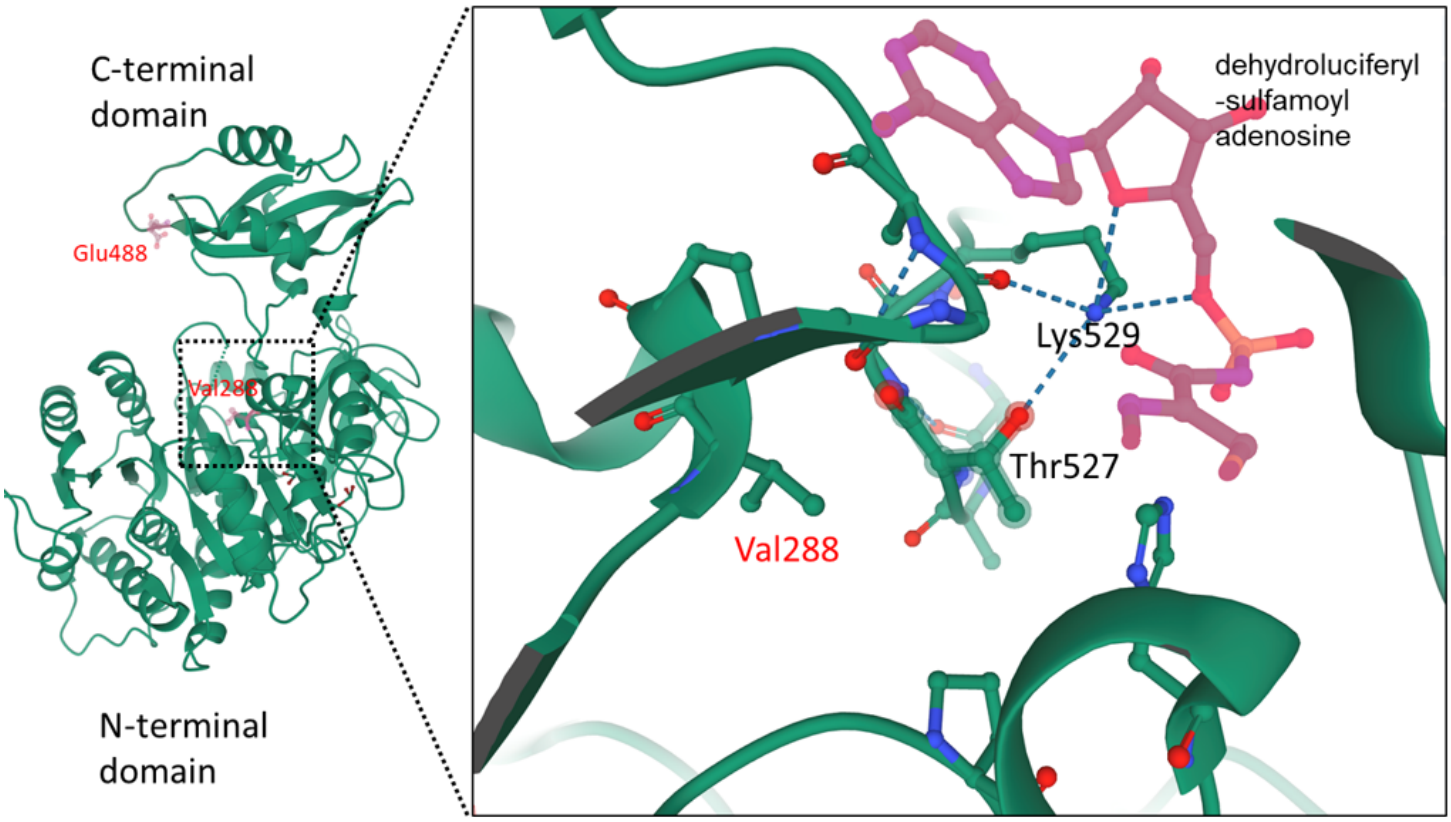

Figure 6

The three-dimensional structure of Photinus pyralis luciferase and the locations of mutations in the luciferase CR The active sites with a substrate analog (dehydroluciferyl-sulfamoyl adenosine) are indicated in the right box. The amino acid substitutions (red) in the luciferase CR were indicated. This figure was created based on the crystal structure (Protein Data Bank ID code 4G36) of Photinus pyralis luciferase.