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Sequence analysis

Structural distinctions of fast and slow bacterial luciferases revealed by phylogenetic analysis

Anna A. Deeva[1,*], Evgenia A. Temlyakova[2], Anatoly A. Sorokin[2,3], Elena V. Nemtseva[1,4] and Valentina A. Kratasyuk[1,4]

[1]Laboratory of Bioluminescent Biotechnologies, Siberian Federal University, Krasnoyarsk, Russian Federation, [2]Mechanism of Cell Genome Functioning Laboratory, Institute of Cell Biophysics RAS, Moscow Region, Pushchino, Russian Federation, [3]Moscow Institute of Physics and Technology, Dolgoprudny, Russian Federation, and [4]Laboratory of Photobiology, Institute of Biophysics SB RAS, Krasnoyarsk, Russian Federation

*To whom correspondence should be addressed.

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Abstract

Motivation: Bacterial luciferases are heterodimeric enzymes that catalyze a chemical reaction, so called bioluminescence, which causes light emission in bacteria. Bioluminescence is vastly used as a reporter system in research tools and commercial developments. However, the details of the mechanisms that stabilize and transform the reaction intermediates as well as differences in the enzymatic kinetics amongst different bacterial luciferases remain to be elucidated.

Results: Amino acid sequences alignments for 21 bacterial luciferases (both α- and β-subunits) were analyzed. For α-subunit, containing the enzyme active center, 48 polymorphic amino acid positions were identified. According to them, the sequences fell into two distinct groups known as slow and fast based on the decay rate of the bioluminescence reaction. The differences in the enzyme active site induced by structural polymorphism are analyzed.

Availability and implementation: Three-dimensional models of Photobacterium leiognathi luciferase and Vibrio harveyi luciferase (with reconstructed mobile loop) are freely available at PMDB database: PM0080525 and PM0080526, respectively.

Contact: adeeva@sfu-kras.ru

Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction

Luminous bacteria are known as the most abundant and ubiquitous bioluminescent organisms. Their traditional classification was rooted in the morphological peculiarities, metabolism and ecological distribution of light emitting species. Currently, according to the sequence analysis of 16S rRNA and *gyrB* genes, all known luminous bacteria are assigned to three *Gammaproteobacteria* families: *Vibrionaceae*, *Shewanellaceae* and *Enterobacteriaceae* (Dunlap and Urbanczyk, 2013). In spite of several studies, many details about the mechanism of bacterial bioluminescence including the differences in reaction kinetics are yet to be known.

Although the investigations has identified the sequence of about 21 bacterial luciferases, but could only solve the crystal structure of luciferase from *Vibrio harveyi* (Campbell *et al.*, 2009). Bacterial luciferase consists of α- and β-subunit folded into the same type of $(\beta/\alpha)_8$ or TIM barrel structure and encoded by *luxA* and *luxB* genes. In addition to the structure similarities, α- and β-subunits share about 32% of sequence identity, indicating that *luxB* gene might have evolved as a result of *luxA* gene duplication (Baldwin *et al.*, 1979). However, α-subunit is larger due to additional 29 amino acid residues, which constitutes a mobile loop flanking the active site of the enzyme and playing an important role in binding to the substrates (Campbell and Baldwin, 2009).

All known bacterial luciferases catalyze the same oxidative reaction (Fig. 1), where R-CHO and R-COOH are a long-chain aliphatic aldehyde and corresponding carboxylic acid, $FMNH_2$ and FMN—

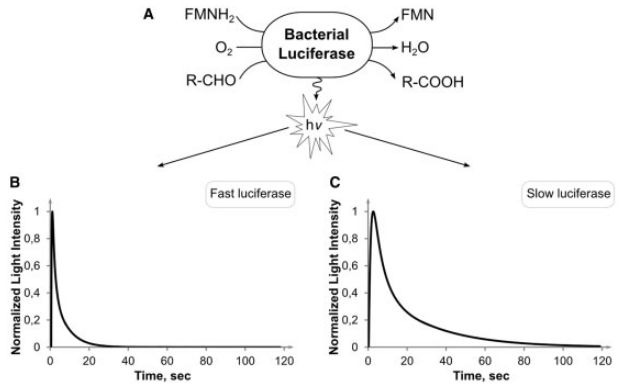

Fig. 1. The scheme of chemical reaction catalyzed by bacterial luciferase (**A**) and typical light emission kinetics for fast (**B**) and slow (**C**) decay luciferases

reduced and oxidized flavin mononucleotide respectively. Interaction of luciferase with the substrates causes the electronically excited species formation and following light emission. The emitted light then slowly decays in a first-order kinetics manner (Fig. 1B and C). The maximum light intensity of the reaction is proportional to the amount of the enzyme, and the decay depends on the rate constant of the enzyme and the aldehyde chain length. Light intensity and decay kinetics vary among luciferases from different species. However, two types of luciferases slow and fast, can be distinguished by the decay patterns (enzyme turnover time) (Fig. 1) (Nealson and Hastings, 1979). Luciferases from *Photobacterium* species are fast decay enzymes (the rate is within 0.64–1.0 s^{-1}), while the luciferase from *Vibrio* and *Photorhabdus* are slow (0.12 s^{-1}) (Nealson and Hastings, 1979; Tinikul and Chaiyen, 2014). The reasons for the kinetic differences are associated with binding to the substrates (Tinikul and Chaiyen, 2014), duration of time that it takes to form the intermediates in the reaction (Abu-Soud *et al.*, 1993), and the decay rate of the hydroperoxy flavin intermediate upon stabilization by the binding pocket environment (Lin *et al.*, 2002). So far only a few luciferases from limited range of bacterial species are studied. However, analysis of new luminous bacterial genomes can assist to better understand the bacterial bioluminescence.

The current research aimed to identify the conservative motifs that are involved in the active site formation of bacterial luciferases. We analyzed the phylogenetic relationship of luciferase amino acid sequences from 21 luminous bacteria. Our data showed two clades of the luciferases that relate bacteria, which share similar reaction kinetics. We also identified the residues that are important to form the bacterial groups and discussed the structural differences between the enzymatic active sites as well as the mechanisms to stabilize the flavin intermediates between two groups.

2 Materials and methods

We studied amino acid sequences of luciferases from 21 luminous bacteria: *A.fischeri* ES114, *A.logei* KCh1 and *A.salmonicida* NCMB 2262 from *Aliivibrio* genus; *Candidatus Photodesmus katoptron* Akat1; *P.aquimaris* NBRC 104633, *P.damselae* BT-6, *P.kishitanii* NCMB 844, *P.leiognathi* lnuch.13.1, *P.l. subsp. mandapamensis* ATCC 27561, *P.phosphoreum* MIE from *Photobacterium* genus; *Ph.asymbiotica* and *Ph.luminescens* ATCC 29999 from *Photorhabdus* genus; *S.hanedai* NCIMB 2157 and *S.woodyi* ATCC 51908 from *Shewanella* genus; *V.albensis* VL 426, *V.azureus* NBRC 104587, *V.campbellii* ATCC BAA-1116, *V.chagasii* SB-52, *V.harveyi* NBRC 15634, *V.orientalis* ATCC 33934 and *V.vulnificus* ATCC 43382 from *Vibro* genus. We performed the phylogenetic analysis on the amino acid sequences of α- and β-subunits using the Jalview program package (version 2.8.1, (Waterhouse *et al.*, 2009)). The dataset was analyzed with the multiple sequence alignment software MAFFT (Katoh and Standley, 2013) and BLOSUM 62 as substitution matrix, a gap open penalty of 1.53 and a gap extension penalty of 0.123 were used. ProtTest (Abascal *et al.*, 2005) enabled to identify LG + G as the best-fit amino acid replacement model for the evolution of both subunits. This model was used to build phylogenetic trees with the PhyML software (Guindon *et al.*, 2010). To find the roots of reconstructed trees we added an outgroup. Moreover, the ape package (version 3.1-4, (Paradis *et al.*, 2004)) enabled us to perform other analysis in an open source environment R/Bio-conductor (Gentleman *et al.*, 2004).

In order to build the tertiary structure of other bacterial luciferases—using the Swiss-MODEL server (Biasini *et al.*, 2014)—we applied the crystal structure of *V.harveyi* luciferase (PDB ID: 3FGC) as a template. Then we optimized the obtained structures for 1 ns at 300 K and a constant pressure of 1 atm, using a model of explicit water SPC/E and CHARMM27 force field in GROMACS (version 5.0.3, (Pronk *et al.*, 2013)). We also analyzed the enzymatic active sites by using VMD (Humphrey *et al.*, 1996). The tertiary structures for other slow luciferases in vicinity of active center were found unchanged if compare with that of *V.harveyi* luciferase. The enzymes among the fast group also demonstrated very similar architecture of the binding pocket, but different from the *V.harveyi* one.

3 Results and discussion

3.1 Conservative regions

The amino acid sequence analysis revealed that the pairwise sequence identity among 21 species is 53–99% for α-subunit and 43–98% for β-subunit (Supplementary Table S1). This finding is in a good agreement with previously reported amino acid identity levels that were obtained for 6 species of *Photobacterium*, *Photorhabdus* and *Vibrio* genus (Meighen, 1991). α-subunits were found to have highly conserved amino acid sequences with 138 identical positions. Eight of them: αGlu^{43}, αHis^{44}, αHis^{45}, αArg^{107}, αTyr^{110}, αAsp^{113}, αArg^{125}, αGlu^{175} are known to be critical for catalytic activity of the enzyme (Hou *et al.*, 2014; Huang and Tu, 1997; Madvar and Naderi-Manesh, 2007; Moore *et al.*, 1999; Xin *et al.*, 1991). According to the crystal structure of *V.harveyi* luciferase, the active center (a cavity within 6 Å distance from FMN binding site) consists of 35 amino acid residues where 20 of them are conserved. Such conservative amino acids gathering allows forming the proper structure of the substrate-binding pocket.

Another substantial part of α-subunit conserved residues were found on the α/β-interface: 25 identical amino acids can be observed within 4 Å distance from the β-subunit surface. More than a third of β-subunit conserved regions are also contacting with α-subunit (25 positions). That confirms a proposed important function of the β-subunit—the stabilization of the α-subunit active conformation (Campbell *et al.*, 2009).

3.2 Two groups of luciferases

Phylogenetic analysis of α-subunit amino acid sequences revealed two clades of luciferases with 65.01 and 53.99% identity within each group (Fig. 2). These groups significantly correspond to the previous classification of luciferases as slow or fast according to their kinetic properties. Phylogenetic analysis of luciferase α-subunit

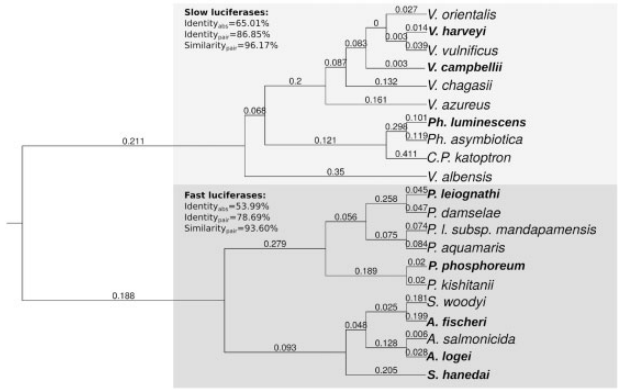

Fig. 2. Phylogenetic tree for α-subunit amino acid sequences of bacterial luciferases. Bacteria in each clade share similar reaction kinetics. The names of bacteria with experimentally confirmed enzyme kinetics are in boldface

sequences helped to predict the type of enzyme with unknown kinetics. According to the tree the luciferase from *Candidatus Photodesmus katoptron* probably belongs to slow type, as well as the rest of luciferases from *Vibrio* species, while luciferases of *Aliivibrio* and *Shewanella* are of fast type.

Exact and partial matches found in specific positions of the studied sequences characterize two observed clades (Supplementary Table S2). Exact match refers to a residue that is strictly conserved among a group. For instance, all fast luciferases contain Leu, while all slow luciferases have Ala at position 183 without an exception (αLeu^{183} versus αAla^{183}). Partial match refers to less than four residues of the same type (according to polarity, side-chain group type, etc.) in one position showing the tendency for the same clade formation. For example, all fast luciferases have Arg in contrast to Asn of slow luciferases at a position 26 except for *Ph.asymbiotica* and *Ph.luminescence* having αLys^{26}.

We have identified 22 exact matches on the α-subunit, which constitute an inherent feature of the found clades. Vicinity of the active site (the area within 6 Å distance from FMN molecule bound to the active site and close to it) contains 11 out of 22 exact matches. Four of them (αAla^{74}, αAla^{75}, αCys^{106}, αVal^{173} were previously described as critical for proper functioning of slow *V.harveyi* luciferase (Fisher *et al.*, 1995; Hou *et al.*, 2014; Lin *et al.*, 2004)).

In addition to 22 exact residue matches specific for two groups of α-subunit sequences, 26 partial residue matches were found. Two of them were experimentally identified as important residues for luciferase functioning: αSer^{227} (Chen and Baldwin, 1989) and αLys^{286} (Campbell and Baldwin, 2009). The latter is located on the enzyme mobile loop and stabilizes the reaction intermediates. αSer^{227} is important to form the tertiary structure of the enzyme. Inspecting the crystal structure, we observed that residues meeting partial residue matches spread across the structure unlike the residues identified as exact matches. The observed tendency can indicate that residues with partial matches are necessary to preserve the tertiary structure.

A number of previous studies have tried to convert a slow luciferase into fast type by changing a sequence of segment (residues 166–233) near the active center with a template sequence from fast luciferase (Valkova *et al.*, 1999) or site directed mutagenesis of αGlu^{175} residue (Hosseinkhani *et al.*, 2005). Valkova *et al.* recorded changes in the kinetic properties of luciferase, but simultaneously observed a substantial loss in the enzyme activity (up to 0.03% of the wild type activity). According to our findings, the substituted part (67 a.a.) consists of 24 conservative residues, 6 amino acids that meet exact matches (i.e. are supposed to define slow or fast type of kinetics) and 3 residues that are described here as partial matches. However, the rest of the substituted residues (34 a.a.) are different from the wild type enzyme. This difference might have caused tertiary structure distortion and less enzymatic activity. Regarding αGlu^{175}, we found that it is conserved among all studied luminous bacteria; therefore, this residue is unlikely to be responsible for the type of kinetics. Mutation of αGlu^{175} induced higher structure flexibility and destabilized the reaction intermediates. These changes intensified the dark pathways of the reaction and finally increased the kinetics of the enzyme. It is noteworthy that natural fast luciferases are highly active, and some of them even more active than the slow ones (Katsev *et al.*, 2004).

The β-subunit of bacterial luciferase is assumed to stabilize the α-subunit active conformation through hydrogen bonds and a number of specific hydrophobic interactions. Experiments revealed an important role of interaction between αPhe^{272} and βTyr^{151} for the activity of *V.harveyi* luciferase (Campbell *et al.*, 2009). We found that αPhe^{272} meets the exact residue match: αTyr in all fast luciferases versus αPhe in slow ones. Interestingly, the β-subunits also undergo βTyr-βPhe substitutions, that probably indicates a similar stabilization mechanism conserved among all luminous bacterial species including aromatic stacking interaction between amino acid side chains.

Phylogenetic tree for β-subunit exhibit distinct clades for slow and fast luciferases as well as for α-subunit (Supplementary Figure S1). However only 7 exact and 19 partial matches were identified through β-subunit sequences alignment analysis (Supplementary Table S3).

The mechanisms for origin of fast and slow luciferases from the only ancestor are poorly understood yet. Some correlation of kinetics type could be found only with ecological niches. Species having slow luciferases are mostly free-living, while fast decay luciferases are typical for symbiotic luminous bacteria (Dunlap and Urbanczyk, 2013). However, it was shown that phylogenies of luminous bacteria and their host organisms exhibited no meaningful topological congruence (Dunlap *et al.*, 2007). To address functional evolution of bacterial luciferases an investigation of changes on the lineages with consensus tree reconstruction should be done (Arenas and Posada, 2010).

3.3 Analysis of luciferases active centers

The fact that many of α-subunit exact matches are located in the active site or close to it allows examining all differences that might appear in the structure of this part of the enzyme. We have reconstructed 3D-structure for *P.leiognathi* luciferase as the representative of fast group and made structure optimization with molecular dynamics. Mapping of active centers revealed TIM barrel structure preservation for both fast and slow luciferases. Moreover, the structure of the binding pocket varied due to distinctions in amino acid composition. Additionally we have identified two significant differences in the active center structure for slow and fast enzymes caused by four exact amino acids substitutions.

We observed the phenylalanine shifting ($\alpha Phe^{6} \leftrightarrow \alpha Ile^{6}$, $\alpha Leu^{8} \leftrightarrow \alpha Phe^{8}$) (Fig. 3A). αPhe^{6} (in *V.harveyi* slow luciferase) and αPhe^{8} (in modelled *P.leiognathi* fast luciferase) are found at the si-face side of the isoalloxazine moiety. In both cases they form the active site essential for protein-ligand binding which involves hydrophobic and stacking interactions. Substitution to hydrophobic residues such as leucine and isoleucine is also very common and preserves the hydrophobic surfaces in the active site.

Furthermore, we studied the utilization of alternative sulphur-containing amino acid ($\alpha Ala^{74} \leftrightarrow \alpha Met^{74}$, $\alpha Cys^{106} \leftrightarrow \alpha Val^{106}$) that

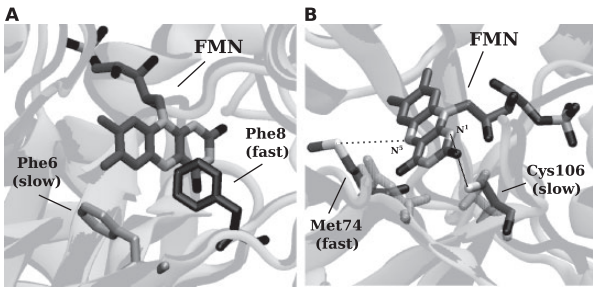

Fig. 3. Peculiarities identified in active centers of slow and fast luciferases caused by two pairs of exact residue matches: (**A**) phenylalanine shifting ($\alpha Phe^{6} \leftrightarrow \alpha Ile^{6}$, $\alpha Lue^{8} \leftrightarrow \alpha Phe^{8}$) and (**B**) utilization of alternative sulphur-containing amino acid ($\alpha Ala^{74} \leftrightarrow \alpha Met^{74}$, $\alpha Cys^{106} \leftrightarrow \alpha Val^{106}$)

stabilize the flavin derivatives (Fig. 3B). Bioluminescent reaction includes a step where the addition of O_2 molecule and deprotonation of N^1 results in hydroperoxy flavin formation in complex with enzyme (Hou *et al.*, 2014). Abu-Soud *et al.* showed that αCys^{106} is involved into this reaction step, because its mutation to αVal^{106} leads to extremely unstable complex of luciferase with hydroperoxy flavin intermediate and reduces the bioluminescence activity (Abu-Soud *et al.*, 1993). However, a double mutant luciferase with αGly^{75} and αVal^{106} instead of αAla^{75} and αCys^{106} showed high enzyme activity and a stable intermediate, but fast luminescence decay (Lin *et al.*, 2002). This is in good accordance with our findings that αAla^{75} and αCys^{106} refer to exact matches of slow group, whereas αGly^{75} and αVal^{106} belong to exact matches of fast group. Inspecting the modeled active sites of fast luciferases for sulfur-containing residues that could manage the same function as αCys^{106}, we observed αMet^{74} residue. It is one of highly conserved amino acids among fast luciferases group (exact match). Therefore we can conclude that αMet^{74} roles as a substitute to αCys^{106} in fast luciferases which is also able to form a hydrogen bond through its sulphur group. It is likely that αMet^{74} interacts with N^5 atom, while αCys^{106} probably contacts with N^1 (Fig. 3B). Consequently αMet^{74}, αGly^{75} and αVal^{106} probably form an important part of the active site that stabilizes the intermediates in fast luciferases, similar to αAla^{74}, αAla^{75} and αCys^{106} in slow ones.

To our knowledge the existence of two evolutionary conserved binding platforms could not be related with all variations in metabolism and luminous system regulation. The important physiological role of luminescence reaction is proposed to be as a secondary respiratory chain that is active when oxygen or iron levels are too low for the cytochrome system to operate (Dunlap and Urbanczyk, 2013). It permits cells to continue growing even under microaerobic conditions, such as in animal gut tracts. Previously it was obtained some correlation between type of kinetics and sensitivity of bacterial strains to hydrogen peroxide (Katsev *et al.*, 2004). But causal relationship in this observations and connection with the luciferase polymorphic forms is had to be understood on the basis of whole picture on luminous bacteria evolution and metabolism. The occurrence of two binding platforms probably plays an important role relying on the whole picture of luminous bacteria phylogeny and metabolism.

4 Conclusion

Study of the conservative motifs critical for the interaction of the bacterial luciferase with substrates and intermediates stabilization is far reaching in terms of understanding the mechanism of bioluminescence. In this study, we presented a phylogenetic analysis of amino acids important for light emitting reaction. Our data suggested the conservative positions in structure of all known bacterial luciferases, and amino acids causing the division into fast and slow types of this enzyme. We found two sets of critical amino acids exactly matching each types of luciferases and probably responsible for kinetic decay rate. Through protein structure homology modelling, we observed the formation of two different binding platforms that probably stabilize the reaction intermediates. Two evolutionary fixed types of reaction kinetics could indicate the appearance of two different functions carried by bacterial luciferases that never been discussed before from the structural point of view.

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Conflict of Interest: none declared.

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