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Bifunctional role of leucine 300 of firefly luciferase in structural rigidity

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ABSTRACT

Firefly luciferase is susceptible to thermal inactivation, thereby its intracellular half-life decreased. Previous reports indicated that $L^{300}R$ mutation (LRR mutant) in $E^{354}R/Arg^{356}$ double mutant (ERR mutant) from *Lampyris turkestanicus* luciferase has increased its thermal stability and rigidity through induction of some ionic bonds with Asp 270 and 271. Disruption of the deduced ionic bonds in an ultra-rigid mutant of firefly luciferase did not reverse the flexibility of the protein. In this study, we investigated the effects of this residue to find the truth behind an extraordinary increase in thermal stability and rigidity of luciferase after replacement of leucine 300 by arginine based on previous reports. For this purpose, $L^{300}R$, $L^{300}K$ and $L^{300}E$ mutations were performed to compare the effects of these mutations on the native firefly luciferase. In spite of increase of intrinsic fluorescence of the mutants a slight increase in thermostability and retention of kinetic properties was observed. Based on our results, we can conclude that $L^{300}R$ mutation in LRR mutant accompanying with alteration in a flexible loop (352–359) increased thermostability and rigidity of luciferase.

1. Introduction

Firefly luciferase is a peroxisomal enzyme that converts a cyclic substrate luciferin to an excited state oxyluciferin in the presence of ATP, Mg^{+2} and O_2 [1,2]. When excited state oxyluciferin molecules return to ground state, light is produced with high quantum yield [3,4]. Luciferase from *Photinus pyralis* produces light at pH ≈ 7.6 *in vitro,* in the range of green-yellow (λ_{max} 557 nm) and red (λ_{max} 620 nm) at pH ≈ 5.4 [5–7]. Naturally, most firefly species emit light in yellow-green and few in red. Firefly luciferase-based assay is a highly sensitive technique and has wide applications such as ATP assay [8–10], *in vivo* imaging [8], gene reporters [11], Pyrosequencing [12] and luciferase-based split biosensors [13].

In order to exploit luciferase in different applications following strategies usually applied including: optimization and stabilization of kinetic properties through rational and random mutations [14,15], enzyme inhibitors removing by addition of detergents [16], Coenzyme A (CoA) and other thiol compounds [17,18], pyruvate orthophosphate dikinase (PPDK) [19], and luciferin regenerating enzyme (LRE) to recycle adenosine monophosphate (AMP) and oxyluciferin [20–23] as luciferase reaction products, respectively.

Luciferase is a mesophilic enzyme which exhibit low temperature stability *in vivo* and *in vitro*. Thus, many studies have been performed to overcome this problem. For this purpose, luciferases sequences and structures have been compared with those of many thermophile proteins and based on the data obtained, different experiments have been designed to increase luciferase thermostability [14,24–27]. According to the aforementioned studies in thermostable proteins, guanidinium group of arginine residue situated at the protein surface loops, participate in ionic and hydrogen interactions which in turn lead to unexpected increase in thermal stability [14,27]. For example, in acetylcholine esterase 14 hydrophobic residues situated at surface loops and their replacement by arginine resulted in increase in thermal stability [28]. Mutation in the most flexible region of firefly luciferase ($D^{474}K$ and $D^{476}N$) shows that $D^{474}K$ mutation became much more flexible than wild type although $D^{476}N$ didn't have any significant difference [29]. Moreover, $H^{489}P$ mutation within most flexible regions of luciferase improved its thermostability while maintaining its catalytic efficiency compared to that of wild type luciferase. Rigidity of $H^{489}P$ mutant is greatly strengthened. $D^{476}P$ mutation decreased its thermostability while $S^{307}P$ mutation decreased kinetic stability and enhanced thermodynamic stability [30]. In another study,

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$E^{354}R$ mutation in a flexible loop of *L. turkestanicus* luciferase and *P. pyralis* luciferase has increased their thermal stability with a clear shift in bioluminescence spectra [31–34]. Saturation of positive charges by additional Arg substitution in this flexible loop (ERR mutant) brought about with a bioluminescence spectrum with only a single peak in red region [35]. Substitution of some surface amino acids including Gln, Ile, Leu or Arg located at other exposed loops of luciferase from *L. turkestanicus* increases thermostability and optimum temperature of both green-emitter and red-emitter mutants [14,36]. Previous studies proved that surface flexible loops of luciferase play a key role in the enzyme thermostability. In other words, the further research has revealed that substitution of arginine by hydrophobic amino acids increases ionic interactions and hydrogen bonds which results in enzyme rigidity and also stability. In addition, based on the previous study, $L^{300}R$ mutation (in $E^{354}Q/Arg^{356}$ double mutant (ERR)) is less active than others, although this mutation increased structural rigidity. Additionally, it has been suggested that thermal stability and activity in $L^{300}R$ mutant are related inversely. According to what previous reported, $L^{300}R$ has increased structural stability and conversely decreased activity of ERR mutant from *L. turkestanicus* luciferase [14]. Furthermore, above mentioned study demonstrated that after replacement of leucine 300 with arginine in luciferase from *L. turkestanicus* brought about salt bridges between arginine 300 and glutamate 270 and 271. Software analysis in the last study has showed that ionic and hydrogen interactions can be formed by other amino acids such as E300, K303 and Y304 [14]. Deletion of the deduced salt bridges in LRR mutant through mutation of 270 and 271 residues did not restore the original flexibility and activity of firefly luciferase [37].

The aim of this study was investigation of the effects of replacement of leucine 300 (situated at the surface loop of native firefly luciferase from *L. turkestanicus*) with other amino acids on thermal stability and rigidity, in comparison with L300R mutation in addition to alteration in a flexible loop (352–359) which reported earlier [14,36,37]. In this study, leucine 300 (uncharged) in native *L. turkestanicus* luciferase replaced by different polar amino acids including lysine (positively charged), glutamate (negatively charged) and arginine (positively charged) to analyze each substitutions effects on function and structure of this enzyme compared to LRR mutant with two other mutations in other positions [triple mutant $L^{300}R$ with $E^{354}R/Arg^{356}$ or $E^{354}Q/Arg^{356}$].

2. Material and methods

2.1. Materials

Isopropyl-D-thiogalactopyranoside (IPTG) and Kanamycin from Sigma-Aldrich, ATP from Roche, Luciferin potassium salt from Resem BV Netherlands, PrimeSTAR HS DNA polymerase and *Dpn*I from Takara; Plasmid extraction, Gel extraction and polymerase chain reaction (PCR) purification kits from Bioneer crop, Ni-NTA Sepharose column and pET-28a from Novagen were provided. All tests were repeated in triplicate.

2.2. Site-directed mutagenesis

Quick-change PCR was used for site-directed mutagenesis of a vector carrying luciferase cDNA. For this, a vector harboring wild type (recombinant native luciferase from *L. turkestanicus*) was used as template. In order to construct the single mutant, the forward and reverse primers were designed and synthesized. The following sequences are forward primers and the underlined shows mutated codons:

$L^{300}K$ (F) 5′- CCTATTTTCATTCTTTGCCAA**AAG**CACCAAGGTCGACAAAATACG-3′ (44mer)

$L^{300}E$ (F) 5′- CCTATTTTCATTCTTTGCCAAAAGCACC**GAA**GTCGACAAATACG-3′ (44mer)

$L^{300}R$ (F) 5′- CCTATTTTCATTCTTTGCCAAAAGCACC**CGC**GTCGACAAATACG-3′ (44mer)

PCR was performed using PrimeSTAR HS DNA polymerase under following condition: at first the master mix were added to two micro tubes then forward primers was added to one micro tube and the other one take the reverse primer, initial denaturation at 98 °C for 15 s, 6 cycles (95 °C for 1 min, 58 °C for 1 min and 68 °C for 10 min); then the two micro tubes were mixed. Next, for 24 cycles (95 °C for 1 min, 58 °C for 1 min and 68 °C for 10 min) and final extension 68 °C for 15 min. After completion of the reaction, the PCR product was treated with *Dpn*I in 37 °C overnight. Then, *Dpn*I-treated PCR product was cleaned up by PCR purification kit. Subsequently, the purified PCR product was transformed to $DH_{5\alpha}$ competent cells by chemical method and colony screening performed on LB-agar-kanamycin plates.

2.3. Mutation validation by sequencing

In order to sequence determination of the mutants, pET-28a vectors containing desired mutations were isolated and sequenced by using of T7 promoter and terminator universal primers, automatic sequencer (Macrogen, Korea).

2.4. Expression and purification of native and mutant luciferases

After validation, native and mutated luciferase plasmids were transformed to *E. coli* BL21 competent cells for recombinant-protein expression and purification. Mutated and native histidine-tailed luciferases in pET-28a vector were expressed in LB medium with 50 μg/ml kanamycin. For this purpose, at first, 10 ml of LB medium was inoculated by one BL21 colony containing native or mutated luciferase, and incubated overnight at 37 °C, 220 rpm. Then, 1 ml of the seeding was added to 200 ml of LB medium with kanamycin and incubated at 37 °C at 220 rpm. Subsequently, when OD_{600} reached ~0.6–0.8, IPTG to the final concentration 1 mM was added to culture medium and incubated at 22 °C for 16 h with 220 rpm shaking. Then after the cell pellets were collected by centrifugation 10,000*g* for 10 min at 4 °C. The pellets were suspended in 5 ml lysis buffer, (50 mM Tris-HCl (pH 7.8), 300 mM NaCl, 10 mM imidazole, 1 mM PMSF). The suspension was sonicated on ice to disturb bacterial cells. Centrifugation 15,000 g for 20 min, at 4 °C was done to collect supernatant, and then luciferase enzymes both native and mutants were purified by Ni-Sepharose affinity chromatography column, as reported earlier [14,30,36]. Protein concentration was determined by Bradford assay using coomassie blue and bovine serum albumin as standards.

2.5. Kinetic parameters measurements

Km factor for both of luciferase substrates, ATP and Luciferin, were determined by bioluminescence assays at 25 °C (Sirius tube luminometer, Berthold Detection System) using Line weaver–Burk plot for native and mutant luciferases. K_m factor for ATP was evaluated by addition 5 μl diluted enzyme to 95 μl of cocktail solution [10 mM $MgSO_4$, 4 mM ATP and 2 mM luciferin, 50 mM Tris-HCl, pH 7.8] but with different concentration of ATP (final concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, 2.5, 3.5, or 4.5 mM). LH_2 kinetic constant was also determined in the same way, though the cocktail included different concentrations of luciferin (final concentrations of 0.015, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, or 2.0 mM) each time. In addition, specific activity values were measured. Reactions were started by injecting 2.5 μl of fresh enzyme to 25 μl of cocktail solution and 22.5 μl Tris-HCl buffer (50 mM) at 25 °C. The emissions of light were measured during 1 s [14].