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# Relationship between stability and flexibility in the most flexible region of *Photinus pyralis* luciferase

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ABSTRACT

Firefly luciferase is a protein with a large N-terminal and a small C-terminal domain. B-factor analysis shows that its C-terminal is much more flexible than its N-terminal. Studies on hyperthermophile proteins have been shown that the increased thermal stability of hyperthermophile proteins is due to their enhanced conformational rigidity and the relationship between flexibility, stability and function in most of proteins is on debate. Two mutations (D474K and D476N) in the most flexible region of firefly luciferase were designed. Thermostability analysis shows that D476N mutation doesn't have any significant effect but D474K mutation destabilized protein. On the other hand, flexibility analysis using dynamic quenching and limited proteolysis demonstrates that D474K mutation became much more flexible than wild type although D476N doesn't have any significant difference. Intrinsic and ANS fluorescence studies demonstrate that D476N mutation is brought about by structural changes without significant effect on thermostability and flexibility. Molecular modeling reveals that disruption of a salt bridge between $D^{474}$ and $K^{445}$ accompanying with some H-bond deletion may be involved in destabilization of D474K mutant.



## 1. Introduction

Bioluminescence is a fascinating process in which living organisms convert chemical energy into light. Several reports have shown that bioluminescence occurs in many different organisms including bacteria, fungi, algae, fish, squid, shrimp and insects [1–4]. Luminous organisms produce light by an enzymatic reaction of a luciferase (enzyme) with a luciferin (substrate). Among luciferases, basic and applied research mainly focused on the North American firefly *Photinus pyralis* luciferase. Firefly luciferase (EC 1.13.12.7) oxidizes a benzothiazole substrate (beetle luciferin) in the presence of magnesium ions, ATP and molecular oxygen [5]. The product, oxyluciferin, is generated in an excited state which then decays to the ground state with the emission of a photon.

Firefly luciferase has been studied for the last 50 years and proven to be a useful enzyme. It has been used in many bioanalytical fields from ATP detection methods to in vivo imaging [6]. Moreover, the three-dimensional structure of luciferase from *P. pyralis* in the absence of substrate [7] and in complex with bromoform [8] has been solved.

Protein function is intimately linked to protein flexibility and any interaction between a protein and another molecule requires the protein to be able to change its conformation. This conformational change may be very small, involving only the rearrangement of a few amino acid side chains, or it may be large and even involve folding of the entire protein. Potentially, a perturbation that changes the flexibility of a protein may interfere with its function. How function, flexibility and stability are connected is still heavily debated. A deeper understanding of the interplay among these properties is not only of basic scientific interest but will also have implications for protein design and applied protein science [9].

In thermophilic organisms, proteins have evolved to resist high temperatures. The thermophilic proteins are generally more rigid than their mesophilic homologues at similar temperatures [10,11].

Protein molecules exhibit varying degrees of flexibility throughout their three-dimensional structures, with some segments showing little mobility while others may be so disordered as to be unresolvable by techniques such as X-ray crystallography [12]. The atomic displacement parameters (B-factor) determined by high resolution X-ray crystallographic studies represent smearing of atomic electron densities around their equilibrium positions due to thermal motion and positional disorder. Analysis of B-factor, therefore, is likely to provide newer insights into protein dynamics, flexibility of amino acids and protein stability. Direct relationship between high flexibility and high B-factor has been established [13,14]. Molecular dynamics studies have suggested that protein unfolding might be initiated at sites that are prone to large thermal fluctuations. Therefore the pattern of B-factor determined by high-resolution X-ray crystallographic studies might contain information regarding protein stability. The correlation between experimentally observed B-factor and stability,

*Abbreviations:* ATP, Adenosine triphosphate; ANS, 1-Anilino-8-naphthalene sulfonate; CD, Circular dichroism; Ni-NTA, Nickel nitrilotriacetic acid; *Ppy*, *Photinus pyralis*; Trp, Tryptophan.

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unlike the contributions of various other interactions, has not been examined in any great detail [15].

Thermostability of proteins is an important issue when they are applied as catalysts. Numerous studies have been performed to identify principles of thermostability and, subsequently, to apply those in rational or data-driven protein engineering [16,17]. Different mutagenesis methods have been used to increase the thermostability of firefly luciferases. One of these successful methods was random mutagenesis that identified specific positions that increase firefly thermostability [18–21]. In some works the 3D structure of firefly luciferase was used as a basis for rational design of thermostable mutants, for example, the mutagenesis of solvent exposed residues [22], introduction of disulfide bonds [23,24], and the comparative analysis of the selected residue microenvironment [25]. In other works, earlier defined positions were used for construction of multi-point mutants [20,26,27]. Among these mutations, F465R mutation [22] being present in the C-terminal domain of the *P. pyralis* luciferase.

According to our best of knowledge, it is the first time that B-factor based analysis is used for the design of thermostable mutants in firefly luciferase. In this study, in order to investigate the relationship between flexibility and stability in the most flexible regions (according to residues B-factor analysis) of the *P. pyralis* luciferase, two mutants (D474K and D476N) were constructed and evaluated for their thermal stability, structural flexibility, kinetic and structural properties.

## 2. Materials and methods

The following reagents and kits were used. Expand long template PCR system (Roche), Dpn1 (Roche), Isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, ATP (Roche), D-luciferin potassium salt (Synchem), ANS (8-anilino-naphthalene-1-sulfonic acid) and plasmid extraction kit (Bioneer CO., South Korea), DNA ladders (1 Kb, 100 bp; Fermentas), Ni-NTA spin kit(QIAGEN Inc.), pET16b vector (Novagen) and trypsin (Fluca).

Reproducibility of the data presented in this manuscript was confirmed by repeating the experiments at least three times. The data presented here are typical experimental data.

### 2.1. X-ray structure analysis and luciferase B-factor map determination

B-fitter software [28] and WHAT IF (http://swift.cmbi.ru.nl/servers/html/index.html) [29] server were used to determine the amino acids with highest B-factor to select appropriate sites for site-directed mutagenesis. Suitable residues for substitution were proposed by Rosetta Design Software in its flexible backbone mode [30].

### 2.2. Site-directed mutagenesis

Substitution mutagenesis was performed using the Quick Change site-directed mutagenesis method described by Fisher and Pei[31]. Two pairs of overlapping primers (30–40 mers) were designed as follows:

D474K primer F:
5′ GCAGGTCTTCCCAAAGATGACGCCGGTGAACTTC 3′
D474K primer R:
5′ GAAGTTCACCGGCGTCATCTTTGGGAAGACCTGC 3′
D476N primer F:
5′ GTGGCAGGTCTTCCTGACGATAACGCCGGTGAACTTC
D476N primer R:
5′ GAAGTTCACCGGCGTTATCGTCAGGAAGACCTGCCAC.

The plasmid containing the native *Ppy* luciferase was used as a template and two step quick change method using expand long template PCR system was carried out [32]. In step one, two extension reactions were performed in separate tubes; one containing forward primer and the other one containing the reverse primer. Subsequently, the two reactions were mixed, and the standard quick change method was carried out on the mixture. After amplification, plasmids containing staggered nicks were generated. The products were treated with Dpn1 in order to digest native parental plasmids, subsequently; the digestion products were purified using a clean-up kit to remove the redundant primers, then, this product transformed to *E. coli* XL1-blue by chemical method [33] for each mutation.

### 2.3. Sequencing

pET28a (+) vectors containing native and mutant luciferases were sequenced using an automatic sequencer (MWG) by the T7 promoter and T7 terminator universal primers.

### 2.4. Protein expression and purification

Five milliliters of LB medium containing 50 μg/ml ampicillin with a fresh bacterial colony harboring the expression plasmid was inoculated and grown at 37 °C overnight. Then 200 ml of medium with 500 μl overnight cultures was inoculated and grown at 37 °C with vigorous shaking until the $OD_{600}$ reached 0.6–0.9 at 600 nm (A600), as reported earlier [34]. Then, IPTG and lactose were added to the solution to final concentrations of 1 mM and 4 mM respectively and the mixture incubated at 22 °C overnight with vigorous shaking. The cells were harvested by centrifugation at 5000 *g* for 15 min. The cell pellet was resuspended in lysis buffer [50 mM Tris–HCl, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF (add fresh) (pH 7.8)].

Purification of $His_6$-tagged fusion protein was performed with the Ni-NTA spin column as described by the manufacturer (QIAGEN).

### 2.5. Kinetic properties

Luciferase activity was measured using Sirius tube luminometer (Berthold Detection System). Maximal intensity of the light emitted during the enzymatic reaction at saturating concentrations of substrates was used as a measure of the luciferase activity. Assay was initiated by injecting 50 μl of complex solution (containing 4 mM ATP, 2 mM luciferin and 0.01 M $MgSO_4$ in 50 mM Tris–HCl buffer, pH 7.8) into 5 μl enzyme solution. The bioluminescence intensity was registered at room temperature (22–25 °C). Activity was expressed in relative light units (RLU/s) of the luminometer. ATP and luciferin kinetic parameters were measured at 25 °C. To estimate LH2 $K_m$, 50 μl of assay reagent containing 10 mM $MgSO_4$ and 4 mM ATP in 50 mM Tris–HCl (pH 7.8) was mixed with 40 μl of various concentrations of luciferin (0.0025–5 mM) in a tube. The reaction was initiated by adding 10 μl of enzyme, and light emission was recorded over 10 s. The estimation of ATP kinetic constants was performed in a similar way. Various concentrations (40 μl) of ATP (from 0.004 to 8 mM) were mixed with 50 μl of assay reagent, including 10 mM $MgSO_4$ and 1 mM luciferin in 50 mM Tris–HCl (pH 7.8). The reaction was initiated by adding 10 μl of enzyme, and light emission was recorded over 10 s. Apparent kinetic parameters were calculated by Hanes plots. The decay times of native and mutant luciferases were measured in 15 min and compared with each other. The residual activity for each enzyme was reported as a percentage of the original activity. Approximate protein concentrations were calculated using a Bradford assay [35], and relative specific activities (enzyme activity vs protein concentration) were also calculated. To obtain the optimal temperature of activity for native and mutant luciferases, activities were measured in the range of 5–45 °C. These experiments were repeated with 5 °C temperature intervals. Moreover, the optimum pH of activity for both enzymes was measured by incubation of enzyme in a mixed buffer in the pH range of 5–10.5.