Biochimica et Biophysica Acta 1854 (2015) 65–72

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

The role of proline substitutions within flexible regions on thermostability of luciferase

Haoran Yu [a,b,c,1], Yang Zhao [d,1], Chao Guo [a,b,c], Yiru Gan [a,b,c], He Huang [a,b,c,*]

[a] Department of Biochemical Engineering, School of Chemical Engineering & Technology, Tianjin University, Tianjin 300072, PR China
[b] Key Laboratory of Systems Bioengineering, Ministry of Education of China, Tianjin 300072, PR China
[c] Collaborative Innovation Center of Chemical Science and Engineering, Tianjin PR China
[d] National Institutes for Food and Drug Control (NIFDC), Beijing 100050, PR China

ARTICLE INFO

Article history:
Received 6 August 2014
Received in revised form 19 October 2014
Accepted 21 October 2014
Available online 30 October 2014

Keywords:
Thermostability
Rational design
Flexibility
Proline
Luciferase

ABSTRACT

Improving the stability of firefly luciferase has been a critical issue for its wider industrial applications. Studies about hyperthermophile proteins show that flexibility could be an effective indicator to find out weak spots to engineering thermostability of proteins. However, the relationship among flexibility, activity and stability in most of proteins is unclear. Proline is the most rigid residue and can be introduced to rigidify flexible regions to enhance thermostability of proteins. We firstly apply three different methods, molecular dynamics (MD) simulation, B-FITTER and framework rigidity optimized dynamics algorithm (FRODA) to determine the flexible regions of *Photinus pyralis* luciferase: Fragment 197–207; Fragment 471–481 and Fragment 487–495. Then, introduction of proline is used to rigidify these flexible regions. Two mutants D476P and H489P within most flexible regions are finally designed. In the results, H489P mutant shows improved thermostability while maintaining its catalytic efficiency compared to that of wild type luciferase. Flexibility analysis confirms that the overall rigidity and local rigidity of H489P mutant are greatly strengthened. D476P mutant shows decreased thermosatbility and the reason for this is elucidated at the molecular level. S307P mutation is randomly chosen outside the flexible regions as a control. Thermostability analysis shows that S307P mutation has decreased kinetic stability and enhanced thermodynamic stability.

1. Introduction

Firefly luciferase catalyzes the oxidation of luciferin in the presence of ATP, Mg^{2+}, and molecular oxygen. This enzyme can efficiently convert chemical energy into light with high quantum yield [1]. The most widely used luciferase reporter gene is that of *Photinus pyralis* (*P. pyralis*, North American Firefly). Wild-type *P. pyralis* luciferase is thermolabile, with an in vitro half-life for activity of the order of 2–3 min at 37 °C, which limits the wide industrial applications of this enzyme [2]. The design of proteins with enhanced thermostability is one of the major goals of protein engineering. Many attempts have been made by site directed mutagenesis to increase thermostability of firefly luciferase [3–5].

The critical point of designing thermostable proteins is to pinpoint the weak spots or "unfolding nucleus". Once the weak spots were identified, further thermostability could be achieved through optimizing weak spots regions [6]. Recently, flexibility has been proved to be an effective indicator to find out weak spots. A protein engineering approach named RFS (rigidify flexible sites) has been widely used to enhance thermostability of proteins, which contains two steps: predict flexible sites and rigidify these sites [7].

However, the relationship among flexibility, stability and activity is complex. Flexibility is required by numerous proteins in their folded state to accomplish their function such as ligand binding, activity modulation and macromolecular interactions. Thermophiles generally lack activity under ambient conditions because low temperature leads to a reduced motion of the protein. Rigidity is needed to maintain integrity of the native folded structure, whereas a certain degree of flexibility is required to activity. A deeper understanding of the interplay among stability, activity and flexibility is not only of basic scientific interest but also beneficial for development of criteria to design proteins with better properties.

Determining flexible regions of proteins would be useful to decipher and eventually control their biological function. Protein molecules exhibit various degrees of flexibility throughout their 3-D structures. Some regions show little mobility while others are so disordered as to be unresolved by techniques such as X-ray crystallography [8]. There are many computational programs available to analyze the flexibility of protein. Nevertheless, each of the methods has its own advantages and disadvantages. For example, atomic positional fluctuations

* Corresponding author at: Department of Biochemical Engineering, School of Chemical Engineering & Technology, Tianjin University, Tianjin 300072, PR China. Tel./fax: +86 22 2740 9598.
E-mail address: huang@tju.edu.cn (H. Huang).
[1] These authors contributed equally to this study and share first authorship.

http://dx.doi.org/10.1016/j.bbapap.2014.10.017
1570-9639/

information can be derived from X-ray crystallographic B-factors. But there is an important difference between B-factor and solution protein flexibility [9]. Molecular dynamics (MD) simulation is a commonly used method to study protein dynamics [10]. However, molecular dynamics (MD) is complex, computationally expensive and their use requires a certain degree of expertise [11]. Coarse-grained methods such as FRODA (framework rigidity optimized dynamics algorithm) are a fast and cheap alternative to MD simulation. It can explore the large-amplitude motions of larger systems up 160 times faster than MD simulation [12]. Nevertheless, performance of FRODA on different types of protein motions is still largely unknown because it is only tested on a few selected proteins [13]. Combinational use of numerous methods would be an effective solution to precisely determine flexibility of a protein molecule.

In this study, in order to investigate the relationship among stability, activity and flexibility, we introduced several proline residues within flexible regions of *P. pyralis* luciferase. Introduction of proline was expected to rigidify flexible regions because its rigid pyrrolidene ring constrains main chain dihedral angel Φ to $-63 \pm 15°$ and decreases conformational freedom of Cα-N rotation [14]. However, it is not easy to specifically pinpoint the suitable position for replacement of proline because proline misses amide hydrogen and inappropriate substitution will break the hydrogen bridge of WT (Wild Type) enzyme. Additional criteria were suggested to solve this problem. Flexible sites were generally detected in the regions without compacted secondary structures such as β-turns. Sequence statistics collected by Guruprasad and Rajkumar and relative studies indicated that proline is preferred at $i + 1$ position of Type I and II β-turns and at the *i* position in Type II β-turns [15,16]. Additionally, since proline is missing amide hydrogen atom, we believe if the wild type residue amide proton is involved in H-bonding, then substitution with proline will lead to destabilization of the protein. We call this assumption as "H-bonding criteria".

According to our best of knowledge, it is the first time that proline residues were introduced within flexible regions to enhance thermostability of *P. pyralis* luciferase. In this study, three computational approaches, MD simulation, FRODA and B-FITTER were used to understand the flexibility of luciferase molecule. In order to investigate the relationship between flexibility and stability, three mutants (S307P, D476P and H489P) were constructed and their thermal stability, flexibility change, kinetic and structural properties were evaluated. As a result, a more thermostable mutant of *P. pyralis* luciferase was constructed successfully without hampering catalytic activity.

2. Materials and methods

2.1. Materials

Vector pGL2-Control, D-luciferin were from Promega (USA). Plasmid pET28a was obtained from Novagen (USA). The restriction enzymes (*Dpn* I, *Bam* HI, *Hind* III) and fast Pfu DNA polymerase were obtained from Takara (Japan) and Transgene (China). Ni-NTA agarose for the His-tag protein purification was purchased from GE Healthcare (England). The competent *E. coli* DH5 α and *E. coli* BL21 (DE3) were purchased from Biomed (China). Bacterial culture media were bought from Oxoid (England). The chemicals used in this study were of analytical grade

2.2. Site-directed mutagenesis and purification of enzymes

The wild type luciferase gene was PCR-amplified from vector pGL2-Control (GenBank: X65324.2). The amplified fragment were digested by *Bam* HI and *Hind* III and then cloned into the multicloning site of the pET-28a vector (Novagen, USA) to produce the recombinant plasmid pET28aLuc as described by us before [17]. The luciferase variants used in this study were constructed using the modified QuikChange™ method [18]. The PCR elongation time was 2.5 min, and the target PCR products were purified using Biomed Gel Extraction Kit. The purified DNA was digested with *Dpn* I for 1 h and then transformed into competent *E. coli* DH5 α. The mutant plasmids were extracted and verified by DNA sequencing (Invitrogen, Shanghai). The verified plasmids were transformed into competent *E. coli* BL21 (DE3). A single colony was inoculated to 4 mL LB liquid medium containing 50 μg/mL of kanamycin. The culture was incubated overnight at 37 °C and then were induced by adding 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and cultured at 160 rpm, 20 °C for 18 h to express luciferase variants. Suspend the cells in lysis buffer containing 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, 0.5 mg/mL lysozyme, pH 7.4 after centrifuging at 4600 *g* for 10 min; crushing cells with ultrasonics, then centrifuging at 6670 *g* for 30 min. The supernatant was filtered through a 0.45 μm filter and then purified through immobilized metal ion affinity chromatography (IMAC). His-tagged recombinant luciferase variants were purified using Ni Sepharose 6 Fast Flow (GE Healthcare, England) according to the manufacturer's instructions. 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to test the result of chromatography. Final concentration of luciferase was measured through Bradford method [19]

2.3. Activity assay and determination of kinetic parameters

Luciferase activity was determined using GloMaxTM 20/20 luminometer (Promega, USA). Assay was initiated by injecting 5 μL luciferase solution into 100 μL of complex solution (containing 0.3 mM luciferin, 1 mM ATP and 10 mM $MgSO_4$ in 25 mM HEPES buffer, pH 7.8). The intensity of light was registered at room temperature (22–25 °C). Light emission was recorded over 10 s with delay of 2 s Activity was expressed in the relative light unites (RLU). The kinetic parameters of LH2 (luciferin) and ATP were determined from bioluminescence activity assays as described by Ali Moradi et al. [20]. To estimate K_m of LH2, 50 μL of assay buffer containing 1 mM EDTA, 2 mM ATP and 10 mM $MgSO_4$ in 25 mM HEPES (pH 7.8) was mixed with 50 μL various concentrations of luciferin (0.0025–1 mM) in a 1.5 mL tube. The reaction was initiated by injecting 10 μL purified luciferase (10 μg/mL). The determination of ATP kinetic constants was conducted in a similar way. Various concentration of ATP (0.004–3 mM) were mixed with 50 μL of assay buffer containing 1 mM EDTA, 10 mM $MgSO_4$ and 0.6 mM luciferin in 25 mM HEPES (pH 7.8). The reaction was initiated by adding 10 μL of enzyme (10 μg/mL) and light emission was recorded. Kinetic parameters were calculated from Lineweaver-Burk plots using Origin 7.5 software (Origin Lab, USA).

2.4. Measurement of thermostability

To study the half-lives of thermal inactivation of the luciferase variants, the purified luciferase (10 μg/mL) in 50 mM Trsi-HCl buffer containing 100 mM NaCl, 2 mM EDTA, 1 mM DTT in, PH 7.8 were incubated at 35 °C for different time intervals from 0 to 60 min, and then cooled on ice for 10 min. Their enzyme activities were assayed under room temperature (25 °C) as described above and the remaining activity was recorded as percentage of the original activity. The data were fitted to first-order plots and analyzed, with the first-order rate constant (k_d) measured by linear regression of ln (*remaining activity*) versus the incubation time (t). The time required for the residual activity to be reduced half ($t_{1/2}$) of the luciferase variants at 35 °C was calculated by the equation, $t_{1/2} = \ln 2/k_d$.

The thermal activities of luciferase variants were evaluated by measuring residual activity at different temperatures. The enzyme solutions (10 μg/mL) were incubated from 25 to 50 °C for 5 min and then cooled on ice for 10 min. The enzyme activities were assayed at room temperature.

The melting temperature (T_m) of the luciferase variants was measured by circular dichroism (CD) measurements using a J-810 spectrometer, USA, equipped with a Julabo temperature control system.