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Implication of Disulfide Bridge Induced Thermal Reversibility, Structural and Functional Stability for Luciferase

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Abstract

Firefly luciferase is a relatively unstable protein and commonly loses its activity at room temperature because of structural changes. The structural and functional stability of this protein is critical for its enzymatic applications. Different approaches are applied to increase the stability of this enzyme such as designing of covalent cross-links (disulfide bonds). In this study, luciferase mutants containing one or two disulfide bonds were compared to the native protein for their fortheir structural, thermodynamic, and functional properties. Mutant forms of *P. Pyralis* luciferase A²⁹⁶C-A³²⁶C and A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C were used. Thermodynamic and biophysical studies were carried out using UV-Vis, fluorescence, circular dichroism, luminescence spectroscopy and differential scanning calorimetry (DSC). We observed that both mutant forms of the protein were more stable than the wild-type enzyme. However, the single disulfide bond containing mutant was structurally and functionally more stable than the mutant protein containing two disulfide bonds. Furthermore, the enzymatic activity of the single disulfide bond containing mutant protein was 7-folds greater than the wild type and the double disulfide bond proteins. The A²⁹⁶C-A³²⁶C mutation also increased the reversibility and disaggregation of the protein. The enhanced activity of the single disulfide bond mutant protein was contributed to the expansion of its active site cleft, which was confirmed by bioinformatics tools.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

Keywords

Bioluminescence; differential scanning calorimetry; disulfide bridge mutants; luciferase; stability; thermal reversibility

INTRODUCTION

Firefly luciferase catalyzes oxidative decarboxylation of luciferin (LH₂) in the presence of Mg²⁺, O₂ and ATP which leads to generation of light during a multi-step reaction. Initially luciferin and ATP react with luciferase and produce a triplet complex. An anhydride acid is created between carboxyl group of luciferin and AMP (luciferinadenylate) that causes the release of pyrophosphate. During some intermediate steps the adenylate is oxidized into cyclic dioxetanone peroxide. Decarboxylation of the produced peroxide leads to the appearance of excited oxyluciferin which generates a photon while returning to its basic state [1]. Quantum yield of this reaction is the highest among known bioluminescence systems compared with the amounts of utilized luciferin. Although the used luciferin in insects is the same, the emitted wavelengths are different from green 530 nm to red 635 nm determined by the enzyme sequence [2]. Recently, great amount of knowledge has been gained about firefly luciferin structure in the absence of substrate with crystallography techniques. This enzyme consists of a large N-terminal domain (1-436) which is connected to a small C-terminal domain (440-550) with a flexible loop (436-440) [3]. During bioluminescence reaction enzyme structure incurs a considerable conversion in which the N- and C-terminals approximate towards each other to hold the substrate like a sandwich, and N- and C-terminals form a multilayer aBaBa and B+a structure, respectively. The surfaces of two domains lie upon each other and generate a crevice containing some conserved amino acids, which are assumed to play a role in the active site [4].

Bioluminescence has varieties of applications in molecular biology studies, medicine, design of biological sensors, and reporters. Some of these applications are including reporter systems [5, 6], high sensitive recognition of ATP in microbial contaminations [7], *in-vivo* studies on protein interactions, marker in gene transformations and gene therapy, and vast use in *in-vivo* nonaggressive diagnostic and therapeutic methods for tumors and metastatic cancers [8, 9]. Bioluminescence based methods have many advantages compared with fluorescence ones, such as higher sensitivity, less complexity, low aggressiveness, less background light in biological studies, and easiness of assay. Being a multipurpose, rapid and reversible protein makes this enzyme a unique protein for biological and medical studies.

Firefly luciferase is rather unstable and commonly loses its activity at room temperature. This is mainly attributed to structural alterations forced by the appearance of inner hydrophobic areas of the protein, which impacts the analytical assay procedures [10-13]. Consequently structural and functional stability of this enzyme is critical for its application. Many studies have been carried out to overcome such problems, specifically the thermostability of luciferase [14-16]. Adding luciferase stabilizing compounds like glycerol, bovine serum albumin, and P-mercaptoethanol only partly overcome these instabilities [17,

18]. Having many industrial and diagnostic applications, many mutations of luciferase have been studied for improvements of structural and catalytic characteristics [10-13].

Luciferase is a proxysomal enzyme and enters into the proxysome of firefly lantern cells after translation [19]. Keller *et al* presented the translocation of expressed luciferase into proxysomes of mammalian cells [20]. It is assumed that despite the presence of sulfhydryl residues in the protein, generation of disulfide bonds are blocked because of reducing condition of cytosol. Another report determined the unique presence of a luciferase enriched with cysteine residues secreted by a marine copepod [21]. This enzyme is capable of generation of disulfide bonds that are essential for its activity.

Increasing the stability of proteins and enzymes is a goal of protein engineering. There are different approaches to accomplish this goal such as increasing the weak intermolecular interactions like hydrogen bonds, hydrophobic interactions, and polar interactions which are caused by covalent forces. The decreasing of probable unfolding conformations of the protein with substitution of glycine for other amino acids like proline, or design of covalent bonds like disulfide bonds have been also performed [22]. It is assumed that disulfide bonds stabilize proteins and their omission leads to a decrease in thermostability. Thus, stabilizing effects of disulfide bonds are established upon configuration entropy in which configurations for unfold protein is decreased and resulting free formation energy is increased [23]. Many disulfide bonds are generated in different proteins by site directed mutagenesis [24-27]. Some mutations were stable, while others were not [25-28]. Instability of such proteins depends on multiple factors, and the main factor is special hindrance pressure on the folded protein. This is usually caused by unsuitable sites for mutations [29, 30].

The reversibility of an enzyme is critical for function and stability of the protein. There are different factors that cause irreversible denaturation of proteins such as size and number of domains, secondary structure content and composition within domains, interaction of domains, conformation of domains, and sequence similarity in domains. Some side reactions occur during denaturation that makes the irreversible form of the protein. One side reaction may be aggregation. For instance yeast hexokinase B, in which aggregation causes the irreversibility of this enzyme [31]. Here we implicated the disulphide bridges in the modulation of luciferase structure and function. Using the MODIP server [32] and also we investigated the effects of disulfide bonds in stability, thermal reversibility, and activity of two mutant forms of firefly luciferase compared with the native enzyme.

METHODS

Protein Expression and Purification

All mutant sequences were confirmed by DNA sequencing. The selected mutants were constructed and successfully expressed in *E. coli* (origami 2). Five milliliters of TB medium containing 50 mgml⁻¹ kanamycin and 12.5 mgml⁻¹ tetracycline with a fresh bacterial colony harboring the desired expression plasmid was inoculated and grown at 37°C overnight. Cultures were diluted in fresh medium and grown at 37°C with vigorous shaking until the OD₆₀₀ reached 0.9. The IPTG and lactose were then added to the cultures at a final concentration of 1 mM and 4 mM, respectively, and incubated at 22°C overnight with

vigorous shaking. The cells were harvested by centrifugation at 5,000 xg for 15 min. The cell pellets were resuspended in lysis buffer: 50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF (add fresh) at pH 8.0. The purification of His₆-tagged fusion protein was performed using the Ni-NTA spin columns as recommended by the manufacturer (Qiagen) [33]. The mutant luciferases with one and two additional disulphide bridges have been reported earlier [34, 35]. The thiol content of these proteins is display in Table 1.

Enzyme Dialysis

Luciferase solutions should be dialyzed because of high concentration of glycerol and other salts in the elution buffer. The protein solutions were dialyzed before performing experiments. Imidazole is one of the most undesirable salts in the solution interfering with protein function. Dialysis was performed overnight at 4°C with continuous stirring 120-200 rpm.

Bioluminescence Emission Spectra

Bioluminescence spectra were recorded from 400 to 700 nm using a Cary-Eclipse fluorescence spectrometer (Varian). For this recording, 100 µl of solution of purified luciferase was added to 300 µl of 50 mM Tris-base buffer (pH 7.8) in a quartz cell. The buffer mixture contained 2 mM ATP, 5 mM MgSO₄ and 1 mM luciferin as substrate. Conditions of using this recording were 25°C and pH 7.8. During the time course of each measurement no significant loss of light was observed. The slit width and scan rate were adjusted to optimize instrument response.

Specific Activity

Specific activity carried on a luminometer Cary-Eclipse luminescence spectrometer (Varian). Five µl of purified enzyme was added to 5 µl of buffer mixture and results reported as RLU⁻¹ mg⁻¹ for activity measurements. The concentration of enzymes was determined by Bradford method.

Fluorescence Measurements

Fluorescence studies were carried out by a spectrophotometer Varian 100 Bio-Model. Intrinsic fluorescence was determined using 20 µg ml⁻¹ luciferase, with excitation wavelength 295nm, slit emission 10 nm, and slit excitation 10 nm. Emission spectra were recorded at 300 to 700 nm. Extrinsic fluorescence was studied using 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic fluorescent probe. The concentration of ANS was 9.8 µM, and the molar ratio of protein to ANS was 1:30. Emission of ANS was recorded at 400-650 nm, and excitation wavelength was 350 nm.

Circular Dichroism Measurements

Study of firefly luciferase was done at far-UV CD by Aviv circular dichroism spectropolarimeter model 215. Protein concentration was 2 mg ml⁻¹ in 20 mM Tris-base buffer (pH 7.8) using a cell with 1 mm path length. The background was corrected against

buffer blank, and all spectra were collected from 200 to 260 nm. For calculation of molar ellipticity, 62 kDa was used for molecular weight using the following equation:

θ_{obs} is the observed ellipticity in the defined wavelength, c is protein concentration (mg/ml) and L is the distance of light (cm). We used CDNN software for computing the percent of secondary structure.

$$[\theta]_{\lambda} = (100 \times (MRW) \times \theta_{obs} / cl)$$

Thermal Aggregation

Time periods of thermal aggregation were performed for luciferase by measuring absorbance at 360 nm in Cary-100 Bio Varian spectrophotometer using a quartz cuvette with 10 mm path length. Temperatures were controlled within ± 0.1 °C by a Cary temperature controller. Aggregation was followed for 85 min at 40°C.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed on Nano-DSC π with two constant cells with a volume of 328 μ l. The pressure of 2 atmospheres was adjusted for preventing bubble formation. For reversibility measurements, the samples were heated at a rate of 2°C per min from 25°C to 90°C. The scan performance was then done when the sample thermogram passed the T_m (melting point) by 2°C. The samples were rescanned when they became cold. All samples were normalized by subtracting baseline thermogram using Cpcalc software.

Modeling of 3D Protein Structure Using Modeller

In this study, homology modeling was used as the best way for 3D structural modeling of the target enzyme and their site directed mutants. Version 9.9 of Modeller (1) used as 3D modeling machine and for S-S bonds, we used our own written Python script. As a template for homology modeling firefly luciferase (PDB ID: 1BA3) was used.

Measuring Intra Molecular Distance and Manipulating 3D Protein Structures

DeepView/Swiss-PDB Viewer and pymMOL Viewer softwares were used for measuring the distance between atoms of selected residues of different domains. The Discovery Studio 2.5 (Accelrys Company) was used as a tool for manipulating 3D structures.

Investigation of Thermodynamic Feature by HAMDAM Software

Many of the structural aspects of proteins were computed by using HAMDAM software version 1.2 [36]. The software predicts various structural and bio-thermodynamic features of query protein just by using amino acid sequence information. Briefly, HAMDAM scans the protein sequence via sliding windows recognizer and using standard tables of amino acid properties. The represented values of parameters were computed by HAMDAM for current query sequences are qualitative (dimension-less) and quantitative interpretations require higher levels of computation.

RESULTS

Expression and purification of native and mutant luciferases were performed as previously reported [1, 2]. The selected mutants (A²⁹⁶C-A³²⁶C, and A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C) were successfully expressed in *E. coli* (origami 2). Wild type and all mutant luciferases were similar with respect to expression levels and yields of purification. The protein expression yields for wild type and all mutants were the same. The purification of His₆-tagged fusion luciferases was performed by affinity (Ni-NTA Sepharose) chromatography. The purified wild type and mutant luciferases had purities more than 95% on the basis of the SDS-PAGE analysis in which luciferases were present as a band of 62 kDa (Fig. 1).

Measuring Bioluminescence Emission Spectra

The *in vitro* bioluminescence spectra of the wild type and disulfide mutants of luciferase were measured at pH 7.8 and 25°C. As reported earlier, the wild type and mutant forms of luciferase exhibited a single peak at 560 nm, A²⁹⁶C-A³²⁶C mutant at 570 nm, and A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C mutant at 590 nm, respectively (data not shown). Results showed that the mutant forms exhibited a red shift compared with the wild type form. Thus, introduction of disulfide bridges resulted in a red shift in the mutant forms of the enzyme.

Specific Activity

Measurements of specific activity indicated that A²⁹⁶C/A³²⁶C mutant displays 7.2-folds greater activity compared with the wild type, and A²⁹⁶C/A³²⁶C-P⁴⁵¹C/V⁴⁶⁹C mutant showed a 40% decrease in activity compared to the wild type of enzyme (Table 2).

Intrinsic Fluorescence

In this study, for removing the effect of tyrosine and phenylalanine, excitation of enzyme was performed at 295 nm. Luciferase has 2 Trp residues in its N-terminal domain; Trp 417 and Trp 426. Our results showed that the wild type luciferase displays a single peak in fluorescence spectrum with a maximum at 340 nm. In the mutant forms of luciferase emission intensity increased without red or blue shift compared with wild type protein. However, A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C mutant compared with A²⁹⁶C-A³²⁶C mutant showed an increase in emission intensity when compared to wild type enzyme. These results indicated that polarity of microenvironment in disulfide bridge mutants was changed, and resulted in the alteration of structure and solvent exposure of Trp residues of luciferase in the mutant forms.

ANS Fluorescence

The 8-anilino-1-naphthalene sulfonate (ANS) is a hydrophobic dye in aqueous solution and bind with high affinity to solvent exposed hydrophobic patches on proteins. Our results showed that the ANS fluorescence of the wild type and mutant forms was different, and fluorescence intensity in the mutant forms was less than the wild type form (Fig. 2). Thus, indicating that the hydrophobic patches in mutant forms of luciferase were less accessible on the protein surface compared to the wild type protein. Thus, disulfide bonds may make a more compact structure where the hydrophobic patches are more hidden compared to native luciferase structure

Circular Dichroism (CD)

Far UV-CD spectra of the wild type and mutant forms of luciferase were performed in Tris-base buffer (20 mM, pH 7.8 at 25°C). These results indicated that the secondary structure content of the disulfide luciferase mutants were different from the wild type enzyme, and A²⁹⁶C-A³²⁶C mutant contained more alpha-helix structure compared with the wild type protein (Table 3).

Enzyme Aggregation

The enzyme aggregation experiments were performed by monitoring absorbencies at 360 nm after 50 min incubation at 40°C using UV-VIS spectrophotometry. These results indicated that A²⁹⁶C-A³²⁶C mutant aggregated less than the other forms of the protein (Fig. 3).

Differential Scanning Calorimetry

Figure 4a,b,c shows excess heat capacity thermogram of wild type without reversibility in heat denaturation behavior, while denaturation for A²⁹⁶C-A³²⁶C and A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C mutants brought about by 55% and 39% reversibility in heat denaturation, respectively. Table 4 shows the thermodynamic parameters of wild type, A²⁹⁶C-A³²⁶C, and A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C mutant luciferases. These data show the significant difference of free energy (ΔG) between wild type and mutant form of luciferase. The maximum stability belonged to A²⁹⁶C-A³²⁶C mutant luciferase, and the wild type had the lowest amount. The A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C mutant luciferase showed a moderate stability (see T_m in Fig. 4). These results were compatible with the aggregation studies and confirmed each other. Denaturation of wild type form was irreversible, and mutant forms containing disulfide bridges showed more reversibility upon denaturation.

Comparison of Predicted 3D Structural Model of the Two Mutants with Native Luciferase

As a result of measuring distance between selected residues in C- and N-terminal domains of the wild type enzyme and two mutant forms of firefly luciferase, it was observed that the distance between C-terminal and N-terminal domains in the active site cleft in 3D structure of protein with four mutations and two new S-S bonds (A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C), was shorter than the distance for the wild type 3D structural model of the target enzyme. However, the distance between C-terminal and N-terminal domains in the active site cleft in 3D structural model of protein with two mutations and one new S-S bond (A²⁹⁶C-A³²⁶C) was greater than the wild type form of the target enzyme. As a result of thinning the cleft between N and C terminal domains in mutant enzyme with two new S-S bonds, the entering of substrate would be harder than the wild type form. However, this cleft for the mutant with one new S-S bond was expanded with faster substrate exchange. This hypothesis was compatible with experimental data. As a result of experimental observations, activity of the mutated derivative with one S-S bond was increased and the activity for mutant with two S-S bonds was decreased. Measured distances between residues in N- and C-terminal domains for 3D structural models of the wild type enzyme and two mutants are summarized in Table 5. Thus, the observed differences between the two mutants could be resulted from differences in S-S bonds. None of the S-S bonds were located between N and C terminal

domains. However, under these conditions, S-S bonds could alter the distance between C and N-terminal domains (Table 5).

Thermodynamic Feature by HAMDAM Software

The results obtained from HAMDAM software indicated a good agreement with experimental results (Table 6).

DISCUSSION

The disulfide bridge is one of the best strategies for increasing thermostability and improving luciferase function using site directed mutagenesis [35]. Here we investigated two mutants which contained a disulfide bridge (A²⁹⁶C-A³²⁶C) in the vicinity of active site [34]. Creation of a disulfide bridge in the A²⁹⁶C-A³²⁶C mutant resulted in more secondary structure and compactness of the enzyme. Mutation of this position increased the thermostability of luciferase. Thus, incorporation of a disulfide bridge in the vicinity of active site not only increased the function but also increased the stability at higher temperatures. These results are consistent with the ability of disulfide bridges improving the stability with decreasing configurational entropy of the unfolded state.

The fluorescence studies indicated that both mutant forms of luciferase had less hydrophobic accessible area than the wild type protein. Thus, lower hydrophobic patches may indicate more rigidity of the mutant proteins' structure. In addition, A²⁹⁶C-A³²⁶C mutant form was the most compact form compared with P⁴⁵¹C-V⁴⁶⁹C and A²⁹⁶C-A³²⁶C mutant. Thus, more alpha structures were created in the single disulfide bond mutant form. The fluorescence study also verified these results. Thus, the microenvironment of Trp residues were altered with the incorporation of disulfide bridges, and Trp residues moved towards the hydrophobic environment. Thus, they are not accessible for ANS binding resulting in lower ANS fluorescence. These results are in agreement with the fluorescence spectra.

To study thermostability, the aggregation test and differential scanning calorimetry evaluations were carried out. The DSC data showed that the single disulfide bridge mutant form was more stable than other forms of the enzyme. Unfolding process at higher temperatures takes longer times which are related to conformational stability created by disulfide bridges. Thus, A²⁹⁶C-A³²⁶C and A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C mutants are more stable than the wild type luciferase.

CONCLUSION

Our results indicate that the existence of two disulfide bridges caused compaction of luciferase structure. Thus, proper distance between N-terminal and C-terminal is required for functioning of the enzyme. The changes in this distance decreased the activity of luciferase. However, the created pressure by introduction of two disulfide bridges altered this distance and decreased activity. Conformational distortion may create new interactions generating intermediates between folding and unfolding states. Thus, A²⁹⁶C-A³²⁶C/ P⁴⁵¹C-V⁴⁶⁹C mutant form is less thermostable and less reversible than the A²⁹⁶C-A³²⁶C mutant form, which was verified by aggregation tests. The decrement of aggregation is one of the

strategies for increasing the reversibility. It is proposed that creation of two disulfide bridges in the A²⁹⁶C-A³²⁶C/ P⁴⁵¹ C-V⁴⁶⁹ mutant form, causes compaction that makes a more rigid conformation, impacting function and thermostability. The more reversibility for A²⁹⁶C-A³²⁶C mutant is compatible with more disaggregation, and this mutant form is more stable, reversible, and functional form of the enzyme.

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Biography

Author profile: Professor Ali A. Moosavi-Movahedi is a professor at the University of Tehran. He obtained his Ph.D. focusing on thermodynamics of catalase -surfactant interactions. He remains involved in study of protein structure function relationship. In addition to this work, He has worked on the structure and function of bioactive peptides and proteins.

REFERENCES

- [1]. Branchini BR, Ablamsky DM, Murtiashaw MH, Uzasci L, Fraga H, Southworth TL. Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem.* 2007; 361:253–262. [PubMed: 17181991]
- [2]. Seliger HH, McElroy WD. Pectral emission and quantum yield of firefly bioluminescence. *Arch. Biochem. Biophys.* 1960; 88:136–141. [PubMed: 14444706]
- [3]. Wet J, De R, Wood KV, Helinski DR, DeLuca M. Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 1985; 82:7870–7873. [PubMed: 3906652]
- [4]. Conti E, Franks NP, Brick P. Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure.* 1996; 4:287–298. [PubMed: 8805533]
- [5]. Gu MB, Mitchell RJ, Kim BC. Whole-cell-based biosensors for environmental biomonitoring and application. *Adv. Biochem. Eng. Biotechnol.* 2004; 87:269–305. [PubMed: 15217109]
- [6]. Naylor NH. Reporter gene technology: the future looks bright. *Biochem.Pharmacol.* 1999; 58:749–757. [PubMed: 10449183]
- [7]. Spielman H, Jacob-Muller U, Schulc P. Simple assay of pmol of ATP, ADP and AMP in signal somatic cells using purified luciferin luciferase. *Anal. Biochem.* 1981; 113:172–176. [PubMed: 7270882]
- [8]. Contag CH, Spilman SD, Contag PR, Oshiro M, Eames B, Dennery P. Visualizing gene expression in living mammals using a bioluminescent reporter. *Photochem. Photobiol.* 1997; 66:523–531. [PubMed: 9337626]

- [9]. Baggett B, Roy R, Momen S, Morgan M, Tisi L, Morse D, Gillies RJ. Thermostability of firefly luciferases affects efficiency of detection by *in vivo* bioluminescence. *Mol. Imaging*. 2004; 3:324–33. [PubMed: 15802049]
- [10]. Riahi-Madvar A, Hosseinkhani S. Design and characterization of 55 novel trypsin-resistant firefly luciferases by site-directed mutagenesis. *Protein Eng., Des. Sel.* 2009; 22:655–663. [PubMed: 19661129]
- [11]. White PJ, Squirrell DJ, Arnaud P, Lowe CR, Murray JAH. improved thermostability of the North American firefly luciferase: Saturation mutagenesis at position 354. *Biochem. J.* 1996; 319:343–350. [PubMed: 8912666]
- [12]. Law GHE, Gandelman OA, Tisi LC, Lowe CR, Murray JAH. Mutagenesis of solvent-exposed amino acids in *Photinuspyralis* luciferase improves thermostability and pH-tolerance. *Biochem. J.* 2006; 397:305–312. [PubMed: 16551268]
- [13]. Tisi LC, White PJ, Squirrell DJ, Murphy MJ, Lowe CR, Murray J. Development of a thermostable firefly luciferase. *Anal. Chim. Acta.* 2002; 457:115–123.
- [14]. Tafreshi NK, Hosseinkhani S, Sadeghizadeh M, Sadeghi M, Ranjbar B, Naderi-Manesh H. The influence of insertion of a critical residue (Arg356) in structure and bioluminescence spectra of firefly luciferase. *J. Biol. Chem.* 2007; 282:8641–8647. [PubMed: 17197704]
- [15]. Moradi A, Hosseinkhani S, Naderi-Manesh H, Sadeghizadeh M, Alipour BS. Effect of charge distribution in a flexible loop on the bioluminescence color of firefly luciferases. *Biochem. J.* 2009; 48:575–582.
- [16]. Hongwei L, Jin zhong L, Gregory AH, Dongfeng P. Non-invasive imaging of firefly luciferase reporter gene expression using bioluminescence imaging in human prostate cancer models. *Biotechnol. Appl. Biochem.* 2007; 46:179–184. [PubMed: 17073822]
- [17]. Arakawa T, Timasheff SN. The stabilization of proteins by osmolytes. *Biophys. J.* 1985; 47:411–414. [PubMed: 3978211]
- [18]. Mehrabi M, Hosseinkhani S, Ghobadi S. Stabilization of firefly luciferase against thermal stress by osmolytes. *Int. J. Biol. Macromol.* 2008; 43:187–191. [PubMed: 18555523]
- [19]. Gould SG, Keller GA, Subramani S. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.* 1987; 105:2923–2931. [PubMed: 3480287]
- [20]. Keller GA, Gould S, Deluca M, Subramani S. Firefly luciferase is targeted to peroxisomes in mammalian cells. *Proc. Natl. Acad. Sci.* 1987; 84:3264–3268. [PubMed: 3554235]
- [21]. Stepanyuk GA, Xu H, Wu C-K, Markova SV, Lee J, Vysotski ES, Wang B-C. Expression, purification and characterization of the secreted luciferase of the copepod *Metridia longa* from Sf9 insect cells. *Protein Expr. Purif.* 2008; 61:142–148. [PubMed: 18595733]
- [22]. Clarke J, Hounslow AM, Bond CJ, Fersht AR, Daggett V. The effects of disulfide bonds on the denatured state of barnase. *Protein Sci.* 2000; 9:2394–2404. [PubMed: 11206061]
- [23]. Betz SF. Disulfide bonds and the stability of globular proteins. *Protein Sci.* 1993; 2:1551–1558. [PubMed: 8251931]
- [24]. Mitchinson C, Wells JA. Protein engineering of disulfide bonds 90in subtilisin BPN'. *Biochemistry.* 1989; 28:4807–4815. [PubMed: 2504281]
- [25]. Matsumura M, Becktel WJ, Levitt M, Matthews BW. Stabilization of phage T4 lysozyme by engineered disulfide bonds. *Proc. Natl. Acad. Sci. USA.* 1989; 86:6562–6566. [PubMed: 2671995]
- [26]. Yutani K, Ogasahara K, Sugino Y. Effect of amino acid 95substitutions on conformational stability of a protein. *Adv. Biophys.* 1985; 20:13–29. [PubMed: 3914832]
- [27]. Li Y, Coutinho PM, Ford C. Effect on thermostability and catalytic activity of introducing disulfide bonds into *Aspergillusawamoriglucoamylase*. *Protein Eng. Des. Sel.* 1998; 11:661–667.
- [28]. Liu HL, Ford C, Reilly PJ. Mutations to alter *Aspergillusawamoriglucoamylase* selectivity. IV. Combinations of Asn20→Cys/Ala27→Cys, Ser30→Pro, Gly137→Ala, 311→314loop, Ser411→Ala and Ser436→Pro. *Protein Eng. Des. Sel.* 1999; 12:163–172.
- [29]. Betz SF, Marmorino JL, Saunders AJ, Doyle DF, Young GB, Pielak GJ. Unusual Effects of an Engineered Disulfide on Global and Local Protein stability. *Biochem. J.* 1996; 35:7422–7428.

- [30]. Mansfeld J, Vriend G, Dijkstra BW, Veltman OR, Van den Burg B, Venema G, Ulbrich-Hofmann R, Eijssink VG. Extreme Stabilization of a Thermolysin-like Protease by an Engineered Disulfide Bond. *Biochem. J.* 1997; 272:11152–11156.
- [31]. Strucksberg KH, Rosenkranz T, Fitter J. Reversible and irreversible unfolding of multi-domain proteins. *Biochim. Biophys. Acta.* 2007; 1774:1591–1603. [PubMed: 17964867]
- [32]. Dani VS, Ramakrishnan C, Varadarajan R. MODIP revisited: re-evaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins. *Protein Eng.* 2003; 16:187–193. [PubMed: 12702798]
- [33]. Dombkowski AA. Disulfide by Design™: a computational method for the rational design of disulfide bonds in proteins. *Bioinformatics.* 2003; 19:1852–1853. [PubMed: 14512360]
- [34]. Imani M, Hosseinkhani S, Ahmadian Sh. Nazari M. Design and introduction of a disulfide bridge in firefly luciferase: increase of thermo stability and decrease of pH sensitivity. *Photochem. Photobiol. Sci.* 2010; 9:1167–1177. [PubMed: 20593108]
- [35]. Nazari M, Hosseinkhani S. Design of disulfide bridge as an alternative mechanism for color shift in firefly luciferase and development of secreted luciferase. *Photochem. Photobiol. Sci.* 2011; 10:1203–1215. [PubMed: 21494742]
- [36]. Hadi-Alijanvand H, Rouhani M, Proctor EA, Dokholyan NV, Moosavi-Movahedi AA. A Folding Pathway-Dependent Score to Recognize Membrane Proteins. *PLoS ONE.* 2011; 6(3):e16778. [PubMed: 21390303]

Figure 1.
SDS-PAGE electrophoresis of luciferases. The purified wild type and mutants samples have purities of >95%. 'M' is MW markers.

Figure 2.

(a): ANS fluorescence intensity vs maximum wave-lengths of luciferase. The ANS concentration was 9.8 μM and the molar ratio of luciferase to ANS was 1:30. The ANS emission was recorded at 400-650 nm and the excitation wavelength was 350 nm. (b): Intrinsic fluorescence intensity vs maximum wavelengths were obtained at pH7.8 and 25°C, using 20 $\mu\text{g ml}^{-1}$ luciferase and the excitation wavelength of 295nm. Emission spectra were recorded at 300 to 700 nm. 1) *Ppy*, 2) $\text{A}^{296}\text{C-A}^{326}\text{C/P}^{451}\text{C-V}^{469}\text{C}$, 3) $\text{A}^{296}\text{C-A}^{326}\text{C}$. The slit of emission and excitation was adjusted to 10 nm.

Figure 3.

The aggregation curve of luciferase (absorbance 360 nm vs times) at 40°C in 50 mM Tris-base buffer, pH 7.8. 1) Ppy, 2) A²⁹⁶C-A³²⁶C/P⁴⁵¹C-V⁴⁶⁹C, 3) A²⁹⁶C-A³²⁶C.

Figure 4.

DSC profiles of luciferases. (a): *Ppy*, (b): A²⁹⁶C-A³²⁶C, and (c): A²⁹⁶C-A³²⁶C/p⁴⁵¹C-V⁴⁶⁹C. The protein concentration was 1mg/ml and pressure was adjusted to 2 atm. The thermal reversibility was checked by rescanning the sample which was cooled after warming up about 2°C above the T_m . Curve 1) forward process, Curve 2) reversible process.

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Table 1

Thiol content of various Ppy derivatives are obtained according to OD.412 upon treatment with Ellman's reagent (all the data repeated at least 3 times).

samples	Mol of SH/ mol of protein	
	-DTT	+DTT
<i>Ppy</i>	4	4
A ²⁹⁶ C-A ³²⁶ C	3.91	6.07
A ²⁹⁶ C-A ³²⁶ C/P ⁴⁵¹ C-V ⁴⁶⁹ C	4.02	8.06

Table 2

Specific activity of the wild type and mutants of enzyme

Specific Activity (10^{10} *RLU s ⁻¹ /mg)	Samples
2.7	<i>Ppy</i>
19.6	A ²⁹⁶ C/A ³²⁶ C
1.18	A ²⁹⁶ C-A ³²⁶ C/P ⁴⁵¹ C-V ⁴⁶⁹ C

Table 3

Secondary structure content of the wild type and mutant forms of luciferase

Secondary structure	<i>Ppy</i>	A ²⁹⁶ C-A ³²⁶ C/P ⁴⁵¹ C-V ⁴⁶⁹ C	A ²⁹⁶ C/A ³²⁶ C
α-Helix-	7	8.24	12.61
β(sheets+turns)	63.3	58.09	49.84
Random coil	29.69	33.66	37.53

Table 4

Thermodynamic parameters of the wild type and mutant forms of luciferase

Samples	H _{Tm} (kJ/mol)	S _{Tm} (kJ/mol.K)	T _m (K)	G _{Tm} (kJ/mol)
<i>Ppy</i>	351.12	0.54	315.55	180.218
A ²⁹⁶ C-A ³²⁶ C	623.14	0.299	318.05	527.903
A ²⁹⁶ C-A ³²⁶ C/P ⁴⁵¹ C-V ⁴⁶⁹ C	467.742	0.354	316.35	355.878

Table 5

Distances(Angstrom) between selected residues in N and C terminal domains in the active site cleft for structural model of the wild type luciferase and two mutated derivatives via pymMOLViewer and DeepView/ Swiss-PDB Viewer software.

(A ²⁹⁶ C-A ³²⁶ C)	(A ²⁹⁶ C-A ³²⁶ C/P ⁴⁵¹ C V ⁴⁶⁹ C)	<i>Ppy</i>	Selected residues in the active site cleft
22.89	22.61	22.69	Thr ²⁵¹ , Lys ⁵²⁹
19.72	19.44	19.59	Ile ³⁵¹ , Lys ⁵²⁹
25.23	24.97	25.02	Arg ²¹⁸ , Lys ⁵²⁹
16.24	15.93	16.05	Gly ³¹⁵ , Lys ⁵²⁹
17.17	16.87	16.97	Phe ²⁴⁷ , Lys ⁵²⁹
13.6	12.8	13.4	Gly ³⁴¹ , Lys ⁵²⁹

Table 6

Parameter 1: Mean polarity; the higher value indicates the most hydrophobic core. Parameter 2: Free energies of transfer from water to octanol; the lower value indicates the higher hydrophobicity. Parameter 3: Free energies of transfer from water to a hydrophobic environment-water interface; the lower value indicates more hydrophobic helices in unfold state. Parameter 4: Stability based on residues' ASA; the higher value indicates the higher stability. Parameter 5: non-polar surface area

Parameter 5	Parameter 4	Parameter 3	Parameter 2	Parameter 1	Samples
2456	692	32	909	652	<i>Ppy</i>
2421	702	27	915	684	A ²⁹⁶ C-A ³²⁶ C/P ⁴⁵¹ C-V ⁴⁶⁹ C
2412	707	16	918	701	A ²⁹⁶ C/A ³²⁶ C