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Implication of Arg213 and Arg337 on the kinetic and structural stability of firefly luciferase

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ABSTRACT

Possible roles of two different Arginine (Arg; R) 213 and 337 on kinetic and structural stability of *Photinus pyralis* luciferase have been investigated using thermal and chemical denaturation studies. This enzyme is highly sensitive to protease digestion and temperature, which limits its fieldability, particularly for *in vivo* imaging. In order to generate more stable luciferases against trypsin digestion, site-directed mutagenesis was conducted to block two representative tryptic sites on the surface of N-terminal domain, via substitution of Arg213 and Arg337 by methionine (Met; M) and glutamine (Gln; Q), respectively [A. Riahi-Madvar, S. Hosseinkhani, Protein engineering, design and selection 22 (2009) 655–663]. The improvement of mutant enzymes stability against protease hydrolysis may be attributed to the more rigidity of the enzyme structure upon mutations, as can be deducted from elevated levels of $m_{U\text{-}N}$ values and decrease of activation energy. Furthermore, mutation at position 337 which is accompanied with more alteration on the basic kinetic properties relative to mutation at position 213, revealed the high values of the ΔG_{H_2O}, half-time of inactivation at 30 °C and T_m for R337Q where Arg213 is maintained in structure. Based on the results, it can be concluded that whilst Arg213 affects structural stability, Arg337 is critical for kinetic stability.

1. Introduction

Firefly luciferase catalyzes the oxidation of luciferin in presence of ATP, Mg^{2+} and molecular oxygen to produce photon in the visible range (λ_{max} = 560) with high quantum yield [1–4]. Among various species of firefly luciferases that have been sequenced and crystal structure of them determined [5–9], majority of the studies have been done on *Photinus pyralis* luciferase; which is a single polypeptide chain, containing 550 amino acids. Tertiary structure of this enzyme obtained in the absence of substrate, shows that it folds in a large N-terminal and a small C-terminal domain that are connected together by a short link without any intra- or interchain disulfide bonds on the structure [8]. Although position of the luciferase active site have been suggested that is located around the cleft between two domains and the role of some important conserved residues involving active site have been revealed [10,11], the exact relationship between structure-function for the bioluminescence reaction has not been clearly known [8,12].

The ease in detection, versatility and quick response to transcriptional change [10] made this enzyme a very useful reporter gene in molecular biology [13–16], as well as a reporter protein for *in vivo* bioluminescence imaging [16], which is used in determining the efficiency of gene and drug therapies [17], tumor growth [18] and metastasis [19].

Despite many application of this enzyme in various fields, inherent instability reduces its intracellular half-life and has deleterious effect on luciferase-based assay [20–24]. The most undesirable instability of the luciferase is low thermostability [25] and high susceptibility to protease digestion [26]. In a recent study, site – directed mutagenesis was used to introduce some point mutations on the surface of *P. pyralis* luciferase, in order to generate more stable luciferases against protease hydrolysis. Mutations caused some changes in structural and functional properties that accompanied with improvement in thermostability and resistance against trypsin and chymotrypsin digestion; which was remarkable for both mutants; R213M [Arg213 substituted by Met] and R337Q [Arg337 changed to Gln] [27]. In this study, thermal and chemical denaturation studies were conducted to investigate the roles of R^{213} and R^{337} on kinetic and structural stability of the enzyme.

2. Materials and methods

2.1. Materials

Transformed wild-type (WT) (cloned on pET16b) and mutants (R213M and R337Q) enzymes (cloned on pET28a) into

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Escherichia coli BL21, were kindly provided by Dr. Hosseinkhani (Tarbiat Modares University, Tehran, Iran). As recently reported, the R337Q mutant was accompanied by additional substitution at position of 331 where phenylalanine is converted to leucine [27]. Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin, which were purchased from Sigma. ATP was obtained from Roche and D-luciferin potassium salt from Synchem. Affinity column of Ni-NTA resin for His6-tagged proteins was purchased from Qiagen. All other chemicals were of analytical reagent grade and obtained from Merck.

2.2. Protein expression and purification

Overexpression was carried out in *E. coli* BL21 in the presence of 1 mM of IPTG at 22 °C, the cells were harvested when OD_{600} reaches to 1.3 ± 0.2. Purification of the His6-tagged fusion luciferases was performed by affinity (Ni-NTA-sepharose) chromatography, as described earlier [24]. Except for phosphate buffer (Na_2PO_4) was used instead of Tris–HCl. Purity of the enzymes was analyzed using 12% SDS-PAGE gel electrophoresis. Approximate protein concentrations were calculated using a Bradford assay in which bovine serum albumin (BSA) was used as the standard [28]. Moreover, active form of the enzymes was proven by the light emitted upon addition of substrate to purified luciferases; which could be seen by naked eye in a dark room.

2.3. Kinetic stability measurement

Kinetic stability of enzymes was measured, by comparison of remaining activity of the WT and mutants enzymes after denaturation using urea and heat as denaturant. Enzyme activity was recorded using Sirius tube luminometer (Berthold Detection System, Germany).

2.3.1. Luciferase assay in the presence of urea

Chemical kinetic stability of the purified enzymes was analyzed with concentrations ranging from 0 to 6 M urea at 25 °C (optimum temperature for enzymes activity) and optimum pH for each enzyme (7.8 for WT and R213M and 7.3 for R337Q) in phosphate buffer. The activity was measured by addition of 10 μL of the treated enzymes (at final concentration of 10.0 μg/mL) to 10 μL of substrate solution (10 mM $MgSO_4$, 1 mM luciferin, 2 mM ATP, 50 mM Tris–HCl, pH 7.8, except for R337Q that was 7.3.) in luminometer cuvette. The residual activities were reported as a percentage of the control (urea concentration = 0). C_m for wild-type and mutant luciferases is calculated as the concentration of urea that reduces enzyme activity to 50% of the original one.

2.3.2. Thermal inactivation studies

Ten μg/mL sample of each purified enzymes (in phosphate buffer) were incubated at different temperatures (23, 30 and 37 °C) in a water-bath circulation system for different time intervals. Samples were then removed and cooled on ice (for 2 min); the remaining activity of enzymes was then measured by adding 10 μL substrate solution to 10 μL of each treated enzyme. Activity of the enzyme solution kept on ice was considered as the control (100%). Remaining activity was reported as a percentage of the control activity [29]. Half-time ($T_{1/2}$) of inactivation were calculated as the time needed for decreases of remaining activity to 50% that was obtained from thermal inactivation at 30 °C for 60 min.

2.3.3. Activation energy measurements

To obtain activation energy for wild-type and mutant luciferases, enzymes and substrate solution were incubated for 5 min at temperatures ranging from 20 to 40 °C (in a water-bath circulation system) before the activity was measured. It should be noted, firefly luciferase always emits light in glow form. Activation energy (E_a) was obtained from graphs of the natural logarithm value of each luciferase activity expressed in RUL/S *vs.* $1/T$, known as Arrhenius plot [30,31].

2.4. Structural stability measurement

2.4.1. Protein preparation

Intrinsic fluorescence was determined at final protein concentration of 20 μg ml^{-1} in phosphate buffer; for which the final pH of each sample was adjusted to 7.8 except for R337Q which was set to 7.3. Since imidazole interrupts all fluorescence data, by producing noise throughout the spectra; its elimination is necessary for fluorescence studies. In order to eliminate imidazol, protein solution were exhaustively dialyzed against dialysis buffer solution (50 mM phosphate buffer, 1% glycerol, 1 mM EDTA, 50 mM NaCl, 0.05% β-mercaptoethanol) for 20 h at 4 °C.

2.4.2. Chemical denaturation studies

Chemical denaturation studies and data analysis were carried out as outlined earlier [32,33]. Fluorescence spectra were recorded on Cary-Eclipse luminescence spectrophotometer (Varian) apparatus at 25 °C. Intrinsic fluorescence was determined at final protein concentration 20 μg mL^{-1} in phosphate buffer, using an excitation wavelength of 295 nm and the emission spectra were recorded between 300–400 nm (excitation and emission slits were set to 5 and 10 nm, respectively). Fluorescence spectra of enzymes were recorded after 45 min incubation of enzymes in the final concentration of urea ranging from 0 to 6 M, used as a denaturant. Both the change in fluorescence intensity and the shift in maximum wavelength of fluorescence were recorded to monitor the unfolding transition. Denaturation curves were plotted with the ratio of fluorescence intensities with emission wavelength of λ_{max} (340 nm) of the proteins against the denaturant concentrations; further analysis of the data was performed as described by Pace and co-workers [34,35]. The data obtained from denaturation curves were analyzed to obtain $[Urea]_{50\%}$, $m_{U\text{-}N}$ and ΔG_{H_2O}. In order to evaluate the stability of mutant proteins relative to that of the wild-type form, difference in conformational stability was calculated, using the relationship: $\Delta(\Delta G_{H_2O}) = \Delta G_{H_2O\text{-}WT} - \Delta G_{H_2O\text{-}M}$, where WT and M refer to wild-type and mutant enzymes respectively.

2.4.3. Thermal fluorescence studies

Steady-state fluorescence measurements were conducted with a Cary-Eclipse luminescence spectrophotometer (Varian) with thermostated cell holders where the temperature was kept constant by a circulating water bath. The excitation wavelength was set at 295 nm and emission wavelength of 340 nm was scanned at 5 °C intervals over temperature range of 25–80 °C at final protein concentrations of 20 μg mL^{-1} in phosphate buffer. T_m (°C), as the temperature which fluorescence intensity of Trp reached to half were calculated from the thermal fluorescence curves.

Refolding was followed by monitoring the changes in the intrinsic fluorescence of luciferases (after cooling) at 25 °C. The fluorescence emission was recorded between 300 and 400 nm with an excitation wavelength of 295 nm [36].

2.5. Bioinformatics study

Three dimensional structure models of mutant luciferases were obtained using *P. pyralis* luciferase (PDB code 1LCI) as the PDB template in Swiss Model Alignment Interface protein modeling server [37]. The possible hydrogen (H) bond and salt bridges between R^{213} and R^{337} and other residues in WT and related residues which