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Roles of trehalose and magnesium sulfate on structural and functional stability of firefly luciferase

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ABSTRACT

Firefly luciferase is widely used in many analytical techniques. However, the enzyme is unstable, so that its relative inactivation results in low sensitivity of those techniques. In this study, we have investigated the effects of $MgSO_4$ and trehalose on the structural stability and function of luciferase from *Photinus pyralis* using circular dichroism (CD), conventional and stopped-flow fluorescence spectroscopy and bioluminescence assay. The secondary structural content, compactness and its melting temperature are also studied, which showed that the stability of luciferase increased in the presence of additives. Measurements of refolding rate constants under conditions that favor folding, show that $MgSO_4$ accelerates the folding of enzyme, on the contrary, refolding rate constant decreases in the presence of trehalose which can be attributed to its high viscosity. Finally, combined with remaining activity assay we concluded that magnesium sulfate and trehalose can be used for short- and long-term stabilization, respectively.

1. Introduction

Firefly luciferase from *Photonis pyralis* is a single polypeptide chain of 550 amino acid residues and molecular weight of 62 kDa (E.C.1.13.12.7). This enzyme catalyses monooxygenase reaction in which D-luciferin is converted to oxyluciferin, using ATP, O_2 and also Mg^{2+} as cofactor. This is a two-step process that is accompanied by emission of yellow-green photon of light at 560 nm. First step is followed by the formation of enzyme luciferyl adenylate intermediate. In the second step luciferyl adenylate reacts with molecular oxygen, producing enzyme-bond excited state product. This excited state product decays to ground state by emitting light [1–3]. The crystal structures of firefly luciferases from *P. pyralis* and Japanese firefly (*Luciola cruciata*) have been obtained [1,3]. Luciferase from *P. pyralis* folds into two globular domains, a large N-terminal domain consisting of residues 4–435 and C-terminal domain consisting of residues 44–544. N-terminal can be further divided into three sub-domains, A–C. N-terminal domain comprising a β-barrel and two β-sheets, which arranged between three α-helices, forming αβαβα structure, C-terminal domain consists of three α-helices and five β-strands [1,2]. There is a linker between two domains, connecting residues 435 and 441 [2]. This enzyme is well known as a fundamental agent in several research areas. Some of its applications are: ATP-assay with high sensitivity in bacterial contamination, using as a reporter gene [4], pyrosequencing [5] and bioluminescence imaging [6].

Firefly luciferase is relatively unstable and its activity decreases at room temperature significantly. Also irreversible aggregation due to the exposure of its hydrophobic sites followed by structural changes, causes its further inactivation [7–9]. Obviously, this phenomenon can affect the sensitivity of analytical assays performing by luciferase. So, the structural stabilization of enzyme is essential for retaining its application.

It seems that site directed mutagenesis strategy is a good means for this purpose, however, there are reports that some mutant proteins with higher structural stability show lower activity relative to that of native protein and *vice versa* [10–13]. Therefore, structural stabilization of enzyme should be done to improve function at extreme environment. In other words, it was shown that sucrose; sorbitol and proline as additives can stabilize the structure and function of firefly luciferase against thermal stress [14]. In addition, the use of additives for stabilization of different kinds of enzymes has been recommended [15–17].

Surface free energy increase is the main proposed mechanism of the mentioned stabilizers. So, they can increase the surface free energy of protein in solvent. As the protein–solvent interface increases upon denaturation, the surface free energy would increase due to the surface increment which is unfavorable.

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Therefore the equilibrium of "Native ↔ Unfold" move toward the favorable energy, to the native form [18].

Trehalose is a non-reducing disaccharide, composed of two D-glucopyranose and is present in some organisms. It has interesting physico-chemical properties which draw researchers' attention [19]. It can be found in insects' hemolymph [20], some desert plants [21], yeast and fungi [20,22]. It has been shown that trehalose can increase the stability of folded state of proteins against conditions that favor denaturation [23–26]. Furthermore, it has cryoprotective property in some freeze-tolerant organisms [23,27]. It also can protect the structure of membrane structure in dry state [27].

$MgSO_4$ acts as a stabilizer in which its stabilization property determined by a competition phenomenon between two factors, "salt exclusion and salt binding effect". Increasing the surface tension of solvent results in salt exclusion of SO_4^{2-} (preferential hydration) whereas salt binding effect refers to Mg^{2+} affinity for ionic residues and peptide bonds. The extent of change in preferential hydration during denaturatrion of protein is determined by a delicate balance between these factors. In other words, as protein–solvent interaction increases during denaturation, these factors increase concomitantly, but dominant factor is the high exclusion of SO_4^{2-} ions, which yield in protein stabilization [18,28].

In present study, we have examined the effects of two different kinds of additives ($MgSO_4$ as a salt and trehalose as a sugar) on the structural stability and function of firefly luciferase using circular dichroism (CD), conventional and stopped-flow fluorescence spectroscopy and bioluminescence assay.

2. Materials and methods

2.1. Reagent preparation

2.1.1. Buffers and chemicals preparation

Affinity column of Ni-NTA resin for His_6-tagged proteins was purchased from Qiagen. Tris, KH_2PO_4 and K_2HPO_4 were purchased from CarloErba, glycerol, $MgSO_4$, trehalose, β-mercapto ethanol, ammonium sulfate, imidazole were purchased from Merck. Dialysis membrane, di-thio erytheritol (DTE) and guanidine hydrochloride (GdmCl) were purchased from Sigma. Phenyl methyl sulfonyl fluoride (PMSF) from Boehrienger and NaCl from Panreac. Buffers for affinity column were prepared according to the Qiagen manual: Washing buffer: NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 20 mM, pH 8.0. Elution buffer: NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 250 mM, pH 8.0. Lysis buffer: NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 10 mM, pH 8.0. Dialysis buffer comprising: Tris–HCl buffer, pH 7.8, NaCl 150 mM, DTE 1 mM, β-mercapto ethanol 1 mM, ammonium sulfate 1 mM and glycerol 5% (w/v). Substrate solution: tricine–NaOH buffer 50 mM, ATP solution 4 mM, luciferine solution 2 mM, $MgSO_4$ solution 10 mM, pH 7.8 [7,14].

2.1.2. Additive preparation

Two additives dissolved in dialysis buffer at pH 7.8, as additive stocks: $MgSO_4$ at 2.4 M and trehalose at 2.0 M.

2.1.3. Enzyme preparation

Firefly luciferase from *P. pyralis* prepared in our laboratory. Its gene was cloned into pET-16b vector with a His_6-tagged and transfected to host bacterium *Escherichia coli* BL21. After over-expression, firefly luciferase was purified by affinity chromatography "Ni-NTA" resin [29]. All the steps in preparing and purification, handled in ice carefully. After addition of 20–30% glycerol to the protein solutions, a sample of 50–70 μl from each fraction, stored for further analysis, such as Bradford assay for protein concentration assay [30] and SDS-PAGE for its purity verification. Analysis showed that they had purity of more than 95% (data not shown). The enzymes were stored at −20 or −80 °C freezer.

2.1.4. Enzyme dialysis

Before all the experiments, luciferase should be dialyzed due to high concentration of glycerol and other salts present in elution buffer. One of the most unfavorable salts in the solution is imidazole, interrupting all the far-UV circular dichroism data, by producing noises through the spectra. Dialysis has done two times for 8–10 h at 4 °C in 1 l dialysis buffer and constant stirring 120–200 rpm.

2.2. Methods

2.2.1. Measurements of bioluminescent kinetics

2.2.1.1. Remaining activity measurements. Bioluminescence activities are measured by Sirius tube luminometer, connected to a PC, in which data were analyzed on its own software (Berthold Detecton System, Germany). Bioluminescence was measured by mixing of 100 μl enzyme solution (6 μg/ml) with 50 μl of substrate solution, reported in RUL/S (relative light unit per second). Remaining activities of luciferase were measured at different time intervals in time course of 60 min in the absence and presence of additives at different concentrations (0–1.2 M) at 35 °C. Luciferase was incubated with different concentrations of additives for 5 min, and then tubes were placed in water-bath circulation system in order to control the temperature easily. At regular intervals samples were removed and placed at 25 °C for 2 min then the remaining activity were measured. Remaining activities were calculated using percentages of Initial activity of enzyme at 25 °C as control (100% activity), as reported earlier [7,14,31].

2.2.1.2. Activation energy measurements. Activities of enzyme in the presence of additives at constant concentration of enzyme (8 μl/ml) and additives (0.7 M), pH 7.8, were measured. Temperature was varied from 20 to 45 °C. Luminometer cuvettes were pre-loaded with substrate solution, placed into water-bath for 5 min at the same degree for luciferase incubation, which means each assay measured at the same temperature for incubated luciferase. In order to obtain activation energy, natural logarithm of luciferase activity in RUL/S against 1/T, plotted in a graph, called Arrhenius plot [32,33]. Also, thermal sensitivity of the luciferase was determined by incubating luciferase at 20–45 °C and its activity was measured.

2.2.1.3. Circular dichroism measurements. Spectropolarimetry study of firefly luciferase has done at both far-UV and near-UV CD regions, for secondary and tertiary structural studies, respectively, by JASCO Spectropolarimeter J-715. Data analysis and smoothing performed on its own software J-715 using fast Fourier-transform enabling users to reduce and smooth the noisy spectra, preventing data distortion. The results were reported in molar ellipticity, $[\theta] = (\theta \times 100MRW/cl)$, where c is the sample concentration, l is the length of the cuvette cell, MRW "mean amino acid residue weight" calculated for firefly luciferase (113) and θ is the ellipticity measured by spectropolarimeter in degree at wavelength of λ. $[\theta]$ is reported in ($^\circ$ cm^2 dmol^{-1}) [34]. Thermal plots of luciferase in the presence and absence of additives obtained at 222 nm, temperature for thermal scanning, varied between 20 and 85 °C at rate of 2 °C/min. Far-UV and near-UV CD spectropolarimetry were performed at different concentrations of additives 0–1.2 M. Luciferase concentration was adjusted to 0.2 mg/ml for far-UV CD in 1 mm cuvette and 1.5 mg/ml for near-UV CD in 1 mm cuvette (0.2 mg/ml in 10 mm cuvette) at room temperature 25 °C (pH 7.8).