Journal of Photochemistry and Photobiology B: Biology 144 (2015) 1–7

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

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

Impact of trifluoroethanol-induced structural changes on luciferase cleavage sites

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ARTICLE INFO

Article history:
Received 30 July 2014
Received in revised form 11 January 2015
Accepted 12 January 2015
Available online 22 January 2015

Keywords:
Firefly luciferase
Trifluoroethanol
Proteolysis
Aggregation
Secondary structure

ABSTRACT

Induction of structural changes in firefly luciferase were identified by proteolytic digestion, activity measurement and spectroscopic tools upon treatment with 2,2,2-trifluoroethanol (TFE). Our results show that the conformation and function of luciferase change according to TFE concentration. Limited addition of TFE (below 10%) alters the tertiary structure and proteolytic rate with a similar digestion pattern, without noticeable changes in the secondary structure. Conformational changes result in loss of enzymatic activity. More addition of TFE (between 20% and 30%) disrupts the tertiary structure, and consequently the activity completely disappears without recovery upon dilution. Furthermore, at high protein concentration, significant aggregation is observed in this range of TFE concentration. A further increase in TFE concentration (above 30%) induces more helical structure, which is more resistant to tryptic attack. Overall, in spite of large conformational changes of luciferase induced by TFE, the prime-sites of proteolytic cleavage are still located at the same chain segments.

1. Introduction

Bioluminescence enzyme, 'luciferase', is a family of proteins that can be isolated from a large variety of organisms [1–3]. Firefly luciferase is a monomeric enzyme of 62 kDa that produces excited state oxyluciferin from D-luciferin in the presence of Mg^{2+}-ATP and molecular oxygen. Relaxation of oxyluciferin is accompanied by radiation of visible light, usually in the green to yellow region, typically 550–570 nm [3–5]. The basic reaction research mainly focused on the firefly *Photinus pyralis* luciferase [4]. According to crystal structure, *P. pyralis* luciferase is composed of a large N-terminal domain and a smaller C-terminal domain connected by a flexible linker region [6], and the presumed active site is surrounded by residues predominantly located at the N-terminal domain except for one or a few residue(s) in the C-terminal domain [7,8].

It has been demonstrated that the alcoholic co-solvents destabilize the tertiary structure of proteins [9,10] and stabilize the secondary structures [11,12]. 2,2,2-trifluoroethanol (TFE) is an important co-solvent in studying of protein structure [13]. Like conventional denaturants such as urea and guanidine hydrochloride, TFE destroys tertiary structure of proteins. Regarding secondary structure of proteins, however, generally differs from the aforementioned denaturants [12,14]. TFE is also known to induce non-native partially folded states in proteins and to increase the helical content of the proteins. By addition of TFE to aqueous solutions, peptides with a predisposition for helical secondary structure can often be induced to undergo a transition from random coil to α-helix [11,12,14,15]. In fact, the induction of α-helical structures are dependent on the inherent structural preference of the amino acid sequence [16,17]. Currently, the detailed mechanism for disruption of polypeptide chains by TFE is not clear, however, it is well reported that the major mechanisms by which TFE modulates protein structures are weakening the hydrophobic interactions, increasing intramolecular hydrogen bonds and disrupting water networks [18,19].

According to previous studies [20–22], two distinct regions of *P. pyralis* luciferase were found sensitive to proteolytic degradation. Digestion of the *P. pyralis* luciferase using trypsin yields two major fragments with molecular masses about 40 and 30 kDa and some other peptides in low amounts. The aim of the current research was to investigate the conformational aspects and helical propensity of *P. pyralis* luciferase, in aqueous TFE and elucidate the mechanism of TFE-induced conformational changes. Contrasting the flexibility regions in the enzyme will help to identify more protease-sensitive regions and possibly facilitate the design of more stable forms of luciferase, to improve luciferase application in broad range of *in vitro* and *in vivo*. Insights into the structure and dynamic of the firefly luciferase in aqueous TFE were obtained through

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http://dx.doi.org/10.1016/j.jphotobiol.2015.01.008
1011-1344/

proteolysis studies, spectroscopy and activity measurements in the presence of various TFE concentrations.

2. Materials and methods

All experiments were performed in triplicate and the results were presented as their mean value. The experimental error was never over 10%.

2.1. Materials and chemicals

The gene encoding wild-type *P. pyralis* luciferase (pET-luc) was used to express *P. pyralis* luciferase as reporter earlier [21–23]. The Ni–NTA Sepharose was purchased from Qiagen Inc (Germantown, USA). ATP was purchased from Roche (Basel, Switzerland). D-Luciferin potassium salt was obtained from Synchem Corp. TFE was prepared from Merck (San Diego, CA, USA). All the other chemicals were of analytical reagent grade and were obtained from Sigma (St. Louis, USA) or Fluka (Ronkonkoma, NY, USA).

2.2. Expression and purification of luciferase

The bacteria containing the recombinant vector for *P. pyralis* luciferase was grown at 37 °C on LB-ampicillin (50 μg/ml) medium, until the cell density reached an absorbance of ~0.8–1 at 600 nm (A_{600}), and then induced with lactose (4 mM) for 12 h at 22 °C. After centrifuging the cell bacteria was resuspended in lysis buffer (50 mM Tris–HCl, 10 mM imidazole (pH 7.8), 300 mM NaCl, and 1 mM PMSF (freshly added)), lysed by sonication and total protein extracted. The cell lysate clarified by centrifugation was added to Ni–NTA Sepharose column. After washing with an imidazole step gradient (30, 60 mM), the recombinant luciferase protein was eluted with 250 mM imidazole [21–24]. Protein purity was estimated by SDS–PAGE and protein concentration was determined by Bradford method [25].

2.3. Limited proteolysis of firefly luciferase in TFE

Proteolysis of *P. pyralis* luciferase was performed by trypsin, added at enzyme/substrate (E/S) ratio of 1:100 or chymotrypsin with E/S ratio 1:250 [21]. The reaction was carried out at 25 °C for 15 min and started by 0.5 mg/ml of purified luciferase, either in the absence and presence of TFE up to 50% (v/v) concentration. At the end of incubation time, aliquots were stopped by adding 1 mM PMSF. Samples were then placed in SDS loading buffer and boiling for 5 min. Cleaved peptides were resolved on SDS–PAGE (12.5% acrylamide) and stained with coomassie brilliant blue R-250 (Serva Fine Chemicals, Westbury, NY, USA).

2.4. Spectroscopic studies

The purified luciferase was dialyzed in 50 mM Tris buffer pH 7.8, containing 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM Ammonium Sulfate, and 2% (v/v) glycerol; at 4 °C, to exude imidazole. The structural studies were performed on the dialyzed proteins.

2.4.1. Circular dichroism measurements

CD spectra were obtained on a JASCO J-715 spectropolarimeter (Japan) at ambient temperature of 25 °C. The final protein concentration for far-UV CD was 0.2 mg/ml in the absence and presence of TFE, and pH was maintained at 7.8. The results were expressed as molar ellipticity, $[\theta]$(degree cm^2 $dmol^{-1}$), based on a mean residue molecular weight (MRW) assuming its average weight for firefly luciferase protein. The mean residue ellipticity $[\theta]$ was calculated from the formula $[\theta]_\lambda = (\theta(100\,MRW)/(cl)$, where θ is measured ellipticity in degrees wavelength λ, c is the protein concentration in mg/ml, and l is the optical path length in centimeter.

2.4.2. Intrinsic fluorescence and ANS binding measurements

Fluorescence emission spectra of purified luciferase were recorded in the absence and presence of TFE using a Cary-Eclipse fluorescence spectrophotometer (Varian Inc., Mulgrave, Australia) at an excitation wavelength of 295 nm. The protein concentration was adjusted to 0.02 mg/ml. Measurements were taken at 25 °C, pH 7.8, and the fluorescence emission spectra were scanned between 300 and 400 nm. ANS binding studies were performed in the TFE/water mixture at pH 7.8. The final luciferase concentration was also 0.02 mg/ml, and the molar ratio of protein to ANS was 1:30. The ANS emission spectra were scanned from 400 to 600 nm with an excitation wavelength of 350 nm.

2.4.3. Bioluminescence emission spectra measurements

Bioluminescence emission spectra of the luciferase were measured using Cary-Eclipse fluorescence spectrophotometer (Varian Inc., Mulgrave, Australia) from 400 to 700 nm wavelengths, as reported earlier [21–24]. A volume of 200 μl of the substrate solution (50 mM Tris–HCl, pH 7.8, 4 mM ATP, 2 mM luciferin, 10 mM $MgSO_4$) was added to 200 μl of purified protein incubated with TFE, in a quartz cell. The spectra were automatically corrected for the photosensitivity of the equipment.

2.5. Aggregation measurements

Aggregation measurements were carried out by a UV spectrophotometer (SCINCO UV S-2100, NY, USA) using the absorption wavelength 405 nm (A_{405}) with purified luciferase at a final concentration of 1.5 mg/ml, pH 7.8, in the presence of different concentrations of TFE.

2.6. Measurements of luciferase activity

The luciferase activity was measured at 25 °C by Sirius Single Tube Luminometer (Berthold Detection Systems, GmbH). Assays were initiated by 10 μl of substrate solution containing 4 mM ATP, 2 mM luciferin, 10 mM $MgSO_4$ in 50 mM Tris–HCl (pH 7.8), that mixed with 10 μl of luciferase enzyme incubated with different concentrations of TFE. The luciferase activity (RLU/Sec) was recorded in aliquots taken at different time intervals. Reactivation experiment was carried out by 100-fold dilution of samples into 50 mM Tris–HCl buffer, pH 7.8, incubated for 10 min at 4 °C.

2.7. Protease assay in aqueous organic solvent media

The protease activity of trypsin in the absence and presence of TFE was determined by a UV spectrophotometer (SCINCO UV S-2100, NY, USA) at 25 °C in 50 mM Tris buffer, pH 7.8, in 0.5 ml reaction volume, as reported earlier [26]. The reaction was carried out with 0.5% casein as substrate and 100 μg/ml trypsin enzyme. After 15 min, the enzymatic activity of protease was stopped by incubation with 0.5 ml of 10% trichloroacetic acid (TCA) for 30 min, subsequently, after centrifuging, absorbance of the supernatant was measured at 280 nm. In all experiments, co-solvent mixtures were prepared by mixing required amounts of the components (TFE, Tris buffer and casein substrate) and the enzyme, to determining protease activity in the presence of TFE.