Critical Role of Glu175 on Stability and Folding of Bacterial Luciferase: Stopped-flow Fluorescence Study

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Bacterial luciferase is a heterodimeric enzyme, which catalyzes the light emission reaction, utilizing reduced FMN (FMNH2), a long chain aliphatic aldehyde and O2, to produce green-blue light. This enzyme can be readily classed as slow or fast decay based on their rate of luminescence decay in a single turnover. Mutation of Glu175 in α subunit to Gly converted slow decay Xenorhabdus Luminescence luciferase to fast decay one. The following studies revealed that changing the luciferase flexibility and lack of Glu-flavin interactions are responsible for the unusual kinetic properties of mutant enzyme. Optical and thermodynamics studies have caused a decrease in free energy and anisotropy of mutant enzyme. Moreover, the role of Glu175 in transition state of folding pathway by use of stopped-flow fluorescence technique has been studied which suggested that Glu175 is not involved in transition state of folding and appears as surface residue of the nucleus or as a member of one of a few alternative folding nuclei. These results suggest that mutation of Glu175 to Gly extended the structure of Xenorhabdus Luminescence luciferase, locally.

Keywords: Anisotropy, Bacterial luciferase, Stopped-flow fluorescence, ϕ-value

Introduction

Protein folding like any chemical process consists of two fundamental component, thermodynamics and kinetics; thermodynamics is related to the stability of the folded state and kinetics is related to the pathway of folding (Serrano et al., 1992). The free energy of folding is generally quite small being typically some 5 to 15 Kcal mol⁻¹ (Privalov, 1979). This is the difference between the free energy of the non-covalent interactions in the folded state of protein and free energy of non-covalent in the unfolded state (Privalov, 1979).

Predicting whether a protein is stable requires the calculation of the energies of two states. It is difficult to calculate these two energies, to overcome this problem an experimental strategy is used primarily based on protein engineering. The first step is to identify those side-chains that their interactions appear to be important in stabilizing the protein, which can be detected by site direct mutagenesis. The change of stability of the protein is then measured using biophysical methods such as stopped-flow fluorescence. This step includes measurement of unfolding or refolding rate constant and equilibrium constants of mutant and wild type proteins for determining the structure of transition state at site of mutation using ϕ-value analysis (Serrano et al., 1992).

The ratio of the changes in the activation energy of unfolding and the free energy of unfolding on mutation is measured for obtaining ϕ-value parameter. The ϕ-values typically range from 0 to 1. A value of ϕ = 0 implies that the structure at the site of mutation is as folded as in the folded state as is in the folded state. In the other hand, ϕ = 1 shows that the structure of the site of mutation is as unfolded in the transition state as it is the unfolded structure (Fersht et al, 1992). Fractional ϕ-values indicate partial formation of structure but, in general, there is not a linear relationship between ϕ-value and extent of structure formation. In this case, the mutant residue affects the transition state stability to a lesser degree than the native protein stability, this means that the residue in question either belongs to one of a few alternative folding nuclei or forms only a part of its native contacts within the nucleus (this residue is at the surface of the nucleus) (Finkelstein and Ptitsin, 2002).

In this study, bacterial luciferase from Xenorhabdus Luminescence was used. Bacterial luciferase emits and generates the emission of green-blue light through the oxidation of its two substrates, reduced flavin mononucleotide...
Bacterial luciferase can be classified in two distinct groups based on their luminescence decay rate; slow decay and fast decay with the luciferase from \textit{Xenorhabdus luminescens} (XL) having slow decay rate (Nealson and Hastings, 1979). The studies have revealed that Glu175 has a critical role in control of catalytic activity and luminescence decay. By mutation of Glu175 to Gly, luciferase was converted from a slow XL luciferase to a luciferase with fast decay rate (Hosseinkhani et al., 2005). Structural and molecular modeling studies shows that the phosphate group of FMNH$_2$ is engaged in a network of seven intermolecular hydrogen bonds with the enzyme. Three hydrogen bonds with the main chain NH groups of Glu175 and Ser176 and two hydrogen bonds with the hydroxyl groups of Ser176 and Thr 179. Conversion of Glu175 to Gly (a highly flexible residue) probably changes the peptide backbone conformation. Moreover, increase of fluorescence intensity and decrease of T$_{1/2}$ are the other effects of this mutation (Riahi Madvar et al., 2005). This fact indicates that Glu175 residue is likely involved in aldehyde binding and the mechanism of turnover of intermediates (Riahi Madvar et al., 2005).

Attention to this fact that Glu175 is a residue in a connecting loop between $\alpha$-helix 5 and $\beta$-strand 5 in $\alpha$ subunit within bacterial luciferase, it seemed that mutation of this residue has effect on flexibility, stability and folding pathway of enzyme. In present work, changes of stability of enzyme upon mutation of Glu175 to Gly by use of circular dichroism and change of transition state of folding pathway by use of stopped-flow fluorescence technique were investigated.

**Materials and methods**

**Materials.** The phosphate buffer solution (pH = 7.0) was prepared by mixing appropriate amounts of NaH$_2$PO$_4$ and Na$_2$HPO$_4$, obtained from Carlo Erba (Italy). Gdn.HCl (Guandine hydrochloride) was purchased from Merck. BSA (bovine serum albumin) and all other compounds were of analytical reagent grade purchased from Sigma.

**Expression and enzyme purification.** The pT7-5 plasmid containing parental or mutant XL lux A was transformed in to \textit{E. coli} BL21 whose chromosomal DNA contains the IPTG (isopropyl $\beta$-D-thiogalacto pyranosid) -inducible T7 RNA polymerase. Gene expression and purification of luciferase was performed according to previous methods (Hosseinkhani et al., 2005). The purified wild type and mutant enzymes had purities of greater than 95% based on analysis by SDS-polyacrylamid gel electrophoresis. Expression and purification of functionally active mutant luciferase rules out its misfolding (Giansalas-Miguel et al., 1972).

**Protein concentration.** Concentrations of native and mutant forms of luciferase were determined by the Bradford method (Bradford, 1976).

![Fig. 1. Topology diagram. Cartoon showing the secondary structural elements of the two luciferase subunits. $\alpha$-strands and $\alpha$-helices are represented by arrows and cylinders, respectively. The ($\alpha$/B)$_8$ core is drawn flat along the middle with the loop insertions drawn above and below the core. $\beta$ wraps around and hydrogen bonds to $\beta1$ to form the closed barrel. The numbers refer to the beginning and end of each secondary structural element (Fisher et al., 1996).](image-url)
Circular Dichroism (CD) measurements. CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with protein concentrations varying from 0.2 (far-UV) to 2 mg/ml (near-UV). The results were expressed as molar ellipticity, [θ] (deg cm$^2$ dmol$^{-1}$), based on a mean amino acid residue weight (MRW) assuming its average weight for bacterial luciferase to be equal to 115. The molar ellipticity was determined as [θ] = -(0 × 100 MRW)/(c l), where [θ] is the protein concentration in mg/ml, c is the light path length in centimeters, and l is the measured ellipticity in degrees at wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming [θ]$_{222}$ = 7820 deg cm$^2$ dmol$^{-1}$ (Schippers and Dekkers, 1981), and with JASCO standard 1-naphthroic acid at 291 deg cm$^2$ dmol$^{-1}$ (Uray, 1985). Noise in the data was smoothed using the JASCO J-715 software, including the fast Fourier-transform noise reduction routine, which allows enhancement of most noisy spectra without distorting their peak shapes (Protasevich et al., 1997).

Optical parameters measurements. Optical parameters are consist of dielectric transition dipole strength ($\mu_e^2$ - D) and rotational strength (R). The change in dipole moment of a chromophore is called electric transition dipole moment; $\mu_e$. The magnitude of this was obtained from the absorption spectrum and by equation (1) (Uray, 1985):

$$|\mu_e|^2 \cdot \Delta \lambda = 1.63 \times 10^{38} \left( \epsilon_0 \Delta \lambda / \epsilon_a \right)$$

With $\Delta \lambda$ being the dipole strength, $\epsilon_0^0$ the molar extinction coefficient at the absorption maximum and $\Delta \lambda$, the half-band width in nm at $\epsilon_0^0$/e.

Rotational strength, in direct analogy to dipole strength, is measured from the area of the circular dichroism band. It can be either positive or negative since the CD band may be either positive or negative. The rotational strength, $R_e$, is calculated from the far-UV-CD band by the equation (2) (Uray, 1985):

$$R_e = 1.23 \times 10^{-20} \left( |\epsilon_0^0| / \Delta \lambda \right)$$

Where $\epsilon_0^0$ is the molar ellipticity at the band maximum and, $\Delta \lambda$, the half-band width at $|\epsilon_0^0|/e$. The ratio $R_e$/$D_e$ referred as anisotropy (indicated the difference absorption left and right circularly polarized light) of enzyme (Uray, 1985).

Fluorescence measurements. Equilibrium unfolding as a function of [Gdn.HCl] was monitored by fluorescence spectroscopy. Native and mutant luciferase was incubated in 30 mM sodium phosphate and 10 mM 2-mercaptoethanol at different concentrations of Gdn.HCl (final pH 7) at 25°C. Samples were allowed to equilibrate for 1 h. The tryptophan fluorescence was measured for each sample with an excitation at 296 nm and emission at 340 nm. The protein concentration after unfolding transition was 10 μM (Serrano et al., 1992). The fluorescence emission spectrum of the enzyme was performed in a Perkin-Elmer fluorescence spectrometer LS50B. The fluorescence emission was scanned between 290 and 440 nm with an excitation wavelength of 296 nm (Hosseinkhani et al., 2004).

Stopped-flow kinetic measurements. All experiments were performed at 25°C and pH 7.0. Stopped-flow fluorescence measurements were carried out with a Biologic μ-SFM-20 using a 0.8 cm cuvet (FC-08) and data were collected and analysed with the Biokine analysis software.

Unfolding was initiated by rapidly diluting 1 volume of folded protein, 1.3 mg/ml in 50 mM sodium phosphate and 10 mM 2-mercaptoethanol, pH 7.0, into 10 volume of concentrated Gdn.HCl. The final concentration of Gdn.HCl solutions was 1.5 M and that for protein 0.13 mg/ml. Unfolding was followed by monitoring the changes in the intrinsic fluorescence of bacterial luciferase (excitation at 296 nm wavelength; emission wavelength was 320 nm (Serrano et al., 1992).

Unfolding curves analysed by a nonlinear regression procedure using the Biokine analysis software and fitted to an equation describing a single exponential decay with linear drift and offset according to eq. (3):

$$F(t) = A_0 \exp(-k_t) - m_0 + c$$

Where $F(t)$ the fluorescence at time t, $A_0$ is the amplitude, $k_t$ the rate constant, m, the slope of drift and c an offset. The drift, when present, is very small and result from baseline instability.

Equilibrium and kinetic measurements. The rate and equilibrium constants along the folding for any individual protein are in principle directly measurable by experiments. These measurements may be converted into to free energies. The free energies of ground states are calculated from equilibrium thermodynamics using the standard equation $\Delta G = -RT \ln K_e$, in this equation $K_e$ in the presence of Gdn.HCl is calculated from equation (5):

$$K_e = (F_u - F_{da}) / (F_{da} - F_0)$$

Where $F_{da}$ is the observed fluorescence and $F_0$ and $F_u$ are the values of the fluorescence of the unfolded and folded forms of protein, respectively (Fersht et al., 1992). The free energy of transition state may be calculated from transition state theory using the standard equation $k = (K_e T/b) \exp(-\Delta G_2/RT)$. Where k is the rate constant, $K_e$ the Boltzman constant, b the Plank constant and $\Delta G_2$ the free energy of activation or the difference in energy between the transition and ground state (Fersht et al., 1992). $\phi$-value is calculated according to equation (6):

$$\phi = (\Delta G_u - \Delta G_0) / (\Delta G_u - \Delta G_0 - \Delta G_{fe} - \Delta G_{se})$$

in which $\Delta G_{fe}$ is the difference in energy of transition state of unfolding relative to the folded state between wild-type and mutant enzyme and $\Delta G_{se}$ is the difference in energy of the unfolded and folded state between native enzyme and mutant one (Evans and Polanyi, 1935; Eyring, 1935).

Results and discussion

Conversion of Glu175 to Gly by random mutagenesis brought about changes in basic kinetic properties of bacterial luciferase (Hosseinkhani et al., 2005). This effect has been interpreted by the position of Glu175 in forming hydrogen bond with phosphate group of FMN and also in connecting loop between α helix5 and β strand3 (residue 166-233) (Gibson and Hasting, 1962; Fisher et al., 1996). Therefore, it seems that mutation of this residue changes the structural and
thermodynamic properties and folding pathway of bacterial luciferase.

By conversion of Glu175 to Gly, secondary and tertiary structures (Fig. 2 and Fig. 3) were decreased. These results mean that mutant enzyme has more flexible structure than native one. Thermodynamic parameters are calculated from thermal denaturation curves with considering that the process of thermal denaturation of native enzyme and mutant one is reversible, (Fig. 4). Obtained results indicate a decrease in $T_m$ and $\Delta G_{25}^0$ (Table 1) which show thermal stability of enzyme is decreased upon mutation. Moreover, enhancement of electric transition dipole momentum indicates that upon mutation, the structure of enzyme is slightly unfolded. In the other word, increase of electric transition dipole momentum and decrease in rotational strength means that the structure of mutant enzyme is more flexible than native enzyme (Urray, 1985).

Since, these results reflect that mutant enzyme has more extended structure than native one, anisotropy as one of the optical parameters was measured to demonstrate that replacement of Glu175 by Gly is responsible for it. A material property is isotropic when it does not depend on how the sample is turned; it is anisotropic when it does depend on the orientation of the sample with respect to some external frame. Such anisotropy of a property is due to the arrangement of the building blocks and structure of the material. From morphology point of view, the structural elements responsible for anisotropy are not in the size of polypeptides, but their shape and, in particular, their orientation (Kocks et al., 1998). In the other word, all of the chromophore of anisotropic molecule (in this case, polypeptide), do not absorb the left and right circularly polarized light, similarly (Fasman, 1996). As indicated in Table 1, upon replacement of Glu175 to Gly, anisotropy of bacterial luciferase decreased. It means that, mutant enzyme has a lesser $\Delta \theta$ than native one where $\Delta \theta$ is the difference absorption of right-handed and left-handed polarized light (considering that, rotational strength for both enzyme are negative, so $\varepsilon_R > \varepsilon_L$ totally (Urray, 1985). This fact indicates that in spite of native enzyme with an anisotropic structure, by

Table 1. Thermodynamic and optical parameters of native and mutant bacterial luciferase. Data were obtained by use of thermal profiles and far-UV CD spectrum and UV spectrum of native and mutant enzyme

<table>
<thead>
<tr>
<th></th>
<th>$\Delta G_{25}^0$ (kcal/mol)</th>
<th>$T_m$ (°C)</th>
<th>$\lambda$ (nm)</th>
<th>$\varepsilon$ (cm$^2$ mol$^{-1}$)</th>
<th>$\mu_i^2 - D$</th>
<th>$R_i$</th>
<th>$R/D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>11.76</td>
<td>50.2</td>
<td>224</td>
<td>4.84x10$^7$</td>
<td>3.5x10$^{-23}$</td>
<td>-17.6x10$^{-77}$</td>
<td>5x10$^{-3}$</td>
</tr>
<tr>
<td>Mutant</td>
<td>6.22</td>
<td>48.4</td>
<td>230</td>
<td>5.28x10$^7$</td>
<td>3.9x10$^{-23}$</td>
<td>-14.2x10$^{-77}$</td>
<td>3.6x10$^{-3}$</td>
</tr>
</tbody>
</table>
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this mutation, structure of enzyme locally converted to isotropic structure respect to $\Delta \varepsilon$. That is to say, with replacement of a charged amino acid by small one, many hydrogen bonds are disrupted, so the structure of mutant enzyme, converted to isotropic form. This substitution decreased the total anisotropy of native enzyme.

Local change of anisotropy of an enzyme is established by calculation of $\phi$-value as mentioned in material and methods. The $\phi$-value analysis approach allows the energetic contribution of side chain to be mapped out by comparing the wild type with individual mutants where conservative changes are introduced side chains (Anil, et al., 2005). This parameter extracted from unfolding curve of native and mutant enzyme by use of intrinsic fluorescence (Fig. 5.) and stopped-flow fluorescence (Fig. 6.) techniques. Figure 5 shows that with increase the Gdn.HCl concentration, the intensity of intrinsic fluorescence of native enzyme is more decreased than mutant enzyme. By use of these curves $\Delta G$ (for each enzyme) and $\Delta \Delta G$ (between them) are calculated (Table 2). Following, $\Delta G^2$ and $\Delta \Delta G^2$ are extracted from stopped-flow fluorescence curves as shown in Table 2. As shown in Table 2, calculated $\phi$-value for native and mutant enzyme is 0.29. It means that in mutant form during unfolding process at the transition some bonds between Glu175 with other residue are broken. Therefore, it can be concluded at transition state, in fact we have not native form and not mutant one. As a result, we can say that Glu175 is a surface residue of the nucleus, or as a member of one of a few alternative folding nuclei. It may be suggested by conversion of Glu175 (bulky amino acid) to Gly (small amino acid), structural compactness of luciferase slightly decreased. This change is responsible for converting the luminescence decay of enzyme from slow decay to fast decay. In the other word, weakening of substrate binding to enzyme changed it to a luciferase with significantly more rapid decay rate.

In conclusion, the results presented in this study suggest that mutation of Glu175 to Gly, extended the structure of X. luminescence luciferase, locally and Glu175 does not participate in central nucleus of enzyme on folding pathway.

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References


