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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 O_2, 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 lake 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 suggesting 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 $\varphi = 0$ implies that the structure at the site of mutation is as folded in the transition state as it is in the folded state. In the other hand, $\varphi = 1$ 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

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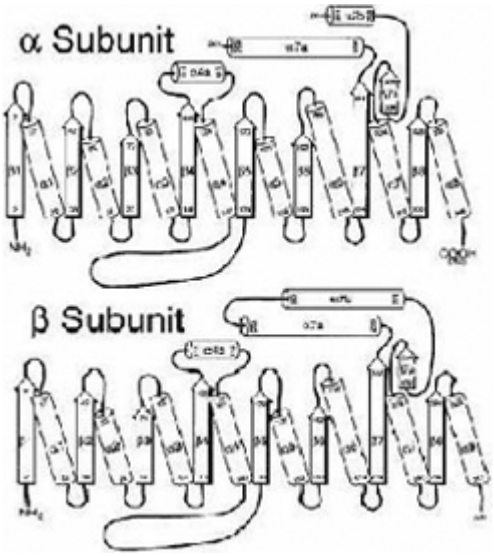

Fig. 1. Topology diagram. Cartoon showing the secondary structural elements of the two luciferase subunits. β-strands and α-helices are represented by arrows and cylinders, respectively. The $(\alpha/\beta)_8$ core is drown flat along the middle with the loop insertions drown above and below the core. β8 wraps around and hydrogen bonds to β1 to form the closed barrel. The numbers refer to the beginning and end of each secondary structural element (Fisher *et al*., 1996).

($FMNH_2$) and long chain aliphatic aldehyde. The final products of bacterial luciferase reaction are oxidized flavin mononucleotide (FMN), the corresponding long chain fatty acid and water (Hastings and Gibson, 1963; Szittner and Meighen, 1993; Lin *et al.*, 2001).

Bacterial luciferase is a 79 kD heterodimeric enzyme comprised of two non-identical subunits, named α and β which the former has 360 amino acid with six tryptophan and the later has 327 amino acid with two tryptophan (Szittner and Meighen, 1990). The α and β subunits have similar molecular weight of approximately 40 and 39 kD, respectively. The two subunits assemble noncovalently at an interface through vander waals interactions, hydrogen bonds and salt bridges (Cline and Hasting, 1974; Fisher *et al*., 1996). Each subunit of the heterodimeric enzyme form an $(\alpha/\beta)_8$ barrel motif (Meighen and Mackenzie, 1973). The α and β subunits have identical topologies with the most outstanding loop of the $(\alpha/\beta)_8$ motif existing between β7 and α7 (Fig. 1) (Fisher *et al*., 1996). The catalytic properties are believed to be controlled by α subunit (Szittner and Meighen, 1993; Lin *et al*., 2001). Most of the kinetic and physical properties of luciferase , such as the decay rate of luminescence , the specifity for different chain length aldehydes and the affinity for flavin and flavin analogues are attributed to the α subunit (Cousineau and Meighe, 1976). However, the presence of β subunit is known to be essential for high quantum yield and stability of enzyme (Nealson, 1978).

Bacterial luciferase can be classified in two distinct groups based on their luminescence decay rate; slow decay and fast decay with the luciferase from *Xenorhabdus Luminescence* (XL) having slow decay rate (Nealson and Hasting, 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 Glu 175 to Gly (a highly flexible residue) probably changes the peptide backbone conformation. Moreover, increase of fluorescence intensity and decrease of T_m 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 turn over of intermediates (Riahi Madvar *et al*., 2005).

Attendant to this fact that Glu175 is a residue in a connecting loop between α-helix 5 and β-strand 5 in α 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_2PO_4 and Na_2HPO_4, obtained from Carlo Erba (Italy). Gdn.HCl (Guanidine 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 *E. coli* BL21 whose chromosomal DNA contains the IPTG (isopropyl β-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 (Gunsalus-Miguel *et al*., 1972).

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

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] = (θ × 100 MRW)/(cl), where c is the protein concentration in mg/ml, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291}$ = 7820 deg cm^2 $dmol^{-1}$ (Schippers and Dekkers, 1981), and with JASCO standard nonhydroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5}$ = 7910 deg cm^2 $dmol^{-1}$ (Takakuwa *et al*., 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 electric transition dipole strength (μ_i^2 = D) and rotational strength (R). The change in dipole moment of a chromophore is called electric transition dipole moment; μ_i. The magnitude of this was obtained from the absorption spectrum and by equation (1) (Urray, 1985):

$$| \mu_i^2 | = D_i = 1.63 \times 10^{-38} (\varepsilon_i^0 \Delta_i)/\lambda_i \quad (1)$$

With D_i being the dipole strength, ε_i^0 the molar extinction coefficient at the absorption maximum and Δ_i the half band width in nm at ε_i^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 for $\varepsilon_L > \varepsilon_R$ or negative when $\varepsilon_L < \varepsilon_R$. The rotational strength, R_i, is calculated from the far UV-CD band by the equation (2) (Urray, 1985):

$$R_i = 1.23 \times 10^{-42} [\theta_i^0] \Delta_i/\lambda_i \quad (2)$$

Where θ_i^0 molar ellipticity at the band maximum and, Δ_i is the half-band width at $[\theta_i^0]$/e. The ratio R_i/D_i referred as anisotropy (indicated the difference absorption left and right circularly polarized light) of enzyme (Urray, 1985).

Fluorescence measurements. Equilibrium unfolding as a function of [Gdn.HCl] was monitored by fluorescence spectroscopy. Native and mutant luciferase was incubated in 50 mM sodium phosphate and 10 mM 2-mercaptethanol at different concentration 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 spectra of the enzyme performed in a Perkin-Elmer luminescence 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-mercaptethanol, 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):

$$F(t) = A_o \exp(-k_u t) - mt + c \quad (3)$$

Where F(t) the fluorescence at time t, A_o is the amplitude, k_u, the rate constant, m, the slop of the 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 in to free energies. The free energies of ground states are calculated from equilibrium thermodynamics using the standard equation $\Delta G = -RT\ LnK_u$. In this equation K_u in the presence of Gdn.HCl is calculated from equation (5):

$$K_u = (F_F - F_{obs})/(F_{obs} - F_U) \quad (5)$$

Where F_{obs} is the observed fluorescence and F_U and F_F 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, $k = (K_B T/h)\ EXP\ (-\Delta G_{\ddagger}/RT)$. Where k is the rate constant, K_B the Boltzman constant, h the Plank constant and $\Delta G_{\ddagger}$ the free energy of activation or the difference in energy between the transition and ground state (Fersht *et al*., 1992). φ-value is calculated according to equation (6):

$$\varphi = (\Delta G_{\ddagger} - G_F) / (\Delta G_u - \Delta G_F) = \Delta\Delta G_{\ddagger\text{-}F}/\Delta\Delta G_{u\text{-}F} \quad (6)$$

in which $\Delta\Delta G_{\ddagger\text{-}F}$ is the difference in energy of transition state of unfolding relate to the folded state between wild-type and mutant enzyme and $\Delta\Delta G_{u\text{-}F}$ 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 strand5 (residue 166-233) (Gibson and Hasting, 1962; Fisher *et al*., 1996). Therefore, it seems that mutation of this residue changes the structural and

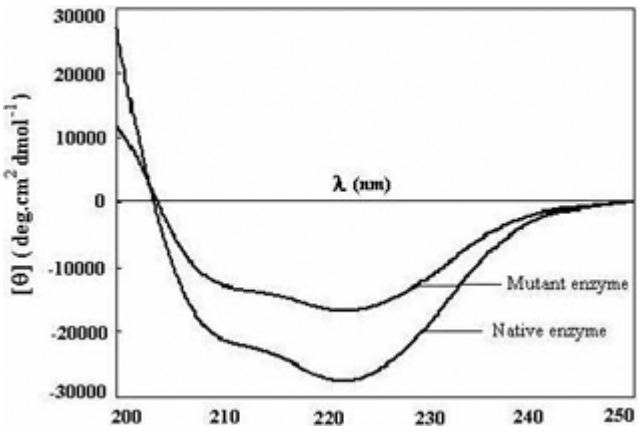

Fig. 2. Far-UV CD spectra of native and mutant bacterial luciferase in 50 mM-phosphate and 10 mM 2-mercaptethanol (pH 7) at 25°C.

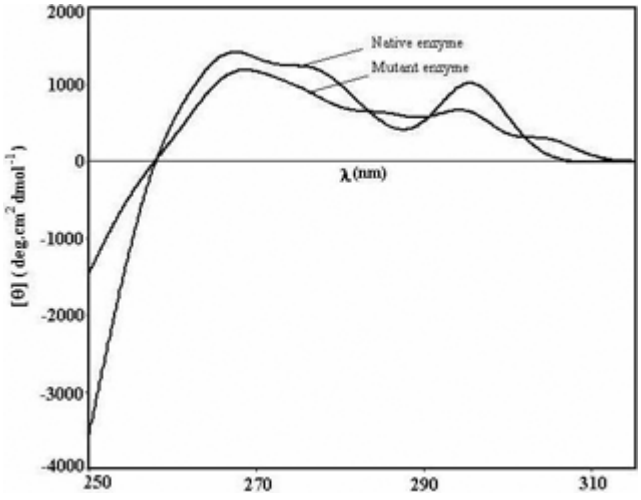

Fig. 3. Near-UV CD spectra of native and mutant bacterial luciferase in 50 mM-phosphate and 10 mM 2-mercaptethanol (pH 7) at 25°C.

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 ΔG^0_{25} (Table 1) which show thermal stability of enzyme is

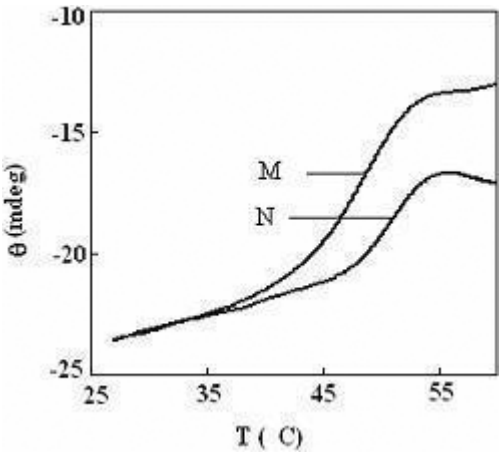

Fig. 4. Thermal denaturation profiles of native (N) and mutant (M) luciferase. Spectra were taken at 25-70°C by far-UV CD in phosphate buffer (0.05 M, pH 7.0). The concentration of protein was 0.15 mg/ml.

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 $\Delta\varepsilon$ than native one where $\Delta\varepsilon$ 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

	ΔG_{15} (kcal/mol)	T_m (°C)	λ (nm)	ε (cm^2 mol^{-2})	$\mu_i^2 = D$	R_i	R_i/D
Native	11.76	50.2	224	4.84×10^5	3.5×10^{-35}	-17.6×10^{-37}	5×10^{-3}
Mutant	6.22	48.4	230	5.28×10^5	3.9×10^{-35}	-14.2×10^{-37}	3.6×10^{-3}

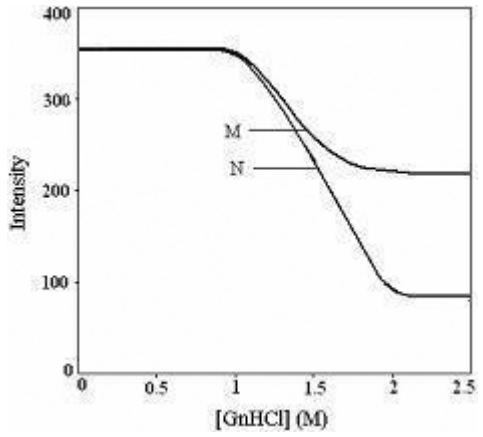

Fig. 5. Gdn.HCl-induced unfolding of native (N) and mutant (M) luciferase. The calculations are described under material and methods.

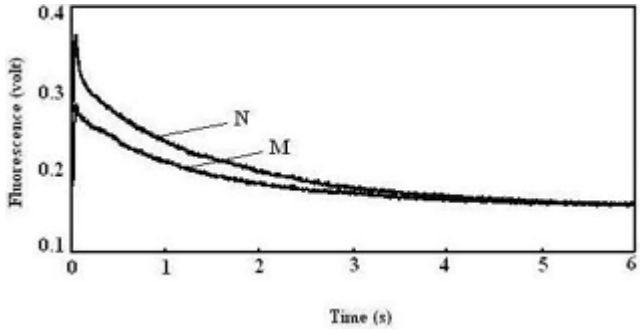

Fig. 6. Unfolding kinetic trace of native (N) and mutant (M) luciferase measured by stopped-flow fluorescence. Unfolding of protein (1.3 mg/ml) was initiated by rapidly diluting 1 volume of protein in 50 mM-phosphate and 10 mM 2-mercaptethanol (pH 7) into 10 volume of concentrated Gdn.HCl solution containing 50 mM-phosphate and 10 mM 2-mercaptethanol (pH 7) and 1.5 M Gdn.HCl. The unfolding was followed by the monitoring the change in the intrinsic fluorescence of native and mutant luciferase. (Excitation at 290 nm wavelength; emission wavelength 320 nm).

this mutation, structure of enzyme locally converted to isotropic structure respect to Δε. 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 φ-value as mentioned in material and methods. The φ-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 florescence (Fig. 5.) and stopped flow-florescence (Fig. 6.) techniques. Figure 5 shows that with increase the Gdn.HCl concentration, the intensity of intrinsic

Table 2. Rate constant, free energy and equilibrium free energy of native and mutant enzyme. ΔG and $\Delta G^{\ddagger}$ were extracted from florescence spectroscopy and stopped- flow fluorescence spectroscopy, respectively. Spectra were taken at 25°C in phosphate buffer (0.05 M, pH 7.0)

	Intrinsic flourescence		Stoppedfolw flourescence	
	K (equ)	ΔG (kcal/mol)	k	$\Delta G^{\ddagger}$ (kcal/mol)
Native	0.77	0.14	1.25±0.007	0.15
Mutant	2.96	-0.64	1.86±0.003	0.39

florescence of native enzyme is more decreased than mutant enzyme. By use of these curves ΔG (for each enzyme) and ΔΔG (between them) are calculated (Table 2). Following, $\Delta G^{\ddagger}$ and $\Delta\Delta G^{\ddagger}$ are extracted from stopped-flow florescence curves as shown in table (2). As shown in Table 2, calculated φ-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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