

# Implication of a critical residue (Glu175) in structure and function of bacterial luciferase

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**Abstract** Structural properties of a bacterial luciferase mutant, evolved by random mutagenesis, have been investigated. Bacterial luciferases (LuxAB) can be readily classed as slow or fast decay luciferases based on their rates of luminescence decay in a single turnover assay. By random mutagenesis, one of the mutants generated by a single mutation on LuxA at position 175 (E175G) resulted in the “slow decay” *Xenorhabdus luminescens* luciferase was converted into a luciferase with a significantly more rapid decay rate [Hosseinkhani, S., Szittner, R. and Meighen, E.A. (2005) *Biochemical Journal* 385, 575–580]. A single mutation (E175G), in a loop that connects  $\alpha$  helix 5 and  $\beta$  sheet 5 brought about changes in the kinetic and structural properties of the enzyme. Enhancement of tryptophan fluorescence was observed with a lower degree of fluorescence quenching by acrylamide upon mutation. Near- and far-UV circular dichroism spectra of the native and mutant forms suggested formation of an intermediate structure, further supported by 8-anilino-1-naphthalene-sulphonic acid (ANS) fluorescence which indicated lower exposure of hydrophobic residues as a result of mutation. Fluorescence quenching studies utilizing acrylamide indicated a more accessible fluor for the native form. Thus, the E175G point mutation appears to change the enzymatic decay rate by inducing a substantial tertiary structural change, without a large effect on secondary structural elements, as revealed by Fourier transform IR spectroscopy. Overall, the mutation caused structural changes that go beyond the simple change in orientation of Glu175.

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**Keywords:** Bacterial luciferase; Random mutagenesis; Decay rate; Fluorescence quenching; Intrinsic fluorescence; 8-Anilino-1-naphthalene-sulphonic acid; Circular dichroism

## 1. Introduction

Bacterial luciferase catalyzes the light emission reaction, utilizing reduced FMN (FMNH<sub>2</sub>), a long chain aliphatic aldehyde and O<sub>2</sub>, to produce green-blue light [1]. This reaction is dependent on the sequential formation of several enzyme-

flavin intermediates (Scheme 1). Luciferase (E) interacts with FMNH<sub>2</sub> to form the EF complex, which subsequently reacts with O<sub>2</sub> to yield an oxygenated enzyme-flavin complex (EFO). This complex either decomposes by a dark side pathway with a rate constant to yield oxidized flavin mononucleotide (FMN) (F) and H<sub>2</sub>O<sub>2</sub>, or interacts with aldehyde (A) to form an EFOA complex [2]. Decay of EFOA can occur by dissociation of the aldehyde followed by decomposition via the dark pathway, or the reaction goes to completion with the emission of blue-green light at 490 nm characterized by the rate constant  $k_L$  (Scheme 1).

The three-dimensional structure of *Vibrio harveyi* bacterial luciferase has been solved at a resolution of 1.5 Å [3]. Each subunit of the heterodimeric (LuxAB) enzyme forms an ( $\alpha/\beta$ )<sub>8</sub> barrel motif [4]. A large difference in the luminescence decay rates has been used as a taxonomic basis to class luminescent bacteria into two clearly distinct groups, “slow” and “fast” luciferases [5,6]. Characterization of strains of the *Xenorhabdus luminescens* (XL) has shown that this species contains a luciferase with a slower decay than that of *V. harveyi* luciferase [7].

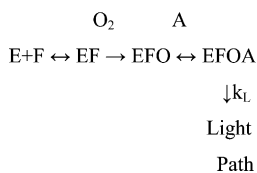
In bacterial luciferase, the  $\alpha$  subunit (coded by LuxA) is primarily responsible for kinetic properties; however, the presence of the  $\beta$  subunit is essential for high catalytic efficiency [8,9]. A chimaera containing a replacement of 17% of its LuxA (residues 166–233) sequence with that of *Photobacterium phosphoreum* (PP) luciferase was converted from a “slow” X1 luciferase to a luciferase with properties more similar to PP luciferase, including a rapid luminescence decay rate [10]. Random mutagenesis in this area showed by conversion of Glu175 to Gly (a highly flexible residue) a “slow” X1 luciferase was converted to a fast luciferase similar to PP luciferase [11]. Recent studies using molecular modeling and site-directed mutagenesis have shown phosphate docking to apo-luciferase is not possible without changing the conformation of the side chain of the Glu175 residue whose carboxylate group is projected into the phosphate-binding site [12,13]. Glu175 is a residue in a connecting loop between  $\alpha$  helix 5 and  $\beta$  strand 5 in  $\alpha$  subunit within bacterial luciferase [14]. The spectral properties of the aromatic amino acid residues of proteins provide extremely useful information, as they serve as both structural elements and intrinsic probes. In the absence of X-ray crystallographic data, spectroscopic techniques in conjunction with the application of molecular biology techniques furnish attractive tools for the study of protein structure.

In the present report, we investigate the structural properties of mutant (E175G) luciferase by different spectroscopic techniques. Changes in far- and near-UV circular dichroism

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**Abbreviations:** FMN, flavin mononucleotide; FMNH<sub>2</sub>, reduced FMN; XL, *Xenorhabdus luminescens*; PP, *Photobacterium phosphoreum*; ANS, 8-anilino-1-naphthalene-sulphonic acid; CD, circular dichroism



Scheme 1.

(CD) spectra, FTIR spectroscopy, intrinsic and extrinsic fluorescences suggest a significant change in tertiary structure and a little change in secondary structure upon conversion of Glu175 to Gly.

## 2. Materials and methods

### 2.1. Materials

Bovine serum albumin (BSA) was purchased from Sigma or Boehringer–Mannheim. All other compounds were of analytical reagent grade and prepared from Sigma.

### 2.2. Expression and enzyme purification

The pT7-5 plasmid containing parental or mutant *X. luminescens luxA* was transformed into *E. Coli* BL21 whose chromosomal DNA contains the IPTG (isopropyl  $\beta$ -D-thiogalactopyranoside)-inducible T<sub>7</sub> RNA polymerase. Gene expression and purification of luciferase was performed according to previous methods [11,15]. The purified wild type and mutant enzymes had purities of greater than 95% based on analyses by SDS–polyacrylamide gel electrophoresis. Expression and purification of functionally active mutant luciferase ruled out its misfolding.

### 2.3. Protein concentration

Concentration of native and mutant forms of luciferase was determined by the Bradford method [16].

### 2.4. Fluorescence measurements

Tryptophan fluorescence was measured on a Perkin–Elmer luminescence spectrophotometer LS 50B apparatus. The excitation wavelength was set at 295 nm and the emission spectra were obtained. Extrinsic fluorescence studies were carried out as outlined earlier [17], using 8-anilino-1-naphthalene-sulphonic acid (ANS) as a fluorescence probe. Measurements were taken on the same spectrofluorometer as used for intrinsic fluorescence studies. All experiments were carried out at 25 °C with ANS and protein concentrations of 30 and 1  $\mu$ M in 50 mM phosphate buffer. An excitation wavelength of 350 nm was used.

### 2.5. Dynamic quenching

Fluorescence quenching was carried out by the addition of 2 M acrylamide and KI (as an ionic quencher) to protein solutions (20  $\mu$ g/ml) at an excitation wavelength of 295 nm and an emission wavelength of 340 nm in a Perkin–Elmer luminescence spectrophotometer LS 50B. Quenching data were analyzed in terms of the Stern–Volmer constant,  $K_{sv}$ , which was calculated from the ratio of the unquenched and the quenched fluorescence intensities,  $F_0/F$ , using the relationships  $F_0/F = 1 + K_{sv}[Q]$ . Q is the molar concentration of the quencher [18].

### 2.6. Infrared spectroscopy

FTIR spectra were recorded on a Nexus 870 FT-IR spectrophotometer with a DTGS detector. The samples were placed in a cell. Typically, 1  $\mu$ M of protein mixture was injected into the cell and a spectrum recorded. The cell was then drained and the protein deposited on the crystal was dried. Second derivative spectra were calculated by a method reported earlier [19].

### 2.7. CD measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with protein concentrations about 0.2 and 1.5 mg/ml for far- and near-UV regions, respectively. Results are expressed as molar ellipticity  $[\theta]$  (deg cm<sup>2</sup> dmol<sup>-1</sup>), based on a mean amino acid residue weight (MRW) of 115 for luciferase. The molar ellipticity was determined as  $[\theta]_l = (\theta \times 100\text{MRW})/(cl)$ , where  $c$  is the protein concentration in mg/ml,  $l$  is the light path length in centimeters, and  $\theta$  is the measured ellipticity in degrees at a wavelength  $\theta$ . The instrument was calibrated with (+)-10-camphorsulphonic acid, assuming  $[\theta]_{291} = 7820$  deg cm<sup>2</sup> dmol<sup>-1</sup>, and with JASCO standard non-hydroscopic ammonium (+)-10-camphorsulphonate, assuming  $[\theta]_{290.5} = 7910$  deg cm<sup>2</sup> dmol<sup>-1</sup> [20]. The data were smoothed using the fast Fourier-transform noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes. The free energy of unfolding ( $\Delta G$ ) was calculated as a function of temperature by assuming a two-state model:  $\Delta G_{\text{NU}}^0 = -RT \ln(\theta_{\text{N}} - \theta_{\text{obs}}/\theta_{\text{obs}} - \theta_{\text{U}})$ . In this equation  $\theta_{\text{N}}$ ,  $\theta_{\text{obs}}$ ,  $\theta_{\text{U}}$  are molar ellipticities for native, observed at each temperature and unfolded forms.

## 3. Results

Mutation of Glu175 using a random but directed manner showed its critical role in control of luminescence decay and also classification of bacterial luciferase to slow and fast decay [11]. Native and mutant luciferases were purified to homogeneity.

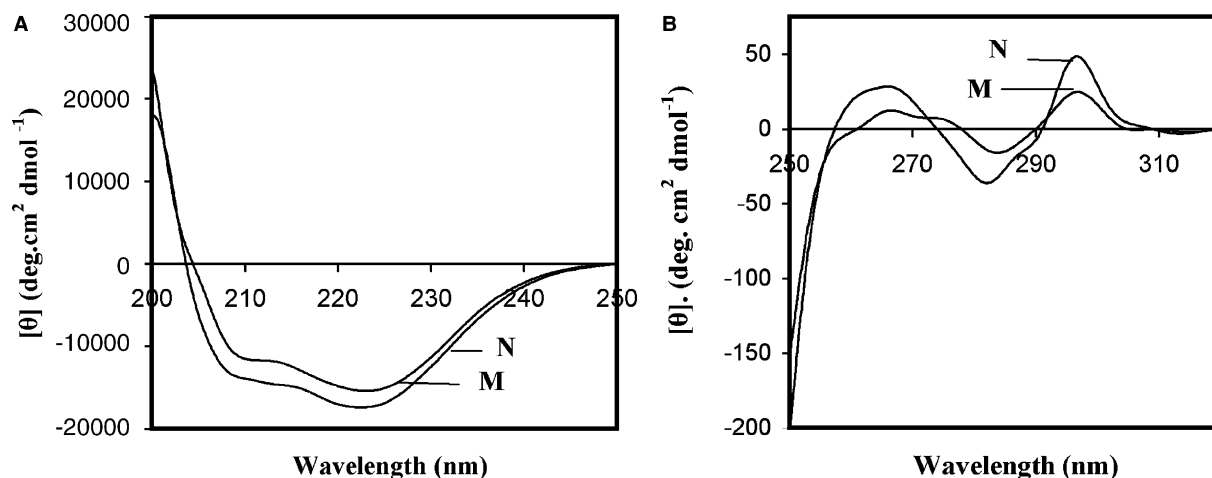


Fig. 1. Far-UV CD spectra (A) and near-UV CD spectra (B) for native (N) and mutant (M) luciferases. The concentration of protein used for the far-UV CD spectrum (185–250) was 0.15 mg/ml and for near-UV CD spectrum (250–350) was 1.5 mg/ml. Protein was equilibrated in phosphate buffer (0.05 M, pH 7.0), at 25 °C. Each spectrum represents the average of five scan.

### 3.1. CD spectra of native and mutant luciferases

CD spectra of native and mutant (E175G) luciferases obtained in phosphate buffer, pH 7.0, are shown in Fig. 1. As indicated in Fig. 1A, the far-UV CD spectra of luciferase show a little decrease in negative ellipticity at 208 and 222 nm upon replacement of Glu 175 by Gly. According to molar ellipticity helix content of native and mutant luciferases is 69.6 and 66.7, respectively. Near-UV CD spectra indicate that the defined tertiary structure of the proteins is decreased by conversion of Glu 175 to Gly (Fig. 1B). Change of tertiary structure is also confirmed by a decrease in  $T_m$  of 5.1 °C and also its  $\Delta G$  about 3 kcal/mol, obtained by thermal denaturation profile of native and mutant forms (Fig. 2).

### 3.2. Fluorescence measurements

As indicated in Fig. 3 an increase of fluorescence intensity observed upon mutation of luciferase. Loss of hydrophobic patches on the mutant protein surface was also confirmed by fluorescence measurements using ANS (Fig. 4). ANS fluores-

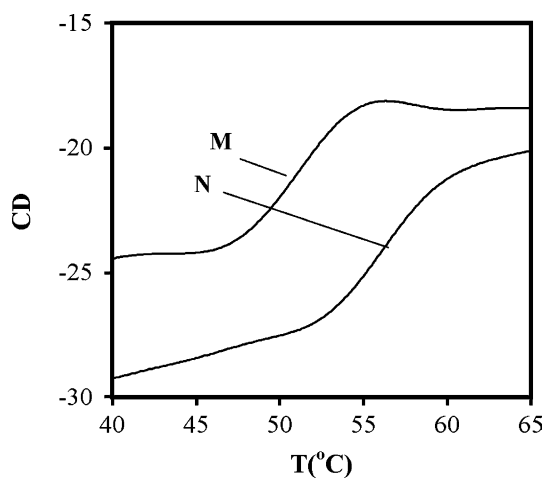


Fig. 2. Thermal denaturation profiles of native (N) and mutant (M) luciferases. Spectra were taken at 25–70 °C by far-UV CD in phosphate buffer (0.05 M, pH 7.0). The concentration of proteins was 0.15 mg/ml.

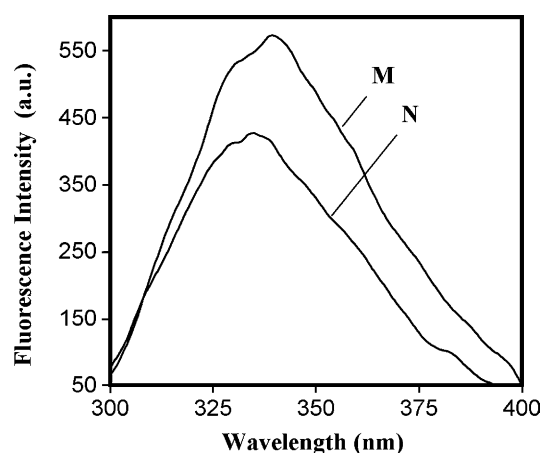


Fig. 3. Intrinsic fluorescence spectra of native (N) and mutant (M) luciferases. Spectra were taken at 25 °C in phosphate buffer (0.05 M, pH 7.0). The concentration of proteins was 1  $\mu$ M. The excitation wavelength was 295 nm.

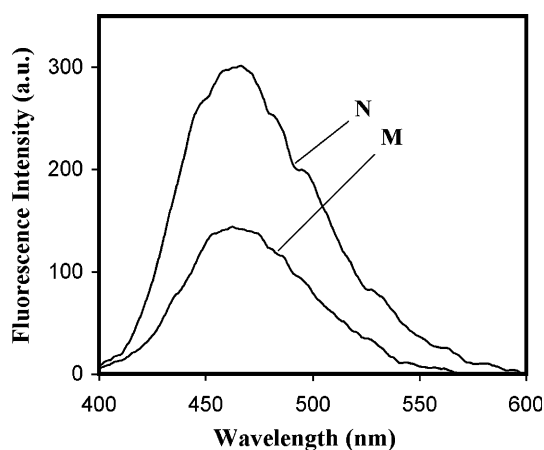


Fig. 4. Fluorescence spectra of ANS in presence of native (N) and mutant (M) luciferases. Spectra were taken at 25 °C in phosphate buffer (0.05 M, pH 7.0). The sample contained 1  $\mu$ M protein and 30  $\mu$ M ANS. The excitation wavelength was 350 nm.

cence is clearly enhanced upon interaction with native XI luciferase, as observed earlier [21].

### 3.3. Fluorescence quenching by quenchers

The results of quenching experiments allowed us to assess the relative solvent exposure of different types of fluorophores [22]. Fig. 5A shows the Stern–Volmer plots for quenching of native XI luciferase and its mutant form (E175G) by acrylamide. It is evident that native XI luciferase can be quenched quite effectively by acrylamide, which indicates higher accessibility of tryptophan to quencher molecules and exposure of fluorophores to the solvent [22]. Stern–Volmer plots for quenching of native XL luciferase and its mutant form by KI show the addition of potassium iodide gave rise to quenching in the fluorescence intensity of mutant luciferase more than native form (Fig. 5B).

### 3.4. IR spectra

Fig. 6A shows the original absorbance of native and mutant luciferases at pH 7.0. In  $^1\text{H}_2\text{O}$ , the amide I band showed maximum at  $1652\text{ cm}^{-1}$  for both native and mutant luciferases. The band at  $1658\text{ cm}^{-1}$  has been previously assigned to the C=O stretching vibration of the side chain amide groups. The most obvious difference was the intensity of the band around  $1658\text{ cm}^{-1}$ , which was visibly smaller in the mutant luciferase. We suppose that this was likely to be due to a change in the extinction coefficient, because no shifts occurred. Fig. 6B compares the second derivative spectrum of native luciferase at pH 7.0 with those of mutant (E175G) at the same pH. The bands at 1658, 1666 and  $1672\text{ cm}^{-1}$  have been assigned to  $\alpha$ -structures,  $\beta$ -turns and  $\beta$ -sheets, respectively. Mutation of luciferase seemed to cause a very small modification of amount of  $\alpha$ -structures,  $\beta$ -turns. A shift of 1672 band shows a change in  $\beta$ -sheet content of protein upon mutation.

## 4. Discussion

Structural effects of a single mutation (E175G) of a loop in the  $\alpha$  subunit that connects  $\beta$  strand 5 to  $\alpha$  helix 5 are investigated. In a recent study, random mutagenesis experiments

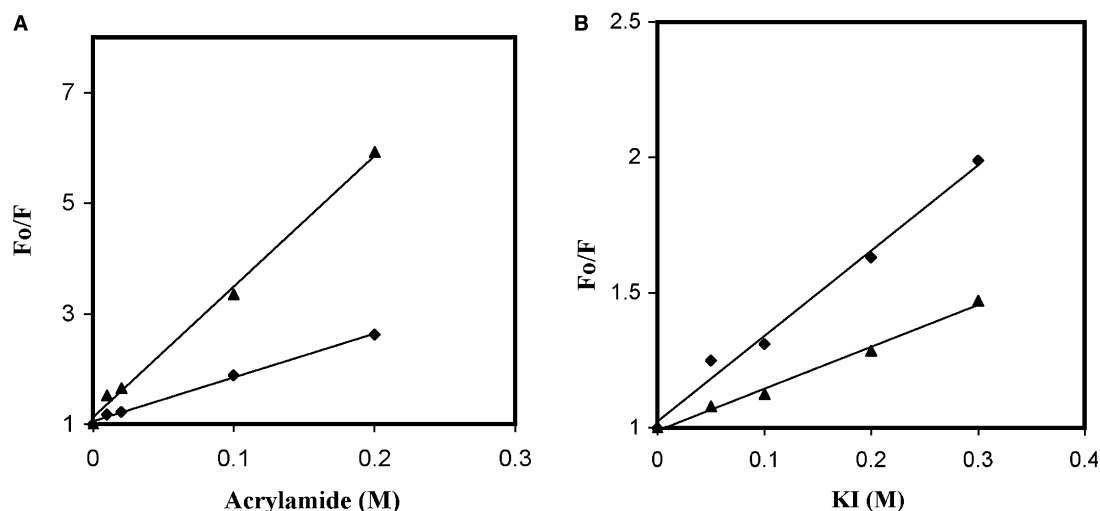


Fig. 5. The Stern–Volmer plots of native ( $\blacktriangle$ ) and mutant ( $\blacksquare$ ) luciferases obtained by quenching with acrylamide (A) and KI (B). The excitation and emission wavelengths were 295 and 340 nm, respectively. The protein was dissolved in 0.05 M phosphate at pH 7.0 and the protein concentration was 20  $\mu\text{g/ml}$  in all cases.

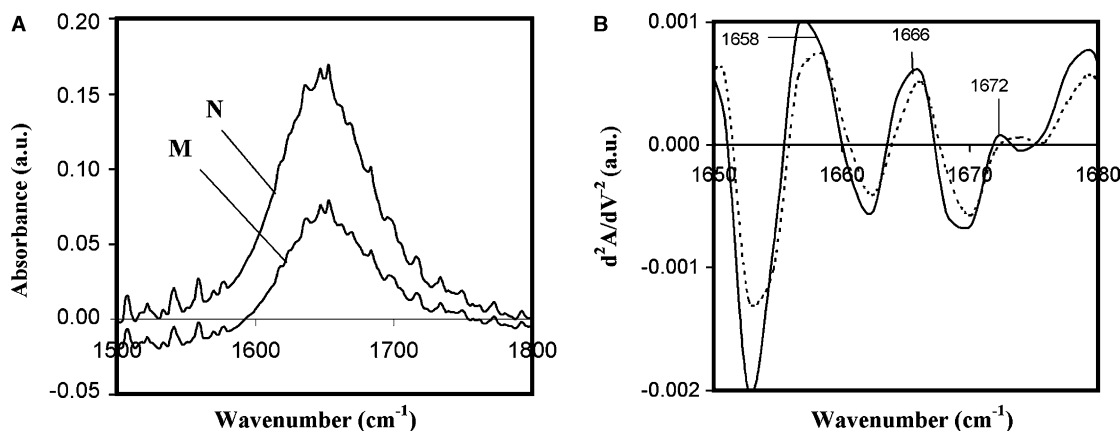


Fig. 6. Infrared spectra the original absorbance (A: native, N; mutant, M) and second derivative spectrum (B: —, N; ----, M) of native (N) and mutant (M) luciferases. Spectra were taken at 25  $^{\circ}\text{C}$  in phosphate buffer (0.05 M, pH 7.0). The concentration of proteins was 1  $\mu\text{M}$ .

were designed to engineer new luciferases and identify active site residues not only by screening for decay rate changes but also for changes in stability, efficiency and aldehyde specificity [11]. This type of mutagenesis has great advantages in use of bacterial luciferase as a sensor. The most notable property of mutant (E175G) luciferase was its rapid rate of luminescence decay compared to that of wild-type XL luciferase. The difference between the fast luminescence decay of the E175G mutant and the slow decay of XL luciferase indicated that the Glu175 residue in the central region (residues 166–233 of LuxA) is likely involved in aldehyde binding and the mechanism of turnover of the EFO and EFOA intermediates [11].

Luciferase from *X. luminescens* contains eight tryptophan, six in the  $\alpha$  subunit and two in the  $\beta$  subunit [7]. Amongst them, tryptophan 194 is in close contact with Glutamate 175 as indicated in crystal structure. Therefore, as shown in Fig. 3, mutation of Glu175 to Gly caused a clear increase in intrinsic fluorescence, suggesting that Trp(s) are being located in a more hydrophobic environment. A red shift in the emission spectra was also seen suggesting that other factors are being involved in fluorescence emission. In fact the overall

fluorescence spectrum of the *X. luminescens* luciferase appeared to be comprised of a set of individual intrinsic emitters with the emission maxima ranging from 330 to 345 nm, as reported earlier [23]. Mutation of Glu175 to Gly alters a negative charge ( $pK \sim 4.32$ ) to a non-polar, small side chain. Thus, at pH 7.0, at which most of the experiments were carried out, there is clearly the possibility of charge reduction as confirmed by an increase in the pI (IEF electrophoresis) of the native protein upon mutation of the Glu 175 (data not shown). Using Expsy program and theoretical calculation, a net charge of  $-27$  for native and  $-26$  for mutant enzymes were obtained. It may therefore be suggested that the change in intrinsic fluorescence (Fig. 3) occurs as a result of protein mutation due to changes in the net charge, by reduction of negative charges in the protein molecules. Alternatively, the results presented may be explained in terms of the quenching effect of charged carboxyl group. Thus for the native enzyme, it is proposed that presence of glutamate side chain near to tryptophan residue (S) results in fluorescence quenching. For the mutant enzyme, in the other hand, the situation is reversed due to changes brought about by replacement of Glu by Gly. A

similar pattern in the dependence of the fluorescence, observed here, has been reported for anticoagulation factor I from *Agkistrodon acutus* venom [24].

The far-UV and near-UV CD spectra of the native and mutant (E175G) forms were taken which reflected substantial changes in tertiary structure of the protein with small changes in its secondary structure (Fig. 1). The type of changes observed in the CD and IR spectra upon mutation of luciferase suggests conformational changes and possible formation of an intermediate structure (Figs. 1 and 6). A clear change in conformational stability of the protein was observed upon mutation and conversion of Glu to Gly (a highly flexible residue). This was suggested by lowering of ellipticity and a considerable change in  $T_m$  of 5.1 °C.

Loss of tertiary structure was accompanied with loss of hydrophobic pockets on protein surface upon mutation as confirmed by ANS binding as a hydrophobic reporter group (Fig. 4). Decrease of hydrophobic patches on mutant luciferase surface compared with native form cannot support formation of an intermediate structure like molten globule [25]. This is probably due to the fact ANS itself carries a negative charge at the pH of the experiment. Previous results indicate that at neutral pH the net charge of the exterior of luciferase is negative [8], therefore it should be noted; conformational changes upon mutation probably could increase negative charges on protein surface (E175G luciferase). It is therefore suggested that had it not been for such repulsive electrostatic interactions, the typically higher enhancement would have been obtained.

Mutation of Glu175 to Gly (a highly flexible residue) has changed the protein conformation, as confirmed by different spectroscopic evidences. Structural and molecular modeling studies show that phosphate group is engaged in a network of seven intermolecular hydrogen bonds with enzyme. The electron dense phosphate site on luciferase is within hydrogen-bonding distance to the main chain amide hydrogen atoms of Glu 175 and Ser 176, the hydroxyl group of Thr 179, and the positively charged guanidinium group of Arg 107 on the LuxA subunit [14]. Three hydrogen bonds are formed with the guanidinium moiety of Arg107, two hydrogen bonds with the main chain NH groups of Glu175 and Ser176, and two hydrogen bonds with the hydroxyl groups of Ser176 and Thr179 [13]. Higher decay rate constant of mutant (E175G) luciferase was interpreted by weakening of flavin-enzyme interaction and faster decomposition of EFO intermediates [11]. Another experiment related to this study shows conversion of Glu175 to Gly changes luciferase flexibility (Fig. 5). Mutation of Glu 175 not only caused changes in the fluorescence spectral features but also altered the intrinsic fluorescence quenching properties in response to externally added quencher. The variations of the quenching constant ( $K_q$ ) derived from Stern–Volmer plot (Fig. 5A) for mutant form reflect the lower accessibility of these intrinsic groups to quencher (acrylamide) compared with native form. This is in a good agreement with the fact of higher fluorescence intensity of mutant form. Quantitative measurements of the fluorescence quenching by KI provided more specific information on the structure of protein (Fig. 5B).

It is known that  $(\beta/\alpha)_8$  barrels are very stable structures and that mutation of the loop connecting the  $\beta$  strands to the  $\alpha$  helices generally does not have any large effect on the overall structure of protein [26]. However, it was necessary to confirm that substitution of Glu175 by Gly in a loop between  $\alpha$  helix 5 and  $\beta$  strand 5 in  $\alpha$  subunit does not perturb the tertiary struc-

ture. Therefore, it could be concluded, against present knowledge of  $(\beta/\alpha)_8$  barrels stability, a single mutation of a loop in a  $(\beta/\alpha)_8$  barrels (bacterial luciferase) has a significant effect on the overall structure of protein tertiary structure.

In conclusion, the results presented in this study suggest mutation of Glu175 to Gly with subsequent change of luciferase flexibility and lack of the usual Glu-flavin interactions are responsible for the unusual kinetic properties of the E175G mutant XL luciferase.

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