Effect of Charge Distribution in a Flexible Loop on the Bioluminescence Color of Firefly Luciferases[†]

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ABSTRACT: Firefly luciferase is a monooxygenase that catalyzes the ATP-dependent conversion of firefly luciferin into a luciferyl-adenylate, which is oxidized to an electronically excited oxyluciferin in a multistep reaction and produces visible light with a remarkable quantum yield. The bioluminescence color of firefly luciferases is determined by the luciferase structure and assay conditions. Among different beetles, only luciferase from *Phrixotrix* railroad worm (Ph_{RE}) emits red bioluminescence, naturally. The presence of Arg353 in Ph_{RE} luciferase, which corresponds to the deleted residue in the other luciferases, is an important distinctive structural feature of it. Insertion of Arg356 into a green-emitter luciferase (*Lampyris turkestanicus*), corresponding to Arg353 in *Phrixotrix hirtus*, changed the emitted light from green to red. To further clarify the effect of this position on the light shift mechanism, four residues with similar sizes but different charges (Arg, Lys, Glu, and Gln) were inserted into *Photinus pyralis* luciferase, using site-specific insertion mutagenesis. Insertion of a residue with a positive side chain (Arg356 and Lys356) changed the light color to red, while insertion of a residue with a negative side chain (Glu356) had little effect on color. Insertion of a neutral residue (Gln356) at this position was performed without any change in bioluminescence spectra. Insertion of positively charged residues in this loop took place with a series of structural changes which were confirmed by fluorescence spectroscopy and homology modeling. Homology modeling reveals the appearance of a bulge in a flexible loop (T352−P359) upon mutation which shifts to the left side with a color change from green to red.

Bioluminescence (BL),[1] the conversion of chemical energy into light by living organisms, is beautifully illustrated by the flashing light produced by the firefly. Basic research mainly focused on the North American firefly, *Photinus pyralis*, has progressed toward a very good understanding of the chemical transformations leading to light emission (*1, 2*).

Luciferase from the North American firefly *P. pyralis* (EC 1.13.12.7) is a monooxygenase that catalyzes ATP-dependent conversion of firefly luciferin into a luciferyl-adenylate, which is oxidized to electronically excited oxyluciferin in a multistep reaction. Relaxation to the ground state results in the production of yellow-green light ($\lambda_{max} \approx 560$ nm) with a remarkable quantum yield (*3*−*6*).

Bioluminescence in beetles is characterized by a wide range of colors: green-yellow in fireflies (*7, 8*), green-orange in click-beetles (*9, 10*), and green-red in railroad worm (*11, 12*). This variety of bioluminescence colors was attributed to the luciferase structures, since luciferin is the same in all species (*13*).

The chemical basis for color determination in beetle luciferases is complex and dependent on a number of factors, including pH, temperature, and the chemistry of the luciferin, and it is clear from recent studies that particular amino acids in the luciferase primary structure are key determinants of the color of light emitted in the bioluminescence reaction (*13*−*17*). A great deal of effort was directed to elucidating the mechanism of variation in bioluminescence color from a unique emitter, oxyluciferin. However, four mechanisms have been proposed to explain different colors in luciferases: (I) the solvent effect, orientation, and polarizability of the active site microenvironment around the excited oxyluciferin (*18, 19*), (II) the degree of rotation of excited oxyluciferin along the C_2-C_2' bond under the control of the active site conformation (*20, 21*), (III) the effect of basic residues on tautomerization between a ketonic (red emitter) and enolic (yellow-green light emitter) species of excited oxyluciferin (*22*−*24*), and (IV) controlling the resonance-based charge delocalization of the anionic keto form of the oxyluciferin excited state (*25*).

Different studies have been performed in determining the bioluminescence mechanism by X-ray crystallography, random (*26, 27*) and site-directed mutagenesis (*28*), and a combination of chimeragenesis and site-directed mutagenesis (*29, 30*). The crystal structures of luciferase without substrates from the North American firefly (*P. pyralis*) and wild-type and mutant forms (red emitter) of the Japanese firefly (*Luciola cruciata*) in complex with a high-energy intermediate analogue, DLSA, have been resolved (*31, 32*). Crystallographic data indicated that the degree of molecular rigidity

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[1] Abbreviations: *Ppy*, *Photinus pyralis*; Arg356/Lys356/Glu356/Gln356, mutant luciferase containing an additional Arg, Lys, Glu, or Gln at position 356; BL, bioluminescence; SOE-PCR, splicing overlap extension polymerase chain reaction; Ni-NTA, nickel-nitrilotriacetic acid; DLSA, 5′-*O*-[*N*-(dehydroluciferyl)sulfamoyl]adenosine; RLU, relative light unit.

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of the excited oxyluciferin, which is controlled by a transient movement of Ile288 (in *L. cruciata*) determines the color of bioluminescence during the emission reaction (*32*). Among all beetle luciferases, the *Phrixotrix railroad* worm luciferase is the only luciferase that naturally emits red light through two cephalic lanterns (λ_{max} = 628 nm) in addition to the yellow-green bioluminescence (λ_{max} = 542 nm) emitted through 11 pairs of lateral lanterns along the body. *Phrixotrix hirtus* red (Ph_{RE}) luciferase is 46−49% identical to firefly luciferases. Ph_{RE} luciferase with a naturally red emitting ability is a unique model for investigating the relationship of luciferase structure with BL color (*33*). Multiple-sequence alignments of the primary structure of Ph_{RE} with other firefly luciferases, which emit green light, showed the presence of Arg353 in Ph_{RE} luciferase, corresponding to a missing residue in other firefly luciferases.

To investigate the role of the missing residue (Arg353), it was inserted into the corresponding position (Arg356) in *Lampyris turkestanicus* luciferase. Upon insertion of Arg, the color of emitted light was changed to red and the optimum temperature of activity was also increased. Comparison of native and mutant luciferases using homology modeling revealed a significant conformational change of a flexible loop in the red mutant. Movement of the flexible loop brought about a new ionic interaction concomitant with a change in the polarity of the emitter site, thereby leading to red emission (*17*).

In this study, to understand the effect of this residue on bioluminescence color in other species, we have inserted it in the corresponding position (Arg356) in North American firefly lucifersae (*P. pyralis*), and its effect on the BL spectra and kinetic and structural properties were analyzed. To analyze the role of Arg356 in this loop further, other residues (Lys, Glu, and Gln) with similar sizes but different charges were inserted in the same position. The insertions of these residues show that basic residues (Arg356 and Lys356) have a critical role in the BL color shift to red.

EXPERIMENTAL PROCEDURES

Reagents. The following reagents and kits were used. Isopropyl β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma. ATP was purchased from Roche. D-Luciferin potassium salt was obtained from Synchem Corp. Restriction enzyme, *Pfu* polymerase, and T4 ligase were purchased from Fermentase. The plasmid extraction kit, the gel purification kit, and the PCR purification kit were obtained from Bioneer Corp. The Ni-NTA spin kit was purchased from Qiagen Inc., and the pET28a(+) vector was obtained from Novagen.

Insertion Mutagenesis. Insertion mutagenesis was performed using splicing overlap extension polymerase chain reaction (SOE-PCR) (*34*). Two pairs of primers were used for this purpose: F-Cloning containing the *Bam*HI restriction site (5′-C GTT GGA TCC ATG GAA GAC GCT AAA AAC ATA AAG-3′) and R-Cloning containing the *Hin*dIII restriction site (5′-C AGC AAG CTT TTA CAA TTT GGA CTT TCC GCC-3′). The overlapping primers, F Mutant and R Mutant, containing the Arg, Lys, Glu, and Gln codon insertions were as follows: F Mutant (5′-ATT ACA CCC GAG GGG AGA GAT GAT AAA C-3′, 5′-ATT ACA CCC GAG GGG AAG GAT GAT AAA C-3′, 5′-ATT ACA CCC GAG GGG GAA GAT GAT AAA C-3′, and 5′-ATT ACA CCC GAG GGG CAA GAT GAT AAA C-3′), R Mutant (5′-C ATC TCT CCC CTC GGG TGT AAT CAG AAT AG-3′, 5′-C ATC CTT CCC CTC GGG TGT AAT CAG AAT AG-3′, 5′-C ATC TTC CCC CTC GGG TGT AAT CAG AAT AG-3′, and 5′-C ATC TTG CCC CTC GGG TGT AAT CAG AAT AG-3′).

The plasmid containing the native *P. pyralis* luciferase was used as a template. Two PCRs for performing primary amplification of the two DNA fragments to be spliced were carried out using F Mutant, R-Cloning and F-Cloning, and R Mutant by *Pfu* polymerase under the following conditions: initial denaturation at 94 °C for 5 min, a 30 cycle (94 °C for 1 min, 52 °C for 1 min, and 72 °C for 90 s), and a final extension for 10 min at 72 °C. Subsequently, primary PCR products were purified using a cleanup kit to remove redundant primers. The resulting fragments from primary PCRs were mixed in a 1:1 molar ratio so that the amount of DNA added to a second PCR mixture was around 100 ng.

The second PCR was performed in two steps: the first step performed with 10 cycles similar to the first PCR condition only with a difference in the extension time (42 °C for 1 min). At the end of this step, F and R cloning primers added to each tube, and PCR continued like the first PCR. The mutagenesis products digested with *Bam*HI and *Hin*dIII were inserted into the *Bam*HI−*Hin*dIII restriction sites of digested/dephosphorylated pET28a(+) high expression vector, and ligated mixtures were transformed into competent cells of *Escherichia coli* BL21 by electroporation.

Protein Expression and Purification. Five milliliters of LB medium containing 50 μg/mL kanamycin with a fresh bacterial colony harboring the expression plasmid was inoculated and grown at 37 °C overnight. Then 200 mL of medium with 500 μL overnight cultures was inoculated and grown at 37 °C with vigorous shaking until the OD_{600} reached 0.6. Then, IPTG was added to the solution to a final concentration of 1 mM and the mixture incubated at 22 °C overnight with vigorous shaking. The cells were harvested by centrifugation at 5000*g* for 15 min. The cell pellet was resuspended in lysis buffer [50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF (add fresh) (pH 7.8)]. Purification of His_6-tagged fusion protein was performed with the Ni-NTA spin column as described by the manufacturer (Qiagen). The color of the emitted light of the purified firefly luciferase reaction was obvious after addition of luciferin and Mg-ATP to purified luciferases.

Sequencing. pET28a(+) vectors containing native and mutant luciferases were sequenced using an automatic sequencer (MWG) by the T7 promoter and T7 terminator universal primers.

Measurement of Bioluminescence Emission Spectra. BL spectra were recorded using a Cary-Eclipse luminescence spectrophotometer (Varian) from 400 to 700 nm, as reported previously (*35*). A volume of 300 μL of 50 mM Tris-HCl buffer (pH 7.8) and mix buffer [50 mM Tris-HCl, 100 mM glycine, and 100 mM succinic acid (pH 5.5)], including 2 mM ATP, 5 mM $MgSO_4$, and 1 mM luciferin, was added to 100 μL of a purified luciferase solution (∼50 pg) in a quartz cell. Gate and delay times, detector voltage, scan rate, and slit width were adjusted to optimize instrument response. The spectra were automatically corrected for the photosensitivity of the equipment.

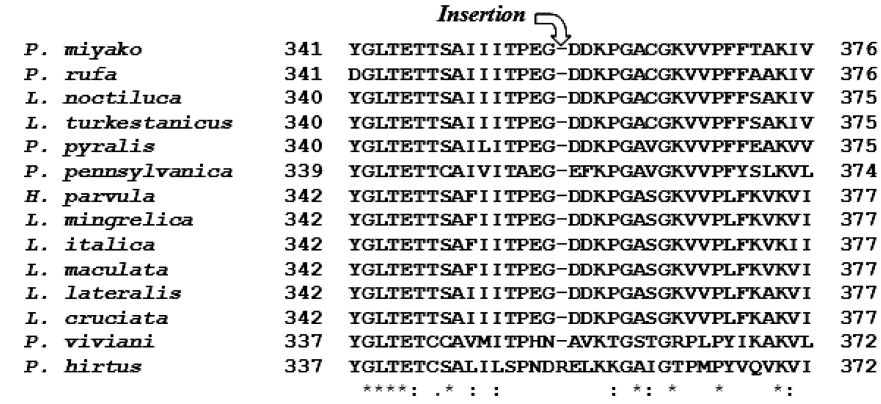

FIGURE 1: GenBank accession numbers for the amino acid sequences used for multiple-sequence alignment are as follows: *Pyrocoelia miyako* (L39928), *Pyrocoelia rufa* (AY447203), *Lampyris noctiluca* (X89479), *La. turkestanicus* (AY742225), *P. pyralis* (MI5077), *Photuris pennsylvanica* (U31240), *Hotaria parvula* (E10030), *Luciola mingrelica* (S61961), *Luciola italica* (DQ138966), *Lampyroidea maculata* (DQ137139), *Luciola lateralis* (X66919), *Luciola cruciata* (M26194), *Phrixotrix vivianii* (AF139644), and *Ph. hirtus* (red-bioluminescence eliciting luciferase, AF139645).

Kinetic Properties. ATP and luciferin kinetic parameters were measured at 25 °C. To estimate LH_2 K_m, 50 μL of assay reagent containing 10 mM $MgSO_4$ and 8 mM ATP in 50 mM Tris-HCl (pH 7.8) was mixed with 40 μL of various concentrations of luciferins (0.0025−5 mM) in a tube. The reaction was initiated by adding 10 μL of enzyme, and light emission was recorded over 10 s (Sirius tube luminometer, Berthold Detection System). The estimation of ATP kinetic constants was performed in a similar way. Various concentrations (40 μL) of ATP (from 0.004 to 8 mM) were mixed with 50 μL of assay reagent, including 10 mM $MgSO_4$ and 1 mM luciferin in 50 mM Tris-HCl (pH 7.8). The reaction was initiated by adding 10 μL of enzyme, and light emission was recorded over 10 s. Apparent kinetic parameters were calculated by Lineweaver−Burk plots. The decay times of native and mutant luciferases were measured in 90 s and compared with each other. The residual activity for each enzyme was reported as a percentage of the original activity. Approximate protein concentrations were calculated using a Bradford assay (*36*), and relative specific activities (enzyme activity vs protein concentration) were also calculated. To obtain the optimal temperature of activity for native and mutant luciferases, activities were measured in the range of 5−45 °C. Moreover, the optimum pH of activity for both enzymes was measured by incubation of enzyme in a mixed buffer in the pH range of 5−10.5.

Thermal Inactivation and Thermal Stability Studies. To study thermal inactivation, the purified luciferases (10 μg/mL) were incubated in the range of 20−45 °C for 5 min. Enzyme activities were measured at room temperature (25 °C), and the remaining activity was recorded as percentage of the original activity after incubation for 2 min in ice. The time courses of thermostability of purified luciferases were measured by incubating the enzyme in 50 mM Tris-HCl (pH 7.8) at 35 °C. At regular intervals (0−60 min), samples were removed and cooled on ice (2 min), and the remaining activity was determined. The activity of the enzyme solution kept on ice was considered as the control (100%).

Fluorescence Measurements. The purified luciferases were dialyzed in dialysis buffer containing 50 mM Tris-HCl, 1% glycerol, 1 mM EDTA, 50 mM NaCl, and 0.05% β-mercaptoethanol (pH 7.8) at 4 °C. Fluorescence studies were carried out on a Cary-Eclipse luminescence spectrophotometer (Varian). Intrinsic fluorescence was determined using 20 μg/mL protein and an excitation wavelength of 295 nm. Emission spectra were recorded between 300 and 400 nm (*37*). Extrinsic fluorescence studies were carried out with 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescence probe. Measurements were taken on the same spectrofluorometer that was used for intrinsic fluorescence studies. All experiments were carried out at 25 °C. The final concentration of the ANS in the enzyme solutions was 30 μM, and the molar ratio of protein to ANS was 1:50. The ANS emission was scanned between 380 and 700 nm with an excitation wavelength of 350 nm (*38, 39*).

Dynamic Quenching. Fluorescence quenching was carried out via the addition of 2.0 M KI (as an ionic quencher) to dialyzed protein solutions (20 μg/mL) at an excitation wavelength of 295 nm and an emission wavelength of 300−450 nm in a Cary-Eclipse luminescence spectrophotometer (Varian). Quenching data were analyzed in terms of the Stern−Volmer constant, K_{SV}, which was calculated from the ratio of the unquenched (F_0) and quenched fluorescence intensities (F) using the relationship $F_0/F = 1 + K_{SV}[Q]$, where [Q] is the molar concentration of the quencher (*40*).

Bioinformatics Study. P. pyralis firefly luciferase [Protein Data Bank (PDB) entry 1LCI] is used as the PDB template in the SwissModel Alignment Interface protein modeling server (*41*). With submission of multiple-sequence alignment of luciferase sequence family and template, three-dimensional structural models of native and mutant luciferases were obtained. The fit between the three-dimensional structures of models was evaluated with SWISS-PDB Viewer by calculating the root-mean-square deviation after iterative fitting. The pK_a and salt bridges of native and mutant proteins were also calculated with MacroDox (Northrup) (*42*) and the WHAT IF Web server, respectively (*43*).

RESULTS

Construction, Expression, and Purification of the Native and Mutant Luciferases. The multiple-sequence alignment among firefly luciferases and the red-emitting railroad worm showed in *P. pyralis*, as in others species, Arg356 is missed (Figure 1). According to results for *La. turkestanicus*, the insertion of residues Arg356, Lys356, Glu356, and Gln356 into *P. pyralis* was performed by PCR insertion mutagenesis.

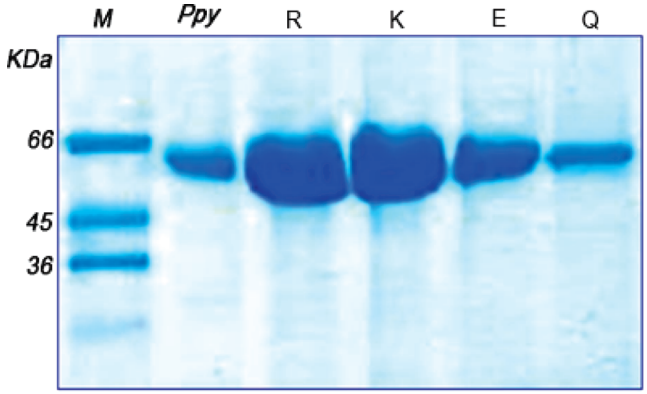

FIGURE 2: SDS−PAGE of purified native (*Ppy*) and mutant (R356, K356, E356, and Q356) luciferases after purification via affinity Ni-NTA column chromatography. Lane M contained the molecular markers.

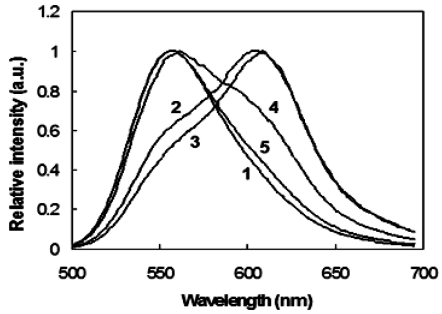

FIGURE 3: Bioluminescence emission spectra produced by the native from *P. pyralis* (1) and mutant [(2) Arg356, (3) Lys356, (4) Glu356, and (5) Gln356] luciferases by a luciferase-catalyzed reaction at pH 7.8.

After cloning and transformation, amino acid insertion was confirmed by sequencing (data not shown).

To further purify and characterize native and mutant forms, overexpression of luciferase was carried out in the BL21 host, and the purification of His_6-tagged fusion luciferases was also performed by affinity (Ni-NTA Sepharose) chromatography. The purified native and mutant luciferases had purities of >95% on the basis of the analysis by SDS−PAGE in which luciferases were present as a band of ~62 kDa (Figure 2). Upon addition of D-luciferin to purified luciferases, visible light was observed in the dark by the naked eye at pH 7.8 and 5.5.

Bioluminescence Emission Spectra. The in vitro bioluminescence spectra of native and mutant luciferases appear in Figure 3. Luciferase mutants Arg356, Lys356, and Glu356 display a bimodal spectrum. Arg356 and Lys356 mutants have a maximum in the red region (608 nm) and a smaller shoulder at 557 nm in the green region, while the Glu356 mutant exhibits a maximum in the green region (563) and a shoulder in the red region (591 nm). The native form and Gln356 mutant exhibit a similar spectrum with only a peak at 557 nm. At a lower pH (pH 5.5), the native form and all mutants show a red shift with only one peak at 613 nm.

Study of the Kinetic Constants, Decay, and Thermostability. Lineweaver−Burk plots were used to estimate the apparent K_m values for luciferin (LH_2 K_m) and ATP (ATP K_m). The results [relative specific activity, optimum pH and temperature, and K_m (for LH_2 and ATP)] are listed in Table 1.

The K_m values of Arg356 and Gln356 mutants for luciferin increased 1.5 and 2 times, respectively, while that for Glu356 decreased up to 50%. The K_m values for all mutants for Mg-ATP were almost the same as that of the native form (*Ppy*).

The time of light decay was measured by injection of a 50 μL cocktail [2 mM luciferin, 4 mM ATP, and 10 mM $MgSO_4$ in 50 mM Tris-HCl (pH 7.8)] to 50 μL of native and mutant purified enzymes. According to the result, the decay time for mutant luciferases was slower than that for the native form (Figure 4). A clear improvement in thermostability was observed for the Gln356 mutant luciferase compared to other mutants and native luciferase as indicated in Figure 5A,B. The optimum temperature for mutant luciferases did not exhibit any change (25 °C) (Table 1). Optimum pH values for mutants with insertion of an ionizable side chain (Arg356, Lys356, and Glu356) were increased from 8 to 8.5, while the Gln356 mutant did not exhibit any change in optimum pH, like native luciferase.

Intrinsic Fluorescence. To further discriminate the spectroscopic contributions of the Trp residues, the conformational changes in mutant and native (*Ppy*) forms were evaluated by measuring the intrinsic fluorescence intensity. Fluorescence spectroscopy is a useful technique for following tertiary structure transitions in proteins because the intrinsic fluorescence of tryptophanyl residues is particularly sensitive to the polarity of microenvironments along the transition. In this study, to eliminate possible contributions from Tyr residues, the illumination with 295 nm light exclusively excites tryptophan residues, and therefore, this spectrum is ascribed to tryptophan. *P. pyralis* luciferase has two tryptophans, Trp417 and Trp426; these Trp residues are located in a large N-domain. They are not surface residues, although they are near the protein surface; the tryptophan aromatic groups are immersed in the folds of the protein globule that contains water molecules. As shown in Figure 6, the fluorescence spectrum of the native form (*Ppy*) had a broad spectrum with a maximum at 341 nm at pH 7.8. However, an increase in the emission intensity without a red and blue shift related to the native form was observed for mutant forms, while according to Figure 6, Lys356, Gln356, Arg356, and Glu356 exhibited increases and decreases in intensity compared to the native form (*Ppy*). The results were indicative of the conformational changes in mutant forms versus the native form, presumably resulting from unfolding (Arg356 and Glu356) and compacting structure (Lys356 and Gln356). Therefore, this result of intrinsic fluorescence may be useful in illustrating the potential structural changes taking place in the enzyme induced by insertion of new residues.

ANS Fluorescence. It is known that ANS, a nonfluorescent hydrophobic dye in aqueous solution, binds to hydrophobic patches on proteins with greater affinity, resulting in a detectable blue shift in its fluorescence spectrum. Figure 7 shows differential ANS fluorescence spectra of *P. pyralis* and mutant luciferases. Maximum and minimum ANS emission intensities are observed for Lys356 and Glu356, respectively. From ANS binding data, we can deduce that the whole conformation of Glu356 is compact and hydrophobic patches are buried; however, in Lys356, hydrophobic patches are exposed to allow the binding of ANS, which in turn results in enhanced ANS fluorescence intensity.

Quenching Study with KI. The quenching experiment provided information about the relative solvent exposure of fluorophores. Greater exposure of a fluorophore leads to more effective fluorescence quenching. Figure 8 depicts the Stern−

Table 1: Kinetic Properties of the Native (*Ppy*) and Mutant Luciferases

	LH_2 K_m (μM)[a]	ATP K_m (μM)[b]	relative specific activity (%)[c]	λ_{max} (nm)[d] at pH 7.8	optimum temperature (°C)	optimum pH
Ppy	11	120	100	557 (66)	25	8
Arg356	15	100	88	608, 557* (94)	25	8.5
Lys356	9	110	82	608, 557* (69)	25	8.25
Glu356	6	105	197	563, 593* (91)	25	8.5
Gln356	18	105	94	557 (71)	25	8

[a] The error associated with the K_m values falls within ±10% of the value. [b] The error associated with the K_m values falls within ±10% of the value. [c] The specific activity measurements are estimated to be ±5% of the value. [d] Bioluminescence emission spectra were recorded as described in Experimental Procedures. Bandwidths (nanometers) at 50% of the emission maxima are given in parentheses, and asterisks identify minor peaks.

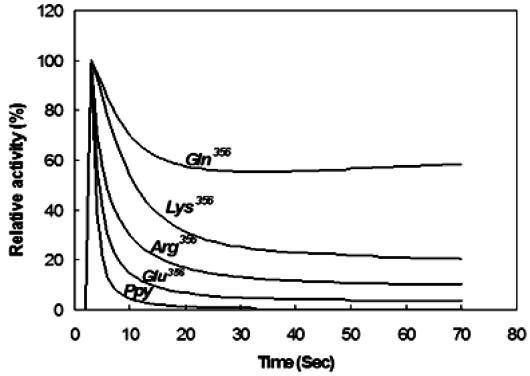

FIGURE 4: Comparison of decay times of native (*Ppy*) and mutant luciferases. For further details, see Experimental Procedures.

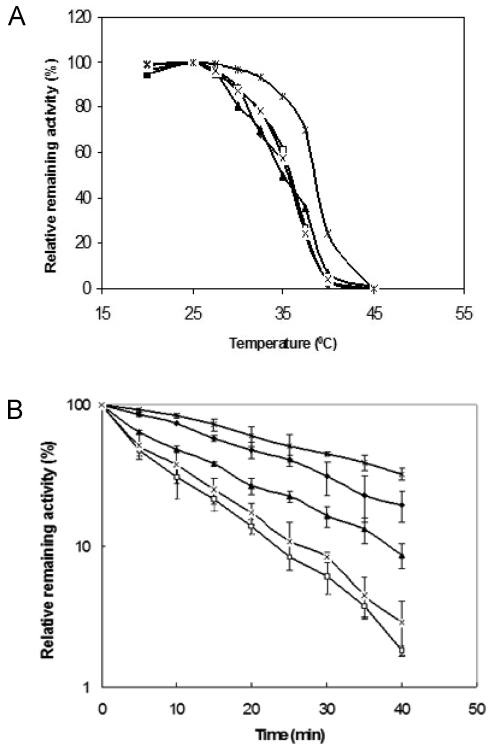

FIGURE 5: Comparison of thermal inactivation (A) and thermal stability (B) of native (◆) and mutant [Arg356 (□), Lys356 (▲), Glu356 (×), and Gln356 (∗)] luciferases. The error associated with each point falls within ±5% of the value. For further details, see Experimental Procedures.

Volmer plot of *Ppy* and mutants for fluorescence quenching by KI at pH 7.8. As indicated in Figure 8, insertion of Lys356 into the protein changes its conformational integrity and exposes the fluorophore to the quencher, while in Glu356, the conformation is compact and fluorophores are buried.

Bioinformatic Study. The crystal structure of *P. pyralis* firefly luciferase was used as a template to elucidate the

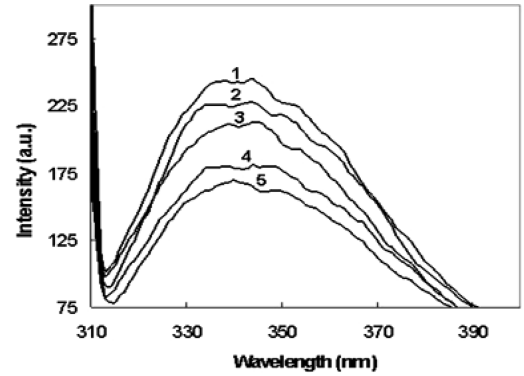

FIGURE 6: Intrinsic fluorescence spectra of native (3) and mutant [(1) Lys356, (2) Gln356, (4) Arg356, and (5) Glu356] luciferases. Spectra were recorded at 25 °C in 0.05 M Tris-HCl buffer (pH 7.8). The concentration of proteins was 0.02 mg/mL. The excitation wavelength was 296 nm. For further details, see Experimental Procedures.

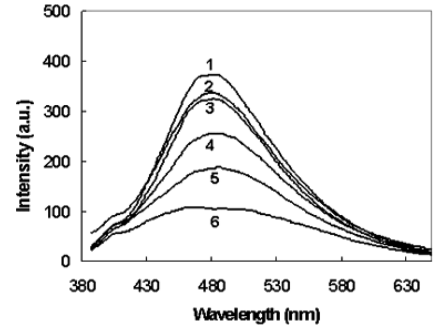

FIGURE 7: Fluorescence spectra of ANS (6) in the presence of native (2) and mutant [(1) Lys356, (3) Arg356, (4) Gln356, and (5) Glu356] luciferases. Spectra were recorded at 25 °C in 0.05 M Tris-HCl buffer (pH 7.8). The sample contained 0.02 mg/mL protein and 30 μM ANS. The excitation wavelength was 350 nm.

structure of the mutant luciferases. Superposition of the three-dimensional structures of native and mutant luciferases revealed a clear conformational change in the flexible loop with formation of a bulge upon insertion of a residue (Figure 9). Moreover, the insertion of these residues has disrupted some interactions and created new ionic and hydrogen bonds as revealed by calculation of the geometry of ionic bonds. A bulge in the left side of the flexible loop is observed for Lys356 and Arg356 mutants. It is interesting to note that a clear shift in the bulge position is observed when the color shifts from green (Gln) to red (Lys). On the other hand, insertion of His at the same position produced a bulge similar to that with the Gln residue (data not shown).

DISCUSSION

Differences in bioluminescence color are attributed to variations in the luciferase structure (*33*), amino acid

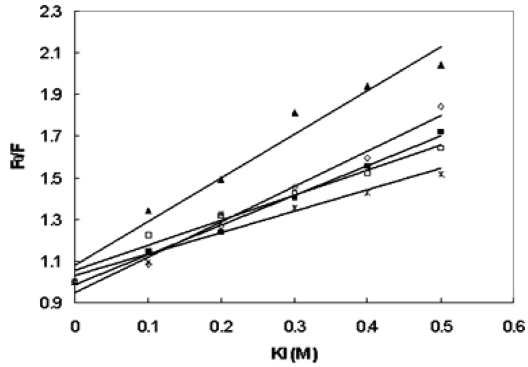

FIGURE 8: Stern−Volmer plots of native (◇) and mutant [Arg356 (■), Lys356 (▲), Glu356 (□), and Gln356 (∗)] luciferases obtained by quenching with KI. The excitation and emission wavelengths were 295 and 340 nm, respectively. The protein was dissolved in 0.05 M Tris-HCl buffer (pH 7.8), and the protein concentration was 20 μg/mL in all cases.

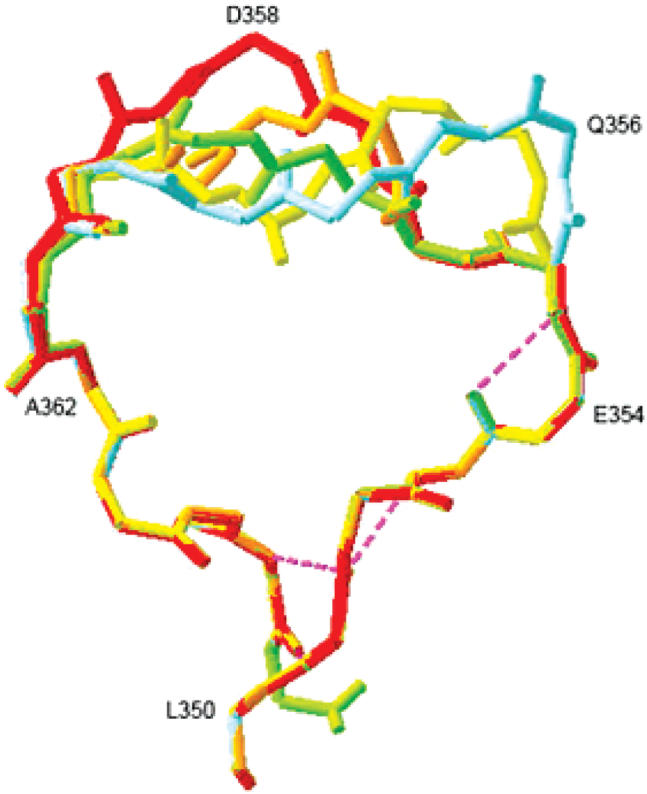

FIGURE 9: Superposition of flexible loop (residues 350−365) models from native (green) and mutant [Lys356 (red), Arg356 (orange), Glu356 (yellow), Gln356 (blue)] luciferases. The insertion of new residues yields a longer flexible loop in comparison with that of the native luciferase.

substitutions (*12, 13, 44−46*), in vitro substitutions of analogues of luciferin and ATP (*1, 20*), and modification of assay conditions like changes in assay pH (*47, 48*). The multiple-sequence alignment of *Ph. hirtus*, the only native species with red bioluminescence, and the other green-emitter luciferases showed in the luciferase from firefly *P. pyralis* like *La. turkestanicus* luciferase Arg356, which corresponds to Arg353 in *Ph. hirtus*, is missed. Insertion of this residue (Arg356) into *La. turkestanicus* luciferase brought about a change in the emitted color from green to red (*17*). For further clarification of the effect of this position and geometry of the loop of residues 352−359 on the color of the light, four different residues (Arg, Lys, Glu, and Gln) were inserted at position 356 of *P. pyralis* luciferase. These residues have similar side chain lengths but different charges at the optimum pH of luciferase activity (pH 7.8). It is obvious that any change in primary sequence can be accompanied by a change in both structure and kinetic properties. Via insertion of these residues into *P. pyralis*, a series of changes in structure and kinetic parameters were observed (Table 1). The comparison of bioluminescence emission spectra of native and mutant luciferases revealed that positively charged residues (Arg356 and Lys356) are more important in the bioluminescence color shift to red, and insertion of these residues resulted in a spectrum with a major peak at 608 nm and a minor shoulder at 557 nm (Figure 3). On the other hand, insertion of Glu354 into *P. pyralis* luciferase leads to a minor shift in bioluminescence spectra. The emission spectrum of Glu354 was bimodal with major and minor peaks at 557 and 591 nm, respectively. The insertion of Gln354 did not affect the emission spectra, and its bioluminescence spectra were similar to those of the native (*Ppy*) luciferase. It has been suggested that the bioluminescence color is determined by the relative contribution of yellow-green and red wavelengths of emission peaks, each of which corresponds to the different forms of excited oxyluciferin (enol−keto tatumerization or two forms of resonance hybrids) that is ultimately controlled by the protein structure (*24, 25*). All mutant and native luciferases are sensitive to pH changes, so a decrease in pH from 7.8 to 5.5 results in green to red shift with maximum emission light at 613 nm (data not shown). On the other hand, a model of the flexible loop for mutant luciferases revealed a different configuration for Arg356 and Lys356 mutant luciferases (Figure 9). Therefore, we may conclude that the insertion of positively charged residues (Arg356 and Lys356) can induce new ionic and hydrogen bonds and as a result displaces the flexible loop and exposes the active site to water, resulting in red light emission; a similar result was obtained for *La. turkestanicus* luciferase (*17*). Also, according to a recently suggested alternative mechanism for interpreting bioluminescence color in firefly luciferases, the green light is produced by minimizing charge delocalization and fixing the charge on the phenolate ion of oxyluciferin (*25*). Accordingly, the key luciferase residues for the luciferin binding site are absolutely conserved among the luciferases and therefore have major roles in this mechanism. It is also reported that the natural red bioluminescence of *Ph. hirtus* has been found intriguing as this luciferase also contains all of the essential proposed residues for green light emission, and even deletion of this residue (Arg353) did not affect the emission color (*49*). Apparently, the results obtained in this investigation may explain the effect of additional Arg residues in *Ph. hirtus* luciferase as well as insertion of positively charged residues in the mutant form of *P. pyralis* in the production of the red color through long-range effects and altering the local polarity of the emitter site which is confirmed by fluorescence spectroscopy. The latter is important as the recent findings show the interaction of this position with another important loop in the luciferase structure (*50*). Another interpretation can be considered here; insertion of basic residues may bring about a decrease in the pH of the emitter microenvironment.

Moreover, the insertion of these residues affected other kinetic properties of luciferases. The rate of decay of native and mutant luciferases was measured, which shows slower

rates of decay for mutant luciferases; thus, this can probably be due to slower decomposition of the intermediate state in the firefly luciferase reaction. The other basic kinetic properties of the mutant enzymes were relatively similar to those of native form. The K_m value for ATP was almost constant, but the K_m values of Arg356 and Gln356 mutants for luciferin were slightly increased, indicating lower affinity of the substrates for the mutant enzymes, and for Glu356 were decreased.

To understand the effect of additional residues in the induction of long-range effects on the local polarity of the emitter site, purified native and mutant luciferases were compared by fluorescence spectroscopy. As indicated in Figure 6, the fluorescence intensity (compared to native) is increased for Lys356 and Gln356 and decreased for Arg356 and Glu356 mutant luciferases. Any changes in the enzyme conformation and oxidation states usually affect the tryptophan fluorescence of the protein (*38*). Hence, insertion of these residues, at position 356, may bring about alteration of the microenvironment of tryptophan residues of the enzyme, resulting in a fluorescence intensity changes. As shown in Figure 6, a considerable increase and a considerable decrease in emission intensity were observed for Lys356 and Glu356 mutants, respectively, suggesting substantial alteration of their conformations, which indicates displacement of Trp(s) to a more hydrophobic (Lys356) or polar (Glu356) environment. The enhancement of fluorescence is a result of the decreased degree of local freedom of rotation of tryptophan molecules due to the location in the interior hydrophobic core of the enzyme molecule. That is to say, insertion of residues Arg356, Lys356, Glu356, and Gln356 would result in different states of structural changes. Moreover, the quenching effect of the positive or negative charge of an additional residue on Trp fluorescence may also occur. However, changes in the luciferase structure upon mutation were confirmed by spectroscopic evidence, which may show that changes in a remote area exert long-range effects through the alteration of polypeptide packing that in turn could modify the structure of the luciferase active site and may alter stabilization of the luciferin phenolate ion, similar to some other luciferase mutants (*24*). Both tryptophans, Trp417 and Trp426, are not surface residues, although they are near the protein surface; the tryptophan aromatic groups are immersed in the folds of the protein globule that contain water molecules (*51*). Therefore, any change in loop can be associated with conformational changes and therefore lead to quenching of these tryptophans. It can be concluded that the increase in the intrinsic fluorescence of the Lys356 mutant is probably due to a charge effect on structure. On the other hand, according to the extrinsic fluorescence of ANS, one may conclude that the hydrophobic patches of the Lys356 mutant are more exposed compared to those of the native form. To reveal the accessibility of the Trp residues in the luciferases, quenching of the tryptophan fluorescence of *P. pyralis* luciferases with KI was carried out. Fluorescence quenching with ions provides information about the polarity of the environment around fluor(s) (*52*). The tryptophan fluorescence of the Lys356 mutant was more effectively quenched with KI which confirmed the results of ANS fluorescence. The nonconserved Trp426 is more accessible to KI ions. The analysis of the three-dimensional structure of *P. pyralis* luciferase shows that the entrance to the cavity, where Trp417 is located, contains positively charged His419, Arg393, and Lys372 and negatively charged Glu370 and Asp413 residues, making Trp417 inaccessible for charged particle. Trp426 is more accessible for hydrate KI ions since it is located closer to the protein surface than Trp417. All the fluorescence results described above show the insertion of positive charges in the 356-deleted position (in *P. pyralis* compared to *Ph. hirtus* luciferase), brought about with different extents of structural changes, which is more pronounced in the Lys356 mutant and produces more displacement of the flexible loop and higher solvent accessibility of the emitter site.

In conclusion, the result presented in this article indicates that the insertion of basic amino acids (with a positively charged R group) into a flexible loop changes the color of light to red. Insertion of basic (positively charged) residues through long-range interactions changes the polarity of the emitter site and produces a bulge in the left side of the flexible loop due to differences in charge distribution and pK_a values of ionizable residues in the flexible loop. Insertion of these residues in addition to the previous findings may explain the importance of this position in the diversion of luciferases to red and green emitters.

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