The Influence of Insertion of a Critical Residue (Arg\textsuperscript{356}) in Structure and Bioluminescence Spectra of Firefly Luciferase*

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The firefly bioluminescence reaction, which uses luciferin, Mg-ATP, and molecular oxygen to yield an electronically excited oxyluciferin, is carried out by luciferase and visible light is emitted. The bioluminescence color of firefly luciferases is determined by the luciferase structure and assay conditions. Among different beetle luciferases, those from Phrixothrix railroad worm emit either yellow or red bioluminescence colors. Sequence alignment analysis shows that the red-emitter luciferase from Phrixothrix hirtus has an additional Arg residue at 353, which is absent in firefly luciferases. We report here the construction and purification of a mutant at residue Arg\textsuperscript{356}, which is not conserved in beetle luciferases. By insertion of an additional residue (Arg\textsuperscript{356}) using site-specific insertion mutagenesis in a green-emitter luciferase (Lampyris turkestanicus) the color of emitted light was changed to red and the optimum temperature of activity was also increased. Insertion of this Arg in an important flexible loop showed changes of the bioluminescence color and the luciferase reaction took place with relatively retention of its basic kinetic properties such as $K_{m}$ and relative activity. Comparison of native and mutant luciferases using homology modeling reveals a significant conformational change of the flexible loop in the red mutant. Movement of flexible loop brought about a new ionic interaction concomitant with a change in polarity of the emitter site, thereby leading to red emission. It is worthwhile to note that the increased optimum temperature and emission of red light might make mutant luciferase a suitable reporter for the study of gene expression and bioluminescence imaging.

Luciferases are the enzymes that catalyze the light-emitting reactions in bioluminescent organisms. In beetles they catalyze a two-step oxidation of luciferin in the presence of ATP, Mg\textsuperscript{2+}, and molecular oxygen to produce light, oxyluciferin, CO\textsubscript{2}, and AMP (1, 2).

The bioluminescence color of fireflies has a wide range from green to red depending on the species: fireflies emit in the yellow-green light (3, 4), click beetles emit in the green-orange (4, 5), and railroad worms emit in a wide range from green to red (6, 7). Although chemical reaction catalyzed by all beetle luciferases and the substrate (luciferin) are identical, these variations in color of emission are still observed (8).

One of the main basis for differences in the luciferases bioluminescence color is the property of the emitter in the microenvironment localized in the enzyme active site. The observed bioluminescence color of pH-sensitive luciferases (but not in click beetle and railroad worm luciferases) shifts to a red region in acidic medium (9, 10). Differences in bioluminescence color are also attributed to the luciferase structure (11, 12). The construction of chimeric proteins (13, 14) and site-directed and random mutagenesis studies have revealed some important residues and regions involved in bioluminescence color (8, 11, 13, 14). Given these results, four mechanisms have been proposed to explain color variations in beetle luciferases.

1) The interaction between solvent and emitter (solvent effect) molecules that influences the difference of the energies between the ground and excited states and therefore maximum luminescence (16). The interaction energy depends on the dynamic characteristics of the interaction such as the rate of reorientation of the solvent molecules around the excited emitter molecules (8).

2) The interaction of basic residues of luciferase with oxyluciferin assist in oxyluciferin tautomerization (keto and enol). This implies that the BL spectra of beetle luciferases are determined by the ratios between enol (green-yellow emitter) and keto (red emitter) forms of excited oxyluciferin. The change in color under the acidic pH medium (red shift) in pH-sensitive luciferases can be explained by this mechanism (9, 17).

3) The active site conformation, which could have an effect on the freedom of rotation of the oxyluciferin thiozolinic rings (18, 19). On the other hand, it affects the rotation of excited oxyluciferin along the C\textsubscript{2}-C\textsubscript{2} bond. According to a recently proposed mechanism, luciferase modulates emission color by controlling the resonance-based charge delocalization of the anionic keto form of the oxyluciferin-excit state (20).

The crystal structure of luciferase without substrates from the North American firefly (Pho\textit{ruitus pyralis}) has been resolved (21), the detailed mechanism for the bioluminescence color change is still unclear. Recently, the crystal structure of wild type and mutant forms (red-emitter) of Japanese firefly (\textit{Luciola cruciata}) in complex with a high-energy intermediate analogue,
**Insertion of Arg\textsuperscript{356} in Firefly Luciferase**

DLSA\textsuperscript{3} has been obtained. Crystallographic data indicated that the degree of molecular rigidity of the excited oxyluciferin, which is controlled by a transient movement of Ile\textsuperscript{288} (in *L. cruciata*), determines the color of bioluminescence during the emission reaction (22). Among all beetle luciferases, the *Phrixothrix* railroad worm luciferase is the only luciferase that naturally emits red light through two cephalic lanterns (\(\lambda_{\text{max}} = 628 \text{ nm}\)) in addition to the yellow-green bioluminescence (\(\lambda_{\text{max}} = 542 \text{ nm}\)) emitted through 11 pairs of lateral lanterns along the body. *Phrixothrix hirtus* red (Ph\textsubscript{\textit{R}}) luciferase shows 46–49\% identity with firefly luciferases. Ph\textsubscript{\textit{R}} luciferase with a naturally red-emitting ability is a unique model to investigate the relationship of luciferase structure with BL color (14, 23). The sequence of multiple alignments of primary structure of Ph\textsubscript{\textit{R}} with other firefly luciferases, which emit green light, showed the presence of Arg\textsuperscript{353} in Ph\textsubscript{\textit{R}}, corresponding to a missed residue in other firefly luciferases.

To identify the effect of this residue (Arg\textsuperscript{353}) on bioluminescence color, we have inserted it in the corresponding position (Arg\textsuperscript{356}) using site-specific mutagenesis in the Iranian firefly luciferase (*Lampyris turkestanicus*) (24) and the effect of this residue on the BL spectra, kinetic properties, and structure were analyzed. BL spectra and molecular modeling results show that Arg\textsuperscript{356} has a critical role in determining BL color.

**EXPERIMENTAL PROCEDURES**

**Reagents**

The following reagents and kits were used: isopropyl \(\beta\)-D-thiogalactopyranoside, kanamycin, and ATP (Roche), \(\delta\)-luciferin potassium salt (Sigma), restriction enzyme (Fermentase), *Pfu* polymerase, plasmid extraction kit, gel purification kit, and the PCR purification kit (Bioneer Co.), Ni-NTA spin kit (Qiagen Inc.), and the pET28a vector (Novagen).

**Insertion Mutagenesis**

Insertion mutagenesis was performed using splicing overlap extension-PCR (25). Two pairs of primers were used for this purpose: F-Cloning containing the BamHI restriction site (5\textsuperscript{\textprime} CGTGTGGATCCATGGAAGATGCAAAAAATATTATG-3\textsuperscript{\textprime}), R-Cloning containing the HindIII restriction site (5\textsuperscript{\textprime} CCAGCTTTTACAAATTTAGATTATTTTTTCCCCATC-3\textsuperscript{\textprime}). The overlapping primers, F-Mutant and R-Mutant, containing the Arg codon insertion, respectively, are: 5\textsuperscript{\textprime} CACCCGAAGGGAGAGATGATAAAACCAGGAGCATGTG-3\textsuperscript{\textprime} and 5\textsuperscript{\textprime} GTTTATCATCTCTCCCTCGGGTGTTAATTTAATTTGC-3\textsuperscript{\textprime}.

The cDNA for *L. turkestanicus* had been previously cloned in pQE30 plasmid (24). It was subcloned in pET28a plasmid in this study. The plasmid including the native luciferase was used as template. Two PCRs to perform 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 \(^\circ\text{C}\) for 5 min, a 25 cycle (94 \(^\circ\text{C}\) for 1 min, 68 \(^\circ\text{C}\) for 1 min, and 72 \(^\circ\text{C}\) for 1:30 min), and a final extension for 5 min at 72 \(^\circ\text{C}\). Subsequently, primary PCRs were purified using a clean up 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 was around 100 ng. PCR conditions were the same as primary PCRs except that it was carried out for 10 cycles without primers. Finally, F- and R-cloning primers were added to each tube and the next step of PCR was performed. The mutagenesis products digested by BamHI/HindIII were inserted into the BamHI/HindIII restriction sites of digested/dephosphorylated pET28a high expression vector and ligated mixtures were transformed into competent cells of *Escherichia coli* BL21 by electroporation.

**Multiple Sequence Alignment**

Multiple sequence alignments were performed by utility ClustalW program of amino acid sequences of firefly luciferases and red-emitting railroad worm luciferase.

**Protein Expression and Purification**

Ten ml of 2\times YT medium containing 50 \(\mu\text{g/ml}\) kanamycin with a fresh bacterial colony harboring the expression plasmid was inoculated and grown at 37 \(^\circ\text{C}\) overnight. Then 300 ml of medium with 500-\(\mu\text{g/ml}\) overnight cultures was inoculated and grown at 37 \(^\circ\text{C}\) with vigorous shaking until the \(A_{600}\) reaches 0.6. After which, isopropyl \(\beta\)-D-thiogalactopyranoside was added to a final concentration of 1.5 \(\text{mM}\) to the solution and incubated at 22 \(^\circ\text{C}\) overnight with vigorous shaking. The cells were harvested by centrifugation at 5000 \(\times\) \(g\) for 15 min. The cell pellet was resuspended in lysis buffer (50 mM NaH\textsubscript{2}PO\textsubscript{4}, 300 mM NaCl, 10 mM imidazol, pH 8) and stored at \(-70\) \(^\circ\text{C}\) for the next step.

Purification of His\textsubscript{6}-tagged fusion protein was performed by the Ni-NTA spin column as described by the manufacturer (Qiagen). The color of emitted light of firefly luciferase reaction was obvious by luciferin addition to purified luciferases. Pictures of glowing colonies on LB-Agar plate and the mixture containing luciferases were taken by Konica 400 ASA film. *P. pyralis* luciferase was also purified under the same condition.

**Sequencing**

pET28a containing native and mutant luciferase were sequenced using an automatic sequencer (MWG, Germany) by the T7 promoter and T7 terminator universal primers.

**Determination of Kinetic Parameters**

ATP and luciferin kinetic parameters were measured at 25 \(^\circ\text{C}\). To estimate LH\(2\) \(K_{\text{m}}\) 10 \(\mu\text{M}\) of assay reagent containing 20 \(\text{mM}\) MgSO\textsubscript{4}, 2 \(\text{mM}\) ATP in 50 \(\text{mM}\) Tricine, pH 7.8, was mixed with 50 \(\mu\text{L}\) of various concentrations of luciferin (from 0.01 to 2 \(\text{mM}\)) in a well of a 96-well plate. The reaction was initiated with injection of 50 \(\mu\text{L}\) of diluted enzyme and light emission was recorded over 5 s (Orion microplate Luminometer, Berthold Detection System, Germany). The estimation of ATP kinetic constant was performed in a similar way but various concentrations of ATP were mixed with assay reagent containing 20 \(\text{mM}\) MgSO\textsubscript{4}, 2 \(\text{mM}\) luciferin in 50 \(\text{mM}\) Tricine, pH 7.8. Apparent kinetic parameters were calculated by Lineweaver-Burk plots.

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\(3\) The abbreviations used are: DLSA, 5\textsuperscript{\textprime} O-[N-(dehydroxyfurfuryl)-sulfamoyl]adenosine; Ph\textsubscript{\textit{R}}, *Phrixothrix hirtus* red light emitter; *L*uc, recombinant native luciferase; *L*uc(Arg), recombinant mutant luciferase containing Arg insertion; BL, bioluminescence; Ni-NTA, nickel-nitrilotriacetic acid; Tricine, N-\(\{2\)-hydroxy-1,1-bis(hydroxymethyl)ethyl\]-glycine.
The decay times of native and mutant luciferase were measured in 20 min and compared with luciferase from *P. pyralis*. The residual activity for each enzyme was reported as a percentage of the original activity. Approximate protein concentrations were calculated using a Bradford assay (26) and relative specific activities (enzyme activity/protein concentration) were also calculated.

**Measurement of Bioluminescence Emission Spectra**

BL spectra were recorded using a Cary-Eclipse luminescence spectrophotometer (Varian) from 400 to 700-nm wavelengths. A volume of 300 μl of Tricine buffer, pH 7.8 and 5.5, including 2 mM ATP, 5 mM MgSO4, and 1 mM luciferin, was added to 100 μl of purified luciferase solution (around 50 pg) in a quartz cell. The spectra were automatically corrected for photosensitivity of the equipment.

**Thermostability, Optimum pH, and Temperature**

The purified luciferases (10 μg/ml) were incubated at 22, 27, 32, and 37 °C for 10 min. Enzyme activities were measured at room temperature (25 °C) and the remaining activity were recorded as percentage of the original activity. To obtain optimum temperature of activity for native and mutant luciferase, activities were measured in the 20–37 °C range. Moreover, optimum pH of activity for both enzymes was measured by incubation of enzyme in a mixed buffer at the pH 5.5–9.5 range, respectively.

**Structural Studies**

**Intrinsic Fluorescence**—The purified luciferases were dialyzed in dialysis buffer containing 50 mM Tris-HCl, pH 7.8, 1% glycerol, 1 mM EDTA, 50 mM NaCl, 0.05% glycerol, 1 mM EDTA, 50 mM NaCl, 0.05% mercaptoethanol and 1 mM luciferin, was added to 100 μl of purified luciferase solution (around 50 pg) in a quartz cell. The spectra were automatically corrected for photosensitivity of the equipment.

**Circular Dichroism (CD) Measurements**—CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with purified and dialyzed 0.2 mg/ml protein concentrations (far UV). Protein buffer was the same as fluorescence measurements. The results were expressed as molar ellipticity, [θ] (degree cm2 dmol−1), based on a mean amino acid residue weight (MRW) assuming its average weight for firefly luciferase. The molar ellipticity was determined as [θ] = (θ × 100 MRW)/(εl), where ε 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 [θ]291 = 7820 degree cm2 dmol−1 (27), and with JASCO standard nonhydroscopic ammonium (+)-10-camphorsulfonate, assuming [θ]290.5 = 7910 degree cm2 dmol−1 (28). Noise in the data was smoothed using JASCO J-715 software, including the fast Fourier transform noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes (29).

**Model Building and Analysis**

BLASTP (30) search against the Protein Data Bank was used to obtain *L. turkestanicus* luciferase (Q5UFR) homologues. *P. pyralis* firefly luciferase (PDB code 1LCI) shows 84% identity and 91% similarity with Q5UFR and therefore is useful as the PDB template in the SwissModel Alignment Interface protein modeling server (31). With submission of multiple sequence alignment of luciferase sequence family and template, three-dimensional structure models of native and mutant luciferase were obtained. The fit between the three-dimensional structure of PDB 1LCI and models were evaluated in the SWISS-PDB Viewer by calculating the root mean square deviation after iterative fitting. The spatial position of ligand (5′-O-[n-(dehydrolicerif)-sulfamoyl]adenosine) was made by superposition of model structures with red-emitter Japanese firefly (*L. cruciata*) luciferase (S286N mutant) complexed with a high-energy intermediate analogue (PDB code 2D1T). Accessible surface areas of native and mutant proteins were also calculated by the GETAREA program (32) and salt bridges were calculated by WHAT IF Web server (33).

**RESULTS**

**Construction, Expression, and Purification of the Native and Mutant Luciferases**—To identify the residue involved in the determination of BL color in luciferases, a multiple sequence alignment among firefly luciferases and the red-emitting railroad worm luciferase was performed (Fig. 1). Multiple sequence alignment showed the presence of Arg356 in PhPy luciferase, which corresponds to the deleted residue in firefly luciferases. As indicated in Fig. 1, Arg356 is missing in *L. turkestanicus*, as all the other green emitting luciferases. Therefore, we inserted this residue in the same position in luciferase from *L. turkestanicus* using PCR insertion mutagenesis. After cloning and transformation, the presence of Arg356 was confirmed by sequencing.

Upon addition of D-luciferin, the photographs of the isopropyl β-D-thiogalactopyranoside-induced bright bacteria cells containing native and mutant luciferases were taken in a dark room at pH 7.8 and 5.5. For further purification and characterization of native and mutant forms, overexpressions of luciferases were carried out in the BL21 host. The purification of the His tag fusion luciferases was also performed by affinity (Ni-NTA-Sepharose) chromatography. The purified native and mutant luciferases had purities of more than 95% based on analyses by SDS-PAGE in which luciferase was present as a band of about 62 kDa. The color of the reaction upon addition of substrate to purified luciferase could be seen with the naked eye in the dark room (Fig. 2).

**Bioluminescence Emission Spectra**—The *in vitro* bioluminescence spectra of native and mutant luciferases are indicated in Fig. 2. It is evident that rLuc(Arg) displays a bimodal spectrum with a maximum in the red region (at 615 nm) and a smaller shoulder at 560 nm in the green region, whereas the native luciferase exhibits a spectrum with the peak at 555 nm. The mutant form bioluminescence spectrum at the lower pH (pH 5.5) also has a shoulder in the green region, as indicated in Fig. 2; however, the shoulder in the green region was smaller at
acidic pH. The emission spectrum for native luciferase was slightly changed under acidic condition.

Influence of the Arg Insertion on the Kinetic Constants, Decay, and Thermostability—Lineweaver-Burk plots were used to estimate the apparent \( K_m \) values for luciferin and ATP. The results (relative activity, optimum pH, and optimum temperature of activity in addition to \( K_m \) toward LH2 and ATP) are shown in Table 1. The mutation has affected the performance of the enzyme, and whereas the \( K_m \) value for ATP is approximately similar to native, that for LH2 is around 1.5 times higher in rLuc(Arg).

The decay time of light emission for mutant luciferase was somewhat faster than the native form and is compared with \( P.\ pyralis \) (Fig. 3). A clear improvement in thermostability was also observed for mutant luciferase compared with the native form as indicated in Fig. 4. However, as indicated in Table 1, the astonishing finding was that the optimum temperature for the activity of the mutant enzyme is increased to 34 °C, which is 10 °C higher than native luciferase. In addition, optimum pH of the mutant also was slightly higher.

CD and Fluorescence Spectra of Native and Mutant Forms of Luciferase—Circular dichroism (CD) spectra of native and mutant forms of luciferase obtained in phosphate-buffered saline, pH 7.0, are shown in Fig. 5. The far UV CD spectra of the native and mutant forms of luciferase show subtle changes in the secondary structure of the protein. The content of the secondary structure elements of the native and mutant forms are also indicated in Fig. 5. An apparent increase of helical structure has occurred in luciferase upon addition of Arg356 to the primary structure. However, change of the helical structure is accompanied by a small decrease of unordered structures. On the other hand, as indicated in Fig. 6 an increase of fluorescence intensity has been observed for mutant (rLuc(Arg)) luciferase. Even small changes in the enzyme conformation have proven to affect the tryptophan fluorescence of proteins (34).

Molecular Modeling—The crystal structure of \( P.\ pyralis \) firefly luciferase was used as template to elucidate the structure of the mutant \( L.\ turkestanicus \) luciferase. The sequence identity and sequence similarity were 84 and 91%, respectively. Superposition of the three-dimensional structures of native and mutant luciferase revealed quite similar structures, with an exception in the flexible loop conformation that was clearly changed (Fig. 7). Moreover, the insertion of this residue has disrupted some interactions and brought new ionic and hydrogen bonds as revealed by calculation of the geometry of ionic bonds.

DISCUSSION

Emission of red bioluminescence is unusual among beetle luciferases. Generally, differences in bioluminescence color are
caused by natural species variations in the luciferase structure (23); amino acid substitutions introduced by mutagenesis techniques (8, 11–15); and in vitro substitutions of analogues of luciferin (19) and ATP (2, 35) for the natural substrates and modification of assay conditions like changes of assay solution pH and temperature (10, 37).

The cDNA encoding of luciferase from the glowworm *L. turkestanicus* has been isolated using reverse transcriptase-PCR and functionally expressed in *E. coli* (24). As indicated in Fig. 1, Arg<sup>356</sup> in *L. turkestanicus* luciferase is absent as well as other luciferases. Bioluminescence catalyzed by *L. turkestanicus* as in *P. pyralis* luciferase in vivo and at pH 7.8 in vitro is yellow-green with an emission maximum of about 555 nm.

The insertion of an additional residue (Arg<sup>356</sup>) in a critical region in *L. turkestanicus* luciferase brought about changes in both kinetic and structural properties of the enzyme. It is noteworthy that the native (rLuc) and mutant (rLuc(Arg)) luciferases catalyzed the emission of yellow-green and red light, respectively, and the corresponding emission spectra were approximately similar to those at pH values of 7.8 and 5.0 for native luciferase (Fig. 2). Apparently, insertion of an additional Arg is responsible for the bimodal spectrum of bioluminescence.

![FIGURE 3](image3.png)

**FIGURE 3.** Comparison of decay times of rLuc and rLuc(Arg) with *P. pyralis* luciferase. Faster decay of bioluminescence signal for mutant luciferase (rLuc(Arg)) is clear.

![FIGURE 4](image4.png)

**FIGURE 4.** The comparison of rLuc and rLuc(Arg) thermostability. Mutant form of rLuc(Arg) shows remarkably greater thermostability in comparison with the native form. For further detail see “Experimental Procedures.”

![FIGURE 5](image5.png)

**FIGURE 5.** Far-UV CD spectra for rLuc and rLuc(Arg) luciferase. The concentration of protein used for the far UV CD spectrum (200–250) was 0.2 mg/ml. The protein was equilibrated in Tris-HCl buffer (0.05 M, pH 7.8), at 25 °C. Each spectrum represents the average of five scans. The content of secondary structure elements of native and mutant forms are also indicated in the inset. For further details see “Experimental Procedures.”

![FIGURE 6](image6.png)

**FIGURE 6.** Intrinsic fluorescence spectra for native (rLuc) and mutant (rLuc(Arg)) luciferase. Spectra were taken at 25 °C in Tris-HCl buffer (0.05 M, pH 7.8). The concentration of proteins was 0.01 μg/ml. The excitation wavelength was 296 nm. For further details see “Experimental Procedures.”

| Mutants | Luciferin $K_m$ (μM) | ATP $K_m$ (μM) | Relative specific activity % | $\lambda_{max}$ (nm) pH 7.8 | $\lambda_{max}$ (nm) pH 5.5 | Optimum Temperature °C | pH |
|---------|---------------------|---------------|-----------------------------|-----------------------------|-----------------------------|------------------------|-----|
| rLuc    | 16 135              | 100           |                             | 555                         | 560                         | 24                     | 8   |
| rLuc(Arg)| 24 142              | 81.6          |                             | 616, 555<sup>a</sup>        | 618, 560<sup>a</sup>        | 34                     | 8.5 |

<sup>a</sup> Identify of minor peaks.

**TABLE 1** Comparison of the rLuc and rLuc(Arg) kinetic properties

The concentration of protein used for the far UV CD spectrum (200–250) was 0.2 mg/ml. The protein was equilibrated in Tris-HCl buffer (0.05 M, pH 7.8), at 25 °C. Each spectrum represents the average of five scans. The content of secondary structure elements of native and mutant forms are also indicated in the inset. For further details see “Experimental Procedures.”

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mutant luciferase. The orange appearance of glowing colonies can be interpreted through bimodal emission spectra of mutant forms also (Fig. 2). Therefore, it may be 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 different forms of excited oxyluciferin (enol-keto tautomerization or two forms of resonance hybrids) that is ultimately controlled by the protein structure. As indicated in Fig. 2 the presence of an additional Arg made the \textit{L. turkestanicus} luciferase more sensitive to pH changes compared with the native form. Arg\textsuperscript{356} has been added in a region containing a flexible loop, 352-TECGDDKP359. Structural and molecular modeling studies show that the flexible loop is engaged in a network of many intermolecular hydrogen and ionic bonds with other residues in the backbone. As shown in \textit{FIGURE 7}, insertion of Arg\textsuperscript{356} in this flexible loop changes the peptide backbone conformation. On the other hand, calculation of geometry of ionic bonds revealed induction of a new ionic bond between Glu\textsuperscript{354} and Arg\textsuperscript{334} in the mutant luciferase. Therefore, it may be suggested that insertion of a new Arg and induction of a new ionic bond displaces the flexible loop exposing the active site to water and therefore resulting in red light emission, thereby opening the active site to the pH will be made. A similar result has been reported for another single mutant of firefly luciferase (38).

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 (20). 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 \textit{P. hirtus} has been found intriguing because this luciferase also contains all of the essential proposed residues for green light emission (20). Apparently, the results obtained in this investigation may explain the effect of additional Arg in \textit{P. hirtus} luciferase as well as the mutant form of \textit{L. turkestanicus} in production of the red color through long-range effects and altering the local polarity of the emitter site (39).

Moreover, this mutation has affected other kinetic properties of luciferase. The time course of the light emitted by both luciferases was also measured that shows relatively faster decay of bioluminescence signal for mutant luciferase (Fig. 3). Therefore, it may be concluded that insertion of an Arg in the polypeptide backbone could cause faster decomposition of the intermediate state in the firefly luciferase reaction. Change in rate of luminescence decay was also observed for a single mutant of bacterial luciferase (40). As indicated in Fig. 3, the glow-type luminescence signal of the native and mutant forms of \textit{L. turkestanicus} is more stable than the flashing pattern of \textit{P. pyralis} luciferase. The optimum temperature of activity for mutant luciferase (34 °C) was much higher than the native form (24 °C). This finding is very intriguing as mutant luciferase indicates a brighter and more stable signal at physiological temperature in the case of using the red-emitter luciferase (rLuc(Arg)) as a reporter for bioluminescence imaging and also in \textit{vivo} diagnostic applications. Luciferase is a useful reporter gene \textit{in vivo} and has been used for non-invasive imaging of tumor growth (41), metastasis (42), as a reporter for efficiency of gene transfer and therapy (43, 44), and gene expression (43, 45). It should be noted that increased light production and sensitivity have been observed in the tumors bearing thermostable luciferase (36). The other basic kinetic properties of the mutant enzyme were relatively similar to the native form. The \(K_m\) values for ATP and luciferin were slightly increased, indicating lower affinity of the substrates to the mutant enzyme. Lower affinity was concomitant with lower relative activity (81%).

It is worthwhile to note that the expression of an active mutant luciferase under the same condition as the native form suggests formation and refolding of a properly folded luciferase without aggregation, which has been confirmed by different spectroscopic techniques. Moreover, the far UV CD spectra of the native and mutant forms of luciferase show subtle changes in secondary structure of the protein upon insertion of an additional Arg\textsuperscript{356} in the protein backbone (Fig. 5).

As indicated in Fig. 6 an increase in fluorescence intensity is observed for the mutant luciferase. Any changes in the enzyme conformation and oxidation states usually affect the tryptophan fluorescence of protein (34). Hence, insertion of an Arg residue may bring about alteration of the microenvironment of tryptophan residues of the enzyme, resulting in a fluorescence intensity change. As shown in Fig. 6 a considerable increase in emission intensity was observed in the mutant, suggesting substantial alteration in its conformation, which indicates displacement of Trp(s) to a more hydrophobic environment. The enhancement of emission under this condition is a result of the decreased 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 an Arg moiety (Arg^{356}) would result in a different pattern of structural changes. Moreover, the quenching effect of the positive charge of an additional residue on Trp(s) fluorescence may also occur. It may be proposed that, if it had not been for such a quenching effect of positive charge, higher fluorescence intensities for the mutant form would have been obtained. It is therefore suggested that an increase of intrinsic fluorescence (Fig. 6) occurs as a result of protein rigidity due to partial compactness of the mutant enzyme compared with the native enzyme in the same condition. And so, the higher optimum temperature of activity of mutant luciferase may be interpreted through higher rigidity of its structure. However, changes in the luciferase structure upon mutation were confirmed by spectroscopic evidence, which 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 as proposed by Branchini et al. (20).

In conclusion, the result presented in this article indicates that insertion of Arg^{356} in the central cavity of L. turkestanicus luciferase, similar to the native form of Phrixothrix railroad worms red-emitter luciferase (Ph_{RE}), plays a critical role in determination of the color of emitted light. Insertion of this additional residue increased the polarity of the emitter site. Moreover, expression of an active refolded mutant luciferase may explain the effect of this position on diversion of firefly luciferases into two distinct groups of red and green emitters.

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