Supplementary Data

Methods

Design and construction of Disulfide Bridges

To investigate the effect of disulfide bonds in structure and function of luciferase, sites for insertion of disulfide bridges were selected using the program Disulfide by Design Version 1.2 and MODIP ((http://caps.ncbs.res.in/iws/modip.html) server. [1, 2] These programs use the backbone coordinates from three-dimensional model to select residue pairs on the basis of the calculated C_β- C_β distances. Subsequently, S_γ positions with ideal or nearly ideal geometries were generated for the selected pairs. Energy minimization procedures were used to select acceptable conformations. The coordinates of pairs of cysteine residues in disulfide bridges (total of 73 pairs, data not shown) in known protein x-ray structures were collected, in the second step energy minimization to estimate the strain associated with formation of disulfide bond calculated and locations for the four sites were selected (Table S1).

Protein Expression and Purification

All mutant sequences were confirmed with sequencing. The selected mutants were constructed and could successfully be expressed in *E. coli* (origami 2). Five millilitres of TB medium containing 50 mg mL^{-1} kanamycin and 12.5 mg mL^{-1} tetracycline with a fresh bacterial colony harboring the expression plasmid was inoculated and grown at 37 ºC overnight. Then 200 mL of medium with 500 mL overnight. Cultures was inoculated and grown at 37 ºC with vigorous shaking until the OD_{600} reached 0.9. Then, IPTG and lactose were added to the solution to a final concentration of 1 mM and 4 mM, respectively, and the mixture incubated at 22 ºC 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 8.0)]. Purification of His_6-tagged fusion protein was performed with the Ni-NTA spin column as described by the manufacturer (Qiagen).

Thiol titration

The wild type *Ppy* and mutants at concentration of 2 $mgml^{-1}$ were denatured by Gu.HCl in 100 mM sodium phosphate (pH 8.0) and 2 mM EDTA with or without 50 mM dithiothreitol (DTT) [3, 4] The denatured proteins dialyzed against denaturing solution to remove DTT. A small portion of the resulting dialyzed samples was used to determine the protein concentrations by the Bradford assay (Emamzadeh et al, 2006). Thiol titration was carried out by mixing 30 parts of the samples with one part of 4 $mgml^{-1}$ of dithionitrobenzoic acid (DTNB) in denaturing solution for 15 min at room temperature and then the absorbance was measured at 412 nm with a molar absorbance value of 13,600 M^{-1} cm^{-1}. [3]

Measurement of Bioluminescence Emission Spectra

BL spectra were recorded using a Cary-Eclipse luminescence spectrophotometer (Varian) from 400 to 700 nm, as reported previously. [5] A volume of 300 μL of 50 mM Tris-HCl buffer (pH 8.0 and 7.2) including 2 mM ATP, 5 mM $MgSO_4$, and 1 mM luciferin, was added to 100 μL of a purified

luciferase solution [50 µg in elution buffer 50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, and 1 mM PMSF (add fresh) (pH 8.0)] in a quartz cell. For recording the spectra at 37 °C the thermal mode of spectrophotometer was selected. During the time course of each measurement no significant loss of light was observed. 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.

Kinetic Properties

ATP and luciferin kinetic parameters were measured at 25 °C. To estimate LH2 K_m, 50 μL of assay reagent containing 10 mM $MgSO_4$ and 4 mM ATP in 50 mM Tris-HCl (pH 8.0) was mixed with 40 μL of various concentrations of luciferin (0.0025-5 mM) in a tube. The reaction was initiated by adding 10 μL of enzyme (it was injected in the luminometer chamber during the signal recording), and light emission was recorded over 10 s (Orion II Microplate Luminometer, Berthold Detection Systems). 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 8.0). The reaction was initiated by adding 10 μL of enzyme, and light emission was recorded over 10 s. Apparent kinetic parameters were calculated by Hanes–Woolf plots. The decay times of wild-type and mutant luciferases were measured in 15 minute 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, [6] and relative specific activities (enzyme activity vs protein concentration) were also calculated. To obtain the optimal temperature of activity for wild-type and mutant luciferases, activities were measured in the range of 5-45 °C. Moreover, Optimum pH of activity for the enzymes was measured by injecting 10 µl of a luciferase solution (pH 8.0) to a cell containing 10 µl of 10X cocktail (containing 20 mM ATP, 50 mM $MgSO_4$ and 10 mM luciferin in 50 mM Tris-HCl buffer (pH 8.0)) and 180 µl mix buffer (100 mM glycine, 100 mM succinic acid and 50 mM Tris-HCl) over a range of pH (5–12). The light emission was recorded 1 s at room temperature (25 ºC). It should be noted that the activities have not been corrected for spectral response of the detector.

Thermal Inactivation and Thermal Stability

To study thermal inactivation, the purified luciferases (20 μg mL^{-1}) 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 10 min in ice. The time courses of thermostability of purified luciferases were measured by incubating the enzyme in 50 mM Tris-HCl (pH 8.0) at 32 °C. At regular Intervals (0-45 min), samples were removed and cooled on ice (10 min), and the remaining activity was determined. The activity of the enzyme solution kept on ice was considered as the control (100%). (In these experiments the enzyme was added manually with subsequent transferring of the reaction tube into the (Sirius single tube luminometer, Berthold Detection Systems).

Results

Construction, Expression, and Purification of the Wild-type and Mutant Luciferases According to the result of MODIP server and disulfide-by design software all residue pairs are rapidly assessed for proximity and geometry consistent with disulfide formation. The output displays residue pairs meeting the appropriate criteria, the estimated chirality and torsion angle are based on the best possible orientation of putative mutant cysteine Sγ atoms, as determined by an energy minimization, the selected mutants were (C^{81}-$A^{105}C$, $L^{306}C$-$L^{309}C$, $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$, $P^{451}C$-$V^{469}C$/ $A^{296}C$-$A^{326}C$, and C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$) constructed and could successfully be expressed in *E. coli* (origami2). Wild type and all mutant luciferases were similar with respect to expression levels and yields of purification. The protein yields for wild type and all mutants except $L^{306}C$-$L^{309}C$ were the same and it was 2-3 mg ml^{-1} (about 20 mg L^{-1} of culture) but for the $L^{306}C$-$L^{309}C$ mutant was 0.5 mg ml^{-1}. The purification of His_6-tagged fusion luciferases was also performed by affinity (Ni-NTA Sepharose) chromatography. The purified wild-type and mutant luciferases had purities more than 95% on the basis of the analysis by SDS-PAGE in which luciferases were present as a band of 62 kDa (see Figure S1).

Correct formation of engineered disulphide bridge

Mutant enzymes were expressed in the Origami2 (DE3) strain for suitable formation of disulphide bridge. Although, in expressing proteins containing disulphide bridges the mismatched formation of disulphide between other free cysteines and insolubility of proteins are very likely, the presence of activity in mutant enzymes confirmed proper refolding. Incubation of wild-type firefly luciferase with DTNB (Ellman reagent) shows all luciferase cysteines are buried inside of non-accessible area of luciferase. Since all cysteines aren't accessible to Ellman reagent, all mutants and wild-type luciferases treated with denaturant agent GuHCl. The profile of the reaction of DTNB molecules with wild-type and mutant enzymes in the presence of denaturant are similar (Table S2). These results showed that the introduced Cys residues formed disulphide bridge instead of remaining free thiol groups to react with DTNB. Hence the released TNB concentrations were similar to wild-type *P. pyralis* luciferase. Meanwhile, DTNB assay under denaturing and reducing conditions (with DTT) for mutant proteins brought about with a twice release of TNB compared to wild-type which confirms formation of disulphide bond in the absence of a reducing agent, for C^{81}-$A^{105}C$ mutant show one free cysteine of protein involved in disulfide bridge.

Near CD spectra of wild-type and mutant forms of luciferase

As indicated in (Figure S2) the near-UV CD spectra of the wild-type and mutant forms of luciferase show changes in the tertiary structure of the mutants. The near-UV CD spectra of C^{81}-$A^{105}C$, $P^{451}C$-$V^{469}C$, and $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$, and $L^{306}C$-$L^{309}C$ mutants show increase in positive ellipticity,

whereas C^{81}-$A^{105}C$/$P^{451}C$-$V^{469}C$, and $A^{296}C$-$A^{326}C$ mutants show decrease in positive ellipticity, compared to wild-type structure.

Biochemical properties

Subtle changes in optimum pH for mutant luciferases ($L^{306}C$-$L^{309}C$, $P^{451}C$-$V^{469}C$, and $A^{296}C$-$A^{326}C$), was also observed (Figure S3) but for C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$ and C^{81}-$A^{105}C$, $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$ mutant luciferases display an increase from 8 to 8.5 and 9 respectively. While the optimum temperature of $L^{306}C$-$L^{309}C$, $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$ increased 5 ˚C and for C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$, C^{81}-$A^{105}C$, and $A^{296}C$-$A^{326}C$ mutants increased 10˚C, and 15 ˚C compared to wild-type enzyme respectively and it was not affected in $P^{451}C$-$V^{469}C$ (Figure S4). All mutants displayed typical flash-like light emission kinetics with the kinetic profile faster than WT, except for $A^{296}C$-$A^{326}C$ mutant with a slightly higher decay time that resulted in a significance increase of the integrated specific activity.

References

1. V. S. Dani, C. Ramakrishnan and R. Varadarajan, MODIP revisited: re-evaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins, *Protein eng.,* 2003, **16**, 187-193.
2. A. A. Dombkowski, Disulfide by Design™: a computational method for the rational design of disulfide bonds in proteins, *Bioinformatics*, 2003, **19**, 1852-1853.
3. A. Habeeb, Reaction of protein sulfhydryl groups with Ellman's reagent, *Method. Enzymol.,* 1972, **25**, 457-464.
4. S. Pollitt and H. Zalkin, Role of primary structure and disulfide bond formation in beta-lactamase secretion, *J. Bacteriol.,* 1983, **153**, 27-32.
5. A. R. Emamzadeh, S. Hosseinkhani, M. Sadeghizadeh, M. Nikkhah, M. J. Chaichi and M. Mortazavi, cDNA cloning, expression and homology modeling of a luciferase from the firefly Lampyroidea maculata, . *J. Biochem. Mol. Biol.,* 2006, **39**, 578-585.
6. M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.,* 1976, **72**, 248-254.

Table S1: Thiol content of various Ppy Derivatives are determined according to OD.412 upon treatment with Ellman reagent (all the data repeated at least 3 times).

Ppy derivatives	Mol of SH/ mol of protein	
	-DTT	+DTT
Ppy	4	4
C^{81}-$A^{105}C$	3.2	5.2
$L^{306}C$-$L^{309}C$	3.96	6
$P^{451}C$-$V^{469}C$	4.08	6.2
$A^{296}C$-$A^{326}C$	3.91	6.07
C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$	2.91	7.01
$A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$	4.02	8.06

Table S2: Parameters for disulfide bridges in *P. pyralis* firefly luciferase

	C^{81}-$A^{105}C$	$L^{306}C$-$L^{309}C$	$P^{451}C$-$V^{469}C$	$A^{296}C$-$A^{326}C$
Energy	4.83	1.73	3.92	0.73
Chi 3	+ 125.32	- 73.76	- 76.75	+ 85.24
Loop Size, residues	24	3	18	30

Table S3. Half-life in minute for wild-type and mutants at the temperatures tested**.**

Firefly Luciferase	$t_{1/2}$
Ppy [a]	2
$E^{354}K$ [a]	12
Hotaria parvula (wt) [b]	18
$A^{217}L$ [b]	40
$E^{356}R$ [b]	20
$V^{368}A$ [b]	18
$E^{356}R$/ $V^{368}A$ [b]	60
Ppy [c]	0.58
rLucx4ts ($T^{214}A$, $I^{232}A$, $F^{295}L$ and $E^{354}K$) [c]	5.95
Luciola cruciata [d]	5
$T^{217}I$ [d]	30
Ppy [e]	5
Insertion of Q^{356} [e]	25
Ppy [f]	10
$P^{451}C$-$V^{469}C$ [f]	20
$L^{306}C$-$L^{309}C$ [f]	60
$A^{296}C$-$A^{326}C$ [f]	30
C^{81}-$A^{105}C$ [f]	18
C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$ [f]	15
$A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$ [f]	35
Lampyris turkestanicus [g]	2
$E^{354}K$ [g]	40
$E^{354}R$ [g]	55
$E^{354}Q$/Arg^{356} [g]	10
$E^{354}K$/Arg^{356} [g]	20
$E^{354}R$/Arg^{356} [g]	20
Lampyris turkestanicus [h]	2.6
$E^{354}Q$/R [h]	4.3
$E^{354}Q$/R-$Q^{35}R$ [h]	6
$E^{354}Q$/R-$I^{232}R$ [h]	11
$E^{354}Q$/R-$I^{182}R$ [h]	5
$E^{354}Q$/R-$L^{300}R$ [h]	4.5

a) Purified luciferases (*Photinus pyralis*) and mutant were incubated in 50 mM potassium phosphate buffer, pH 7.5, containing 10 % saturated ammonium sulphate, 1 mM DTT and 0.2% (w/v) BSA, inactivation at 40 °C.

b) Five hundred microliters of purified luciferase (*Hpa*) in 50 mM sodium phosphate (pH 7.8) containing 10 % saturated ammonium sulfate and 0.2% (w/v) BSA was incubated at 45 ºC.

c) Heat inactivation at 45 ºC assays were performed with the enzymes (*Ppy*) buffered in 20 mM tricine (pH 7.8), 2mM $MgSO_4$, 1mM EDTA and 1mM DTT.

d) The purified wild-type (*Luciola cruciata*) and mutant were incubated at 50 ºC in 0.05 M phosphate buffer (pH 7.8) containing 10 % saturated ammonium sulfate and 0.2 % (w/v) BSA.

e) Purified luciferases (*Ppy*) and mutant were measured by incubating the enzyme in 50 mM Tris-HCl (pH 7.8) at 35 °C.

f) Purified luciferases (*Ppy*) and mutant were measured by incubating the enzyme in 50 mM Tris-HCl (pH 7.8) at 32 °C.

g) The purified luciferases (*L. turkestanicus*) were incubated at 32 ºC in 50 mM Tris-HCl pH 7.8 containing 10 % saturated ammonium sulfate and 0.2 % (w/v) BSA.

h) Assay was performed in 50 mM Tris-HCl (pH 7.8) at 35 ºC.

Figure S1. SDS-PAGE of purified Wild-type and mutant Luciferases after purification via affinity Ni-NTA column chromatography.

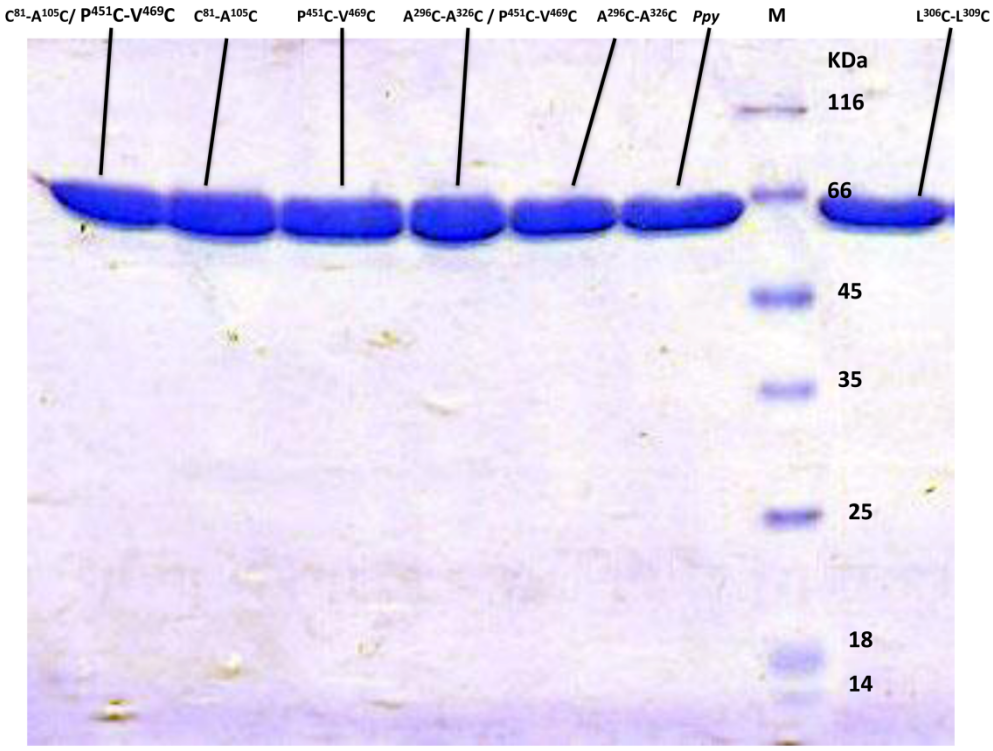


Figure S2. Near-CD spectra for the wild type and mutant forms of luciferases.

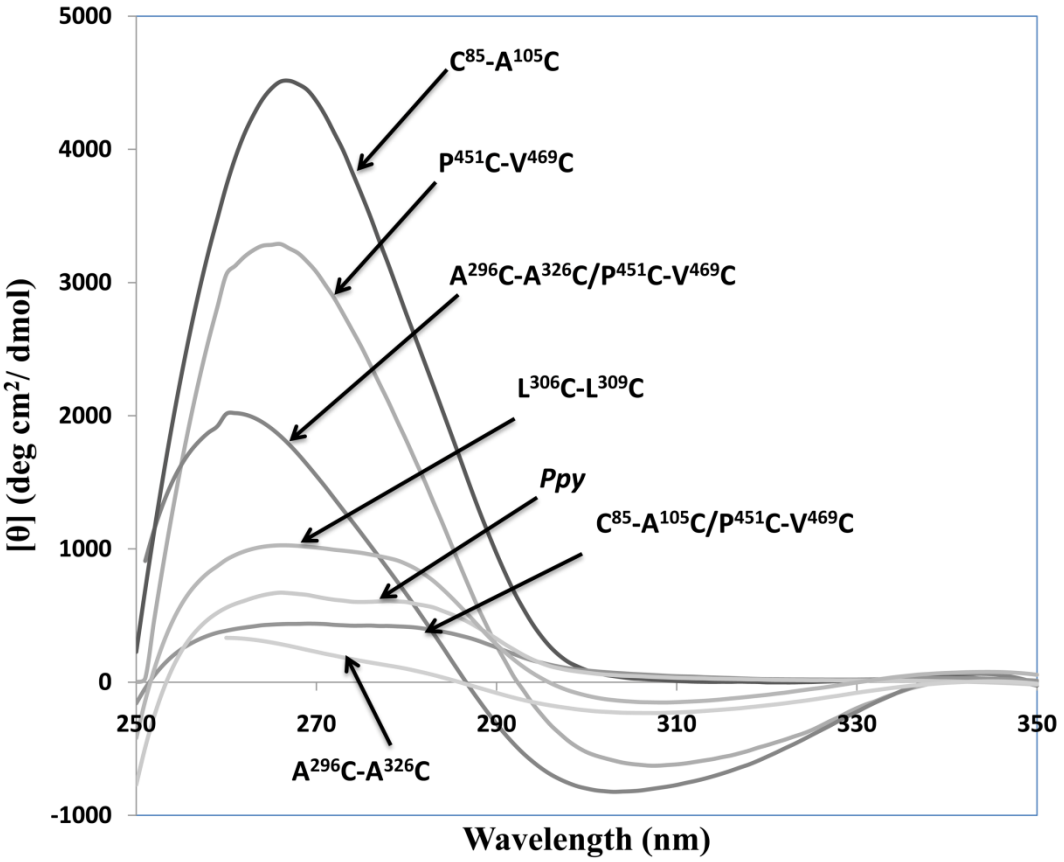


Figure S3. pH profile of wild-type (♦) and mutant (C^{81}-$A^{105}C$ (■), $P^{451}C$-$V^{469}C$ (▲), A^{296C}-$A^{326}C$ (*), $L^{306}C$-$L^{309}C$ (×), C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$ (+), and $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$ (•) luciferases.

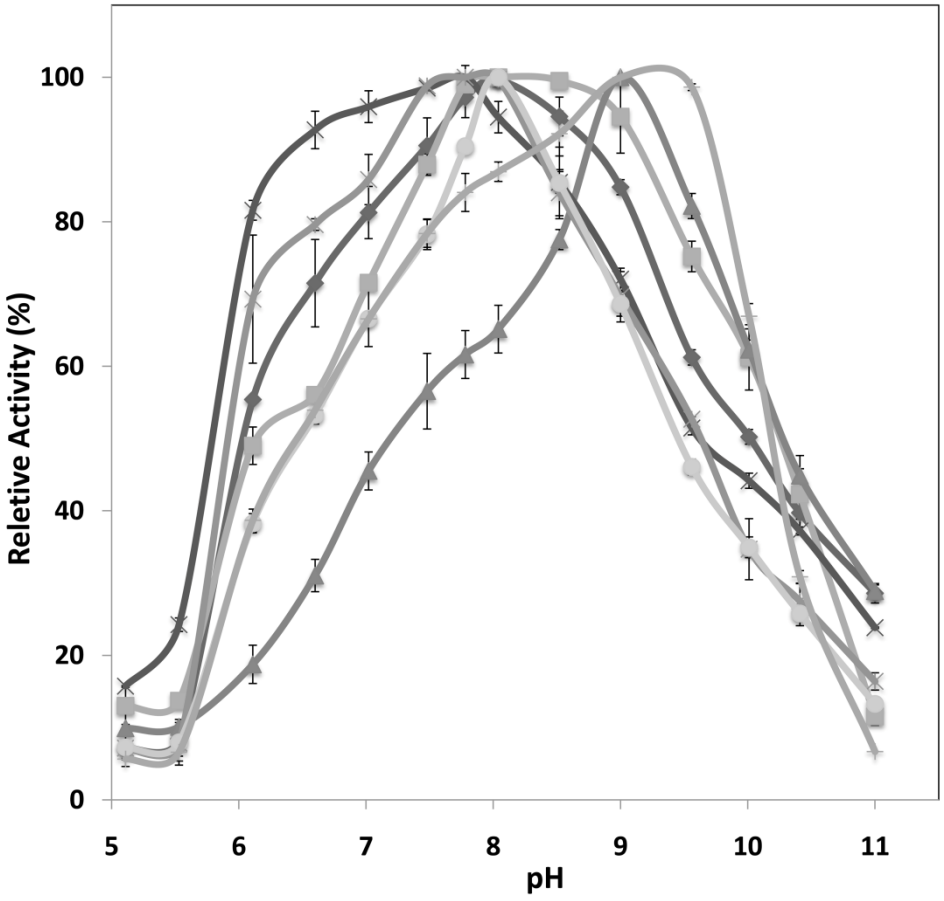


Figure S4. Temperature profile of wild-type (♦) and mutant (C^{81}-$A^{105}C$ (■), $P^{451}C$-$V^{469}C$ (▲), A^{296C}-$A^{326}C$ (*), $L^{306}C$-$L^{309}C$ (×), C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$ (+), and $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$ (•) luciferases.

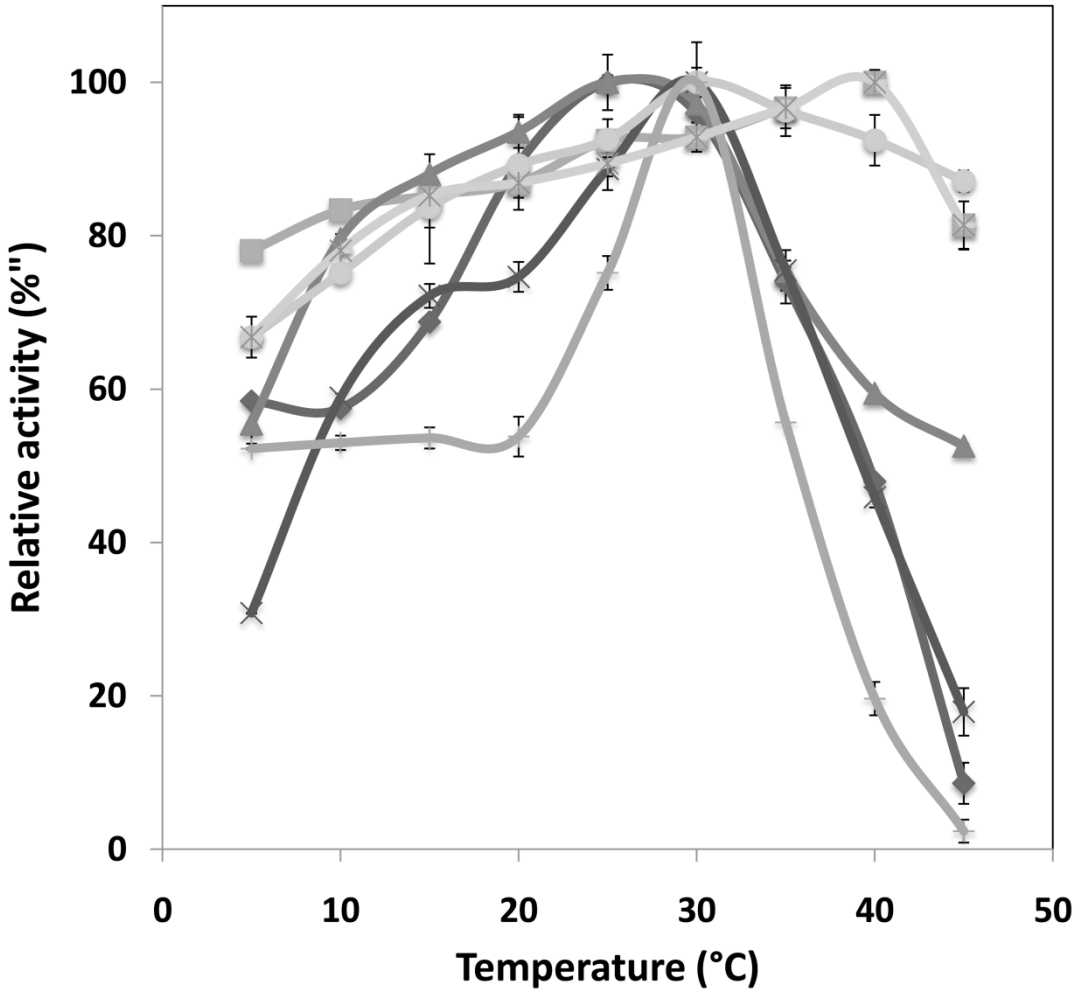


Figure S5. Normalized light emission kinetic profiles of *Ppy* and the mutants. Each curve is a mean of three measurements. Flash heights and integrated intensities were within 10% of the mean value. Reactions were conducted at the saturating concentrations of the substrates as described in Materials and Methods.

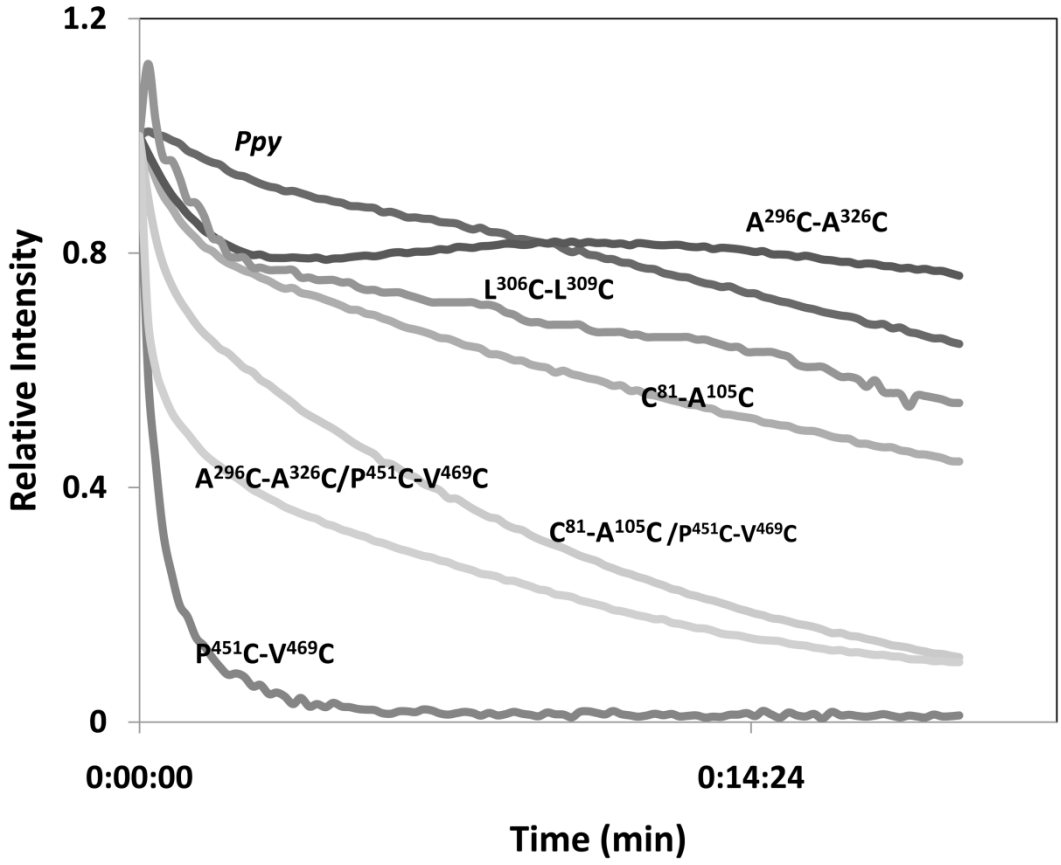


Fig S6. The maximum intensity emission plotted as a function of urea concentration in wild-type (♦) and mutant (C^{81}-$A^{105}C$ (■), $P^{451}C$-$V^{469}C$ (▲), $L^{306}C$-$L^{309}C$ (×), C^{81}-$A^{105}C$/ $P^{451}C$-$V^{469}C$ (*), and $A^{296}C$-$A^{326}C$/ $P^{451}C$-$V^{469}C$ (●)) at 25 ºC. Fluorescence spectra of wild-type and mutant luciferases were reported after 120 min incubation in the presence of various concentration of urea (M).

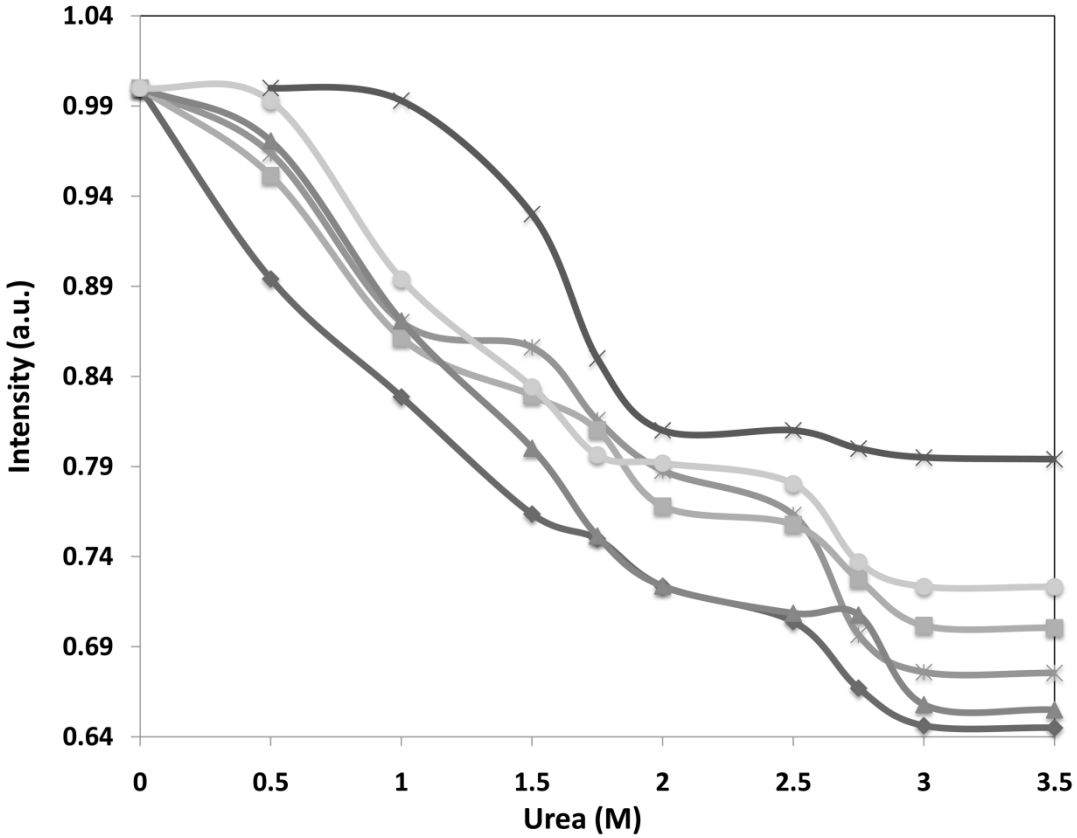
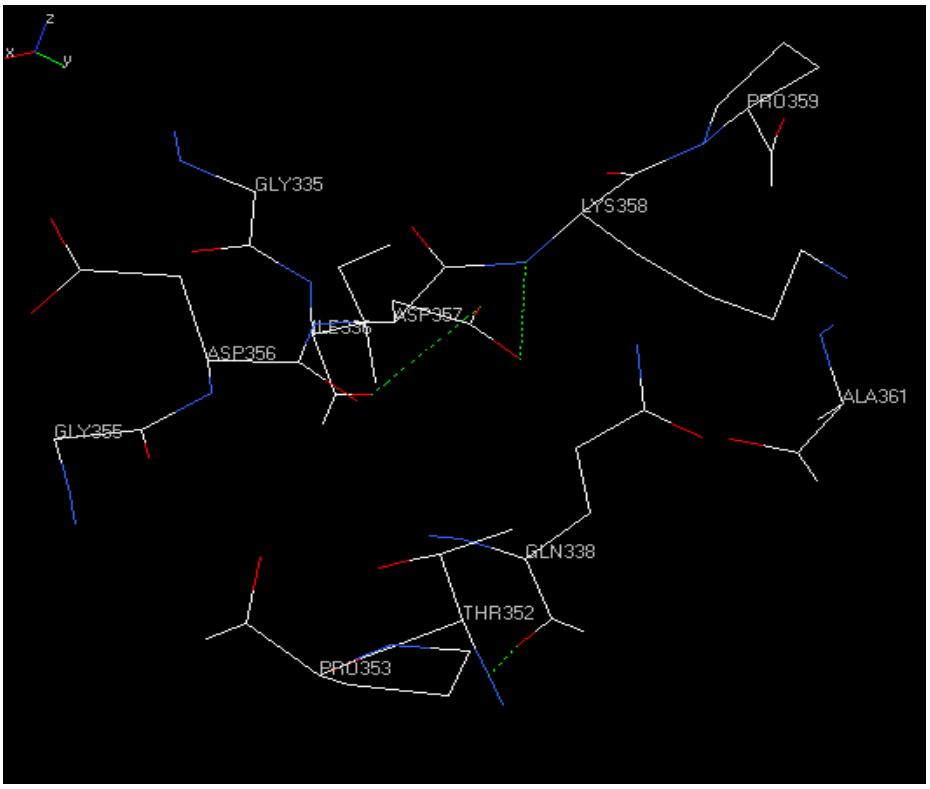


Fig. S7 Comparative analysis of hydrogen bonds network in the charged amino acids of flexible loop between (350-365) and neighboring amino acids in wild-type (A), and $P^{451}C$-$V^{469}C$ mutant. A strong hydrogen bonds (showed by green arrow), and week hydrogen bond (showed by orange arrow).

A



B