Mutagenesis of solvent-exposed amino acids in *Photinus pyralis* luciferase improves thermostability and pH-tolerance

G. H. Erica LAW^{*1}, Olga A. GANDELMAN^{*}, Laurence C. TISI^{*†}, Christopher R. LOWE^{*} and James A. H. MURRAY^{*}

^{*}Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, U.K., and [†]Lumora Ltd, c/o Institute of Biotechnology, Tennis Court Road, Cambridge CB2 1QT, U.K.

Firefly luciferase catalyses a two-step reaction, using ATP-Mg²⁺, firefly luciferin and molecular oxygen as substrates, leading to the efficient emission of yellow-green light. We report the identification of novel luciferase mutants which combine improved pH-tolerance and thermostability and that retain the specific activity of the wild-type enzyme. These were identified by the mutagenesis of solvent-exposed non-conserved hydrophobic amino acids to hydrophilic residues in *Photinus pyralis* firefly luciferase followed by *in vivo* activity screening. Mutants F14R, L35Q, V182K, I232K and F465R were found to be the preferred substitutions at the respective positions. The effects of these amino

acid replacements are additive, since combination of the five substitutions produced an enzyme with greatly improved pH-tolerance and stability up to 45 °C. All mutants, including the mutant with all five substitutions, showed neither a decrease in specific activity relative to the recombinant wild-type enzyme, nor any substantial differences in kinetic constants. It is envisaged that the combined mutant will be superior to wild-type luciferase for many *in vitro* and *in vivo* applications.

Key words: bioluminescence assay, firefly luciferase, imaging, *Photinus pyralis*, protein engineering.

INTRODUCTION

Beetle luciferases catalyse the efficient transfer of chemical energy into light via a two-step process, utilizing ATP-Mg²⁺, firefly luciferin (D-LH₂) and molecular oxygen, yielding oxyluciferin (LO):

A wide range of novel *in vitro* and *in vivo* applications of beetle luciferases has been developed, including ultrasensitive detection of pathogens, DNA sequencing and the use of the luciferase gene as a genetic reporter or for imaging [1,2]. This reflects the high quantum yield of the bioluminescence reaction (defined as the number of photons emitted per molecule of luciferin consumed) and also the fact that the metabolite, ATP, is a substrate in the bioluminescence reaction, so allowing a wide range of assays to be coupled to biochemical processes. Furthermore, recombinant beetle luciferases have been shown to be fully functional when expressed in a wide variety of cells.

Both the native and recombinant forms of the most extensively studied beetle luciferase from *Photinus pyralis* ('firefly luciferase'), demonstrate a number of undesirable properties with respect to application in assays and research. In particular, the enzyme inactivates readily at elevated temperature [3] and exhibits a large red-shift in its bioluminescence spectrum at low pH and under destabilizing conditions [4,5]. As standard PMTs (photomultiplier tubes) are less sensitive to red light, the red-shift is an undesirable trait in applications where the pH fluctuates. For some applications, such as whole-animal imaging, red luminescence is desirable because of increased penetration of living tissues; however, luciferases emitting red light (particularly at low pH) display lower quantum yields (0.5 at pH 6 compared with 0.88

at pH 7.8 [4,6]). In addition, active-site mutations that result in red-light emission are accompanied by losses in specific activity [7,8].

Numerous protein-engineering studies have been carried out aimed at isolating variants of the enzyme with improved properties. The most common approach has been to apply random mutagenesis to the entire firefly luciferase gene, followed by screening of mutants for specific properties. Firefly luciferase lends itself particularly well to activity screens owing to the ease with which its bioluminescence activity can be followed.

With respect to mutants that show decreases in rates of inactivation at elevated temperatures, point mutations that have been identified include T217I in *Luciola cruciata* luciferase [9], where the equivalent position in *P. pyralis* luciferase is 215; E354K in *P. pyralis* luciferase [3] and also T214A, I232A and F295L in *P. pyralis* luciferase [10]. Furthermore, it has been shown that combining such point mutations can have cumulative effects on thermostability, although the increased stability can be accompanied by a decrease in specific activity [10]. With regard to luciferase mutants that show an increased pH-tolerance, both the *Hotaria parvula* firefly luciferase mutant E356R/V368A [11] and *P. pyralis* luciferase mutant T214A/I232A/F295L/E354K [10] have been shown to exhibit improved thermostability with no red-shift at acidic pH, but with reduced specific activity.

In spite of these successful attempts to identify thermostable mutants, random mutagenesis followed by screening has a number of limitations. First, the process is not truly random, as some parts of a gene are more susceptible to mutagenesis than others. Secondly, a point mutation can only change the nature of the amino acid coded to a limited subset of the 19 other amino acids. Finally, the approach is time-consuming if a large sequence space is to be searched in order to find mutants with the desired properties. This is especially the case when multiple mutations are required to generate a mutant with the desired properties.

Abbreviations used: CCD, charge-coupled device; DTT, dithiothreitol; D-LH₂, D-luciferin; Ni-NTA, Ni²⁺-nitrilotriacetate; PMT, photomultiplier tube; RLU, relative light units; rLuc, recombinant luciferase; SDM, site-directed mutagenesis; WT, wild-type.

¹ To whom correspondence should be addressed (email E.Law@biotech.cam.ac.uk).

In the present study, we describe a semi-random mutagenesis approach to the luciferase gene to find mutants with useful properties. In a previous study where alanine substitutions were made at positions 14, 35, 182, 226, 232, 376 and 465, which are occupied by bulky hydrophobic solvent-exposed residues, it was demonstrated that the mutant I232A had a significantly reduced rate of inactivation compared with WT (wild-type) enzyme at 37 °C [12]. Additionally, it was found that five of these positions (Phe¹⁴, Leu³⁵, Val¹⁸², Ile²³² and Phe⁴⁶⁵), which are all non-conserved and do not participate in secondary-structure formation, were amenable to mutagenesis to alanine without loss of bioluminescence activity.

The present study explores the substitution of these five amino acids with hydrophilic residues. It was anticipated that mutagenizing these positions to hydrophilic residues, in particular to charged groups, could increase structural stability by establishing more favourable local interactions. The mutants generated were selected for brightness and thermostability, then purified and characterized for their biochemical and biophysical properties. We report mutants that are more pH-tolerant, show a reduction in red-shift at low pH and exhibit improved stability at temperatures up to 45 °C with no associated loss of activity. We believe that these luciferases, particularly the combined mutant in which all five mutations are incorporated ('×5'), would perform better than the WT enzyme in situations where the pH fluctuates, e.g. in *in vivo* imaging applications.

MATERIALS AND METHODS

Materials

D-LH₂ potassium salt was obtained from Europa Bioproducts. Plasmid pPW601L, derived from pPW601a [3], with additional cloning sites [13], encoding for the WT *P. pyralis* luciferase gene was used in the random SDM (site-directed mutagenesis) experiments. Plasmid pET16b (Novagen) was used for the expression of N-terminally His₁₀-tagged luciferases, and pET16b-luc was obtained by ligating the WT *P. pyralis* luciferase (EC 1.13.12.7) gene into pET16b [13].

SDM, screening and selection of mutants

Selective random SDM was carried out using the QuikChange™ SDM kit (Stratagene) according to the manufacturer's protocol. Plasmid pPW601L was subjected to five rounds of mutagenesis with each using a pair of the following partially degenerate mutagenic primers: 5'-GGCCCGGC**CA(CAG)(AG)(N)**TATCCTCTAGAGG-3' and 5'-CCTCTAGAGGATA**(CT)(CTG)TGGTGGCCGGGCC-3'** (HaeII) for F14X; 5'-GGCTATGAAG**GcGTA-CGCC(CAG)(AG)(N)**GTTCCTGG-3' and 5'-CCAGGAAC**(N)(CT)(CTG)GGCGTAGCgCTTCATAGCC-3'** (HaeII) for L35X; 5'-GAATACGATTTT**(CAG)(AG)(N)**CCAGAaagCTTTGATCG-3' and 5'-CGATCAAAG**GctTCTGG(N)(CT)(CTG)**AAAA-TCGTATTC-3' (HindIII) for V182X; 5'-GGCAATCAAATC**(CAG)(AG)(N)**CCGGATACTGCG-3' and 5'-CGAGTATCCGG**(N)(CT)(CTG)**GATTTGATTGCC-3' for I232X and 5'-CCCCAACATC**(CAG)(AG)(N)**GACGCGGGCGTGGCAGG-3' and 5'-CCTGCCACGCCCGCGTC**(N)(CT)(CTG)**GATGTTGGG-3' for F465X (lower-case letters represent silent changes to modify a endonuclease site used to facilitate screening, boldface type represents the mutated codon, and the endonuclease used for screening is shown in parentheses). XL2-Blue ultracompetent cells (Stratagene) were used as cloning hosts for the generation and selection of mutants from SDM. Mutants were screened and selected for brightness and apparent thermostability by imaging

for light emission, using a CCD (charge-coupled device) camera (Syngene Optics), from colonies at room temperature, and after incubation at 42 °C, using an *in vivo* colony screen [14]. Colonies grown overnight at 37 °C were lifted on to a nylon membrane (Hybond N; Amersham Biosciences), and these were assayed for light emission by spraying the colonies with 0.1 M citrate buffer, pH 5.0, containing 1 mM D-LH₂. For each position, 80 random colonies were screened in the first round, resulting in the selection of between ten and 12 mutants for the second round of screening, which were sequenced.

The desired point mutant for each position was generated by SDM on pET16b-luc using the following primers: 5'-GGCCC-GGC**CaCCACGCTATCCTCTAGAGG-3'** and 5'-CCTCTAGAGGATAG**CGTGGTGGCCGGGCC-3'** (HaeII) for F14R; 5'-GGCTATGAAGAGATACGCCCCGGTTCCTGG-3' and 5'-CCAGGAACCTGGGCGTATCTCTTCATAGCC-3' for L35Q; 5'-GAATACGATTTTAAACCAGAAagCTTTGATCG-3' and 5'-CGATCAAAGctTCTGGTTTAAATCGTATTC-3' (HindIII) for V182K; 5'-CGCAcGCCAGAGATCCTATTTTGGCAATCAAATCAAACCGG-3' and 5'-CCGGTTTGATTGATTGCCAAA-AATAGGATCTCTGGCgTGCG-3' (SphI) for I232K and 5'-CCCAACATCCGCGACGCcGGCGTGGCAGG-3' and 5'-CCTGCCACGCCgGCGTCGCGGATGTTGGGG-3' (BglII) for F465R (highlighted bases were as explained above). Plasmid pET16b-luc×5, containing all five mutations, was constructed sequentially using the aforementioned primers. Primer synthesis and DNA sequencing were carried out using facilities at the Departments of Biochemistry and Genetics (University of Cambridge) respectively.

Expression and purification of His₁₀-tagged luciferases

His₁₀-tagged WT luciferase and mutants were expressed from pET16b-luc in *Escherichia coli* BL21(DE3)pLysS host cells (Novagen). Cultures were grown in LB (Luria-Bertani) medium supplemented with 100 µg·ml⁻¹ carbenicillin and 50 µg·ml⁻¹ chloramphenicol at room temperature (23 ± 2 °C) until an *D*₆₀₀ of 0.8–0.9 was reached. They were then induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) for 6–8 h at the same temperature, after which cells were harvested by centrifugation at 15 000 g for 20 min at 4 °C and stored overnight at –80 °C. Cell pellets were resuspended in lysis buffer, which consists of buffer A [10 mM phosphate buffer, pH 8.0, 2.7 mM KCl, 0.3 M NaCl, 10 mM 2-mercaptoethanol, 20 % (v/v) glycerol, 1× EDTA-free protease cocktail inhibitor (Roche)] supplemented with 2 % (v/v) Triton X-100 and 20 mM imidazole. A volume of 5 ml of lysis buffer was used/g (wet weight) of cells. Benzonase nuclease (Novagen) was added to a final concentration of 125 units·g⁻¹ (wet weight) of cells. Crude cell extract was obtained by centrifugation at 20 000 g for 1 h at 4 °C.

His₁₀-tagged luciferases were purified using Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose (Novagen) on a 1.5 cm diameter column with a 2.5 ml bed vol. at 4 °C. Crude cell extract was loaded on to the column and this was then washed with 4 column vol. of buffer A containing 50 mM imidazole. Luciferases were eluted in 2.5 ml fractions of buffer A containing 200 or 300 mM imidazole. Fractions with the highest luciferase activity and purity based on activity measurement and amino acid analysis (Department of Biochemistry, University of Cambridge) respectively were desalted on a PD-10 column (Amersham Biosciences) into storage buffer [TEM (0.1 M Tris/acetate, pH 7.8, 10 mM MgSO₄ and 2 mM EDTA) containing 10 % (v/v) glycerol and 2 mM DTT]. These were stored in 50 or 100 µl aliquots at –80 °C. Total protein concentrations were estimated using the method of Bradford [15], using the Coomassie Blue protein assay reagent kit from

Pierce according to the manufacturer's protocol, with BSA as the standard.

Luciferase activity assays

Dilution of enzyme and activity assay buffer

Luciferases were diluted from the purified stock solution into pre-chilled TEM containing 2 mM DTT to obtain the required concentration, unless specified otherwise. As specified, a final concentration of either 2 or 10 % (v/v) glycerol was added to the diluted enzyme solution. The activity assay buffer consisted of TEM with various concentrations of ATP, D-LH₂ and sometimes CoA and DTT, which are defined in each experiment.

Methods of luciferase activity measurement

Luciferase activity was measured on a Labsystems Luminoskan Ascent luminometer and recorded in RLU (relative light units). All activity and spectral measurements were carried out at $23 \pm 2^\circ\text{C}$ unless specified otherwise. Experiments used either (i) injection mode, involving the automatic injection of assay buffer and allowing determination of flash height, or (ii) manual mode, involving manual mixing of assay buffer with enzyme in wells of a 96-well microtitre plate (Labsystems) and allows the integration of light intensity emitted over a specified period of time.

Kinetic constants, specific activity, bioluminescence spectra, effect of pH on activity, rates of thermal inactivation and fluorescence emission spectra

K_m values for ATP were determined by assaying 80 pmol of enzyme in TEM containing 200 μM D-LH₂ and concentrations of ATP equivalent to 0.1–10 times the K_m of WT luciferase. K_m values for D-LH₂ were determined in a similar way, except that 56 pmol of enzyme and a saturating ATP concentration of 1 mM were used. Flash heights were used to derive the kinetic constants according to the method of Hanes [16]. PMT voltage was set at 550 and 520 V for measurements used to determine the K_m values for D-LH₂ and ATP respectively.

Bioluminescence spectra were recorded 45 s after initiating the reaction using a PerkinElmer LS50B spectrophotometer. Sensitivity of the PMT was corrected by calibrating with Lucifer Yellow and using the known corrected spectra from Molecular Probes.

In experiments investigating the effects of pH on activity, enzymes were diluted in TEM adjusted to the required pH, containing 2 % (v/v) glycerol. Any loss of activity in the diluted enzyme solution over the course of the experiment was corrected for by making parallel measurements of the total activity of the enzyme at pH 7.8.

For the measurements of rates of inactivation, enzymes were diluted into 50 mM potassium phosphate, pH 7.8, 10 % (v/v) glycerol and 2 mM DTT. Aliquots of 30 μl of 0.2 μM enzyme solutions were incubated at temperatures between 37 and 52 $^\circ\text{C}$ for various lengths of time up to 120 min. These aliquots were then chilled on ice before activity measurements. Activity was determined using flash intensity measurements by injecting 100 μl of TEM containing 1.05 mM ATP, 210 μM D-LH₂ into wells containing 5 μl of the 0.2 μM enzyme solution. PMT voltage was set at 760 V, and rates of inactivation were calculated from sets of data that exhibited an apparently first-order reaction, which were then used to construct an Arrhenius plot.

Fluorescence emission spectra were obtained using a Varian Cary Eclipse spectrophotometer at $23 \pm 2^\circ\text{C}$ and scanning at 600 nm $\cdot$ min⁻¹ as described in Table 3. Excitation was carried

Figure 1 Structure of *P. pyralis* luciferase

The five residues selected for mutagenesis and the two tryptophan residues are indicated. Residues that have been shown or modelled to form the active site [8] are in black. This Figure was generated using the RasMol program [17] based on the crystal structure *P. pyralis* luciferase reported by Franks et al. [18].

out at 290 nm with both the excitation and emission slit widths set at 5 nm. PMT voltage was at 950 V.

RESULTS AND DISCUSSION

Mutagenesis, screening and selection of bright and/or thermostable luciferase mutants

Phe¹⁴, Leu³⁵, Val¹⁸², Ile²³² and Phe⁴⁶⁵ in *P. pyralis* luciferase (Figure 1) were chosen for mutagenesis as these have been shown previously to be amenable to changes without affecting the catalytic activity [12]. These were mutagenized randomly to eight hydrophilic amino acids using semi-random SDM. Transformed *E. coli* colonies expressing the mutant luciferases were screened at room temperature, with and without prior incubation at 42 $^\circ\text{C}$.

For each position, 80 colonies were picked randomly in the primary screen. Of these, between 10 and 12 mutants that exhibited varying brightness and/or thermostability were selected for the secondary screen and sequenced (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/397/bj3970305add.htm>). The second round of screening allowed the selection of the brightest and/or most apparently thermostable mutant at each position

Figure 2 Bioluminescence of *E. coli* colonies expressing WT and the single Ile²³² mutant luciferases

Each set of five colonies expressing WT or Ile²³² mutant luciferases was screened for brightness and thermostability using 1 mM D-LH₂ in citrate buffer, pH 5.0. Clone numbers and mutations are indicated. Bioluminescence was integrated over a period of 5 s, at room temperature (**A**) and after colonies were incubated at 42 °C for 40 min (**B**).

Figure 3 Bioluminescence of *E. coli* colonies expressing WT and the five single mutant luciferases

Light was integrated over a period of 5 s from each set of seven colonies. The assay was carried out on colonies at room temperature and after incubation at 42 °C. Results are means $\pm$ S.E.M.

(illustrated for position 232 in Figure 2). The increase in contrast for Figure 2(B) made the mutants appear brighter after the heat treatment relative to the unheated colonies. The absolute luminescence of all mutants was lower after the heat treatment (results not shown). Using this screening method, the preferred mutations at each position were found to be F14R, L35Q, V182K, I232K and F465R (Figure 3). It was notable that four of the five mutations selected had a positively charged substitution, the exception being L35Q. Furthermore, at each position, the proportion of mutants with similar substitutions selected from the first screen was greater than that expected from the number of codons for these residues. Previous observations showed that, when colonies of *E. coli* were assayed for light emission in the same way, red bioluminescence was observed (P. J. White and L. C. Tisi, unpublished work). Thus

the selection of brighter mutants *in vivo* might also select for mutants that lack the red-shift, due to the lower sensitivity of the CCD camera to red light.

Examination of the crystal structure of firefly luciferase [18] shows that Leu³⁵ is the only position among the five that has a positively charged amino acid within a 5 Å (1 Å = 0.1 nm) radius, whereas all positions but Ile²³² have at least one negatively charged residue within this radius. This suggests that the addition of a positively charged group at positions 14, 182 and 465 is favourable for increased light production under the conditions used for screening, which is probably a result of stabilizing charge-charge interactions effected, presumably, by high local negative charge densities.

Construction, expression and purification of luciferase mutants

Using SDM, F14R, L35Q, V182K, I232K, F465R and the $\times 5$ mutant were individually constructed in pET16b-luc, which expresses an N-terminal His₁₀-tag. These, together with His₁₀-tagged WT luciferase of *P. pyralis* were expressed in *E. coli* BL21(DE3)pLysS cells and purified using Ni-NTA resin. Fractions with a purity of > 90 %, as determined by amino acid analysis, were obtained and used for subsequent characterization. WT luciferase was shown to have a similar or higher purity to rluc (recombinant luciferase) obtained from Promega when analysed using SDS/PAGE (results not shown). However, the specific activity of WT was only ~68 % of that of rluc (Table 1). This agrees with previous findings that showed His-tagged luciferase to have a lower specific activity than that of recombinant WT luciferase [19,20]. The mutants exhibited specific activities similar to that of WT, as might be expected from mutating non-conserved residues.

Kinetic analysis of luciferase mutants

K_m values for D-LH₂ and ATP determined for the mutants and WT showed no significant difference (Table 1). The rise times for these reactions were also observed to be the same (results not shown), suggesting no change in kinetics under the same conditions. k_{cat} values obtained were similar between mutants and WT. These

Table 1 Biochemical characteristics for luciferase enzymes

The specific activity is determined by measuring flash height and was carried out by injecting TEM containing 200 μM D-LH₂ and 1 mM ATP into wells containing 1.52 μg of enzyme. k_{cat} values were calculated from data used to determine the K_{m} values for ATP. A PMT voltage of 520 V was used for the specific-activity measurements. Results are means $\pm$ S.E.M.

Enzyme	Specific activity ($\times 10^{-6}$ RLU $\cdot$ mg $^{-1}$)	K_{m} for LH ₂ (μM)	K_{m} for ATP (μM)	k_{cat} ($\times 10^{-10}$ RLU $\cdot$ s $^{-1}$)
rluc	3.25 $\pm$ 0.20	6.6 $\pm$ 1.0	65.5 $\pm$ 7.8	6.14 $\pm$ 0.01
WT	2.27 $\pm$ 0.13	13.9 $\pm$ 2.4	61.7 $\pm$ 3.2	4.31 $\pm$ 0.25
F14R	1.99 $\pm$ 0.06	18.5 $\pm$ 0.8	64.1 $\pm$ 5.2	3.99 $\pm$ 0.13
L35Q	2.04 $\pm$ 0.05	15.3 $\pm$ 0.1	62.9 $\pm$ 2.4	4.04 $\pm$ 0.07
V182K	2.23 $\pm$ 0.04	14.7 $\pm$ 1.5	71.8 $\pm$ 5.1	4.48 $\pm$ 0.07
I232K	2.50 $\pm$ 0.03	15.2 $\pm$ 0.5	72.4 $\pm$ 1.2	4.99 $\pm$ 0.06
F465R	1.97 $\pm$ 0.01	16.5 $\pm$ 2.3	64.6 $\pm$ 0.5	3.94 $\pm$ 0.02
$\times 5$	1.86 $\pm$ 0.02	15.5 $\pm$ 0.9	76.3 $\pm$ 2.3	3.79 $\pm$ 0.05

observations are consistent with the unchanged specific activity for WT and mutants observed above.

Effect of pH on the colour of bioluminescence and luciferase activity

The colour of bioluminescence for these mutants at three different pH values was also determined. These measurements were carried out under the conditions of saturating substrates in the presence of CoA. Similar spectral profiles were seen for the normalized bioluminescence spectra of WT and mutants, with a single maximum at 556 nm, at pH 7.8 (Figure 4). The same profiles were observed at pH 9.0 (results not shown). At pH 6.5, a subsidiary maximum at approx. 610 nm was observed for WT luciferase. This appears to be due to the formation of a red-emitting species. It was seen that the contribution of this second species is reduced for all mutants, with the exception of L35Q, the only uncharged amino acid substitution. For $\times 5$, the subsidiary peak is completely absent. As a result, the absolute light emitted at pH 6.5 showed a nearly 2-fold increase for $\times 5$ relative to WT luciferase, whereas all of the other mutants showed moderate increases in light emission (Table 2). As these mutations (i) are far from the active site, (ii) are non-conserved within the family of firefly luciferases, and (iii) do not alter the K_{m} for either D-LH₂ or ATP, this suggests that the reduction in the proportion of red-emitting enzyme might be due to resistance to conformational changes under acidic conditions.

The bioluminescence activity of the luciferase mutants over the pH range 6.2–9.4 was measured by using the light integrated over 5 s upon initiation of the reaction. The $\times 5$ mutant showed a significantly higher level of bioluminescence in the acidic range (Figure 5A). Activity over the same pH range was also measured by monitoring flash heights. It was seen using this method that the optimal pH for both WT and $\times 5$ luciferase was approx. 8.0 (Figure 5B), which agreed with the reported range of 7.7–8.1 for WT luciferase, determined using a number of different instruments and buffer systems [21–23]. Thus altering the five surface residues at the selected positions did not change the optimal pH for the bioluminescence reaction. The apparent increase in the optimal pH seen in Figure 5(A) is due to the way luciferase activity was measured, which integrates the light emitted after the flash, and does not reflect the initial turnover of active luciferase molecules. However, a long integration time is employed in many applications. The increase in $\times 5$ mutant bioluminescence activity at low pH (Figure 5) can be only partly attributed to the decrease in PMT sensitivity to red light, since results from the

Figure 4 Normalized bioluminescence spectra of luciferases at pH 6.5 (A) and 7.8 (B)

Enzyme (0.31 nmol) was assayed with 1 ml of TEM containing 200 μM D-LH₂, 1 mM ATP, 270 μM CoA and 2 mM DTT, adjusted to pH 6.5 and 7.8. Spectra were recorded from 450 to 650 nm and scanned at 200 nm $\cdot$ min $^{-1}$ with a slit width of 10 nm. PMT voltage was 900 V. Spectra obtained were corrected for the baseline and sensitivity of the PMT. Experiments were subject to a pH accuracy of ± 0.05 unit and timing accuracy of ± 5 s. The spectrum for L35Q in (A) is identical with that of WT.

Table 2 Bioluminescence activity of luciferases at pH 6.5 and 7.8

The values presented were from the bioluminescence spectra shown in Figure 4. Error associated with activity is $\pm 8\%$ of the value.

Enzyme	Relative bioluminescence activity (%)	
	pH 7.8	pH 6.5
WT	100	25
F14R	123	38
L35Q	115	27
V182K	122	35
I232K	102	32
F465R	109	40
$\times 5$	102	49

corrected spectral measurements also showed a similar increase in activity for some mutants relative to WT luciferase (Table 2).

The red-shift observed in firefly luciferase has been investigated extensively, but no theory can, at present, fully explain the phenomenon. It has been observed previously in our laboratory that many thermostable luciferase mutants show a lack of red-shift at acidic pH [24]. This suggests that the red-shift observed at acidic

Figure 5 Effects of pH on luciferase activity using integrated light measurements (A) and flash height measurements (B)

Luciferase solution (20 μ l at 0.42 μ M) was assayed by the manual mixing (A) with 180 μ l of TEM, 1.11 mM ATP, 222 μ M D-LH₂ and 300 μ M CoA over a range of pH values between 6.0 and 9.5. Bioluminescence was integrated over 5 s using the luminometer. The lag time between initiation of the reaction and recording of light emission was $\sim$ 5 s. For flash height measurements (B), enzymes and activity assay buffer used were identical, except that CoA was omitted and the activity assay buffer was injected into wells containing the enzyme. Measurements at each pH were carried out in triplicate. A PMT voltage of 550 V was used in both sets of measurements. Results are means $\pm$ S.E.M. for triplicate measurements.

pH is related to certain conformational changes in the luciferase which change the characteristics of the active site. Thus mutations that confer thermostability may prevent such red-shift. In addition to the red-shift observed at low pH, factors contributing to the pH-dependence of light emission include the change in enzyme kinetics, the inactivation of the enzyme by acid and the quantum yield.

We attribute both the greater activity and the absence of red-shift at acidic pH to the stabilizing effect of the mutations, primarily due to electrostatic interactions. We note that the stabilization must be a result of the local environment of the mutated site and not the net charge of the protein; since the pI of His₁₀-tagged WT luciferase is 7.2, the overall charge on the protein is positive at neutral and acidic pH, and its magnitude therefore increased by the mutations.

Thermal inactivation of luciferase mutants

First-order thermal inactivation rate constants for luciferase mutants were obtained by incubating aliquots of enzyme solutions at temperatures between 43 and 52 °C. At temperatures below this

Figure 6 Arrhenius plot showing the dependence of rates of inactivation on temperature for WT and mutant luciferases

range, rates were found not to be first-order, while, above 52 °C, the rate of inactivation was too high to follow.

The Arrhenius plot for these first-order rate constants is shown in Figure 6. Mutants L35Q, V182K and F14R exhibit a similar inactivation profile to that of WT luciferase. I232K and F465R inactivate substantially more slowly than WT luciferase, especially at the lower range of temperatures. A clear additive effect is seen in x5, which appears to be significantly more stable than any of the single mutants.

Closer analysis of the Arrhenius plot can give us insight into the stabilizing effects conferred by the mutations. The first-order kinetics indicate a single rate-determining step at any given temperature. We observed that it is not possible to fit a single straight line through all the data points for each mutant, indicating different rate-determining steps at high and low temperatures.

Transition state theory [25] can be applied to the rate determining step at a given temperature. The gradient of the Arrhenius plot is proportional to the activation enthalpy of this step. At lower temperatures, there is an increase in activation enthalpy for all mutants except V182K, and a cumulative effect in x5. This is consistent with stabilization by strong electrostatic interactions. The entropic cost associated with the fixing of the side chains in such interactions probably accounts for the convergence of the plots at high temperatures.

A comparison of the half-lives obtained for the x5 mutant and WT at different temperatures shows that x5 has a half-life (33 min) six times greater than that of WT (5 min) at 43 °C. This decreases to 1.5 times at 50 °C (0.6 min for x5 mutant compared with 0.4 min for WT). In the temperature range 37–42 °C, we observed qualitatively that x5 is much more stable than WT, although, since the kinetics do not obey a simple rate law, the improved stability cannot be quantified. We expect x5 to confer significant advantages over WT in applications operating at temperatures below approx. 45 °C such as *in vivo* imaging, especially considering the increased pH-tolerance of the mutant.

Fluorescence study of WT and x5 luciferase at 43 °C and pH 6.5

Fluorescence emission spectra were obtained for WT and x5 luciferase after subjecting to either thermal inactivation at 43 °C for 20 min or dilution in a buffer at pH 6.5. On heat treatment, WT luciferase showed an approx. 4.0 nm increase in the wavelength of the emission maximum (Table 3), consistent with additional exposure of tryptophan residues to aqueous environment resulting from partial unfolding of the protein. The x5 mutant showed a 1.4 nm increase after the same treatment. The ratio of maximum fluorescence intensities before and after heat treatment for WT

Table 3 Fluorescence emission maxima of WT and $\times 5$ luciferase enzymes under different conditions

Fluorescence emission spectra were recorded between 300 and 400 nm. For thermal inactivation experiments, 160 μ l aliquots of 0.2 μ M enzymes in 50 mM phosphate buffer, pH 7.8, 10% (v/v) glycerol and 2 mM DTT were incubated at 43 °C for 20 min, and then equilibrated at room temperature before measurement. For pH experiments, 160 μ l aliquots of 0.2 μ M enzymes in TEM at the specified pH supplemented with 10% (v/v) glycerol and 2 mM DTT were allowed to equilibrate for 10 min before measurement. Results are means $\pm$ S.E.M. of emission maximum for three or more three scans. All measurements were carried out at 23 ± 2 °C.

Conditions	Fluorescence emission maximum (nm)	
	WT	$\times 5$
0 min pre-incubation at 43 °C	338.7 $\pm$ 0.1	339.0 $\pm$ 0.2
20 min pre-incubation at 43 °C	342.7 $\pm$ 0.4	340.4 $\pm$ 0.1
pH 7.8 (0 min)	338.8 $\pm$ 0.6	338.9 $\pm$ 0.6
pH 7.8 (10 min after dilution)	339.0 $\pm$ 0.3	338.5 $\pm$ 0.4
pH 6.5 (0 min)	338.2 $\pm$ 0.5	337.9 $\pm$ 0.2
pH 6.5 (10 min after dilution)	337.6 $\pm$ 0.7	337.7 $\pm$ 0.6

luciferase is 4:1 compared with 2:1 for $\times 5$, suggesting that a larger proportion of WT luciferase underwent structural changes that increased exposure of tryptophan residues to the aqueous environment. The reduction in wavelength shift and lower ratio of maximum fluorescence intensities support the increase in structural stability of $\times 5$ (see the Supplementary Figure 1 at <http://www.BiochemJ.org/bj/397/bj3970305add.htm> for fluorescence spectra).

At pH 6.5, neither the WT nor the $\times 5$ enzyme showed a shift in their emission maxima (Table 3) and the ratio of maximum fluorescence intensities at pH 7.8 to that at pH 6.5 for both enzymes is 1:1. This shows that any structural changes there might be to the enzyme at pH 6.5 do not affect the tryptophan environment. However, as the two tryptophan residues are not located in the active site (see Figure 1), conformational changes affecting the active site, which could account for the lower activity of WT at acidic pH, might well be undetectable using this technique. We also note that both tryptophan residues are located in the N-terminal domain of the protein, so that only unfolding processes in this domain could be followed. As we were unable to show any structural differences between WT and $\times 5$ at pH 6.5 using fluorescence measurements, we attempted CD aimed at measuring the secondary structure of the enzymes; however, these attempts were unsuccessful owing to the high background caused by stabilizers which were required to maintain the enzyme in solution.

Conclusion

A targeted approach was employed for the mutagenesis of luciferase with the aim of identifying mutants with improved characteristics for the purpose of application. This approach combined structure and sequence data to identify non-conserved positions in firefly luciferase that were likely to accept a hydrophilic substitution. This resulted in the successful identification of mutants with enhanced pH tolerance and increased resistance to thermal inactivation.

Kinetic analysis of these mutants showed similar kinetic constants to those of WT, which is significant, as it has been found previously that thermostable luciferase mutants have reduced specific activity. Bioluminescence spectra at pH 7.8 and 9.0 were also unchanged, but, at pH 6.5, some of the point mutants showed reduced formation of red-emitting species and an increase in activity. The $\times 5$ mutant exhibited an absence of such species

due to the additive effect of the mutations. Analysis of thermal-inactivation profiles showed a substantial increase in half-life for the $\times 5$ mutant relative to that of WT at temperatures up to 45 °C, and indicated that the stabilization is primarily due to electrostatic interactions.

The use of the $\times 5$ mutant in applications operating at acidic or fluctuating pH, such as *in vivo* medical imaging, or at elevated temperatures up to 45 °C, is expected to provide assays with greatly increased sensitivity and reliability.

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