Improved thermostability of the North American firefly luciferase: saturation mutagenesis at position 354

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We have used random chemical mutagenesis and a simple genetic screen to generate and isolate a thermostable mutant of luciferase from the North American firefly (*Photinus pyralis*). A single G-to-A transition mutation, resulting in the substitution of a glutamate for a lysine residue at position 354 in the protein sequence, was shown to be responsible for this enhanced thermostability. Replacement of Glu-354 with all possible amino acid residues was achieved using directed mutagenesis, and produced mutant enzymes with a range of thermostabilities. The mutations E354K and E354R conferred the largest increases in thermostability, suggesting that side-chain size and hydrophobicity, as well as charge, may also be important contributors to the overall thermostability of the polypeptide chain at this position. Unusually for such mutations, biochemical studies suggest that this position is on the surface of the protein and exposed to solvent.

INTRODUCTION

Firefly luciferase (EC 1.13.12.7) catalyses the oxidation of a benzothiazole substrate (beetle luciferin) in the presence of magnesium ions, ATP and molecular oxygen [1]. The product, oxyluciferin, is generated in an excited state which then decays to the ground state with the emission of a photon. The biochemistry of the enzyme and the chemistry of the reaction have been extensively studied over several decades [2–4]. More recently, the cloning of the gene for luciferase from several species of firefly [5–9] and the determination of the crystal structure of firefly luciferase [10] has opened up new opportunities to study the enzymology of this enzyme using molecular biological techniques.

As well as being an extremely sensitive genetic reporter in molecular biology [11,12] the North American firefly luciferase is used extensively for measuring microbial contamination [13], and over the last decade or so there has been intense commercial interest in developing bioluminescence-based technologies as a replacement for more conventional screening techniques. However, several factors limit further application and development of this technology, including the low stability of the enzyme both *in vitro* and *in vivo*, a peak wavelength of emitted light that is not optimal for detection using conventional photomultiplier tubes, a low turnover number and a high K_m for the substrate ATP. We have been attempting to improve the usefulness of this enzyme using an approach based on random mutagenesis and simple *in vivo* screen for enzyme activity [14].

Previously, Kajiyama and Nakano have used random mutagenesis and screening to isolate mutants of luciferase from the Japanese fireflies *Luciola cruciata* and *Luciola lateralis*. They showed that single amino acid replacements can affect either the thermostability of the protein or the wavelength of emitted light [15,16]. The North American firefly luciferase is almost exclusively the enzyme of choice for commercially available *in vitro* and *in vivo* systems. We have used an *in vivo* screen in *Escherichia coli* to isolate the first reported thermostable mutant of luciferase from the North American firefly. Using directed mutagenesis we have made all possible substitutions at position 354 in the polypeptide chain and characterized the thermostability of these mutants. This work has highlighted the importance of this position in determining the stability of the polypeptide chain and resulted in the generation of an enzyme with improved thermostability.

MATERIALS AND METHODS

Plasmids and *E. coli* strains

The bacterial expression vector pDR540 was obtained from Pharmacia Biotech, St Albans, U.K. Plasmid pT7-7 [17] was already available in the laboratory. Plasmid pGEM-*luc* was obtained from Promega Corporation, Madison, WI, U.S.A. The phagemid pBluescript II SK(+) was obtained from Stratagene, La Jolla, CA, U.S.A. *E. coli* strain BL21 (DE3) [hsdS gal (λcIts857 ind 1 Sam 7 nin 5 lacUV5 gene 1)] [18] was used for the expression of luciferase from pT7-7-derived plasmids. *E. coli* strain JM109 [recA 1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB)] [19] was used for the expression of luciferase from pDR540-based plasmids.

Reagents

Coenzyme A, ATP and wild-type North American firefly luciferase (L9009) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Beetle luciferin (potassium salt) was obtained from Promega Corporation, Madison, WI, U.S.A.

Oligonucleotides

Oligonucleotide primers for sequencing and site-directed mutagenesis were synthesized in the Department of Biochemistry,

Abbreviations used: DTT, dithiothreitol; RLU, relative light units.

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University of Cambridge, using an Applied Biosystems model 380A DNA synthesizer.

Mutagenesis and screening for thermostable mutants

Plasmid pPW304 was treated according to the method of Kironde et al. [20], with 0.8 M hydroxylamine/0.1 M sodium phosphate/1 mM EDTA, pH 6.0, for 2 h at 65 °C. The mutagenized plasmid was desalted on a G50 DNA-grade Nick column (Pharmacia) followed by transformation into *E. coli* BL21 (DE3). *E. coli* cells transformed with mutated plasmid were grown overnight at 37 °C, transferred to nylon filters and soaked with luciferin solution as described below. Colonies were selected on the basis of brightness and isolated for further characterization.

Assay of luciferase activity

Bioluminescence emitted by *E. coli* cells expressing luciferase was monitored by transferring colonies to nylon filters (Hybond N, Amersham) and then soaking with sodium citrate buffer, pH 5.0, containing D-luciferin (1.0 mM) at room temperature [21]. Standard luciferase assays *in vitro* were performed at 21 °C using 100 μl of assay buffer [20 mM Tricine, pH 7.8, containing 1.0 mM $MgSO_4$, 0.1 mM EDTA, 33 mM dithiothreitol (DTT), 270 μM coenzyme A, 470 μM D-luciferin and 530 μM ATP]. The bioluminescence reaction was initiated by injecting the assay cocktail into a microtitre plate well containing enzyme (1–5 μl) and measured using a Labsystems Luminoskan RS plate luminometer.

Preparation of crude cell extracts

Cell-free extracts were prepared as described in the Promega technical bulletin no. 101. Aliquots of *E. coli* cultures in Eppendorf tubes were lysed in cell culture lysis reagent [25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol (DTT), 2 mM EDTA, 10 % (v/v) Triton X-100, 2.5 mg/ml BSA and 1.25 mg/ml hen egg lysozyme] for 10 min at room temperature. The lysed extracts were centrifuged at 16000 ***g*** for 2 min and then stored on ice prior to assay.

Purification of firefly luciferase

Purification of the recombinant wild-type enzyme was achieved in four steps as outlined below. Purification of the mutants E354K and E354R, to more than 85 % homogeneity followed a similar protocol, but with dialysis into buffer C after step 2, and the omission of step 3. All steps were performed at 4 °C unless otherwise stated.

Step 1: preparation of crude extract

E. coli JM109 cells expressing the recombinant wild-type or mutant luciferases were grown and harvested as described above. A 1.5 g (wet weight) batch of cells was resuspended in 20 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 50 mM NaCl, 1 mM DTT and 1.2 mM PMSF (buffer A). The cells were broken by disruption in an MSE soniprep 150 sonicator (amplitude 14 μm). The cell lysate was then centrifuged at 30000 ***g*** for 30 min.

Step 2: fractionation with ammonium sulphate

The supernatant of the crude extract (20 ml) was subjected to fractionation with ammonium sulphate. The fraction that precipitated between 30 and 55 % satn. contained luciferase activity. The precipitated material was resuspended in 3.5 ml of buffer A and desalted on a Pharmacia PD10 column equilibrated in 10 mM sodium phosphate buffer (pH 7.5) containing 0.5 mM DTT (buffer B).

Step 3: chromatography on hydroxyapatite

The material from step 2 was loaded on to a column of hydroxyapatite (bed volume 40 ml) equilibrated in buffer B, at room temperature. The column was then eluted with a 180 ml linear gradient of 10 to 200 mM sodium phosphate containing 0.5 mM DTT. Fractions (3 ml) containing high luciferase activity were then pooled and dialysed overnight against one litre of 50 mM Tris/HCl buffer, pH 8.0, containing 0.5 mM DTT (buffer C).

Step 4: chromatography on MonoQ

The enzyme was applied to a Mono Q anion-exchange column (HR 10/10) at room temperature and eluted with a linear gradient of 0 to 500 mM NaCl in buffer C (flow rate 4 ml/min; 2 ml fractions). The peak fraction containing luciferase activity was dialysed against 250 ml of 25 mM sodium phosphate buffer, pH 7.5, containing 0.5 mM DTT and 12 % (v/v) glycerol for long-term storage at −20 °C.

DNA sequencing

Double-stranded DNA sequencing was performed by the dideoxy chain termination method [21] with [α-^{35}S]dATP and electrophoresis in 8 M urea–6 % (w/v) polyacrylamide gels. Automatic sequencing was undertaken by Melanie Murphy (CBDE, Porton Down, Wilts., U.K.), using a DNA model 373A automated sequencer (Applied Biosystems).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Clontech Transformer site-directed mutagenesis kit (version 2.0). The plasmid pPW601a was used in site-directed mutagenesis experiments. DNA oligonucleotide primers were designed to destroy a unique *Ava*I site within the *luc* gene. The presence of this restriction enzyme site was used to select against plasmids that had not undergone mutagenesis.

PAGE

Electrophoresis in the presence of SDS was performed by the method of Laemmli [22], with a 3 % (w/v) polyacrylamide stacking gel and a 10 % (w/v) polyacrylamide running gel. Protein was detected by staining with Coomassie Brilliant Blue R250. The purity of purified enzyme preparations was determined by scanning densitometry. SDS/PAGE gels were scanned using a Millipore XRS scanner and analysed using BioImage band analyser software.

Protein determination

Protein was determined by the method of Bradford [23], with BSA as standard.

Time-dependent inactivation studies

Eppendorf tubes containing cell-free extracts of luciferase or purified luciferases were incubated in 50 mM potassium phosphate buffer, pH 7.5, containing 10 %-satd. ammonium sulphate, 1 mM DTT and 0.2 % (w/v) BSA. At given times a tube was

removed and cooled in an ice-water bath prior to assay. The remaining activity was expressed as a percentage of the original activity.

Emission spectra

Emission spectra were measured on a Perkin Elmer LS50B spectrofluorimeter and corrected for the spectral sensitivity of the photomultiplier tube. The intensity maximum for each spectrum was normalized. Bioluminescence spectra of purified luciferases were measured in 50 mM Tris-acetate at pH 7.0 and pH 7.8. Each buffer also contained 1 mM EDTA, 5 mM $MgSO_4$, 0.2 mM D-luciferin, 0.5 mM ATP and 0.02 % (w/v) BSA.

RESULTS

Construction of expression plasmids

The gene for the North American firefly luciferase is available from Promega on the plasmid pGEM-*luc*. The *luc* gene was subcloned from this plasmid into the *E. coli* expression vectors pT7-7 and pDR540 to give pPW304 and pPW601 respectively (Figures 1 and 2). Plasmid pPW601 contains the strong *tac* promoter under control of the lactose repressor and expression may be induced in the presence of isopropyl β-D-thiogalactopyranoside. Plasmid pPW304 has the *luc* gene under control of the T7 RNA polymerase promoter and this provides the basis for a very high level of gene expression in *E. coli* cells expressing T7 RNA polymerase. Expression of luciferase from pPW601 results in the synthesis of a protein of wild-type sequence, while expression from pPW304 results in the synthesis of a fusion protein with four extra N-terminal amino acids. The *Xho*I site in pPW601 was removed to create pPW601a thereby leaving a single restriction site for the endonuclease *Ava*I. This site was the target for the site-directed mutagenesis experiments.

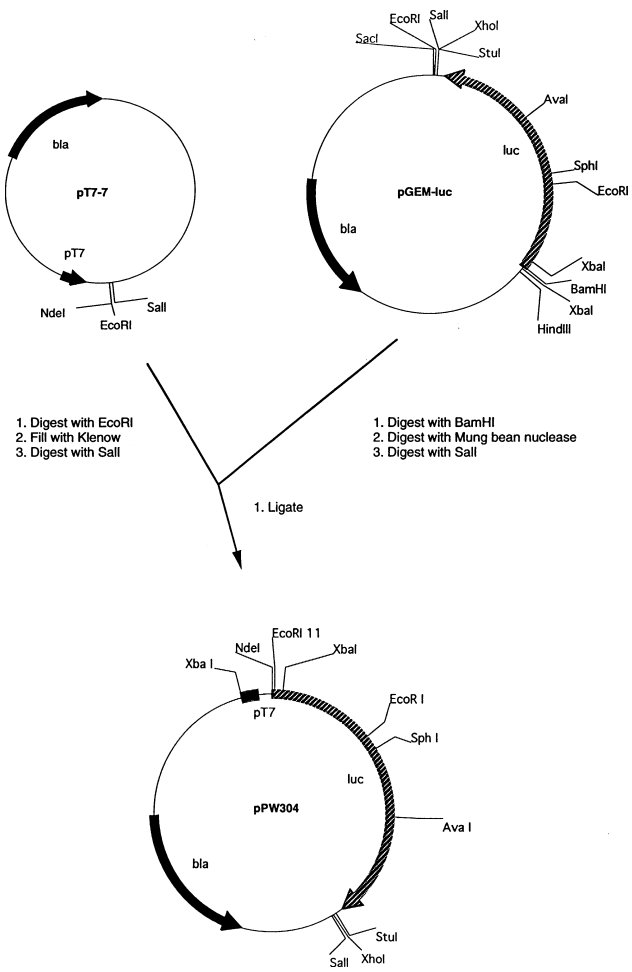

Figure 1 Construction of plasmid pPW304

This plasmid was subjected to random chemical mutagenesis.

Screening, isolation and characterization of a thermostable mutant

The plasmid pPW304 was treated with hydroxylamine as described in the Materials and methods section. This DNA was then used to transform *E. coli* BL21(DE3) cells which were then plated on to LB agar containing 50 μg/ml ampicillin in 90-mm-diam. Petri dishes and grown overnight at 37 °C. The following morning Petri dishes containing 100–300 colonies were overlaid with nylon filters and the colonies transferred to fresh LB agar plates. These plates were then incubated for 2–4 h at 37 °C after which the filters were then removed and soaked in D-luciferin as described in the Materials and methods section. Bioluminescence emitted by colonies expressing luciferase was visualized by eye in a dark room. Those colonies emitting the brightest light were picked for further analysis. Approximately 4000 *E. coli* colonies were screened this way and one clone (TT-1) was selected for further study. The thermostability of the luciferase isolated from this strain was compared in cell-free extracts with that isolated from *E. coli* cells expressing luciferase from untreated plasmid pPW304. After incubation at 37 °C the control luciferase was almost completely inactivated, while luciferase activity from clone TT-1 still showed 80 % activity (Figure 3).

Mapping experiments were undertaken in order to identify the mutation or mutations responsible for the increased thermostability. Plasmids pPW304 and pPTT-1 were subjected to a series of restriction enzyme digests. DNA fragments isolated from these digests were purified and re-ligated to each other in order to create hybrid plasmids. *E. coli* BL21(DE3) cells were then transformed with these plasmids and luciferase activity assayed *in vivo* and *in vitro* after overnight growth at 37 °C. These mapping experiments indicated that the mutation(s) responsible for increased thermostability lay within a 677 nt region of *luc* DNA bounded by the restriction sites *Sph*I and *Eco*RV. One further mapping experiment involving the exchange of fragments generated by an *Ava*I digest of each plasmid could not be completed as it was found that one of the two *Ava*I restriction sites on the plasmid pTT-1 was no longer present. Further restriction analysis indicated that the missing site lay within the *luc* coding region. These results demonstrated that the target mutation(s) occurred within this restriction site (CCCGAG).

To confirm that a mutation lay within the *Ava*I site in *luc*, a 591 nt *Eco*RI/*Ava*II restriction fragment from pPW304 and pTT-1 was subcloned into the sequencing vector pBSK(+). Dideoxy sequencing of one strand confirmed that a single base change from G to A at position 1060 in the *luc* gene had occurred in the fragment from TT-1. This change is consistent with the type of mutation expected to arise from treatment of DNA with hydroxylamine, and results in the amino acid substitution glutamate to lysine at position 354 in the primary sequence of luciferase. This change, while destroying an *Ava*I site, generates a new and unique *Sty*I restriction site [CCAAGG]. The presence of this new site in pTT-1 was confirmed by restriction analysis.

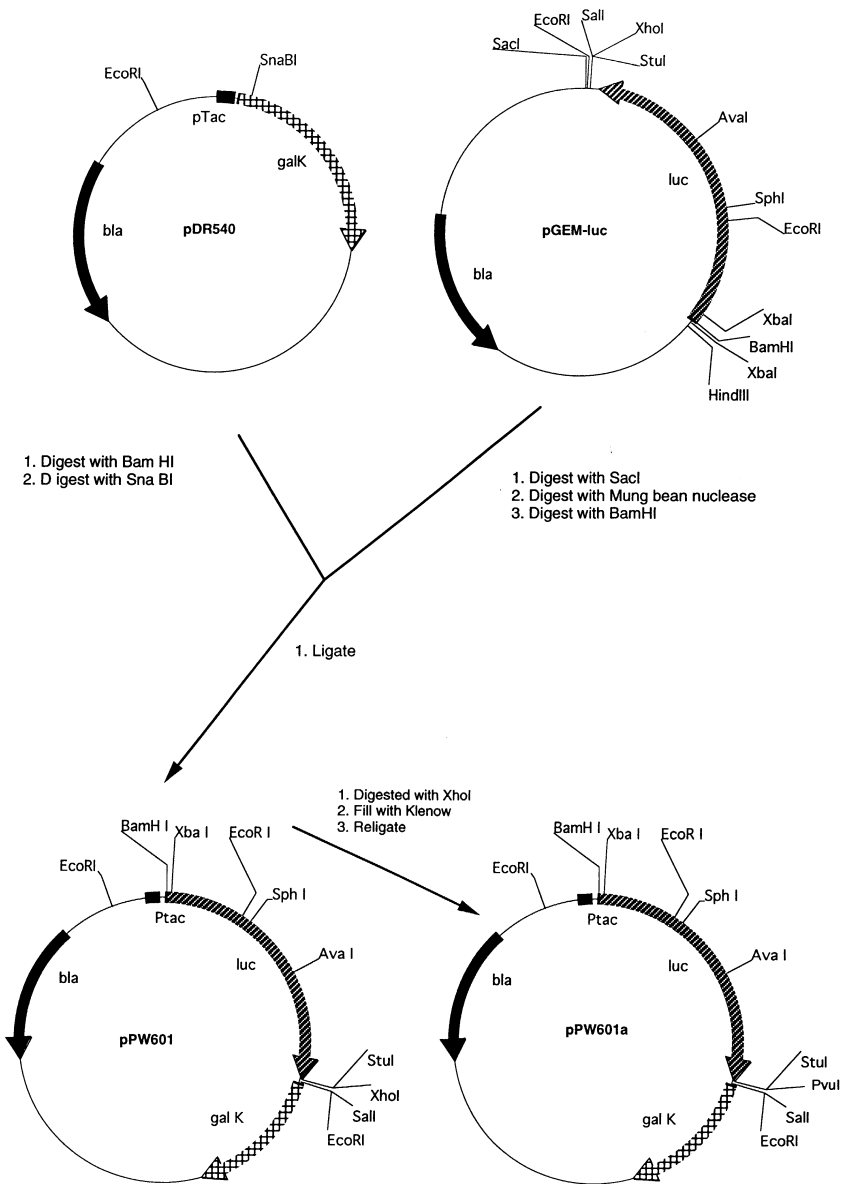

Figure 2 Construction of plasmid pPW601a

This plasmid was used in the site-directed mutagenesis experiments.

Expression of luciferase from pPW304 pTT-1 results in the synthesis of a fusion protein with four extra N-terminal amino acids. In order to exclude any possible effects of these extra amino acids on the activity or thermostability of luciferase, the *luc* gene was cloned into pDR540 to create pPW601 (Figure 2). Expression of luciferase from this plasmid results in the synthesis of an enzyme with a wild-type sequence. The unique *Xho*I site present in the polylinker of this plasmid was then removed to create pPW601a. This plasmid contains a single recognition site for the endonuclease *Ava*I, which simplified the subsequent site-directed mutagenesis.

Site-directed mutagenesis was used to unambiguously show that a single amino acid change from glutamate to lysine increased the thermostability of luciferase. A mutagenic oligonucleotide (5′-CATCCCCCTTGGGTGTAATCAG-3′) was used to create a single G to A transition at position 1060 in the *luc* gene in the plasmid pPW601a, to create plasmid pPW601a E354K. The fidelity of the mutagenic procedure was confirmed by dideoxy sequencing.

Saturation mutagenesis at position 354

To investigate the effect of other amino acid substitutions at position 354, site-directed mutagenesis was used to replace the glutamate with the remaining 18 possible amino acids. Five degenerate and two specific mutagenic oligonucleotides were used to create the mutations (Figure 4). The thermostabilities of these mutants were compared with the recombinant wild-type and the mutant E354K (Figure 5 and Table 1). The mutants show very different rates of inactivation at 40 °C. The most thermostable are those with the substitutions E354K or E354R, while the least stable luciferases contain the amino acids gluta-

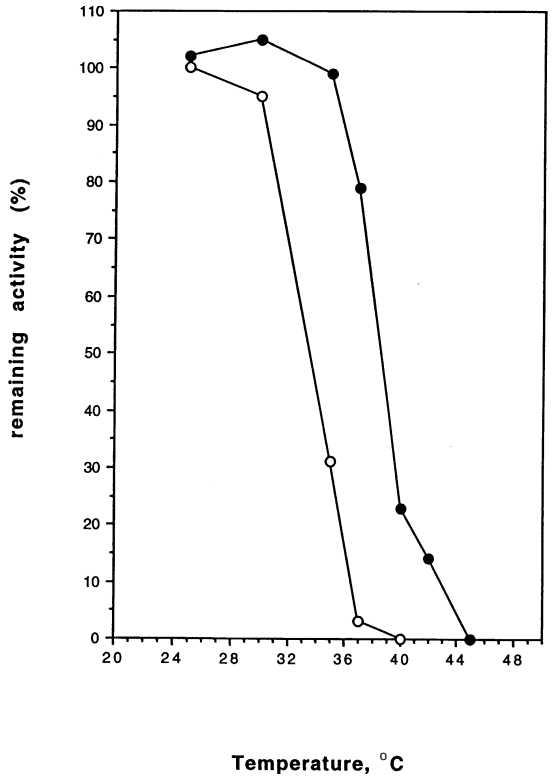

Figure 3 Thermal inactivation of recombinant wild-type (○) and mutant TT-1 (●) luciferases

Crude cell extracts were prepared in lysis buffer as described in the Materials and methods section. The extracts were incubated at various temperatures for 20 min and remaining actvities were measured after cooling in an ice-water bath.

	amino acid substitution
L I T P E G D D K 5'-CTG ATT ACA CCC GAG GGG GAT GAT AAA C-3'	wild-type
5'-CTG ATT ACA CCC NNN GGG GAT GAT AAA C-3'	any
5'-CTG ATT ACA CCC TNA GGG GAT GAT AAA C-3' T	Y,C,S,F,L
5'-CTG ATT ACA CCC GNC GGG GAT GAT AAA C-3'	G,A,V,D
5'-CTG ATT ACA CCC TGT GGG GAT GAT AAA C-3' T	C,F
5'-CTG ATT ACA CCC ATG GGG GAT GAT AAA C-3'	M
5'-CTG ATT ACA CCC AAC GGG GAT GAT AAA C-3' C	N,H
5'-CTG ATT ACA CCC AAG GGG GAT G-3'	K

Figure 4 Sequences of the oligonucleotide primers used for site-directed mutagenesis

The first sequence is wild-type and shows the amino acid associated with each codon. The *Aval* restriction site is underlined. N = A+G+C+T.

mate, aspartate, proline or glycine at position 354. The order of thermostability of these least stable mutants was determined at 37 °C (results not shown).

Comparison of the thermostability of the recombinant wild-type and mutant luciferase E354K

The recombinant wild-type luciferase and the most thermotolerant mutant luciferases, E354K and E354R, were purified

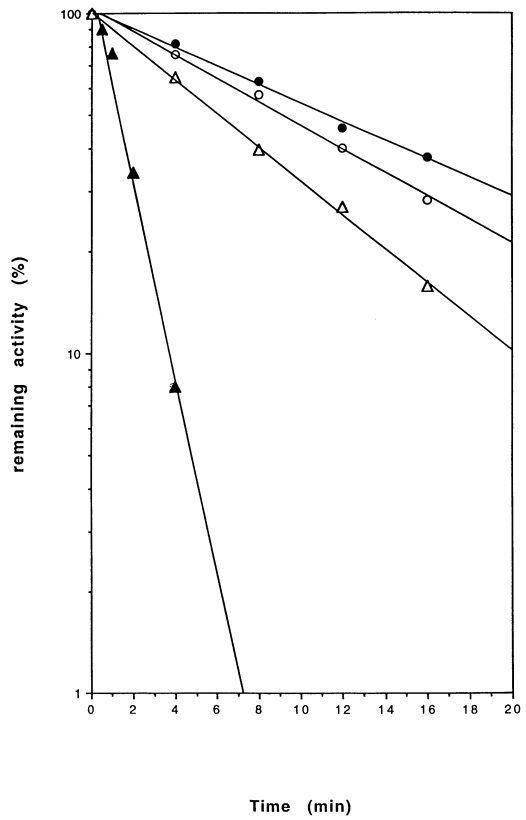

Figure 5 Thermostability of mutant luciferases

Crude cell extracts were prepared in lysis buffer as described in the Materials and methods section. At given times a tube was removed and cooled in an ice-water bath prior to assay. The remaining activity was expressed as a percentage of the original activity. Time courses for the inactivation of recombinant wild-type (▲), E354A (▲), E354I (○) and E354K (●) luciferases are shown.

and their thermal stability compared. The mutant luciferase E354K was significantly more thermostable than the recombinant wild-type enzyme over the range of temperatures tested. Arrhenius plots of the first-order rate constants for the thermal inactivation of each enzyme showed that, for equivalent rates of inactivation, the mutant enzyme had nearly a 2 °C increase in thermostability (Figure 6). A similar increase in thermostability was observed for the mutant luciferase E354R (results not shown).

In vitro emission spectra of recombinant wild-type and mutant luciferases

It was observed that some of the amino acid replacements resulted in a change in the colour of emitted light from *E. coli* cells expressing luciferases. To confirm that these changes could be seen *in vitro* the spectrum of bioluminescence emitted by the reaction catalysed by the purified recombinant wild-type and mutant luciferases was determined in a Tris-acetate buffer at pH 7.0 and 7.8 (pH 7.8 results not shown). The most significant changes were associated with the mutant E354I where the peak wavelength increased 3.5 nm from 558.5 to 562 nm and the spectral distribution contained a greater contribution coming from longer wavelengths (Figure 7). Each spectral measurement was repeated immediately after the first run in order to determine

Table 1 Comparison of the biochemical properties of the recombinant wild-type luciferase and luciferases with amino acid substitutions at position 354

$t_{\frac{1}{2}}$ values were determined in cell-free extracts at 40 °C as described in the Materials and methods section. Specific activities were determined for purified enzymes. Abbreviation: nd, not determined.

Amino acid	$t_{\frac{1}{2}}$ (min)	$10^{-10} \times$ Specific activity (RLU/mg)
K	12.0	5.2
R	12.0	3.7
L	9.7	4.1
I	9.3	4.3
N	9.0	4.2
V	8.6	3.4
W	8.0	4.8
A	6.1	4.3
Y	5.8	3.3
M	5.4	3.5
F	5.4	4.2
H	5.2	4.1
T	4.8	2.8
Q	4.5	2.9
C	4.0	3.6
S	3.7	2.7
E	1.0	2.1
D	< 1.0	nd
P	< 1.0	nd
G	< 1.0	nd

the effects of luminescence decay and no differences in the measured peak wavelength were observed.

Characterization of the mutant luciferases

The purification of the recombinant wild-type firefly luciferase is summarized in Table 2. The enzyme was purified 75-fold in 15% overall yield using this procedure. The specific activity of the purified enzyme was 2.1×10^{10} relative light units (RLU)/mg, which is similar to the value of 3.8×10^{10} we have determined for homogeneous preparations of wild-type North American firefly luciferase obtained from Sigma. The specific activities of the purified mutants E354K and E354R were 5.2×10^{10} and 3.7×10^{10} RLU/mg respectively (Table 1). Attempts to purify mutants with the substitutions aspartate, proline or glycine at position 354 were unsuccessful due to large losses of enzyme activity throughout the purification procedure.

DISCUSSION

Random chemical mutagenesis with hydroxylamine has been used to generate a thermostable mutant of the North American firefly luciferase. Subsequent sequencing and site-directed mutagenesis experiments showed that a single base change of G to A within the *luc* coding sequence was the mutation responsible for the increased thermostability of the enzyme. This mutation results in the amino acid change glutamate 354 to lysine in the primary sequence.

In order to understand the importance of this position in relation to thermostability and function of the enzyme, all possible mutants of luciferase at position 354 were generated. Analysis of the thermal inactivation of these mutants indicated that the replacement of glutamate with any other amino acid except glycine, proline or aspartate resulted in a protein with increased thermostability.

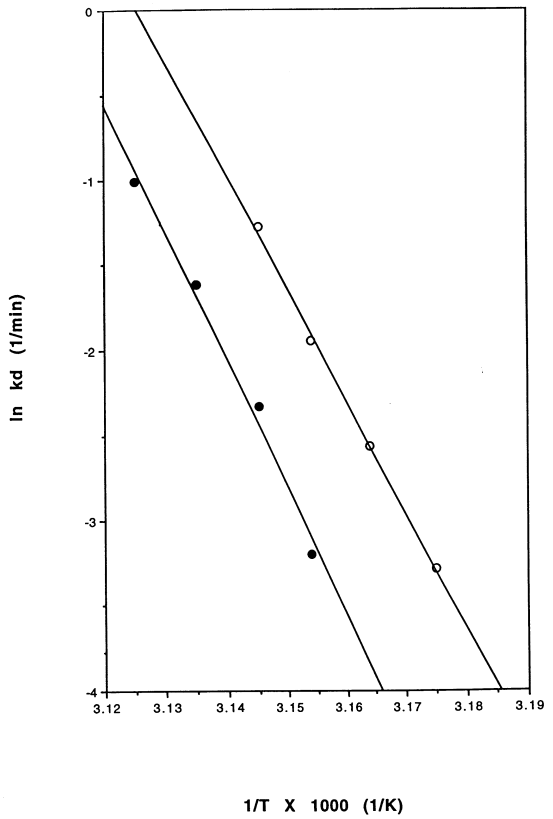

Figure 6 Arrhenius plots for the thermal inactivation rate constants of the recombinant wild-type (○) and E354K (●) luciferases

Eppendorf tubes containing purified luciferase (20 μg/ml) were incubated at various temperatures as described in the Materials and methods section. At given times, a tube was removed and cooled in an ice-water bath prior to assay. The first-order inactivation rate-constant (k_d) at each temperature was determined from the slope of a semi-logarithmic plot.

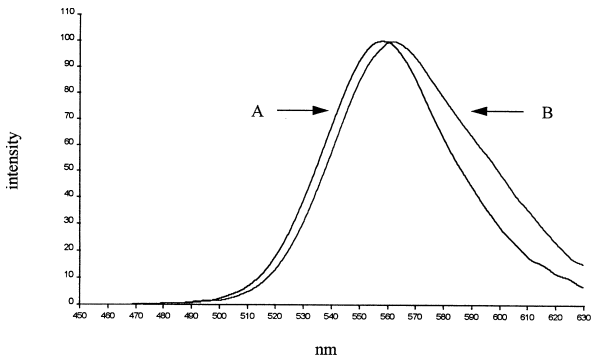

Figure 7 Emission spectra of recombinant wild-type and mutant luciferases

Emission spectra were measured on a Perkin Elmer LS 50B spectrofluorimeter. Bioluminescence spectra of the purified luciferases were measured in 50 mM Tris-acetate, pH 7.0. Each buffer also contained 1 mM EDTA, 5 mM $MgSO_4$, 0.2 mM D-luciferin, 0.5 mM ATP and 0.02% (w/v) BSA. A, recombinant wild-type; B, E354I.

These results suggest there is a correlation between the charge of the side chain of the residue at position 354 and the thermostability of the enzyme. Substitution with an amino acid with a neutral side chain, other than glycine or proline, results in a protein that is less thermostable than either E354K or E354R,

Table 2 Purification of recombinant wild-type luciferase

Step	Total protein (mg)	Total activity ($\times 10^{-10}$ RLU)	Specific activity ($\times 10^{-10}$ RLU/mg)	Purification (fold)	Yield (%)
Crude extract	460	13	2.8	1	100
30–60%-satd. $(NH_4)_2SO_4$	230	7.3	3.2	1.1	56
Hydroxyapatite	26	6.8	26.0	9.3	52
MonoQ	0.92	1.9	210.0	75	15

but more thermostable than the recombinant wild-type enzyme. Indeed it would seem that the loss of a negative charge at this position is the most important criterion for increased thermostability. However, other parameters may be important. The thermostabilities of mutant enzymes with the aliphatic residues alanine, valine, leucine or isoleucine substituted at position 354 suggest that increases in thermostability may be related to increases in the size or hydrophobicity of the amino acid side chain. It is likely that a combination of these factors is responsible for determining the stability of the polypeptide chain at this position.

Kajiyama and Nakano [16] have used a similar mutagenesis and selection procedure to isolate a thermostable mutant of luciferase from the Japanese firefly, *Luciola cruciata*. They showed that a single amino acid substitution T217I was found to be responsible for the increased thermostability of the mutant enzyme. Other amino acid replacements at Thr-217, in particular the hydrophobic amino acids isoleucine, valine and leucine, also stabilized the enzyme against thermal inactivation. They suggested that amino acids at this position may contribute to the major hydrophobic core of the protein. This hypothesis has subsequently been shown to be correct, since in the crystal structure of the North American firefly luciferase, the equivalent amino acid (Ala-215) is buried in the core (E. Conti and P. Brick, personal communication).

The forces that stabilize proteins can be divided broadly into hydrophobic interactions and electrostatic interactions [24]. The hydrophobic effect is considered to be significant and is probably the major contributor in stabilizing the folded structures of globular proteins. Electrostatic interactions, on the other hand, are usually considered to be much less important. Protein engineering of T4 lysozyme has shown that long-range electrostatic interactions and salt-bridges contribute little to protein stability. An exception is the electrostatic interaction of helix dipoles with charged groups in their vicinity, and this type of association can result in significantly enhanced stability [25].

From our experiments, and analysis of the crystal structure of the enzyme, it is possible to make some deductions about the physical location and structure of amino acid side chains at position 354 in the North American firefly luciferase. We have observed that glutamate 354 can be replaced with all other amino acids, except glycine, proline and aspartate, without compromising thermostability, suggesting that structural constraints based on size and charge at this position are minimal. This is unlikely to be the case for a residue that is buried in the core of the protein. Also, the mutants E354K and E354R elute from an anion-exchange column at a much lower salt concentration than the recombinant wild-type luciferase (results not shown), suggesting that there may be a specific interaction between the charged side-chains and the positively charged chromatography matrix. Amino acid replacements had little effect on the specific activity of the purified enzymes, indicating that E354 is not an active-site residue. These observations are consistent with position 354 being located on the surface of the protein in a region of flexible structure and freely accessible to solvent. Indeed, this hypothesis has been shown to be correct. The crystal structure of the North American firefly luciferase has been determined recently [10] and shows position 354 to be part of a segment of the polypeptide that is on the surface of the protein connecting two strands of β-sheet, and in a region of poorly ordered structure. The role of turns in determining the structure and stability of proteins is poorly understood, but it is likely that preferred backbone dihedral angles and local environmental effects will exert significant influence on these parameters [26]. The poor electron density in this region makes it difficult to model the effect of amino acid substitutions; however, our experimental data have shown that the polypeptide chain at position 354 is sufficiently flexible to accommodate many different amino acid substitutions, and is important in determining the overall thermostability of the protein.

Three substitutions at this position resulted in proteins with significantly reduced thermostabilities and it is interesting to note that two of them, glycine and proline, have side chains that impart special properties to the flexibility of the polypeptide backbone. Glycine has no side chain, which gives the polypeptide chain great flexibility while proline restricts the flexibility of the polypeptide backbone [27]. Taken in context with the surrounding sequence, these mutations result in a Pro-Pro or Gly-Gly dipeptide, which will have an even greater effects on the flexibility of the polypeptide.

The chemical basis for colour 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 sequence are key determinants of the colour of light emitted in the bioluminescence reaction [15,28–31]. Also, McCapra et al. [32] have proposed that the planarity of the rings in the excited-state oxyluciferin could influence the energy of the photon. We have also observed that amino acid substitutions at position 354 can effect small changes in the peak wavelength of emitted light. These shifts were observed by eye in the bioluminescence emitted by *E. coli* cells expressing mutant luciferases and were confirmed *in vitro* using a spectrofluorimeter. The maximum peak shift observed was associated with the mutation E354I where peak wavelength was increased by 3.5 nm. These changes are small and are unlikely to be due to interactions with the excited-state product but are more likely to be associated with longer-range influences on local structure.

We conclude that random mutagenesis and a visual screen can be a powerful method for identifying variants of firefly luciferase with improved characteristics. In particular, we have shown that replacement of Glu-354 can produce an enzyme with enhanced thermotolerance, and we have characterized the effects produced by all possible side-chain substitutions at this position.

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