Random mutagenesis of bacterial luciferase: critical role of Glu¹⁷⁵ in the control of luminescence decay

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Bacterial luciferases (LuxAB) can be readily classed as slow or fast decay luciferases based on their rates of luminescence decay in a single turnover assay. Luciferases from *Vibrio harveyi* and *Xenorhabdus* (*Photorhabdus*) *luminescens* have slow decay rates, and those from the *Photobacterium* genus, such as *Photobacterium fischeri*, *P. phosphoreum* and *P. leiognathi*, have rapid decay rates. By substitution of a 67-amino-acid stretch of *P. phosphoreum* LuxA in the central region of the LuxA subunit, the 'slow' *X. luminescens* luciferase was converted into a chimaeric luciferase with a significantly more rapid decay rate [Valkova, Szittner and Meighen (1999) *Biochemistry* **38**, 13820–13828]. To understand better the role of specific residues in the classification of luciferases as slow and fast decay, we have conducted random

mutagenesis on this region. One of the mutants generated by a single mutation on LuxA at position 175 [E175G (Glu¹⁷⁵ → Gly)] resulted in the 'slow decay' *X. luminescens* luciferase being converted into a luciferase with a significantly more rapid decay rate. These results indicate the importance of Glu¹⁷⁵ in LuxA as a critical residue for differentiating between 'slow' and 'fast' luciferases and show that this distinction is primarily due to differences in aldehyde affinity and in the decomposition of the luciferase–flavin–oxygen intermediate.

Key words: bacterial luciferase, bioluminescence, decay rate, error-prone PCR, fluorescence quenching, random mutagenesis.

INTRODUCTION

Bacterial luciferases catalyse the concomitant oxidation of FMNH₂ and a long-chain aldehyde into FMN and the corresponding long-chain fatty acid [1]. This reaction, shown in Scheme 1, depends on the sequential formation of several enzyme–flavin intermediates. Luciferase (E) interacts with FMNH₂ to form the EF complex, which subsequently reacts with O₂ to yield an EFO (oxygenated enzyme–flavin) complex. This complex either decomposes by a dark side pathway with a rate constant *k_D* to yield oxidized FMN (F) and H₂O₂ or interacts with aldehyde (A) to form an EFOA (EFO–aldehyde) complex. Decay of EFOA can occur by dissociation of the aldehyde followed by decomposition through the dark pathway or the reaction is completed with the emission of blue–green light at 490 nm characterized by the rate constant *k_L* (Scheme 1). The standard luciferase assay is quite unusual in that the enzyme undergoes only a single turnover as the FMNH₂ substrate is oxidized chemically at a rate faster than the decay of the EFOA intermediate [2], allowing direct measurement of decay of light intensity.

The three-dimensional structure of *Vibrio harveyi* bacterial luciferase has been solved at a resolution of 1.5 Å (1 Å = 0.1 nm) [3]. Each subunit of the heterodimeric (LuxAB) enzyme forms an (α/β)₈ barrel motif. The two non-covalently bound subunits interface by means of two α-helices in the N-terminal half of each subunit to form a parallel four-helix bundle. As the enzyme was crystallized without bound substrates, inferences can only be made with regard to the crucial residues involved in catalysis. A mobile region located in the LuxA subunit appears to be important, as substrates protect this region from proteolytic cleavage [4].

A large difference in the luminescence decay rates has been used as a taxonomic basis to divide luminescent bacteria into two

clearly distinct groups [5,6]. More than 3000 strains of luminescent bacteria from the *Vibrio* and *Photobacterium* genera could be classified as containing luciferases with either a slow or a fast decay of luminescence. The luminescent species with 'slow' luciferases were all *V. harveyi* strains, whereas *Photobacterium fischeri*, PP (*P. phosphoreum*) and *P. leiognathi* species all contained 'fast' luciferases. A more recent characterization of strains of XL (*Xenorhabdus luminescens*) has shown that this species contains a luciferase with a slower decay than that of *V. harveyi* luciferase [7].

A previous study [8] of chimaeric luciferases has been instrumental in delineating the regions crucial for catalysis and thermostability. In bacterial luciferase, the LuxA subunit is believed to control the primary kinetic properties of luciferases [9,10]. Luciferases containing chimaeric LuxA subunits also seem to be capable of identifying key regions involved in luminescence. An important first step towards characterizing the active-site residues is the identification of the region responsible for the classification of luciferases as 'slow' or 'fast' on the basis of their decay rates. In [8], a set of chimaeric luciferases containing portions of the C-terminal half of XL LuxA were replaced with the corresponding sequences from PP LuxA. A chimaera containing a replacement of 17% of its LuxA (residues 166–233) sequence with that of PP luciferase was converted from a 'slow' XL luciferase into a luciferase with properties more similar to PP luciferase, including a rapid luminescence decay rate. To identify the residues that play an important role in controlling and determining decay rates, we have initiated experiments that use directed molecular evolution approaches such as random mutagenesis and DNA shuffling to generate mutants. Kinetic analysis of one of these mutants shows that Glu¹⁷⁵ on LuxA has a critical role in determining the decay rate of luminescence for luciferases.

Abbreviations used: EFO complex, oxygenated enzyme–flavin complex; EFOA complex, EFO–aldehyde complex; IPTG, isopropyl β-D-thiogalactoside; PP, *Photobacterium phosphoreum*; XL, *Xenorhabdus luminescens*.

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Scheme 1 Intermediates in the bacterial bioluminescence reaction

EXPERIMENTAL

Chemicals

FMN was the highest-grade product available from Fluka (Ronkonoma, NY, U.S.A.). The concentration of FMN was determined using a molar absorption coefficient of $12\,200\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 450 nm. FMNH₂ was prepared by passing H₂ gas through an FMN solution containing Pt-asbestos. All aliphatic aldehydes were purchased from Aldrich, and myristic acid was from Sigma. BSA was purchased from either Sigma or Boehringer Mannheim (Montreal, QC, Canada). The phosphate buffer solution (pH 7.0) was prepared by mixing appropriate amounts of NaH₂PO₄ and K₂HPO₄, obtained from Baker. All compounds were reagent grade.

Random mutagenesis

XL *luxAB*, which has a unique *Xba*I site in the middle of *luxA* and also has two unique but silent restriction sites, *Sal*I and *Kpn*I, inserted in the 3'-end of *luxA*, was used for the expression of luciferase. Using the *Xba*I and *Sal*I sites, the 200 bp fragment was excised and transferred into pT7-5 for random mutagenesis experiments.

Mutations within this *luxA* region were introduced by error-prone PCR [11,12]. The reaction mixture contained 10 ng of template plasmid DNA, 50 pmol each of the primers, the *Xba*I site (5'-GGGGTCTAGAGGTGGCGCACCGGTTT-3') and the *Sal*I site (5'-AATTGAGTCATGGTCGACAGATGTTATA-3'), 2 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 0.5 mg/ml BSA, 0.2 mM each of dNTP and 1 unit of *Taq* DNA polymerase. Then, 30 cycles of amplification were performed (94°C for 1 min, 55°C for 1 min and 72°C for 30 s). To increase the frequency of misincorporated bases, PCR amplifications were performed by limiting one of the four dNTPs to 14 μM, increasing Mg²⁺ to 5 mM and adding 200 μM dITP.

Subcloning and selection of mutated PCR fragments

Amplified fragments were digested at *Xba*I and *Sal*I restriction sites. The fragments (200 bp) were then ligated into the original pT7-5 plasmid containing *luxAB*, previously cut by the same enzymes. The resulting ligation mixtures were then transformed into *Escherichia coli* BL21 and individual colonies were screened using *in vivo* light measurements. Dim and dark colonies were analysed by nucleotide sequencing of the extracted DNA between the *Xba*I and *Sal*I sites. The luminescence of colonies containing the mutated plasmids was monitored during growth.

Expression and enzyme purification

The pT7-5 plasmid containing parental or mutant XL *luxA* was transformed into *E. coli* strain BL21, whose chromosomal DNA contains the IPTG (isopropyl β-D-thiogalactoside)-inducible T₇ RNA polymerase. The cells were grown in the appropriate

medium containing 50 μg/ml ampicillin at 30°C, and IPTG was added to a final concentration of 0.6 mM when the cell density reached D_{660} 1.2–1.3. After induction with IPTG, the cell culture was allowed to grow for 1 h before luciferase extraction took place. The medium was centrifuged, and the cells were suspended in 10% of the original culture volume of 50 mM phosphate buffer (pH 7.0) and 20 mM 2-mercaptoethanol before lysis by ultrasonication. For cells with low luminescence, suspension was in 1% of the original culture volume before lysis. Purification of luciferase was performed according to previous methods [13]. Protein concentrations were estimated using the Bio-Rad protein determination kit with BSA as a standard [14].

Bioluminescence assay

For *in vitro* measurements, two assays were used; the standard flavin injection assay and the dithionite assay [15]. In the standard assay, 1 ml of catalytically reduced FMN ($5 \times 10^{-5}\text{ M}$) was rapidly injected into 1 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.2% BSA, 10 μl of 0.1% (v/v) decanal in propan-2-ol and the enzyme [16]. In the dithionite assay, 10 μl of 1 M Na₂S₂O₄ was mixed with 1 ml of phosphate solution (0.05 M, pH 7.0) containing $5 \times 10^{-5}\text{ M}$ FMN and the enzyme before the injection of 1 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.01% (v/v) decanal. The light emission in both assays was measured with a custom-built luminometer [17]. All luminescence assays were performed at room temperature ($23 \pm 2^\circ\text{C}$). The rate of luminescence decay was determined from the time elapsed between 80 and 40% of the maximum light intensity. Specific activities of luciferases were expressed in terms of LU (light unit)/mg. One LU is equivalent to 4×10^9 quanta s⁻¹. *In vivo* luminescence in *E. coli* cells was measured after vortex-mixing 1 μl of neat decanal into 1 ml of cell culture.

Steady-state fluorescence measurements

Fluorescence studies were performed on a Hitachi F-3010 fluorimeter equipped with a 150 W xenon short-arc lamp and a 5 mm path length quartz cuvette. The wavelength dispersion was 3 nm for excitation and 5 nm for emission, and the excitation wavelength was 296 nm. The samples were incubated in 50 mM phosphate buffer (pH 7.0) and 20 mM 2-mercaptoethanol at $25 \pm 0.2^\circ\text{C}$.

Molecular graphic model

The 1.5 Å resolution crystal structure of *V. harveyi* apo-luciferase determined under low salt conditions was retrieved from the Protein Data Bank (PDB entry 1LUC). The apo-luciferase crystal structure was used throughout the molecular modelling experiments. By the conversion of Glu¹⁷⁵ into a glycine residue, a graphic model of the FMN-luciferase complex was obtained in SWISS-pdb viewer (version 3.7).

RESULTS

Previous work on bacterial luciferase has established that the catalytic properties are primarily dictated by the LuxA subunit in the heterodimeric enzyme (LuxAB). Hybrid luciferases containing subunits from different species have been shown to have catalytic properties almost identical with those of the parental luciferase containing the same LuxA subunit even though the LuxB subunits could differ in amino acid sequence at more than 100 positions [18]. The generation of luciferases with chimaeric LuxA subunits has provided an alternative approach, which has

Table 1 Luminescence of *E. coli* cells containing mutant luciferases

In vivo luminescence of 1 ml of *E. coli* cells containing the respective luciferases grown at 30 °C to a $D_{660} \geq 2.5$. The activity is given as a percentage of the native *luxAB* expressed in *E. coli*.

No.	Native	Mutation	% activity
1	Glu ¹⁷⁵ (GAA)	Gly (GGA)	0.9
2	Thr ¹⁹⁸ (ACT)	Ser (AGT)	84
3	Lys ²⁰² (AAA)	Arg (AGA)	95
4	Asp ²³² (GAC)	Gly (GGC)	63
5	Met ¹⁹⁰ (ATG)	Thr (ACG)	29
6	Glu ¹⁷⁵ (GAA)/Asn ¹⁹⁹ (AAC)	Gly (GGA)/Asp (GAC)	0.1

Table 2 Specific activities and quantum yields of the purified mutant luciferase (E175G) as a function of aldehyde chain length

Specific activities of mutant luciferases with aldehydes of different chain lengths are close to 1% of native luciferases.

Aldehyde chain length	Specific activity (quanta · s ⁻¹ · mg ⁻¹)	Quantum yield
7	1.8×10^{10}	4.5×10^{10}
8	8.3×10^{10}	1.9×10^{11}
9	3.5×10^{11}	8.4×10^{11}
10	3.3×10^{11}	7.9×10^{11}
11	1×10^{11}	3.7×10^{11}
12	7.1×10^{11}	5.9×10^{11}

the potential of defining the region within LuxA containing the key residues controlling the catalytic properties [8].

Random mutagenesis of the 200 bp region of LuxA (residues 166–233) was accomplished by the incorporation of the wrong bases during PCR by limiting one of the dNTPs and adding dITP as well as by increasing the Mg²⁺ concentration. This approach is useful since a defined chemical mechanism using specific residues or regions in LuxA has not yet been proposed [8]. Among the 80 clones that were sequenced, approx. 20 contained at least one base substitution in this 200 bp region and appeared dim on Luria–Bertani agar plates. Most of the mutants were single A to G substitutions, whereas a few mutants had an additional substitution (Table 1).

The average light intensity of the mutants during the exponential phase of growth (D_{660} 0.5–1.0) was then compared with that for cells containing the wild-type construct. Most of the 20 mutants exhibited luminescence intensities lower than that of the wild-type (Table 1). *E. coli* cells containing LuxAB mutated at residue 175 [E175G (Glu¹⁷⁵ → Gly)] had low light emission (close to 1%) and different kinetic properties in comparison with those containing native XL luciferase, whereas most other mutants exhibited basic kinetic properties, including decay rates similar to native luciferase. Therefore only the XL luciferase with a single mutation at residue 175 (E175G) was further purified and investigated.

Specific activities and quantum yields as a function of aldehyde chain length

Since the E175G mutant luciferase was stable and had adequate activity in extracts, it could be readily purified to homogeneity. Table 2 compares the specific activities and quantum efficiencies of the E175G mutant luciferase with those of the wild-type enzyme for different aliphatic aldehydes.

Figure 1 Decay rates of luminescence as a function of aldehyde chain length

The decay rate for mutant (E175G) luciferase (■) is similar to that of the PP luciferase and much faster than XL luciferase (▲). The decay rates were determined by the standard assay. For details, see the Material and methods section.

Dependence of the decay rate of luminescence on aldehyde chain length

The constant for luminescence decay (k_L) reflects the rate of turnover of luciferase in the light pathway. As the enzyme undergoes a single catalytic cycle, the light or quantum yield can be deduced by integrating the light intensity produced during the reaction, which is equal to I_0/k_L , where I_0 represents maximum light intensity. Figure 1 shows that the rate constants for luminescence decay of E175G with different aldehydes are significantly increased over those of the wild-type luciferase.

Dependence of the decay rate of luminescence on aldehyde concentration

Responses of the native and E175G luciferases to increasing decanal concentrations are shown in Figure 2(A). The native XL luciferase shows maximum activity at 10 μ M decanal. In contrast, the mutant E175G requires a higher aldehyde concentration for maximum activity (20 μ M) and inhibition of activity occurs at higher decanal concentrations. Similar results were also obtained for activity against dodecanal. The dependence of the rates of luminescence decay on decanal concentration is also shown in Figure 2(B). In contrast, E175G shows a remarkably similar pattern to PP luciferase (results not shown) with the decay rate decreasing at higher aldehyde concentrations.

Decay of the EFO enzyme intermediate

The decay rate of the intermediate EFO complex formed before aldehyde binding was directly measured (Figure 3). The decay rate for the EFO complex (k_D) of the E175G mutant (0.6 s^{-1}) is much higher than the decay rate for the EFO complex of XL luciferase (0.03 s^{-1}). Values for the mutant are in close agreement with the rate constants measured for the EFO enzyme intermediate of PP luciferase [8].

Specificity and interactions of flavin

To analyse the interactions with flavin more directly, the dissociation constants K_d for FMNH₂ were measured by the dithionite assay. Analysis of the data (results not shown) gave a K_d for the mutant luciferase (1.2 μ M) for FMNH₂ quite similar to that of the XL native luciferase (1.4 μ M). To investigate the interactions

Figure 2 Luminescent light intensity (A) and decay rates (B) as a function of decanal concentration, determined for the E175G mutant (■) and wild-type luciferases (▲)

The percentage activity on the y-axis denotes the percentage of the maximum luminescent light intensity determined for each luciferase.

Figure 3 Decay rates of the EFO intermediates of native (■) and mutant (▲) luciferases

The decay rate of the EFO intermediate of mutant luciferase is very rapid relative to XL luciferase. The assay was performed by the injection of 1 ml of 50 μ M catalytically reduced FMN into a 1 ml mixture of the enzyme in 50 mM phosphate buffer with 0.2% BSA, and 1 ml of 0.001% decanal was injected at subsequent time intervals.

Figure 4 Fluorescence spectra of the native and mutant luciferases showing their different affinities for FMN

The addition of 10 μ M FMN in the presence of 50 μ M myristic acid (dotted line) quenches the fluorescence of XL luciferase to a much greater extent than that of the E175G mutant luciferase. The protein concentration was 2 μ M, and the enzyme was buffered in 50 mM phosphate (pH 7) and 20 mM 2-mercaptoethanol.

of native and mutants with flavin directly, the quenching of the intrinsic fluorescence of the purified luciferases by FMN was compared. Figure 4 shows that the emission spectra of the luciferases are very similar and that the quenching by FMN is much weaker for the mutant (E175G) than the parental XL luciferase. The fluorescence of XL luciferase is quenched by 40% by FMN, whereas the fluorescence of the mutant is quenched to less than 17%. The lower extent of quenching reflects a weaker binding of FMN as the quenching of the mutant (similar to PP luciferase) can be increased by adding higher concentrations of FMN.

DISCUSSION

Although the crystal structure of *V. harveyi* luciferase has been elucidated, the binding sites for the FMN and fatty aldehyde substrates are not yet clearly delineated in bacterial luciferases [19,20]. All luciferase mutants with altered kinetics in the bioluminescence reaction, selected by Cline and Hastings [21], had lesions in the LuxA subunit. In a similar investigation, one of the mutants, namely AK-20, had shown slightly enhanced thermal stability and FMNH₂ binding affinity, with a different aldehyde chain length specificity when compared with the wild-type luciferase [20]. Moreover, molecular aspects of different decay rates in bacterial luciferases are still not known.

Directed evolution has rapidly emerged as a powerful new strategy for improving the characteristic of enzymes in a targeted manner. By coupling various methods for generating large variant libraries of genes (random mutagenesis, DNA shuffling etc.)

together with high-throughput screens that select for specific properties of an enzyme such as thermostability, catalytic activities and substrate specificity, it is now possible to optimize a biocatalyst for specific applications [22–24].

In the present study, random mutagenesis of 200 nt in the C-terminal region of LuxA provides a directed approach towards the random mutagenesis of a limited region involved in the decay mechanism in luciferase. Among numerous dim mutants, a mutant (E175G) was remarkably different from the others and, surprisingly, in many aspects, closely resembled the properties of PP luciferase even though the sequence is XL luciferase. The most notable property of E175G luciferase was its rapid rate of luminescence decay when compared with native XL luciferase. The fast decay rate arose from a low aldehyde binding affinity and a rapid rate of decay of the EFO complex through a dark pathway. Both these properties of E175G closely paralleled the same properties of PP luciferase. The difference between the fast luminescence decay of the E175G mutant and the slow decay of XL luciferase indicates that the Glu¹⁷⁵ in the central region (residues 166–233 of LuxA) is probably involved in aldehyde binding and the mechanism of turnover of the EFO and EFOA intermediates. A striking similarity between the luminescence decay rates of E175G and PP luciferase with aldehydes of different chain lengths strengthened this conclusion.

Analyses of thermal stability showed that the E175G mutant and native XL luciferase had very similar properties, with inactivation rates at 35 °C being very slow and much slower than PP luciferase (results not shown). Therefore the basic elements of luciferase stability are still unchanged and mutation had not caused any major perturbation in the tertiary and/or quaternary structure of the enzyme [20].

Structural and molecular modelling studies show that the phosphate group is engaged in a network of seven intermolecular hydrogen bonds with the enzyme. Three hydrogen bonds are formed with the guanidinium moiety of Arg¹⁰⁷, two hydrogen bonds with the main chain NH groups of Glu¹⁷⁵ and Ser¹⁷⁶ and two hydrogen bonds with the hydroxy groups of Ser¹⁷⁶ and Thr¹⁷⁹ [19]. Conversion of Glu¹⁷⁵ into a glycine (a highly flexible residue) probably changes the peptide backbone conformation. In other words, Glu¹⁷⁵ provides an excellent environment for the 'right' juxtapositioning of FMN, and its change to glycine weakens aldehyde binding and possibly flavin binding by causing an increase in the decomposition of the EFO intermediate. Recent studies using molecular modelling and site-directed mutagenesis showed that phosphate docking to apo-luciferase was not possible without changing the conformation of the side chain of the Glu¹⁷⁵ whose carboxylate group is projected into the phosphate-binding site [19,25]. That is, phosphate binding to luciferase induces a conformational transition in the Glu¹⁷⁵ side chain by displacing the Glu¹⁷⁵ carboxylate from the phosphate-binding site into a solvent-exposed location [19,26]. Therefore it may be suggested that conversion of Glu¹⁷⁵ into a glycine residue and the subsequent lack of the usual glutamine–flavin interactions is responsible for the unusual kinetic properties of the E175G mutant XL luciferase (Figure 5). Although there were major changes in most of the properties related to aldehyde binding, changes related to flavin binding were much less pronounced.

Experiments shown here were designed to engineer new luciferases and identify active-site residues not only by screening for decay rate changes but also for changes in stability, efficiency and aldehyde specificity. For example, a shift in the colour of light emission in the reaction catalysed by luciferase would be a great benefit to the expanding application of the bacterial *lux* system as a sensor. Such changes have been accomplished in other luminescent or fluorescent systems by altering side chains

Figure 5 View of the modelled complex of FMN with the α subunit of bacterial luciferase

The FMN ligand and the selected protein residues lining the binding site, as discussed in the text, are shown in ball-and-stick representation. Intermolecular hydrogen bonds formed by the FMN molecule are shown as broken lines. Glu¹⁷⁵ is replaced by a glycine residue.

in close contact with the chromophore as shown for firefly and related luciferases [27,28] and green fluorescent protein [29,30].

Results presented in this paper have indicated that the presence and geometry of Glu¹⁷⁵ in the central region of LuxA plays a critical role in luciferases in determining the decay rate of luminescence. Identification of this mutant by a random but directed approach shows that this general method will also be effective in identifying the other residues involved in a variety of properties.

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