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A Cysteine-Free Firefly Luciferase Retains Luminescence Activity

Janet R. Kumita, Lily Jain, Ekaterina Safroneeva, and G. Andrew Woolley[1]
Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario M5S 3H6, Canada

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A mutant of *Photinus pyralis* luciferase in which all four native cysteine residues are converted to serines retains about 10% of wild-type activity. This mutant should prove useful as a starting point for the introduction of biophysical probes of conformational changes associated with enzyme function. The activities of the cysteine-free mutant and others in which two or three cysteines are converted to serines suggest, however, that small chemical changes can have substantial and interdependent effects on bioluminescence. The introduction of probes should therefore be approached cautiously. © 2000 Academic Press

Firefly luciferase (EC 1.13.12.7) from the species *Photinus pyralis* is a 62 kDa enzyme that catalyzes emission of yellow-green light (λ_{max} = 560 nm) upon reaction of D-luciferin, ATP, and molecular oxygen [1–4]. Firefly luciferase is a single polypeptide that is active in a monomeric form; it performs its function with extraordinary efficiency, exhibiting a quantum yield of 0.88 ± 0.25 [5].

The remarkable properties of firefly luciferase have led to its use as a tool in a variety of settings [6, 7]. These include the ultra-sensitive detection of ATP [8, 9], a reporter gene for monitoring transcriptional activity [10, 11], a biosensor for chemical toxins [12], a probe for monitoring protein folding *in vivo* [13, 14], and a probe for the actions of local anesthetics [15].

Aside from these practically useful attributes, the luciferase enzyme presents an opportunity for understanding, in molecular detail, how nature produces bioluminescence [1]. A variety of mutagenesis studies, in particular those by Branchini *et al.*, have provided a working model for the luciferase active site and have revealed aspects of how particular residues affect enzyme activity [16–26]. However, several aspects of the enzyme mechanism have remained unclear even with the recent elucidation of an X-ray crystal structure of firefly luciferase [2, 15].

Several studies have suggested that significant conformational changes in the enzyme accompany the process of conversion of chemical energy to light [27–29]. In particular, closing of the active site pocket around substrate has been proposed to account for the lag observed in light emission after ATP is added to a mixture of luciferase and luciferin [1, 2, 21, 28]. To better understand the nature of such conformational changes and their role in luciferase function, site specific probes of conformational change, operating on relevant time scales, are required. A convenient approach for the site-specific introduction of biophysical probes, is the introduction of cysteine residues via mutagenesis followed by reaction with thiol-specific reporter groups. Thiol-specific electron spin resonance probes have been particularly successful for probing enzyme motion accompanying activity [30–33].

For this approach to be successful, interference from native cysteine residues in the protein must be removed, and nonnative cysteines must be added without compromising enzyme activity. *Photinus pyralis* luciferase contains four native cysteine residues—Cys^{81}, Cys^{216}, Cys^{258}, and Cys^{391}. Several studies have addressed whether these cysteine residues are essential for activity.

Early chemical modification experiments showed that N-tosyl-L-phenylalanine inhibited luciferase [34]. Loss of enzyme activity was associated with the loss of approximately two sulfhydryl groups. Later *p*-mercuribenzoate, *N*-ethylmaleimide (NEM), and iodoacetamide were all shown to inhibit luciferase activity [35]. The presence of luciferin afforded some protection from inactivation. In particular, substrate was found to protect Cys^{216} and Cys^{391} from reaction with NEM [21]. Chemical modification with the fluorescent iodoacetamide derivative *N*-(iodoacetyl)-*N′*-(5-sulfo-1-naphthyl) ethylenediamine also led to inactivation [29]. In this case too, substrate provided some protection from inactivation, but the effect appeared rather

[1] To whom correspondence should be addressed. Fax: 416-978-0675. E-mail: awoolley@chem.utoronto.ca.

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nonspecific as all four cysteines were approximately equally protected.

Interestingly, chemical modification of luciferase with methyl methanethiosulfonate did not lead to inactivation but instead to a luciferase with a red-shifted emission spectrum [35]. This result suggested that certain cysteine modifications could be tolerated and that the size and/or nature of the modifying group was important in determining the outcome of cysteine modification.

Individual cysteine to alanine mutations have been reported at Cys^{216} and Cys^{391} of *P. pyralis* luciferase [21] and at all cysteine sites of the related *Luciola mingrelica* luciferase [36]. Each of these single cysteine to alanine mutation was reported to be active. Finally, Ohmiya and Tsuji [22] have shown that each of the four cysteines of *P. pyralis* luciferase can be individually mutated to serine resulting in proteins with 42–86% of wild-type activity.

Taken together these studies lead to the conclusion that no cysteine residue is essential for luciferase activity, in the sense of being required for the chemical mechanism. Nevertheless alteration of cysteine, e.g. by chemical modification or mutagenesis can have major effects on enzyme activity, presumably by interfering with substrate binding, altering the local environment of the active site, inhibition of some conformational change or a combination of the above. Since all the cysteines appear reactive to varying degrees, the introduction of biophysical probes at new sites requires removal of all of them. Although single cysteine to serine mutations were shown to be tolerated, it was not clear whether conversion of all four cysteines to serines would result in an active enzyme.

We report here that replacement of all four cysteine residues with serine residues in *P. pyralis* luciferase results in active enzyme although activity is about 10% of wild-type. We also compare the activity of various cysteine mutants containing, single, double, and triple cysteine replacements.

MATERIALS AND METHODS

All restriction endonucleases were obtained from New England Biolabs (NEB). All other enzymes were obtained from Boehringer Mannheim unless otherwise noted. Phosphorimaging was performed on a Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and results were analyzed in ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA). Bioluminescence was measured on a FB-12 Femtomaster Luminometer (Zylux Corp., Maryville, TN). Primer synthesis and DNA sequencing was performed by the Hospital for Sick Children Biotechnology Service Centre, Toronto, Canada. The luciferase assay reagent was a modified version of the one described in the Promega TNT coupled Reticulocyte Lysate Systems protocol. The following was dissolved in a 25 mM glycyl-glycine (Sigma) buffer of pH 7.8: 10 mM $MgSO_4$ (Caledon), 0.1 mM EDTA (ACP Chemicals Inc.), 33 mM DTT (Bioshop, Burlington, ON, Canada), 270 μM coenzyme A (Sigma), 470 μM luciferin (Sigma) and 530 μM ATP (Sigma).

Plasmid preparation and expression of luciferase. The P-cite 4a(+)-luciferase construct was prepared by digesting the P-cite 4a(+) vector (Novagen) with *Bam*HI/*Not*I, followed by the insertion of *P. pyralis* (Pp) luciferase cDNA. The cDNA for Pp luciferase was obtained from the T7 luciferase control DNA in the Promega TNT kit. The cDNA was amplified using PCR with VENT polymerase (NEB) and primers which introduced a *Bam*HI site at the 5′ end of the luciferase gene and a *Not*I site at the 3′ end. The P-cite 4a(+)-luciferase construct was then used to transform *E. coli* DH5α cells (Gibco BRL) and the plasmid DNA was isolated using the standard alkaline-lysis technique. This DNA was used for site-directed mutagenesis and protein expression.

Luciferase was expressed using the T7 polymerase TNT Coupled Reticulocyte Lysate System (Promega). The lysate contained [^{35}S]methionine (Amersham) to allow for detection of expressed luciferase after SDS–PAGE. Relative amounts of expressed protein in each band were quantified by phosphorimaging.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the Gene Editor kit (Promega). A ssDNA template of luciferase was obtained by performing alkaline denaturation on the dsDNA template isolated previously. The mutagenesis reactions were performed with the introduction of five primers, the Bottom Strand Selection Oligomer (Promega), **C81S** (5′ C GTC GTA T**C**C AGT GAA AAC TCT CT*G* CA*G* TTC TTT ATG C 3′), **C216S** (5′ CCG CAT AGA ACT GCC T**C**C GTC AGA TTC TCG CA*C* GCC AGA GAT CC 3′), **C258S** (5′ CTC GGA TAT TTG ATA T**C**T GGA TTT CG 3′) and **C391S** (5′ GGC GAA TTA T**C**T GTC AGA GG*G* CCT ATG ATT ATG TCC GG 3′). Mutations that result in amino acid sequence changes (cysteine to a serine) are denoted by bold underline. Mutations that result in the introduction or removal of restriction endonuclease sites, but do not cause changes in the amino acid sequence, are denoted in italic underline.

After the mutant strand synthesis and ligation, the plasmids were transformed into BMH71-18 *mutS* competent cells (Promega), followed by transformation into JM109 competent cells (Promega). The plasmid DNA was isolated from these cells using the alkaline-lysis technique. The presence or absence of mutations were determined by restriction endonuclease digestions with *Pst*I (present in C81S mutant), *Sph*I (absent in C216S mutant), *Eco*RV (presence of third site in C258S mutant) and *Ava*II (absence of one site from C391S mutant). Quadruple mutants (as determined by restriction digests) were sequenced (HSC Biotechnology Service Centre, Toronto, Canada).

Measurement of bioluminescence. For each sample, 5 μL of the translation reaction was combined with 100 μL of the luciferase assay reagent in a 1.5 mL Eppendorf tube. The tube was inserted into the luminometer and after a 0.2 s delay, relative light units (RLU)/second were measured over a course of 10 seconds. Measurements were carried out in triplicate on each translation reaction. Sample size reports the number of luminometer readings that were carried out. Thus, the activities reported reflect average relative activities from several translation reactions. Control reactions were performed with each translation reaction to permit comparisons, relative to wild type, from day to day. To ensure that no luminescence occurred due to components of the translation mixture other than luciferase (including [^{35}S]methionine) readings of the translation blank (minus template DNA) were also carried out.

RESULTS AND DISCUSSION

To facilitate efficient *in vitro* transcription, translation and subsequent purification of luciferase, the coding sequence for luciferase was inserted into the P-cite 4a(+)-vector. Expression of the construct resulted in a [^{35}S]methionine-labeled protein corresponding to 65 kDa (the P-cite vector introduced an N-terminal S-tag

TABLE I

Comparison of Activity for Multiple Cysteine Mutations

Mutant identification	Sample size	Sites of mutation	% Relative activity
Mutant A	6	C216S	52 ± 14
Mutant B	9	C81S, C391S	44 ± 7
Mutant C	6	C258S, C391S	21 ± 3
Mutant D	11	C216S, C258S, C391S	19 ± 4
Mutant E	9	C81S, C216S, C391S	67 ± 8
Mutant F	9	C81S, C258S, C391S	20 ± 2
Mutant G	15	C81S, C216S, C258S, C391S	6.5 ± 1.4

to the native luciferase). This construct was then used to produce cysteine to serine mutants. Restriction digestions identified a quadruple mutant, along with numerous partial mutants (i.e., double or triple) (Table I). Sequencing of the quadruple mutant confirmed the restriction digestion results. To determine the relative activity of each mutant, bioluminescence was measured using a luminometer and the relative light units obtained were corrected for the amount of protein expressed as determined by phosphorimaging.

The activity of luciferase expressed from the P-cite 4a(+) vector and that expressed using the T7-luciferase control DNA supplied with the *in vitro* transcription/translation kit from Promega were found to be indistinguishable. Thus, the presence of an N-terminal S-tag sequence and thrombin cleavage site (Fig. 1) is well tolerated. The S-tag sequence was used to purify the protein from the translation mixture (data not shown). This result is important because a variety of changes to the N-terminus of luciferase have been found to be deleterious for luciferase activity [19].

The relative bioluminescence data for each mutant are collected in Table I. All mutants, including the cysteine-free mutant (mutant G), are active to varying

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-38
MATTHM KETAAAKFERQHMDS PDQGTLVPRGS MADIGSMEDAKNIKKGPAPFYPLEDGTAGEQL
27
HKAMKRYALVPGTIAFTDAHIEVNITYAEYFEMSVRLAEAMKRYGLNTNHRIVVCSENSLQFFM
91
PVLGALFIGVAVAPANDIYNERELLNSMNISQPTVVFVSKKGLQKILNVQKKLPIIQKIIIMDS
155
KTDYQGFQSMYTFVTSHLPPGFNEYDFVPESFDRDKTIALIMNSSGSTGLPKGVALPHRTACVR
219
FSHARDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLICGFRVVLMYRFEEELFLRSLQDYKI
283
QSALLVPTLFSFFAKSTLIDKYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPGIRQGYGLTETT
347
SAILITPEGDDKPGAVGKVVPFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNPEATNA
411
LIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLIKYKGYQVAPAELESILLQHPNIFDAGVAGLPD
475
DDAGELPAAVVVLEHGKTMTEKEIVDYVASQVTTAKKLRGGVVFVDEVPKGLTGKLDARKIREI
539
LIKAKKGGKSKL
```

FIG. 1. The protein sequence of S-tagged luciferase from *P. pyralis*. The S-tag sequence is underlined and italicized; the thrombin cleavage site is underlined. The N-terminal methionine residue and the four cysteine residues are indicated in bold underline. Calculated mol wt: 64831.77.

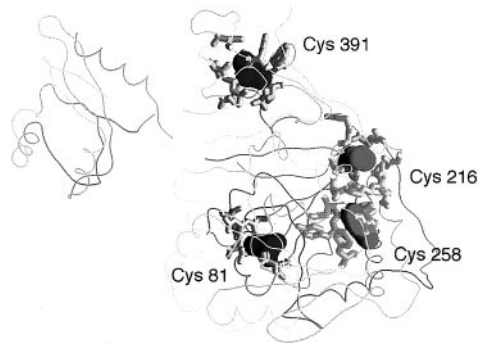

FIG. 2. Three-dimensional structure of *P. pyralis* luciferase (protein data bank ID = 1LCI). The four native cysteines are shown in surface representation. Surrounding residues (where at least one atom is within 4 Å of the cysteine) are shown as sticks. The protein backbone is represented with a ribbon. The figure was prepared using GRASP [38].

degrees. The only single mutant obtained was C216S (mutant A), which gave an activity relative to wild-type of 52%. Taking into consideration the difference in assay methods, this value compares reasonably well with the relative activity value of 42% for C216S reported by Ohmiya and Tsuji [22]. This single mutant and mutants B (double) and E (triple) all displayed retention of 50% activity or greater. Other double or triple mutants, i.e., mutants C (double), D (triple) and F (triple) (containing C258S mutations) showed a decline to approximately 20% activity.

The replacement of cysteine by serine is a relatively minor structural change but serine is significantly less hydrophobic than cysteine [37]. Figure 2 shows the locations of the four native cysteines in the luciferase crystal structure. Atoms found within 4 Å of the sulfur atom of the cysteine side chain are tabulated in Table II. A simple count of heteroatoms versus carbon atoms at each site reveals that the environments of Cys^{258} and Cys^{391} are considerably more hydrophobic than those of Cys^{81} or Cys^{216}. Thus cysteine to serine mutations might be ex-

TABLE II

Atoms within 4 Å of Cysteine Sulfur Atoms

Cysteine residue number	Atoms within Å of γS, but not part of the Cys residue	Number of carbons − number of heteroatoms
Cys 81	*N*Ser82, *O*Ala105, OH_2O69	−3
Cys 216	*C*Thr21 *Cα*Ala22, *Nε2*Glu25 *O*Gly20 *Nε*Arg188, *O*His212, *N*Ala22	−3
Cys 258	*Cβ*Arg223, *Cδ1*Ile231, *Cγ1*Ile231, *Cε2*Phe260, *O*Gly254	3
Cys 391	*Cε*His419, *Cγ*His419, *Cα*His419, *Cγ2*Val374, *C*Leu390, *O*Leu390, *O*Leu418, *Nδ1*His419	2

pected to be less well tolerated at the Cys^{258} and Cys^{391} sites. Of the mutants that we have analyzed, double and triple mutants with lowest activity all contained the C258S mutation along with a C391S mutation (mutants C, D, and F). Conversely, mutant E, a triple mutant where Cys^{258} is intact shows 67% activity.

Cys^{258} is located on the C-terminal end of the α-helix containing His^{245}, a residue which has been shown to be important for luciferase activity [16, 17]. A disruption at the Cys^{258} site may alter this α-helix enough to affect the interactions of His245 with the substrate, thereby decreasing the enzyme activity. Interestingly, Ohmiya and Tsuji [22] report that the single mutation of C258S results in 86% activity relative to wild type. Thus, the effects of cysteine to serine mutations on luciferase activity appear to be interdependent. This finding underscores the sensitivity of the luminescence reaction to alterations in luciferase structure and dynamics.

The activity of the cysteine-free luciferase mutant (mutant G), confirms that cysteine residues are not essential for *P. pyralis* luciferase activity. Although the activity of the quadruple mutant luciferase is significantly lower than the control luciferase, the activity is still easily detected with a standard luminometer. Thus, the cysteine-free construct should be suitable as a starting point for the introduction of biophysical probes in a site-specific manner. Importantly, however, the relative activity of any new luciferase mutant bearing such a probe must be compared to the cysteine-free enzyme. The sensitivity of the luminescence reaction means that one cannot assume the introduction of such probes will be nonperturbing.

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