

Mutagenesis of firefly luciferase shows that cysteine residues are not required for bioluminescence activity

Yoshihiro Ohmiya^{a,*}, Frederick I. Tsuji^{1,b}

^aDepartment of Biochemistry, Faculty of Education, Shizuoka University, Ohya 836, Shizuoka 422, Japan

^bMarine Biology Research Division 0202, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA

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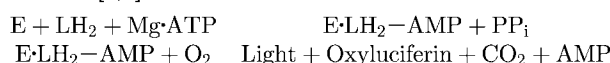
Abstract Conflicting reports exist in the literature as to whether sulfhydryl groups are essential for firefly luciferase activity. Inactivation of *Photinus pyralis* luciferase with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and site-directed mutagenesis demonstrate that the cysteine residues are not absolutely required for activity. However, loss of *P. pyralis* luciferase activity is observed when any of the 4 cysteine residues is replaced with serine.

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1. Introduction

Firefly luciferase (E) is a 62 kDa enzyme (oxygenase), which catalyzes the reaction leading to yellow-green light emission in fireflies [1,2]. In the presence of adenosine 5'-triphosphate (ATP) and Mg²⁺, the substrate, luciferin (LH₂), is activated to form luciferyl adenylate, which is then oxidized by molecular oxygen to yield light, oxyluciferin, CO₂ and adenosine 5'-monophosphate (AMP). LH₂ is a benzothiazole compound which serves as a common substrate in the firefly luminescence reaction [3,4].



Employing sulfhydryl-modifying reagents, earlier investigators have found that the luciferase from the North American firefly, *Photinus pyralis* (Pp), possesses 2 sulfhydryl groups, located at or near the luciferin binding site, that are essential for catalytic activity [5–8]. However, a subsequent study showed that, while chemical modification of sulfhydryl groups results in loss of activity, the sulfhydryl groups are not essential for activity [9]. These conflicting reports have left unanswered the question as to whether sulfhydryl groups are really needed for firefly luciferase activity.

Firefly luciferase is an enzyme that has been studied extensively and has served as an important reporter in monitoring gene expression in eukaryotic cells. In this study, the question

of the essentiality of sulfhydryl groups in firefly luciferase has been reexamined using a combination of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and site-specific mutagenesis to modify the cysteine residues in Pp luciferase [10] and in the luciferases of the Japanese fireflies, *Hotaria parvula* (Hp) [11] and *Pyrocoelia miyako* (Pm) [11]. The results show that, even though modification of the cysteine residues can cause significant changes in activity, sulfhydryl groups are not absolutely required for activity.

2. Materials and methods

2.1. Chemicals

Firefly D(–)luciferin-Na was purchased from Wako Pure Chemicals, Osaka, Japan, and *N*-tosyl-L-phenylalanine chloromethyl ketone was obtained from Sigma Chemical.

2.2. Expression and purification of Hp and Pm luciferases

Partially purified recombinant Hp and Pm luciferases were prepared as previously described [11]. The cDNA coding for either Hp or Pm luciferase was inserted into the Pharmacia high expression vector, pTrc-99A, and the vector used to transform *E. coli* LE392. The transformed cells were grown in LB medium containing 0.5 mM IPTG. The cells were harvested by centrifugation, resuspended in TE buffer (30 mM Tris-HCl/10 mM EDTA), and disrupted by sonication. The sonicated cell suspension was centrifuged at 12000×g and the luciferase in the supernatant purified by ammonium sulfate fractionation (30–65%), followed by anion exchange chromatography and gel exclusion chromatography. The most active fractions were pooled and stored at –70°C until used.

2.3. Modification of the cysteine residues by TPCK and assay of luciferase activity

TPCK was used to modify the sulfhydryl groups of partially purified recombinant Pp, Hp and Pm luciferases, as described [7]. The reaction mixture consisted of 1 µg of luciferase (1.6×10^{–11} mol), 5 mg of BSA and 0.5 µg of TPCK (1.4×10^{–9} mol) in 0.5 ml of sodium phosphate buffer, pH 7.8. The control consisted of the same mixture without the added TPCK. Luminescence activity was measured by transferring a 20 µl aliquot of the reaction mixture to a well of a 96-well culture plate and injecting 200 µl of 25 mM glycylglycine buffer, pH 7.8, containing 1.7×10^{–5} M firefly luciferin, 2×10^{–4} M ATP and 15 mM MgSO₄. The light intensity was measured for 10 s with an ATTO (Tokyo) Model AT-1000 photomultiplier plate reader. All assays were done in triplicate and the values were averaged. The activity, expressed in relative light units, was calculated as the ratio of the light intensity of the enzyme mixture/light intensity of the control mixture.

2.4. Site-specific mutagenesis of cysteine residues and assay of partially purified mutant luciferases

The plasmid, pU-PpL, containing the gene for Pp luciferase, was constructed by digesting pRSV/L [10] with *Hind*III/*Bam*HI and inserting the luciferase cDNA into the *Hind*III/*Bam*HI digestion site of pUC19 [12]. The amino acid residue, Cys²¹⁰, of Pp luciferase was replaced with valine by PCR in which a specific base substitution was introduced as a mismatch between a PCR primer and the target sequence [13,14]. The mutation was confirmed by sequencing the pUC-PpL216V luciferase gene by a modified dideoxynucleic acid

*Corresponding author. Fax: (81) (54) 237-3354.

¹Corresponding author. Fax: (1) (619) 534-7313.

Abbreviations: ATP, adenosine 5'-triphosphate; AMP, adenosine 5'-monophosphate; PP_i, pyrophosphate; IPTG, isopropyl-β-D-thiogalactoside; Pp, *Photinus pyralis*; Hp, *Hotaria parvula*; Pm, *Pyrocoelia miyako*; PCR, polymerase chain reaction; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; Val or V, valine; Ile or I, isoleucine; Lys or K, lysine; Cys or C, cysteine; Ser or S, serine

Table 2

Bioluminescence activities of *Photinus pyralis* wild-type and serine mutant luciferases after incubation with TPCK

Luciferase	Activity (counts)		Relative activity (%)
	0 h	4 h	
Wild-type	2.50×10^4	1.84×10^4	73.6
C81S	3.05×10^4	2.11×10^4	69.2
C216S	1.18×10^4	1.10×10^4	93.2
C258S	8.15×10^4	3.05×10^4	37.4
C391S	6.22×10^4	5.72×10^4	92.0

An aliquot (0.01 ml) of the luciferase solution was treated with TPCK (0.04 mM) for 4 h and the activity determined as described in Section 2.3. Values are the means of 3 replicate experiments.

TPCK. PpC216V/K141I was found to be inactivated at a rate considerably slower than wild-type luciferase. The fact that PpC216V/K141I retains activity suggests that Cys²¹⁶ is not essential for activity, unless a loss of activity due to the C216V substitution has been compensated by the K141I substitution. Interestingly, replacements of the adjacent Thr²¹⁷ with isoleucine in Lc luciferase [22] and Ala²¹⁷ with either isoleucine, leucine or valine in Ll luciferase [23] have been found to increase thermal stability of the enzymes. Mutagenesis has also been shown to cause mutant luciferases to produce different colors of light [24,25] and PpL luciferase to lose activity when the 12 C-terminal amino acid residues are removed [26,27].

The other 3 cysteine residues in Pp luciferase, Cys⁸¹, Cys²⁵⁸ and Cys³⁹¹, are seen to be conserved (Table 1), except that in *Photuris pennsylvanica* luciferase the amino acid residue homologous to Cys³⁹¹ is tyrosine. Table 1 also shows that the content of cysteine residues in all of the firefly luciferases is relatively high [6–8] compared to Pp luciferase, which has 4. In the present study, the 4 cysteine residues, Cys⁸¹, Cys²¹⁶, Cys²⁵⁸, and Cys³⁹¹, in Pp luciferase were each replaced with serine, a close chemical analogue of cysteine. Unlike TPCK, which can be used to modify exposed and accessible cysteine residues, mutagenesis may be used to modify both accessible and inaccessible cysteine residues in Pp luciferase. The mutant luciferases were expressed, purified and the amount present estimated by Western-blotting with anti-Pp luciferase. A 10 ng sample of each mutant luciferase was assayed, yielding the following activities (in relative light units): wild-type, 6.52×10^5 (100%); C81S, 3.62×10^5 (55.2%); C216S, 2.77×10^5 (42.2%); C258S, 5.64×10^5 (86.0%) and C391S, 4.64×10^5 (70.7%). Since the mutant luciferases are all active, the results indicate that Cys⁸¹, Cys²¹⁶, Cys²⁵⁸ and Cys³⁹¹ are not absolutely required for activity. In a further experiment, aliquots (0.01 ml) of the wild-type and serine mutant luciferases were mixed with TPCK (0.04 mM) and their activities determined (in relative light units) after 0 and 4 h of incubation. The results (Table 2) show that, while the activities are not uniform, all of the mutant luciferases were active, with two, C216S and C391S, showing activities close to their initial values.

The present results demonstrate that cysteine residues are not essential for firefly luciferase activity. This conclusion is consistent with the findings of a recent X-ray crystallographic study of Pp luciferase, which show the presence of Glu³⁸⁹,

Gly⁴²¹, Asp⁴²², Ser⁴²⁰, Tyr³⁴⁰, Glu³⁴⁴, Ser¹⁹⁸, Lys²⁰⁶, and Tyr⁴⁰¹, but not cysteine or histidine residues, around the cleft forming the active site [28]. Further, P. Brick (personal communication) has recently informed us that all 4 cysteine residues (Cys⁸¹, Cys²¹⁶, Cys²⁵⁸ and Cys³⁹¹) are buried and located some distance from the active site, and presumably not involved with catalytic activity. A corollary to this observation is the question of the accessibility of the cysteine and histidine residues to TPCK. Changes in luciferase activity resulting from TPCK treatment or cysteine mutagenesis, therefore, must have other causes, such as an alteration in the tertiary structure of the molecule affecting the active site.

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