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

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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.

Key words: *Photinus pyralis*; Oxygenase; Active site; Protein modification; Amino acid sequence homology

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 Mg^{2+}, the substrate, luciferin (LH_2), is activated to form luciferyl adenylate, which is then oxidized by molecular oxygen to yield light, oxyluciferin, CO_2 and adenosine 5′-monophosphate (AMP). LH_2 is a benzothiazole compound which serves as a common substrate in the firefly luminescence reaction [3,4].

$$E + LH_2 + Mg{\cdot}ATP \qquad E{\cdot}LH_2{-}AMP + PP_i$$
$$E{\cdot}LH_2{-}AMP + O_2 \quad Light + Oxyluciferin + CO_2 + AMP$$

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.

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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

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 $12000 \times g$ 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 $MgSO_4$. 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 *Hin*dIII/*Bam*HI and inserting the luciferase cDNA into the *Hin*dIII/*Bam*HI digestion site of pUC19 [12]. The amino acid residue, Cys^{216}, 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-PpLC216V luciferase gene by a modified dideoxynucleic acid

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sequencing method [15]. One amino acid residue, Lys^{141}, was found to be replaced by isoleucine due to a PCR error and this luciferase was designated PpC216V/K141I. Following the same PCR procedure, the expression plasmids pUC-PpLC81S, pUC-PpLC216S, pUC-PpLC258S and pUC-PpLC391S were constructed, which allowed for the cysteine residues, Cys^{81}, Cys^{216}, Cys^{258} and Cys^{391}, in Pp luciferase to be replaced with serine.

The above plasmids were used to transform *E. coli* HB101. Each transformant was grown in LB medium overnight at 37°C, followed by the addition of IPTG to a concentration of 0.5 mM and the incubation continued for an additional 24 h at 25°C. The cells were harvested by centrifugation, resuspended in TE buffer, disrupted by sonication and the suspension centrifuged. The expressed luciferase in the supernatant was purified by fractional precipitation with ammonium sulfate (30–65%), anion exchange chromatography and gel exclusion chromatography, as mentioned above. The most active fractions were pooled and stored at −70°C until used. Besides PpC216V/K141I, the other partially purified luciferases were Pp (wild-type), PpC81S, PpC216S, PpC258S and PpC391S. Luminescence activity was determined by transferring 20 μl of an ATP solution (10 pmol) to a well of a 96-well culture plate and injecting 200 μl of 25 mM glycylglycine buffer (pH 7.8) containing 10 ng of enzyme, 1.7×10^{-5} M firefly luciferin and 15 mM $MgSO_4$. Modification of PpC216V luciferase with TPCK and assay for luminescence activity were carried out as mentioned above.

3. Results and discussion

Previous studies have shown that Pp luciferase is inactivated by *p*-mercuribenzoate [5], *N*-ethylmaleimide [6–8] and TPCK [7]. In the presence of 4 molar excess of TPCK, Pp luciferase gradually loses 70% of its activity over a period of 4.5 h, whereas in a 40-fold molar excess of TPCK the enzyme is completely inactivated in 4 h [7]. The luciferase, however, is not inactivated by TLCK, the lysine analogue of TPCK [7]. The inactivation by TPCK was reported to be due to the modification of sulfhydryl groups. Fig. 1 shows the time course of inactivation of Hp (a), Pm (b) and wild-type Pp (c) luciferases at room temperature in a 87.5-fold molar excess of TPCK. The residual activities after 2 h of incubation for Pp and Pm luciferases were 30% and 40%, respectively, whereas after 18 h of incubation, the activities were zero. With Hp luciferase, the residual activity was close to 65% after 2 h of incubation and the enzyme still had significant activity even after 18 h of incubation (data not shown). The results suggest that, besides Pp luciferase, other firefly luciferases may be inactivated similarly by TPCK, albeit at a slower rate. In other studies, TPCK has been reported to inactivate papain by alkylating a sulfhydryl group [16] and α-chymotrypsin by

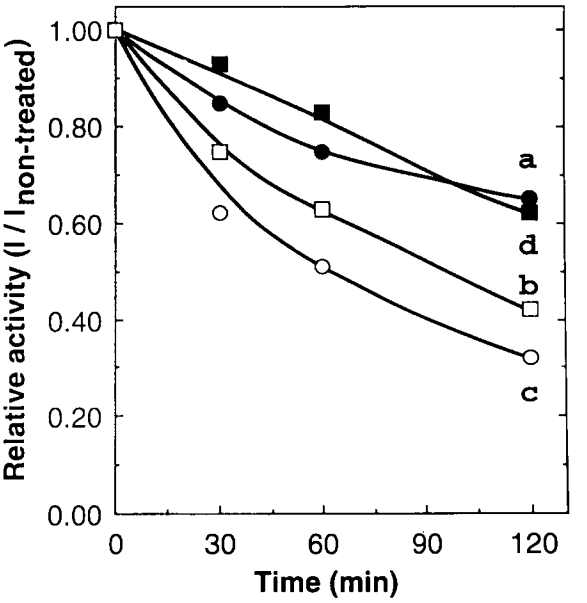

Fig. 1. Time course of inactivation of Hp (a), Pm (b), wild-type Pp (c) and PpC216V/K141I (d) luciferases by TPCK. Details of the experimental procedures are described in Section 2.3.

alkylating a histidine residue [17]. Pp luciferase contains 4 cysteine and 14 histidine residues [10]. Thus, since TPCK is capable of reacting with both cysteine and histidine residues, it is problematical whether the inactivation of Pp luciferase is due to the reaction of TPCK with cysteine or histidine residue, or both.

Multiple alignment of the amino acid sequences of luciferases from 7 species of fireflies shows that there is significant homology among the cysteine residues (Table 1) [11]. However, Cys^{216} of Pp luciferase is seen not to be highly conserved. In *Luciola cruciata* (Lc) [19,11] and *L. lateralis* (Ll) [20] luciferases, as well as in *L. mingrelica* (Lm) [21,11] and Hp [11] luciferases, the amino acid residue homologous to Cys^{216} of Pp luciferase is Val^{218}, whereas in Pm luciferase the amino acid residue is Cys^{217} [11]. Consequently, it was of interest to replace Cys^{216} of Pp luciferase with valine and to determine the activity of the mutant luciferase. As mentioned in Section 2.4, due to a PCR error, PpC216V/K141I, instead of PpC216V, was produced. Fig. 1d shows the time course of inactivation of PpC216V/K141I luciferase by

Table 1
Cysteine residues in luciferases of seven species of fireflies

Species	Location in primary structure											
P. pyralis			C^{81}					C^{216}		C^{258}		C^{391}
P. miyako	C^{62}		C^{82}		C^{129}			C^{217}	C^{223}	C^{259}		C^{392}
L. cruciata	C^{63}	C^{64}	C^{83}	C^{87}			C^{165}			C^{260}	C^{284}	C^{393}
L. lateralis	C^{63}	C^{64}	C^{83}	C^{87}						C^{260}	C^{284}	C^{393}
H. parvula	C^{62}		C^{82}	C^{86}		C^{146}	C^{164}			C^{260}	C^{284}	C^{393}
L. mingrelica	C^{62}		C^{82}	C^{86}		C^{146}	C^{164}			C^{260}	C^{284}	C^{393}
P. pennsyl.	C^{60}		C^{80}		C^{127}		C^{162}		C^{221}	C^{257}		

The full species names are as follows: *Photinus pyralis, Pyrocoelia miyako, Luciola cruciata, Luciola lateralis, Hotaria parvula, Luciola mingrelica and Photuris pennsylvanica.* Cysteine is denoted by the standard single letter code, C. A cysteine residue occurring only once in a given position in a luciferase molecule is not shown, these being C^{94} and C^{363} in *P. miyako* and C^{38} in *P. pennsylvanica.* The cysteine residues in the luciferases of the taxonomically related click beetle, *Pyrophorus plagiophthalamus* (Elateridae), are not shown, but they occur at C^{60}, C^{69}, C^{80}, C^{114}, C^{127}, C^{160}, C^{194}, C^{213}, C^{310}, C^{311}, C^{335}, C^{388} and C^{460} [18]. This beetle, although not a true firefly, uses the same luciferin as substrate for light emission as true fireflies [3]. The amino acid sequence of *P. pennsylvanica* luciferase (GenBank accession number, U31240) was kindly provided by Dr. Franklin R. Leach.

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 Cys^{216} 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 Thr^{217} with isoleucine in Lc luciferase [22] and Ala^{217} 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, Cys^{81}, Cys^{258} and Cys^{391}, are seen to be conserved (Table 1), except that in *Photuris pennsylvanica* luciferase the amino acid residue homologous to Cys^{391} 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, Cys^{81}, Cys^{216}, Cys^{258}, and Cys^{391}, 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 Cys^{81}, Cys^{216}, Cys^{258} and Cys^{391} 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 Glu^{389}, Gly^{421}, Asp^{422}, Ser^{420}, Tyr^{340}, Glu^{344}, Ser^{198}, Lys^{206}, and Tyr^{401}, 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 (Cys^{81}, Cys^{216}, Cys^{258} and Cys^{391}) 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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