Firefly Luciferase: Alteration of the Color of Emitted Light Resulting from Substitutions at Position 286

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Firefly luciferase transduces chemical energy for the production of light.[1] Interestingly, different species of fireflies, and even different anatomical regions of a single firefly, can emit light of different colors, athough their luciferases all use the same substrate.[2] Light production involves the net conversion of firefly luciferin to oxyluciferin; an excited state oxyluciferin (di)anion is believed to actually emit the observed light.[3]

That the wavelength of emitted light is controlled broadly by the structure and conformation of the protein bound to luciferin is suggested by a few different lines of evidence, including altered pH dependence of the color of emitted light in the presence of a "stretched-out" ATP analogue[3b] and the absence of evidence for any covalent enzyme−substrate intermediate. Kajiyama and Nakano identified five sites in *Luciola cruciata* luciferase at which a single amino acid change could substantially alter the wavelength of emitted light.[4] That these five sites were widely separated throughout the sequence of the protein further supports the idea that numerous alterations of protein structure can influence the emission wavelength. The tendency for luciferase point mutants to emit longer wavelengths than the wild type is consistent with nonspecific alterations that destabilize the substrate−luciferase interaction, e.g. by raising the ground state energy of the system.

To further define the nature of light emission, we have studied *Luciola mingrelica* luciferase, a thermolabile species.[5,6] Suppression of a TAG codon[7] introduced at position 286 was effected using several misacylated suppressor tRNAs,[8] affording proteins in which Ser286 was replaced with other amino acids. Presently we demonstrate (i) the first direct incorporation of glycosylated and phosphorylated amino acids into a nascent enzyme, (ii) that replacement of Ser286 in *L. mingrelia* luciferase can result in alteration of the wavelength of emitted light, and (iii) that the efficiency of light emission by the derived luciferase mutants can be temperature dependent and affected differentially by alteration of Ser286.

Table 1. Emission Characteristics of Luciferase Elaborated in *E. coli* Containing Suppressor tRNAs[11]

suppressor tRNA isoacceptor present in *E. coli* strain	luciferase plasmid harbored	luciferase emission wavelength (nm)	color
serine	pTrcLuc-St286	582	yellow-green
leucine	pTrcLuc-St286	621	red-orange
glutamine	pTrcLuc-St286	609	orange
tyrosine	pTrcLuc-St286	617	red-orange
lysine	pTrcLuc-St286	608	orange
phenylalanine	pTrcLuc-St286	608	orange
	pTrcLuc-St286		
	pTrcLuc	583	yellow-green
serine	pTrcLuc	583	yellow-green

The luciferase gene of *L. mingrelica*,[5,9] was cloned into expression vector pTrc-99A[10] under the control of a *trc* promoter. Site-specific mutagenesis was used to change Ser codon AGT at position 286 to stop codon TAG. Substitution of other naturally occurring amino acids for serine at position 286 was assayed initially using *Escherichia coli* strains, each of which contained a specific suppressor tRNA recognized by a different endogenous aminoacyl-tRNA synthetase.[11] Following transformation of these strains with plasmid pTrcLuc-St286, the spectrum of light emitted by each of the elaborated luciferases was measured.[12,13] Both wild-type luciferase and luciferase elaborated by the *E. coli* strain containing suppressor $tRNA^{Ser}$ produced light with an emission maximum at about 583 nm. Each of 10 other amino acid substitutions afforded a luciferase whose emitted light exhibited an altered λ_{max}; several representative examples are given in Table 1. As shown, all of the modified luciferases emitted light at longer wavelengths than wild type. The greatest difference was for the luciferases containing Ser286 (582 nm) and Leu286 (621 nm) (Figure 1); this corresponds to an energy difference of approximately 3 kcal/mol.

Additional substitutions at position 286 were introduced by *in vitro* translation of a luciferase mRNA derived from pTrcLuc-St286. In common with luciferase expression in *E. coli*, luciferase production in the cell-free system obtained only when an aminoacylated suppressor tRNA was present (Table 2).[14,15]

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(2) (a) Seliger, H. H.; McElroy, W. D. *Proc. Natl. Acad. Sci. U.S.A.* **1964**, *52*, 75. (b) Wood, K. V.; Lam, Y. A.; Seliger, H. H.; McElroy, W. D. *Science* **1989**, *244*, 700.
(3) Light emission is apparently due to an oxyluciferin dianion at pH > 6.7 that produces yellow-green light; protonation at lower pH affords a red emission band. See, e.g.: (a) White, E. H.; Steinmetz, M. G.; Miano, J. D.; Wildes, P. D.; Morland, R. *J. Am. Chem. Soc.* **1980**, *102*, 3199. (b) Rosendahl, M. S.; Leonard, N. J.; DeLuca, M. *Photochem. Photobiol.* **1982**, *35*, 857.
(4) Kajiyama, N.; Nakano, E. *Protein Eng.* **1991**, *4*, 691.
(5) Devine, J. H.; Kutuzova, G. D.; Green, V. A.; Ugarova, N. N.; Baldwin, T. O. *Biochim. Biophys. Acta* **1993**, *1173*, 121.
(6) This luciferase is homologous with *L. cruciata* luciferase at four of the five sites capable of controlling the wavelength of light emitted by the latter species, including Ser286.
(7) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182. (b) Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1991**, *202*, 301. (c) Bain, J. D.; Wacker, D. A., Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389. (d) Bain, J. D.; Diala, E. S.; Glabe, C. G.; Wacker, D. A.; Lyttle, M. H.; Dix, T. A.; Chamberlin, A. R. *Biochemistry* **1991**, *30*, 5411. (e) Bain, J. D.; Switzer, C.; Benner, S. A.; Chamberlin, A. R. *Nature* **1992**, *356*, 537. (f) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621.
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(10) Purchased from Pharmacia Biotech.
(11) Promega Corporation Interchange In Vivo Mutagenesis System. See: (a) Kleina, L. G.; Masson, J.-M.; Normanly, J.; Abelson, J.; Miller, J. H. *J. Mol. Biol.* **1990**, *212*, 705. (b) Normanly, J.; Kleina, L. G.; Masson, J.-M.; Abelson, J.; Miller, J. H. *J. Mol. Biol.* **1990**, *212*, 719.
(12) For elaboration and assay of luciferase in *E. coli*, the cells harboring pTrcLuc or pTrcLuc-St286 were cultured overnight at 25 °C in 3 mL of Luria-Bertan broth containing 50 μg/mL ampicillin and 1 mM IPTG. The cells were harvested by centrifugation, resuspended in 100 μL of lysis buffer (100 mM K phosphate, pH 7.8, containing 1 mM EDTA and 1 mg/mL lysozyme), incubated at 25 °C for 30 min, and then frozen in dry ice. The frozen pellets were allowed to thaw at 25 °C; following centrifugation 20 μL of the cleared lysate was added to 100 μL of a commercial luciferase assay solution (Promega), and the spectrum of emitted light was recorded at pH 7.8 on a fluorescence spectrophotometer.
(13) No luciferase activity resulted when pTrcLuc-St286 was transformed into *E. coli* lacking a suppressor tRNA (cf. Table 1), consistent with the insertion of amino acid 286 from a suppressor tRNA.
(14) The use of misacylated yeast suppressor $tRNA^{Phe}_{CUA}$s assured that no reactivation of the suppressor tRNAs occurred following amino acid transfer into protein, as this tRNA has been shown not to be a substrate for any *E. coli* aminoacyl-tRNA synthetase.[7a]

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Table 2. Emission Characteristics of Luciferases Synthesized in a Cell-Free System[a]

expression plasmid	suppressor tRNA	suppression (%)	specific activity[b]	emission wavelength (nm)
pTrcLuc		*c*	100	583
pTrcLuc-St286	$tRNA^{Phe}_{CUA}$	*d*	*d*	*d*
pTrcLuc-St286	$tRNA^{Phe}_{CUA}$-Ser (**1**)	11	109	584
pTrcLuc-St286	$tRNA^{Phe}_{CUA}$-PhnSer (**2**)	7.5	29	584
pTrcLuc-St286	$tRNA^{Phe}_{CUA}$-GlcSer (**3**)	6	36	585
pTrcLuc-St286	$tRNA^{Phe}_{CUA}$-Phe	27	27	608
pTrcLuc-St286	$tRNA^{Phe}_{CUA}$-Val	*e*	*e*	621

[a] Transcription and translation were carried out in an *E. coli* S30 system.[7b] [b] Relative to that of the wild type. [c] Not applicable. [d] No detectable light production. [e] Not determined.

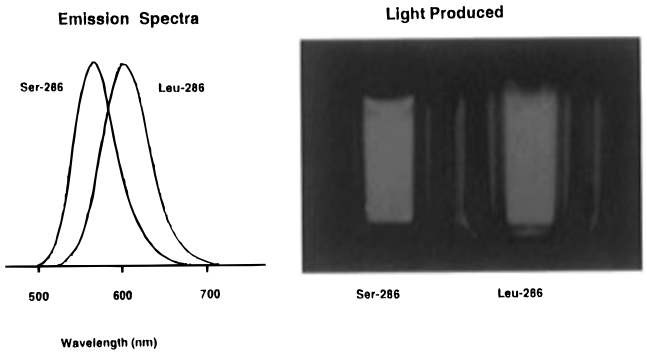

Figure 1. Alteration of the wavelength of light emitted by firefly luciferase upon substitution of Leu286 for Ser286.

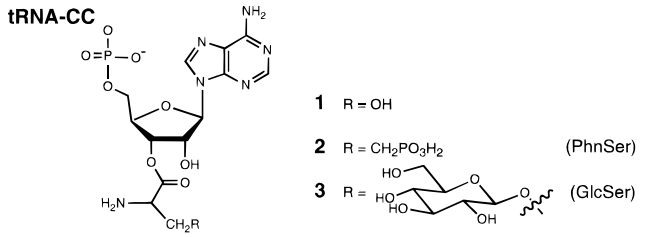

Figure 2. Misacylated suppressor tRNAs used for introduction of amino acids into position 286 of luciferase.

The misacylated suppressor tRNAs were obtained as described,[8] by T4 RNA ligase-mediated ligation of aminoacylated pdCpA derivatives to a yeast suppressor $tRNA^{Phe}_{CUA}$ transcript lacking the 3′-terminal nucleotides (pCpA) normally present. In addition to $tRNA^{Phe}_{CUA}$s activated with serine, valine, and phenylalanine, we also prepared the tRNAs activated with the serine phosphonate (**2**; PhnSer) and glucosylated serine (**3**; GlcSer) analogues shown in Figure 2.[16]

The luciferase prepared in a cell-free system using $tRNA^{Phe}_{CUA}$ misacylated with serine had the same light emission properties as wild-type luciferase (Table 2), reflecting the incorporation of serine into position 286. Likewise, the use of phenylalanyl-$tRNA^{Phe}_{CUA}$ afforded a species whose light emission (λ_{max} 608 nm) occurred at the same wavelength as the Phe286 analogue elaborated in *E. coli* (cf. Tables 1 and 2). A luciferase analogue having Val286 exhibited the same wavelength of emitted light (621 nm) as an analogue having Leu286 (cf. Tables 1 and 2), an observation that presumably reflects the similar nature of these amino acids. Interestingly, the luciferase analogues having serine phosphonate and glucosylserine analogues at position 286 emitted light at the same wavelength as wild-type luciferase.[17] The observation that replacement of Ser286 with a negatively charged (PhnSer) or large and highly polar (GlcSer) amino acid has no effect on the emission wavelength argues strongly that Ser286 is not involved in specific interactions with the luciferin substrate. The data appear more consistent with conformational changes promoted by amino acids such as leucine and valine whose presence at position 286 may induce alterations in protein structure locally through the introduction of new hydrophobic interactions which ultimately affect the environment around the bound luciferin substrate.[18] Although the structure of firefly luciferase has not been well defined as yet, a more interesting issue is the prospect for altering the properties of luciferase through alterations that effect specific interactions, e.g., in direct stabilization of the putative oxyluciferase dianion responsible for light production.[3] Such stabilization might well lead to differences in the energy of light production much larger than those observed here.

The serine analogues introduced into firefly luciferase constitute the first report of the ribosomally mediated introduction of glycosylated and phosphorylated amino acid analogues directly into proteins.[19] Glycosylation and phosphorylation are modifications associated with the appearance of specific properties in proteins. The carbohydrate portion of specific proteins, for example, can be important in biological recognition processes.[20] Phosphorylation is an important mechanism for the regulation of the activity of certain enzymes, as well as biological processes such as protein synthesis, receptor activation, and carcinogenesis.[21] The strategy employed here for the introduction of phosphate and sugar moieties at a specific position has some obvious advantages relative to the use of protein kinases and glycosyl transferases, and may contribute importantly to an understanding of protein function and the mechanisms that control posttranslational modifications.

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Supporting Information Available: Text and schemes describing the syntheses of serine phosphonate and *O*-glucosylserine derivatives (10 pages). See any current masthead page for ordering and Internet access instructions.

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(15) In addition to the dependence of luciferase production on the presence of an aminoacylated suppressor tRNA (Table 2), three of the activated suppressor tRNAs (containing phenylalanine, valine, and glucosylserine (**3**)) were used for the elaboration of dihydrofolate reductase (DHFR) from a mRNA containing a UAG codon at position 10. The derived DHFRs, which formed only in the presence of the activated suppressor tRNAs, were degraded with Glu-C endopeptidase, affording a peptide encompassing amino acids 1−17 of DHFR. These peptides were shown to be identical with authentic standards, as judged by HPLC analysis in systems highly sensitive to alteration of polypeptide structure.

(16) The aminoacylated pdCpA derivatives were prepared by chemical synthesis;[8] the syntheses of the serine phosphonate and *O*-glucosylserine derivatives are provided as supporting information.

(17) Normalization of light intensity for the amount of luciferase protein actually produced in each case revealed that the luciferases containing serine phosphonate and glucosylated serine produced light somewhat less efficiently than the wild type (Table 2). At present, it is not clear whether this is due to incorrect folding of some of the modified proteins, or to an intrinsic property such as lesser population of the excited state or (partial) decay of the oxyluciferin dianion through a dark reaction.

(18) Consistent with this interpretation, the thermostability of wild-type luciferase (58% loss of light production at 37 °C vs 25 °C) was much greater than that of luciferase containing Val286 (95% loss of light production).

(19) See, however, ref 7c for introduction of PhnSer into a short peptide.

(20) See, e.g.: (a) Baenziger, J. U. *FASEB J.* **1994**, *8*, 1019. (b) Lasky, L. A. *Annu. Rev. Biochem.* **1995**, *64*, 113.

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