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The structural origin and biological function of pH-sensitivity in firefly luciferases†

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Firefly luciferases are called pH-sensitive because their bioluminescence spectra display a typical red-shift at acidic pH, higher temperatures, and in the presence of heavy metal cations, whereas other beetle luciferases (click beetles and railroadworms) do not, and for this reason they are called pH-insensitive. Despite many studies on firefly luciferases, the origin of pH-sensitivity is far from being understood. This subject is revised in view of recent results. Some substitutions of amino-acid residues influencing pH-sensitivity in firefly luciferases have been identified. Sequence comparison, site-directed mutagenesis and modeling studies have shown a set of residues differing between pH-sensitive and pH-insensitive luciferases which affect bioluminescence colors. Some substitutions dramatically affecting bioluminescence colors in both groups of luciferases are clustered in the loop between residues 223–235 (*Photinus pyralis* sequence). A network of hydrogen bonds and salt bridges involving the residues N229-S284-E311-R337 was found to be important for affecting bioluminescence colors. It is suggested that these structural elements may affect the benzothiazolyl side of the luciferin-binding site affecting bioluminescence colors. Experimental evidence suggest that the residual red light emission in pH-sensitive luciferases could be a vestige that may have biological importance in some firefly species. Furthermore, the potential utility of pH-sensitivity for intracellular biosensing applications is considered.

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Introduction

Bioluminescent beetles emit a wide variety of colors ranging from the green to the red.[1] The bioluminescence color is determined by the luciferase structures, which influence the active site microenvironment around the excited emitter. Different bioluminescence colors, such as those occurring in click beetles and railroadworms, are usually displayed by different luciferase isozymes.[1,2] In the Jamaican click beetle, the bioluminescence color is determined by the co-expression of different isozymes.[3] Although firefly luciferases usually elicit bioluminescence in the yellow-green region, they can shift the spectrum to the red at acidic pH, a condition that has been called pH-sensitivity[4,5] (Fig. 1). Higher temperatures,

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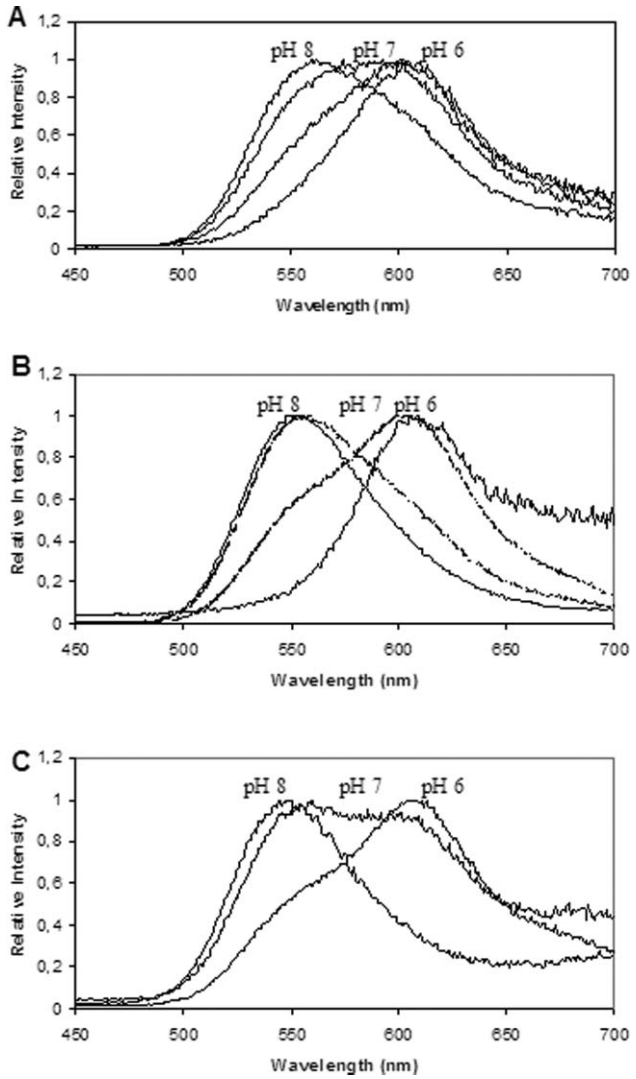

Fig. 1 Bioluminescence spectra of pH-sensitive luciferases according to Viviani *et al.*:[40] (A) *Macrolampis* sp_2; (B) *Photinus pyralis* and (C) *Cratomorphus distinctus*. Reproduced from: V. R. Viviani, T. L. Ohelmeyer, F. G. C. Arnoldi and M. R. Brochetto-Braga, A new firefly luciferase with bimodal spectrum: identification of structural determinants of spectral sensitivity in firefly luciferases, *Photochem. Photobiol.*, 2005, **81**, 843–848 © American Society for Photobiology and Blackwell Publishing.

divalent heavy metal cations (Cu^{+2} and Zn^{+2}) and denaturants such as urea can also shift the spectrum of firefly luciferases to the red, probably by the same underlying mechanism of pH-sensitivity. On the other hand, although a click beetle luciferase was reported to undergo some spectral shift upon changing pH,[2] click beetle and railroadworm luciferases do not display the typical red-shift observed for firefly luciferases in the pH range from 6 to 8, in the temperature range from 20–35 °C, and in the presence of divalent heavy metal cations, and therefore they have been called pH-insensitive.[5]

The primary sequences of several beetle luciferases[6–17] are known. The three-dimensional structure has been solved for the North-American firefly luciferase, in the absence of substrates,[18] and more recently for the Japanese *Luciola cruciata* firefly luciferase in the presence of either the luciferyl–adenylate analogue DLSA, or oxyluciferin and AMP.[19] The structure of firefly luciferases shows a main N-terminal domain connected to a smaller C-terminal domain by a short and flexible hinge. Luciferin binding-site residues were identified by modeling studies,[20,21] site-directed mutagenesis[22–25] and by direct inspection of the three-dimensional structure in the presence of analogues.[19] However, several other residues distributed seemingly randomly over the primary structure of firefly luciferases are known to severely affect bioluminescence colors, many of them resulting in red mutants.[26–31]

Despite many studies about the structure and function in firefly luciferases, the structural origin of the curious pH-sensitivity remains unknown. Furthermore, the possible biological and evolutionary significance of pH-sensitivity has been neglected. In this manuscript, we review the relationship between luciferases structure, pH-sensitivity and bioluminescence color based on recent results obtained by comparative studies of pH-sensitive and pH-insensitive luciferases, and discuss the possible evolutionary origin, biological functions and applications of this unique property.

Nature of the emitters and mechanisms of bioluminescence color

Three basic mechanisms have been proposed to determine bioluminescence colors in the luciferase active site (I) non-specific solvent effects;[31] (II) specific effects of interacting residues[32] and (III) the conformation of the active site affecting the rotation of oxyluciferin thiazinic rings.[33]

The solvent effect of the active site was suggested to account for short-range shifts in the bioluminescence spectrum of beetle luciferases.[34] A later development of this hypothesis, the orientation polarizability hypothesis, predicts that the ability of solvent molecules to relax around the excited emitter may affect bioluminescence color.[31]

Among the specific-effects, the tautomerization of excited oxyluciferin, assisted by basic residues in the active site, was originally proposed to explain green (enol and enolate forms) and red (keto form) bioluminescence in firefly luciferases.[35] However, recent studies with dimethyloxyluciferin, which can not undergo tautomerization, indicate that the tautomerization hypothesis is no longer an exclusive mechanism for green and red bioluminescence color determination.[36]

Recent theoretical and experimental studies suggested that the polarization of phenolate and enolate groups, under influence of interacting residues in the luciferase active site,[37] and the consequent charge delocalization between the thiazinic rings,[38] can generate different emitters. Ugarova and coworkers[30] found evidence that there could be three emitting species in firefly bioluminescence spectrum, instead of two: (I) the keto red emitter; (II) the enol orange emitter and (III) the enolate green light emitter (Fig. 2). According to studies of luciferyl–adenylate chemiluminescence in aqueous environment, in the presence of BSA and detergents, red chemiluminescence is usually associated with polar and less organized environments whereas green chemiluminescence requires more hydrophobic and structured environments[39] (Fig. 3).

Fig. 2 Firefly bioluminescence emitters, according to Ugarova *et al.*[30] Reproduced with permission from: Bioluminescence spectra of native and mutant firefly luciferase as a function of pH, N. N. Ugarova, L. G. Maloshenok, I. V. Uporov and M. I. Koksharov, Bioluminescence spectra of native and mutant firefly luciferase as a function of pH, *Biochemistry (Moscow)*, **70** © Pleiades Publishing, Inc.

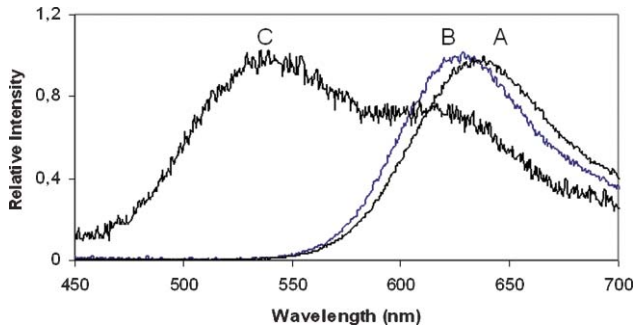

Fig. 3 Chemiluminescence spectra of luciferyl–adenylate in water:[39] (A) in 0.1 M Tris-HCl buffer pH 8.5; (B) in DMSO in the presence of 10% triethanolamine and (C) in DMSO in the presence of 10% potassium *tert*-butoxide.

The bioluminescence spectrum of fireflies determined by a ratio between green and red emitters

The bioluminescence spectra of all firefly luciferases undergo the typical red shift at lower pHs (Fig. 1), higher temperatures, and in the presence of divalent heavy metal cations, high concentrations of phosphate and denaturants.[5] However, the magnitude of the red-shift varies from luciferase to luciferase[40] (Fig. 1). In the more green-shifted luciferases, the emission spectrum still contains considerable amount of green light at pH 6. On the other hand, in the more red-shifted ones, even at alkaline pH there is still considerable amount of red light. Therefore, it is likely that the bioluminescence color in fireflies is determined by the ratio between green, red and, possibly, by orange light emitters (Fig. 2) as proposed by Ugarova *et al.*[40] This ratio could be determined by basic residues interacting with excited oxyluciferin, as well as with residues involved in keeping an appropriate active site structure, affecting the degree of exposure of the active site to the solvent. Since the inflection point of pH-sensitivity is close to pH 7, and divalent heavy metal cations such as copper and zinc have high affinity for histidine and cysteine, it is likely that residues such as histidine and cysteine could be involved in the promotion of pH-sensitivity. Differently from pH-sensitive luciferases, the pH-insensitive luciferases produce a single bioluminescence color, suggestive of a single emitter.

pH-sensitivity associated with conformational changes

Besides affecting the bioluminescence spectra, pH also affects the kinetics and substrate affinities of firefly luciferases.[40,41] Using firefly luciferases that produce different colors, we have found that the more blue-shifted firefly luciferases display lower K_M values for luciferin than the red-shifted ones (Table 1), indicating a higher affinity for luciferin. However, there is an increase in the K_M value for luciferin upon increases of pH[41] (Table 1) suggesting that at acidic pH the protonation of some luciferase active site group may attenuate some negative charge repelling luciferin or generate a new positive charge increasing the affinity for luciferin. Furthermore, the desprotonation of the luciferin phenol/enol groups at higher pH, generating negative charges, may decrease luciferin affinity if the luciferin binding site includes a negative charge.[42]

At alkaline pH firefly luciferases display a typical flash followed by a rapid decay with high decay rates (k_d) (Table 1). However, at pH 6 and in phosphate buffer, the kinetics becomes much slower with slow decay rates (Table 1). Phosphate ions are strong reversible inhibitors of firefly luciferases.[43] CoA, which is known to stimulate bioluminescence activity of firefly luciferases and to change the kinetics from a flash-like to a glow-type at pH 8, has little effect at pH 6. The slow-type kinetics could be related to the reversal of the inhibition caused by dehydroluciferin and L-luciferin adenylates by CoA,[44] allowing to sustain the luminescence reaction for longer times. Furthermore, it is known that CoA promotes conformational changes of the C-terminal domain in CoA-ligases.[45,46] Circular dichroism studies showed that at acidic pH, firefly luciferase undergoes conformational changes indicative of a lack of α-helix structure (results not shown). Altogether, these findings indicate that firefly luciferases undergo considerable conformational changes at different pHs, and that the ability to produce red bioluminescence could be related with the same conformational changes responsible for the change of kinetics. However, the residues involved in such conformational changes remain unknown.

Structural basis for pH-sensitivity

Originally pH-sensitivity was associated to the presence of basic residues assisting oxyluciferin tautomerization in the active site.[32]

Table 1 Effect of pH on the emission spectra, K_M and decay rates (k_d) of bioluminescence of three firefly luciferases

		λ_{max}/nm		K_M LH_2/μM		K_M ATP/μM		$k_d/10^{-4}$ s^{-1} [b]	
Luciferase	pH_{opt}	pH 8	pH 6	pH 8 [T][a]	pH 6	pH 8 [T][a]	pH 6	pH 8 [T][a]	pH 6
Cratomorphus	8.0	548	610	10 [10]	4	110	22	7 [290]	9
P. pyralis	7.9	555	608	12 [19]	5	12 [19]	5		
Macrolampis	8.2	569	606	19 [34]	30	83	38	6 [670]	2

[a] pH measurements were performed in 0.1 M sodium phosphate buffer at pH 8 and 6, or [T] 0.1 M Tris buffer pH 8. [b] k_d = Decay rate = $\ln[I_0/I_1]/\Delta t$ where I_0 = intensity at the peak of bioluminescence, I_1 = intensity after Δt.

After cloning and comparing the sequences of several pH-insensitive luciferases, we suggested that pH-sensitivity could be associated to interacting residues involved in keeping a closed active site conformation for green light emission.[15,47] We then suggested that pH-sensitivity could be related to higher active site flexibility, allowing the production of two emitters depending on the conformation, whereas pH-insensitivity could be related to higher rigidity, allowing the production of a single emitter.[47] However, the structural origin of such differences of flexibility was until recently unknown.

pH-sensitivity could be related to one or more of the following factors: (I) higher core hydrophilicity; (II) higher content of histidines and other basic residues near pH 7; (III) higher content of flexibility conferring residues, mainly glycine; (IV) increased length of surface loops. On the other hand, similar to the thermophylic enzymes, pH-insensitivity could result from one or more of the following factors:[48] (I) higher core hydrophobicity; (II) additional networks of hydrogen bonds; (III) enhanced secondary structure propensity; (IV) ionic interactions; (V) increased packing density and (VI) decreased length of surface loops.

There is no evident difference in the histidine content between pH-sensitive and pH-insensitive luciferases (Table 2). However, there is an evident difference in the content of glycine, which is higher in the pH-sensitive luciferases relative to the insensitive ones (Table 2). Furthermore, a slightly higher hydrophobic character was found in the region between residues 220–344 of pH-insensitive luciferases, which consists of most of the subdomain B and contains several active site residues. This higher content of hydrophobic residues in pH-insensitive luciferases might help to stabilize and to make the active site structure of these luciferases more rigid than in the pH-sensitive luciferases. On the other hand, in firefly luciferases there must be a set of residues which upon influence of pH or temperature changes could trigger conformational changes in these flexible structures, giving rise to pH-sensitivity. Lower content of glycine and higher content of hydrophobic residues could in part be responsible for higher rigidity and pH-insensitivity. Site-directed mutagenesis studies are attempting to identify the structural basis for pH-sensitivity.

Substitutions affecting bioluminescence colors and pH-sensitivity

pH-sensitive (firefly luciferases)

In firefly luciferases several substitutions result in red mutants, which are pH-insensitive (Table 3). Furthermore, in a few cases single point mutations also resulted in green-shifted pH-insensitive luciferases. Kajiyama and Nakano showed that the single mutant V239I in *Luciola cruciata* produced a green pH-insensitive mutant.[26] The natural substitution of the conserved E354 by asparagine in *Macrolampis* luciferase was shown to be responsible for the shoulder in the red region.[40] The double mutant E354R/V368A in *Hotaria parvula* luciferase resulted in pH-insensitive luciferase.[29] Shapiro *et al.* showed that the double mutant S293P/L287I in *Photinus pyralis* luciferase results in an almost pH-insensitive luciferase.[49] Similarly, Branchini *et al.* showed that the multiple *P. pyralis* luciferase mutant T214A/A215L/I232A/F295L/E295K results in a pH-insensitive luciferase.[50] It is remarkable that in several cases the mutants involve the substitution of polar residues by more hydrophobic ones. Besides becoming pH-insensitive, these luciferases also became thermostable, suggesting that red light emission is associated with thermal instability.[29] This indeed seems to be the case since higher temperatures are known to shift the spectrum to the red in firefly luciferases.

pH-insensitive luciferases (click beetle and railroadworm luciferases)

In contrast, much fewer substitutions were found to affect the bioluminescence spectra of the pH-insensitive luciferases.[51]

Table 2 Amino-acid content in pH-sensitive and pH-insensitive luciferases

Luciferase	λ_{max}/nm	His	Cys	Basic	Acid	Polar	Hydrophobic	Gly
pH-sensitive								
Photuris pennsilvanica	538	13	7	62	58	29	50	43
Cratomorphus distinctus	548	10	8	59	66	29	53	49
Pyrocoelia miyako	550	17	9	57	65	29	52	46
Lampyris noctiluca	550	14	9	57	64			49
Photinus pyralis	562	14	4	60	64	28	54	45
Macrolampis sp	569	15	9	56	64	28	54	47
Luciola lateralis		8	7	66	66	29	51	53
Luciola cruciata		8	8	62	63			52
Hotaria parvula	568	13	8	60	62	28	52	50
Luciola mingrelica	570	15	8	61	67	29	52	49
pH-insensitive								
Railroadworms								
Ragophtalmus ohbai	550	10	10	66	63	29	51	39
Phrixotrix vivianii	546	13	7	53	57	26	53	43
Phrixotrix hirtus	623	15	9	59	61	29	51	40
Click beetles								
Pyrearinus termitilluminans	534	14	10	59	60	28	50	40
Pyrophorus plagiophtalmus								
• Green isozyme	546	13	13	63	65	29	51	38
• Yellow-green isozyme	560	13	13	62	64	29	51	38
• Yellow isozyme	575	13	13	61	65	29	51	38
• Orange isozyme	593	13	13	62	64	29	51	39

Table 3 Mutants affecting bioluminescence colors and pH-sensitivity in beetle luciferases

λ_{max} pH [half-bandwith][a] / nm												
pH-sensitive						pH-insensitive						
Cratomorphus			*Macrolampis*			*Pyrearinus*			PxGR		PxRE	
	pH 8	pH 6		pH 8	pH 6		pH 8	pH 6		pH 8	pH 6		pH 8	pH 6
WT	548 [71]	610 [95]	**WT**	569 [99]	606 [77]	**WT**	534 [65]	534[78]	**WT**	546 [71]	546 [75]	**WT**	623 [55]	623 [62]
F227Y	550		**Y227A**	603 [89]	—	**V227A**	541 [67]	—	**Y227A**	575 [97]	—	**Y227A**	620 [55]	—
			Y227F	618/569	—	**V227F**	590 [87]	560	**Y227F**	548 [72]	—			
G228A		—	**G228A**	587	—	**G228de**	587	—	**G228A**	560 [77]	—			
N229T		—				**T229N**	546 [73]/541		**T229N**	574 [86]	574 [69]	**T229N**	611 [57]	605 [63]
						T229de	587	—						
V239I														
G246A	—					**A246G**	546 [77]		**A246G**	553 [76]	553	**A246G**	621 [66]	614 [59]
F260L	548 [72]	552 [109]				**L260F**	555							
S286N	—													
H310A			**H310A**	564 [99]	607 [77]									
			H310R	573 [105]	607									
E311A	—		**E311A**	600	611									
G320A	—		—			**A320G**	536							
E354N	556 [86]	603 [62.5]	**N354A**	558 [83]	606 [77]	—								

[a] Standard deviation: ±2.5 nm.

Among the conserved active site residues, only the substitution of R215 resulted in a dramatic red shift in the green-yellow emitting railroadworm luciferases, but not in the red emitting luciferases.[51–54] Other active site residues whose substitutions dramatically affected the bioluminescence spectra of pH-sensitive luciferases had no effect on the pH-insensitive ones.[51] Furthermore, until very recently no single mutant was found to display the large 40–50 nm red-shifts observed in firefly luciferases, nor resulted in pH-sensitive enzymes.[51]

Conserved residues differing between pH-sensitive and pH-insensitive luciferases

Comparison of the amino-acid sequences of pH-sensitive and pH-insensitive luciferases revealed a set of conserved residues differing between these groups of luciferases which could be involved in pH-sensitivity determination[15] (Fig. 4). Indeed, site directed-mutagenesis showed that some of these substitutions considerably affected the bioluminescence colors (Table 3). The major effects were observed for the substitution N229T, G246A, and F257L in the pH-sensitive luciferases, and the corresponding T226A, A243G and L257F in the pH-insensitive luciferases[47,52,55] (Table 3). Notably, these substitutions involve changes of polar residues in the pH-sensitive luciferases by more hydrophobic and larger residues, which may affect flexibility and packing in the pH-insensitive ones. The substitutions at position 229 displayed the largest effects in both groups of luciferases, resulting in dramatic red-shifts, with the exception of the red-emitting PxRE luciferases which had a modest 10 nm blue-shif.[47] The substitution at position 246 resulted in slightly red-shifted spectra in both groups of luciferases, and a slight decrease of pH-sensitivity in firefly luciferases.[52] The effect of these substitutions in pH-insensitive luciferases was further investigated by double mutants.[52] Although the double mutants showed their spectra further shifted to the red in relation to the single mutants, the magnitude of the shifts was lower than that expected for an additive effect, indicating some degree of cooperation between these substitutions. Furthermore, until very recently no single or double substitution conferred pH-sensitivity in the pH-insensitive luciferases. More recently we also investigated the effect of other substitutions F257L, which decreased the pH-sensitivity in *Cratomorphus* firefly luciferase.[55] According to the closed conformation of *Luciola cruciata* luciferase complexed with the luciferyl–adenylate analogue, DLSA, the corresponding residue Y260 makes a labile hydrogen bond with S286, which disappears in the open conformation.[19]

The influence of the loop 223–235

Noteworthy, the residues that had the most dramatic effects on the bioluminescence spectra of both pH-sensitive and pH-insensitive luciferases (Table 3) were found to be clustered in the loop between residues 223–235[55] (Fig. 5 and 6), indicating that this loop plays a major role in bioluminescence color and pH-sensitivity determination.[55] Although this loop is not directly involved in the active site, it may indirectly affect the structure of the active site. The residues 227YGN229 (227F/V/YGR229) affect dramatically the bioluminescence spectra of both pH-sensitive and pH-insensitive luciferases. Although the substitution V227A was originally suggested to be one of the key substitutions responsible

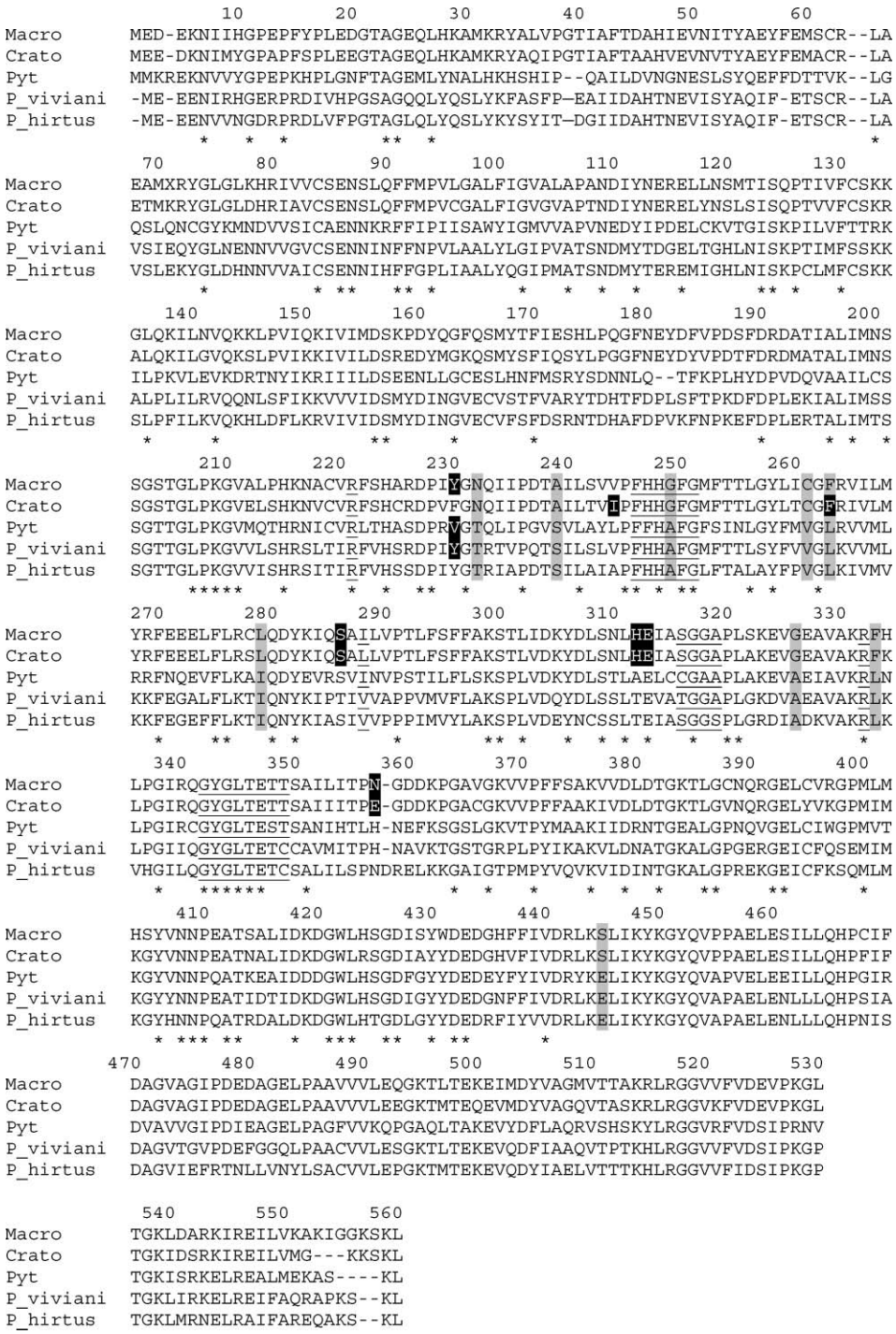

Fig. 4 Multialignment of pH-sensitive and pH-insensitive luciferases: (gray shadow) conserved residues differing between pH-sensitive and pH-insensitive luciferases; (Crato) *Cratomorphus distinctus*; (Macro) *Macrolampis* sp_2; (Pyt) *Pyrearinus termitilluminas*; (P.viviani) *Phrixotrix vivianii* green emitting luciferase; (P.hirtus) *Phrixotrix hirtus* red emitting luciferase; (black shadow) residues whose substitution affects pH-sensitivity; (underlined) luciferin-binding site residues; (*) invariant residues.

for red shifts in the Jamaican click beetle isozymes, the substitution V224A in *Pyrearinus* click beetle luciferase had very little effect on the emission spectrum. On the other hand, other substitutions at the variable 227 position were found to cause dramatic red- and time-dependent shifts in beetle luciferases. Especially, the substitution V227F in *Pyrearinus* click beetle luciferase was the first mutant found to confer pH-sensitivity to a pH-insensitive luciferase.[55] Modeling studies suggest that the residues Y227 and N229 may function as anchorage points for this flexible loop to the protein core (Fig. 5). The large and hydrophobic residues such as phenylalanine, tyrosine and valine at position 227 appear to stick into a hydrophobic pocket of the luciferase. The residue N229 in

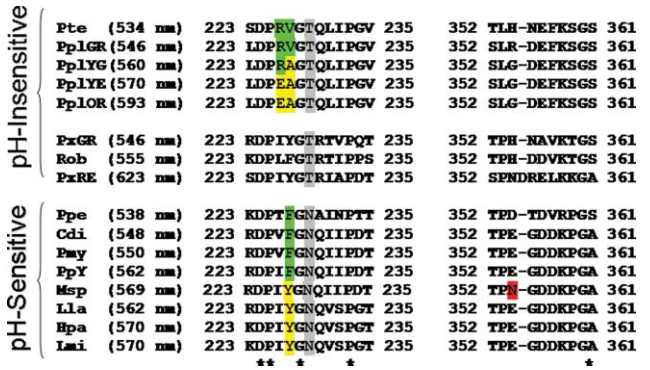

Fig. 5 Multialignment of the loop 223–235 in beetle luciferases according to Viviani *et al.*:[55] (Cdi) *Cratomorphus distinctus*; (Hpa) *Hotaria parvula*; (Lla) *Luciola lateralis*; (Lmi) *Luciola mingrelica*; (Msp) *Macrolampis* sp_2; (Pmi) *Pyrocoelia miyako*; (Ppe) *Photuris pennsylvanica*; (Ppy) *Photinus pyralis*; (Ppl) *Pyrophorus plagiophtalamus* (GR) green emitting isozyme; (YG) yellow-green emitting isozyme; (YE) yellow emitting isozyme; (OR) orange emitting isozyme; (Pte) *Pyrearinus termitilluminas*; (PxGR) *Phrixotrix vivianii* green emitting luciferase; (PxRE) *Phrixotrix hirtus* red emitting luciferase; (Rob) *Ragophtalmus ohbai* luciferase. Reproduced from: V. R. Viviani, A. J. Silva Neto, F. G. C. Arnoldi, J. A R. G. Barbosa and Y. Ohmiya, The influence of the loop between residues 223–235 in beetle luciferases bioluminescence spectra: a solvent gate for the active site of pH-sensitive luciferases, *Photochem. Photobiol.*, 2007, © American Society for Photobiology and Blackwell Publishing.

firefly luciferases participates in a developed network of hydrogen bonds with S286 and E311[40] (Fig. 6), whereas the corresponding residue T229 in pH-insensitive luciferases is less polar and displays a less developed network of hydrogen bonds with its surroundings. The invariant residue G228 is also involved in the network of hydrogen bonds and may affect the flexibility of the loop.

Not surprisingly the substitution of all these interacting residues was found to cause dramatic red shifts on the bioluminescence spectra of firefly luciferases.[40,50] According to modeling studies, the invariant residues E311 and R337 coordinate the interaction of the loops between residues 223–235, 352–361 and the residue S286 (Fig. 7), all of them are important structural elements for bioluminescence colors.[37,47] The salt bridge formed between the conserved E354 and H310 at acidic pH,[40] could influence the position of the adjacent and partially buried residue E311, exposing it and disrupting the associated network of interactions with N229, S284 and R337. In pH-insensitive luciferases, however, the corresponding network of hydrogen bonds is less developed, and several residues within this region, including T229 and T284 are slightly more hydrophobic than the corresponding residues in the pH-sensitive luciferases, suggesting that this region could be more rigid due to better hydrophobic packing. This network of residues participate in a wall that shields the benzothiazolyl side of the luciferin-binding cavity.

A proposed mechanism for pH-sensitivity

Ultimately, the conformation of the luciferase active site determines bioluminescence spectra, by affecting the microenvironment polarity, specific interactions and even the rotation of oxyluciferin thiazolic rings (Fig. 8). The recently solved three-dimensional structure of *Luciola cruciata* luciferase and its mutants in the presence of the DLSA analogue, or oxyluciferin and AMP,

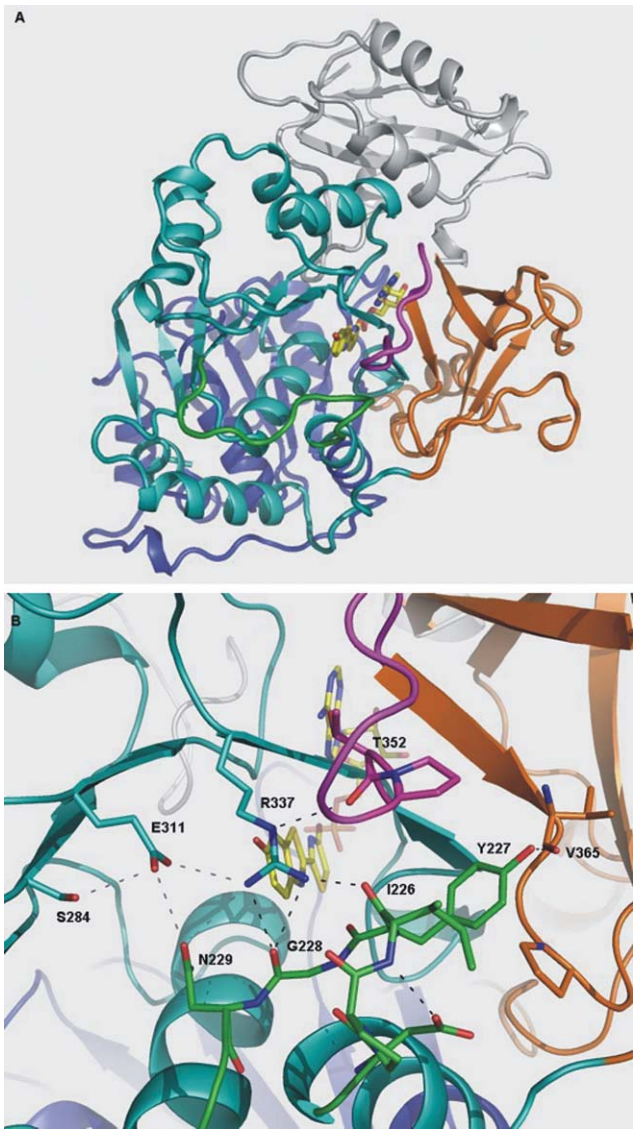

Fig. 6 Three-dimensional model of *Macrolampis* firefly luciferase based on *Luciola cruciata* three-dimensional structure[19] (A) showing the loop between residues 223–235 in green; (B) zoom of the loop 223–235.

showed that luciferase may assume two conformations: an open conformation and a closed one.[19] Not surprisingly, some interactions responsible for keeping the closed conformation in *Luciola cruciata* luciferase model have been previously identified as important determinants of bioluminescence color in other beetle luciferases.[40,52] The authors suggested that hydrophobic and rigid environment in the closed conformation could be responsible for yellow-green bioluminescence.[47]

According to the proposed mechanisms of bioluminescence color, access of the solvent molecules to the active site is one of the main determining factors. It may affect specific interactions of active site residues and the protonation state of excited oxyluciferin (Fig. 8). The main access is provided by the entrance of the luciferin binding site from the N-terminal side facing the C-terminal domain (Fig. 8). Zako *et al.*[56] clearly showed that the removal of the C-terminal domain in firefly luciferase results in a weak red emitting luciferase. Ugarova and coworkers also showed

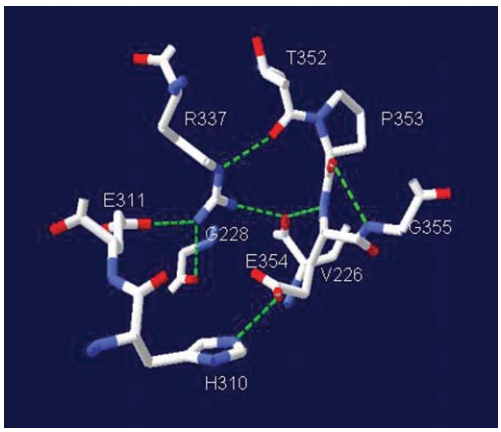

Fig. 7 Network of residues involved in the putative pH-sensor of *Cratomorphus distinctus* firefly luciferase according to Viviani *et al.*[40] Reproduced from: V. R. Viviani, T. L. Ohelmeyer, F. G. C. Arnoldi and M. R. Brochetto-Braga, A new firefly luciferase with bimodal spectrum: identification of structural determinants of spectral sensitivity in firefly luciferases, *Photochem. Photobiol.*, 2005, **81**, 843–848 © American Society for Photobiology and Blackwell Publishing.

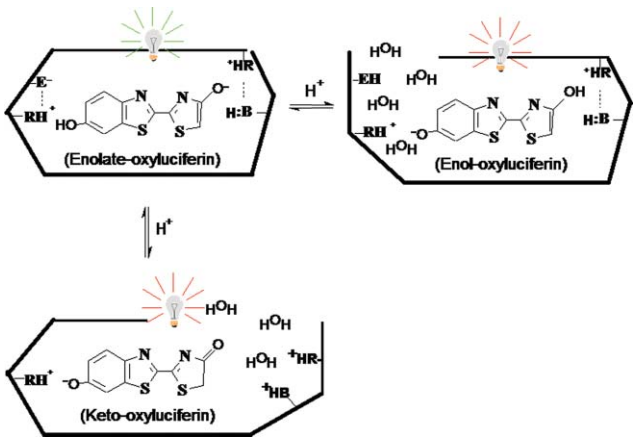

Fig. 8 Putative active site mechanisms of pH-sensitivity.

that the substitution H431Y in *Luciola mingrelica* luciferase results in a red light emitting mutant by affecting the flexibility of the hinge connecting the C-terminal and N-terminal domains.[31]

However, we proposed an alternative model in which the network involving the residues E311, R337 and the loops between residues 223–235 and 351–360 may constitute another gate for water in the pH-sensitive luciferases (Fig. 8), which could be opened in the lateral surface through a pH-dependent mechanism, exposing the benzothiazolyl side of the luciferin binding site[55] (Fig. 8). Protonation of basic residues such as H310 at the surface of the protein, could affect the ionic interactions and the hydrogen bonds of adjacent residues E311 and R337, exposing them and beginning a disrupting process of the remaining hydrogen bonds (G228, N229, S286) in a zipper-like manner, culminating with the opening of a channel to the bottom of the luciferin binding site. The consequent polarization of oxyluciferin phenolate, generating red bioluminescence, is consistent with the recently proposed mechanisms of bioluminescence color determination.[5] In contrast, in the pH-insensitive luciferases, the bottom of the luciferin binding site could be more rigid and better packed due to steric and hydrophobic interactions adding over the hydrogen bond network. Alternatively, the compactness of the active site, which is determined by the flexibility of its segments, could also influence its ability to exclude water from the active site. In any case, the identities of the specific residues directly involved with pH-sensitivity determination remain a fertile area of investigation of beetle luciferases structure and function.

The evolutionary origin of pH-sensitivity

pH-sensitivity is found only in fireflies luciferases. Although fireflies (Lampyridae) and railroadworms (Phengodidae/Ragophtalmidae) were originally clustered within the superfamily Cantharoidea, distant from click beetles (Elateridae), it is remarkable that railroadworms and click beetle luciferases are functionally more similar by being pH-insensitive. More recently, molecular data based on the mitochondrial genome of the bioluminescent click beetle *Pyrophous divergens*, starworms and fireflies suggested that railroadworms and starworms could be closer to click beetles than previously suspected.[57] These results suggest that bioluminescence in Elateroidea may have arisen three times independently. Although green bioluminescence is predominant among present day beetles, suggesting this color evolved earlier, this trend could be the mere result of selective pressure for green color, considering that most organisms are visually sensitive to green and insensitive to red.

Ultimately, bioluminescence color is dictated by the chemistry of the reaction. In this regard, red chemiluminescence is easier to obtain under mild non-enzymatic circumstances. Studies of luciferyl–adenylate chemiluminescence in aqueous medium, indeed suggest that red chemiluminescence could proceed under more primitive conditions, since it requires less organized environments to occur.[39] Efficient luciferyl–adenylate green chemiluminescence appears only in aprotic solvents in the presence of strong bases, indicating the need for a more structured environment for green bioluminescence. Furthermore, under such circumstances, green light emission appears mixed with red light, resulting in bimodal spectra, which are qualitatively similar to the bimodal spectra of pH-sensitive luciferases (Fig. 3).

Therefore, instead of being just a side-effect of denaturation, the red component of bioluminescence spectra in the pH-sensitive luciferases may have a biological meaning. It could be a vestigial condition carried from the early stages of bioluminescence evolution in firefly luciferases. It is possible that during the very first stages of evolution, bioluminescence in the firefly luciferase ancestrals was in the red region, and later developed to green bioluminescence, through bimodal intermediary stages, as the active site evolved to be more structured for bioluminescence. This is supported by the observation that luciferase-like enzymes in non-bioluminescent beetles and impaired C-terminal deletion mutant luciferases, also produce a weak red chemiluminescence in presence of D-luciferin and ATP.[58] Alternatively, red bioluminescence in pH-sensitive luciferases could be the result of the gradual flexibilization of the luciferase structure during the evolution, but the selective pressure underlying such flexibilization is unclear. This rises the possibility that pH-sensitivity may have a biological function for fireflies. The Brazilian firefly *Macrolampis* sp_2 displays an unusual color variation among individuals in the field, and a time dependent-red

shift in the same individual in the laboratory. We cloned their luciferase and found that it displays a bimodal spectrum, which is very sensitive to temperature and pH variation.[40] We suggested that in this case, color modulation could be achieved by a pH/thermal-sensitive mechanism.

Potential biotechnological uses of pH-sensitivity

For bioanalytical applications, pH-sensitivity has been considered an undesirable side-effect of firefly bioluminescence, because it reduces the efficiency of the signal detection in the blue region, where most photodetectors are usually more sensitive. The exception is the desirable use of red-shifted bioluminescence in mammalian cells.[59] As we have seen, the bioluminescence spectrum of firefly luciferases expressed in cells is very sensitive to intracellular pH changes. Intracellular changes of pH, divalent heavy metal cations and phosphate ions are important indicators of major physiological and pathological processes. In many cells, intracellular acidification anticipates apoptosis.[60] When fireflies are dying often they emit an orange-reddish bioluminescence. Similarly, when bacteria are submitted to stress, such as when supplied with luciferin in acidic buffer, or when they are exposed to higher temperatures (Fig. 9), they emit reddish color. Therefore, it would be in principle possible to use the spectral sensitivity of firefly luciferases as a bioindicator of cellular stress, probing intracellular changes of pH, and other physical-chemical conditions. Another possibility would be to use firefly luciferases spectral changes to probe for the presence of toxic divalent heavy metal cations such as copper and mercury. By using the ratio between green and red emissions, it would be in principle possible to quantify such changes. This would offer an additional advantage for firefly luciferase as a single dual reporter gene, since it would be possible to use it for simultaneously measuring intensity (gene expression) and spectral (pH, heavy metal cations) parameters.

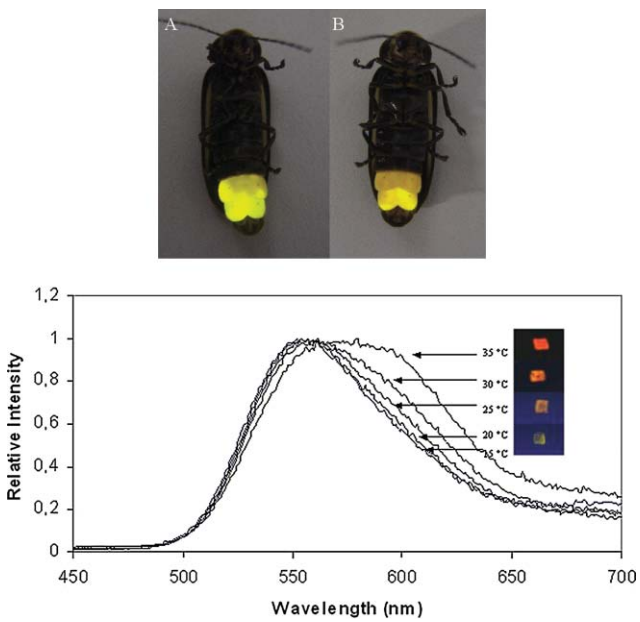

Fig. 9 (Upper panel) Time-dependent shift of the bioluminescence spectrum of *Macrolampis* sp_2 firefly (A) at the beginning of glow; (B) after 10 min glowing; (Lower panel) Temperature effect on the bioluminescence color of bacterial colonies expressing *Macrolampis* luciferase.

Abbreviations

(Cdi) *Cratomorphus distinctus*; (Hpa) *Hotaria parvula*; (Lla) *Luciola lateralis*; (Lmi) *Luciola mingrelica*; (Msp) *Macrolampis* sp_2; (Pmi) *Pyrocoelia miyako*; (Ppe) *Photuris pennsylvanica*; (Ppy) *Photinus pyralis*; (Ppl) *Pyrophorus plagiophtalamus* (GR) green emitting isozyme; (YG) yellow-green emitting isozyme; (YE) yellow emitting isozyme; (OR) orange emitting isozyme; (Pte) *Pyrearinus termitilluminas*; (PxGR) *Phrixotrix vivianii* green emitting luciferase; (PxRE) *Phrixotrix hirtus red emitting luciferase*; (Rob) *Ragophtalmus ohbai luciferase*; DLSA (5′-*O*-[*N*-(dehydroluciferyl)-sulfamoyl]adenosine).

Acknowledgements

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