# Effect of the substitutions G216N/A217L and S398M on thermal stability, activity and bioluminescence color of *L. mingrelica* firefly luciferase

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## 1. Introduction

Insufficient thermostability of wild-type (WT) firefly luciferases often limits their application. The substitution A217L greatly increases thermal stability of many firefly luciferases, for example, *Luciola lateralis*, *Luciola cruciata* and *Photinus pyralis* luciferases [1]. However, for *Hotaria parvula* firefly luciferase (98% sequence identity with *Luciola mingrelica* (Lml) luciferase) the A217L mutation dramatically decreased catalytic activity more than 1000-fold [1]. We have analyzed the environment of A217 in the 3D-structure of Lml and compared it with that in other enzymes in order to propose possible additional mutations that would retain the high thermal stability of the mutant A217L while preserving the high level of activity.

The 7Å environment of A217 is identical in *L. mingrelica* and *H. parvula* luciferases, thus it was safe to assume that in both these highly homologous enzymes the single substitution A217L would lead to the loss of activity.

The neighboring residue G216 and the more remote S398 appeared to be the key positions that distinguish the environment of A217 in a small subgroup of luciferases including *L. mingrelica* and *H. parvula* from that of *L. cruciata*, *L. lateralis*, *P. pyralis* and most others. In other beetle luciferases the position 216 is occupied with a residue having a side group (in contrast to G216 in Lml) and the position 398 is generally occupied with methionine (Table I).

**Table I**. Sequence alignment (regions 214-223 and 393-403) of the 21 species of beetle luciferases

| Luciferase | Genbank number | 212 216 223 393 398 403 | % identity |
|---|---|---|---|
| **Luciola mingrelica** | **S61961** | ITHEG**A**VTRFSH...CVKGP**S**LMLGY... | 100 |
| Hotaria parvula | L39929 | ITHEG**A**VTRFSH...CVKGP**S**LMLGY... | 98.0 |
| Luciola italica | DQ138966 | ITHEG**T**VTRFSH...CVKGP**S**LMLGY... | 95.3 |
| Lampyroidea maculata | DQ137139 | ITHE**AT**VTRFSH...CVKGP**S**LMKGY... | 93.4 |
| Luciola cruciata | P13129 | LTHE**NT**VTRFSH...CVKGP**M**LMKGY... | 80.6 |
| Luciola lateralis | X66919 | LTHE**NA**VTRFSH...CVKGP**M**LMKGY... | 81.7 |
| Photinus pyralis | P08659 | LPHR**TA**CVRFSH...CVRGP**M**IMSGY... | 66.7 |
| Lampyris turkestanicus | AY742225 | LTHK**NI**CVRFSH...CVKGP**M**IMKGY... | 66.7 |
| Lampyris noctiluca | X89479 | LTHQ**NV**CVRFSH...CVKGP**M**IMKGY... | 65.8 |
| Cratomorphus distinctus | AY633557 | LSHK**NV**CVRFSH...YVKGP**M**IMKGY... | 65.4 |
| Pyrocoelia pectoralis | Q7M4K3 | LTHM**NV**CVRFSH...CVKGP**M**IMKGY... | 65.6 |
| Pyrocoelia miyako | L39928 | LTHM**NV**CVRFSH...CVKGP**M**IMKGY... | 64.5 |
| Photuris pennsylvanica | D25415 | ISHR**NT**TYRFSH...CFTGP**M**IMKGY... | 60.2 |
| Photuris pennsylvanica | U31240 | LTHK**NI**VARFSH...YFKGD**M**IMKSY... | 53.9 |
| Phrixothrix hirtus | AF139645 | ISHR**SI**TIRFVH...CFKSQ**M**LMKGY... | 47.1 |
| Phrixothrix vivianii | AF139644 | LSHR**SL**TIRFVH...CFQSE**M**IMKGY... | 52.5 |
| Rhagophthalmus ohbai | AB255748 | LTHR**NL**SVRFVH...CFKSE**I**IMKGY... | 49.3 |
| Pyrearinus termitilluminans | AF116843 | QTHR**NI**CVRLTH...CIWGP**M**VTKGY... | 48.6 |
| Pyrophorus mellifluos vGR | AF545853 | QTHQ**NI**CVRLIH...CVGGP**M**VSKGY... | 48.7 |
| Pyrophorus plagiophthalamus dGR | Q7M4K2 | QTHR**NV**CVRLIH...CIKGP**M**VSKGY... | 48.2 |
| Pyrophorus plagiophthalamus vYE | S29352 | QTHQ**NI**CVRLIH...CIKGP**M**VSKGY... | 47.7 |

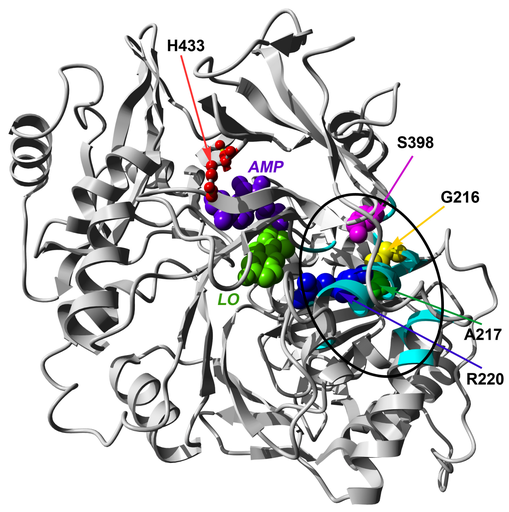


**Figure 1.** The structure of WT firefly luciferase showing the location of mutations. 7 Å microenvironment of A217 is indicated by ellipse.

We decided to eliminate these differences to make the A217 environment in in *L. mingrelica* luciferase similar to that of *Luciola cruciata* luciferase and thus possibly prevent the loss of activity in the case of the substitution A217L [2].

## 2. Results

- the mutant G216N/A217L increased thermal stability 18-fold (42°C) and 28-fold (45°C) compared with WT Lml
- however, it decreased activity to ~10% of WT
- the bioluminescence emission maximum showed a red shift from 566 to 611 nm (similar to the mutant H433Y [3] located 23 Å away from the position 217)
- the addition of the third substitution S398M effectively restored the catalytic and spectral properties of the mutant to the values similar to WT while retaining high thermal stability

A more detailed information about the characterized mutants is given in Table II.

Figure 2 shows the bioluminescence spectra of the mutants as well as the sensitivity of their spectra to pH and temperature. Figure 3 depicts *in vivo* bioluminescence in *E. coli*.

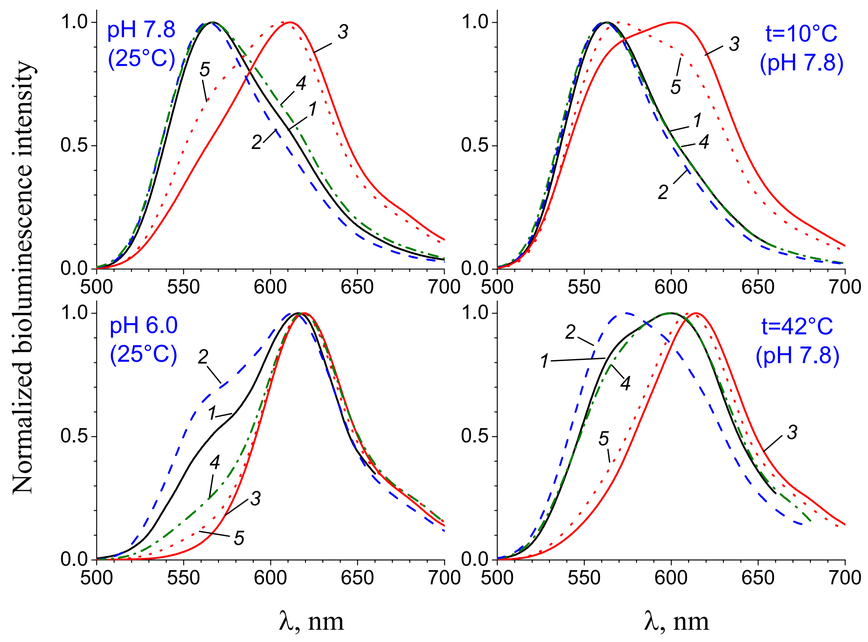


**Figure 2.** Bioluminescence spectra of WT (*1*) luciferase and the mutants S398M (*2*), G216N/A217L (*3*), G216N/A217L/S398M (*4*) and H433Y (*5*) at pH 7.8 and pH 6.0 (25°C) or at 10°C and 42°C (pH 7.8).

**Table II**. Catalytic and bioluminescent properties of mutant luciferases

| Enzyme | Relative specific activity, % | $K_m$, μM | | $\lambda_{max}$ (***half-width***), nm | | Half-life, min (42°C) |
|---|---|---|---|---|---|---|
| | | $LH_2$ | ATP | pH 7.8 | pH 6.0 | |
| WT | 100 ± 10 | 74 ± 8 | 170 ± 20 | 566 (***78***) | 616 (***79***) | 8.4 ± 0.8 |
| G216N/A217L | 10 ± 1 | 105 ± 10 | 1100 ± 100 | 611 (***82***), 570$^{sh}$ | 619 (***58***) | 165 ± 17 |
| S398M | 106 ± 10 | 47 ± 3 | 108 ± 15 | 564 (***72***) | 613 (***96***) | 8.0 ± 0.8 |
| G216N/A217L /S398M | 60 ± 6 | 47 ± 3 | 80 ± 8 | 566 (***84***) | 618 (***63***) | 160 ± 17 |
| H433Y | 100 ± 10 | 83±8 | 280±30 | 607 (***90***), 570$^{sh}$ | 619 (*57*) | 7.2 ± 0.6 |

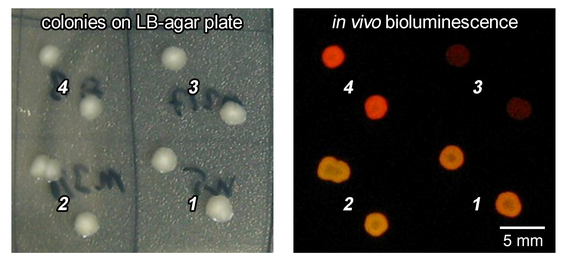


**Figure 3.** *In vivo* bioluminescence of *E. coli* colonies producing WT (*1*) luciferase and the mutants S398M (*2*), G216N/A217L (*3*), G216N/A217L/S398M (*4*).

## 3. Conclusions

The substitution of both G216 and S398 to the more conservative N216 and M398 is required for the high activity and green emission of the mutant A217L in *L. mingrelica* luciferase. It is possible that the presence of the non-conservative G216 (destabilizing Gly in α-helix) and S398 (creates an internal cavity compared with M398) lowers the local conformational stability near the residue 217 (Fig. 4). As a result of the more flexible microenvironment, the substitution A217L may lead to the displacement of R220, which is crucial for high activity and green emission of luciferase [4].

The red shift of the mutant G216N/A217L indicates that in spite of the increase in the overall protein stability the local microenvironment became more flexible leading to the lower emission energy of oxyluciferin. It is further confirmed by the fact that lowering temperature (decreasing the structure flexibility) significantly increases the contribution of green emission (Fig. 2).

The substitutions G216N, A217L, S398M fill in internal cavities in luciferase (Fig. 4). The 1st and 2nd mutations contribute to the thermal stability and the 3rd one improves the local conformational stability.

Rational protein design of residue microenvironment can be an effective strategy when a single mutation in one enzyme does not lead to the desirable effect reported for the mutation of the homologous residue in another enzyme.

The triple mutant G216N/A217L/S398M showed significantly improved thermal stability, high activity and bioluminescence spectrum close to that of the WT enzyme [2]. The improved characteristics of this mutant could make it a useful and efficient tool for a variety of *in vitro* and *in vivo* applications.

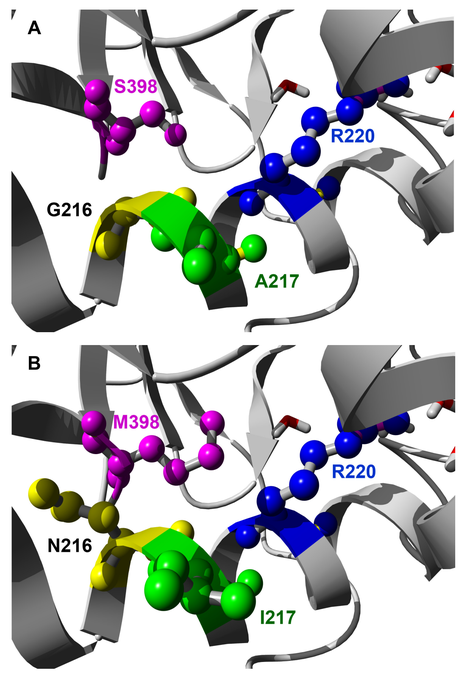


**Figure 4.** Residues 216, 217, 220 and 398 in the structures of *L. mingrelica* (A) and *L. cruciata* (B) luciferases.

## Literature cited

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