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Approaches to engineer stability of beetle luciferases

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Abstract: Luciferase enzymes from fireflies and other beetles have many important applications in molecular biology, biotechnology, analytical chemistry and several other areas. Many novel beetle luciferases with promising properties have been reported in the recent years. However, actual and potential applications of wild-type beetle luciferases are often limited by insufficient stability or decrease in activity of the enzyme at the conditions of a particular assay. Various examples of genetic engineering of the enhanced beetle luciferases have been reported that successfully solve or alleviate many of these limitations. This mini-review summarizes the recent advances in development of mutant luciferases with improved stability and activity characteristics. It discusses the common limitations of wild-type luciferases in different applications and presents the efficient approaches that can be used to address these problems.

Mini Review Article

Introduction

Firefly luciferase catalyzes the two-step oxidation of firefly luciferin in the presence of ATP, Mg^{2+}, and molecular oxygen which is accompanied by the emission of visible light [1,2]. This reaction is the same for all bioluminescent beetles but historically the enzyme from *Photinus pyralis* fireflies was the first to be extensively studied, so all representatives of this enzyme family are often called "firefly luciferases". The peak of the light emission varies from 538 to 623 nm for the enzymes from different species or for the mutant luciferases but the yellow-green bioluminescence is the most common [3]. Beetle luciferases demonstrate a notable quantum yield (45-60%), which is the highest among bioluminescent systems [6]. Firefly luciferases show bright bioluminescence, low background signal, high catalytic efficiency, substrate specificity and high sensitivity to ATP. This makes them a widely used tool in a variety of *in vitro* and *in vivo* applications: in ATP-related assays from direct ATP measurements to estimation of bacterial contamination and pyrosequencing [4,5], in *in vivo* molecular imaging and as a genetic reporter in molecular biology [6-8]. This enzyme was also shown to be a promising tool for molecular sensing of protein-protein interactions and different analytes [9-11], in analytical assays based on real time monitoring of polynucleotide amplification [12] and a label for immunoassays [13].

Many novel beetle luciferases with promising properties have been reported in the recent years [14-16]. Some of them were developed into *in vivo* reporters which are superior to the commonly used *P. pyralis* luciferase (Ppl) [17]. However, the applications of wild-type (WT) beetle luciferases are often limited by insufficient stability of these enzymes at elevated temperatures above 30°C. Therefore, the development of thermostable forms of luciferase is often required [18,19] and this problem arises for the recently cloned promising enzymes. For example, the most commonly used Ppl looses half of its activity within 15 min at 37°C and some of the newly cloned luciferases inactivate even faster [19]. Thermal stability of luciferases is most crucial for *in vitro* assays: immunoassays and pyrosequencing are usually conducted at 37°C [5] and assays based on polynucleotide amplification require luciferase to be stable at least at 50°C (preferably at temperatures >60°C) [12]. This problem is less pronounced in common *in vivo* applications since the *in vivo* half-life of Ppl is around 3-4 h at 37°C in mammalian cells [20], which is usually sufficient to monitor gene expression and for molecular imaging. However, more stable luciferases significantly improve the *in vivo* bioluminescence signal and provide more sensitive detection [19,21]. If intracellular processes are needed to be monitored at higher *in vivo* temperatures then the thermostability becomes crucial since Ppl inactivates within 5-20 minutes *in vivo* at 40-45°C in eukaryotic cells [22,23]. High thermostability of enzyme can also be highly beneficial for evolving other types of stability and new enzyme functionalities [24] such as a recent work on changing luciferase substrate specificity [25] or the popular trend to develop multi-color luciferases [26].

Another problem that often needs to be addressed is denaturation or inhibition of firefly luciferase at conditions of a particular assay. For example, in hygiene monitoring the inhibition from the extractants used for releasing intracellular ATP is a common problem [4]. The activity of luciferase during monitoring of *in vivo* bioluminescence can be affected by various intracellular factors including pH, proteases, pyrophosphate, reactive oxygen species, etc [27-29]. The latter can affect not only the sensitivity of detection but the interpretation of results as well.

A large number of works have been reported that describe the development of mutant luciferases with enhanced properties that showed improved stability towards the action of temperature and other factors. Like with the general field of protein engineering these works followed structure-based rational design approach [30] or random mutagenesis / selective screening approach [31]. Both strategies gave many successful examples of luciferase stabilization. However, the random mutagenesis approach can be very efficient in case of luciferase because colony libraries of mutant luciferases can be

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rather easily screened for activity (emitted light) in the presence of different factors which is often quite cumbersome for many other enzymes [31,32].

This mini-review discusses the recent results in engineering stable and active beetle luciferases, describes the types of stability required in different applications and compares the strategies that can be efficiently used to achieve a desirable level of luciferase stability. The major enhanced variants of beetle luciferases discussed here are summarized in the Supplementary Table 1.

Thermal stability of wild-type beetle luciferases

Firefly luciferases can be relatively stable *in vitro* in solution at low temperature in the presence of stabilizing compounds, though at low concentration without protective additives up to 99% of the enzyme can be lost due to the protein adsorption on the container surface [33]. However, even in the presence of stabilizing compounds Ppl luciferase inactivates within 6-20 minutes at 37-42°C [18,34,35]. Similar stabilities were reported for most other beetle luciferases [26,36]. The inactivated luciferase is almost unable to restore activity after cooling and usually aggregates [22]. It can be effectively reactivated only in the presence of different chaperone systems [37]. The detailed mechanism of luciferase inactivation in solution is still unknown and may vary for enzymes from different species. The knowledge of the inactivation and unfolding mechanism is necessary for the definite prediction of mutations that would increase thermostability; otherwise, the particular stabilization approach may be found not efficient because of the different factors defining the thermostability [30]. In several works different unfolding intermediates of Ppl were analyzed [38,39]. It was shown that *Luciola mingrelica* luciferase undergoes two-step inactivation with a homodimer dissociation step [40] unlike the Ppl enzyme. The crystal structures of luciferase [41,42] show that this enzyme consists of a big N-domain (1-436 aa) and a small C-domain (~443-544 aa) which are connected by a flexible loop. The N-domain is further composed of two distinct subdomains: A (1-190) and B (191-436) stacked together via a strong hydrophobic interface (Fig. 1).

Regarding this structure, the most interesting were the results of Frydman *et al* [38] who had investigated the unfolding of Ppl by chemical denaturation with subsequent protease treatment. They have shown that the middle subdomain "B" (192-435 aa) is significantly less stable that the other two and that it is the first to unfold under denaturing conditions. It may be assumed that the intrinsically low stability of the second subdomain is the "bottleneck" that determines the stability of the whole protein. Therefore, it is not surprising that almost all stabilizing mutations reported in the literature are located in this subdomain or on the interface between the middle subdomain with the first and third subdomains. It is further confirmed by our recent finding [43] that the structurally destabilizing mutation E457K in C-domain doesn't affect the thermostability of the WT luciferase but causes the 3-fold decrease in stability of the highly thermostable mutant [44] stabilized by four mutations in the middle subdomain. Thus, the effect of the deleterious mutation E457K in the third subdomain is only noticeable when the second subdomain is sufficiently stabilized. The similar picture was observed for thermolysin-like protease whose inactivation is governed by the unfolding of the N-terminal domain [30].

Rational design of thermostable luciferases

Relative improvements in stability at 37°C can be achieved by the addition of stabilizing compounds [5,45,46] but the effect is limited and the resultant solution may be incompatible with the particular application. The mutagenesis approach which increases the intrinsic stability allows to achieve much higher stabilization without changing the assay conditions. Before the 3D-structure of luciferase was obtained the only viable strategy to increase the thermostability was random mutagenesis. Several stabilizing mutations were identified by this approach in the early 1990s: the substitution of A217L in *Luciola cruciata* and *Luciola lateralis* luciferases [47,48] and the substitutions T214A, I232A, F295L, E354K in Ppl [34]. The identified positions were further extensively analyzed by site-directed mutagenesis to identify the most efficient substitutions. The major part of the following work was focused on developing thermostable multi-point mutants that would include these and other previously identified positions. Branchini *et al* have constructed a 5-point mutant of Ppl (T214A/A215L/ I232A/F295L/E354K) which showed a 44-fold improvement of half-life from 15 min to 11.5 h at 37°C. These mutations were further combined with the green and red emitting mutants to give a thermostable mutant pair for the dual-color imaging [18,26]. Even more striking example was reported by Murray *et al* [49] who have combined almost all previously known single thermostabilizing mutations in the highly stable 12-point mutant of Ppl. This mutant had a half-life of 15 min at 55°C whereas WT luciferase inactivates within seconds at these conditions.

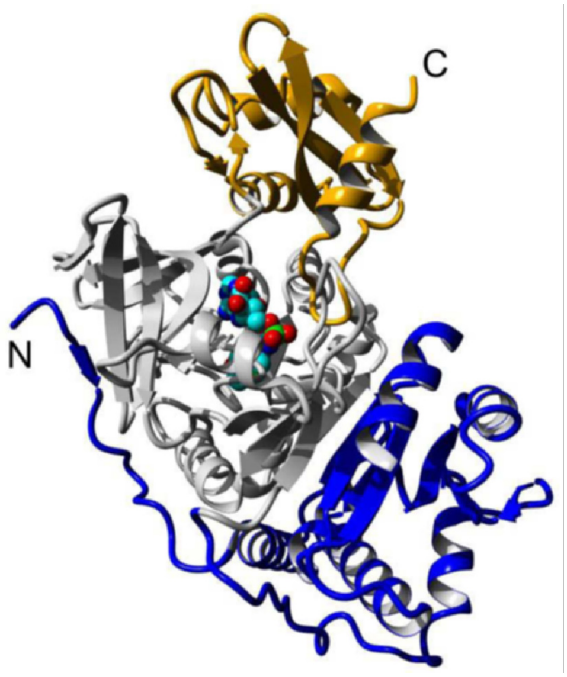

Figure 1. Structure of beetle luciferases (*L. cruciata* firefly luciferase in complex with DLSA [42]). Subdomains A, B and C are depicted in blue, grey and orange, respectively

However, the mutant possessed only 15% of the original activity which shows one of the downsides of this approach: in case of combining many individual mutations it may require additional extensive and laborious analysis by site-directed mutagenesis to identify the mutations which will retain the high activity in addition to high stability. Another limitation of this approach is that the mutations obtained for one particular enzyme often can not be directly applied to another homologous enzyme. For example, the mutation A217L was discovered in *L. cruciata* luciferase and was successfully applied to *L. lateralis* and *P. pyralys* luciferases to give