Mutation of a Protease-sensitive Region in Firefly Luciferase Alters Light Emission Properties*

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Luciferase (EC 1.13.12.7) from the North American firefly, Photinus pyralis, is widely used as a reporter enzyme in cell biology. One of its distinctive properties is a pronounced susceptibility to proteolytic degradation that causes luciferase to have a very short intracellular half-life. To define the structural basis for this behavior and possibly facilitate the design of more stable forms of luciferase, limited proteolysis studies were undertaken using trypsin and chymotrypsin to identify regions of the protein whose accessible and flexible character rendered them especially sensitive to cleavage. Regions of amino acid sequence 206–220 and 329–341 were found to be sensitive, and because the region around 206–220 had high homology with other luciferases, CoA ligases, and peptidyl synthetases, this region was selected for mutagenesis experiments intended to determine which of its amino acids were essential for activity. Surprisingly, many highly conserved residues including Ser198, Ser201, Thr202, and Gly203 could be mutated with little effect on the luminescent activity of P. pyralis luciferase. One mutation, however, S198T, caused several alterations in enzymatic properties including shifting the pH optimum from 8.1 to 8.7, lowering the $K_m$ for Mg-ATP by a factor of 4 and increasing the half-time for light emission decay by a factor of up to 150. While the S198T luciferase was less active than wild type, activity could be restored by the introduction of the additional L194F and N197Y mutations. In addition to indicating the involvement of this region in ATP binding, these results provide a new form of the enzyme that affords a more versatile reporter system.

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The kinetics of light emission can be modulated by coenzyme A, but this is not a required substrate (3, 4). Luciferase can also function as a ligase (5) as the AMP group of the enzyme-luciferyl-AMP complex can be transferred to ATP to produce diadenosine tetraphosphate.

Despite many studies, relatively little was known about the structure of luciferase or the nature of its active site until recently. Cloning and sequencing of luciferase from the firefly (6) and its homologues from several beetles (reviewed in Ref. 7) have shown that these enzymes are related to certain acyltransferases (8, 9). Alignment of these related sequences reveals conserved residues that may be important for enzymatic activity. For example, the high degree of homology in the 198–207 region of luciferase led to speculation that this region participates in acyladenylate formation (9). The recent publication of the crystal structure of luciferase (10) will promote understanding of the significance of the conserved amino acids.

Luciferase is highly susceptible to proteolysis in vitro and has a very short half-life in vivo (11). For different applications, this can create either an advantage or a disadvantage. Rapid intracellular turnover enables the level of the luciferase signal to respond rapidly to changes in transcription of the luciferase reporter gene, which enables kinetic studies that are impossible with the more stable reporter enzymes chloramphenical acetyltransferase or β-galactosidase. However, the same differences in protein stability among the reporters complicate their use as controls for the expression of luciferase and create a need for careful attention to the timing of signal variations. This paper describes new forms of luciferase with altered luminescent properties that may allow some of these problems to be circumvented.

EXPERIMENTAL PROCEDURES

Limited Proteolysis—Proteolysis of luciferase was carried out as described previously (11). Firefly luciferase (0.5 mg/ml) was suspended in assay buffer (50 mM HEPES, pH 7.7, 10 mM MgSO4) and then incubated at 23 or 37 °C with 1 μg/ml trypsin or chymotrypsin for varying lengths of time. The reaction mixtures were then subjected to SDS-polyacrylamide gel electrophoresis; the gels were electroblotted to Immobilon P (Millipore Corp.), and luciferase and its proteolytic fragments were detected by staining with Coomassie Blue. Bands were subjected to sequence analysis by automated Edman degradation on an Applied Biosystems model 470A gas-phase sequencer with on-line model 120A

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phenylthiohydantoin analyzer.

Construction of Plasmids—Luciferase-expressing plasmids were derived by removing the luciferase gene from pSV035 (a generous gift of Dr. Donald Helsinki) by cutting with HindIII/BamHI and inserting it into pUC13 cut with the same enzymes to generate pLuxF3–13 (WT).1 All mutagenic oligonucleotides were obtained from Genosys, and the restriction enzymes were obtained from New England Biolabs or Boehringer Mannheim. Partial digestions of plasmids were carried out by cutting DNA with 4-fold serial dilutions of restriction enzymes from 1/2 unit/μg to 16 units/μg in conditions recommended by the manufacturer, electrophoresing the DNA on an agarose gel, and eluting the linearized plasmid with GeneClean (BIO 101, Inc.). This DNA was recut with EcoRI and completely cleaved with BstEIII, and various double-stranded oligonucleotides were ligated into the plasmid. For pLuxF93 and pLuxF105-X, oligonucleotides containing a mixture of 64 different sequences were synthesized to span the MunI and BstEIII sites. This mixture was inserted into pLuxF44, which had been partially cleaved with MunI and then completely cleaved with BstEIII. Because the luciferase (S199A) originally encoded by pLuxF44 was completely inactive, colonies derived from the parent plasmid that might contaminate the novel clones could be easily eliminated by assaying for luminescence (11). Plasmids recovered from colonies displaying luciferase activity were sequenced to determine which amino acid changes were compatible with activity.

Luciferase Purification—For larger quantities of luciferase, E. coli cultures were grown at 37 °C in 500 ml of rich broth and harvested at late growth phase. Bacteria were pelleted (5000 × g, 25 min, 4 °C) and resuspended in 50 ml of 10 mM Tris-HCl (pH 8) containing 1 mM EDTA and 25 μg/ml phenylmethylsulfonyl fluoride. The suspended cells were lysed by adding 2.5 ml of a stock aqueous solution of lysozyme (10 mg/ml) and incubating at 0 °C for 5 min. Tween 20 was added (final concentration 0.2%, v/v) to the suspension, which was then incubated at 0 °C for an additional 5 min and centrifuged (25,000 × g, 30 min, 4 °C). The resulting supernatant, termed the crude extract, slowly lost activity on storage at 4 °C. To prevent this, the crude extract was immediately fractionated by subjecting it to 41% followed by 61% saturated ammonium sulfate (45 min stirring at 5 °C and centrifugation at 20,000 × g, 30 min, 4 °C). The pellet protein from the 61% saturated ammonium sulfate treatment was redissolved in 0.5–3.0 ml of NME (50 mM sodium N-morpholinopropanesulfonate buffer [pH 7.2] containing 2 mM EDTA) containing 0.8 mM ammonium sulfate and 2% (v/v) glycerol. This protein mixture could be stored at 5 °C for several days without loss of activity. A 0.8-ml aliquot of the protein solution was centrifuged twice (20,000 × g, 10 min, 5 °C) and applied to a 1.6 × 50-cm Sephacryl S-200 column pre-equilibrated at 5 °C with NME buffer containing 0.4 mM ammonium sulfate. The column was eluted with the same buffer at a flow rate of 0.6 ml/min. The eluate was monitored by A280, and 1.2-ml fractions were collected. Aliquots (10 μl) of each fraction were assayed for luciferase activity expressed in relative light units by using a flash height-based assay similar to that described previously (12). Fractions containing ~80% of the total recovered bioluminescent activity were pooled and concentrated to ~0.8 ml with a Centricon-30 (Amicon, Inc.) device. Glycerol was added (final concentration 2%, v/v) to the concentrated protein solutions, which could be stored at 5 °C for several weeks without significant activity loss.

Luciferase Activity Measurements—For flash height measurements, data were acquired and stored with a Strawberry Tree, Inc. A/D converter and a Macintosh SE computer. Initial flash height measurements were made with a customized version of the workbench software. Assays were initiated by injecting 120 μl of 10 mM Mg-ATP in 25 mM glycylglycine buffer (pH 7.8) into 400 μl of the same buffer containing 30 μl of t-luciferase and luciferase solutions (4 μl). For the integration-based assays, coenzyme A (0.1 mM final concentration) was included in the assay mixture. After injection of ATP and a 30-s delay, light emission was measured for 30 s to generate the integrated value. The kinetic properties of luciferase were evaluated with various concentrations of luciferin (1–75 μM) at pH 8.7 using 25 mM Tris with 0.1% (v/v) glycerol. Kinetic measurements were also made with varying concentrations of Mg-ATP (0.15 μM–2 mM) by injecting luciferin solution to initiate the reaction.

The ability of mutants in this region to retain activity led us to study additional changes. Positions 194, 196, 197, 198, and 203 could be changed while alanine, and the resultant proteins were assayed for function. Amino acids 198, 201, 202, and 203 could be changed while retaining 10–100% of WT luminescent activity, but the S199A or G200A changes resulted in a complete loss of activity (<0.01% remaining).

RESULTS

Partial proteolysis of luciferase was undertaken to identify regions of the protein whose particular sensitivity to proteolysis would denote that they were flexible, accessible, and possibly near the active site. Cleavage with trypsin or chymotrypsin yielded similar product patterns with initial products of about 45 and 30 kDa followed by a number of smaller peptides (Fig. 1). The major bands were sequenced from electroblots to determine the N terminus of each fragment. The primary cleavages, which generated the initial bands at early times, occurred in two ranges of sequence: residues 206–220 or residues 329–341 (Fig. 2). Bands appearing later included C-terminally shortened versions of the original peptides as well as the products of new cleavages in the ranges of 31–34 and 383–446. In all cases analyzed, the sites of cleavage conformed to the canonical selectivity of the protease employed.

In considering the suitability of each region for detailed mutagenesis studies, attention was given to local homologies with other proteins. Since the region around residue 200 is highly homologous to all other luciferases, a variety of CoA ligases, and several antibiotic and polypeptide synthetases, this region was chosen for study in greater detail. Alignments for eight different luciferases and nine CoA ligases/acyl synthetases are shown in Fig. 3. Six of the luciferases are identical in 16 consecutive residues. The other sequences show more variability, but all are identical for 6 of 10 amino acids (corresponding to positions 198–207). To determine whether amino acids in this region could be changed without loss of function, each amino acid from 198 to 203 was individually mutated to alanine, and the resultant proteins were assayed for function. Amino acids 198, 201, 202, and 203 could be changed while retaining 10–100% of WT luminescent activity, but the S199A or G200A changes resulted in a complete loss of activity (<0.01% remaining).

The abbreviation used is: WT, wild type.
greater than 10% of wild-type activity whereas S198P was about 500-fold less active than WT. To rationalize the properties of these novel proteins, three of the more active variants, L194F/N197Y/S198T, L194F/M196L, and S198T were partially purified and studied in detail.

The effect of pH on the bioluminescence activity of highly purified native luciferase, partially purified WT (putatively the same sequence as native but prepared in E. coli in parallel to the mutant luciferases (13)), and mutant luciferases was investigated using flash height-based assays performed over the pH range 6.5–9.0. The results are shown in Fig. 5, and the pH optima data are summarized in Table I. L194F/M196L and WT have identical pH optima of 8.1. The other four luciferases, all of which have the S198T change, had pH optima shifted to 8.7–8.8. The observation that the single point mutant S198T had maximal activity at pH 8.7 indicated that this single conservative amino acid change was sufficient to cause the alkaline pH shift also found in L194F/S198T, L194F/N197Y/S198T, and L194F/M196L/S198T/S201T (Table I and Fig. 5).

Because pH had such a dramatic effect, the mutant luciferases were evaluated at pH 7.8 and 8.7 to enable a comparison of relative specific activities. Luciferase protein levels were estimated using Western blot analysis. The flash height and integration-based specific activity data at pH 7.8 and 8.7 for WT and the pH optimum-shifted mutants are presented in Table I. The ratios of pH-dependent specific activity measured by integration assays or by flash height-based measurements varied by more than a factor of 10 among the proteins. Among the four S198T-containing mutants, L194F/N197YS198T, L194F/M196L, and S198T had the highest specific activity at pH 7.8 and 8.7 using either assay method.

The time dependence of bioluminescence intensity for crude and purified luciferase. In fact, at pH 7.8, the light emission from the purified S198T protein rose only slowly to its maximum value over 22 min and decayed to 40% of this value after approximately 3 h (data not shown). The decreased bioluminescence decay rate of both the S198T and S198A retained greater than 10% of wild-type activity whereas S198P was about 500-fold less active than WT. To rationalize the properties of these novel proteins, three of the more active variants, L194F/N197YS198T, L194F/M196L, and S198T were partially purified and studied in detail.

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obtained after 90 s at pH 7.8 and 8.7, respectively (data not shown). The N197Y mutation increased bioluminescence efficiency and decay rates relative to both L194F/S198T and S198T.

The effects of the amino acid changes in L194F/N197Y/S198T and S198T on substrate binding were investigated by determining $K_m$ values for substrates luciferin and Mg-ATP at pH 8.7. For native and WT luciferases at pH 8.7, the $K_m$ values for Mg-ATP were 146 ± 22 and 180 ± 20 μM, respectively. The $K_m$ values for L194F/N197Y/S198T and S198T were 44 ± 4 and 39 ± 4 μM, indicating that the mutants bind Mg-ATP more tightly than the unaltered enzyme. The $K_m$ for luciferin with the native enzyme was 11.5 ± 2 μM and was essentially unchanged in the mutants.

An evaluation of the mutants for proteolytic and thermal stability compared with the WT or native enzyme revealed only minor differences (data not shown). L194F/N197Y/S198T was found to be a slightly more stable protein than the other enzymes studied, including WT and native luciferases, based on modest improvements in stability to chymotrypsin hydrolysis and incubation at 42 °C (data not shown).

To determine the usefulness of the L194F/N197Y/S198T protein as a reporter in mammalian cell systems, its expression level was examined in HepG2 cells, and no differences were found between mutant and WT. The ability to generate stable cell lines upon co-transfection with pSV2neo was also examined, and again no difference could be detected between the mutant and WT.

Because WT and L194F/N197Y/S198T have different pH optima, the two proteins could potentially be distinguished in the same cell. This situation was simulated by adding progressively more L194F/N197Y/S198T to a constant amount of WT and measuring light intensity at both pH 6.6 (which favors WT) and pH 9.0 (which favors L194F/N197Y/S198T). The ratio of activities at these pH levels provides an accurate reflection of the relative amounts of the two proteins (Fig. 7). Because the decay kinetics for the proteins differ, a calibration curve of known ratios should be run to ensure accuracy.

**DISCUSSION**

Firefly luciferase has been a very useful reporter protein because of the ease with which it is detected and its quick response to changes in transcription. Nevertheless, because there are times when altered properties would be desirable, we attempted to identify regions in which mutagenesis might lead to improved functional properties. The strategy of combining partial proteolysis with knowledge of protein family homologies was successful in allowing us to generate functionally interesting mutations.

The $K_m$ results for L194F/N197Y/S198T and S198T provide an experimental basis for the involvement of the region around amino acid 200 in Mg-ATP binding. The single conservative change, S198T, results in a mutant luciferase with unaltered affinity for luciferin but a higher affinity for Mg-ATP than WT. This suggests that Ser$^{198}$ and perhaps the conserved domain comprised of residues Ala$^{199}$-Val$^{208}$ is directly involved in Mg-ATP binding but not luciferin binding. Supporting this further is the observation that a T204D mutation in Luciola mingrelica increases the $K_m$ for ATP by a factor of 8 while leaving the specific activity unchanged (14). The homologous region of the Bacillus brevis tyrocidine synthetase protein has also been implicated in ATP binding based on mutational studies (15). Photoaffinity labeling suggested that ATP binding occurred at

**TABLE I**

| Enzyme          | Specific activity$^a$ | Decay time (to 20% initial activity)$^b$ | pH optimum$^c$ |
|-----------------|-----------------------|-----------------------------------------|----------------|
|                 | Flash height          | Integrated                              |                 |
|                 | pH 7.8 pH 8.7         | pH 7.8 pH 8.7                            |                 |
| Native          | 35,000 20,000         | 49,600 33,500                            | 17.0 10.5       |
| WT              | 2980 1420             | 20,000 16,000                            | 10.2 9.2        |
| L194F/N197Y/S198T | 390 1381             | 2100 13,700                              | 104.0 103.0     |
| S198T           | 59 256                | 333 3176                                 | >3 h 1900       |
| L194F/S198T     | (497)$^d$             | (6160)$^d$                               | —$^f$          |
| L194F/M196L     | 8 43                  | 161 770                                  | 8.7            |
| L194F/M196L/S198T/S201T | 1090 746          | 15,300 13,300                            | 15.0 12.0       |
| L194F/M196L/S198T/S201T | 5 61              | 82 193                                   | 8.8            |

$^a$ Enzyme activities were determined by the standard flash height assay or 30-s integration assays. The pH 8.7 assays were performed in 25 mM Tris-HCl buffer. Luciferase protein values were determined by Western blot analysis. Specific activities are the average of three values obtained from three separate batches of protein. Flash height-based relative specific activities were measured over the pH range of 6.5–9.0.

$^b$ Decay rates were measured from maximum initial flash heights.

$^c$ Specific activities are reported relative to the value obtained for each protein at pH 7.8.

$^d$ These values were measured with a final concentration of 0.2 mM ATP.

$^e$ After 3 h, the light emission had decayed to only 40% of the maximum value, which was recorded at 22 min.

$^f$ Decay times were not calculated, but slow decay kinetics similar to those obtained for L194F/N197Y/S198T were observed.
Indeed, Ser198 is absolutely conserved in 38 sequences that include the luciferase (Gly200) can be changed to alanine in the positions. Furthermore, one of the positions that we find to be surprising was that changes could be tolerated in so many positions. Though they are distant in the primary structure. This region of the protein likely constitutes a portion of the ATP binding domain.

Given the highly conserved nature of the 198–207 region, it was surprising that changes could be tolerated in so many positions. Furthermore, one of the positions that we find to be critical in luciferase (Gly200) can be changed to alanine in the homologous position of the B. brevis tyrocidine synthetase with only a minor loss of activity (15). In addition to the G200A mutation, five further changes were also made (15). One of these is identical to the G203A change we made. In both cases, no effect was seen on activity. We have not made the other mutations described (15) but note that these authors found that altering the highly conserved Lys206 was detrimental to activity. Whereas the single mutations provide useful information, the compensating effects among some multiple mutations are particularly interesting. For example, L194F/N197Y/S198T has three changes relative to WT, L194F/S198T has two of the same changes, and S198T has only the single change shared by these proteins. The introduction of the single S198T change results in a significant loss of activity at pH 7.8, and the additional L194F change causes an even greater loss of activity. However, when the N197Y mutation is introduced in addition to the first two, significant activity is restored.

Some properties of these novel proteins are dramatically affected. The pH optima of several of the luciferases shift from 8.1 to 8.7. This effect is due to the S198T mutation as it occurs in all proteins with the change and in none without it. The mechanistic rationale for this change is not yet known, but it is noteworthy that there are two charged residues in close proximity to amino acid 198, Lys206 and Glu344, both of which are absolutely conserved in 38 sequences that include the luciferases and acyladenylate-forming enzymes (10). Indeed, Ser198 is hydrogen bonded to Glu344 in the crystal structure (10). This is near the other region of luciferase, 329–341, which we observed to be hypersensitive to proteases and is also highly conserved among luciferases. Other luciferases have been observed to have altered pH optima (17), but these are shifted toward more acidic pH levels.

Interestingly, each of the mutants with the altered pH optimum also has altered emission decay kinetics. The decay of L194F/N197Y/S198T is more than 10-fold slower than WT while S198T decays over 1000 times more slowly. The increased affinity for ATP may be related to the altered luminescent decay. Addition of CoA to the luciferase reaction is thought to have a similar effect (18) on WT luciferase. The mutant luciferases examined here are also stimulated by addition of CoA (data not shown). The slow, steady decay of luminescence of L194F/N197Y/S198T is particularly useful in situations in which large numbers of samples prevent rapid assaying and in laboratories without access to rapid flash instrumentation.

The luciferase mutations described in this work not only provide insight into how luciferase carries out its luminescent reaction but also provides an improved reporter system. Whereas transfection experiments in which gene expression is quantitated frequently contain internal controls, these controls are often flawed because of the different kinetic response and stability of the tested and control reporters. By using two versions of the same reporter, more reliable controls can be generated. For example, the activity of WT and mutant luciferase expressed under the control of two independent promoters can be separated by measuring the ratio of luminescence at pH 6.6 and 9.0. This can provide an accurate measure of the relative concentrations of WT and L194F/N197Y/S198T. Since the in vivo stability of the proteins is nearly identical, the two luciferases provide excellent controls for one another.

The results of proteolysis and mutagenesis studies presented
here are fully consistent with the crystal structure of firefly luciferase and provide some clues as to the enzymatic mechanism. Studies employing these data as a basis should provide more insight into the mechanism of the protein and possibly allow further improvements in its use as a reporter gene.

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