FIREFLY LUCIFERASE: MODIFICATION AND CLONING

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PREFACE

The active site of firefly luciferase has been studied by chemical modification using several ATP analogs or amino acid (-NH_2, His, and Trp) specific chemical reagents, as well as by physical modification resulting from UV irradiation and low pH treatment. Two peptides labeled by the Lys residue specific reagent thiourea dioxide have been isolated and sequenced. One of these two peptides, GLTGK, is related to the ATP binding site of firefly luciferase. The Lys residue within the peptide GLTGK is a highly conserved residue for ATP binding sites among several ATP requiring proteins. The *Photuris* pennsylvanica luciferase has been partially purified and characterized. The luciferase gene from *Photuris* firefly has been cloned and sequenced. The amino acid sequence of *Photuris* luciferase, deduced from the nucleic acid sequence has high homology with that of the *Photinus* and *Luciola* fireflies, as well as with click beetle (*Pyrophorus plagiophthalamus*). The research described in the thesis provides evidence to identify the active site of firefly luciferase by modification and gene cloning.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
DEPC	diethyl pyrocarbonate
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EAB	enzyme assay buffer
EDB	enzyme dilution buffer
FL	firefly luciferase
FSBA	5' [*p*-(fluorosulfonyl)-benzoyl] adenosine
HPLC	high performance liquid chromatography
LH_2	D-luciferin
NBS	*n*-bromosuccinimide
NBT	nitro blue tetrazolium
NHPB	phosphate buffer
o-PA	*o*-phthalaldehyde
PABA	*p*-aminobenzoic acid
PLP	pyridoxal 5'-phosphate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel eletrophoresis
TB 1	Tricine complete buffer
TB 2	TB 1 with DTT

TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TUD	thiourea dioxide

CHAPTER I

INTRODUCTION

Bioluminescence is the emission of light by living organisms. Many living organisms, including bacteria, fungi, insects, plants, animals, etc., produce light for survival, reproduction and other purposes. Bioluminescence is a class of chemiluminescent reactions catalyzed by specific enzymes. Basically, bioluminescence is produced during the oxidation of the substrate luciferin catalyzed by the enzyme luciferase (Campbell, 1988).

Fireflies emit a bright light in the evening as signals to communicate with each other for mating. Firefly luciferase has the highest efficiency of any chemiluminescence reaction, yielding a 0.9 quantum efficiency (other bioluminescence and chemiluminescence reactions have efficiencies of 0.01 or less) (Seliger et al., 1959). The study of firefly luciferase is important for both theoretical and practical reasons. Elucidation of the conformational changes of firefly luciferase that occur during substrate binding and catalysis will help us to understand how the efficient energy conversion is achieved and what the chemical basis of the high quantum yield is.

Firefly luciferase has many basic and practical applications in medicine, public health, industrial and molecular biological techniques. Since ATP is required to form luciferyl adenylate in the enzymatic reaction, and given the extreme specificity of the enzyme for ATP, firefly luciferase can be used for measurement of ATP in a variety of samples. The applications include determining antibiotic susceptibility following antibiotic therapy; determining erythrocyte and sperm viability; determining the number of bacteria in soil, water, milk, food and drinks; etc. (Kricka, 1988). Firefly luciferase is also an

important reporter gene widely used in molecular biology, e.g. as a reporter for monitoring promoter activity, as a model for elucidation of the signals required for targeting proteins to peroxisomes, etc (Gould and Subramani, 1988).

Properties of Firefly Luciferase

Firefly luciferase (*Photinus pyralis*) has been studied extensively since the late 1940s (DeLuca, 1976). Five reviews on firefly luciferase are by Leach (1981), McElroy and DeLuca (1985), Campbell (1988), Wood et al., (1989) and Kricka (1988).

Firefly luciferase is an euglobulin and is extremely hydrophobic. The active luciferase from *Photinus pyralis* (the North American firefly) has a molecular weight of 1.2 $\times 10^5$, is comprised of two apparently identical subunits, and has a pH optimum of 7.8 and a temperature optimum of 25 °C.

McElroy and DeLuca's group suggested that there are two luciferins, one MgATP, two ATPs, and one luciferyl adenylate bound to the molecule of firefly luciferase (*P. pyralis*). Firefly luciferase contains two -SH groups that influence active-site conformation. The monomer is apparently active, but the enzyme exists as a dimer in most solutions (Wood et al., 1989). Firefly luciferase has an anion-binding site and is inhibited by AMP, ATP, PP_i, and some other anions. There is a large conformational change during firefly luciferase catalysis, specifically a change from 37% to 14% helix upon addition of substrates (DeLuca and Marsh, 1967).

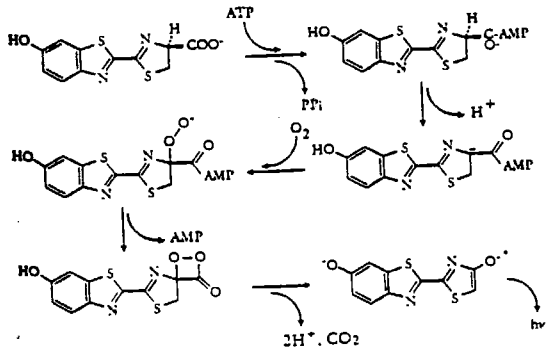
Firefly luciferase catalyzes the reaction represented by the following equation, where FL = firefly luciferase and LH_2 = luciferin:

$$FL + LH_2 + MgATP \text{ <-----> } FL\text{-}LH_2\text{-}AMP + PP_i$$

$$FL\text{-}LH_2\text{-}AMP + O_2 \text{ ------> } FL + \text{Oxyluciferin} + AMP + CO_2 + \text{light}$$

The reactions catalyzed by firefly luciferase are similar to those involved in activations of amino acids (Berg, 1956), fatty acids (Berg, 1956) and vitamins such as lipoic acid, (Leach, 1970) and biotin, (Lane et al., 1964), resulting in the formation of an enzyme-

bound acyl adenylate intermediate. The current understanding of the reaction mechanism is shown in the following scheme:



In the first step, the carboxylate of luciferin reacts with MgATP to form an acyl-AMP complex. One atom of O_2 then forms a bond with this carbon, and the other atom displaces the AMP to form a peroxylactone. Bond rearrangement in the peroxylactone relaeases the carboxylate as CO_2 (Wannlund, et al., 1978). The oxyluciferin thus generated is in an electronically excited state. A photon of light is then emitted from the excited-state oxyluciferin upon its trasition to the ground state. The oxyluciferin does not have to be released from the enzyme for light production.

DeLuca and McElroy suggest that two ATPs may be required for each luciferyl adenylate produced. The function of the second ATP molecule is not known. There may be two kinetically distinguishable active sites for ATP on the *P. pyralis* luciferase that differ tenfold in K_ms (DeLuca and McElroy, 1984). These two sites display different time courses of light production. The time course of the enzymatic reaction (either a flash or steady production of light) depends upon the ATP concentration (DeLuca and McElroy, 1984). At saturating concentrations of ATP, light emission occurs from both sites. Site I_0 (for the initial peak of light emission) is rapidly inactivated by product and/ or AMP and PP_i. At low concentrations of ATP, where only constant-light production occurs, there is a slow inhibition by product. Some other nucleotides or analogs can enhance or reduce the enzyme activity of firefly luciferase (Ford et al., 1992). Most analogs have little effect on the time course of light production at low ATP concentration, but there is marked

enhancement (up to a eight-fold increase) of the activity at high ATP concentration. These effects of nucleotides in changing the enzyme activity and the time course of light production are probably achieved through allosteric sites. When the substrates and luciferase are mixed, light production begins in 25 ms and maximum light emission occurs at about 300 ms. There is a slow release of oxyluciferin (DeLuca, 1976). ATP may function both as a substrate and as a regulator. The observation of two active sites has not been confirmed and is suspect since the two-site hypothesis was based only on kinetic measurements.

Active Sites of Firefly Luciferase

The active-site amino acid residues of firefly luciferase (*P. pyralis*) have been studied by using luciferin and ATP analogs or specific amino acid residue-modifying reagents.

Early experiments demonstrated that two sulfhydryl groups of firefly luciferase were essential for the enzymatic reactions leading to light emission (DeLuca et al., 1964). In the presence of a competitive inhibitor, dehydroluciferyl adenylate, these two sulfhydryls were not titrated with the sulfhydryl-modifying reagent *p*-chloromercuribenzoate. These two sulfhydryl groups were labeled with *N*-[1-^{14}C]ethylmaleimide (Travis and McElroy, 1966). A labeled peptide was isolated, sequenced, and identified as S-*C-E-G-N-A-G-S-Q-K. The -SH involvement in the firefly luciferase reaction has been reexamined (Alter and DeLuca, 1986). Firefly luciferase contains three classes of -SH groups defined by reactivities and patterns of substrate protection. Class SH-III consists of three-SHs not involved in enzyme activity. Class SH-II contains two-SHs whose modification resulted in 0-60% inactivation depending on the reagent used. Class SH-I contains two-SH groups that are protected by either dehydroluciferyl adenylate or dehydroluciferin. Their modification by most reagents results in complete loss of enzymatic activity, but methyl methanethiosulfonate produces an

modified enzyme that causes emission of red light rather than the normal yellow-green light. The enzyme thus modified is catalytically active, but has a distorted active site and operates by a different mechanism (Vellom et al., 1988).

There are two identical noninteracting luciferin-binding sites per molecule of firefly luciferase (*P. pyralis*). Occupancy of an ATP-binding site by either ATP or AMP changes the conformation of luciferase and makes the active site more hydrophobic, thus increasing luciferin binding. A luciferin analog, 2-cyano-6-chlorobenzothiazole, labels a luciferin-binding site and inactivates the enzyme (Lee and McElroy, 1971). A peptide containing this label has been isolated and sequenced after tryptic digestion. The peptide has the following structure: PyroE-X-G-A-V-(B)-I-L, where X is the amino acid, possibly a Tyr, that is labeled by the analog.

MgATP is the nucleotide substrate for firefly luciferase (Lee et al., 1970). Uncomplexed ATP is also bound to luciferase and is a competitive inhibitor with respect to MgATP. Mg^{2+} is not bound to luciferase. The MgATP and luciferin bind in a random order to luciferase. cAMP, dAMP and dATP are all competitive inhibitors with respect to ATP. Moyer and Henderson examined 12 naturally occurring nucleotide triphosphates for activity as substrates or inhibitors of firefly luciferase. dATP was 1.7% as active as ATP and all others (XTP, UTP, GTP, TTP, dUTP, CTP, dGTP, ITP, dITP and dCTP) were less than 0.1% as active. None of these nucleotides tested were potent inhibitors under their experimental conditions (Moyer and Henderson, 1983).

A lysine residue was found at one nucleotide-binding site. By using the affinity label 5'-[ρ-(fluorosulfonyl)benzoyl]adenosine, a labeled peptide with an amino acid sequence of K*-G-Q-B-S-K was isolated. MgATP blocked the inhibition of firefly luciferase by this analog, thus indicating that the analog is bound to the MgATP site (Lee et al., 1981).

The firefly luciferase (*P. pyralis*) gene has been cloned and sequenced (de Wet et al., 1987). Unfortunately, none of the three peptide sequences described above (the -SH-

containing one, the luciferin-binding one, and the one from the nucleotide-binding site) are found in the amino acid sequence deduced from the base sequence (Hill et al., 1986). The mystery of this severe discrepancy has not been explained and needs to be solved.

The -SH containing sequences were reexamined by labeling with *N*-[^{14}C] ethylmaleimide (Vellom and DeLuca, 1988). Two sequences were found. They are T-A-C-V-R (Cys 216) and G-E-L-C-V-R (Cys 391), which are found in the sequence deduced from the cloned gene. By using oligonucleotide-directed mutagenesis of the *luc* cDNA, the above two active cysteines were replaced with alanine. Either of these two mutant luciferases (with one cysteine substituted by an alanine) produced a twofold increase in maximum light intensity. One of them displayed a twofold increase in K_m for luciferin. No significant difference in emission spectrum at pH 8 was detected for either mutant. Therefore, neither of these cysteine residues is acting as a general base responsible for control of the emission spectrum in firefly luciferase (Vellom, 1990).

Sala-Newby and Campbell recently studied the C-terminus of the firefly luciferase (*P. pyralis*). They found that stepwise removal of up to seven C-terminal amino acids did not reduce the bioluminescent activity. But the firefly luciferase activity decreased stepwise from 50 to 0.1% when 8-12 amino acids were removed. Replacing amino acids 539-550 or 543-550 by a decapeptide MRSAMSGLHL gives luciferase with 22 or 35% of activity, respectively. Their experiments also showed that there was no significant relationship between the loss of activity and the reduction of affinity for ATP. The C-terminus of firefly luciferase is suggested to be important in the bioluminescence activity of the enzyme (Sala-Newby and Campbell, 1994).

Genes of Firefly Luciferases

The luciferase gene from *Photinus pyralis* fireflies was first cloned and sequenced in 1987 (de Wet et al., 1987). There are 550 amino acids (MW 60,746). The luciferase gene has been widely used as a reporter gene for recombinant DNA experiments (Gould

and Subramani, 1988). Eight beetle luciferases have been cloned and some used as reporter genes (de Wet et al., 1987; Wood et al., 1989; Tatsumi et al., 1989; Tatsumi et al., 1992; Devine et al., 1993).

Comparison of amino acid sequences of luciferase from beetles showed very similar amino acid composition (Ugarova et al., 1994). More than half of the residues are nonpolar and ambivalent amino acids. The total number of charged residues is almost the same for all luciferases. The major differences between various luciferases occur in the number of Trp and Cys residues.

Luciferases from different species of *Luciola* have 80% homology in their C-terminal regions. There is 67% identity between *Luciola mingrelica* and *P. pyralis,* and 43% identity between *L. mingrelica* and green click beetle luciferase. Three different maximum wavelengths of emission are exhibited by *Luciola* luciferases although they have similar protein structures: *L. mingrelica* at 570 nm, *L. cruciata* at 562 nm and *L. lateralis* at 552 nm (Kajiyama et al., 1992).

The amino acid residues related to changes in the bioluminescent spectrum were elucidated during a mutation study of *L. cruciata* luciferase (Kajiyama and Nakano, 1991). Native luciferase displays yellow-green light (λ_{max} = 562 nm). By mutation of Gly-326 to Ser or His-433 to Tyr, a red spectrum shift was obtained (λ_{max} = 609 or 612 nm). Mutants with substitutions Pro-425 to Ser and Ser-286 to Asn show yellow orange (λ_{max} = 592 nm) and orange (λ_{max} = 607 nm) color respectively. With the mutation of Val-239 to Ile, there is a green shift (λ_{max} = 558 nm). It was concluded that increase of hydrophobicity results in the blue shift, whereas increased hydrophilicity leads to a red shift. That substitutions of amino acids can change the color of bioluminescence was also found in four luciferases of snapping beetles (Wood et al., 1989).

The amino acid sequence of the active site should be highly conserved. The amino acid sequences of firefly luciferases were compared to those of other enzymes. Many enzymes that use MgATP for adenylation of carboxylic groups of substrates, such as 4-

coumarate-coenzyme A-ligase, 2,3-dihydroxybenzoate-ATP-ligase, gramicidin S-synthetase I, tyrocidine-synthetase and 4-chlorobenzoate dehalogenase, have sequences similar to those of luciferases (Scholten et al., 1991). A homologous region in all these enzymes was found, which corresponds to the amino acid residues 197-210 of *L. mingrelica* luciferase. The highest homology among all luciferases (> 90%) and between luciferase and 4-coumarate-coenzyme A-ligase (~ 80%) is located in the region of 410-460 amino acid residues (Schroder, 1989). It is suggested that the region is related to a coenzyme A binding site.

Photuris pennsylvanica

When fireflies are collected in the evening, it is amazing to find that some fly faster and flash brighter than others. They move very quickly and are more difficult to catch. This kind of firefly is *Photuris pennsylvanica.*

P. pennsylvanica is a twi-night-active firefly while the common, well-characterized North American species, *Photinus pyralis*, is more active during the twilight hours. *P. pennsylvanica* expresses more aggression behavior and often attacks other species of fireflies, even their own family members. *Photinus* fireflies are killed and eaten by *Photuris* fireflies if placed in the same container. Before mating female *Photuris* fireflies respond to courtship flashes of conspecific males. After mating they become femme fatales by answering the courtship flashes of males of other species (Soucek and Carlson, 1987).

There are many differences in characteristics of light production when comparing *Photinus* to *Photuris* fireflies (Coblentz, 1912).

	Photinus pyralis	*Photuris pennsylvanica*
Light color	yellow green	greenish blue
Light time	long fulmination	quick flash
Flash frequency	20 times/min	120-180 times/min
Maximum radiation	extends to the red	extends to the blue
Spectrum density	richer in red & yellow	less rich in red & yellow

Illuminating power	greater than in *Photuris*	--
Maximum emission	580 nm	550 nm
Luminous efficiency	87%	92%
Fluorescent material content	abundant	smaller amount

The firefly luciferase of *Photuris pennsylvanica* was first partially characterized by Strause and DeLuca in 1981. The luciferase was partially purified by ammonium sulfate fractionation and was compared to the enzyme from *Photinus pyralis*. Firefly luciferases from *P. pennsylvanica* and *P. pyralis* had similar pIs. Antibodies raised against the luciferase from *P. pyralis* inhibited the luciferase activity of *P. pennsylvanica*. It was concluded that the two luciferases are antigenically related (Strause and DeLuca, 1981). There is an isozyme of luciferase in the larval stage of *P. pennsylvanica* which differs from its adult form. The isozymes have similar K_ms, pH profiles, and molecular weights. However, the isoelectric points and the antigenicity (assayed by antibody inhibiting luciferase activity) of the larval and adult luciferases were significantly different. *Photuris* larval luciferase was larger than the *Photinus* enzyme based on their migration in denaturing gels (Wienhausen and DeLuca, 1985).

Although the *Photinus* luciferase was isolated in the 1940s and has been extensively studied, the mechanism, kinetic characteristics of reaction, and role of the protein in the mechanism of bioluminescence are still not completely understood. The active center and amino acids responsible for ATP binding and catalysis have not been identified. The mechanism of how the high quantum yield is achieved during luciferase catalysis is still a mystery and an interesting project.

The amino acid sequences of the previously identified three peptides related to luciferin or nucleotide binding sites cannot be found in the cloning of luciferase gene. This information gap needs to be explained and reexamined.

Using substrate analogs, amino acid residue specific modifying reagents or other methods to modify the enzyme is an efficient approach to reveal the mystery of the active

sites of firefly luciferase. In the following chapters, the modification experiments which were carried out trying to discover some useful information about the active sites of firefly luciferases will be presented.

Photuris luciferase has not been extensively studied. Before 1980, a few papers reported partial characteristics of the crude enzyme, but there have been no recent papers on the subject. There are a lot of differences in bioluminescence properties between *Photinus* and *Photuris* luciferases. Are there differences in catalytic mechanisms between these two luciferases? How closely related are their nucleic acid sequences? Are there any advantages in analytical application of *Photuris* luciferase over the*Photinus* enzyme? Some of these questions will be dealt with in this thesis.

The research reported in this thesis had four major goals:

1. Study of the active sites of *Photinus* luciferase by modification with chemical and physical treatment to select reagents for labeling amino acid residues at the ATP-binding sites.

2. Isolation and sequencing of the peptides from *Photinus* luciferase labeled with a lysine-modifying reagent, thiourea dioxide.

3. Construction of a cDNA library from *Photuris pennsylvanica* tails, with cloning and sequencing of the firefly luciferase gene from this insect.

4. Characterization of the partially purified firefly luciferase from *P. pennsylvanica.*

Subsequent chapters address each of those goals. A final chapter summarizes the data and discusses their place in our current understanding of firefly luciferase.

CHAPTER II

MODIFICATION OF FIREFLY LUCIFERASE

Introduction

Firefly luciferase produces light via the oxidation of luciferin with a quantum yield of 0.9 (Seliger and McElroy, 1959). This high quantum yield (in other bioluminescence and chemiluminescence reactions the yield is 0.01 or less) makes it the most efficient bioluminescence reaction known. The firefly luciferase protein provides a chemical environment that enables achievement of this efficient energy conversion. The marked conformational changes in firefly luciferase during substrate binding and catalysis may provide part of the chemical basis for this high quantum yield (DeLuca and Marsh, 1967). In order to address this mystery, it is important to determine the interactions among the conformation, structure, active site, and mechanism of light production of firefly luciferase during catalysis.

DeLuca and McElroy (1984) suggested that there are two active sites on the *Photinus pyralis* luciferase with K_ms that differ by ten-fold after they found that luciferase displayed two different time courses of light production during assays at low and high concentrations of ATP. Two ATP molecules are believed to bind to produce one luciferyl adenylate. ATP appears to function both as a substrate and as a regulator. The time course of the enzymatic reaction (either a flash or steady production of light) depends upon the ATP concentration (Leach, 1986; Leach and Webster, 1986; Webster et al., 1979; Webster et al., 1981; DeLuca et al., 1979). With low concentrations of ATP (pM to nM) there is a fairly constant output of light. When larger ATP concentrations are used (μM to mM),

there is a flash of light followed by a slowing in light production due to product inhibition (oxyluciferin). There are different opinions of how to explain the two time courses of light production. Not everyone is satisfied with the idea of two different active sites in luciferase. The two time courses of light production may be due to different conformations of luciferase (Ugarova et el., 1994).

For studying the relationship between nucleotide-binding sites and catalytic properties of firefly luciferase, many nucleotide analogs were used to determine their effect on the activity of firefly luciferase (Ford et al., 1992). Analogs that increased the activity of firefly luciferase included dATP, adenosine 5'-O-(3-thio)triphosphate, periodate-oxidized ATP, 3'-deoxyadenosine triphosphate, etc. There was little effect of most analogs on the pattern of the constant-light production, but there was a marked enhancement of the activity by these analogs on the pattern of the flash-light production. Several affinity-labeling reagents for nucleotide-binding sites such as 8-azidoATP and periodate-oxidized ATP, were able to activate firefly luciferase (Ford et al., 1992).

Many nucleotide analogs and nucleotide-binding-site reagents have been used to label active sites of enzymes. 8-Azido-ATP is a photoaffinity label for two distinct ATP sites on rat liver carbamoyl phosphate synthetase I that allowed preliminary localization of these sites (Powers-Lee and Corina, 1987). One of the more successful reagents for the covalent modification of nucleotide-binding proteins has been 5'-fluorosulfonylbenzoyladenosine (FSBA), an analog of ATP that alkylates amino acid residues present in the ATP-binding sites of a wide range of enzymes (Anostario et al., 1990). FSBA was used by Lee et al. (1981) to label a presumed nucleotide binding site of firefly luciferase. Pyridoxal -5'-phosphate, a chemical modification reagent that reacts with lysine residue to form a Schiff base (stablized by adding $NaBH_4$) at or near phosphate-binding sites, reacts with glutamine synthetase causing a loss of activity, which indicates that lysine(s) appear to be at or near the active site of *Escherichia coli* glutamine synthetase (DiIanni and Villafranca, 1989). *o*-Phthalaldehyde (o-PA) modifies the essential reactive

amino acid residues of 5-enolpyruvoylshikimate-3-phosphate synthetase (EPSP synthetase). The results indicated that o-PA inactivated EPSP synthetase by forming two isoindole derivatives formed from the reaction of lysine and cysteine residues with o-PA (Huynh, 1990). Thiourea dioxide, which reacts with the ε-NH_2 of lysine to produce homoarginine, was found to inactivate glutamine synthetase with total loss of activity and concomitant modification of a single lysine residue (Colanduoni and Villafranca, 1985). A tryptophan-specific reagent, *N*-bromosuccinimide (NBS), can specifically modify the nucleotide binding site-Trp^{235} of mitochondrial F_1-ATPase from *Schizosaccharomyces pombe* (Divita et al., 1993). Diethyl pyrocarbonate (DEPC), which reacts preferentially with histidyl residues, was used to modify deoxynucleotide kinase, indicating that histidine played a role in catalytic function (Brush and Bessman, 1993).

The specificity of modification reagents makes their use a viable approach, not only for dissection of the active site, but also for identification of residues essential for enzymatic activity. To identify the active sites of firefly luciferase, the chemical reagents described above, other ATP analogs, and physical treatments were used to modify firefly luciferase. The modification experiments presented in this chapter demonstrated that 1) firefly luciferase is inactivated by FSBA, PLP, o-PA, and TUD and these inactivations are prevented by ATP; 2) NBS and DEPC inhibit the firefly luciferase activity, but neither ATP or luciferin protect; 3) photoaffinity-labeling reagents such as 8-azido-ATP cannot be applied to FL owing to the sensitivity of FL to UV irradiation. On the contrary, 8-azido-ATP protects FL from UV inactivation; 4) FL is rapidly inactivated at pH value below pH 5.5.

Materials and Methods

Reagents

Photinus pyralis luciferase from Sigma (L-9009) and crystalline enzyme prepared in this laboratory were used interchangeably. D-Luciferin (L-9504) was obtained from

Sigma and dissolved at 5 mg/ml in 20 mM Tricine, pH 7.8; the pH was adjusted to 7.5 with NaOH. The solution was stored in foil-wrapped brown bottles and frozen under nitrogen. 5' [*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA), *o*-phthalaldehyde (*o*-PA), pyridoxal 5'-phosphate (PLP), thiourea dioxide (TUD), *n*-bromosuccinimide (NBS), diethyl pyrocarbonate (DEPC), ATP, and ATP analogs were all obtained from Sigma. SDS-PAGE reagents and molecular-weight-protein standards were from either Bio-Rad or Sigma.

Buffers: Tricine complete (TB 1) contained 250 mM Tricine, 50 mM $MgSO_4$, and 5 mM EDTA, pH 7.8; TB 1 contained DTT (5 mM) is TB 2; phosphate buffer contained 0.1 M NaH_2PO_4, pH 7.8, (NHPB); enzyme dilution buffer (EDB) contained 50 mM Tricine, 10 mM $MgSO_4$, 1 mM EDTA, 20 mM DTT, and 0.15 mM bovine albumin (BSA), freshly prepared; enzyme assay buffer (EAB) contained 25 mM Tricine, 5 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM DTT, and 0.18 mM luciferin, freshly prepared.

Firefly Luciferase Purification

Firefly luciferase was purified from 10 g of dried *Photinus pyralis* lanterns purchased from Sigma or collected locally around Stillwater according to the method reported by DeLuca and McElroy (1978) and modified in this laboratory (Hall et al., 1984). Crystallized luciferase produced a single band on Coomassie-stained SDS-PAGE with a MW of approximately 61 kD (Fig. 1). The enzyme was stored at –20 °C in the dark as a 4-12 mg/ml solution in 10% $(NH_4)_2SO_4$ and 1 mM EDTA, pH 8.0.

Luciferase Assays

Luciferase activity assays were performed on a Lumac/3M Model 2010 A Biocounter (ATP concentration, 0.33 μM) or a SAI Technology Model 3000 Luminometer (ATP concentration is between 0.33 μM and 1.1 mM). Assay on Lumac/3M Model 2010 A Biocounter: firefly luciferase was dissolved in TB 1 or NHPB buffer to 16.4 μM. A 2

µl aliquot of FL solution was diluted with 500 µl of EDB. Diluted luciferase (10 µl) was added to a Lumacuvette that contained 490 µl EAB with 0.33 µM ATP, briefly mixed, and placed in the luminometer. Enzyme activity was expressed as Relative Light Units (a RLU is 10^3 counts in the assay time) displayed by the luminometer. The assay was conducted for 10 s and duplicate assays were done for each sample. Assay on SAI Technology Model 3000 Luminometer: 10 µl of diluted luciferase solution was mixed with 390 µl EAB and placed in the luminometer. The reaction was initiated by rapid injection of 100 µl of 1.66 µM or 5.5 mM ATP with a Digital Syringe Diluter. The instrument settings were: sensitivity 7.0; zero setting 4.30; delay 0.5 s; count time 10 s. An aluminum disk with a small hole was used to attenuate the light striking the phototube to 0.00053 of that without attenuation.

The kinetics of the firefly luciferase reaction were determined by using an LKB Model 1251 automatic luminometer controlled by a Laser Turbo PC-401 computer with the parameters of a time constant of 0.5 s, continuous mixing, no delay, and a temperature of 25 °C, with readings taken every 2 s. The reaction mixture contained 170 µl of 28.6 mM Tricine buffer at pH 7.8, 5.7 mM $MgSO_4$, 0.57 mM EDTA, 0.21 mM luciferin, and 0.57 mM DTT; the luciferase amounts used varied between 2 and 20 ng. After 6 s incubation of the enzyme-containing reaction mixture in the chamber, 30 µl of ATP (0.24 µM for the constant-light production assay, 0.24 mM for the flash-light production) was injected with an LKB Model 1291 injector. Each experimental condition was reproduced three times and the average measured values plotted. Standard deviations were < 5%.

Modification Procedures

Modifying reagents were prepared as follows: FSBA was dissolved in DMSO to 20-200 mM; o-PA stocks, 0.5-2 mM, were prepared with 100% ethanol and diluted to 50-200 µM with water; PLP was made 0.65-5.2 mM with 100 mM Hepes (pH 7.8); NBS stock, 80 mM, was prepared with water and adjusted to pH 7.2, then diluted to 0.5-40 mM

with water; DEPC, 2-40 mM, was prepared with 100% ethanol; TUD was dissolved in NHPB to concentrations of 12.5-200 mM.

Luciferase (16.4 μM) solution was made with TB 1 or NHPB. An experimental time course was developed with staggered sampling times, allowing aliquots to be removed and diluted in a minimal amount of time. At room temperature, the inactivation reaction was initiated by addition of 0.1 volume of the appropriate modifying reagent stock into FL solution, to give varying final concentrations of each reagent. The controls were done by addition of the various solvents used for dissolving the modifying reagents. Inactivation was stopped by removing 2-μl aliquots of solution at the time points and quenching by 250 fold dilution in EDB. The activity of FL was assayed immediately by mixing a 10 μl diluted sample with 500 μl EAB and the light production was counted on the Lumac/3M Model 2010 A Biocounter or SAI Technology Model/3000 Luminometer.

Protection by Substrates

The inactivation of FL by various modifying reagents was performed in the presence and absence of MgATP and/or luciferin in order to test for protection by substrates. These experiments were done by using the same procedures as the testing of the various modifying-reagent inactivations described above. ATP (50 mM) and/or luciferin (1.78 mM) were added to the enzyme (16.4 μM FL) 5-30 min prior to initiation of inactivation and the mixture incubated at room temperature. Aliquots were removed and diluted in EDB at varying times after addition of modifying reagents.

UV Inactivation

While FL (16.4 μM) was incubated in a quartz cuvette under UV (254 nm, 0.53 mW/cm^2) on ice, the activity of FL was measured at 0, 5, 10, 20, 40, 60, 90 and 120 min by removing aliquots of 2 μl to the assay buffer. At the same times, 24 μl of sample was taken from the cuvette and mixed with 8 μl of 4 x sample buffer for SDS-PAGE.

The following reagents were used as potential UV protector: 10 mM azido ATP, 50 mM ADP, 50 mM *p*-aminobenzoic acid (PABA), 50 mM sodium phosphate, 50 mM sodium pyrophosphate, 50 mM adenine, 40 mM ATP, 50 mM adenosine, 25 mM tryptophan, 0.1 M potassium phosphate, 12.5 mM tyrosine, 50 mM sodium arsenate, 4 mg/ml lecithin, 50 mM DTT, and 7.1 mM luciferin. Each protecting reagent (3 μl) was added to 30 μl of 16.4 μM FL solution. Each sample was incubated under UV (254 nm, 0.53 mW/cm) for 60 min. An aliquot of each sample was assayed for activity and the remaining sample was analyzed by SDS-PAGE.

Low-pH Inactivation

Tris (30 mM)/Succi/ES buffer for low-pH inactivation of FL was prepared by titrating 60 mM Tris with 0.85 M succinic acid to pH 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, and 4.0, then mixed with an equal volume of enzyme stabilizer/TB 1.

The activity of FL was assayed every 30 min after incubating FL in 30 mM Tris/Succi/ES buffer with different pH values. FL dissolved in TB 1 was treated as the control. Inactivation of FL by high ATP with low pH was performed by incubating FL (16.4 μM dissolved in a low-ion Tricine (3 mM) buffer) with 10 mM ATP (pH 7.5 and 3.2 separately) and measuring the activity of FL at 0, 15, 30 and 60 min. An aliquot of FL sample after 99.9% inactivation was analyzed by SDS-PAGE.

SDS-Polyacrylamide Gel Electrophoresis

Changes in luciferase molecular weight were monitored by discontinuous SDS polyacrylamide gel electrophoresis (Laemmli, 1970). Gels consisted of a 1 to 2 cm stacking gel (4% acrylamide, 2.7% crosslinked) above a 5-6 cm resolving gel (10% acrylamide, also 2.7% crosslinked), 0.75 mm thick. Samples and protein molecular weight standards (Sigma MW-SDS-200 Kit or Sigma MW-SDS-70L Kit) were subjected

to electrophoresis using a mini gel apparatus (Bio-Rad Mini Protean II) at 80 V per gel for 3 hr. Protein bands were visualized by staining with Coomassie R-250.

Results

FSBA Effect Is Different Between Two ATP Binding Sites of Firefly Luciferase

Firefly luciferase (FL) was inactivated by FSBA. Figure 2 shows the time course of inactivation by 2 mM FSBA; there was an 85% decrease in enzyme activity during the 2-h incubation. Either ATP or adenosine (2 mM) protected against the inactivation. The protection was much greater with ATP (71% protection) than with adenosine (26% protection). Luciferin (1.78 mM) partially protected against inactivation (39%).

FSBA inactivation was different depending on the ATP concentration used for activity determination (Fig. 3). The constant-light production activity of FL (at low ATP concentration) was not diminished after 80-min incubation with addition of 1 µl of either 5.6 or 20 mM FSBA to 60 µl of enzyme every 20 min. On the contrary, there was activation from addition of FSBA during the first 80 min of incubation (Fig. 3A). FSBA-treated enzyme had 40% more activity than the enzyme of the FSBA-free control. After 20 min of incubation with 5.6 mM FSBA, 96% of the light production remained . There was 52% of the light production activity remaining when the enzyme was treated with addition of 1 µl of 20 mM FSBA to 60 µl of enzyme every 20 min over a period of 120 min. The flash-light production activity (at high ATP concentration) was diminished by incubating FL with FSBA, with 61% activity remaining after 80 min of incubation and 35% activity remaining after 120 min regardless of which FSBA concentration was used (Fig. 3B). Thus only after 80 min of incubation was the constant-light production activity of firefly luciferase reduced due to FSBA inactivation.

Firefly Luciferase Is Inactivated by o-PA, PLP, and TUD and the Inhibition Is Prevented By ATP

Firefly luciferase was rapidly inactivated by incubation with the amino-reactive reagent *o*-phthalaldehyde (*o*-PA) (Fig. 4A). ATP (50 mM) can partially protect against *o*-PA inactivation, as shown in Fig. 4B. The inactivation by *o*-PA and the protection by ATP were equal when assayed at both high and low ATP concentration (data not shown).

Incubation of firefly luciferase with pyridoxal-5'-phosphate (PLP) caused inactivation of FL. This inactivation was concentration-dependent, with 59, 76, 89, and 96% inhibition produced by PLP concentrations of 0.65, 1.3, 2.6, and 5.2 mM, respectively (Fig. 5A). There was partial protection against the PLP-produced inactivation by 50 mM ATP (Fig. 5B). The characteristics of inactivation by PLP and protection by ATP were similar with respect to the assay at high and low ATP concentration (data not shown).

The thiourea dioxide (TUD) inactivation of firefly luciferase was time- and concentration-dependent (Fig. 6). Thiourea was a much less effective inhibitor, producing only 25% inhibition after 60 min of incubation with 100 mM thiourea as compared to 68% inhibition achieved with that concentration of TUD. There was no inhibition by sodium thiosulfate under the same conditions. The FL activity (60%) was protected from TUD (50 mM) inhibition by ATP (10 mM) (Fig. 7). TUD inhibition occurred similarly in both high and low ATP assays (Fig. 8 and Fig. 9). The FL activity was inactivated 25-98% during 0-180 min incubation with 50 mM TUD. Both the constant-light (at low ATP concentration) and the flash-light production (at high ATP concentration) of FL were reduced to 50% at 30 min incubation with 50 mM TUD.

Firefly Luciferase Is Inactivated By DEPC and NBS Without ATP Protection

Histidine residues in proteins can be modified by diethyl pyrocarbonate (DEPC) treatment. Figure 10 shows the time course and concentration-dependent inactivation of

firefly luciferase by DEPC. FL was rapidly inactivated during the beginning 5 min incubation with 0.5-4 mM DEPC. Hydroxylamine (30 mM) protected 60% of FL activity from 3 mM DEPC inactivation, but could not reverse the inhibition.

The tryptophan-specific reagent *N*-bromosuccinimide was used to inactivate firefly luciferase. There was a concentration- and time- dependent NBS inhibition of FL activity (Fig. 11). There was marked protection against this inactivation by 1.78 mM luciferin, which was found during the early experiments. Luciferin (3.4 mM) was later found to be destroyed by incubation with NBS (3.8 mM); luciferin treated by NBS lost its function as the substrate for FL. Thus the luciferin may be oxidized by NBS and the protection of FL from the NBS inactivation by luciferin may be nonspecific.

Firefly Luciferase Is Inactivated By UV Irradiation

UV irradiation of enzymes with photoactivable nucleotides has been used to label the nucleotide-binding sites. When this procedure was attempted with firefly luciferase (FL), there was significant inactivation of FL in the irradiated control (without nucleotide analog). The time course of FL inactivation by UV irradiation is shown in Fig. 12. Equivalent inactivation was produced on laboratory-purified crystalline FL, the crystalline enzyme from Sigma and the recombinant-produced enzyme from Amgen. The half-life of the enzyme was 15 min and only 1.2% of the activity remained after a 90-min treatment. When the UV-treated sample was analyzed by SDS-PAGE, a molecular weight form higher than the dimer was observed (Fig. 13). The crosslinking and degradation of the enzyme were followed by gel electrophoresis. After 10 min of irradiation there were three bands with molecular weight higher than that of the subunit of FL (62×10^3). Those bands had molecular weights of 255, 210, and 72×10^3. After 10 min irradiation proteins with lower molecular weight also resulted, e.g. 57, 49.5, 48, 43, 39.5 and 30×10^3.

The addition of nucleotides or other compounds that absorbed UV protected FL from irradiation (Table I). For example, 10 mM azido ATP, 50 mM ADP, 50 mM PABA,

40 mM ATP, and 50 mM adenine all protected. The remaining activity after 60 min treatment (inactivation in the control to 7% activity remaining) was 58, 92, 74, 85, and 87, respectively. By SDS-PAGE, it was shown that these compounds could protect FL against degradation or crosslinking caused by UV irradiation.

Firefly Luciferase Is Inactivated at Low pH

Firefly luciferase is inactivated when incubated at pH values below 5.5 for 30 min or longer. Figure 14 shows the stability of firefly luciferase incubated at different pH values. Electrophoresis of the low-pH-treated preparation did not show any degradation of FL (data not shown).

Discussion

In this chapter, a number of modifying reagents were used to modify firefly luciferase in seeking a useful reagent that would act at the ATP-binding site. The time- and concentration-dependent inactivations by these reagents were measured at both low and high ATP concentrations. The protection by ATP from inactivation by these reagents is a criterion for selecting a reagent for further work. Luciferin, another substrate, was also tested as a protector against the inactivation of luciferase by these reagents.

The modification of firefly luciferase by FSBA as originally reported by Lee et al., (1981) was attempted. The same concentration of FSBA (0.09 - 0.56 mM) as used in Lee's experiments did not inactivate FL when low ATP (0.24 μM) was used for the assay. At low concentrations (0.09-1.3 mM of FSBA added to enzyme solution at the beginning of a 80-min incubation), FSBA decreased the FL activity only at the high [ATP] assay (0.24 mM), but not in the low [ATP] assay. On the contrary, low concentration of FSBA enhanced the activity of FL. In previous experiments, it was found repeatedly that low FSBA (0.2 mM) could increased the activity of FL during low [ATP] assay. These items of evidence suggest that low FSBA selectively binds to one site, which may change the

conformation of FL to make the enzyme more active to produce the constant light. These results can be explained by a simple model involving ATP as a regulator. The binding of a nucleotide could induce a conformational change resulting in increased affinity for ATP leading to enzyme activation for producing the constant light. DeLuca and McElroy (1984) believe that two ATP molecules are bound for production of one luciferyl adenylate; ATP appears to function both as a substrate and as a regulator. An allosteric effect by ATP analogs was discovered in luciferase from a European firefly species, *Luciola mingreica*. Three nucleotide-binding sites (two allosteric, located distant from the active site) are postulated for this luciferase species (Filippova and Ugarova, 1983). The activation of FL by low FSBA in the low-ATP assay demonstrates that ATP may act as a regulator to bind one active site, then influence the other active site by allosteric effects. Therefore the ATP-binding site may have a regulatory as well as a catalytic role. High concentrations of FSBA (>1 mM of FSBA added to FL solution with 80 min to 180 min of incubation) inactivated the enzyme in both high- and low- [ATP] assays. The finding that the FSBA effects differs in the low ATP (for the constant-light pattern) and high ATP (for the flash-light pattern) assays may be a useful molecular probe to study the active site of firefly luciferase.

Since the previous study by Lee (Lee et al., 1981) showed a Lys residue is related to the active site of firefly luciferase, a number of $-NH_2$ reagents were tested for modification of firefly luciferase. From the results presented in this chapter, it appears that certain $-NH_2$ groups are involved either at the active sites or in the important conformational changes. Except for the FSBA inactivation, the inactivations of luciferase by these analogs occur similarly in both high- and low- ATP assays. ATP can prevent the inhibition of FL by these analogs, thus suggesting that these analogs are bound to the ATP site.

The phenomenon of emission of red light by luciferase is due to relatively small perturbations of the active site. The spectral shift from yellow-green to red upon decreasing the pH of the reaction medium is likely due to protonation of a basic amino acid

residue whose function is to abstract a proton from C-5 of oxyluciferin (Vellom, 1990). The suggestion has been made that an active-site sulfhydryl (White et al., 1969) or histidine residue might be responsible for the luciferase activity (McElroy et al., 1965). In our experiments, the tryptophan- and histidine- specific reagents NBS and DEPC rapidly inactivated FL. ATP and luciferin failed to protect against the inactivation. The sites modified by NBS and DEPC seem unrelated to the ATP sites. Therefore the importance of Trp and His residues on the luciferase activity needs to be further examined.

8-Azido-ATP was successfully applied to label the ATP-binding sites of the recBCD enzyme of *Escherichia coli.* The initiation of crosslinking 8-azido-ATP to enzyme was performed using a short-wavelength lamp of 254 nm (Julin and Lehman, 1987). Photoaffinity labeling of bovine pancreatic ribonuclease A with 8-azidoadenosine 3', 5'-bisphosphate was studied at longer wavelengths (300 nm) because the irradiation of RNase A with UV at 254 nm led to a rapid inactivation of the enzyme (Wower et al., 1989). In the experiments described here (Fig. 12 and Fig. 13), firefly luciferase was found to be sensitive and unstable when exposed to UV of short wavelength (254 nm). Further experiments need to be done to examine whether firefly luciferase is stable at longer wavelength (>300 nm).

Since there is a linear dependence between loss of activity and degradation or crosslinking (Fig. 12 and Fig. 13), such alterations of the enzyme are suggested to result in the inactivation of FL. The nucleotides or compounds that absorb UV protect the activity of FL against degradation and crosslinking. The most important factor in the UV inactivation of most enzymes is photodegradation of the cystine and tryptophan residues in the protein (Sapezhinskii, 1986). Reactive free radicals are the basis for the photochemical modification of proteins and lead to photodegradation of many amino acid residues, the formation of crosslinks, rupture of peptide bonds, and photo-oxidation of proteins (Sapezhinskii, 1986). When RNase A was irradiated at 254 nm, there was a linear relationship between loss of activity and destruction of cystine (Schultz et al., 1975).

Firefly luciferase (Fig. 13) displays crosslinking of enzyme molecules as evidenced by the production of higher-molecular-weight proteins after UV irradiation. The mechanism of formation of the crosslinks might relate to cystine (CySSCy) formation in firefly luciferase. As another example, disulfide formation was observed by flash photolysis of CySH at pH >6 (Grossweiner, 1976). The degradation of FL occurred after 10 min of UV irradiation, which is another cause of loss of FL activity. There are other examples of photodestruction of enzymes by UV. UV irradiation of acetylcholinesterase caused progressive enzyme inactivation accompanied by a progressively decreasing molecular weight (Bishop et al., 1980). So far, we do not know whether the degradation of firefly luciferase is due to breaking of peptide bonds or loss of aromatic chromophores. The nature of UV-induced conformational changes in the secondary and tertiary structure of proteins needs to be studied to provide more information about the relationship between the function and structure of FL.

Firefly luciferase is inactivated at low pH (below 5.5). SDS-PAGE of the treated samples did not show any differences from the control samples. The pH inactivation of apomyoglobin involves the breaking of a hydrogen bond between His 24 and 119 when the pH is decreased from 6 to 4 (Barrick et al., 1994). Therefore low-pH inactivation of FL may be caused by His protonation to change the conformation of the enzyme.

In this chapter, firefly luciferase was modified by using several group-specific reagents, affinity-labeling reagents and physical treatments (UV irradiation and low pH). The results suggest that $-NH_2$, Trp, and His are involved in structure/function of firefly luciferase. Thiourea dioxide was selected to label the ATP-binding sites of firefly luciferase in the experiments reported in Chapter III.

Figure 1. SDS-PAGE of FL Purification

10% acrylamide, 2.7% crosslinked. Lane 1: protein standard, A = myosin (205 kD), B = galactosidase (116 kD), C = phosphorylase B (97.4 kD), D = albumin, bovine (66 kD), E = albumin, egg (45 kD), F = carbonic anhydrase (29 kD). Lane 2: crude extract. Lane 3: calcium phosphate fraction. Lane 4: $(NH_4)_2SO_4$ fraction. Lane 5: same as Lane 1. Lane 6: column fraction. Lane 7: crystal fraction.

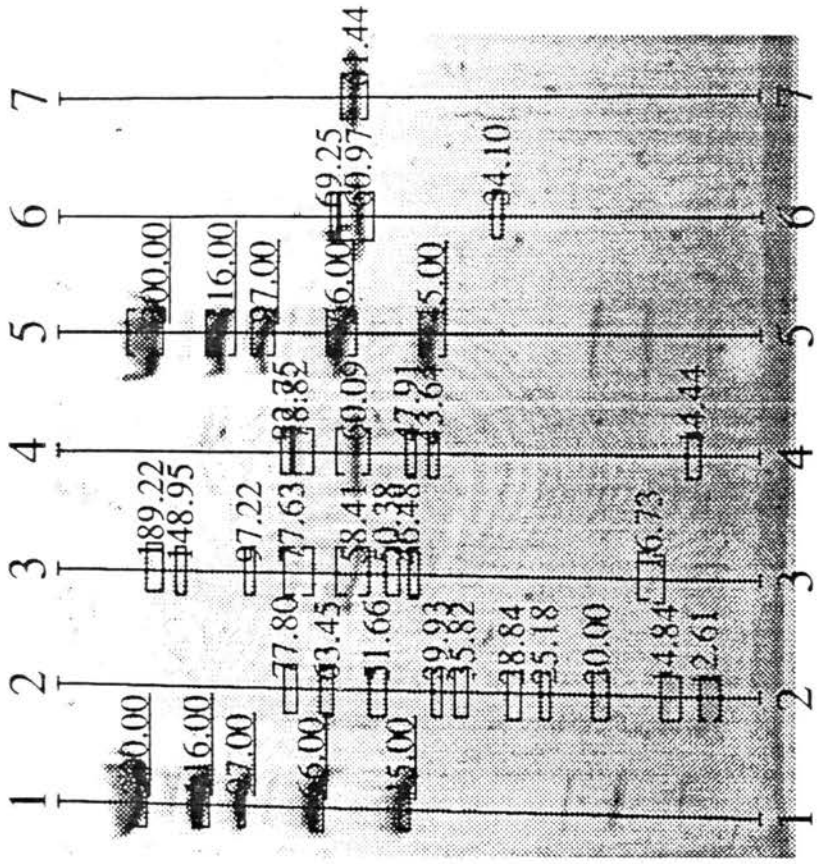

1
2
3
4
5
6
7
0.00
16.00
7.00
6.00
5.00
89.22
48.95
7.22
7.80
3.45
1.66
9.93
5.82
8.84
5.18
0.00
4.84
2.61
6.73
4.44
0.00
16.00
7.00
6.00
5.00
4.10
9.25
0.97
1.44

Figure 2. FSBA Modification and Protection by ATP and Adenine

Firefly luciferase (FL) 16.4 μM was incubated with 2 mM FSBA at room temperature in TC 1, pH 7.8. DMF 2 mM, the solvent of FSBA, was added to the control. ATP (2 mM) or adenine (2 mM) was added to the enzyme for protection prior to the addition of FSBA. Aliquots were removed at the indicated times and assayed in duplicate for FL activity. Low ATP (0.33 μM) was used for assay.

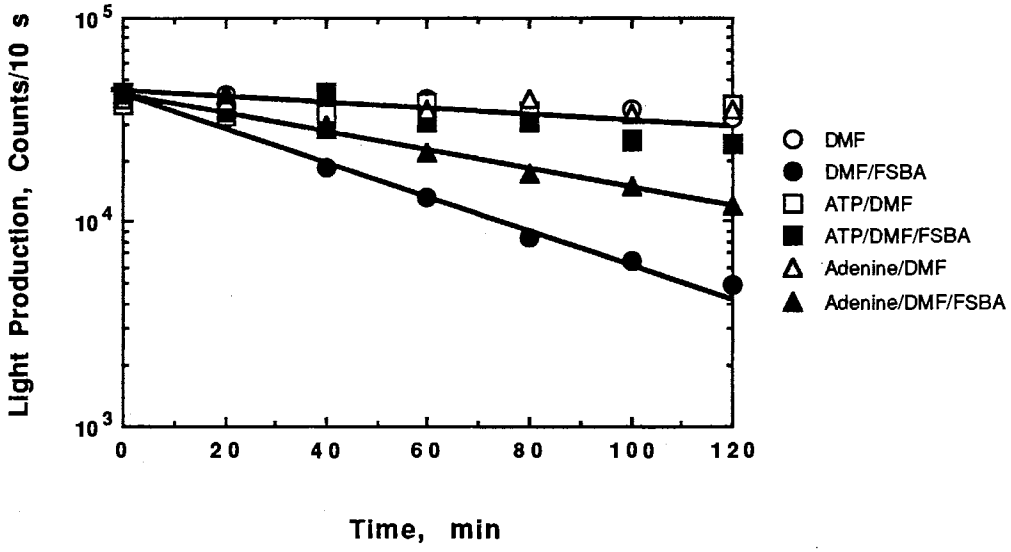
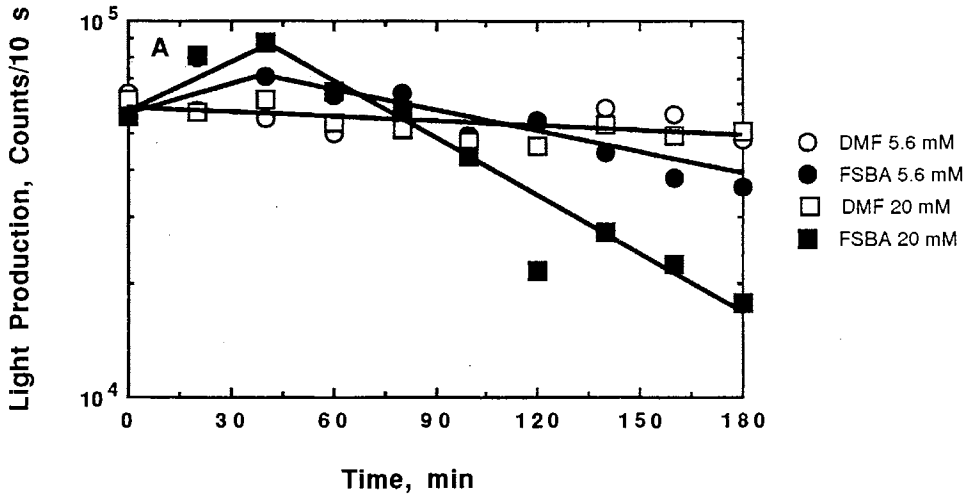

Light Production, Counts/10 s
10^5
10^4
10^3
0
20
40
60
80
100
120
Time, min
DMF
DMF/FSBA
ATP/DMF
ATP/DMF/FSBA
Adenine/DMF
Adenine/DMF/FSBA

Figure 3. FSBA Modification With High and Low [ATP]

To 120 μg of firefly luciferase in 60 μl of 0.1 M sodium phosphate buffer, pH 8.0, was added 1 μl of 5.6 mM or 20 mM FSBA in dimethyl sulfoxide (DMSO) every 20 min over a period of 3 h at room temperature. Small aliquots of the incubation mixture were assayed with high ATP (1.1 mM, Fig. 3A) and low ATP (0.33 μM, Fig. 3B) for enzyme activity at 20-min intervals. A control sample containing luciferase was treated with aliquots of 5.6 mM or 20 mM dimethyl formamide (DMF) in DMSO at 20 min intervals so that the concentration of enzyme, DMF and DMSO were identical with those in the inactivation mixture. The assay was performed by rapid injection of 100 μl of 1.66 μM ATP (for low ATP) or 5.5 mM ATP (for high ATP) to 400 μl of enzyme solution and the light production was measured.


A
10^5
10^4
Light Production, Counts/10 s
0
30
60
90
120
150
180
Time, min
DMF 5.6 mM
FSBA 5.6 mM
DMF 20 mM
FSBA 20 mM

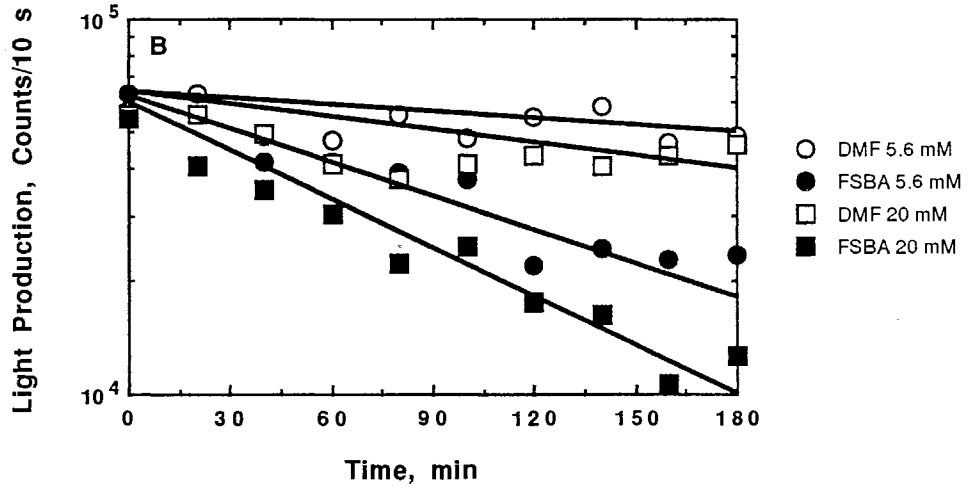
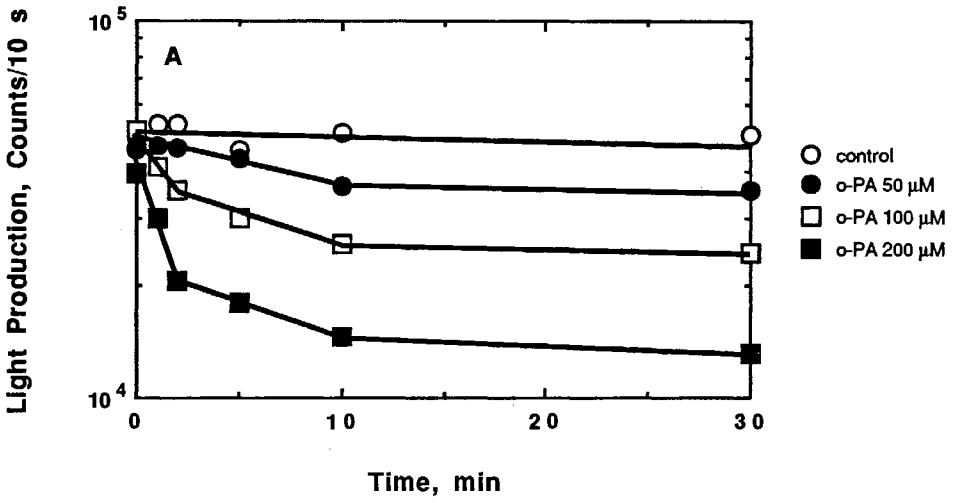

B
10^5
10^4
Light Production, Counts/10 s
0
30
60
90
120
150
180
Time, min
DMF 5.6 mM
FSBA 5.6 mM
DMF 20 mM
FSBA 20 mM

Figure 4. *o*-PA Modification and ATP Protection

Firefly luciferase (16.4 μM) was incubated with varying concentrations of *o*-PA (Fig. 4A) at room temperature in NHPB, pH 7.8. ATP (50 mM)was added to the enzyme 30 min prior to initiation of inactivation by 200 μM *o*-PA (Fig. 4B). Aliquots were removed and assayed in duplicate for FL activity.


A
Light Production, Counts/10 s
10^5
10^4
0
10
20
30
Time, min
control
o-PA 50 µM
o-PA 100 µM
o-PA 200 µM

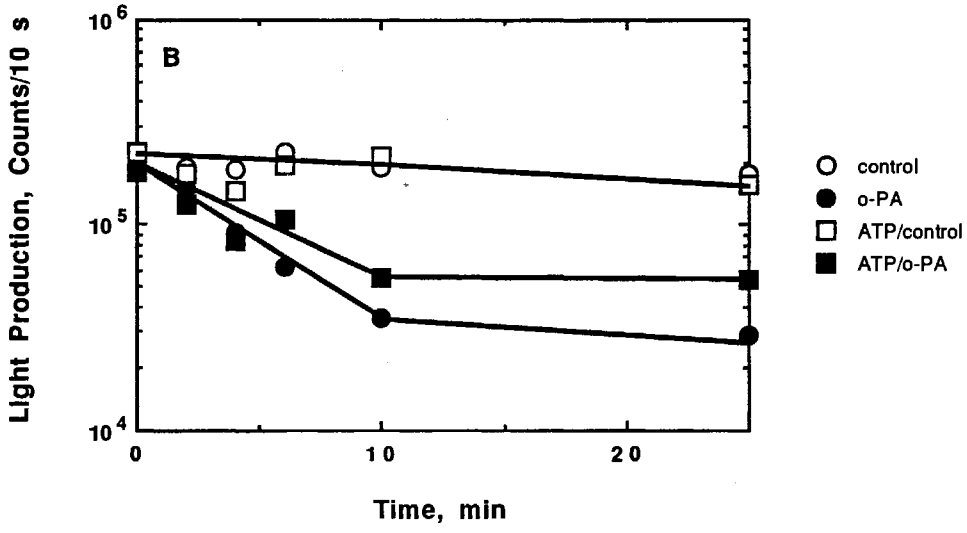
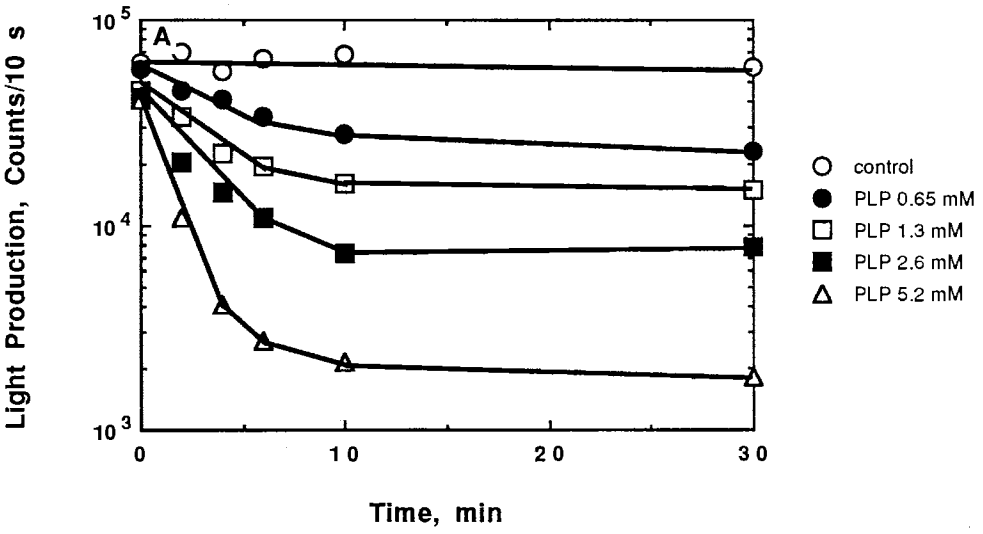

B
Light Production, Counts/10 s
10^6
10^5
10^4
0
10
20
Time, min
control
o-PA
ATP/control
ATP/o-PA

Figure 5. PLP Modification and ATP Protection

The reaction mixture contained 100 mM Hepes (pH 7.8), 16.4 μM enzyme, and PLP at 0.65, 1.3, 2.6, 5.2 mM (Fig. 5A). For substrate protection, FL was incubated with PLP (2 mM) in the presence of 50 mM ATP (Fig. 5B). The mixture was incubated at room temperature. At the time indicated, 2 μl of the mixture was added to EAB with 1 mM $NaBH_4$ to assay the residual activity.


A
Light Production, Counts/10 s
10^5
10^4
10^3
0
10
20
30
Time, min
control
PLP 0.65 mM
PLP 1.3 mM
PLP 2.6 mM
PLP 5.2 mM

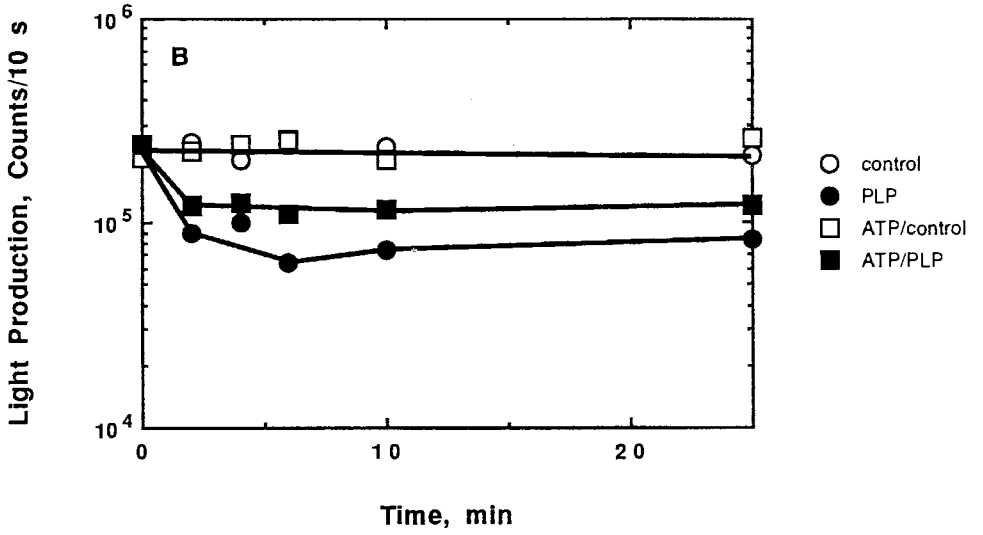

B
Light Production, Counts/10 s
10^6
10^5
10^4
0
10
20
Time, min
control
PLP
ATP/control
ATP/PLP

Figure 6. Rate of Inactivation of Firefly Luciferase by Thiourea Dioxide at Various TUD Concentrations

Firefly luciferase (16.4 μM) was incubated with various TUD concentrations. Aliquots (2 μl) were removed at various time and assayed in duplicate for FL activity.

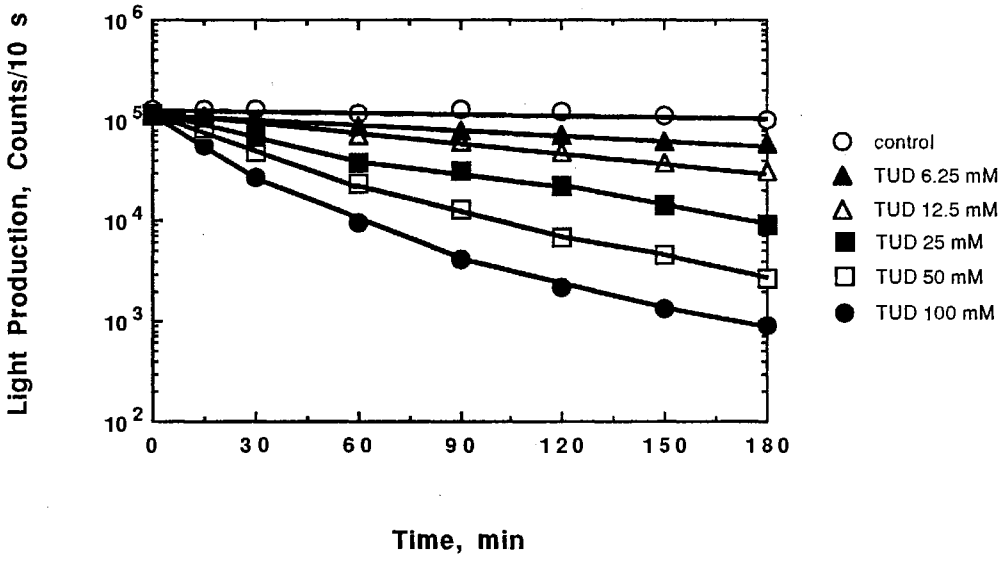

Light Production, Counts/10 s
10 6
10 5
10 4
10 3
10 2
0
30
60
90
120
150
180
Time, min
control
TUD 6.25 mM
TUD 12.5 mM
TUD 25 mM
TUD 50 mM
TUD 100 mM

Figure 7. Protection from TUD Inhibition of FL Activity by ATP

FL was incubated with ATP (10 mM) for 5 min. TUD (50 mM) was then added to the enzyme solution. The activity of FL was measured every 30 min by assaying small aliquots of sample.

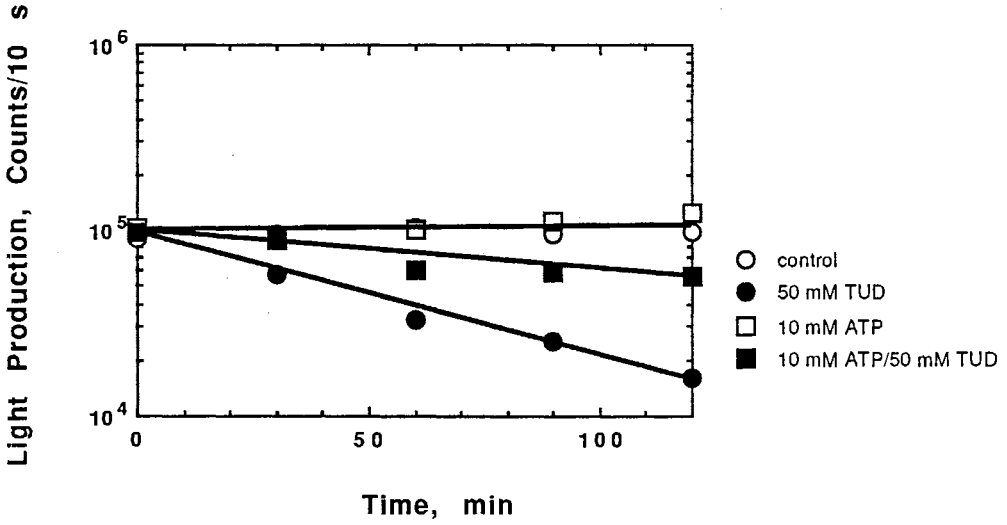
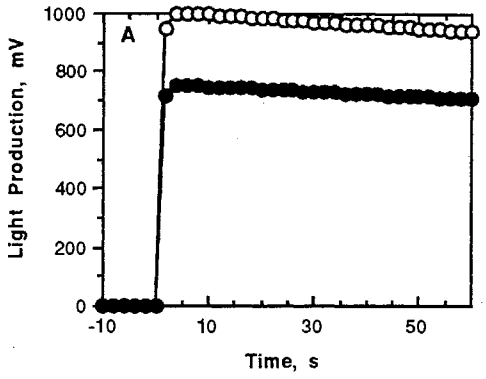
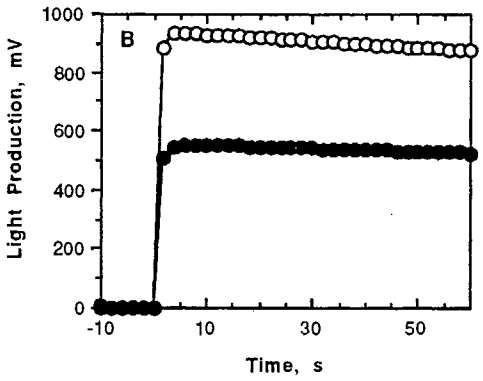
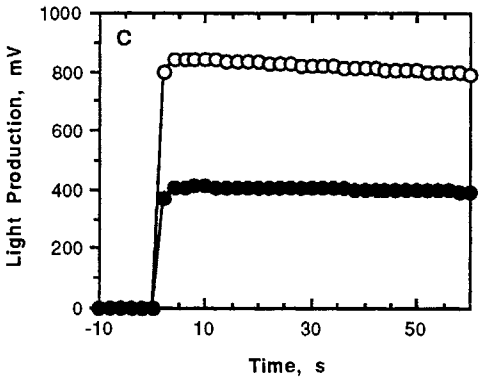

Light Production, Counts/10 s
10^6
10^5
10^4
0
50
100
Time, min
control
50 mM TUD
10 mM ATP
10 mM ATP/50 mM TUD

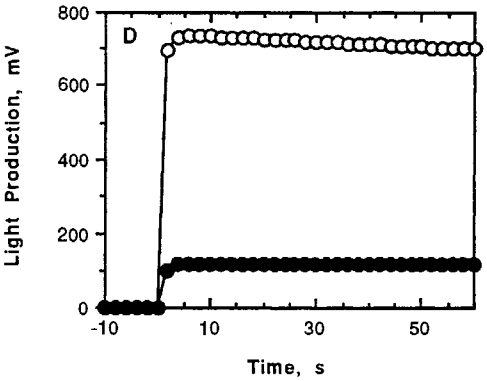
Figure 8. Time Course of Light Production with 0.24 μM ATP during Treatment with TUD.

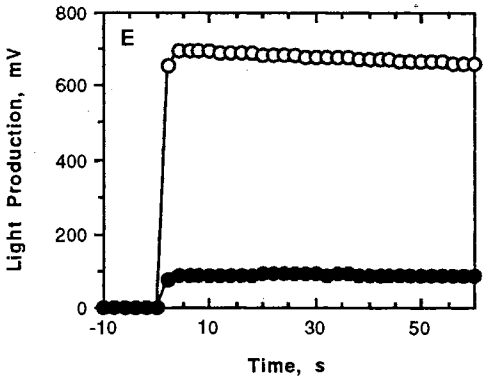
FL was incubated with TUD (50 mM) at room temperature. At various times the inhibition was quenched by removing a 2-μl aliquot which was diluted 1000 times with EDB. The assay was performed with an LKB Model 1251 Automatic Luminometer. The procedures are described in the Materials and Methods section. TUD incubation time (min): 0 (A), 30 (B), 60 (C), 90 (D), 120 (E), 180 (F). The control (-o-) and the TUD- treated (-●-).


A
Light Production, mV
1000
800
600
400
200
0
-10
10
30
50
Time, s


B
Light Production, mV
1000
800
600
400
200
0
-10
10
30
50
Time, s


C
Light Production, mV
1000
800
600
400
200
0
-10
10
30
50
Time, s


D
Light Production, mV
800
600
400
200
0
-10
10
30
50
Time, s


E
Light Production, mV
800
600
400
200
0
-10
10
30
50
Time, s

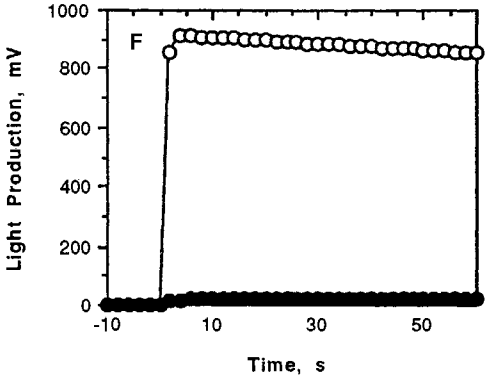
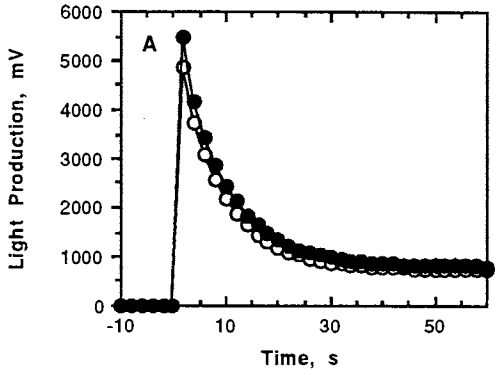
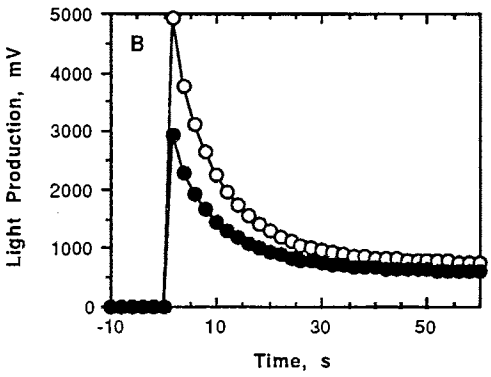
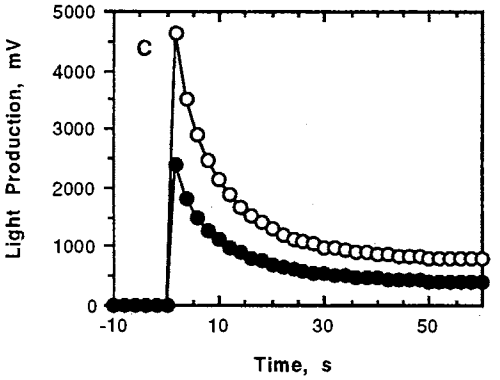

F
Light Production, mV
1000
800
600
400
200
0
-10
10
30
50
Time, s

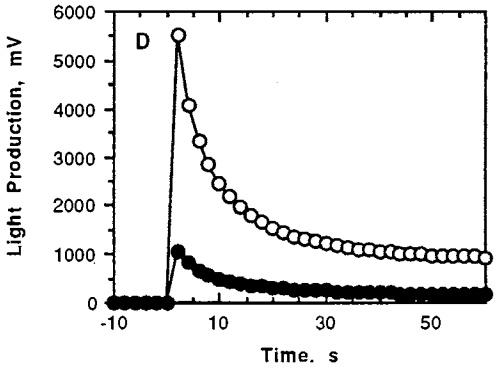
Figure 9. Time Course of Light Production with 0.24 mM ATP during Treatment with TUD.

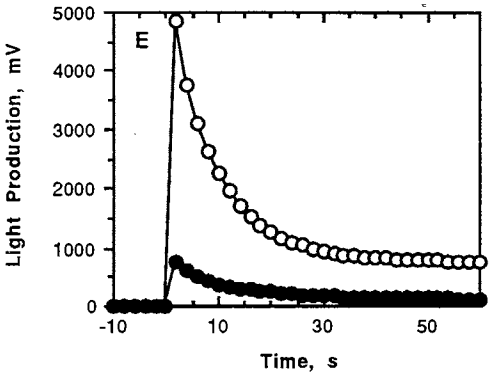
Materials and methods were the same as in Fig. 8. The incubation of TUD (min): 0 (A), 30 (B), 60 (C), 90 (D), 120 (E), 180 (F). The control (-o-) and the TUD- treated (-●-).


A
Light Production, mV
Time, s
6000
5000
4000
3000
2000
1000
0
-10
10
30
50


B
Light Production, mV
Time, s
5000
4000
3000
2000
1000
0
-10
10
30
50


C
Light Production, mV
Time, s
5000
4000
3000
2000
1000
0
-10
10
30
50


D
Light Production, mV
Time. s
6000
5000
4000
3000
2000
1000
0
-10
10
30
50


E
Light Production, mV
Time, s
5000
4000
3000
2000
1000
0
-10
10
30
50

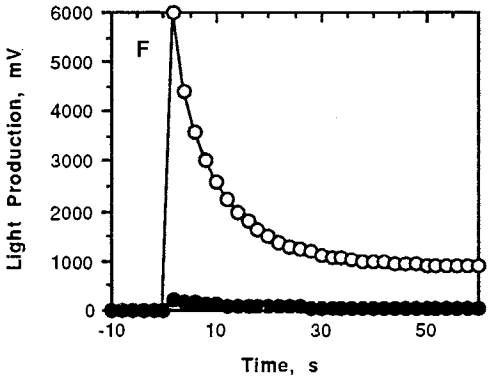

F
Light Production, mV
Time, s
6000
5000
4000
3000
2000
1000
0
-10
10
30
50

Figure 10. Inactivation of Firefly Luciferase by Diethyl pyrocarbonate.

Firefly luciferase (16.4 μM) was incubated with varying concentrations of diethyl pyrocarbonate. At the times indicated, aliquots were withdrawn and assayed for FL activity.

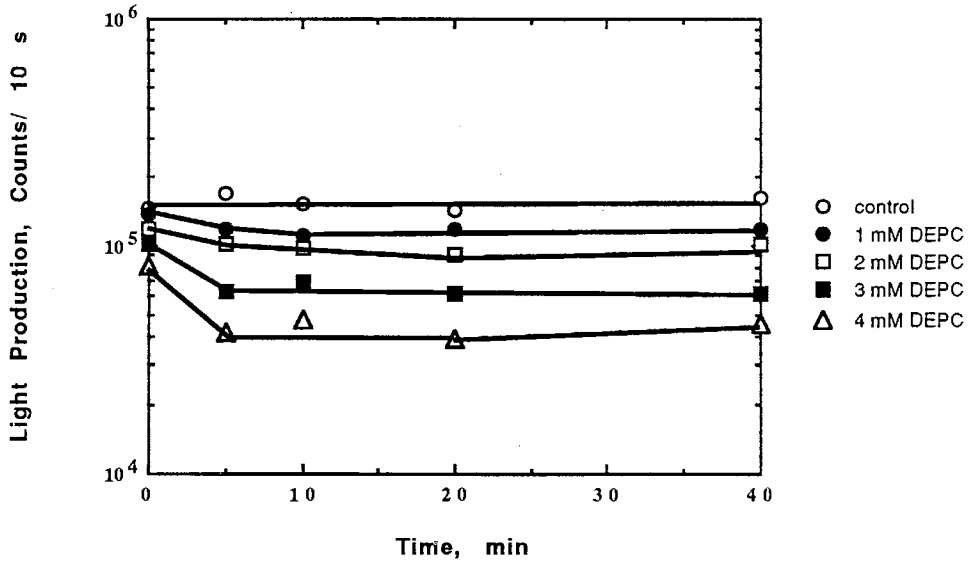

Light Production, Counts/ 10 s
10^6
10^5
10^4
0
10
20
30
40
Time, min
control
1 mM DEPC
2 mM DEPC
3 mM DEPC
4 mM DEPC

Figure 11. Effects of *N*-bromosuccinimide (NBS) Modification on Firefly Luciferase Activity.

Firefly luciferase (16.4 μM) was incubated with the indicated concentrations of NBS at pH 7.8. An aliquot of 2 μl was withdrawn at each time interval and FL activity was determined.

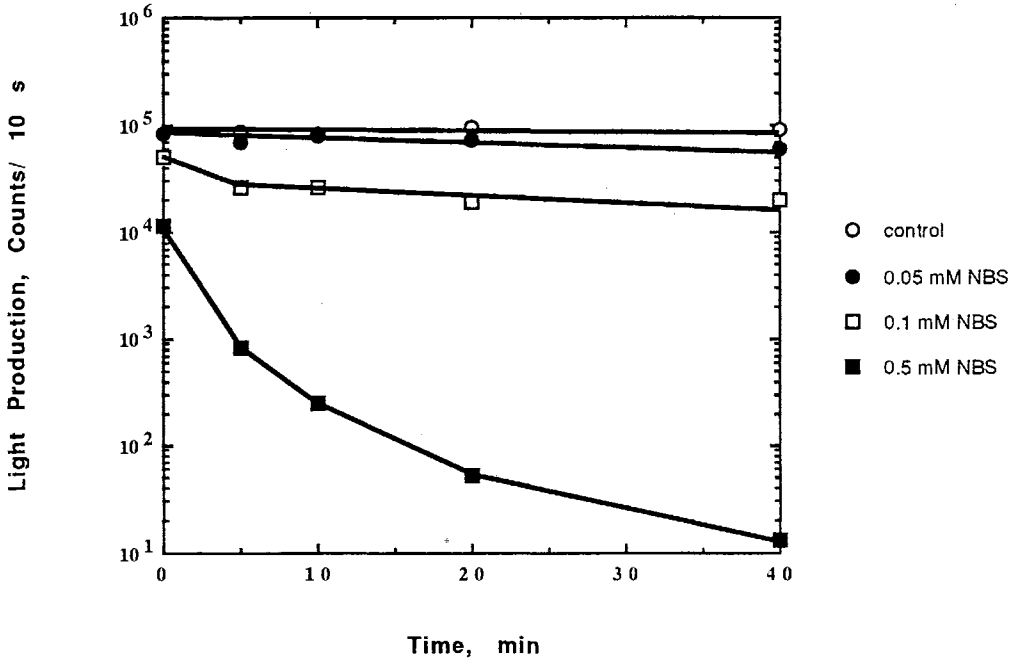

Light Production, Counts/ 10 s
10 6
10 5
10 4
10 3
10 2
10 1
0
10
20
30
40
Time, min
control
0.05 mM NBS
0.1 mM NBS
0.5 mM NBS

Figure 12. Inactivation of Firefly Luciferase by UV Irradiation.

FL (16.4 μM) was incubated under UV (254 nm, 0.53 mW/cm^2) and the activity of FL was measured at the indicated times.

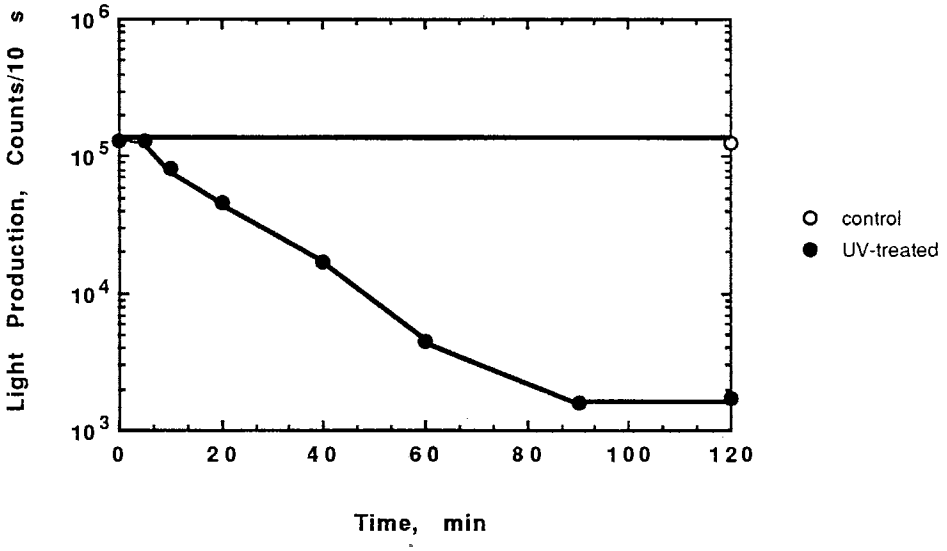

Light Production, Counts/10 s
10^6
10^5
10^4
10^3
0
20
40
60
80
100
120
Time, min
control
UV-treated

Figure 13. SDS-PAGE after Various Time of UV Inactivation

10% acrylamide, 2.7% crosslinked. Lane ST: protein MW standard, a = myosin (205 kD), b = β-galactosidase (116 kD), c = phosphorylase B (97.4 kD), d = albumin, bovine (66 kD), e = albumin, egg (45 kD), f = carbonic anhydrase (29 kD). Lane A: FL without UV treatment. Lane B: 5 min UV irradiation. Lane C: 10 min UV irradiation. Lane D: 20 min UV irradiation. Lane E: 40 min UV irradiation. Lane F: 60 min UV irradiation. Lane G: 90 min UV irradiation. Lane H: 120 min UV irradiation.

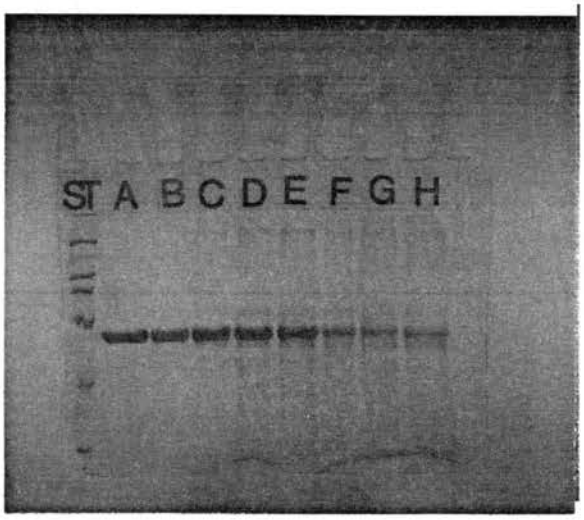
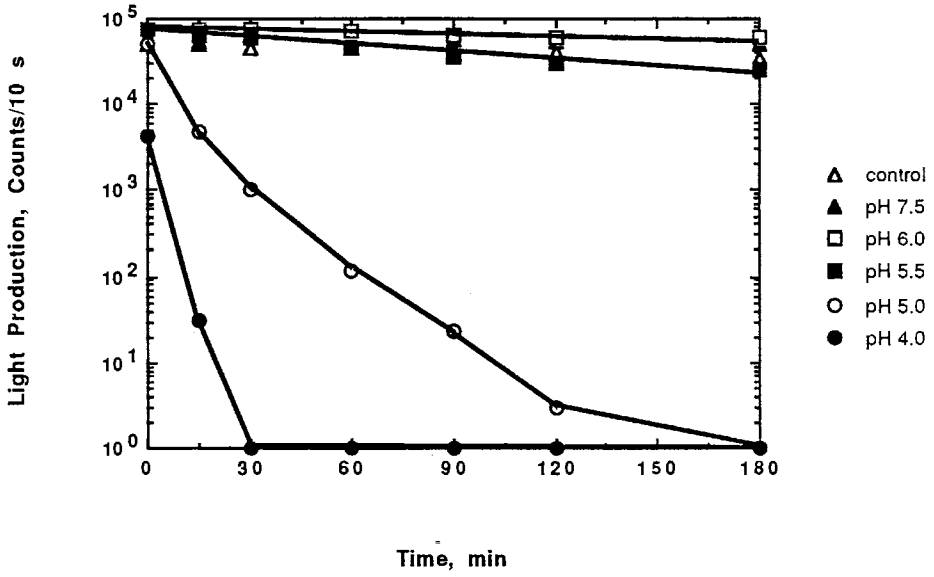

ST A B C D E F G H

Table I. Protection against UV Inactivation

Reagents	% Activity after (UV, 60 min)	Dimer	Cleavage
water	7	+	+
ATP (40 mM)	85	-	-
Adenosine (50 mM)	100	-	-
ADP (50 mM)	92	-	-
Adenine (50 mM)	87	-	-
Azido-ATP (10 mM)	58	-	+
Luciferin (7.1 mM)	18	-	+
Tryptophan (25 mM)	59	-	+
PABA (50 mM)	74	±	-
Tyrosine (12.5 mM)	29	±	+
Ethanol (7%)	11	+	+
P_i (50 mM)	15	+	+
PP_i (50 mM)	14	+	+
Lecithin (4 mg/ml)	7	+	+
DTT (50 mM)	11	+	+
Potassium phosphate (0.1 M)	21	+	+
Arsenate (50 mM)	16	+	+

Figure 14. Stability of Firefly Luciferase at Varying pH Values.

FL (16.4 μM) was incubated in 30 mM Tris/Succi/ES buffer with different pH values and the FL activity was measured at 30-min intervals.


Light Production, Counts/10 s
10 5
10 4
10 3
10 2
10 1
10 0
0
30
60
90
120
150
180
Time, min
control
pH 7.5
pH 6.0
pH 5.5
pH 5.0
pH 4.0

CHAPTER III

IDENTIFICATION OF ^{14}C-TUD-LABELED PEPTIDES OF FIREFLY LUCIFERASE

Introduction

The active site of firefly luciferase has been studied by biochemical means, e.g. use of luciferin and ATP analogs, fluorescent dyes, and specific amino acid-modifying reagents. [1-^{14}C]*N*-Ethylmaleimide (NEM), a sulfhydryl-modifying reagent, was used to label a sulfhydryl-containing peptide that was isolated (Travis and McElroy, 1966). The sequence of this tryptic peptide was S-C-E-G-N-A-G-S-Q-K. The luciferin analog 2-cyano-6-chlorobenzothiazole (CCBT) inactivated firefly luciferase with the incorporation of 1.5 to 2 moles per mole of luciferase. A tryptic peptide labeled by CBB was isolated and its sequence was pyroE-X-A-V-B-I-L with the fluorescent benzothiazole label attached at X, possibly a tyrosine (Lee and McElroy, 1971). Modification of luciferase with the ATP analog 5' [*p*-(fluorosulfonyl)benzoyl]adenosine produced a third peptide whose sequence was K-G-Z-B-S-K (Lee et al., 1981).

The gene for firefly luciferase from the North American firefly, *Photinus pyralis*, has been cloned and sequenced (de Wet et al., 1985). An unexpected result was found. None of the three previously described peptides were present in the deduced amino acid sequence (de Wet, 1986). Since then the -SH containing sequences labeled by the Cys-specific modifying reagent NEM (Vellom, 1990). Two peptides labeled by NEM were determined to be T-A-X (Cys)-V-R and G-E-L-X (Cys)-V-R. Both peptides were found in the sequence derived from the cloned gene with the Cys being at residues 216 and 391. These peptides were protected from NEM modification by either dehydroluciferyl adenylate

or dehydroluciferin. Modification by most -SH reagents resulted in complete loss of enzymatic activity, but methyl methanethiosulfonate-modified enzyme was catalytically active and emitted red light rather than the normal yellow-green light. Thus neither of these two cysteines is essential for activity, but both have roles in determining the color of light produced.

Thiourea dioxide (TUD), a lysine-modifying reagent, was an efficient inactivator of firefly luciferase (see Chapter II). The inactivation of FL by TUD was time- and dose-dependent and was reduced by the substrate ATP. There are other examples of utilization of TUD to modify enzymes. Glutamine synthetase (*E. coli*) was inactivated by TUD with total loss of activity and concomitant modification of a single lysine residue (Colanduoni and Villafranca, 1985). The modified amino acid was identified as homoarginine by amino acid analysis. The inactivation by TUD affected substrate glutamate binding rather than ATP binding. Inactivation of *E. coli* CTP synthetase by TUD involved either an imidazole or an $-NH_2$ group. ATP prevents inactivation, whereas UTP and GTP appear to enhance inactivation (Robertson et al., 1990). Plots of $1/K_{inact}$ versus nucleotide concentration displayed cooperative behavior. Therefore, TUD may modify the sites on the enzyme that are involved in cooperative interactions related to nucleotide binding.

In this chapter, ^{14}C-TUD was used to label firefly luciferase. The data presented here indicates that 1) the incorporation of approximately 2 moles of ^{14}C-TUD per mole of enzyme results in 50% inactivation; 2) ATP prevents 0.6-1.1 moles of ^{14}C-TUD incorporation per mole of enzyme; 3) two peptides labeled with ^{14}C-TUD are isolated by reverse-phase C-18 HPLC; 4) ATP protects one of the two peptides from being labeled with ^{14}C-TUD; 5) amino acids of two ^{14}C-TUD-labeled peptides are found near the C-terminus of the sequence deduced from the cloned gene of *Photinus pyralis* luciferase.

Materials and Methods

Reagents

Firefly luciferase was purified from *Photinus pyralis* tails (locally collected) as previously described in Chapter II. The radioactive thiourea dioxide, ^{14}C-TUD (0.096 mCi/mmole), was synthesized from ^{14}C-thiourea (54.88 mCi/mmole) obtained from Sigma or from New England Nuclear by the procedures described by Bannett (Bannett, 1910). Thiourea (Sigma) 15 g was slowly added during an hour to 230 ml of 6% aqueous H_2O_2, the whole being cooled with ice. The thiourea dissolved, and after an hour the oxidation product crystallized as colorless needles. These were washed with boiling ethanol to remove any unchanged thiourea, and dried in a vacuum. Methylamine was obtained from Eastman Kodak. Trichloroacetic acid (TCA), RNA, iodoacetamide, and TPCK-trypsin were purchased from Sigma. HPLC-grade trifluoroacetic acid (TFA) was purchased from Aldrich or Sigma and acetonitrile from Aldrich. Insta-Gel XF (xylene-free liquid scintillation cocktail) was a Packard product.

High Performance Liquid Chromatography (HPLC)

The HPLC system employed in these experiments consisted of :

Beckman 110 B Solvent Delivery Module pumps

Beckman 421 A Gradient Controller

Beckman 163 Variable Wavelength Detector

FC-80K Gilson Micro Fraction Collector

All buffers for HPLC were made in Waters C18 Sep-Pak Cartridge purified deionized H_2O. Buffers and solvents utilized in HPLC protocols were degassed by helium through vacuum filtration. Raw absorbance data were digitized and stored for later manipulation by the Analog Connection Chrom (a software developed by Strawberry Tree Computer Inc.) on the Macintosh II computer.

^{14}C-TUD Incorporation

FL and ^{14}C-TUD were each dissolved in 0.1 M NaH_2PO_4, pH 7.8 (NHPB). FL (final concentration 16.4 μM) was incubated with ^{14}C-TUD (50 mM, 0.096 mCi/mmole) and the activity of FL was measured at 30 min (approximately 50-60% inhibition) and 100 min (approximately 95% inhibition). Samples were mixed with 300 mM methylamine and incubated for an additional 75 min. After measuring the activity of FL incubated with methylamine, all samples were treated by the following method to determine the incorporation of ^{14}C-TUD.

The modified samples (500 μl) were loaded into separate dialysis tubing (pore diameter 48 Å) and dialyzed against 250 ml dialysis solution containing 10% $(NH_4)_2SO_4$, 1 mM EDTA, pH 7.8, which was changed every four hr for a total of 16 hr. After dialysis, the protein content, biological activity, and radioactivity were measured.

ATP or MgATP protection was done by adding 100 mM ATP or MgATP to 16.4 μM FL before incubating FL with 25 mM ^{14}C-TUD. After incubating FL with ^{14}C-TUD for 30 and 120 min, aliquots were dialyzed against the dialysis solution. The activity, protein content, and radioactivity were quantitated.

Protein and ^{14}C- Measurement

Protein content was measured by using the Pierce Coomassie Protein Assay Reagent. A stock BSA solution was diluted to a known protein concentration series (75-1500 μg/ml) with NHPB and samples were also prepared with NHPB to concentrations which were within the BSA standard range. The diluted standards or samples (0.1 ml) were mixed with 5 ml Coomassie Protein Assay Reagent and the absorbance at 595 nm was read against a NHPB blank.

Radioactivity was determined by adding 10 µl of a ^{14}C-TUD standard series (0.05-2 µg/10 µl NHPB) or 10 µl of each sample to 5 ml Insta-Gel XF and counting on a Scintillation Counter (Packard 1900CA Liquid Scintillation Analyzer).

Preparation of ^{14}C-TUD-Labeled Luciferase for Peptide Isolation

Purified FL was diluted in NHPB buffer to 16.4 µM. For ATP protection, ATP (final concentration 100 mM) was added to the FL sample. ^{14}C-TUD (0.096 mCi/mmole) was added at a final concentration of 20 mM. At varying times, 2.0-µl aliquots were assayed for luciferase activity. Once the inactivation reached 50% (about 90 min), 2-ml samples were loaded into dialysis tubing and dialyzed against 1 liter of dialysis solution. Removal of excess reagents (^{14}C-TUD or ATP) was accomplished by changing dialysis solution ten times (once every four hr). FL without ^{14}C-TUD, for a control, was treated the same as the other samples.

Labeled FL (8.2 µM, 1 ml) and a control were denatured according to the method described by Kathryn et al. (1989). After concentration with a Centricon-30 apparatus, samples were denatured in 200 µl 8 M urea, 0.4 M NH_4HCO_3. Samples were treated with DTT (4.5 mM, 50 °C, 15 min) and iodoacetamide (10 mM, room temperature, 15 min), then dialyzed against 0.1 M NH_4HCO_3, pH 8.0 (300 µl sample per 300 ml dialysis buffer) with one buffer change. Samples were now ready for trypsin digestion.

Isolation and Sequencing of Peptides

TPCK-Trypsin was added to the dialyzed samples (1:20 w/w). Digestion was allowed to proceed for 12 hr at 37 °C. The digested samples (100 or 200 µl each time) were then chromatographed on a C18 column (4.6 × 250 mm, 300 Å) equilibrated in 0.098% TFA/H_2O and 2% acetonitrile. The column was eluted with a linear 2-80% acetonitrile gradient at 0.5 ml/min. The absorbance of the effluent was continuously monitored at 214 nm and 0.5-ml fractions were collected. The radioactivity of collected

fractions was determined on a Packard Scintillation Counter by adding 100 µl of each collected sample to 5 ml of Insta-Gel XF. Radioactive peaks were separately pooled and dried using a "spin vac" concentrator (SVC-100H). The dried ^{14}C-TUD-labeled peptide samples (approximately 0.5-1 mg FL digested peptides) were sequenced in the Molecular Biology Resource Facility of the William K. Warren Medical Research Institute at the University of Oklahoma Health Sciences Center, Oklahoma City.

Results

Inactivation of Firefly Luciferase with ^{14}C-TUD Correlates with the Incorporation of ^{14}C-TUD

To establish a correlation between the inactivation of luciferase and chemical modification by TUD, ^{14}C-TUD was synthesized and used to inactivate the enzyme. Incubation of firefly luciferase with ^{14}C-TUD (50 mM) resulted in 50-60% inactivation in 30 min and 95% inactivation in 100 min. The excess unlabeled ^{14}C-TUD was separated from covalently radiolabeled sample by dialysis against a buffer containing 10% $(NH_4)_2SO_4$, 1 mM EDTA, pH 7.8. Scintillation counting of small portions of the ^{14}C-TUD-modified enzyme confirmed that 1 to 2 moles of ^{14}C-TUD/monomer were covalently bound to the exhaustively dialyzed enzyme with 42-50% inactivation (Table II). By using the dialysis method to remove free radioactive TUD, an enzyme with originally 76 or 97% inactivation was converted to approximately 42 or 56% inactivated form, respectively, during the dialysis. $(NH_4)_2SO_4$ (10%), a component of the dialysis buffer, was used to maintain luciferase solubility and was found to protect luciferase from TUD inhibition (data not shown). $(NH_4)_2SO_4$ may reverse TUD binding which is not specific or not tight.

The Incorporation of ^{14}C-TUD into Luciferase Is Prevented by ATP

As shown in Table III, 2.4 moles of ^{14}C-TUD were incorporated per mole of enzyme in the absence of substrate ATP while only 1.4 moles were incorporated in the presence of MgATP or ATP (100 mM during incubation). Incubation of the enzyme with ^{14}C-TUD in the presence of ATP reduced the inhibition by 50%.

Two Tryptic Peptides of Firefly Luciferase Are Labeled by ^{14}C-TUD and Isolated by HPLC

^{14}C-TUD-labeled luciferase (with 50% inactivation) was denatured in 8 M urea and 0.4 M NH_4HCO_3, treated with 4.5 mM DTT and 10 mM iodoacetamide, and dialyzed in 0.1 M NH_4HCO_3. A sample prepared with addition of ATP during the labeling incubation was treated the same way. After digestion with TPCK-trypsin, the samples were fractionated on a reverse-phase C-18 HPLC column using trifluoroacetic acid (TFA)/acetonitrile as described (Fig. 15). Absorbance profiles (214 nm) for the two samples (with and without ATP during labeling) were nearly identical. The radioactivity profiles (Fig. 16) demonstrated the differential labeling of peptides between these two samples. Two labeled peptides eluting at 38 min (at 22% acetonitrile) and 60 min (at 34% acetonitrile)were found in the absence of ATP and only one labeled peptide was eluted at 60 min when ATP was in the labeling reaction.

Amino Acid Sequences of Isolated Peptides Are Found in the Sequence from the Cloned Luciferase Gene

The two labeled peptides eluted at 38 and 60 min were separately pooled and concentrated by speed vacuum until dry. Amino acid sequencing was done in the Molecular Biology Resource Facility of the Willian K. Warren Medical Research Institute at the University of Oklahoma Health Sciences Center. The sequence of the 38-min peptide

was G-L-T-G-K. The sequence of the 60-min peptide was S-G-Y-V-N-N-P-E-A-T-N-A-L-I-D-K. Both peptide sequences are located near the C-terminus of the translated primary sequence derived from the *Photinus pyralis* cDNA sequence (Fig. 17).

Discussion

The incorporation of approximately 2 moles of ^{14}C-TUD per mole of luciferase resulted in 50% inactivation. When the inactivation was > 90%, the incorporation of ^{14}C-thiourea dioxide into luciferase was more than 5 moles/mole. Extreme dialysis of ^{14}C-TUD-labeled firefly luciferase reduced products of nonspecific interaction of the enzyme with TUD.

Thiourea dioxide incubated with *E. coli* glutamine synthetase leads to inactivation by an irreversible covalent modification. Homoarginine is produced as a result of this reaction as determined by amino acid analysis (Colanduoni and Villafranca, 1985). Colanduoni and Villafranca found 3 molecules of TUD labeled per monomer of enzyme but one molecule could be removed by four 6-hr dialyses treatments. The inactivation of luciferase by TUD was partially reversed during a dialysis process against a solution of 10% $(NH_4)_2SO_4$, 1 mM EDTA, pH 7.8. It is suggested that some amino acids other than lysine react with TUD by noncovalent modification. By employing ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry, Colanduoni and Villafranca showed that thiourea dioxide reacted with several primary amines (methylamine, amino caproic acid, lysine, ornithine) and imidazoles (imidazole, histidine) under basic conditions. Thiourea dioxide was shown to react covalently with lysine to produce homoarginine. The reaction of TUD with imidazole gave a mixture of products that were not further analyzed (Colanduoni and Villafranca, 1985). It is possible that the partial reversible inactivation and reversible incorporation by TUD reflects the involvement of reaction of histidine. The modification of histidine with TUD might be a noncovalent bond, which causes a part of the inactivation

and incorporation of TUD as well as a reversible modification. Therefore, histidine may be one of the important amino acids to correlate with the active sites of firefly luciferase.

Approximately 50% of the inactivation by and incorporation of TUD was prevented by the substrate ATP. The reasons why greater protection by ATP could not be achieved may be: 1) the TUD-labeled sites are not wholly related to ATP-binding sites, or 2) the ATP concentration was not high enough (ATP 100 mM to inhibitor 25 mM).

To identify the TUD labeling sites, modification of luciferase with ^{14}C-TUD was accomplished in the presence and absence of substrate ATP followed by tryptic digestion and reverse-phase HPLC purification of labeled peptides. Two peptides, eluting at 38 and 60 min respectively, were labeled by ^{14}C-TUD. ATP prevented labeling of the former of these peptides. Therefore, the peptide eluted at 38 min and protected by ATP from TUD labeling should be related to an ATP-binding site. The other peptide, eluted at 60 min and labeled by TUD without ATP protection, may not be an ATP-binding site, but may be important for luciferase activity.

The amino acid sequences of these two peptides labeled by ^{14}C-TUD are GLTGK and SGYVNNPEATNALIDK. Both peptides are located near the C-terminus of luciferase. Amino acids near the C-terminus of luciferase appear important in determining the function of the enzyme. By using site-specific mutagenesis it was shown that 8-12 amino acids of the C-terminus are essential for luciferase activity (Sala-Newby and Campbell, 1994). The peptide GLTGK, labeled by ^{14}C-TUD, is located at the position 524-528, which is within a very highly conserved area for different luciferases (Fig. 18). The other peptide SGYVNNPEATNALIDK, located at the position 398-414, is close to the highest homology region (>90%) among all luciferase (residues 410-460) (Ugarova et al., 1992). The identification of the ATP binding site in tyrocidine synthetase 1 (TY1) by selective modification with fluorescein 5'-isothiocyanate (FITC) was reported by Pavela-Vrancic et al. (1994). Two Lys residues (Lys-422 and Lys-505) of TY1 were labeled by FITC and identified to be at the ATP binding sites. When the FITC-labeled peptides of

TY1 were compared with the corresponding fragments within the consensus sequence of several carboxyl-activating proteins such as *N. crassa* acetyl-CoA synthetase, 4-coumarate-CoA ligase from parsley, and *Photinus* luciferase, the ATP-binding site (Lys-505) of TY1 corresponded to Lys-529 of firefly luciferase. The peptide GLTGK (Lys-529) is one of the two TUD-labeled peptides in our experiments. The behavior of these two TUD-labeled peptides located near the C-terminus and at a highly conserved area for various ATP-binding proteins and luciferases, suggests that the two peptides are related to the active sites of firefly luciferase.

Table II. Incorporation of ^{14}C-TUD into Luciferase

^{14}C-TUD exposure time, min	% Inhibition	Incorporation of ^{14}C-TUD/FL mol/mol	% Inhibition after removal of free ^{14}C-TUD
30	76	1.1	42
100	97	1.9	50

Table III. Protection of FL from ^{14}C-TUD Incorporation by ATP

^{14}C-TUD exposure time, min	ATP Addition	% Inhibition after dialysis	ATP Protection %	Incorporation ^{14}C-TUD/FL mol/mol
30	no	30.4		1.4
30	ATP	11.5	18.9	0.8
30	MgATP	21.3	9.1	0.8
120	no	42.7		2.4
120	ATP	23.1	19.6	1.5
120	MgATP	28.3	14.4	1.3

Figure 15. Reverse-Phase C-18 HPLC of Tryptic Fragments of ^{14}C-TUD-Labeled Luciferase

The tryptic peptides of luciferase were applied to a C-18 column (4.6 × 250 mm) and eluted with a linear gradient from 2% acetonitrile and 98% TFA/H_2O (0.1%) to 80% acetonitrile at a flow rate at 0.5 ml/min over 120 min. Two peptides labeled with ^{14}C-TUD that eluted at 38 min and 60 min are indicated.

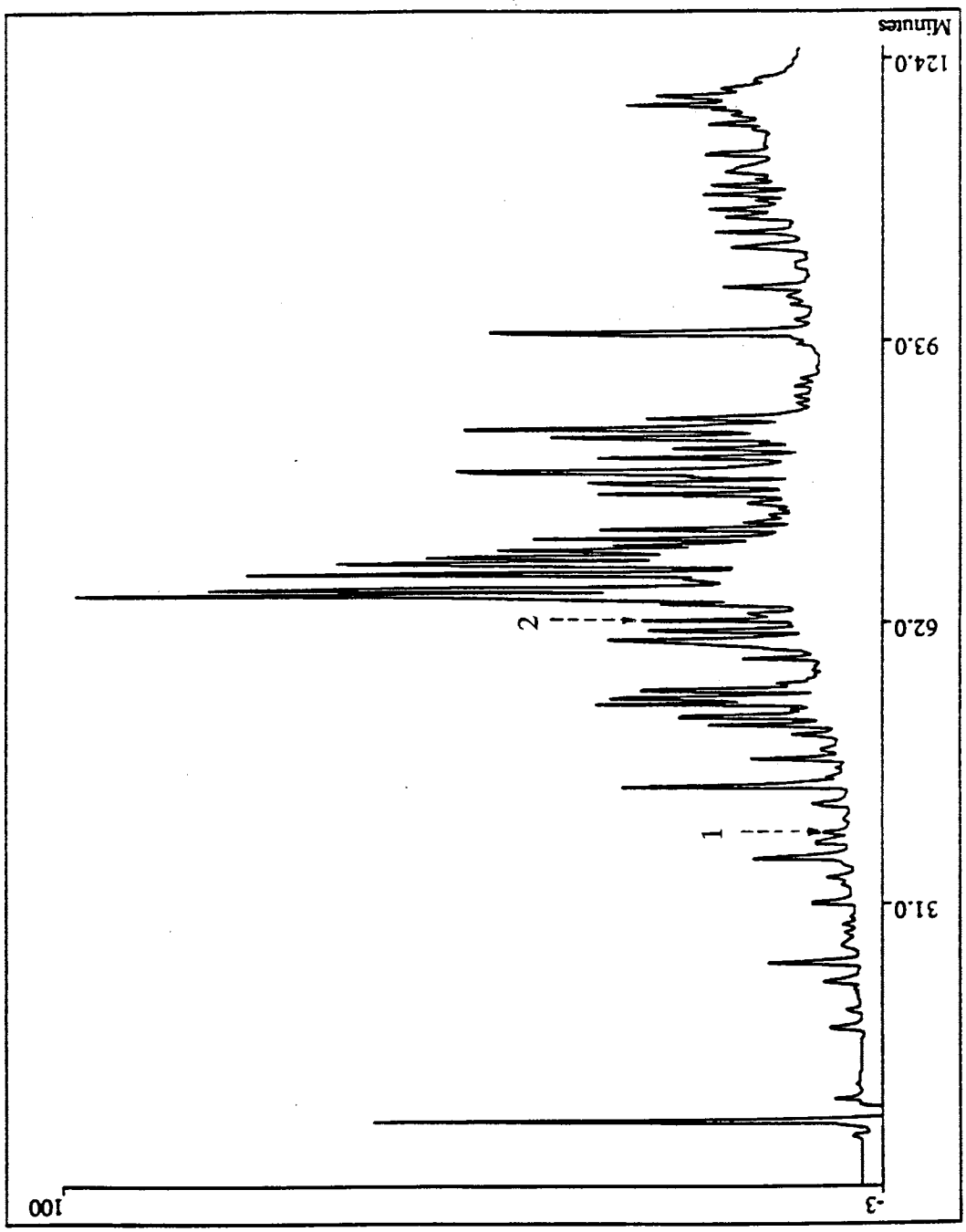

Minutes
124.0
93.0
62.0
31.0
-3
100
1
2

Figure 16. The Radioactive Profiles of HPLC Fractions

The procedures were described in Materials and Methods. A) control sample as background; B) ^{14}C-TUD-inactivated luciferase without ATP; C) ^{14}C-TUD-inactivated luciferase with ATP.

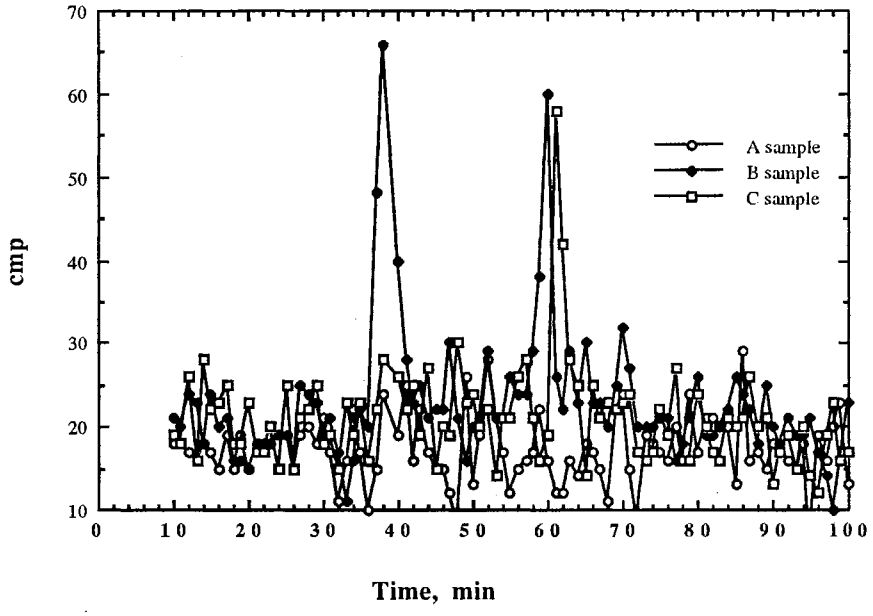

70
60
50
40
30
20
10
cmp
0
10
20
30
40
50
60
70
80
90
100
Time, min
A sample
B sample
C sample

Figure 17. Location of the TUD-Labeled Peptides in the Translated Primary Sequence of *Photinus pyralis* Luciferase (de Wet et al., 1986)

The two ^{14}C-TUD-labeled peptides are underlined.

MEDAKNIKKG	PAPFYPLEDG	TAGEQLHKAM	KRYALVPGTI	AFTDAHIEVN	1-50
ITYAEYFEMS	VRLAEAMKRY	GLNTNHRIVV	CSENSLQFFM	PVLGALFIGV	51-100
AVAPANDIYN	ERELLNSMNI	SQPTVVFVSK	KGLQKILNVQ	KKLPIIQKII	101-150
IMDSKTDYQG	FQSMYTFVTS	HLPPGFNEYD	FVPESFDRDK	TIALIMNSSG	151-200
STGLPKGVAL	PHRTACVRFS	HARDPIFGNQ	IIPDTAILSV	VPFHHGFGMF	201-250
TTLGYLICGF	RVVLMYRFEE	ELFLRSLQDY	KIQSALLVPT	LFSFFAKSTL	251-300
IDKYDLSNLH	EIASGGAPLS	KEVGEAVAKR	FHLPGIRQGY	GLTETTSAIL	301-350
ITPEGDDKPG	AVGKVVPFFE	AKVVDLDTGK	TLGVNQRGEL	CVRGPMIMSG	351-400
YVNNPEATNA	LIDKDGWLHS	GDIAYWDEDE	HFFIVDRLKS	LIKYKGYQVA	401-450
PAELESILLQ	HPNIFDAGVA	GLPDDDAGEL	PAAVVVLEHG	KTMTEKEIVD	451-500
YVASQVTTAK	KLRGGVVFVD	EVPKGLTGKL	DARKIREILI	KAKKGGKSKL	501-550

CHAPTER IV

CHARACTERIZATION OF *PHOTURIS* LUCIFERASE

Introduction

Photuris pennsylvanica is a twi-night-active firefly. Predation by aggressive mimicry is known only for *Photuris* (Lloyd, 1984). When the*Photuris* firefly is compared to the common *Photinus* firefly, the *Photuris* flies higher, faster and later (all night long). It is harder to capture the *Photuris* fireflies in the evening owing to their aggressive action and quick escape.

The properties of light produced by the *Photuris* firefly are also different from the light produced by the *Photinus* firefly. It is easy to distinguish *Photuris* from *Photinus* fireflies, because the *Photuris* emits brighter light and flashes more frequently than the *Photinus*. According to Coblentz (1912), there are many differences in light production between the two species. *Photuris* fireflies produce a greenish blue light with a quick flash at a rate of 120-180 times per minute, and exhibit maximum emission at 550 nm, whereas the *Photinus* fireflies produce a yellow-green light with a long fulminating flash (20 times per minute), and exhibit maximum emission at 580 nm.

A study of *Photuris* luciferase was reported in 1981 (Strause and DeLuca, 1981). An $(NH_4)_2SO_4$ fraction from a crude extract of *Photuris pennsylvanica* tails was characterized with respect to pI, molecular weight and antigenicity. The *Photuris* luciferase has characteristics similar to the *Photinus* luciferase for molecular weight and pI. The antibody raised against the *Photinus* luciferase blocked the *Photuris* luciferase activity. Interestingly, isozymes of *Photuris* luciferases were found in the larvae and in the adult

form. Although they have similar K_ms, pH profiles, and molecular weight, the isozymes exhibit different isoelectric points and antigenicity (Strause and DeLuca, 1981).

The luciferase from *Photuris pennsylvanica* has not been purified and extensively characterized. A characterization of *Photuris* luciferase and comparison to other firefly luciferases will help in studying the mechanism of luciferase catalysis. Since the *Photuris* firefly emits brighter light and flashes more frequently than the common species of *Photinus*, the luciferase from the*Photuris* may have interestingly different properties from the firefly luciferases previously characterized.

As discribed in this chapter, the luciferase from *Photuris pennsylvanica* lanterns was partially purified by the same method used for *Photinus* luciferase purification and characterized. The data presented in this chapter show that 1) the *Photuris* luciferase displays different properties from the *Photinus* luciferase during the purification procedures; 2) the *Photuris* luciferase has the same antigenicity and the same molecular weight as the *Photinus* luciferase; 3) the K_m for ATP of the partially purified *Photuris* luciferase is 7.9 μM and for the purified *Photinus* luciferase is 6.5 μM; 4) the *Photuris* luciferase shows the same time course of light production as does the *Photinus* enzyme; 5) the *Photuris* luciferase is more unstable at 37 °C and pH 9.0 than that of the *Photinus* enzyme; 6) the *Photuris* luciferase is similarly inactivated by thiourea dioxide; 7) etheno-ATP, an activator for the *Photinus* luciferase during high concentration of ATP, is also an activator for the *Photuris* luciferase.

Materials and Methods

Reagents

Luciferin was purchased from Sigma and prepared with 20 mM Tricine buffer to the final concentration of 1 mg/ml (pH 7.5). ATP, thiourea dioxide and 1-N^6-etheno-ATP were obtained from Sigma.

Buffers for luciferase assay are same as described previously: Tricine complete Buffer 1 (250 mM Tricine, 50 mM $MgSO_4$, 5 mM EDTA, pH 7.8), TB 1; TB 1 plus 5 mM DTT , TB 2; enzyme dilution buffer (50 mM Tricine, 10 mM $MgSO_4$, 1 mM EDTA, 20 mM DTT and 0.15 mM BSA, freshly prepared), EDB; enzyme assay buffer (25 mM Tricine, 5 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM DTT and 0.18 mM luciferin, freshly prepared), EAB.

Reagents for Western blots were from Dr. R. Matts' lab. Anti-*Photinus* luciferase antibody was obtained from Promega (pt# E 419 A). Alkaline phosphatase conjugated to rabbit anti-mouse IgG was obtained from ICN. Prestained SDS-PAGE Standard (low range) was a Bio-Rad product. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma.

Firefly Luciferase Purification

Firefly luciferase was purified from 5 g of dried *Photuris pennsylvanica* lanterns collected locally around Stillwater according to the method described before for the *Photinus* luciferase purification (Hall et al., 1984). Because of the unexpected denaturation of the *Photuris* luciferase happened during the final step of $(NH_4)_2SO_4$ crystallization, the supernatant solution of the crystalization stage, still containing part of the luciferase activity, was concentrated using Amico DIAFLO ultrafilters with YM 30 filters to obtain the partially purified *Photuris* luciferase. The partially purified sample had 7-8 bands visualized in an SDS-PAGE gel. Firefly luciferase from *Photinus pyralis* was also purified at the same time. The crystallized *Photinus* luciferase exhibited one band in SDS-PAGE.

Protein Measurement

Protein content was measured by the method described by Read and Northcote (1981). Samples and BSA (for standard) were diluted with Na/P_i buffer separately. The diluted samples or standards (0.25 ml) were mixed with 4.75 ml Coomassie Blue G-250

(prepared with phosphoric acid and ethanol) and the absorbance of individual sample and standard at 595 nm was recorded.

Western Blot Analysis

Western blot analysis was performed using the method described by Matts and Hurst (1989). The partially purified *Photuris* luciferase (0.3-30 ng per well) and the *Photinus* luciferase (30 pg per well) were separated with 10% SDS-PAGE gels (9 × 14 × 0.15 cm; acrylamide:bis acrylamide = 37.5:1). Proteins separated by use of the gels were transferred to a PVDF membrane (Bio-Rad Trans-Blot Transfer Medium) at a current density of 2.5 cm^2 for 40 min. Blots were blocked with TBS (10 mM Tris-H/HCl, pH 7.5 with 150 mM NaCl) containing 5% (W/V) non-fat dry milk (skim milk, Difco Laboratories) for one hr at room temperature. The primary antibody (anti-*Photinus pyralis* luciferase antibody, Promega) 4 µl (5 µg/2.8 µl) was dissolved in 10 ml TBS containing 1% skim milk and was then reacted with the blots at 4 °C overnight. A nonimmuno active rabbit IgG (7 µg/10 ml TBS with 1% skim milk) was used for the negative control. After reacting with the primary antibodies, the blots were washed twice with TBS containing 0.5% Tween-20, once with TBS, and then blocked with TBS contained 5% (W/V) skim milk prior to reaction with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson Laboratory, diluted 1:4000 in TBS containing 0.1% skim milk) for two hr at room temperature. Blots were washed twice with TBS containing 0.5% Tween-20, and twice with TBS. Proteins were then detected by incubating blots in alkaline phosphatase reaction buffer at 35 °C for 1 to 5 min.

Luciferase Assay

Luciferase activity was assayed with a Lumac/3M Model 2010A Biocounter or a SAI Technology Model 3000 Luminometer. The assay procedures were the same as described before. Assay on Lumac/3M Model 2010 A Biocounter: firefly luciferase was

dissolved in TB 1 to 16.4 μM. An aliquot of FL solution (2 μl) was taken and diluted with 500 μl of EDB. Diluted luciferase (10 μl) was mixed with 490 μl EAB containing 0.33 μM ATP in a Lumacuvette and the light production was measured for 10 s in the luminometer. Assay on SAI Technology Model 3000 Luminometer: 10 μl of diluted luciferase was added to a Bio-vial containing 390 μl EAB and placed in the luminometer. The reaction was initiated by rapid injection of 50 μl of ATP (various concentrations) by using a Digital Syringe Diluter. An aluminum disk with a small hole was used to attenuate the light striking the phototube to 0.00053 of that without attenuation.

The kinetics of firefly luciferase were determined on an LKB Model 1251 automatic luminometer controlled by a Laser Turbo PC-401 computer. The assay was performed as described previously. The reaction mixture contained 170 μl of 28.6 mM Tricine buffer at pH 7.8, 5.7 mM $MgSO_4$, 0.57 mM EDTA, 0.21 mM luciferin, 0.57 mM DTT, and luciferase (2-20 ng). After 6 s incubation of the enzyme-containing reaction mixture in the chamber, 30 μl of ATP (final ATP concentration, 0.24 μM-0.24 mM) was injected using an LKB Model 1291 injector.

ATP K_ms Determination

The assay was performed by injecting 50 μl ATP from a series of diluted samples (the final amount range, 0-4000 μmole) into 150 μl of EAB containing 0.6 μg of partially purified *Photuris* luciferase. The light produced was measured on an SAI Technology Model 3000 Luminometer. The purified *Photinus* luciferase (1.3 nM in EAB) was assayed the same way. The ATP K_ms for luciferase were calculated by using the Trinity Software-Enzyme Kinetics V1.11, and the average values determined by the Lineweaver-Burk, Eadie-Hofstee, Johansen-Lumry, Direct-Linear-Plot and Non-Linear-Regression methods are presented.

Time Course of Light Production Measurement

The partially purified *Photuris* luciferase (2 μg of protein for the constant-light production assay or 0.4 μg of protein for the flash-light production assay) was added into a 170 μl-reaction mixture. The light production was assayed immediately after injecting ATP (0.24 μM for high-ATP or 0.24 mM for low-ATP assay) by using an LKB Model 1291 injector. The kinetics of the *Photinus* luciferase solution (20 ng for low ATP or 2 ng for high ATP assay) was determined by the same method. Each experimental parameter was reproduced three times and the average values plotted.

The Stability of Luciferase

The partially purified *Photuris* luciferase (2.5 mg/ml) or *Photinus* luciferase (8.2 μM) was incubated in TB 1 at different pH value (7.8, 5.5, and 9.2) at room temperature or at 37 °C. The activity of luciferase was determined at various times with a Lumac/3M Model 2010A biocounter.

Inactivation Procedures

Inactivation was accomplished by incubation of the *Photuris* luciferase (partially purified, 2.5 mg/ml prepared in a solution containing 10% $(NH_4)_2SO_4$, 1 mM EDTA, pH 8.0) and the *Photinus* luciferase (4.1 μM in the same buffer) with 100 mM thiourea dioxide. The luciferase activity was measured throughout the incubation on a Lumac 3M Model 2010A biocounter.

Activation Procedures

The effect of etheno-ATP on luciferase activity was determined on an LKB Model 1251 automatic luminometer. The partially purified *Photuris* luciferase (4 μg for the constant-light assay or 0.4 μg for the flash-light assay) or the *Photinus* luciferase (20 ng

for the constant light or 2 ng for the flash light) was mixed with 170 µl of the reaction mixture described previously. The rate of light production was measured throughout an assay involving the sequential injection of ATP (0.24 µM for low [ATP] or 0.24 mM for high [ATP]) and etheno-ATP (0.4 mM) respectively at one-minute intervals. The double injection was controlled by an LKB Model 1291 injector.

Results

Properties of *Photuris* Luciferase during Purification Are Different from Those of *Photinus* Luciferase

The purifications of *Photuris* and *Photinus* luciferases are summarized in Table IV. From 5 g of firefly lanterns, the total luciferase activity obtained from purification were 1.36×10^8 for *Photuris* and 9.1×10^{10} for *Photinus*. By measuring the light production of the samples collected during the purification procedures, such as crude extraction of the acetone powder by Tricine buffer, calcium phosphate gel treatment, $(NH_4)_2SO_4$ fractionation, Sephadex G-150 chromatography, and crystallization, the total counts (light production) of each procedure from the *Photuris* samples were always lower than those of the *Photinus* samples. Therefore, the amount of luciferase in the *Photuris* was less than in the *Photinus* fireflies. There was inhibitor in the *Photuris* crude extract. This inhibitor inactivated 76% of the *Photinus* luciferase when the *Photinus* luciferase was incubated with an equal volume of the *Photuris* crude extraction. The calcium phosphate gel treatment removed the inhibitor from the *Photuris* samples.

The crystallization procedure used for *Photinus* luciferase inactivated *Photuris* luciferase (a loss of 97% activity). This suggests the characteristics of the *Photuris* luciferase are different from the *Photinus* luciferase.

The protein content and the luciferase activity of the column fractions collected from the Sephadex G-150 chromatography of the samples were determined. Two peaks (fractions 29 and 38) that exhibited luciferase activity were found in the *Photuris* sample

(Fig. 18A), which indicats the existence of isozymes of the *Photuris* luciferase. Only one peak (fraction 36) was in the *Photinus* sample (Fig. 18B).

Photuris Luciferase Has the Same Antigenicity and the Same Molecular Weight as *Photinus* Luciferase

The partially purified *Photuris* luciferase was separated on SDS-PAGE apparatus, then transferred to a PVDF membrane and blotted with an anti-*Photinus pyralis* luciferase antibody. Figure 19 shows the results of Western blot analysis. The anti-*Photinus* luciferase detected the *Photuris* luciferase with a molecular weight of 61,000 kD (lanes 2, 3, and 4) the same as the *Photinus* luciferase (lane 1). The inactivated crystalline *Photuris* luciferase was also detected by the anti-*Photinus* luciferase antibody (lane 5) and migrated slightly slower than the active enzyme (lanes 2-4).

The K_ms of ATP Are Similar between *Photuris* and *Photinus* Luciferase

The K_ms of ATP, for the partially purified *Photuris* luciferase and the purified *Photinus* luciferase were measured in the presence of luciferin (0.18 mM) and calculated by using the Trinity Software-Enzyme Kinetics V1.11 program. The K_ms of ATP for the partially purified *Photuris* luciferase and the purified *Photinus* luciferase were 7.9×10^{-6} M and 6.5×10^{-6} M, respectively.

Photuris Luciferase Shows a Time Course of Light Production Similar to *Photinus* Luciferase

The time courses of light production for the partially purified *Photuris* and the purified *Photinus* luciferase were determined on an LKB Model 1251 automatic luminometer. The kinetics of the constant-light production at low ATP (0.24 μM) (Fig. 20A) and the flash-light production at high ATP (0.24 mM) (Fig. 20B) were similar between the *Photuris* and *Photinus* luciferases.

Photuris Luciferase Is Less Stable than *Photinus* Luciferase

Figure 21 shows the stability of the partially purified *Photuris* and the purified *Photinus* luciferase at 37 °C, pH 7.8, pH 5.4, and pH 9.0. The remaining activity of the *Photuris* luciferase after a 180-min incubation at 37 °C (pH7-8) or at pH 9.0 (rooom temperature) was about 2% and 35% respectively. Under the same condition, the *Photinus* luciferase retained almost 100% of activity. Both luciferases were stable for 180 min at room temperature when they were incubated at pH 7.8. pH 5.4 inactivated both luciferases rapidly.

Photuris Luciferase Is Inactivated Like *Photinus* Luciferase by Thiourea Dioxide

Thiourea dioxide was used to modify the *Photinus* luciferase and label the ATP-binding sites in Chapter II. When the partially purified *Photuris* and the purified *Photinus* luciferase were incubated with TUD under the same conditions, the activity of both enzymes was decreased to 70% (with TUD 50 mM) and to 50% (with TUD 100 mM) at 180-min incubation.

Photuris Luciferase Is Activated Like *Photinus* Luciferase by Etheno-ATP

Etheno-ATP stimulates the *Photinus* luciferase at high ATP concentration and changes the time course of light production from the flash to the constant pattern (Ford and Leach, 1991). The constant-light production (at the low ATP concentration) by the partially purified *Photuris* and the purified *Photinus* luciferase was not changed when the luciferase was treated with etheno-ATP. When treated with etheno-ATP, the flash-light production (at the high ATP concentration) of both luciferases was changed to a constant-light production that was sustained at a higher level, whearas the flash-light and the lower-light production were still exhibited in the control sample without addition of etheno-ATP.

Discussion

The *Photuris* luciferase has been partially purified and characterized. The properties of the *Photuris* and *Photinus* luciferase were compared. Several properties differed during the purification. The stability of the two luciferases differs but the character of the *Photuris* luciferase was similar to that of the *Photinus* luciferase.

The fluorescent-material content in the *Photuris* lanterns was reported to be less than in the *Photinus* (Coblentz, 1912). During the purification in our experiment, the amount of luciferase from the *Photuris* lanterns seemed much less than from the *Photinus* lanterns, giving only 1% as much total light counts as the *Photinus* lanterns.

The *Photuris* luciferase was partially purified by ammonium sulfate fractionation and partially characterized by Strause and DeLuca (1981). The same pI and antigenicity were shown for the *Photuris* and *Photinus* luciferases. However several properties of the *Photuris* luciferase differ from those of the *Photinus* enzyme. The crystallization of luciferase using $(NH_4)_2SO_4$, a procedure which produce a highly active and homogeneous *Photinus* luciferase, inactivated the *Photuris* luciferase. The inactivation during crystallization was also shown in *Luciola* luciferases (Brovko et al., 1982). The *Photuris* luciferase was less stable at 37 °C and at pH 9.0 than the *Photinus*.

Isozymes of the *Photuris* luciferase were found by Strause and DeLuca (1981). An isozyme of the *Photuris* luciferase (two peaks of activity) was also found during the chromatography reported in Fig. 18.

Although some differences were shown between the *Photuris* and *Photinus* luciferase, the characterization experiments demonstrated that most properties of the two luciferases were similar, such as the same molecular weight, antigenicity (determined by Western blot), the time course of light production at either low or high ATP concentration, and the kinetics of TUD inhibition and etheno-ATP activation.

The *Photuris* luciferase was not crystallized because of an unexpected inactivation. The luciferase isozymes have not been extensively characterized owing to a mistake,

resulting in the mixing of the two peaks after chromatography. To obtain a purified *Photuris* luciferase is an important future project; it will help to reveal the special properties of *Photuris* luciferase, distinct from the other luciferases, and help to explain the brighter light the *Photuris* fireflies create and to understand the significance of isozymes in bioluminescence science.

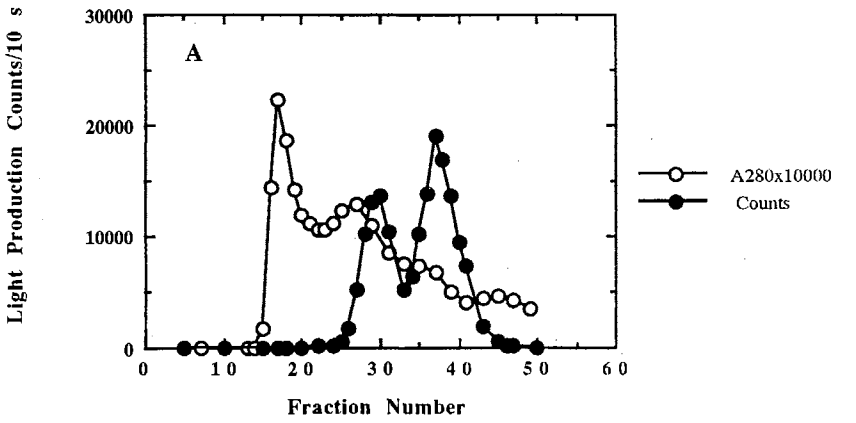
The *Photuris* luciferase gene has not been studied and cloned. The next chapter (V) will describe how the luciferase gene was cloned and sequenced.

Table IV. Purification of FL from*Photuris pennsylvanica* and *Photinus pyralis*

Treatment	Volume (ml)	Protein mg/ml	Protein Total, g	Activity Light/mg	Activity Total Counts	Yield %	Purification
A: *Photuris* fraction							
Crude extract	50	18.4	0.92	$2.47x10^6$	$2.27x10^9$		
Calcium phosphate gel	55	3.6	0.20	$3.56x10^7$	$7.12x10^9$	100	14.4
$(NH_4)_2SO_4$ fraction	5	29.9	0.15	$3.89x10^7$	$5.83x10^9$	100	15.7
Column fraction	92	0.49	0.05	$1.37x10^8$	$6.85x10^9$	100	55.5
Super of crystals	0.8	4.99	0.004	$3.40x10^7$	$1.36x10^8$	5.9	13.7
B: *Photinus* fraction							
Crude extract	50	14.1	0.7	$7.30x10^8$	$5.11x10^{11}$		
Calcium phosphate gel	55	5.1	0.28	$7.43x10^8$	$2.08x10^{11}$	40.7	1.02
$(NH_4)_2SO_4$ fraction	5	32.7	0.16	$8.43x10^8$	$1.35x10^{11}$	26.4	1.15
Column fraction	91	0.75	0.07	$1.83x10^9$	$1.28x10^{11}$	25.0	2.51
Crystals fraction	2.2	12.2	0.026	$3.49x10^9$	$9.10x10^{10}$	17.8	4.78

Figure 18. Column Fractionation of Luciferase Samples

The *Photuris* (Fig. 18A) and *Photinus* (Fig. 18B) samples from $(NH_4)_2SO_4$ induced-precipitation were dissolved in 5 ml of 10 mM Tricine buffer containing 2 mM EDTA, 10% sucrose and 10% $(NH_4)_2SO_4$, pH 7.8. Samples were chromatographed on a Sephadex G-150 column (2.2 × 70 cm) equilibrated with the above buffer. Fractions (5 ml) were collected, the protein content determined as A_{280} and firefly luciferase activity assayed.


A
Light Production Counts/10 s
30000
20000
10000
0
0
10
20
30
40
50
60
Fraction Number
A280x10000
Counts

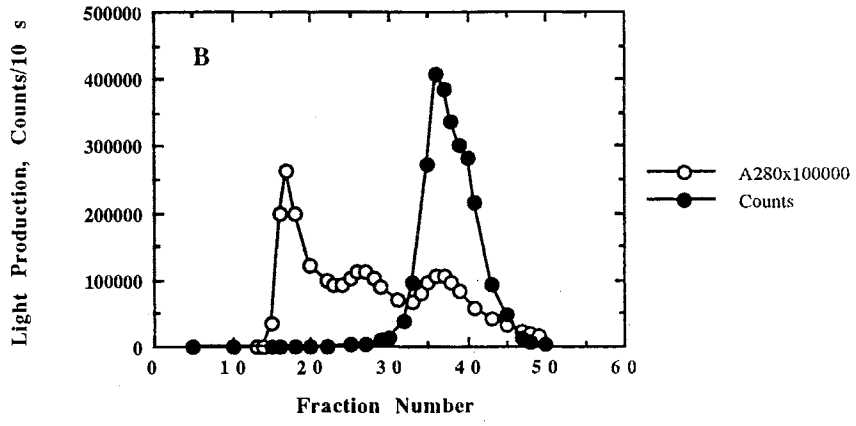

B
Light Production, Counts/10 s
500000
400000
300000
200000
100000
0
0
10
20
30
40
50
60
Fraction Number
A280x100000
Counts

Figure 19. Western Blot Study of Firefly Luciferases

Luciferase samples from *Photuris* and *Photinus* were immunoblotted with the anti-*Photinus pyralis* luciferase antibody as described under "Materials and Methods". Lane 1: *Photinus* luciferase 30 pg. Lane 2-4: the partially purified *Photuris* luciferase 300 pg, 3 ng, 30 ng respectively. Lane 5: the inactivated crystallized *Photuris* luciferase 300 pg. Lane 6: prestained SDS-PAGE standard, phosphorylase B (106 kD), bovine serum albumin (80 kD), ovalbumin (49.5 kD), carbonic anhydrase (32.5 kD), soybean trypsin inhibitor (27.5 kD), lysozyme (18.5 kD).

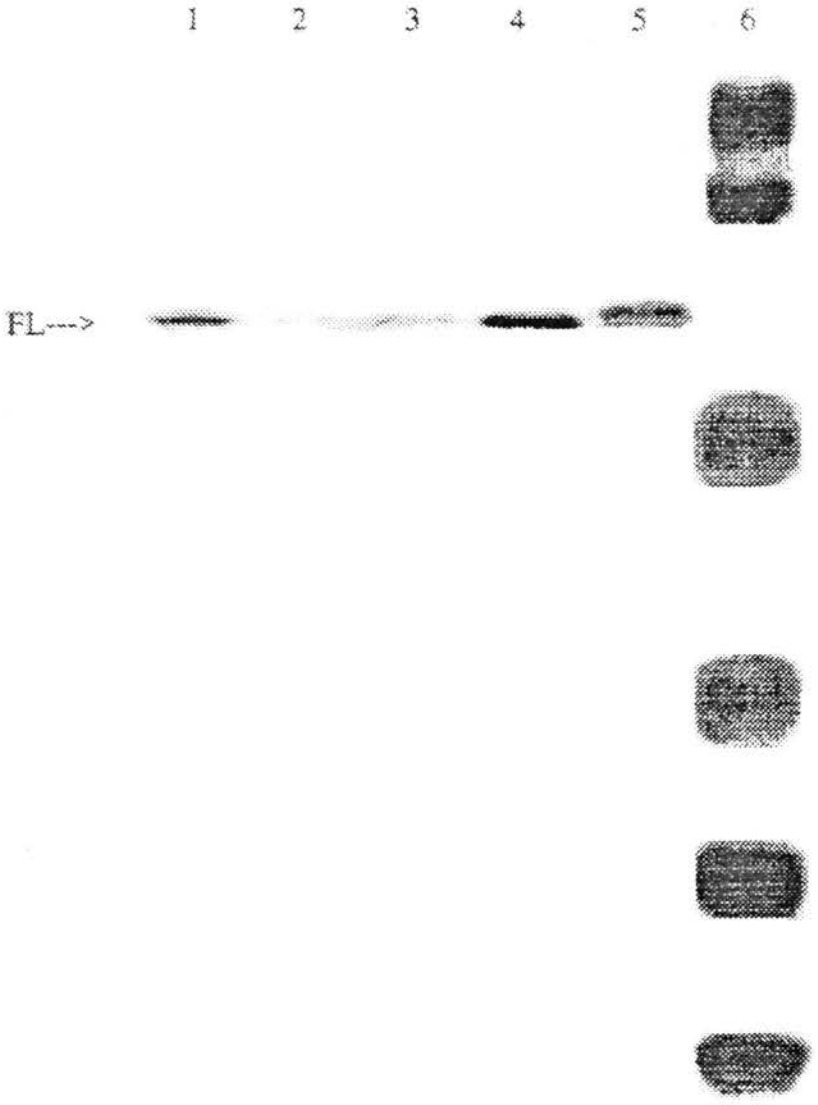
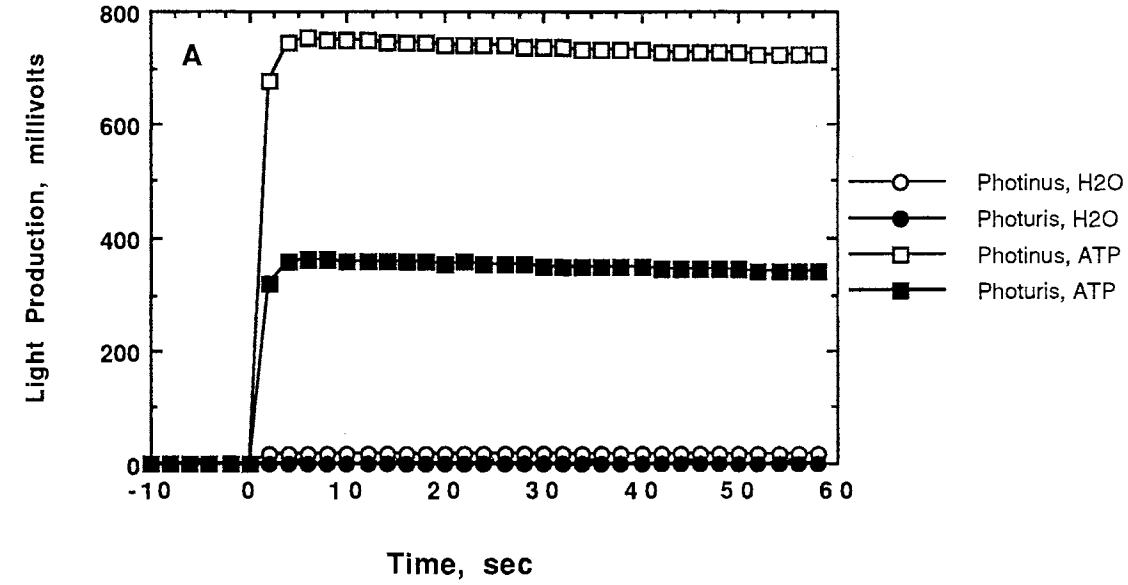

1
2
3
4
5
6
FL--->

Figure 20. Time Course of Light Production of Firefly Luciferases

Partially purified *Photuris* luciferase (2 μg) and *Photinus* luciferase (20 ng) were added to 170 μl of reaction mixture and assayed by using an LKB Model 1251 Automatic Luminometer after injecting 0.24 μM ATP for the constant-light production assay (Fig. A). The flash-light production of *Photuris* luciferase (0.4 μg) and *Photinus* luciferase (2 ng) was assayed in the same way after injecting 0.24 mM ATP into the reaction mixture (Fig. B).


A
Light Production, millivolts
800
600
400
200
0
-10
0
10
20
30
40
50
60
Time, sec
Photinus, H2O
Photuris, H2O
Photinus, ATP
Photuris, ATP

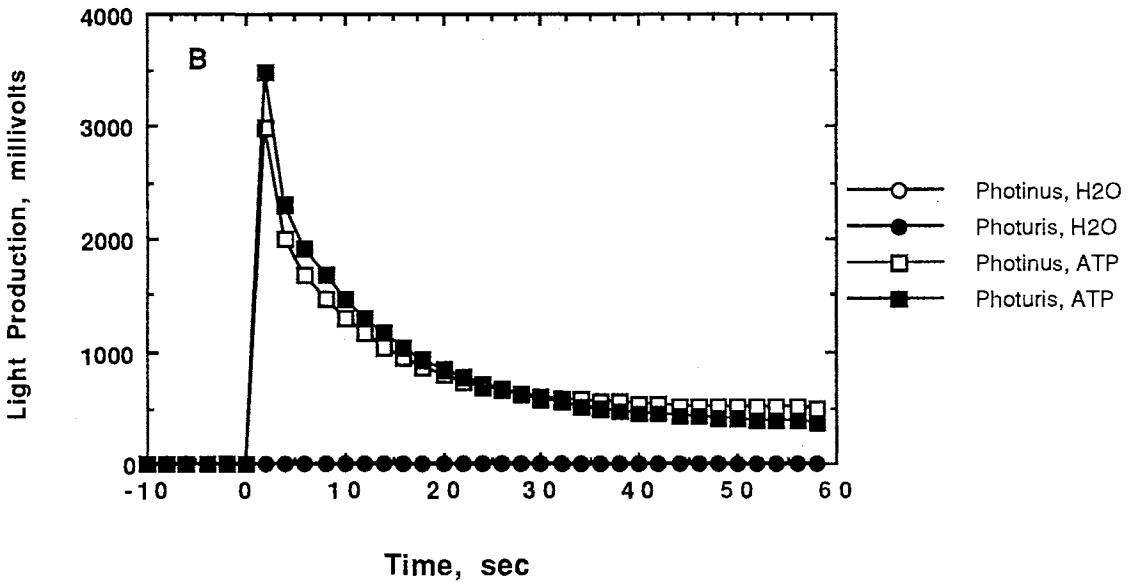
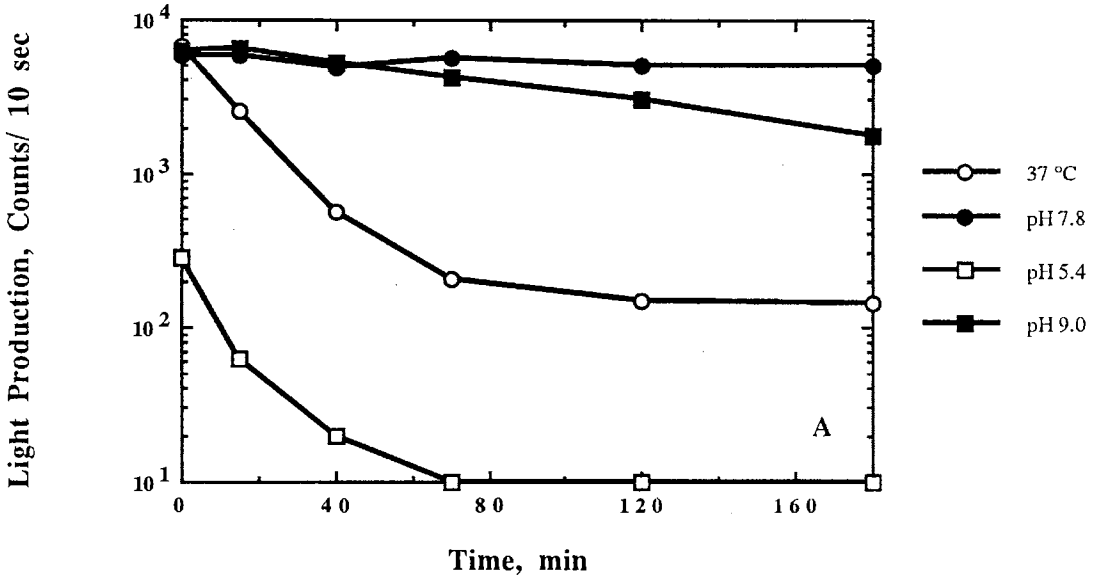
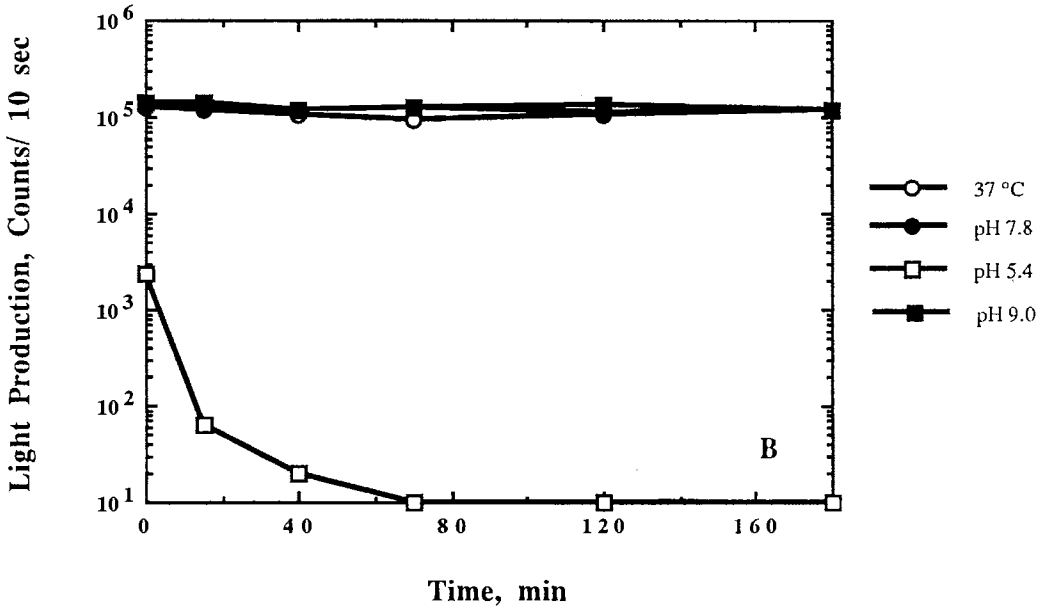

B
Light Production, millivolts
4000
3000
2000
1000
0
-10
0
10
20
30
40
50
60
Time, sec
Photinus, H2O
Photuris, H2O
Photinus, ATP
Photuris, ATP

Figure 21. Variation of Stability of Luciferases

The activity of the partially purified *Photuris* luciferase (2.5 mg/ml), incubated in pH 7.8, 5.5 and 9.2 at room temperature or at 37 °C (pH 7-8), was determined by a Lumac/3M 2010A biocounter (Fig. A). The activity of the *Photinus* luciferase (8.2 μM), incubated under the same conditions, was measured the same way (Fig. B).


Light Production, Counts/ 10 sec
10 4
10 3
10 2
10 1
0
40
80
120
160
Time, min
A
37 °C
pH 7.8
pH 5.4
pH 9.0


Light Production, Counts/ 10 sec
10 6
10 5
10 4
10 3
10 2
10 1
0
40
80
120
160
Time, min
B
37 °C
pH 7.8
pH 5.4
pH 9.0

CHAPTER V

CLONING AND SEQENCING OF THE FIREFLY LUCIFERASE GENE FROM *PHOTURIS PENNSYLVANICA*

Introduction

Firefly luciferase catalyzes the oxidation of luciferin in the presence of ATP, O_2, and Mg^{2+}, producing light (DeLuca and McElroy, 1978). Because of the great sensitivity, ease of use, and cost efficiency of the luciferase assay, the firefly luciferase gene has been widely used in molecular biology (Gould and Subramani, 1988). The firefly luciferase gene has been used to study promoter activity in bacteria, yeast, *Dictyostelium*, plants, viruses, cultured animal cells, and transgenic animals (Gould and Subramani, 1988). Using firefly luciferase as a reporter has many advantages, such as the high sensitivity of bioluminescence measurement, a rapid assay, detectability *in vitro*, and posttranslational modification requirement. Firefly luciferase is 100-1000x as sensitive as the common method using CAT (bacterial chloramphenicol acetyltransferase). Since the firefly luciferase sorts to peroxisomes in eukaryotes, the luciferase gene was a model for elucidating how proteins sort to peroxisomes and the signals needed for targeting proteins into peroxisomes (Keller et al., 1987). Another application of the luciferase gene is to use luciferase in a heterologous gene expression system in mammalian cells (Gould and Subramani, 1988).

The firefly luciferase from the North American firefly, *Photinus pyralis*, is the best-characterized luciferase (DeLuca and McElroy, 1978). In addition, other luciferases from *Luciola mingrelica* in Russia (Filippova and Ugarova, 1979), *Luciola cruciata* (Shimomura

et al., 1977), and *Luciola lateralis* (Kajiyama et al., 1992) in Japan have also been purified and characterized. The reactions catalyzed by these luciferases are similar. All of the beetle luciferases can utilize synthetic *P. pyralis* luciferin to produce light, which means that all of these species synthesize the same or a very similar luciferin (de Wet, 1986). Therefore, there must be extensive conservation in the overall structure of the beetle luciferases because they must have similar substrate binding sites. The antibody raised against the *P. pyralis* luciferase can recognize the luciferases from the six species of fireflies as well as the luciferase from the click beetle *P. plagiophthalamus* (Weinhausen and DeLuca, 1985).

Although luciferases from different fireflies catalyze the same reaction, they have differences in some physicochemical properties. Luciferases isolated from different beetle species are distinguished by maximum wavelength of light emission, e.g. luciferases from *L. mingrelica, L. cruciata*, and *P. pyralis* have λ_{max} between 562-570 nm, whereas *L. lateralis* produces light at λ_{max} 552 nm. All luciferases from the genus *Luciola* are inactivated rapidly at low ionic strength (Brovko et al., 1982), but *P. pyralis* luciferase is crystallized as an active form under the same conditions (McElroy, 1960). Luciferase from *L. lateralis* is more stable than other luciferases at increased temperature and extreme pH. Four luciferases from different organs of the snapping beetle *Pyrophorus plagiophtalamus* catalyze the same reaction as firefly luciferase but they have different emission maxima: green, 546 nm; yellow-green, 560 nm; yellow, 578 nm; and orange, 594 nm (Wood et al., 1982). Since the substrate luciferin is the same for all species of fireflies, the differences in the color of the light must be due to variations in the structure of the enzymes (McElroy and Seliger, 1966).

In 1985 DeLuca et al. isolated cDNA of *Photinus pyralis* luciferase (de Wet et al., 1985) and reported the amino acid sequence of the 550-residue enzyme (de Wet et al., 1987). Since then the deduced amino acid sequences of cDNA of luciferases from eight different beetles have been determined; these are luciferases from *L. cruciata* (Masuda et al., 1989), four different luciferases from firefly *Pyrophorus plagiophthalamus* (Wood et

al., 1989), luciferase from *L. lateralis* (Tatsumi et al., 1992) and from *L. mingrelica* (Kutuzova et al, 1992). Luciferases from beetles have very high amino acid sequence homology (Ugarova et al., 1994). For example, luciferases from *Luciola* have 80% homology. The most coincidence is observed in the C-terminal domain. However, luciferase from *L. mingrelica* has less homology with *P. pyralis* (67%) and much less homology with beetle luciferases (43%). The identification of structure for luciferases from differnt *Luciola* species with different maximum wavelenth suggests that the shift of the bioluminescent spectrum might be caused by some particular amino acids present or absent at a specific position in different species (Ugarova et al., 1994). The mutation experiments done by Kajiyama and Nakano in 1991 demonstrated the relationship between the amino acid sequence of luciferase and the bioluminescence spectrum (Kajiyama and Nakano, 1991). The mutations of native *Luciola cruciata* luciferase with substitutions Gly-326 to Ser or His-433 to Tyr cause the spectrum to be shift from yellow-green (λ_{max} = 562 nm) to the red region (l_{man} = 609 or 612 nm). Shifts to yellow-orange (λ_{max} = 595 nm) and orange (λ_{max} = 595 nm) are obtained when Pro-425 or Ser-286 is substituted for Ser or Asn, respectively. A shift to the green region (λ_{max} = 558 nm) is observed by mutation of Val-239 to Ile. Thus increasing hydrophobicity leads to a blue shift, whereas increasing hydrophilicity causes a red shift (Ugarova et al., 1994).

Since all firefly luciferases catalyze the conversion of the same substrate luciferin to produce light and use ATP, highly conserved sequences must exist for binding substrates in various luciferases. Furthermore the light spectra produced by luciferases are particularly determined by amino acid residues at specific positions. Therefore the cloning and sequencing of luciferases genes from different firefly species will allow us to obtain useful information about the function and structure of firefly luciferase by comparison of the amino acid sequences of different luciferases. Also this will increase knowledge about luciferase at the molecular level. The objective of the research presented in this chapter was to clone and sequence the luciferase gene from the firefly *Photuris pennsylvanica*. By

analyzing and comparing the luciferase sequences from different species, it may be possible to identify the highly conserved regions of amino acids that form the active sites of luciferase.

A cDNA library from the tails of *Photuris pennsylvanica* has been constructed and the luciferase gene has been cloned and sequenced. The data described here show 1) the *Photuris* luciferase gene is expressed in bacterial cells and the activity of luciferase is detected; 2) the inserted size of the luciferase gene is 1.8 kb; 3) the size of the open reading frame is about 550 amino acids; 4) there is high homology of amino acid sequences exits among *Photuris, Photinus, Luciola* and other beetle luciferases.

Materials and Methods

Materials and Reagents

Photuris pennsylvanica fireflies were collected locally around Stillwater at night. The fireflies were frozen in liquid nitrogen and the lanterns were removed for RNA isolation. Luciferin was obtained from Sigma. Reagents for RNA and mRNA isolation were from Stratagene [RNA isolation kit (Cat # 200345, July 6, 1993) and Poly (A) Quik™ mRNA Purification kit (Cat # 200349) November 7, 1989]. Reagents and materials for cDNA synthesis were also obtained from Stratagene [ZAP-cDNA® Synthesis kit (Cat # 200400, 200401 and 200402) July 9, 1993]. Restriction enzymes *EcoR* I, *Xho* I, *Kpn* I and *BamH* I, were purchased from Promega. α-^{32}P-dATP (3000 Ci/mmole) was purchased from New England Biolabs.

Bacterial Strains and Media

E. coli strain XL1-Blue MRF' (Stratagene: *Δ (mcrA) 183, Δ (mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac*, [F' *proAB, lac*^{q}*ZΔM15*, Tn*10* (tet^{r})] was used for λZAP library plating and titering , *E. coli* strain XL1-Blue (Stratagene: *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac*, [F' *proAB, lac*^{q}*ZΔM15*, Tn*10*

(tet^r)] was used for amplification of λZAP library and for recombinant plasmid replication and preparation; *E. coli* strain SOLR™ (Stratagene: e14$^-$ (*mcrA*), Δ (*mcrCB-hsdSMR-mrr*) *171, sbcC, recB, recJ, umuC*::Tn 5 (kan^r), *uvrC, lac, gyrA96, relA1, endA1*, λ^R, [F' proAB, lac^{q}ZΔM15]Su$^-$ (nonsuppressing) was used as a host for *in vivo* excision and cDNA library screening.

The following media were used for propagating *E. coli* and bacteriophage λ: LB (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.4), NZY (per liter: 10 g NZ Amine, 5 g yeast extract, 5 g NaCl, 2 g $MgSO_4.7H_2O$, pH 7.4), bottom agar (LB plus 15 g/liter agar for bacterial colonies or LB plus 12 g/liter agar and 2 g/liter $MgSO_4.7H_2O$ for phage plaques), top agar (NZY plus 7 g/liter agarose), and SM (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, and 0.1% gelatin). *E. coli* strains to be used as hosts for bacteriophage λ were grown to stationary phase in NZY broth plus 0.2% maltose at 37 °C. Phage (1 μl of SM) was mixed with 200 μl of fresh, stationary cells and incubated at 37 °C for 15 min. Liquid top agar (3 ml) at 50 °C was added to the cells, mixed and poured onto a bottom agar plate that had been prewarmed to 37 °C. After the top agar had solidified the plates were inverted and incubated.

RNA and mRNA Isolation

All procedures were done according to the directions provided with the Stratagene RNA isolation kit. The lanterns (1 g, stored frozen in –80 °C) were ground to a powder under liquid nitrogen with a mortar and pestle. The powdered lanterns were homogenized in 10 ml of denaturing solution (guanidinium thiocyanate with β-mercaptoethanol 100 μl /14 ml). Then the homogenized solution was treated with 1 ml of 2 M sodium acetate (pH 4.0), 10 ml of phenol saturated with water and 2 ml of chloroform:isoamyl alcohol mixture (25:24:1). After centrifugation at 10,000 x g, the aqueous solution was mixed with an equal volume of isopropanol to precipitate RNA at –20 °C. The pellet of RNA was obtained by centrifugation at 10,000 x g and, then treated with 3 ml of the denaturing

solution and 3 ml of isopropanol, then centrifuged again to precipitate RNA. The following RNA clean procedures were recommended by Dr. Steve Hartson. The RNA sample (in H_2O) was dissolved in 0.5 ml TE-SDS buffer (10 mM Tris HCl, 1 mM EDTA, 0.1% SDS) and incubated at room temperature for 10 min, then heated at 65 °C for 20 min followed by chilling on ice for 20 min. The purified supernatant RNA was harvested by centrifugation (10,000 x g for 5 min) and stored at –20 °C. The $A_{260/280}$ ratio of the RNA was determined to be about 1.9-2.0 on a Gilford Model 2000 spectrophotometer.

Poly (A) isolation was done as directed by the Stratagene Poly (A) Quik kit. The RNA solution (500 µg) was mixed with 200 µl 10 x sample buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5.0 M NaCl), applied to a Poly (A) Quik column, washed with a high-salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl) and a low-salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl), then eluted with an elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The $A_{260/280}$ ratio of mRNA was > 2.1. From 1 g of lanterns, 1.5 mg of total RNAand 9 µg of poly (A) were obtained.

ZAP-cDNA Synthesis

The procedures for cDNA synthesis were according to the Stratagene ZAP-cDNA® Synthesis Kit directions. The brief procedures were: poly (A) (5 µg) was used to synthesize cDNA using Xho I-oligo (dT) primer and 5'-methyl-dCTP to make the first strand. After second-strand synthesis and ligation to EcoRI adaptors, cDNA was size-fractionated through a Sephacryl S-400 column. The fraction eluted at > 1 kb was ligated into the Uni-ZAP XR vector and packaged with Stratagene's Gigapack II Gold. The resulting library (2/3) was amplified using *E. coli* strain XL1-Blue MRF' and the rest of the primary library (1/3) was excised from ZAP, then converted into pBluescript in SOLR cells. More details of the whole procedures are as following.

First-Strand Synthesis : Poly (A) (5 µg in 25 µl H_2O) was added to 25 µl of first strand synthesis reaction mixture (5 µl 10 x first-strand buffer, 5 µl 0.1 M DTT, 3 µl 10

mM first-strand methyl nucleotide mixture, 2 µl linker-primer (1.4 µg/µl), 6.5 µl DEPC H_2O, 1 µl RNase block I (40 U/µl), 0.5 µl α-^{32}P-labeled dATP (800 Ci/mmol), 2.5 µl M-MuLVRT (20 U/µl). The reaction mixture was incubated at 37 °C for 1 hr then stored on ice for the second-strand synthesis.

Second Strand Synthesis: To 45 µl of first-strand reaction mix, the following reagents were added in order: 40 µl 10 x second-strand buffer, 15 µl 0.1 M DTT, 6 µl 10 mM second-strand nucleotide mixture, 268.3 µl distilled water, 2 µl α-^{32}P-labeled dATP (800 Ci/mmol), 4.5 µl RNase H (1 U/µl) and 19.2 µl DNA polymerase I (5.2 U/µl). The mixture was incubated at 16 °C for 2.5 hr. The reaction was terminated by extraction with 400 µl phenol:chloroform (1:1 v/v). After extraction with another equal volume of chloroform, the cDNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol overnight, then washed with 80% ethanol and dried. The final product was dissolved in 45 µl sterile water.

Blunting the cDNA Termini: 5 µl 10 x buffer # 3, 2.5 µl 2.5 mM dNTP mix and 0.5 µl Klenow fragment (5 U/µl) were added to 42 µl second-strand mix and incubated at 37 °C for 30 min. The mixure was extracted with an equal volume of phenol:chloroform, then precipitated with sodium acetate and ethanol as described above.

Ligating *EcoR* I Adaptors : The methylated cDNA was dissolved in water and ligated to phosphorylated *EcoR* I linkers (7 µl *Eco*R I adaptors, 1 µl 10 x buffer # 3, 1 µl 10 mM rATP, 1 µl T4 DNA ligase (4 Weiss U/µl)). The reaction mixture was incubated at 4 °C overnight and the reaction terminated by heating at 70 °C for 30 min.

Kinasing the *Eco*R I Ends: The kinasing of the adaptor ends was done by adding 1 µl 10 x buffer # 3, 2 µl 10 mM rATP, 6 µl sterile water, and 1 µl T4 polynucleotide kinase (10 U/µl), and incubating at 37 °C for 30 min. The kinasing was inactivated by heating (70 °C, 30 min).

Xho I Digestion : Digestion was accomplished by adding 28 µl *Xho* I buffer supplement and 3 µl Xho I (40 U/µl) to the above reaction mix, and incubating at 37 °C for

1.5 hr. STE (5 µl) was added to the reaction mixture for Sephacryl S-400 column separation.

Size Fractionation : The cDNA was loaded into the prepared spin Sephacryl S-400 column and spun at 400 × g for 2 min. Fractions were collected four times. The size of cDNA was checked by 1% agarose electrophoresis. The fraction containing > 1 kb cDNA was extracted with phenol:chloroform and washed with ethanol as described. The cDNA was quantitated by the ethidium bromide plate assay. A total of 110 ng cDNA (> 1 kb) was obtained.

Ligating cDNA into Uni-ZAP^{TM} XR Vector Arms : The total ligation reaction volume was 5 µl including 2.5 µl cDNA (110 ng), 0.5 µl 10 x buffer # 3, 0.5 µl 10 mM rATP, and 1 µl Uni-ZAP XR vector (1 µg/µl). The test insertion (kit supplement) was done the same as for the sample. The ligation mixture was incubated at 4 °C for two days and stored at –80 °C.

Packaging : The cDNA (1 µl ligation reaction) was immediately added to the Freeze-Thaw extract, then the Sonic extract was added to the Freeze-Thaw extract containing the DNA. The packaging mixture was incubated for 2 hours at 22 °C and ended by adding 500 µl SM buffer and 20 µl chloroform, and stored at 4 °C.

Plating and Titering the Primary cDNA Library : The packaged ligation product (1 µl of 1:1 and 1:10) was mixed with 200 µl of XL1-Blue MRF' cells (A_{600} = 0.5) and incubated at 37 °C for 15 min with gentle shaking, then the 3 ml of top agar (with 15 µl 0.5 M IPTG and 50 µl X-gal 250 mg/ml) was added and the bacteria were plated onto the NZY plates. The plaques were counted after incubating at 37 °C for 7-8 hours.

Amplification of Uni-ZAP XR Library : The packaged mixture (20 aliquots) containing 50,000 plaque-forming bacteriophage was mixed with 600 µl of A = 0.5 host cells (XL1-Blue MRF') and incubated at 37 °C for 15 min. The top agar (same as before) 6.5 ml was mixed with each aliquot of infected bacteria, which was then spread onto a freshly poured 150-mm plate of bottom agar. The harvesting of bacteriophage was done

after 6-8 hr incubation (37 °C) by overlaying 10 ml of SM buffer and stored at 4 °C overnight. After extraction with 5% chloroform followed by centrifugation, the amplified library was stored at 4 °C with 0.3% chloroform. The titer of the amplified library was checked in the same way as that of the primary library.

cDNA Library Screening

in vivo Excision of pBluescript® from Uni-ZAP™ XR(Stratagene ZAP-cDNA™ Synthesis Kit) In a 10-ml Falcon tube, 200 µl XL1-Blue MRF' cells (A_{600} = 1) prepared with 10 mM $MgSO_4$, 100 µl of primary phage stock (1×10^6 phage particles), and 1 µl of ExAssist helper phage (1×10^7 phages) were combined and incubated at 37 °C for 15 min. Then 3 ml of 2 × YT medium was added and the mixture incubated for 2-2.5 hr at 37 °C with shaking. The *in vivo* excision was ended by heating at 70 °C for 20 min and centrifuging (4000 x g, 15 min). The phagemid stock was stored at 4 °C.

Luciferase Expression Screening The phagemid stock (10 µl) was mixed with 200 µl of SOLR cells (A = 1), prepared with 10 mM $MgSO_4$, and incubated 15 min at 37 °C, then plated onto LB-ampicillin plates (50 µg/ml). Twelve plates were used for the first screening and incubated at 37 °C overnight. Each individual plate with 1 mm diameter of colonies was lifted with a piece of labeled nitrocellulose filter. The master plates and lifting filters were incubated at 37 °C for another 2 hr until the colonies came out. The filters with colonies were switched to 22 °C and incubated for another 2 hr. The filters were socked with 1 mM luciferin (in 0.1 M Tris acetate, 10 mM $MgSO_4$, 2 mM EDTA, pH 5.0) for 5 min and wrapped with Saran Wrap®. The positive colonies were identified by exposing to x-ray film overnight. After film development, the positive colonies were picked from the master plates and streaked onto fresh LB-ampicillin plates. The second and third screenings were done the same way as the first screening.

Plasmid (containing luciferase gene) Isolation

Plasmid (SOLR cells) Preparation for Transformation All procedures followed the "A Modified Mini-Alkaline-Lysis/PEG Precipitation Procedure for Preparing DNA Templates for Automated DNA Sequencing" (CORE news, May, 1994). A positive colony was inoculated into 10 ml 2 × YT medium and incubated at 37 °C overnight with 150 rpm shaking. The bacteria were pelleted by centrifugation and resuspended in 200 µl GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0). Lysis was accomplished by adding 300 µl of freshly prepared 0.2 N NaOH/0.1% SDS; then the mixture was neutralized by adding 300 µl of 3.0 M potassium acetate, pH 4.8. After removing the cellular debris, RNase A (DNase-free) was added to a final concentration of 20 µg/ml and the sample was incubated at 37 °C for 20 min. The supernant was extracted with 400 µl phenol (saturated with Tris buffer):chloroform, then 400 µl phenol:chloroform:isoamylalcohol (25:25:1), followed with another 400 µl of chloroform. The total DNA was precipitated by adding an equal volume of 100% isopropanol and washing with 70% ethanol. The pellet was dissolved in 32 µl of deionized H_2O and treated with 8 µl of 4 M NaCl and 40 µl of autoclaved 13% (w/v) PEG-8000. The pellet was washed with 70% ethanol and dissolved in 20 µl of deionized water. The isolated plasmid was quantitated using a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments).

Transformation of Plasmids from the SOLR cells to the XL1-Blue Cells The competent XL1-Blue cells was supplied by Dr. Holly Schaeffer. The methods were recommended by Dr. Holly Schaeffer. DNA (plasmid, 1 µg) was added to 200 µl competent cells and the mixture incubated on ice for 30 min. The cells were heat shocked at 42 °C for 90 s and put back on ice for 1-2 min, then LB broth (1 ml) was added to the cells. The cells were recovered at 37 °C for 45 min and plated on LB-ampicillin plates.

Plasmid (XL1-Blue cells) Preparation for DNA Sequencing The methods for preparing plasmid were the same as "A Modified Mini-Alkaline-Lysis/PEG Precipitation

Procedure for Preparing DNA Templates for Automated DNA Sequencing" described above.

Measurement of Luciferase Gene Size

The plasmid containing the luciferase gene (0.4 μg) was digested with *EcoR* I (0.5 μl 1 mg/ml) and *Xho* I (0.5 μl, 1 mg/ml) in the reaction mix (1 μl 10 x restriction enzyme buffer, 0.5 μl 1 mg/ml BSA, 6.5 μl deionized H_2O) at 37 °C overnight. Another digestion reaction was carried out by adding *BamH* I and *Kpn* I under the same conditions. The digestion was terminated by adding 3 μl stop/load buffer (50% glycerol, 10 mM EDTA, pH 8.0, 0.025% Bromophenol Blue). The digested samples were separated by 1% agarose electrophoresis with DNA 1 kb ladder as a standard and uncut plasmid as a control.

Sequencing of Luciferase Clone

The sequencing of luciferase containing plasmid was done by the CORE Facility in the Department of Biochemistry and Molecular Biology, Oklahoma State University by using the ABI 373A Automatic DNA sequencing system. The sequencing was started at the 5'-terminal and the 3'-terminal using T3 and T7 primer, and continued in two directions using the primers that were designed by the program of Oligo 4.0 and synthesized by the CORE Facility in the Department of Biochemistry and Molecular Biology, Oklahoma State University using a Model 381A Synthesizer. The sequence was compared with other sequences and analyzed by using BLAST, FASTA, and BLITZ.

Results

A cDNA Library of the Tails of *Photuris pennsylvanica* Is Constructed

RNA and mRNA of the tails from *Photuris pennsylvanica* were isolated and the quality of the RNA and mRNA was analyzed by formaldehyde agarose electrophoresis and *in vitro* translation (Ambion Retic Lysate IVT™ Kit Cell-free Translation System)

(experiments not shown). The cDNA library of the tails from *Photuris pennsylvanica* was constructed with 5 μg of poly (A) using Stratagene's ZAP-cDNA ™ Synthesis Kit. The total primary phage library (from 1/5 of ligation) packaged was about 3.15×10^6 (plaques) with a high ratio of recombinant to background (485:1). The total titer for the amplified phage library was 3.6×10^{10} pfu/ml (160 ml).

The Photuris Luciferase Gene Is Expressed in Bacterial Cells and the Activity of Luciferase Is Detected

The screening of the cDNA library was done by using luciferin to detect expressed luciferase in the SOLR cells. Two positive colonies from 10^4 of the primary phage library excised phagemids (12 x 150 mm plates) were found by exposing the colony containing filters to X-ray film for 1 hr. After streaking a single positive colony to a new LB-ampicillin plate, many colonies expressing luciferase were visualized by eye due to the glow from the colonies. Figure 22 displays the spots from the positive XL1-Blue colonies.

The Inserted Size of *Photuris* Luciferase Gene Is 1.8 kb

Restriction digestion with *EcoR* I and *Xho* I and agarose electrophoresis revealed that the insert size of the *Photuris* luciferase gene is 1.8 kb (Fig. 23, lane 3). The restriction digestion by *BamH* I and *Kpn* I exhibited 3 bands (one band showed 3 kb for vector; the other two bands were 1.2 kb and 0.6 kb) in the agarose gel (Fig. 23, lane 5), implying a site for one of these enzymes within the luciferase gene. Neither of these two restriction enzyme sites are found in the midst of the *Photinus* luciferase gene.

Most of the Sequences of the *Photuris* Luciferase Gene Are Identified

The plasmid contained a single extended open reading frame encoding a polypeptide about 550 amino acid residues in length. There was an upstream untranslated region of 61

base pairs and a downstream untranslated region of 106 base pairs were found, including 23 3'-terminal A residues (Fig. 24).

Sequences of the *Photuris* Luciferase Have High Homology with the *Photinus* and *Luciola* Luciferases

The sequences of *Photuris* luciferase were compared with those of the *Photinus* and *Luciola* luciferases. High homology with conservative substitutions permitted, about 51.5% with *Photinus*, 48.9% with *Luciola lateralis* and 47.6% with *Luciola cruciata,* were found (Fig. 25). The long highly conserved fragments of these three luciferases were located at 240-248, 310-324, and 337-347 residues.

Discussion

In this chapter, a cDNA library of lanterns from *Photuris pennsylvanica* fireflies was constructed and the luciferase gene from this species was cloned and sequenced. The inserted size of the luciferase gene was 1.8 kb, including an upstream untranslated region of 61 bases pairs, and a downstream untranslated region of 106 base pairs. Therefore the luciferase gene from *P. pennsylvanica* is about 1.6 kb, coding about 550 amino acids. The amino acid sequences deduced from the nucleic acid sequences were shown to have high homology with that of the luciferases from other beetles.

When the amino acid sequence of luciferase from *P. pennsylvanica* are compared with those of eight different beetles, the amino acid sequences of firefly luciferases are found to be strongly conserved (Fig. 26). There is 31% homology among all these luciferases. The homology of *P. pennsylvanica* with *Photinus pyralis* is 27%, and 21-24% with *Luciola mingrelica, Luciola cruciata*, and *Luciola lateralis*. There is 15% homology with click beetle (*Pyrophorus plagiophthalamus*). There are 32 amino acid residues (6%) that are conserved in all other eight luciferases but not in the preliminary sequence of *P. pennsylvanica* luciferase.

The total number of charged residues are essentially the same for all insect luciferases. The major differences are in the number of Trp residues and Cys residues (Devine et al., 1993). There are two Trp residues in the luciferase of *P. pennsylvanica* , which is the same number as in *P. pyralis* (de Wet et al., 1987). Two or three Trp residues are in four different organs of click beetle (*P. plagiophthalamus*) (Wood et al., 1989), and only one Trp residue in the three species of *Luciola* (Masuda et al., 1989; Tatsumi et al., 1992). The Trp residue located at 420 of luciferase from *P. pennsylvanica* is also in all of the other luciferases. It is the highly conserved Trp residue that was inactivated with NBS and suggested to be related to the luciferin binding pocket (Kutuzova and Baldwin, 1993). The luciferase from *P. pennsylvanica* has seven Cys residues, *P. pyralis* has four Cys residues, and there are 7-8 Cys residues in *Luciola* luciferases and 13 in click beetle luciferase (*P. plagiophthalamus*). The Cys residue located at 391 of luciferase from *P. pennsylvanica* is highly conserved in all of the luciferases. This Cys residue was one of the two Cys residues modified by NEM (Vellom, 1990) and protected by substrates of luciferase.

Comparison of the encoded amino acid sequences of luciferases from three species of *Luciola*, *P. pyralis* and click beetle revealed a high homology (residues 197-210) between all insect luciferases and several MgATP utilizing enzymes which catalyze the adenylation of carboxylic acid-containing substrates with MgATP (Scholten et al., 1991). This highly conserved fragment is also found in the amino acid sequences of *P. pennsylvanica* luciferase.

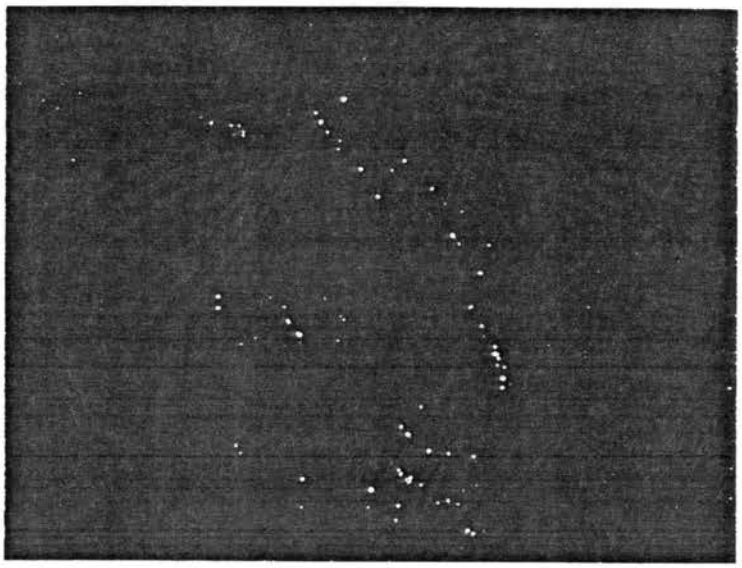
In Chapter III, a peptide GLTGK of *P. pyralis* luciferase labeled with ^{14}C-TUD was suggested to be related to the ATP binding site. The similar peptide GSTGK is also found in the amino acid sequence of luciferase from *P. pennsylvanica* at a similar location near the C-terminus.

Comparison of the amino acid sequence of luciferase from *P. pennsylvanica* with that from other luciferases helps us to determine the active sites of luciferases, to discover

the mechanism of the highly efficient bioluminescence reaction catalyzed by luciferase, and to understand the relationship between function and structure involved in this enzymatic catalysis.

Figure 22. Bioluminescence Detection of *Photuris* Luciferase in XL1-Blue Cells

The cells (on the nitrocellulose filter) were allowed to express luciferase for 2 hr at 22 °C after they were grown overnight at 37 °C. The filters bearing bacteria were soaked in luciferin (1 mM, pH 5.0) for 5 min and then exposed to Polarid film (3000) for 2 hr (top). Two NaOH pellets with luminol to give a chemiluminescence reaction (bottom).



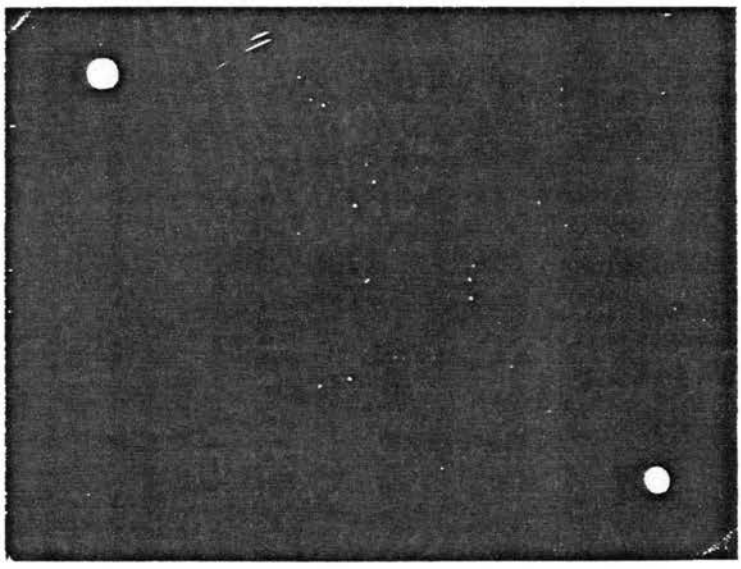


Figure 23. Restriction Analysis of Luciferase-Containing Plasmid

Plasmid (0.4 μg) was digested with *EcoR* I and *Xho* I, and with *BamH* I and *Kpn* I in a restriction mix (0.5 μl of each restriction enzyme 1 mg/ml, 1 μl of 10 x restriction enzyme buffer, 0.5 μl of 1 mg/ml BSA, 6.5 μl deionized H_2O) and the mixture incubated at 37 °C overnight, then electrophoresis was conducted on a 1% agarose gel. Lane 1: uncut. Lane 2: 1 kb DNA ladder. Lane 3: digestion with *EcoR* I and *Xho* I. Lane 4: 1 kb DNA ladder. Lane 5: digestion with *BamH* I and *Kpn* I.

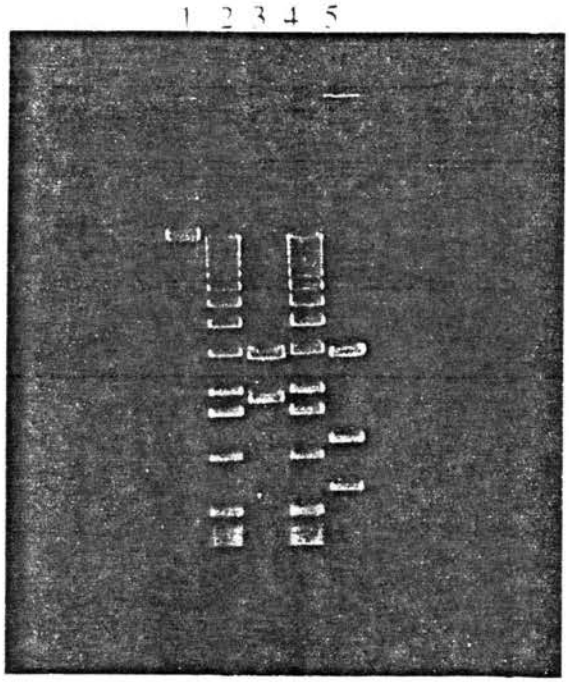

1 2 3 4 5

Figure 24. The 5' and 3' Terminal Sequences of the Luciferase Gene

The initiation codon for the luciferase reading frame is at +62 and the termination codon is at 106 from the 3' terminus with 23 A residues. These are preliminary sequences that have not been confirmed by sequencing both strands.

5' Terminus:

TCCCGGAGCCCTGATGGAGCTCCACCGCGGAGGCGGTCGCTCTAGAACTAGTGG
ATCCCCCGGGCTGCAGGAATTCGGCACGAGCCAGACGGGTCTGGATAGTTTGAT
TTAAATACTTAAAGTTACAAGTTGAAATAATGGAAGATAAAAATATTTTATATG
GACCTGAACCATTTTATCCCTTGGCTGATGGGACGGCTGGAGAACAGATGTTTT
ACGCATTATCTCGGTATGCAGATATTTCAGGATGCATTGCATTGACAAATGCTC
ATACAAAAGAAAATGTTTTATATGAAGAATTTTTAAAATTGTCGTGTCGTCTAG
CGGAAAGTTTTACAAAGTATGGATTAAAACAAAACGACACAATAGCGGTGTGTA
GTGAAAATGGTTTGGAATTTCCCCCCCTTTAATTGCATCATCGTTTCTCGGGGA
TAATTGCAGCACCCGTTAGTGATAACTACATTGACGGGGATTTAATCACAAGCT
TGGGTATTGGTAAACCCACCATATTTTTGGGTCCCCGGATACTTCTCAAAAAGT
CCTGATTGTAAATCTCAATCAATTCGTGGACTTTTTTTTTTTAGCCTAATCGAGC
TAGGGGGTTTCATGCCCAAAACTTTTCTCAACTCCGTTTTACTTGAGTAAGATT
AACCCTCTTCACGAAAGCCGTGGGGGGATGTCCCCGCACTGGTTTTTGGGGCAA
ATACCCAAATTCTGGCGCCCCATGACGTCCCTCGGGCC

3' Terminus:

GATACTTTGGCGGGTGATGAAGCCCTGGTTTATACGGGCTTCTGAGCGGGCCCT
GAAAGTCATCCCTGAGGTGGCCCCCCAGAATTGGCCATGACCTGAGTTTATTAG
GCGTTGTATGAAGTATAAATGTGGAGACTCCAACCATTATCCCAAAGGGGGGTT
CCCCTGTGATTTCCTATTATGCCATGTGCCAATTTAAATGTGACAGGCTGAATC
ATTAATTAAAATAAGGTTATCAGGTTGCACCCTCTGAAATTGGGGGAATACTCT
TACCAACACCCGTATATTGTTGATGCCGCCGTTACTGGAATACCGGATGAAGCC
GCGGGCGAGCTTCCACCTGCAGTTGTTGTAGTACAAGACTGGAAAATATCTAAA
CGGAACAAATCGTACAAAATTTTGTTTCCAGTCAAGTTTCAACAGCCAAATGGC
TACGTGGTGGGGTGAAATTTTTGGATGAAATTCCCAAAGGATCAACTGGAAAAA
TTGACAGAAAAGTGTTAAGACAAATGTTTGAAAAACACAAATCTAAGCTGTAAG
GCTACGTTTCTACGTTATCTATTTATCTATCTACACGTTATCTAATTATGTTTG
AGTTATAATTTATTTTATGTATATGTACAATTATTTATGCTTCTGACTTCATCG
TCCATTAAAAGTGGGGTGCAATAAAAAAAAAAAAAAAAAAAAAAACTCGAGGG
GGGGCCCGGTATCCAGGTCGCATAAGGGC

Figure 25. Comparison of Amino Acid Sequences of Luciferases from *Photuris, Photinus,* and *Luciola*

The entire amino acid sequences of three luciferases, deduced from the DNA sequences, are obtained and compared. High homology is marked with *. 28e00939 = *Photuris pennsylvanica*; Luci-phopy = *Photinus pyralis*; Luci-lucla = *Luciola lateralis*; Luci-luccr = *Luciola cruciata.*

```
                1                                                  50
              **    * ***  ** ****** *** ****   *   ***   * **  ***** * *
28e00939     ME....D.KNILYGP EPFYPLADGT AGEQMFYALS RYADISGCIA LTNAHTKENV
luci_phopy   ME....DAKNIKKGP APFYPLEDGT AGEQLHKAMK RYALVPGTIA FTDAHI.E.VNI
luci_lucla   MENMEND.ENIVYGP EPFYPIEEGS AGAQLRKYMD RYAKL.GAIA FTNALTG..VDY
luci_luccr   MENMEND.ENIVVGP KPFYPIEEGS AGTQLRKYME RYAKL.GAIA FTNAVTG..VDY

               51                                                 100
              * * *  ** ****    ** *  *  **** *** **** *  ** *  *
28e00939      LYEEFLKLSC RLAESFTKYG LKQNDTIAVC SENGLQFFLP LIASLYLGII
luci_phopy    TYAEYFEMSV RLAEAMKRYG LNTNHRIVVC SENSLQFFMP VLGALFIGVA
luci_lucla    TYAEYLEKSC CLGEALKNYG LVVDGRIALC SENCEEFFIP VLAGLFIGVG
luci_luccr    SYAEYLEKSC CLGKALQNYG LVVDGRIALC SENCEEFFIP VIAGLFIGVG

               101                                                150
              **  * * * *** *****  ** * *        *                    * *
28e00939      AAPVSDKYIE RELIHSLGIV KPRIIFWVQE YFSKSTECKS .......... IN.SWTFFFSL
luci_phopy    VAPANDIYNE RELLNSMNIS QPTVVFVSKK GLQKILNVQK KLPIIQKIII.MD.SKTDYQGF
luci_lucla    VAPTNEIYTL RELVHSLGIS KPTIVFSSKK GLDKVITVQK KTVTTIKTIV ILDSKVDYRGY
luci_luccr    VAPTNEIYTL RELVHSLGIS KPTIVFSSKK GLDKVITVQK KTVTAIKTIV ILDSKVDYRGY

               151                                                200
              *       *  ** *              * *  * ** ** * *** * ***
28e00939      KKLSEAGGYH PPPAFLYFSI LILILTNSKF KPNSFNRDDQ VALVMFSSGT
luci_phopy    QSMYTFVTSH LPPGF.....  .....NEYDF VPESFDRDKT IALIMNSSGS
luci_lucla    QSMDNFIKKN TPQGF.....  .....KGSSF KTVEVNRKEQ VALIMNSSGS
luci_luccr    QCLDTFIKRN TPPGF.....  .....QASSF KTVEVDRKEQ VALIMNSSGS

               201                                                250
              **  *** ** * * *  ***    ** *** *  * ******  ******** *
28e00939      TGVSKGVMLT HKNIVARFSH CKDPTFGNAI NPPTAILTVI PFHHGFGMPT
luci_phopy    TGLPKGVALP HRTACVRFSH ARDPIFGNQI IPDTAILSVV PFHHGFGMFT
luci_lucla    TGLPKGVQLT HENAVTRFSH ARDPIYGNQV SPGTAILTVV PFHHGFGMFT
luci_luccr    TGLPKGVQLT HENTVTRFSH ARDPIYGNQV SPGTAVLTVV PFHHGFGMFT

               251                                                300
              **** ***** * **  ***  *** ******   * ******  *** ** *
28e00939      TLGYFTCGFR VSLMHTFEEK LFLQSLQDYK VESTLLVPTL MAFFPKSALV
luci_phopy    TLGYLICGFR VVLMYRFEEE LFLRSLQDYK IQSALLVPTL FSFFAKSTLI
luci_lucla    TLGYLTCGFR IVMLTKFDEE TFLKTLQDYK CSSVILVPTL FAILNRSELL
luci_luccr    TLGYLICGFR VVMLTKFDEE TFLKTLQDYK CTSVILVPTL FAILNKSELL

               301                                                350
              ***** * * ********** **** * ***  *    **** ******* **
28e00939      EKYDLSHLKE IASGGAPLSK EIGEMVKKRF KLNFVRQGYG LTETTSAVLI
luci_phopy    DKYDLSNLHE IASGGAPLSK EVGEAVAKRF HLPGIRQGYG LTETTSAILI
luci_lucla    DKYDLSNLVE IASGGAPLSK EIGEAVARRF NLPGVRQGYG LTETTSAIII
luci_luccr    NKYDLSNLVE IASGGAPLSK EVGEAVARRF NLPGVRQGYG LTETTSAIII

               351                                                400
              **  *  **   ** ***    ****   *** **** ***       * ****
28e00939      TPDTDVRPGS TGKIVPFHAV KVVDPTTGKI LGPNETGELY CIIGGDMIMK
luci_phopy    TPEGDDKPGA VGKVVPFFEA KVVDLDTGKT LGVNQRGELC ..VRGPMIMS
luci_lucla    TPEGDDKPGA SGKVVPLFKA KVIDLDTKKT LGPNRRGEVC ..VKGPMLMK
luci_luccr    TPEGDDKPGA SGKVVPLFKA KVIDLDTKKS LGPNRRGEVC ..VKGPMLMK


               401                                                450
              * **  *** *** *****  *** ****    ** ******* ** *******
28e00939      SYYNNEAATK AIINKDGWLR SGDNAYYDNG GHFYIVDRLK SLNKYKGYQV
luci_phopy    GYVNNPEATN ALIDKDGWLH SGDIAYWDED EHFFIVDRLK SLIKYKGYQV
luci_lucla    GYVDNPEATR EIIDEEGWLH TGDIGYYDEE KHFFIVDRLK SLIKYKGYQV
luci_luccr    GYVNNPEATK ELIDEEGWLH TGDIGYYDEE KHFFIVDRLK SLIKYKGYQV

               451                                                500
              **** * *** *** * ****  * **  **  **** ** **     **  *    *
28e00939      PPAEIEGILL QHPYIVDAGV TGIPDEAAGK LPAADVV.ES LI..TEQIVQ NFV
luci_phopy    APAELESILL QHPNIFDAGV AGLPDDDAGE LPAAVVVLEH GKTMTEKEIV DYV
luci_lucla    PPAELESVLL QHPNIFDAGV AGVPDPIAGE LPGAVVVLES GKSMTEKEVM DYV
luci_luccr    PPAELESVLL QHPSIFDAGV AGVPDPVAGE LPGAVVVLES GKNMTEKEVM DYV

               501                                          544
              ******* * **** * **  *** *****  *  *    *     ****
28e00939      SSQVSTAKWL RGGVKFLDEI PKGSTGKIDR KVLRQMFEKH ....KSKL
luci_phopy    ASQVTTAKKL RGGVVFVDEV PKGLTGKLDA RKIREILIKA KKGGKSKL
luci_lucla    ASQVSNAKRL RGGVRFVDEV PKGLTGKIDG KAIREILKKP VAKM
luci_luccr    ASQVSNAKRL RGGVRFVDEV PKGLTGKIDG RAIREILKKP VAKM
```

Figure 26. Comparison of Amino Acid Sequences of Luciferases from *Photuris*, *Photinus*, *Luciola* (three species), and *Pyrophorus* click beetle (four different light color).

The entire amino acid sequences of nine luciferases, deduced from the DNA sequences, are compared. The sequence of *Photuris* luciferase is compared to the sequences of other luciferases, indicated by: $ = conserved; # = present in at least one other sequence; ~ = conservative substitution. NAFF = North American firefly (*Photinus pyralis*); EEFF = Eastern European firefly (*Luciola mingrelica*); JFF1 = Japnese firefly (*Luciola cruciata*); JFF2 = Japnese firefly (*Luciola lateralis*); CB1 = click beetle, green, CB2 = click beetle, yellow-green, CB3 = click beetle, yellow, CB4 = click beetle, orange, (*Pyrophorus plagiophthalamus*); Pp = *Photuris pennsylvanica*.

Comparison of Amino Acid Sequences from Nine Firefly Luciferases

```
Source  #

NAFF    1   M  -  -  -  E  D  A  K  N  I  K  K  G  P  A  P  F  Y  P  L  E  D  G
EEFF    1   M  E  -  M  E  K  E  E  N  V  V  Y  G  P  L  P  F  Y  P  I  E  E  G
JFF1    1   M  E  N  M  E  N  D  E  N  I  V  V  G  P  K  P  F  Y  P  I  E  E  G
JFF2    1   M  E  N  M  E  N  D  E  N  I  V  Y  G  P  E  P  F  Y  P  I  E  E  G
CB1     1   M  M  K  R  E  -  -  K  N  V  V  Y  G  P  E  P  L  H  P  L  E  D  L
CB2     1   M  M  K  R  E  -  -  K  N  V  I  Y  G  P  E  P  L  H  P  L  E  D  L
CB3     1   M  M  K  R  E  -  -  K  N  V  I  Y  G  P  E  P  L  H  P  L  E  D  K
CB4     1   M  M  K  R  E  -  -  K  N  V  I  Y  G  P  E  P  L  H  P  L  E  D  K
Pp      1   M  -  -  -  E  D  -  K  N  I  L  Y  G  P  E  P  F  Y  P  L  A  D  G
            $           $  #     #  $  #  ~  #  $  $  #  $  #  #  $  #     #  #
Source  #

NAFF    21          T  A  G  E  Q  L  H  K  A  M  K  R  Y  A  L  V  P  G  T  I
EEFF    23          S  A  G  I  Q  L  H  K  Y  M  H  Q  Y  A  K  L  -  G  A  I
JFF1    24          S  A  G  T  Q  L  R  K  Y  M  E  R  Y  A  K  L  -  G  A  I
JFF2    24          S  A  G  A  Q  L  R  K  Y  M  D  R  Y  A  K  L  -  G  A  I
CB1     22          T  A  G  E  M  L  F  R  A  L  R  K  H  S  H  L  P  Q  A  L
CB2     22          T  A  G  E  M  L  F  R  A  L  R  K  H  S  H  L  P  Q  A  L
CB3     22          T  A  G  E  M  L  F  R  A  L  R  K  H  S  H  L  P  Q  A  I
CB4     22          T  A  G  E  M  L  F  R  A  L  R  K  H  S  H  L  P  Q  A  I
Pp      20          T  A  G  E  Q  M  F  Y  A  L  S  R  Y  A  D  I  S  G  C  I
                    #  $  $  #  #     #     #  #     #  #  #     ~     #     #
Source  #

NAFF    41          A  F  T  D  A  H  I  E  V  N  I  T  Y  A  E  Y  F  E  M  S
EEFF    42          A  F  S  N  A  L  T  G  V  D  I  S  Y  Q  E  Y  F  D  I  T
JFF1    43          A  F  T  N  A  V  T  G  V  D  Y  S  Y  A  E  Y  L  E  K  S
JFF2    43          A  F  T  N  A  L  T  G  V  D  Y  T  Y  A  E  Y  L  E  K  S
CB1     44          V  D  V  Y  G  E  -  -  E  W  I  S  Y  K  E  F  F  E  T  T
CB2     44          V  D  V  F  G  D  -  -  E  S  L  S  Y  K  E  F  F  E  A  T
CB3     44          V  D  V  F  G  D  -  -  E  S  L  S  Y  K  E  F  F  E  A  T
CB4     44          V  D  V  F  G  D  -  -  E  S  L  S  Y  K  E  F  F  E  A  T
Pp      40          A  L  T  N  A  H  T  K  E  N  V  L  Y  E  E  F  L  K  L  S
                    #     #  #  #  #  #     #  #  ~     $     $  #  #        #

Source  #

NAFF    61          V  R  L  A  E  A  M  K  R  Y  G  L  N  T  N  H  R  I  V  V
EEFF    62          C  R  L  A  E  A  M  K  N  F  G  M  K  P  E  E  H  I  A  L
JFF1    63          C  C  L  G  K  A  L  Q  N  Y  G  L  V  V  D  G  R  I  A  L
JFF2    63          C  C  L  G  E  A  L  K  N  Y  G  L  V  V  D  G  R  I  A  L
CB1     62          C  L  L  A  Q  S  L  H  N  C  G  Y  K  M  S  D  V  V  S  I
CB2     62          C  L  L  A  Q  S  L  H  N  C  G  Y  K  M  N  D  V  V  S  I
CB3     62          C  L  L  A  Q  S  L  H  N  C  G  Y  K  M  N  D  V  V  S  I
CB4     62          C  L  L  A  Q  S  L  H  N  C  G  Y  K  M  N  D  V  V  S  I
Pp      60          C  R  L  A  E  S  F  T  K  Y  G  L  K  Q  N  D  T  I  A  V
                    #  #  $  #  #  #              #  $  #  #     #  #     #  #  #

Source  #

NAFF    81          C  S  E  N  S  L  Q  F  F  M  P  V  L  G  A  L  F  I  G  V
EEFF    82          C  S  E  N  C  E  E  F  F  I  P  V  L  A  G  L  Y  I  G  V
JFF1    83          C  S  E  N  C  E  E  F  F  I  P  V  I  A  G  L  F  I  G  V
JFF2    83          C  S  E  N  C  E  E  F  F  I  P  V  L  A  G  L  F  I  G  V
CB1     82          C  A  E  N  N  K  R  F  F  V  P  I  I  A  A  W  Y  I  G  M
CB2     82          C  A  E  N  N  K  R  F  F  I  P  I  I  A  A  W  Y  I  G  M
CB3     82          C  A  E  N  N  K  R  F  F  V  P  I  I  A  A  W  Y  I  G  M
CB4     82          C  A  E  N  N  K  R  F  F  I  P  I  I  A  A  W  Y  I  G  M
Pp      80          C  S  E  N  G  L  Q  F  F  L  P  L  I  A  S  L  Y  L  G  I
                    $  #  $  $     #  #  $  $  ~  $  ~  #  #     #  #  ~  $  ~

Source  #

NAFF    101         A  V  A  P  A  N  D  I  Y  N  E  R  E  L  L  N  S  M  N  I
EEFF    102         A  V  A  P  T  N  E  I  Y  T  L  R  E  L  N  H  S  L  G  I
JFF1    103         G  V  A  P  T  N  E  I  Y  T  L  R  E  L  V  H  S  L  G  I
JFF2    103         G  V  A  P  T  N  E  I  Y  T  L  R  E  L  V  H  S  L  G  I
CB1     102         I  V  A  P  V  N  E  G  Y  I  P  D  E  L  C  K  V  M  G  I
CB2     102         I  V  A  P  V  N  E  S  Y  I  P  D  E  L  C  K  V  M  G  I
CB3     102         I  V  A  P  V  N  E  S  Y  I  P  D  E  L  C  K  V  M  G  I
CB4     102         I  V  A  P  V  N  E  S  Y  I  P  D  E  L  C  K  V  M  G  I
Pp      100         I  A  A  P  V  S  D  K  Y  I  E  R  E  L  I  H  S  L  G  I
                    #     $  $  #     #     $  #  #  #  $  $  ~  #  #  #  #  $
```

Source	#																			
NAFF	121	S	Q	P	T	V	V	F	V	S	K	K	G	L	Q	K	I	L	N	V	Q
EEFF	122	A	Q	P	T	I	V	F	S	S	R	K	G	L	P	K	V	L	E	V	Q
JFF1	123	S	K	P	T	I	V	F	S	S	K	K	G	L	D	K	V	I	T	V	Q
JFF2	123	S	K	P	T	I	V	F	S	S	K	K	G	L	D	K	V	I	T	V	Q
CB1	122	S	R	P	Q	L	V	F	C	T	K	N	I	L	N	K	V	L	E	V	Q
CB2	122	S	K	P	Q	I	V	F	C	T	K	N	I	L	N	K	V	L	E	V	Q
CB3	122	S	K	P	Q	I	V	F	C	T	K	N	I	L	N	K	V	L	E	V	Q
CB4	122	S	K	P	Q	I	V	F	C	T	K	N	I	L	N	K	V	L	E	V	Q
Pp	120	V	K	P	T	I	I	F	G	V	R	I	L	L	Q	K	S	T	E	C	K
			#	$	#	#	-	$			#		-	$	#	$			#		

Source	#																			
NAFF	141	K	K	L	P	I	I	Q	K	I	I	I	M	D	S	K	T	D	Y	Q	G
EEFF	142	K	T	V	T	C	I	K	K	I	V	I	L	D	S	K	V	N	F	G	G
JFF1	143	K	T	V	T	T	I	K	T	I	V	I	L	D	S	K	V	D	Y	R	G
JFF2	143	K	T	V	T	A	I	K	T	I	V	I	L	D	S	K	V	D	Y	R	G
CB1	142	S	R	T	D	F	I	K	R	I	I	I	L	D	A	V	E	N	I	H	G
CB2	142	S	R	T	N	F	I	K	R	I	I	I	L	D	T	V	E	N	I	H	G
CB3	142	S	R	T	N	F	I	K	R	I	I	I	L	D	T	V	E	N	I	H	G
CB4	142	S	R	T	N	F	I	K	R	I	I	I	L	D	T	V	E	N	I	H	G
Pp	140	S	I	N	S	I	T	K	F	F	S	L	I	E	L	K	E	A	G	G	Y
		#				#		#				-	-	-		#	#				

Source	#																			
NAFF	161	F	Q	S	M	Y	T	F	V	T	S	H	L	P	P	G	F	N	E	Y	D
EEFF	162	H	D	C	M	E	T	F	I	K	K	H	V	E	L	G	F	Q	P	S	S
JFF1	163	Y	Q	C	L	D	T	F	I	K	R	N	T	P	P	G	F	Q	A	S	S
JFF2	163	Y	Q	S	M	D	N	F	I	K	K	N	T	P	Q	G	F	K	G	S	S
CB1	162	C	E	S	L	P	N	F	I	-	S	R	Y	S	D	G	-	N	I	A	N
CB2	162	C	E	S	L	P	N	F	I	-	S	R	Y	S	D	G	-	N	I	A	N
CB3	162	C	E	S	L	P	N	F	I	-	S	R	Y	S	D	G	-	N	I	A	N
CB4	162	C	E	S	L	P	N	F	I	-	S	R	Y	S	D	G	-	N	I	A	N
PP	160	H	P	P	P	A	F	L	Y	F	S	I	L	P	L	I	L	T	P	S	K
		#									#		#	#	#				#	#	

Source	#																					
NAFF	181	F	V	P	E	S	F	D	-	R	D	K	T	I	A	L	I	M	N	S	S	G
EEFF	182	F	V	P	I	D	V	K	N	R	K	Q	H	V	A	L	L	M	N	S	S	G
JFF1	183	F	K	T	V	E	V	D	-	R	K	E	Q	V	A	L	I	M	N	S	S	G
JFF2	183	F	K	T	V	E	V	-	N	R	K	E	Q	V	A	L	I	M	N	S	S	G
CB1	180	F	K	P	L	H	Y	D	P	-	V	E	Q	V	A	A	I	L	C	S	S	G
CB2	180	F	K	P	L	H	Y	D	P	-	V	E	Q	V	A	A	L	L	C	S	S	G
CB3	180	F	K	P	L	H	Y	D	P	-	V	E	Q	V	A	A	I	L	C	S	S	G
CB4	180	F	K	P	L	H	Y	D	P	-	V	E	Q	V	A	A	I	L	C	S	S	G
Pp	180	F	K	P	P	S	F	-	N	R	D	D	Q	V	A	L	V	M	F	S	S	G
		$	#	#	-	#	#		#	#	#	-	#	#	$	#	-	#		$	$	$

Source	#																			
NAFF	201	S	T	G	L	P	K	G	V	A	L	P	H	R	T	A	C	V	R	F	S
EEFF	203	S	T	G	L	P	K	G	V	R	I	T	H	E	G	A	V	T	R	F	S
JFF1	203	S	T	G	L	P	K	G	V	Q	L	T	H	E	N	T	V	T	R	F	S
JFF2	203	S	T	G	L	P	K	G	V	Q	L	T	H	E	N	A	V	T	R	F	S
CB1	200	T	T	G	L	P	K	G	V	M	Q	T	H	R	N	V	C	V	R	L	I
CB2	200	T	T	G	L	P	K	G	V	M	Q	T	H	Q	N	I	C	V	R	L	I
CB3	200	T	T	G	L	P	K	G	V	M	Q	T	H	Q	N	I	C	V	R	L	I
CB4	200	T	T	G	L	P	K	G	V	M	Q	T	H	Q	N	I	C	V	R	L	I
Pp	200	T	T	G	V	S	K	G	V	M	L	T	H	K	N	I	V	A	R	F	S
		#	$	$	-		$	$	$	#	#	#	$		#	#	#		$	#	#

Source	#																			
NAFF	221	H	A	R	D	P	I	F	G	N	Q	I	I	P	D	T	A	I	L	S	V
EEFF	223	H	A	K	D	P	I	Y	G	N	Q	V	S	P	G	T	A	I	L	T	V
JFF1	223	H	A	R	D	P	I	Y	G	N	Q	V	S	P	G	T	A	V	L	T	V
JFF2	223	H	A	R	D	P	I	Y	G	N	Q	V	S	P	G	T	A	I	L	T	V
CB1	220	H	A	L	D	P	R	V	G	T	Q	L	I	P	G	V	T	V	L	V	Y
CB2	220	H	A	L	D	P	R	A	G	T	Q	L	I	P	G	V	T	V	L	V	Y
CB3	220	H	A	L	D	P	E	A	G	T	Q	L	I	P	G	V	T	V	L	V	Y
CB4	220	H	A	L	D	P	E	A	G	T	Q	L	I	P	G	V	T	V	L	V	Y
Pp	220	H	C	K	D	P	T	F	G	N	A	I	N	P	P	T	A	I	L	T	V
		$		#	$	$		#	$	#		#		$		#	#	#	$	#	#

Source	#																		
NAFF	241	V	P	F	H	H	G	F	G	M	F	T	T	L	G	Y	L	I	C	G	F
EEFF	243	V	P	F	H	H	G	F	G	M	F	T	T	L	G	Y	F	A	C	G	Y
JFF1	243	V	P	F	H	H	G	F	G	M	F	T	T	L	G	Y	L	I	C	G	F
JFF2	243	V	P	F	H	H	G	F	G	M	F	T	T	L	G	Y	L	T	C	G	F
CB1	240	L	P	F	F	H	A	F	G	F	S	I	N	L	G	Y	F	M	V	G	L
CB2	240	L	P	F	F	H	A	F	G	F	S	I	N	L	G	Y	F	M	V	G	L
CB3	240	V	P	F	F	H	A	F	G	F	S	I	N	L	G	Y	F	M	V	G	L
CB4	240	V	P	F	F	H	A	F	G	F	G	I	N	L	G	Y	F	M	V	G	L
Pp	240	V	P	F	H	H	G	F	G	M	F	T	T	L	G	Y	F	T	C	G	F
		#	$	$	#	$	#	$	$	#	#	#	#	$	$	$	#	#	#	$	#

Source	#																		
NAFF	261	R	V	V	L	M	Y	R	F	E	E	E	L	F	L	R	S	L	Q	D	Y
EEFF	263	R	V	V	M	L	T	K	F	D	E	E	L	F	L	R	T	L	Q	D	Y
JFF1	263	R	V	V	M	L	T	K	F	D	E	E	T	F	L	K	T	L	Q	D	Y
JFF2	263	R	I	V	M	L	T	K	F	D	E	E	T	F	L	K	T	L	Q	D	Y
CB1	260	R	V	I	M	L	R	R	F	D	Q	E	A	F	L	K	A	I	Q	D	Y
CB2	260	R	V	I	M	L	R	R	F	D	Q	E	A	F	L	K	A	I	Q	D	Y
CB3	260	R	V	I	M	L	R	R	F	E	Q	E	A	F	L	K	A	I	Q	D	Y
CB4	260	R	V	I	M	L	R	R	F	E	Q	E	A	F	L	K	A	I	Q	D	Y
Pp	260	R	V	S	L	M	Y	T	F	E	E	E	L	F	L	Q	S	L	Q	D	Y
		$	#		#	#	#		$	#	#	$	#	$	$		#	#	$	$	$

Source	#																		
NAFF	281	K	I	Q	S	A	L	L	V	P	T	L	F	S	F	F	A	K	S	T	L
EEFF	283	K	C	T	S	V	I	L	V	P	T	L	F	A	I	L	N	K	S	E	L
JFF1	283	K	C	T	S	V	I	L	V	P	T	L	F	A	I	L	N	K	S	E	L
JFF2	283	K	C	S	S	V	I	L	V	P	T	L	F	A	I	L	N	R	S	E	L
CB1	280	E	V	R	S	V	I	N	V	P	A	I	I	L	F	L	S	K	S	P	L
CB2	280	E	V	R	S	V	I	N	V	P	A	I	I	L	F	L	S	K	S	P	L
CB3	280	E	V	R	S	I	V	N	V	P	A	I	I	L	F	L	S	K	S	P	L
CB4	280	E	V	R	S	I	V	N	V	P	A	I	I	L	F	L	S	K	S	P	L
Pp	280	K	V	E	S	T	L	L	V	P	T	L	M	A	F	F	P	K	S	A	L
		#	#		$		#	#	$	$	#	#		#	#	#		$	$		$

Source	#																		
NAFF	301	I	D	K	Y	D	L	S	N	L	H	E	I	A	S	G	G	A	P	L	S
EEFF	303	I	D	K	F	D	L	S	N	L	T	E	I	A	S	G	G	A	P	L	A
JFF1	303	L	N	K	Y	D	L	S	N	L	V	E	I	A	S	G	G	A	P	L	S
JFF2	303	L	D	K	Y	D	L	S	N	L	V	E	I	A	S	G	G	A	P	L	S
CB1	300	V	D	K	Y	D	L	S	S	L	R	E	L	C	C	G	A	A	P	L	A
CB2	300	V	D	K	Y	D	L	S	S	L	R	E	L	C	C	G	A	A	P	L	A
CB3	300	V	D	K	Y	D	L	S	S	L	R	E	L	C	C	G	A	A	P	L	A
CB4	300	V	D	K	Y	D	L	S	S	L	R	E	L	C	C	G	A	A	P	L	A
Pp	300	V	E	K	Y	D	L	S	S	L	R	E	I	A	S	G	G	A	P	L	S
		#	-	$	#	$	$	$	#	$	#	$	#	#	#	$	#	$	$	$	#

Source	#																		
NAFF	321	K	E	V	G	E	A	V	A	K	R	F	H	L	P	G	I	R	Q	G	Y
EEFF	323	K	E	V	G	E	A	V	A	R	R	F	N	L	P	G	V	R	Q	G	Y
JFF1	323	K	E	V	G	E	A	V	A	R	R	F	N	L	P	G	V	R	Q	G	Y
JFF2	323	K	E	V	G	E	A	V	A	R	R	F	N	L	P	G	V	R	Q	G	Y
CB1	320	K	E	V	A	E	I	A	V	K	R	L	N	L	P	G	I	R	C	G	F
CB2	320	K	E	V	A	E	V	A	V	K	R	L	N	L	P	G	I	R	C	G	F
CB3	320	K	E	V	A	E	I	A	V	K	R	L	N	L	P	G	I	R	C	G	F
CB4	320	K	E	V	A	E	I	A	V	K	R	L	N	L	P	G	I	R	C	G	F
Pp	320	K	E	I	G	E	M	V	K	K	R	F	K	L	N	F	V	R	Q	G	Y
		$	$	-	#	$		#		#	$	#		$			#	$	#	$	#

Source	#																		
NAFF	341	G	L	T	E	T	T	S	A	I	L	I	T	P	E	G	D	D	K	P	G
EEFF	343	G	L	T	E	T	T	S	A	F	I	I	T	P	E	G	D	D	K	P	G
JFF1	343	G	L	T	E	T	T	S	A	I	I	I	T	P	E	G	D	D	K	P	G
JFF2	343	G	L	T	E	T	T	S	A	I	I	I	T	P	E	G	D	D	K	P	G
CB1	340	G	L	T	E	S	T	S	A	N	I	H	S	L	R	D	E	F	K	S	G
CB2	340	G	L	T	E	S	T	S	A	N	I	H	S	L	G	D	E	F	K	S	G
CB3	340	G	L	T	E	S	T	S	A	N	I	H	S	L	G	D	E	F	K	S	G
CB4	340	G	L	T	E	S	T	S	A	N	I	H	S	L	G	D	E	F	K	S	G
Pp	340	G	L	T	E	T	T	S	A	V	L	I	T	P	D	T	D	V	K	P	G
		$	$	$	$	#	$	$	$		#	#	#	#	-		#		$	#	$

```
Source  #

NAFF    361     A V G K V V P F F E A K V V D L D T G K
EEFF    363     A S G K V V P L F K V K V I D L D T K K
JFF1    363     A S G K V V P L F K A K V I D L D T K K
JFF2    363     A S G K V V P L F K A K V I D L D T K K
CB1     360     S L G R V T P L M A A K I A D R E T G K
CB2     360     S L G R V T P L M A A K I A D R E T G K
CB3     360     S L G R V T P L M A A K I A D R E T G K
CB4     360     S L G R V T P L M A A K I A D R E T G K
Pp      360     S T G K I V P F F A V K V V D P T T G K
                # ~ $ # ~ # $ # # # # $ # # $     $ # $

Source  #

NAFF    381     T L G V N Q R G E L - C - V R G P M I M S G
EEFF    383     T L G V N R R G E I - C - V K G P S L M L G
JFF1    383     S L G P N R R G E V - C - V K G P M L M K G
JFF2    383     T L G P N R R G E V - C - V K G P M L M K G
CB1     380     A L G P N Q V G E L - C - I K G P M V S K G
CB2     380     A L G P N Q V G E L - C - V K G P M V S K G
CB3     380     A L G P N Q V G E L - C - I K G P M V S K G
CB4     380     A L G P N Q V G E L - C - I K G P M V S K G
Pp      380     I L G P N Q T G E L Y C I I G G D M I M K S
                  $ $ # $ #   $ $ #   $   # $   $ # # # #

Source  #

NAFF    401   - Y V N N P E A T N A L I D K D G W L H S
EEFF    403   - Y S N N P E A T R E T I D E E G W L H T
JFF1    403   - Y V N N P E A T K E L I D E E G W L H T
JFF2    403   - Y V D N P E A T R E I I D E E G W L H T
CB1     400   - Y V N N V E A T K E A I D D D G W L H S
CB2     400   - Y S N N V E A T K E A I D D D G W L H S
CB3     400   - Y V N N V E A T K E A I D D D G W L H S
CB4     400   - Y V N N V K A T K E A I D D D G W L H S
Pp      402   S Y Y N N E E A T K A I I N K D G W L R S
                $   # $   # $ $ # # # $   # # $ $ $   #

Source  #

NAFF    421     G D I A Y W D E D E H F F I V D R L K S
EEFF    423     G D I G Y Y D E D E H F F I V D R L K S
JFF1    423     G D I G Y Y D E E K H F F I V D R L K S
JFF2    423     G D I G Y Y D E E K H F F I V D R L K S
CB1     420     G D F G Y Y D E D E H F Y V V D R Y K E
CB2     420     G D F G Y Y D E D E H F Y V V D R Y K E
CB3     420     G D F G Y Y D E D E H F Y V V D R Y K E
CB4     420     G D F G Y Y D E D E H F Y V V D R Y K E
Pp      423     G D N A Y Y D N G G H F Y I V D R L K S
                $ $   # $ # $       $ $ # # $ $ $ $ $ #

Source  #

NAFF    441     L I K Y K G Y Q V A P A E L E S I L L Q
EEFF    443     L I K Y K G Y Q V P P A E L E S V L L Q
JFF1    443     L I K Y K G Y Q V P P A E L E S V L L Q
JFF2    443     L I K Y K G Y Q V P P A E L E S V L L Q
CB1     440     L I K Y K G S Q V A P A E L E E I L L K
CB2     440     L I K Y K G S Q V A P A E L E E I L L K
CB3     440     L I K Y K G S Q V A P A E L E E I L L K
CB4     440     L I K Y K G S Q V A P A E L E E I L L K
Pp      443     L N K Y K G Y Q V P P A E I E G I L L Q
                $   $ $ $ $ # $ $ # $ $ $ ~ $   # $ $ #

Source  #

NAFF    461     H P N I F D A G V A G L P D D D A G E L P
EEFF    463     H P N I F D A G V A G V P D P D A G E L P
JFF1    463     H P S I F D A G V A G V P D P V A G E L P
JFF2    463     H P N I F D A G V A G V P D P I A G E L P
CB1     460     N P C I R D V A V V G I P D L E A G E L P
CB2     460     N P C I R D V A V V G I P D L E A G E L P
CB3     460     N P C I R D V A V V G I P D L E A G E L P
CB4     460     N P C I R D V A V V G I P D L E A G E L P
Pp      463     H P Y I V D A G V T G I P D D P A A G K L P
                # $   $   $ # # $   $ # $ $ #     $ $   $
```

Source	#																		
NAFF	482	A	A	V	V	V	L	E	H	G	K	T	M	T	E	K	E	I	V	D	Y
EEFF	484	G	A	V	V	V	M	E	K	G	K	T	M	T	E	K	E	I	V	D	Y
JFF1	484	G	A	V	V	V	L	E	S	G	K	N	M	T	E	K	E	V	M	D	Y
JFF2	484	G	A	V	V	V	L	E	K	G	K	S	M	T	E	K	E	V	M	D	Y
CB1	481	S	A	F	V	V	I	Q	P	G	K	E	I	T	A	K	E	V	Y	D	Y
CB2	481	S	A	F	V	V	K	Q	P	G	K	E	I	T	A	K	E	V	Y	D	Y
CB3	481	S	A	F	V	V	I	Q	P	G	K	E	I	T	A	K	E	V	Y	D	Y
CB4	481	S	A	F	V	V	I	Q	P	G	K	E	I	T	A	K	E	V	Y	D	Y
Pp	483	A	A	D	V	V	-	E	S	L	-	-	I	T	E	Q	I	V	Q	N	F
		#	$		$	$		#					#	$	#			#		

Source	#																		
NAFF	502	V	A	S	Q	V	T	T	A	K	K	L	R	G	G	V	V	F	V	D	E
EEFF	504	V	N	S	Q	V	V	N	H	K	R	L	R	G	G	V	R	F	V	D	E
JFF1	504	V	A	S	Q	V	S	N	A	K	R	L	R	G	G	V	R	F	V	D	E
JFF2	504	V	A	S	Q	V	S	N	A	K	R	L	R	G	G	V	R	F	V	D	E
CB1	501	L	A	E	R	V	S	H	T	K	Y	L	R	G	G	V	R	F	V	D	S
CB2	501	L	A	E	R	V	S	H	T	K	Y	L	R	G	G	V	R	F	V	D	S
CB3	501	L	A	E	R	V	S	H	T	K	Y	L	R	G	G	V	R	F	V	D	S
CB4	501	L	A	E	R	V	S	H	T	K	Y	L	R	G	G	V	R	F	V	D	S
Pp	500	V	S	S	Q	V	S	T	A	K	W	L	R	G	G	V	K	F	L	D	E
		#		#	#	$	#	#	#	$		$	$	$	$	$		$	-	$	#

Source	#																		
NAFF	522	V	P	K	G	L	T	G	K	L	D	A	R	K	I	R	E	I	L	I	K
EEFF	524	V	P	K	G	L	T	G	K	I	D	A	K	V	I	R	E	I	L	K	K
JFF1	524	V	P	K	G	L	T	G	K	I	D	G	R	A	I	R	E	I	L	K	K
JFF2	524	V	P	K	G	L	T	G	K	I	D	G	K	A	I	R	E	I	L	K	K
CB1	521	I	P	R	N	V	T	G	K	I	T	R	K	E	L	L	K	Q	L	L	E
CB2	521	I	P	R	N	V	T	G	K	I	T	R	K	E	L	L	K	Q	L	L	E
CB3	521	I	P	R	N	V	T	G	K	I	T	R	K	E	L	L	K	Q	L	L	E
CB4	521	I	P	R	N	V	T	G	K	I	T	R	K	E	L	L	K	Q	L	L	E
Pp	520	I	P	K	G	S	T	G	K	I	D	R	K	V	L	R	Q	M	F	E	K
		#	$	#	#		$	$	$	#	#	#	#	#	#	#					#

Source	#								
NAFF	542	A	K	K	G	G	K	S	K	L
EEFF	544	P	Q	A	K	M				
JFF1	544	P	V	A	K	M				
JFF2	544	P	V	A	K	M				
CB1	541	S	-	-	-	-	-	S	K	L
CB2	541	S	-	-	-	-	-	S	K	L
CB3	541	S	-	-	-	-	-	S	K	L
CB4	541	S	-	-	-	-	-	S	K	L
Pp	540	H	-	-	-	-	K	S	K	L
								$	$	$

Phorturis pennsylvanica **in comparison with other sequences.**
$ = Conserved
 # = present in at least one other sequence
~ = conservative substitution

NAFF = North American firefly = *Photinus pyralis*
EEFF = Eastern European firefly = *Lucolia mingrelica*
JFF1 = Japnese firefly = *Lucolia cruciata*
JFF2 = Japnese firefly = *Lucolia lateralis*
CB1 = click beetle, green (*Pyrophorus plagiophthalamus*)
CB2 = click beetle, yellow-green (*Pyrophorus plagiophthalamus*)
CB3 = click beetle, yellow (*Pyrophorus plagiophthalamus*)
CB4 = click beetle, orange (*Pyrophorus plagiophthalamus*)
Pp = *Photuris pennsylvanica*

CHAPTER VI

CORRELATION WITH LITERATURE OBSERVATION

Firefly luciferase (FL) catalyzes the oxidative decarboxylation of a unique polycyclic compound, D-luciferin (LH_2), with the concomitant hydrolysis of adenosine 5'-triphosphate (ATP) to produce light (McElroy and Green, 1956). The quantum efficiency of the luciferase reaction is remarkably high (about 0.9) when compared to that of other chemiluminescence and bioluminescence reactions (about 0.1 or less) (Seliger and McElroy, 1960). The efficient bioluminescence reaction of FL indicates that the enzyme must provide a good environment to protect the excited state product from solvent quenching and to give a high conversion of energy to light during catalysis. All firefly luciferases catalyze the oxidation of the same substrate, luciferin, and require ATP as a cosubstrate. There should be highly conserved amino acid sequences at the active site among the different firefly luciferases. Analysis of the relationship between function and structure of FL, and determination of the highly conserved amino acids for the active site will provide useful information to explain how the highly efficient bioluminescence reaction is catalyzed by FL. The research projects presented in this thesis have tried to identify important amino acids that relate to the active site of FL by using several approaches: chemical and physical modifications; molecular cloning and sequencing of the luciferase gene from *P. pennsylvanica*; and comparison of amino acid sequences deduced from the nucleic acid sequences from different cloned luciferases. The possible active sites and important amino acids for FL activity will be discussed according to the observations from this thesis and published information.

Active Site of Firefly Luciferase

Firefly luciferase has been studied extensively by using several Lys-specific reagents. Modification of FL with the ATP analog 5'- [*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) produces a Lys-containing peptide, KGZBSK, labeled by FSBA (Lee et al., 1981). Although this peptide is not found in the protein from the cloned gene, this result, as well as other observations, indicates Lys residues are often at the nucleotide binding sites. In the experiments presented in Chapter II, pyridoxal-5'-phosphate (PLP), *o*-phthalaldehyde (*o*-PA), and thiourea dioxide (TUD) as well as FSBA were used to modify FL. Each of these reagents inactivated FL and the inactivation was prevented by ATP. Two peptides were labeled with ^{14}C-TUD. One of these peptides, whose sequence is GLTGK (amino acids 524-529, in Fig. 27), was protected against TUD labeling by ATP. It is suggested that the Lys within this peptide is close to the ATP binding site.

In a study investigating the ATP binding site of tyrocidine synthetase 1, Pavela-Vrancic et al. (1994) identified two highly conserved Lys residues when compared with consensus sequences of several ATP requiring enzymes, including *Neurospora crassa* acety-CoA synthetase, 4-coumarate CoA ligase from parsley, and firefly luciferase. The TUD labeled-peptide of FL, GLTGK, included one of these highly conserved Lys residues. Comparison of the amino acid sequences deduced from the nucleic acid sequences of luciferases from nine different fireflies (*Photuris pennsylvanica*, *Photinus pyralis*, three species of *Luciola*, four luciferases from click beetles *Pyrophorus plagiophthalamus*) reveals that the TGK portion of this peptide is completely conserved (Fig. 26). The Lys residue within the peptide GLTGK from FL, labeled by TUD, is suggested to be close to the ATP binding site.

Other Important Amino Acids for FL Activity

The data from modification experiments presented in the thesis demonstrate that several amino acids, such as Trp, His and Cys, are important for FL activity. *N*-bromosuccinimide (NBS), a tryptophan-specific reagent, inactivated FL (Fig. 11), but the inactivation of FL with NBS was not prevented by either luciferin or ATP. The comparison of amino acid sequences from nine firefly luciferases (Fig. 26) shows a conserved Trp in all luciferases ($W_{417\text{-}419}$, marked with number 9 in Fig. 27), which has been suggested by Kutuzova and Baldwin (1993) to be in the luciferin binding pocket. Trp may be important for FL activity but how it functions is unknown.

Diethyl pyrocarbonate (DEPC), a His specific modifier, inactivated the FL (Fig. 10) and the inactivation was not prevented by ATP. In Chapter II of the thesis, low pH (< 5) is shown to inactivate the FL rapidly. The low pH inactivation of FL may be caused by His protonation to change the conformation of the enzyme. There are four His residues (H_{211}, H_{220}, H_{244}, and H_{433}), within the luciferase from *P. pyralis*, which are conserved in all nine luciferases (Fig. 26). Thus His residues are important for FL activity, but there is no evidence to address how these His residues relate to the active site or to the catalytic mechanism of FL.

The Cys residues (C_{216} and C_{391}, marked with number 3 in Fig. 27) of FL are not modified by NEM when either ATP or luciferin is added (Vellom, 1990). C_{391} is highly conserved in all nine luciferses (Fig. 26). C_{216} is found in luciferases from *P. pyralis* and in the four enzymes from the click beetle, but not in that from *P. pennsylvanica* or the three species of *Luciola*. Firefly luciferase is inactivated by exposure to UV irradiation (Fig. 12 and Fig. 13). A crosslinking to produce higher molecular weight forms than dimers of FL is suggested to result from a cystine formation during the UV irradiation. Thus Cys is an important amino acid for the structure and conformation of FL.

Nucleotide Binding Sites of FL

Many nucleotide analogs stimulate FL activity (Ford et al., 1992). CoA was also found to activate FL (Rhodes et al., 1958). The enhancement of light production with CoA occurs only with high ATP concentrations (Ford et al., 1992). Airth et al. (1958) concluded that addition of CoA to a reaction mixture stimulates light production through removal of dehydroluciferin from luciferase. Wood (1991) suggested that the enhancement of FL might occur through the formation of a thiol ester with luciferin since the sulfur atom is required and this is consistent with the mechanisms of action of related enzymes. Schröder (1989) found an overall identity of 34% between 4-coumarate:CoA ligase and FL (*P. pyralis*). In a further analysis of the two 4-coumarate:CoA ligase genes in potato, Becker-André et al. (1991) found a common conserved motif of seven amino acids, GELCVRG. This motif is found in *P. pyralis* luciferase at Cys_{391}. MacCabe et al. (1991) found small areas of similarity between firefly luciferase and 4-coumarate:CoA ligase. There are highly conserved quartmers GYVN (G_{400}); pentamers IKYKYI (I_{441}); and an 11-mer SSGSTGLPKGV (S_{198}). The amino acid sequence of FL (Fig. 26) has a sequence similar to the highly conserved amino acids of the above modified CoA binding sites. One of two peptides labeled by TUD, SGYVNNPEATNALIDK (described in Chaper III, marked with number 7 in Fig. 27), is close to the protective CoA binding site - GELCVRG (Becker-André et al., 1991). This peptide also includes the assumed CoA binding site GYVN (MacCabe et al., 1991). Thus this peptide labeled by TUD (not preventable with ATP) is probably related to the CoA binding site of FL.

A site for the acyladenylate intermediate such as the acyl-AMP binding pocket is proposed by Jackowski et al. (1994). It has a glycine-rich AMP-binding signature of SSGSTGxPKGV (S_{198}, marked with number 2 in Fig. 27). The amino acids in this region are highly conserved in the nine luciferases (Fig. 26). The highly conserved amino acids of nine luciferases (Fig. 26) are located throughout the amino acid sequence. Therefore the N-terminal, middle and C-terminal regions of luciferase probably form three

essential areas for the active sites, regulation sites, and important structural sites for the reaction catalyzed by firefly luciferase to produce the highly efficient quantum yield of light.

Figure 27. Structural Motifs Found in Firefly Luciferase

Potentially important amino acid motifs are marked on the *P. pyralis* amino acid sequence. The conserved residues in the four firefly luciferases and the four click beetle luciferases are underlined. The specific sequence elements are delineated by / /, ( ), [ ], and { } and by larger fonts. The numbers indicate important peptides or amino acid residues for FL activity.

MEDAKNIKKG$_{10}$PAPFYPLEDG$_{20}$TAGEQLHK/^{1}AM$_{30}$KRYAL/VPGTI$_{40}$AFTDAHIEVN$_{50}$ITYAEYFEMSV$_{60}$RLAE/^{1}AMKRY$_{70}$GL/NTNHRIVV$_{80}$CSENSLQF FM$_{90}$PVLGALFIGV$_{100}$AVAPANDIYN$_{110}$ERELLNSMN I$_{120}$SQPTVVFVSK$_{130}$KGLQKILNVQ$_{140}$KKLPIIQKI I$_{150}$IMDSKTDYQG$_{160}$FQSMYTFVTS$_{170}$HLPPGFNEY D$_{180}$FVPESFDRDK$_{190}$TIALIMN/^{2}SSG$_{200}$STGLP KG/VAL$_{210}$PHRTA/^{3}C/VRFS$_{220}$HARDPIFGNQ$_{230}$ IIPDTAILSV$_{240}$VPFHHGFGMF$_{250}$TTLGYLICGF26 0RVVLMY/^{5}RFE/E$_{270}$ELFLRSLQDY$_{280}$KIQSALL VPT$_{290}$LFSFFAKSTL$_{300}$IDKYDLSNLH$_{310}$EIASGGA PLS$_{320}$KEVGEAVAKR$_{330}$FHLPGIRQ/^{4}GY$_{340}$GLT{ ^{5}ET}TSA/IL$_{350}$I/^{5}TPE/GDDKPG$_{360}$AVGKVV PFFE$_{370}$AKVVDLDTGK$_{380}$TLGVNQ{^{5}R/^{6}GE}L$_{390}$ (^{3}C)VRGPMIM[^{7}SG$_{400}$Y/VNNPEATNA$_{410}$ LIDK]D/^{8}G[^{9}W]LHS$_{420}$GD/IAYWDEDE$_{430}$H FFIVDRLKS$_{440}$LIKYKGYQV$_{450}$APAELESILLQ$_{460}$H PNIFDAGVA$_{470}$GLPDDDAGEL$_{480}$PAAVVVLEHG$_{490}$K TMTEKEIVD$_{500}$YVASQV/^{5}TTAK$_{510}$KLR/GGVVF VD$_{520}$EVPK/^{7}GL[^{5}TGK/L$_{530}$DAR]KIREI/^{10} LI$_{540}$KAKKGGK[^{11}SKL]/$_{550}$

CHAPTER VII

SUMMARY

Firefly luciferase has been modified using chemical reagents specific for -NH_2, His, and Trp residues. It was also modified by UV and low-pH treatment. The modification experiments revealed that Lys, His, and Trp are important amino acids for firefly luciferase activity. FSBA did not inactivate FL when luciferase activity was determined using low [ATP], but did inactivate the enzyme when the activity was determined at high [ATP]. These observations are consistent with the existence of two active sites. However, firefly luciferase treated by *o*-phthaldehyde, pyridoxal-5-phosphate and thiourea dioxide was inactivated similarly when activity was determined at low and high ATP concentrations. Inactivation with *N*-bromosuccinimide or diethyl pyrocarbonate was not prevented by either ATP or luciferin. The His and Trp residues may be important amino acids for luciferase activity, but not directly related to the substrate binding sites. Firefly luciferase was sensitive to UV irradiation. There were both increases and decreases in molecular weight with UV inactivation. Whether Cys was responsible for crosslinking or Trp and peptide bond cleavage was the main causes for the alteration needs to be examined in the future. Firefly luciferase was inactivated during low-pH (< 5.0) incubation. His residues may be involved in this inactivation.

Thiourea dioxide (TUD) was selected to modify firefly luciferase. The inactivation of luciferase by TUD was dose- and time-dependent. ATP showed about 50% protection against TUD inactivation as well as against TUD incorporation. The inactivation of firefly luciferase by TUD was similar when luciferase activity was determined at low and high ATP concentration. Two peptides labeled by ^{14}C-TUD were isolated using TPCK-trypsin

digestion and HPLC. ATP protects one of the two peptides from labeling by ^{14}C-TUD. The sequences of these two peptides were determined and were found in the deduced amino acid sequence of luciferase (*Photinus pyralis*) cDNA cloning. One labeled peptide, GLTGK, is located near the C-terminus around amino acid residues 525-529 and is protected from labeling by ATP. The other peptide, SGYVNNPEATNALIDK, is also located near the C-terminus (residues 391-414) of luciferase. The Lys residue of the peptide GLTGK is conserved in several ATP-binding proteins and luciferases (Pavela-Vrancic et al., 1994).

The firefly luciferase from *Photuris pennsylvanica* has been partially purified and characterized. Several differences in characteristics between the *Photuris* and *Photinus* luciferases were found. Two peaks of luciferase activity were displayed in the *Photuris* sample during chromatography on a Sephadex G-150 column, whereas only one peak of activity was found in the *Photinus* sample. The *Photuris* luciferase may exist as isozymes. The crystallization procedure that works for *Photinus* inactivated the *Photuris* luciferase. In addition to lower amount of luciferase in the *Photuris* sample, the *Photuris* luciferase was less stable at 37 °C (pH 7.8) and pH 9.0 (room temperature) than the *Photinus* enzyme. Both enzymes were inactivated rapidly at pH 5.0. Most characteristics are similar between these two luciferases, such as the same molecular weight, K_ms of ATP and antigenicity, the same light production pattern at low and high ATP concentrations, and the same kinetic inactivation by TUD and activation by etheno-ATP. The *Photuris* luciferase has not been completely purified owing to an unexpected inactivation upon crystallization and also the isozymes have not been further characterized because of a mistake by mixing the two peaks together after the Sephadex G-150 column chromatography. Future experiments will address these two interesting questions.

A cDNA library from *Photuris pennsylvanica* has been constructed and the luciferase gene has been cloned. Screening of the cDNA library with luciferin revealed the expression of luciferase in bacterial cells. The size of the inserted luciferase gene is 1.8 kb.

The sequence of the luciferase gene is being determined and the preliminary sequences have been compared to those of the other luciferases. There is high homology of amino acids among the luciferases. The conserved amino acids between these nine luciferases are distributed as 11% in the N-terminal third of the sequences, 48% in the central third of the sequences, and 35% in the C-terminal third of the sequences.

Future study on the active sites of firefly luciferases should focus on selection of other ATP analogs and Lys, His, Trp specific reagents to modify luciferase to determine other amino acids that are labeled. Mutation of amino acids within the TUD-labeled peptides, especially GLTGK, should be studied to determine if substitution of amino acids in the peptide will cause decrease of the luciferase activity or reduce the ATP binding. The *Photuris* luciferase should be purified again and further characterized. The potential isozymes of *Photuris* luciferase must be examined and characterized. Such characterization will help to determine if there is any advantages of using *Photuris* luciferase over other luciferases. The recombinant *Photuris* luciferase should be expressed bacterial cells. The purification and characterization of the recombinat *Photuris* luciferase will give more information to understand the function and structure of firefly luciferases.

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