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**ENGINEERING, OVEREXPRESSION, PURIFICATION,
AND ANALYTICAL APPLICATIONS
OF RECOMBINANT BIOLUMINESCENT REPORTERS**

by
Monique E. Verhaegen

A Dissertation
Submitted to the Faculty of Graduate Studies and Research
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
2002



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ENGINEERING, OVEREXPRESSION, PURIFICATION, AND ANALYTICAL APPLICATIONS OF RECOMBINANT BIOLUMINESCENT REPORTERS

ABSTRACT

The initial project entailed the construction of a plasmid suitable for bacterial expression of the *in vivo* biotinylated aequorin photoprotein. The plasmid contained a biotin-acceptor coding sequence fused in-frame with the apoaequorin gene as well as the *birA* gene, encoding biotin protein ligase (BPL), responsible for post-translational biotinylation. The use of the biotin tag facilitated (a) the purification of aequorin from the crude cell extract using an avidin resin and (b) the direct complexation of aequorin with streptavidin for utilization as a reporter molecule. Streptavidin-biotinylated aequorin complexes were used for the quantification of biotinylated hybrids in a hybridization assay, with high detectability and reproducibility. Yields of *in vivo* biotinylated aequorin from one liter of culture were sufficient for 300 000 hybridization assays.

The goal of the second project was to create novel aequorin photoproteins with improved luminescent activity over the native protein, through directed molecular evolution of the apoaequorin gene. Random mutagenesis was accomplished through repeated rounds of DNA shuffling, cloning and expression of mutant proteins, followed by screening and selection of regenerated mutants. Increased ratios of luminescent signals to the mass of total soluble protein were compared as criteria for mutant selection after five rounds of shuffling. Initial data indicated three mutants exhibited 20-60 fold higher luminescence in crude samples. Comparisons utilizing purified proteins indicated the mutant aequorins had identical luminescent activity to the native protein, and selection possibly favoured mutations allowing improved solubility of the overexpressed protein during extraction rather than higher luminescence quantum yield.

The final project involved the *Gaussia princeps* luciferase (GLuc), which has recently been cloned as the smallest known coelenterazine-using luciferase. Presently, there are no analytical studies using GLuc as a bioluminescent reporter. Beginning with GLuc cDNA, a plasmid encoding a biotin acceptor peptide-GLuc fusion protein was constructed. This allowed overexpression in *E. coli* and purification by avidin affinity chromatography. The light emission of purified Gluc was studied at various coelenterazine concentrations to establish the detectability of the new reporter, which was

found to reach attomole levels. Furthermore, the complexation of biotinylated GLuc with streptavidin was used as a detection reagent in a microtiter well-based, bioluminometric DNA hybridization assay.

For C.G

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LIST OF ABBREVIATIONS

A	adenosine
Aeq	aequorin
ALP	alkaline phosphatase
anti-DIG	anti-digoxigenin antibody
amol	attomole
B	biotin
BAeq	biotinylated aequorin
BCCP	biotin carboxy carrier protein
BGLuc	biotinylated <i>Gaussia</i> luciferase
BL	bioluminescent
bp	basepair
B-PER	bacterial protein extraction reagent (Pierce)
BPL	biotin protein ligase
BSA	bovine serum albumin
C	cytosine
CV	coefficient of variation
cDNA	complementary DNA
CHCl ₃	chloroform
CL	chemiluminescent
Da	Dalton
dATP	deoxyadenosine triphosphate
dd H ₂ O	distilled deionized water
DIG	digoxigenin
DIG-dUTP	digoxigenin-11-deoxyuridine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
ds	double stranded
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetra-acetic acid disodium salt
EtBr	ethidium bromide
Eu ³⁺	europium ion
G	guanine
GFP	green fluorescent protein
His	histidine
HRP	horseradish peroxidase
IPTG	isopropyl thiogalactopyranoside
kb	kilobase
K _d	dissociation constant
kD	kilodalton
L	litre
LB	Luri-Bertani
LC	long chain

Luc	luciferase
M	moles/liter (molar)
min	minute
nm	nanometer
mmol	millimole
NHS	N-hydroxysuccinimide ester
nmol	nanomole
NTP	ribonucleoside triphosphate
oligo	oligonucleotide
O/N	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ph	phenol
PMSF	phenylmethyl sulfonyl fluoride
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase PCR
SA	streptavidin
S/B	signal-to-background
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	second
ss	single stranded
T	thymine
<i>Taq</i>	<i>Thermus Aquaticus</i>
U	uracil
μmol	micromole
TdT	terminal deoxynucleotidyl transferase
Trp	tryptophan
Tyr	tyrosine
WT	wild type

CHAPTER 1

GENERAL INTRODUCTION

1.1 NUCLEIC ACID DETECTION

1.1.1 Nucleic Acid Hybridization Assays

Sensitive bioanalytical assays for detection of nucleic acids have become an absolute requirement in many fields encompassing such disciplines as medicine, forensics, and environmental applications. These assays depend on detection techniques that allow the measurement of minute quantities of analyte. Most nucleic acid detection systems require a secondary detection technology, such as some type of external label, since nucleic acids do not have intrinsic properties that allow their detection in a sensitive manner (Kricka, 1999b). Regardless of the detection system used and any required target immobilization steps, most nucleic acid assays are of a probe-based hybridization format. Like many other analytical methodologies, these assays rely on the reaction between an analyte of interest and a specific reagent. In this case, the analyte is the nucleic acid to be determined and the specific reagent is an analyte-specific complementary nucleotide sequence, generally referred to as a probe. The analyte concentration is then deduced by measuring either the amount of analyte-reagent complex formed or the residual reagent (Diamandis *et al.*, 1990a).

Hybridization assays exploit the specificity of Watson-Crick base pairing between adenine (A) and thymine (T) or guanine (G) and cytosine (C) in DNA, with uracil (U) replacing T in RNA. This allows the hybridization of a target nucleic acid and a specific complementary probe. By introducing a labelled probe to the reaction mixture, the analyte of interest can thus be quantified. The majority of nucleic acid hybridization assays to date are heterogeneous in nature, depending on sample separation steps and reagent transfer steps for detection. Reagents are often added in excess and then unbound reagents removed by washings.

The initial step in heterogeneous assays generally involves immobilization of the target sequence on a solid support. This can occur by direct spotting of the nucleic acid on a nitrocellulose or nylon membrane, or the transfer of electrophoretically separated

nucleic acids by Southern or Northern blotting. Alternatively, nucleic acids can be immobilized on microtiter wells through specific affinity methods such as antigen-antibody or biotin-streptavidin interactions. More recently, nucleic acids have been immobilized on microarray chips, by spotting procedures, photolithography methods or other robotic procedures (Arcellana-Panlilio *et al.*, 2002). Regardless of the assay format, the basic principle of a single stranded nucleic acid target binding its complementary probe is always the basis of the hybridization assay. Following the probe binding under optimum conditions, the hybrids are detected by numerous systems depending on the nature of the labelled probe and the assay format, as shown in Figure 1.1.

1.1.1.1 Homogeneous Nucleic Acid Assays

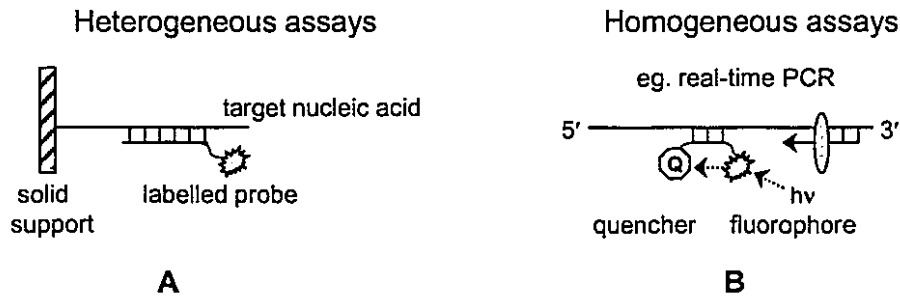
While there are numerous possibilities for heterogeneous hybridization assay formats, there are limited non-separation formats that can be applied to the development of nucleic acid homogeneous assays. Homogeneous assays, requiring no separation steps, are simple, convenient, amenable to automation and would readily allow point-of-care nucleic acid testing. Several dipstick assay formats have already been developed for PCR products generated in HIV-1 assays (Bawa *et al.*, 1995) as well as products generated due to the presence of *Salmonellae* and *Listeria* in foods (Groody, 1994).

The main approach to homogenous assay formats has been the use of fluorescence methods such as fluorescence resonance energy transfer (FRET) systems. This approach often relies on the strategic positioning of a fluorescent label and a second quencher label, either on one or two separate probes. Amplification of the target causes a detectable change in one or both labels, allowing the assay to be performed in one step with no reagent transfer steps necessary for detection (Heller *et al.*, 1993). One representative assay (the TaqMan assay) is shown Figure in 1.1.B (described in section 1.1.4.1.1). Other techniques utilize fluorescence polarization, whereby upon binding of a fluorophore-labelled probe to a target nucleic acid molecule, the polarization of the fluorescence of the bound label increases (Murakami *et al.*, 1991; Checovich *et al.*, 1995).

While homogeneous assays are often rapid and convenient with lower background noise (no non-specific binding to solid phases), they are generally less sensitive than heterogeneous assays and may lack detection levels often required for clinical assays

FIGURE 1.1
STRATEGIES OF NUCLEIC ACID DETECTION

Direct Detection Methodologies



Indirect Detection Methodologies

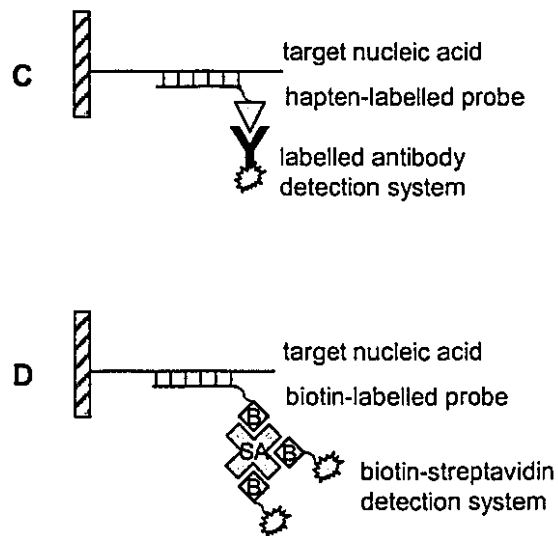


Figure 1.1. Probe-based nucleic acid detection relies on direct or indirect labeling schemes. Direct detection depends on the incorporation of a detectable label into a specific complementary probe. This allows both separation-based heterogeneous assay formats (A) and non-separation homogeneous assay formats (B). Indirect methods employ bridging molecules such as antibodies (C) or streptavidin-biotin systems (D).

(Mansfield *et al.*, 1995). Many novel target and signal amplification systems have now been designed to improve the sensitivity of these assays. Strand displacement amplification (Walker *et al.*, 1996) and exponential rolling circle amplification (Thomas *et al.*, 1999) have both been utilized for these purposes, even allowing quantitative detection in real-time instruments.

1.1.2 Trends in Assay Detection

Over the years, many new developments have arisen in the field of nucleic acid detection. Currently, no consensus exists regarding the best assay formats, labels or detection reactions for nucleic acid assays (Kricka, 1999b). Depending on the overall goal of the nucleic acid assay, various features such as label stability, sensitivity of detection, speed and convenience of the assay format, as well as cost of reagents and detection systems, all play key roles in the assay development. The trend in development of ultrasensitive assays continues, with improvements in sensitivity generally accomplished by improving the detection label, utilizing multiple labels or reducing the background noise of the assay.

Initially, spectrophotometric methods based on early chromogenic labels were replaced by fluorescence methods due to their superior sensitivity of about 10-1000 fold in comparison to absorbance measurements. Initial conventional fluorescent labels such as fluorescein and rhodamine were routinely used, however their use restricted detection limits to the nanomolar range (Smith *et al.*, 1981) due to scattering of excitation light, background fluorescence of samples, quenching and photodestruction.

The incorporation of time-resolved fluorescence using europium chelates improved detection limits to the picomolar range (Diamandis *et al.*, 1988), eliminated high background signals and improved signal-to-noise ratios (Christopoulos, 1995). These methods are based on labels with long lasting fluorescence so that a delay in signal measurement following excitation allows most of the background fluorescence to decay.

Due to their relatively high sensitivity, radionuclides (^{32}P , ^{125}I , ^{35}S) became the popular choice of label in early hybridization assays, but biohazard concerns, waste disposal and the short lifetime of these labels led to the development of sensitive non-radioactive systems that soon replaced them. These non-radioactive detection methods

often incorporating chemiluminescent or bioluminescent molecules (Stults *et al.*, 1992), multiple label systems (Diamandis *et al.*, 1991) or enzymatic labels allowing signal amplification (Evangelista *et al.*, 1991; Christopoulos *et al.*, 1992), equalled and even surpassed the sensitivity of the radioactive labels, routinely allowing detection in the attomolar range. Enzyme labels were also beneficial in that enzyme substrates could be tailored as needed, using colourimetric, fluorescent, or chemiluminescent detection systems.

1.1.3 Detection Strategies

The introduction of improved labels has enabled the development of hybridization assays with increased sensitivity. Regardless of the type of detection method used (colourimetric, fluorescent, chemiluminescent, or bioluminescent), the label must either be measured directly or indirectly as in the case of enzyme products. In direct detection systems, the probe carries a label that allows quantification of the hybrids (Figure 1.1.A). Alternatively, the probe may carry a moiety that allows bridging between the probe and a signal-generating molecule. This is the case when antigen-antibody or biotin-streptavidin systems are used (Figure 1.1.C and 1.1.D), streptavidin serving as a bridge between the probe and the signal-generating molecule. Enzymes are often employed for signal amplification.

Colourimetric detection systems are generally three to four orders of magnitude less sensitive than other non-radioactive methods (Kricka, 1992). Colourimetric assays employing enzymes as labels along with chromogenic substrates are often used in membrane-based assays, such as Southern and Northern blotting. Here, the enzyme label produces insoluble product that precipitates at the site of probe annealing to the target. These assays are simple to perform, produce a visible product and a permanent record.

Fluorescent labels rely on the principle that upon absorption of specific light energy resulting in excitation, the decay to ground state is accompanied by the emission of light of a lower energy and longer wavelength (Tsien *et al.*, 1989). One specific advantage of fluorescence detection is the ability to measure multiple dyes simultaneously in one sample. Three different schemes have been developed to exploit fluorescence as a detection system.

- (i) Direct fluorescence detection relies on measuring total fluorescence from various dyes or the products of enzymatic reactions.
- (ii) Time-resolved fluorescence as mentioned earlier, relies on delayed measurements of long lasting fluorophores (Christopoulos, 1995).
- (iii) Energy transfer and fluorescence quenching methods are often used in homogeneous assay formats, because they allow detection without separation procedures (Heller *et al.*, 1993). These assays rely on the energy transfer between fluorophore and quencher labels, allowing a detectable signal change. Depending on the assay format and hence the distance of the labels, an increase or decrease in fluorescence can be monitored.

Chemiluminescence and bioluminescence detection techniques rely on the emission of light by chemically generated excited states. Assay formats are generally simple, rapid, versatile and provide high sensitivity (Kricka, 1992). These methods often employ chemiluminescent labels such as acridinium esters (Mansfield *et al.*, 1995), photoproteins such as aequorin (Stults *et al.*, 1992), or chemiluminogenic substrates for enzyme labels.

1.1.3.1 Specific Labels

Regardless of the detection type (colourimetric, fluorescent, chemiluminescent, bioluminescent), the following are selected labels and schemes used for sensitive nucleic acid detection and quantification.

1.1.3.1.1 Enzyme Labels

The main advantage to enzyme labels is that apart from allowing signal amplification, many of them also utilize a variety of substrates that can be tailored to the requirements of the assay (See Figure 1.2.A). Enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase (ALP) were the traditional enzymes of choice. HRP has the ability of catalyzing reactions in the presence of H₂O₂ with hydrogen-donor substrates that produce chromophores and can be measured spectrophotometrically (Ishikawa, 1987), fluorophores, and chemiluminescent products such as those from reactions with isoluminol or acridinium esters (Thorpe *et al.*, 1985; Diamandis, 1990b).

FIGURE 1.2
SELECTED DETECTION AMPLIFICATION SYSTEMS

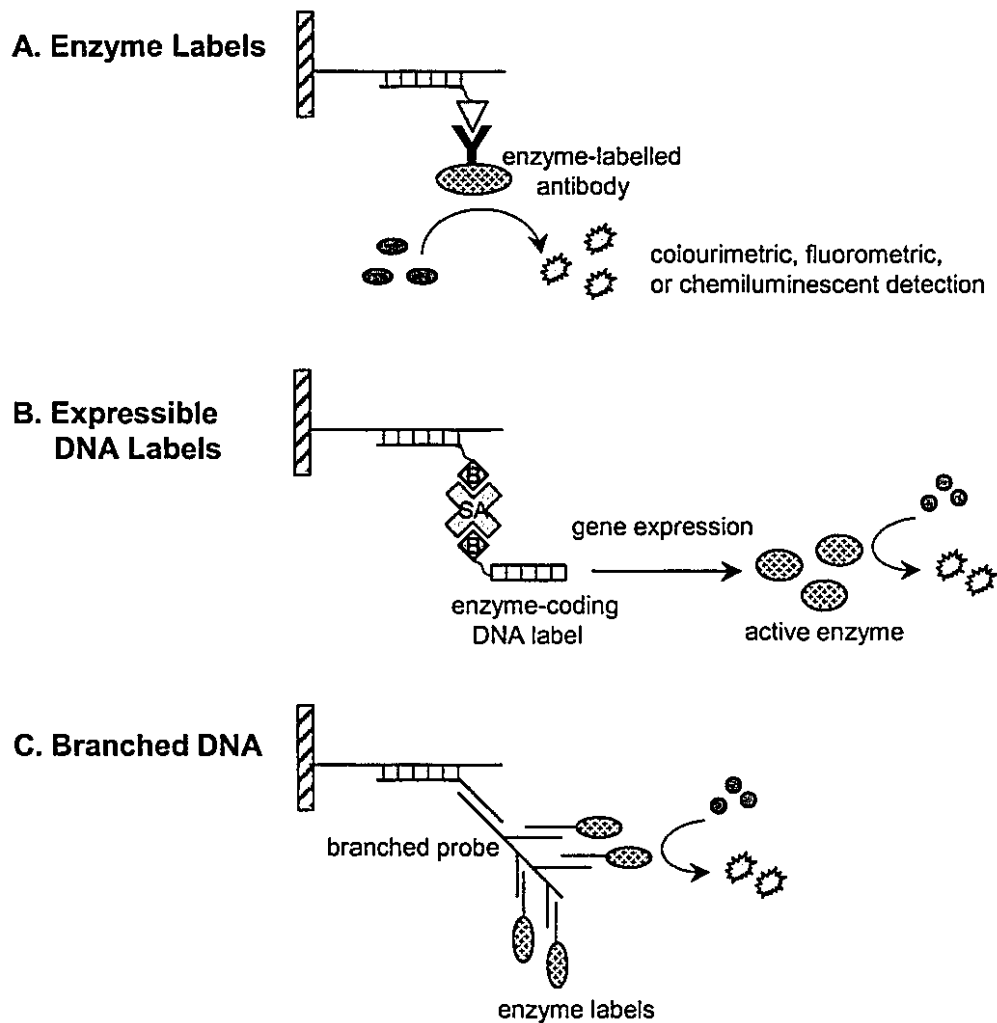


Figure 1.2. Regardless of the detection type (colourimetric, fluorescent, bioluminescent, chemiluminescent), many detection labels and strategies employ signal amplification procedures for increasing the sensitivity. Enzyme systems (A) allow substrate turnover and hence produce many detectable molecules from one enzyme. Expressible DNA labels (B) can provide two-fold amplification, due to the *in vitro* transcription and translation of an enzyme product which can also turnover many substrates. Branched DNA (C) systems allow hybridization of many probes which can also be labelled directly or with enzyme labels.

The ability of ALP to catalyze the hydrolysis of phosphate groups makes it a very versatile label with chromogenic, fluorogenic and chemiluminogenic substrates. Substrates such as *p*-nitrophenyl phosphate produce coloured products that can be measured spectrophotometrically, while other substrates such as diflusal phosphate, form strong fluorescent complexes with Tb^{3+} -EDTA in alkaline solution upon dephosphorylation (Christopoulos *et al.*, 1992). Furthermore, highly stable phosphate analogues of the meta-substituted phenyl dioxetane have been synthesized as ALP chemiluminescent substrates (Voyta *et al.*, 1988). Hydrolytic phosphate cleavage produces a metastable phenolate intermediate, followed by spontaneous breakdown to give an excited-state product, which emits light upon relaxing to the ground state.

1.1.3.1.2 Expressible DNA Labels

As illustrated in Figure 1.2.B, an alternative strategy for high sensitivity entails the use of a gene as a label (instead of a gene product) resulting in amplification of the signal following *in vitro* transcription and translation of the gene to create many protein molecules. By using an enzyme-coding gene as a label there is a combined amplification of both the transcription/translation of the gene, and the enzymatic turnover of the substrate to detectable products. Gene labels encoding the firefly luciferase (Chiu *et al.*, 1996) and the apoaequorin of *Aequorea victoria* (White *et al.*, 1999), allowed target detection limits to reach 5 attomoles and 0.25-0.5 attomoles, respectively. Furthermore, simultaneous detection of two different target DNA sequences has been accomplished by combining cDNAs from both the *Renilla* and firefly luciferase as labels on two separate probes (Laios *et al.*, 2000).

1.1.3.1.3 Inorganic Compounds

Newer labels incorporating inorganic compounds have recently been proposed for nucleic acid hybridization assays (Kricka, 1999b). These trends have included the creation of inorganic microparticle labels termed nanocrystals or quantum dots (Chan *et al.*, 1998; Bruchez *et al.*, 1998), which are very stable, water-soluble and can be as small as 2 nm in diameter. The particles have an outer shell of zinc sulphide surrounding a core

containing a semiconductor material. The particle size and core composition determine the wavelength of emitted light.

1.1.3.1.4 Multilabel Strategies

Aside from enzyme amplification, the incorporation of several labels per hybrid has allowed increased sensitivity. This has been accomplished by the tetravalent interaction of the streptavidin-biotin system. Since streptavidin has the ability to bind four biotin molecules, it can be used to bridge multiple biotins each containing a label (Larsson *et al.*, 1989). Branched DNA technology, as shown in Figure 1.2.C, utilizes probes that have up to 15 branches, with each branch able to bind several labelled probes (Collins *et al.*, 1997). Furthermore, the construction of dendrimers, three-dimensional networks of complementary DNA probes with multiple labels (Nilsen *et al.*, 1997), allows up to 100-fold improvement in sensitivity when compared to single oligonucleotide probes (Vogelbacker *et al.*, 1997).

1.1.3.1.5 Signal Amplification

Various other DNA and RNA signal amplification assay formats have been developed. DNA cleavage methods, consisting of Cycling Probe Technology (ID Biomedicals) and the Invader Assay (Third Wave), rely on continuous cleavage of probes that can then be detected (Fong *et al.*, 2000; Lyamichev *et al.*, 1999). Repeated probe binding and cleavage events can be evoked by one target molecule.

Rolling circle systems can be used for both target and signal amplification. Linear methods of rolling circle amplification are based on the amplification of circular DNA by polymerase extension of a complementary primer, resulting in up to 10^5 tandemly repeated, concatemeric copies of the DNA circle (Lizardi *et al.*, 1998). Utilizing two and three-dimensional DNA arrays, this method can give up to 10 000-fold increase in signal over hybridization of a fluorescent probe (Schweitzer *et al.*, 2001).

1.1.3.2 Labelling Strategies

Labelling schemes for oligonucleotide probes require the modification of the nucleic acid with a detectable component that does not affect the ability of the base

pairing with its target sequence. Traditionally there have been two methods of accomplishing labelling schemes, either by enzymatic reaction or chemical reaction.

Probe labelling by enzymatic means (nick translation, random priming, tailing, PCR) is used to incorporate a labelled derivative into an existing oligonucleotide. Nick translation (Rigby *et al.*, 1977), random priming (Feinberg *et al.*, 1983) and PCR (Saiki *et al.*, 1985) all utilize DNA polymerase I to add modified deoxyribonucleoside triphosphates (dNTPs) to the end of a DNA molecule or to sites within the sequence. Tailing procedures utilize terminal deoxynucleotidyl transferase to add modified dNTPs to the 3' terminal end of a sequence without a template (Kumar *et al.*, 1988).

Various strategies may be used for chemical modifications of unique sites on an oligonucleotide. Certain labels or tags containing nucleophilic groups or photoreactive portions can be coupled directly to oligos using an intermediate activating agent or by radiating with UV light. Initial derivitizations may also be used to add spacer arms containing terminal functional groups for coupling other bridging or detectable molecules. Most chemical derivatization methods employ strategies that produce reactive intermediaries that can then be coupled to nucleophiles, typically species with primary amines (Hermanson, 1996).

In direct detection schemes, the label on the probe must be readily detectable. Indirect detection schemes rely on bridging between a labelled detection probe and additional detection reagents. These detection reagents are most often chemically conjugated to bridging molecules, such as antibodies, haptens or other small molecules that can readily bind the labelled probe.

1.1.4 Target Amplification Strategies

Molecular amplification methods for increasing, exponentially, the concentration of a specific nucleic acid sequence *in vitro* have become fundamental in all aspects of molecular biology including laboratory research, forensics, diagnostics, and environmental applications. The original and still most widely used target amplification system is the polymerase chain reaction (PCR) (Mullis *et al.*, 1987; Saiki *et al.*, 1985). Other nucleic acid amplification methods now include strand displacement amplification (SDA) (Walter *et al.*, 1994), ligase chain reaction (LCR) (Wu *et al.*, 1989), nucleic acid

sequence-based amplification (NASBA/3SR) (Guatelli *et al.*, 1990) and rolling circle amplification (RCA) of padlock probes (Baner *et al.*, 1998).

1.1.4.1 Polymerase Chain Reaction

The principle of PCR is based on the enzymatic amplification of nucleic acid sequences by repeated temperature cycling. In the initial (95°C) heating step, the double stranded (ds) DNA is denatured to form two single strands, upon which two complementary primers bind during a lower temperature annealing step (37 °C-65 °C). The final temperature change to ~72°C promotes the 5'→3' extension of the primers, catalyzed by a DNA polymerase. The primers are positioned so that the newly synthesized strands overlap the binding sites of the opposite oligo primer (Gibbs, 1990). As the cycling continues, the primers bind the original target as well as newly synthesized target, allowing exponential growth of the DNA fragments, reaching a theoretical abundance of 2^n (n = number of cycles).

PCR allows target amplification of single stranded (ss) DNA, ds DNA or RNA sequences. Reverse transcriptase PCR (RT-PCR) permits RNA to be used as a starting template, provided an initial reverse transcription reaction is carried out. A complementary DNA (cDNA) copy, that can be PCR amplified, is created using a reverse transcriptase enzyme that acts as an RNA-directed DNA polymerase. The target material can be a minor fraction of a complex mixture, a discrete molecule or even part of a larger molecule (Mullis *et al.*, 1987). Regardless of the starting material, the resulting amplification products will be of a discrete length defined by the 5' termini of both primers.

Since DNA polymerase catalyzes extension from the 5'→3' direction, the 5' terminus of the primer can be modified to introduce sequence alterations into the final PCR product. This allows the creation of restriction sites, specific mutations, new base sequences, insertions, and regulatory elements such as promoters (Scharf *et al.*, 1986; Sarkar *et al.*, 1989). Furthermore, primers can be used to incorporate binding moieties such as biotin or other detection tags into the amplification products (Chehab *et al.*, 1989). The final products can also be successfully altered through addition of labelled dNTPs, allowing enzymatic incorporation of a label into the PCR product.

Due to its thermal instability, initial PCR studies with DNA polymerase required the addition of enzyme following each denaturation step. The introduction of a heat stable polymerase from the thermophilic bacterium *Thermus aquaticus* (*Taq*), allowed addition of enzyme only once to the reaction (Saiki *et al.*, 1988). Currently, there are many DNA polymerases that have been adapted to automated PCR that allow increased specificity, higher yields or longer templates, such as *Pfu Turbo* or *Herculase* (Stratagene).

1.1.4.1.1 Real Time PCR

PCR has enabled the development of several homogeneous assay formats for real-time detection of amplified products in a closed tube format. The original TaqMan assay as shown in Figure 1.1.B, employs an oligo probe labelled with both a fluorophore and a quencher (Roche Molecular Systems). Upon binding of the probe to the PCR product, the quencher is removed by the 5'→3' exonuclease activity of the DNA polymerase (Holland *et al.*, 1991). Fluorescence can then be monitored in real-time as the PCR is cycling.

Alternatively, molecular beacons, Amplifluors and Scorpion derivatives, have been developed for homogeneous real-time detection. Molecular beacons contain a target recognition loop flanked by a hairpin with a fluorophore and quencher on opposing ends. Binding to the PCR product opens the hairpin, producing fluorescence (Tyagi *et al.*, 1996). Amplifluors and the modified Scorpion version, are beacon variants in that they are PCR primers with a hairpin at the 5' end containing the fluorophore and quencher, thus producing fluorescence upon incorporation into the product (Nazerenko *et al.*, 1997; Whitcombe *et al.*, 1999).

1.1.5 Future Trends in Assay Development

The trend in nucleic acid detection has been towards the development of simple, sensitive and rapid assays. Miniaturization has become essential in handling the tasks associated with simultaneous analysis of many hybridization events. Progress towards better labels and detection reagents may provide alternative routes to assays that previously required amplification strategies or complex assay formats. Progress in target and signal amplification, as well as homogeneous assay formats have greatly improved the speed and sensitivity of detection. Homogeneous PCR-based assays have now even

been performed in silicon microstructures, offering advantages of speed, multiplexing and reduced reagent use (Taylor *et al.*, 1997).

These trends have been fostered by increasing demands for new diagnostic tools, rapid screening of food or biological samples for pathogens, analysis of genetic material from single or rare cells, massive parallel drug discovery strategies and the many applications associated with the Human Genome Project. The identification of increasing numbers of gene specific sequences in the human genome requires high-throughput screening for mutational analysis as well as analyzing the physiological status of gene expression.

The advent of DNA microchips/microarrays allow numerous sequences to be detected in parallel by confining individual analyses to specified positions on a flat surface interacting with the sample (Isaksson *et al.*, 1999). These DNA chips have facilitated methods for screening of multiple mutations in genetic diseases, screening for polymorphisms in the genome and multiple gene expression assays for analyzing copy number differences in genomic DNA or RNA products (Wilgenbus *et al.*, 1999). To date, most micro devices utilize fluorescent detection methods, although other technologies such as bioluminescence and chemiluminescence may play an important role in the future (Kricka, 1999b).

1.2 RECOMBINANT PROTEIN PURIFICATION

1.2.1 Recombinant Protein Expression

Advances in recombinant DNA technology and molecular cloning have recently allowed the production of large amounts of desired proteins from almost any organism. The technique of creating recombinant proteins in some type of prokaryotic or eukaryotic system has proven advantageous over purification from the natural source where the protein may be unstable, modified, or unavailable in large quantities. The ease and convenience of bacterial expression systems under well-defined growth and extraction conditions have favoured *E. coli* as such as system. More recently, the desire for inexpensive production of very large quantities of proteins has led the industry to produce

recombinant proteins in transgenic plants and animals in a type of 'molecular pharming' system (Larrick et al., 2001).

The production of recombinant proteins employs the cloning of a gene into a suitable vector that allows the expression of that gene into a functional protein product. *E. coli* expression vectors are relatively simple and usually only require a regulated promoter and ribosome binding site upstream of the gene of interest. Following transformation into competent cells, expression of the protein can be induced by appropriate means. Since many eukaryotic genes expressed in bacterial systems fold incorrectly or inefficiently and cannot require any post-translational modifications such as glycosylation or phosphorylation, many mammalian systems have now been developed to resolve these problems (Sambrook *et al.*, 1989).

1.2.2 Recombinant Fusion Proteins

Recombinant DNA technology has allowed gene manipulation at the DNA level for creation of fusion partners located at either terminus of the target protein. The ability to genetically fuse a fragment of DNA to the 3'- or 5'- ends of a gene corresponding to the target protein of interest creates recombinant hybrids that can then be expressed as fusion proteins with the attached fusion partner at the N- or C-terminus (Germino *et al.*, 1986; Hearn *et al.*, 2001). Originally these fusion concepts were utilized for the creation of reporter genes in assessing the efficiency and localization of target gene expression in cells (Germino *et al.*, 1986).

The many fusion partners available, varying in molecular size and complexity from large binding proteins to small amino acid tags, have led to many improved applications of recombinant proteins. These fusion partners have rendered recombinant proteins highly selective for separation and purification procedures using affinity labels. They have also been valuable for increasing *in vivo* expression levels, improving stability, increasing solubility, improving cellular localization through trafficking as well as for detection and quantification procedures (Hearn *et al.*, 2001).

Providing an alternative to chemical conjugation of proteins and reporter molecules, gene fusion procedures have now been applied for specifically labelling recombinant proteins. The production of these fusion proteins has many advantages over

traditional conjugation methods. Expression of fusion proteins results in a homogeneous population of conjugates, leading to improved lot-to-lot reproducibility (Lewis *et al.*, 2000) as well as attachment in a site-specific manner. This leads to a beneficial one-to-one population of mono-substituted conjugates, which has been shown to result in better assay performance (Witowski *et al.*, 1993).

Furthermore, chemical conjugation has been shown to interfere with binding abilities or inhibit the activity of some proteins. This was often found to be the case with the luciferases from several bioluminescent organisms, such as the firefly (*P. pyralis*) and the sea pansy (*R. reniformis*) (Kricka, 1988). Although the luciferases would most likely be ideal reporter molecules for assay development, chemical conjugation of the protein generally leads to inactivation (Price *et al.*, 1998). The exploitation of recombinant fusion technology for the creation of bioluminescent-fusion conjugates should preserve the enzyme activity of the luciferases as well as the molecular recognition properties of the binding molecule (Kricka, 1999b). This has been shown in the firefly luciferase-Protein A conjugate (Lindbladh *et al.*, 1991), the *Luciola* luciferase-biotin acceptor molecule (Tatsumi *et al.*, 1998) and the luciferase-RNA binding protein (Kajita *et al.*, 1995).

1.2.3 Recombinant Protein Purification

The purification and isolation of biologically active recombinant proteins from crude cellular lysates or complex mixtures has proven to be a challenging task in current biotechnology efforts. Initially, recombinant proteins were isolated from other molecules based on their characteristic properties such as solubility, ionic charge, molecular size, adsorption properties and binding affinity. Techniques relying on solubility properties cause protein precipitation from solution, dependent on salt concentrations, pH, polarity and temperature. Electrophoretic techniques rely on the migration of ions in an electric field and can take the form of paper electrophoresis or gel electrophoresis (agarose and polyacrylamide).

Chromatographic separations exploit the difference in partition ratios of the solute species between two phases. In these cases, a mobile phase containing the solutes is passed over a stationary phase whereby the solute molecules are exchanged between the phases based on properties of each solute. This causes a continuous separation process,

whereby mixtures separate into bands of pure substances. The many chromatographic techniques are classified according to their mobile and stationary phases (gas or liquid) as well as the nature of the interaction between the solutes being separated and the stationary phase (ion-exchange chromatography, gel filtration chromatography, and affinity chromatography).

1.2.4 Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography whereby the molecule to be purified is specifically and reversibly adsorbed to a complementary binding ligand. This ligand is often immobilized on an insoluble support and the molecules to be purified are passed over this matrix, binding in a manner that allows them to be concentrated upon elution. This purification or separation procedure is then based on the ability of a suitable method for selectively desorbing the bound material from the matrix following removal of the impurities or unwanted species.

The origins of routine affinity chromatography as a separation technique began in 1967 when it was reported that molecules with primary amino groups could be coupled to insoluble polysaccharide supports activated by cyanogen bromide (Axen *et al.*, 1967). This allowed the first general method for coupling ligands to the matrix without destroying binding ability. Ideally, the ligand should have an affinity for the binding substance in the range of 10^{-4} to 10^{-8} M, since dissociation constants greater are too weak for proper separations and those less than 10^{-8} M forbid easy elution and possibly inactivate the molecule of interest (Pharmacia LKB Biotechnology, 1988).

1.2.4.1 Affinity Cassette-Fusion Tags

In recent years, the complexity of recombinant protein purification has been greatly facilitated by DNA techniques allowing the creation of *in vitro* affinity-fusion systems. These systems are based on the fusion of a short fragment of DNA coding for an amino acid sequence that has the ability for strong binding with an associated partner, fused to the 5'- or 3'-terminus of the target protein coding sequence. These recombinant hybrids can then be expressed as fusion proteins with the attached 'affinity tag' at the N-

or C- terminus, providing a molecular recognition site for selective interaction with complementary ligands immobilized on a suitable support (Hearn *et al.*, 2001).

The ultimate goal of these affinity-cassette fusion tags is to simplify the often difficult, costly, time-consuming and inefficient expression and subsequent purification of recombinant proteins. Current trends in biotechnology have demanded the need for simple, cost-effective, large scale bioprocessing techniques, since downstream bioprocessing can account for up to 70-80% of the total production costs (Narayanan, 1994).

1.2.4.2 Affinity Tag Strategies and Systems

The basic strategy used for construction of an affinity-tag fusion protein is shown in Figure 1.3. Generally, the coding region of the gene of interest is ligated in the correct reading frame to a DNA vector containing the affinity coding sequence through use of oligonucleotide primers, PCR procedures and unique restriction enzyme sites. This DNA construct is transfected in suitable host cells, which are then induced to express the fusion protein. Following cell lysis procedures, purification is achieved by basic affinity chromatography techniques.

The crude samples are passed over an affinity column that allows specific binding of the affinity tag to the insoluble support immobilized with a complementary ligand. The immobilized fusion-protein can then be separated from other unwanted cellular components and debris by washing with various buffers that elute unbound molecules. Finally, elution is carried out with a buffer that specifically interrupts the binding of the affinity tagged fusion-protein to the immobilized ligand. The affinity tag can then be cleaved if necessary by engineering either a chemical or enzymatic cleavage site between the affinity tag and desired protein.

Depending on the ultimate goal and specific requirements of the fusion-protein, various cassette affinity-fusion tag systems have been developed or are commercially available. Tags employed for purification of recombinant proteins can be enzymes such as β -galactosidase (Ullman, 1984); polypeptide binding domains such as the albumin-binding domain (Stahl *et al.*, 1989); carbohydrate binding domains such as the maltose binding domain (di Guan *et al.*, 1988); or biotin binding domains (Cronan, 1990).

FIGURE 1.3
PURIFICATION OF AFFINITY-TAG FUSION PROTEIN

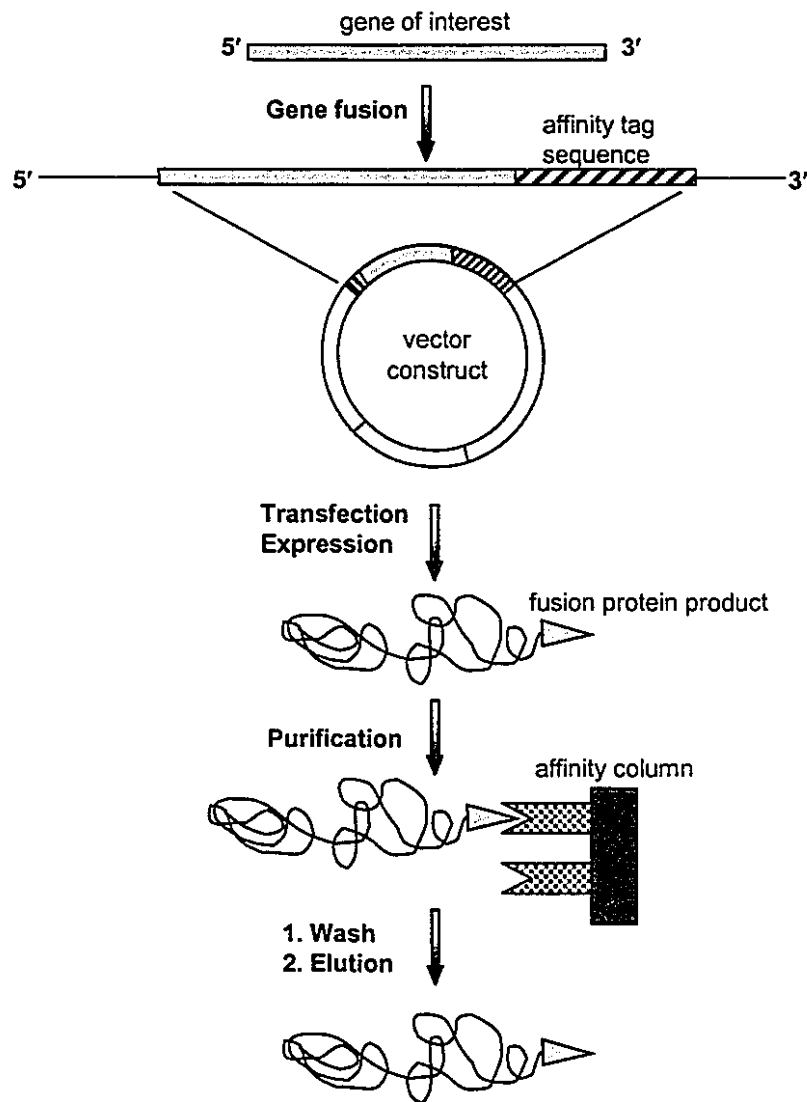


Figure 1.3. Gene manipulation at the DNA level allows expression of fusion partners at the terminus of the target protein. The incorporation of an affinity tag fusion partner provides a molecular recognition site for specific interactions with a binding ligand immobilized on a solid support. This facilitates the purification of recombinant proteins by affinity chromatography methods.

Commonly used small peptide tags include the metal-affinity tags such as the hexa-histidine sequence (Hochuli *et al.*, 1988); biotinylated tags (Schatz, 1993); non-biotinylated streptavidin affinity tags (Schmidt *et al.*, 1996) and antigenic epitopes of either Ca²⁺ or non-Ca²⁺ dependence (Hopp *et al.*, 1988; Field *et al.*, 1988).

1.3 BIOTIN-(STREPT)AVIDIN SYSTEMS

1.3.1 The Biotin-(Strept)avidin Interaction

Biotin (Vitamin H) is a small cofactor necessary for a number of *in vivo* carboxylation reactions. Avidin, a protein found in egg white, and streptavidin a similar protein from *Streptomyces avidinii*, both have the ability to bind biotin with very high affinity (Green, 1975). This non-covalent interaction between (strept)avidin and biotin is one of the strongest in nature with a dissociation constant of $K_d = 10^{-15}$ M (Green, 1975). It is believed that this strong interaction may actually be a natural defense mechanism that inhibits the growth of bacteria requiring biotinylated enzymes.

This specific interaction of (strept)avidin and biotin has become the basis of many bioanalytical techniques. The high affinity and stability of the complex is undisturbed by changes in pH, strong chaotropic agents, harsh reaction conditions and extensive derivatizations (Diamandis *et al.*, 1988). A minimum of guanidine, 6-8 M at pH 1.5 is required for inducing complete dissociation of the avidin-biotin bond (Cuatrecasas *et al.*, 1968; Bodansky *et al.*, 1970). Although the biotin-avidin interaction is not disrupted under denaturing conditions, the four subunits of the protein that are not held together by disulphide bonds, are dissociated in such an environment.

Avidin, a 66 kDa tetrameric protein of four identical subunits, (Green, 1975) is a positively charged glycoprotein. This often leads to side reactions, causing high background problems due to non-specific binding. Streptavidin on the other hand is a neutrally charged tetrameric protein with a molecular mass of about 60 kDa after postsecretory modifications (Bayer *et al.*, 1989) and contains no oligosaccharide groups, leading to better signal-to-noise ratios in assay development (Bayer *et al.*, 1996).

The small size of biotin (244 Da) allows it to be introduced into biomolecules without altering the biological activity. Furthermore, both streptavidin and avidin possess

four biotin binding sites that allow interactions with multiply biotinylated molecules, creating polymers of (strept)avidin and biotinylated moieties (Hsu *et al.*, 1981).

Today, many biotinylated moieties or biotinylation reagents for quick conjugation reactions are readily available commercially. Highly purified streptavidin/avidin or their products derivatized with fluorophores, enzymes, metals, proteins or solid phases are also available commercially. This has encouraged the use of the biotin-(strept)avidin interaction for some of the following applications: immunological assays, flow cytometry, electron microscopy, affinity chromatography, amplification assays, nucleic acid hybridization assays, Western blots, and DNA sequencing. With the cloning and sequencing of the avidin gene from the chicken oviduct (Gope *et al.*, 1987), the streptavidin gene of *Streptomyces avidinii* (Argarana *et al.*, 1986) and the biotin acceptor domain of various species (Cronan, 1990), new techniques such as the creation of fusion proteins will allow further applications of the biotin-(strept)avidin system.

1.3.2 Biotinylation Procedures

Various labelling strategies have been developed that enable the biotinylation of diverse compounds such as proteins, polysaccharides, nucleic acids and other small moieties using active biotin derivatives. Biotin can be incorporated through chemical conjugation of various reactive groups (Becker *et al.*, 1971) through enzymatic means using previously designed nick translation or random priming methods (Feinberg *et al.*, 1983), chemical bonding procedures, intercalation of ds DNA (Isaacs *et al.*, 1977) or even *in vivo* biotinylation procedures (Cronan, 1990).

Currently there are various chemically reactive, biotin-containing reagents that allow biotinylation through various functional groups of resident amino acids, oligosaccharide moieties or reduction of disulphide bonds. One of the most common reactions for biotinylation of reporter molecules is via their ϵ -amino groups of lysines using an N-hydroxy-succinimide ester (NHS-ester) of a biotin analog (Becker *et al.*, 1971). Oligonucleotides can also be biotinylated in this fashion, since probes can be synthesized *in vitro* to contain an active amino group (Connolly, 1987). This reaction creates an amide bond between the reacting amino group and the biotin moiety. Additional spacer arms provided by NHS-long chain (LC)-biotin allows greater physical

separation between the biotin and the labelled group, and has since decreased steric hindrance issues. Various other NHS biotin derivatives, such as the sulfo-derivative allowing solubility in aqueous solutions, are now readily available commercially (Bayer *et al.*, 1996).

In theory, it would be preferable to produce a single biotinylated molecule for assay situations, with the biotin residue situated a distance from the combining site and exposed in a favourable position for optimum interaction with (strep)avidin (Bayer, *et al.* 1996). Unfortunately, there are often unbiotinylated species and impermissible species produced by these chemical biotinylation reactions. This may result in undesirable steric hindrance, chemical interference, deformation or inhibition of active sites, inhibition upon binding (strept)avidin or even non-specific interactions with other assay components (Bayer *et al.*, 1996).

1.3.2.1 *In Vivo* Biotinylation

Biotin, an essential coenzyme required by all organisms and synthesized by plants and most prokaryotes, is covalently attached to the biotin carboxylases and decarboxylases that play a key role in gluconeogenesis, lipogenesis, amino acid metabolism, and energy transduction (Knowles, 1989; Samols *et al.*, 1988). The role of these enzymes is to capture CO₂ from bicarbonate and catalyze the transfer of carboxylate to organic acids to form various cellular metabolites, using the biotin cofactor as a mobile carboxyl carrier (Samols *et al.*, 1988).

The enzyme responsible for the post-translational covalent attachment of biotin, via a lysine amide linkage to its associated protein, is biotin protein ligase (BPL). Although biotinylated proteins are universal in nature, biotinylation is a rather rare modification in the cell, with only one to five species found in different organisms (Cronan, 1990). There is a high degree of conservation throughout evolution with stringent specificity between the interaction of BPL and its protein substrate, even if the two proteins come from widely divergent biological sources (Cronan, 1990; Leon-Del-Rio *et al.*, 1995).

1.3.2.1.1 The Biotin Protein Ligase of *E. coli*

The most widely studied BPL is the multifunctional BirA protein from *E. coli*, encoded by the *birA* gene, whose sole substrate is the biotin carboxy carrier protein (BCCP) subunit of acetyl-CoA carboxylase (Barker, 1981b). This BPL, responsible for the covalent attachment of biotin to a specific lysine residue, also acts as the transcriptional repressor controlling biotin biosynthesis (Barker, 1981b; Eisenberg, 1982). The 35.5 kDa monomeric protein has three distinct domains: an N-terminal DNA binding domain, a central catalytic domain and a C-terminal domain of unknown function (Wilson, 1992).

The mechanism of biotin attachment occurs in a two-step reaction as shown in Figure 1.4. In the first step, BPL catalyzes the attack of an oxygen atom of the biotin carboxylate on ATP to form biotinoyl-AMP. In the presence of the apoprotein form of the biotin-accepting domain of a biotin-requiring enzyme, the nucleophilic ϵ -amino group of the lysine attacks the mixed anhydride carbon atom, thus forming the amide bond between biotin and the lysine side chain. Once formed, the biotin moiety remains attached throughout the lifetime of the protein (Chapman-Smith, 1999).

1.3.2.1.2 Regulation of Biotin Biosynthesis

The biotin operon of *E. coli* contains five structural genes involved with biotin biosynthesis. Transcription of the operon is regulated via BirA by the levels of both biotin (in the form of biotinoyl-5'-AMP) and the unbiotinylated apoprotein substrate. Protein biotinylation and uptake are closely coupled, with little free intracellular biotin, as most is linked covalently to BCCP or non-covalently to BirA as biotinoyl-5'-AMP (Cronan, 1988; Barker, 1981a).

Overexpression of BirA results in super-repression of the operon, whereas overexpression of an apoprotein substrate leads to derepression of the operon (Barker, 1981b; Cronan, 1988). Competition between the apoprotein and DNA operator for the BirA-biotin-5'-AMP complex reflects the switching from biotin ligase to repressor functions. Therefore, the requirement for biotin synthesis is linked to biotin supply as well as apoprotein levels. When all the available apoprotein has been converted to biotinylated protein and no additional biotin is required, the transcription of biosynthetic

FIGURE 1.4
IN VIVO BIOTINYLATION

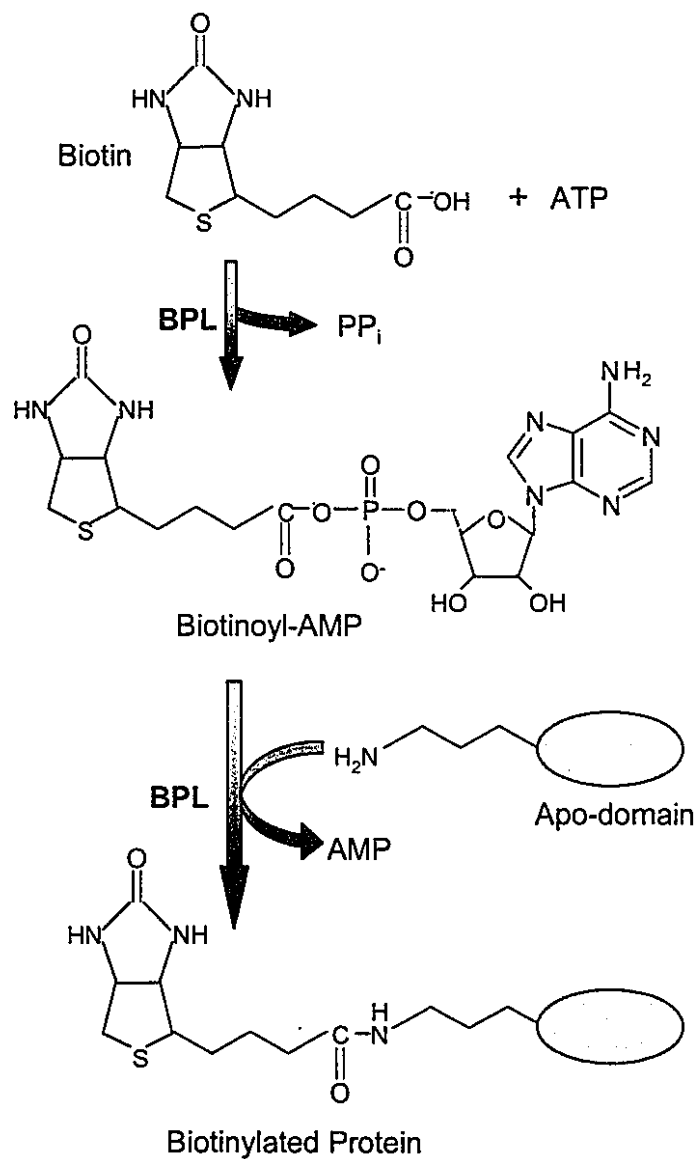


Figure 1.4. Biotin protein ligase catalyzes the addition of a biotin moiety to a specific lysine residue on the target protein. In the first step, BPL catalyzes oxygen attack of the biotin carboxylate on ATP, forming biotinoyl-AMP. A nucleophilic amino group of a lysine then attacks the mixed anhydride carbon atom, forming an amide bond between the lysine side chain and the biotin moiety.

genes is turned off by the presence of the BirA-biotin-5'-AMP complex (Cronan, 1988; Barker *et al.*, 1981b; Beckett *et al.*, 1997).

1.3.3 Biotin as a Purification Tag

The use of biotin as a purification tag has been desirable because of its small size, wide availability of detection reagents and extreme affinity for (strept)avidin. Initially, the only biotin labelling available was through chemical conjugation of a purified molecule, which proved useless as a method for labelling a recombinant protein in a crude lysate. Additionally, the strong affinity of biotin for streptavidin required harsh conditions for elution or separation of the molecules. Until these two matters were resolved, routine applications of biotin as an affinity purification tag for recombinant proteins were uncommon.

1.3.3.1 The Pinpoint System

The PinPoint Protein Purification System (Promega) employs two methodologies that enable a simple system for the purification of recombinant proteins. The gene of interest is cloned into a vector carrying a coding sequence that is biotinylated *in vivo* upon expression. This results in a fusion protein with a naturally biotinylated N-terminus, that can be purified by a one-step affinity chromatography column consisting of monomeric avidin resin. This monomeric avidin has a lower affinity for biotin than the tetrameric form and the protein can be eluted under mild, non-denaturing conditions (Kohanski *et al.*, 1990).

The purification tag used in this system is a 12.5 kDa subunit of the transcarboxylase complex from the *Propionibacterium shermanii* (Cronan, 1990). This peptide sequence contains a highly conserved target sequence to which a single biotin is added at lysine 89 by BPL (Samols *et al.*, 1988). Since the biotin tag is located relatively far from the recombinant protein, activity of the protein is unlikely to be affected when the fusion protein is immobilized on a solid support (Schultz *et al.*, 1993). The biotinylation tag can be removed if necessary, since the PinPoint vector is designed with an in-frame site for endoproteinase Factor Xa cleavage.

1.3.3.2 Monomeric Avidin

The dissociation of tetrameric avidin into monomer units leads to a conformation change accompanied by an increase in K_d for biotin ($\sim 10^{-7}$ M) while still retaining high specificity of binding for biotinylated molecules (Kohanski *et al.*, 1990). The use of monomeric avidin for affinity purification allows elution in a 'soft-release' manner, under mild conditions using competitive elution with biotin. To preserve these lower affinity binding sites, avidin monomers must be immobilized on a matrix such that the subunits are spatially separated (Schultz *et al.*, 1993).

Initially, the most common support for monomeric avidin was agarose, but due to several drawbacks, a newer resin based on a rigid methacrylate polymeric gel filtration matrix was developed. This physically and chemically stable SoftLink Soft Release Avidin Resin (Promega) has a macroporous structure, a narrow particle size distribution, and protein size exclusion limit of 5 kDa. This allows easy access of the sample to the avidin ligand, high flow rates and batch purifications using centrifugation. The avidin resin is immobilized by reductive amination at neutral pH, which produces a stable amine bond linkage with minimal leaching even at pH extremes (Schultz *et al.*, 1993).

The purification of recombinant proteins using the PinPoint vector system in combination with the SoftLink monomeric avidin resin typically yields 1-5 mg of protein per 1 L of culture using a column containing 3-5 mL of resin (Schultz *et al.*, 1993). The resin can be conveniently regenerated and reused by washing with acetic acid followed by a phosphate buffer wash to allow correct folding of the avidin.

1.4 LUMINESCENCE

1.4.1 The Fate of Electronically Excited States

Luminescence procedures are based on molecules that are excited, generating a species whose emission spectrum provides information for qualitative or quantitative analysis. Absorption of energy by a molecule leads to an electronically excited state that may be lost during radiative or non-radiative decay. Radiative decay involves the loss of energy as photons in fluorescence, phosphorescence or chemiluminescence. In non-radiative decay, excess energy is lost to the surrounding molecules creating thermal

motion of the environment. The excitation required for fluorescence and phosphorescence is brought about by the absorption of photons, whereas in chemiluminescence, excitation occurs during the course of a chemical reaction.

Excitation of a molecule occurs through absorption of energy, which excites the molecule from its ground state (S_0) to any of the vibrational states of the higher electronic singlet states (S_1 , S_2). This excited molecule then returns to the ground state in a combination of steps favouring a route that minimizes the excited lifetime. Internal conversion may occur from S_2 to S_1 , as well as vibrational relaxation within each singlet state. Intersystem crossing occurs if the electron spins become unpaired, resulting in crossing to the triplet state. Deactivation by external conversion may occur if there is interaction and energy transfer between the excited molecule and the solvent or other solutes.

Once the molecule occupies the lowest vibrational level of the first excited singlet state or corresponding triplet state, it returns to ground state by means of radiative or non-radiative decay. Light emission, a result of radiative decay, can take several forms depending on the source of excitation or the excited level of decay. Decay from the excited singlet state results in fluorescence or chemiluminescence, whereas decay from the excited triplet state results in phosphorescence.

1.4.2 Chemiluminescence and Bioluminescence

While chemiluminescence (CL) is a result of light emission from a chemical reaction, bioluminescence (BL) is a chemiluminescent reaction from a biological organism. As opposed to fluorescent light emission, chemiluminescent emission occurs from an emitter that is chemically different from the initial substance. The excited molecules are supplied with energy from an enthalpy change during the chemical conversion of reactant A to a product B. The product B is reached via a light-emitting intermediate species B^* that is the electronically excited state relative to product B. Decay of this excited intermediate is responsible for the light emission of a chemiluminescent reaction (Pringle, 1993). Chemiluminescent molecules can only produce a photon once, whereas a fluorescent molecule can be excited over and over again.

Five parameters govern the chemiluminescent reaction: intensity, speed of onset, decay of light intensity, colour and polarization. These factors are then dependent on the rate of chemical reaction, the efficiency in generating electronically excited molecules and the efficiency of the excited molecules in producing photons (Campbell *et al.*, 1993). Only a fraction of the energy supplied to the ground state molecules will be converted to light emission. This phenomenon is described as the quantum yield of the reaction, which relates the photon output to the number of ground state molecules in the reaction. The overall quantum yield of a chemiluminescent reaction, ϕ_{CL} , is defined as:

$$\begin{aligned}\phi_{CL} &= \text{total number of photons emitted/ number of reacting molecules} \\ &= \phi_C \phi_{EX} \phi_F\end{aligned}$$

where: ϕ_C = chemical yield (fraction of molecules in chemiluminescence pathway)

ϕ_{EX} = yield of excited state molecules

ϕ_F = excited state yield (fraction of excited state molecules that emit light)

The ϕ_F value is often influenced by the nature of the surrounding molecules, since quantum yields are often higher in organic solvents than water. The explanation for this is dependent on the fact that nonradiative decay to the ground state is greatly influenced by the environment, with energy from electronically excited states easily transferred to polar molecules (Pringle, 1993).

1.4.3 Analytical Applications

Analytical CL and BL techniques cover a vast array of ultrasensitive applications in fields such as molecular biology, biotechnology, pharmacology, clinical chemistry and environmental chemistry. From an analytical stance, measurement of CL and BL light emission is useful since light output is directly related to the analyte concentration. There are several features that enable CL and BL to be superior to other detection principles involving light (Roda *et al.*, 2000). This luminescence allows superior sensitivity over at least five orders of magnitude, routinely in the attomole range (10^{-18}) and even as low as the zeptomole range (10^{-21}) (Campbell, 1988).

These systems allow light to be measured in the absence of noise, since there are no warm up or drift of light source and detector effects, as well as no interference from light scattering. Furthermore, since the light emission is a direct result of a reaction, the typical background emission of sample components present in fluorescence is avoided,

resulting in lower backgrounds. These systems require no hazardous reagents and since light emission occurs in a time frame of seconds, procedures are generally rapid and simple, often employing single-step methods.

Bioluminescence and chemiluminescence have been applied in the following bioanalytical techniques:

- i) *Enzyme activity and related assays.* Analytical methods utilizing CL or BL systems have been coupled to the determination of enzyme activities and substrates. This is useful not only for species directly involved in the luminescent reaction, but also for reactions that can be coupled to the luminescent one. Methods for the determination of specific analytes such as cholesterol and glucose have been developed by coupling reactions catalyzed by oxygenases to the luminol/H₂O₂/horseradish peroxidase (HRP) system (Campbell, 1988).
- ii) *Flow injection analysis and separative techniques.* The high sensitivity of CL promotes its use in flow injection analysis, column-liquid chromatographic and capillary-electrophoretic separative systems (Kricka, 1999a; Roda *et al.*, 2000).
- iii) *Reactive oxygen species assays.* Chemiluminescence has become a widespread indicator of reactive oxygen species and lipid peroxidation in cells and organs, leading to the study of many pathological conditions (Campbell, 1988). This is due to the discovery that activated phagocytes such as neutrophils emit ultraweak chemiluminescent signals, which can be enhanced by the addition of synthetic or natural compounds.
- iv) *Filter membrane biospecific reactions.* Antibodies and nucleic acids have widely been labelled with CL or BL molecules and used as probes for the detection of proteins in Western blotting and nucleic acids in Southern and Northern blotting. These membrane hybridizations techniques have been used routinely in basic research as well as clinical diagnosis such as the detection of pathogenic organisms in infectious disease (Accotto *et al.*, 1998).
- v) *Immunoassays.* A great variety of CL and BL labels been utilized for the determination of human samples, veterinary samples, and food and agriculture samples in an immunoassay format. Direct labels can be non-enzymatic chemiluminescent molecules such as luminol derivatives, acridinium esters or the

bioluminescent photoproteins. Enzyme labels such as alkaline phosphatase (ALP) or HRP allow signal amplification with higher sensitivity and steady-state light emission (Rongen *et al.*, 1994).

- vi) *Hybridization assays.* All hybridization assays are based on the complementary binding of a specific nucleic acid probe for its target sequence. The probes can be labelled directly or indirectly with some type of hapten molecule that can then be detected by specific ligands or anti-hapten antibodies conjugated to CL or BL signalling molecules. Alternatively, enzymes can be used as labels along with various BL or CL substrates (Kricka, 1991).
- vii) *Recombinant cell-based assays.* Recombinant DNA techniques have allowed the development of genetically engineered cells containing luminescent reporter genes. Reporter gene systems such as the firefly luciferase (Gould *et al.*, 1988), bacterial luciferase (Olsson *et al.*, 1988) or green fluorescent protein (Cubitt *et al.*, 1995) have routinely been used for the study of gene expression and regulation. They have also been widely used in the environmental analysis field, enabling toxicity studies or the study of gene expression in response to specific compounds such as heavy metals (Tauriainen *et al.*, 1999).

1.4.3.1 Kinetics

Analytical applications of CL or BL involve two distinct types of response. The first type of response employs the light-emitting molecule as a direct detection label, which is present in limiting concentrations. This produces a 'pseudo' first-order reaction since the slowest process in the sequence of events leading up to the light emission is the reaction itself (Pringle, 1993). These types of labels produce flash type reactions that proceed through a short initial lag phase followed by a rapid increase. Once the reactants have been used up and can no longer be converted to an excited state, light emission decays. These reactions typically last from one second to tens of seconds (Pringle, 1993).

The second form of chemiluminescent detection utilizes an enzyme capable of catalyzing the light emitting reaction upon addition of appropriate substrate. In quantitative analytical procedures, a limiting amount of enzyme is now detected in the presence of excess substrate. The reaction rate or substrate turnover rate ultimately

determines the light intensity and the kinetic profile reflects the enzyme kinetics. An initial lag phase is present due to formation of the excited state, followed by an increase and then a plateau as the enzyme turns over a constant amount of substrate. These types of glow reactions may remain constant for minutes or even hours (Pringle, 1993). Light emission from an enzyme label may also resemble a flash-type reaction, usually a result of enzyme inhibition by reaction products.

1.4.3.2 Instrumentation

The light emitted from a bioluminescent or chemiluminescent reaction is generally measured by either a luminometer or luminograph. Luminometers, designed to measure the overall light output of a reaction, are relatively simple instruments that may consist of only a suitable reaction vessel, a photomultiplier tube (PMT) and some type of signal processing apparatus. Generally, no wavelength-restricting device is required since the only source of radiation is the reaction between the reagent and analyte. Luminographs on the other hand, can measure the light intensity as well as the spatial distribution of the light emission and are based on bidimensional light detectors such as charge-coupled devices. These instruments can be used to measure light emission in microtiter plates and target surfaces such as gels, blot membranes and thin layer chromatography plates (Roda *et al.*, 2000). These imaging devices can also be used in conjunction with an optical microscope for tissue sections and cells.

Since the light emission from flash-type bioluminescent photoproteins is extremely short lived, measurements of these reactions usually require a luminometer capable of simultaneously injecting a triggering reagent for the reaction. The MLX Microtiter Plate Luminometer (Dynex Technologies) used in the subsequent measurement of bioluminescent reactions, is a general purpose instrument with dual injectors for automated initiation of the reaction, measurement of light emission and calculation of assay results.

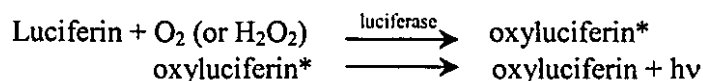
1.5 BIOLUMINESCENT ORGANISMS

1.5.1 The Diversity of Bioluminescent Organisms

There are approximately 30 known bioluminescent systems found in nature, including those of bacteria, worms, insects, crustaceans, fireflies, fungi and fish. Among these systems, there exists much diversity between their chemistries, mechanisms, structures and functions. This high diversity suggests multiple independent origins of bioluminescence over the course of evolution (Buck, 1978; Hastings, 1983). Despite this theory, there is a common principle dictating all bioluminescent systems at the molecular level. Each system involves an exergonic reaction of molecular oxygen with protein and substrate to produce light in the visible region. The enzymes involved in these reactions are oxygenases generically termed luciferases with the substrates termed luciferins (Wilson *et al.*, 1998). The photoproteins are a special class of protein that emit light upon the binding of calcium ions and are usually found in organisms of the phyla Protozoa, Cnidaria or Ctenophora.

While the evolutionary origins of all these bioluminescent systems remains somewhat obscure, various theories have been developed since the 1930's. Originally, it was proposed that bioluminescence emerged from proteins linked to the respiratory chain possessing fluorescent groups (Harvey, 1932). It was later suggested that luciferases evolved to detoxify molecular oxygen in early anaerobic life (McElroy *et al.*, 1962). This was then altered to propose all luminous organisms were derived from oxygenases involved in the metabolism of toxic substances (Seliger, 1993). The latest theory proposes that it is the luciferin substrate with its strong antioxidative properties that is actually the core of evolutionary origins (Rees *et al.*, 1998). This theory suggests that the primary function of the luciferin was to detoxify deleterious oxygen derivatives and that the luciferase enzymes only served to optimize the expression of the endogenous chemiluminescent properties of the luciferin.

Luciferases commonly employ molecular oxygen to oxidize luciferin producing an excited state intermediate. It is this excited state returning to ground state that is responsible for the characteristic light emission of these organisms.



The substrates for the many different bioluminescent reactions are all different in chemical structure depending on the origin of the organism (Hastings, 1996). The production of light also varies, as some organisms emit continuously while others emit a short-lived rapid flash in response to pH changes, the presence of O₂ or the presence of Ca²⁺ (Wilson *et al.*, 1998).

1.5.1.1 Coelenterazine

Coelenterazine is an imidazolopyrazine occurring naturally in many luminous and non-luminous marine organisms (Shimomura *et al.*, 1980). A great many bioluminescent organisms from many phyla rely on coelenterazine as their luciferin, such as fish, protozoans, coelenterates, molluscs and chaetognaths (Rees *et al.*, 1990). Although these organisms utilize the same luciferin substrate, light emission is triggered by different mechanisms at the cellular and molecular level.

The elucidation of the luciferin substrate responsible for the light emission in many organisms was deduced independently for several luminescent systems. The luciferin from *Cypridina hilgendorfi* was one of the first organisms shown to contain an imidazolopyrazine ring system (Kishi *et al.*, 1966). This same structure was then deduced as the chromophore present in the jellyfish *Aequoria victoria* (Shimomura *et al.*, 1973) and in the sea pansy *Renilla reniformis* (Hori *et al.*, 1973). The widespread nature of this same luciferin in many coelenterate organisms led this luciferin to be termed coelenterazine and its oxidized product, coelenteramide (Shimomura *et al.*, 1975).

Light emission from an imidazolopyrazine ring system is dependent on the formation of a carbanion at the C2 centre, which attacks molecular oxygen leading to formation of a hydroperoxide. The hydroperoxide anion then attacks the reactive carbonyl at C3, leading to a transient unstable dioxetanone intermediate. This intermediate immediately undergoes scission to yield CO₂ and an excited state enolate ion. Upon return to the ground state, the enolate ion emits a photon of light (Prendergast, 2000). Light emission from coelenterazine is shown in Figure 1.5.A. In the case of the photoprotein aequorin (as seen in Figure 1.5.B), the coelenterazine is believed to exist as a hydroperoxide stabilized to the apoprotein by hydrogen bonding (Head *et al.*, 2000).

FIGURE 1.5
LUMINESCENCE REACTION OF COELENTERAZINE

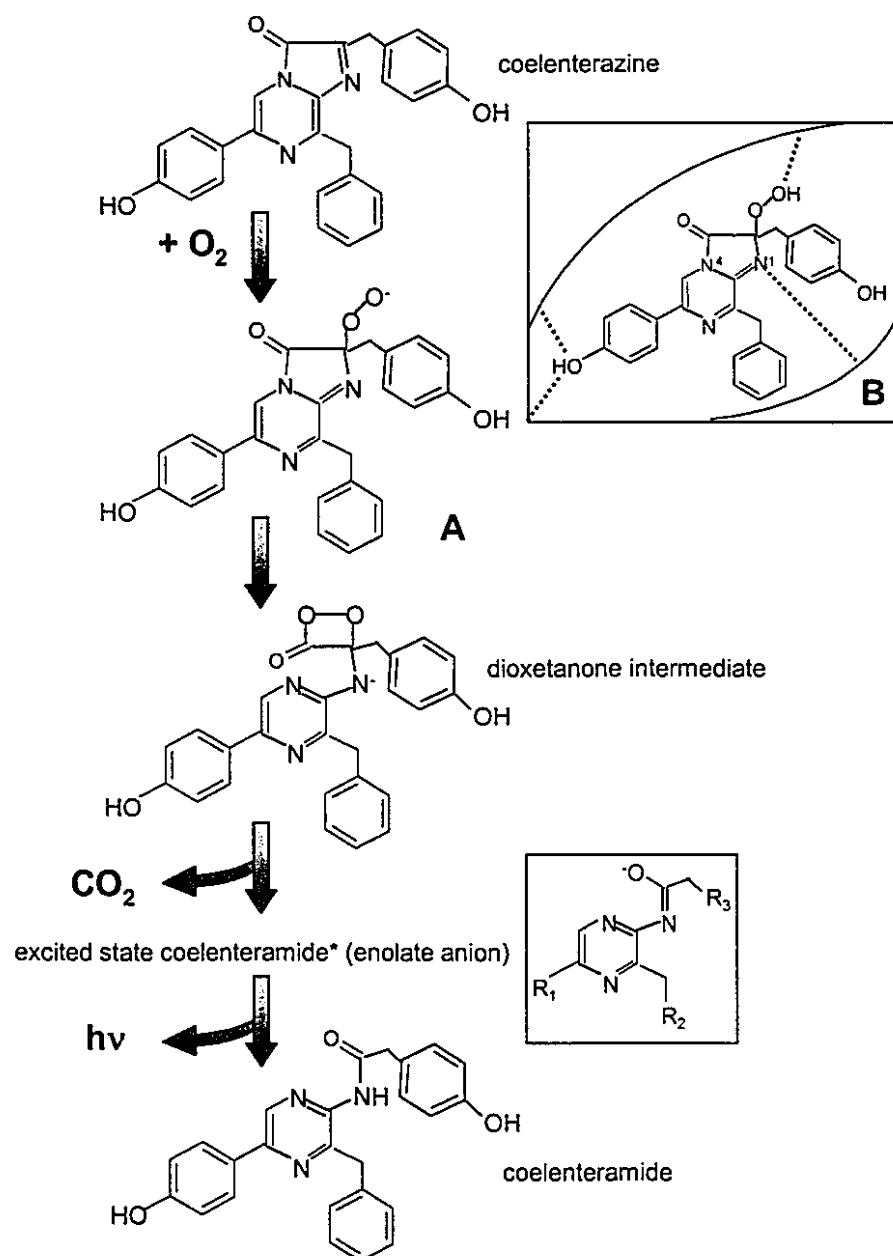


Figure 1.5. Light emission from many bioluminescent organisms is a result of the oxidation of the luciferin coelenterazine (A) to the coelenteramide product via a dioxetanone intermediate. This intermediate undergoes scission to yield CO_2 and an excited state enolate anion that emits light upon falling to ground state. In the case of the photoprotein aequorin, coelenterazine is thought to exist as a hydroperoxide stabilized by hydrogen bonding (B). (Adapted from Prendergast, 2000)

1.5.2 The Photoprotein of *Aequorea victoria*

The calcium-dependent photoprotein aequorin from the hydromedusan jellyfish *Aequorea victoria* was the first photoprotein to ever be isolated (Shimomura *et al.*, 1962). This 22 kDa aequorin complex consists of an apoaequorin protein, the prosthetic chromophore coelenterazine, and some form of molecular oxygen. Upon the binding of Ca^{2+} , aequorin undergoes a conformational change and coelenterazine is oxidized to coelenteramide with a simultaneous emission of blue light ($\lambda_{\text{max}} = 469 \text{ nm}$) and the release of CO_2 (Shimomura *et al.*, 1978; 1962). Characteristic light emission, lasting approximately 3 sec, is a direct result of the excited state of coelenteramide falling to ground state (Shimomura *et al.*, 1973).

Although the light emission from aequorin occurs as a single-turnover event, the active aequorin complex can be regenerated repeatedly and reversibly (Shimomura *et al.*, 1975). Following removal of the coelenteramide, the apoaequorin moiety was shown to be regenerated into active aequorin in the presence of coelenterazine, molecular oxygen, EDTA, and 2-mercaptoethanol, with a yield of $\sim 90 \%$ after 3 hrs. Regeneration did not occur in the absence of molecular oxygen in an evacuated vessel, and furthermore, in the absence of a reducing agent, the luminescence was low, indicating the necessity of protecting the sulfhydryl groups (Shimomura *et al.*, 1975).

While nothing is known about the *in vivo* regeneration of aequorin, it is presumed that the bioluminescence of the jellyfish is regulated by nerve-controlled Ca^{2+} release and sequestration in aequorin-containing cells found in the umbrella region of the jellyfish. Although aequorin is not considered an enzyme, but rather a trapped enzyme intermediate with a single turnover *in vitro*, it is believed that *in vivo* the jellyfish may well be able to control the enzymatic ability of its aequorin photoprotein (Prendergast, 2000).

Apoaequorin has been cloned (Inouye *et al.*, 1985), sequenced (Charbonneau *et al.*, 1985), and more recently the crystal structure has been determined (Head *et al.*, 2000). The single apoaequorin polypeptide chain is composed of 189 amino acids (Inouye *et al.*, 1985), with four characteristic Ca^{2+} -binding EF-hand domains (Head *et al.*, 2000), homologous to those contained within other Ca^{2+} -binding proteins such as calmodulin and troponin (Kendall *et al.*, 1998). The EF-hand motifs consisting of two α -

helices separated by a β -sheet, are arranged in pairs to form a globular protein. The pairs of domains are brought together to form an enclosed hydrophobic binding site that makes up the coelenterazine-binding cavity. The cavity, lined with the side chains of 21 hydrophobic amino acid residues, is situated in the centre of the protein and this conformation inhibits any solvent access to the site (Head *et al.*, 2000).

While the aequorin complex requires some form of molecular oxygen, the state of coelenterazine and the form of the oxygen has been the subject of much debate. Several lines of evidence have suggested that coelenterazine is present in a peroxidized form, attached at the C2 position of the ligand (Shimomura *et al.*, 1975; Musicki *et al.*, 1986), while others have suggested the presence of free coelenterazine with the apoaprotein containing the oxygenated species (Ward *et al.*, 1975). C-NMR studies and the crystal structure have now confirmed that coelenterazine is coupled as a hydroperoxide at the C2 position (Teranishi *et al.*, 1995; Head *et al.*, 2000) as shown in Figure 1.5.B. The hydrophobic residues of the binding cavity are organized in three triads each consisting of a histidine (His), a tyrosine (Tyr) and a tryptophan (Trp), which most likely aid in positioning the coelenterazine in the cavity. The main centres of coelenterazine interacting with the side chains of the apoaequorin are also shown in Figure 1.5. B.

The hydroperoxide at the C2 position is stabilized to the apoaequorin polypeptide by hydrogen bonding to Tyr 184, which is in turn hydrogen-bonded to His 169, which is situated near the C3 centre of coelenterazine and the indole of Trp 173. The second triad consisting of Tyr 132, His 58, and Trp 108 are all either hydrogen bonded or closely associated with the N1 centre of the ligand. The third triad made up of Tyr 82, His 16, and Trp 86 interacts with the *p*-OH group of the phenol ring bonded to C6 of coelenterazine (Head *et al.*, 2000). The amino acid sequence of the apoaequorin polypeptide, including important amino acid side chain residues is shown in Figure 1.6.

1.5.2.1 Possible Ca^{2+} Activation Mechanism

Although, aequorin contains four EF-hand domains, EF hand II is unable to bind Ca^{2+} (Head, 2000), and it has previously been shown that Ca^{2+} binding in only two sites was required for activation (Shimomura, 1995). Mutational studies at each EF-hand motif led to complete losses of Ca^{2+} induced luminescence activity at the EF I domain,

FIGURE 1.6
THE APOAEQUORIN OF *AEQUOREA VICTORIA*

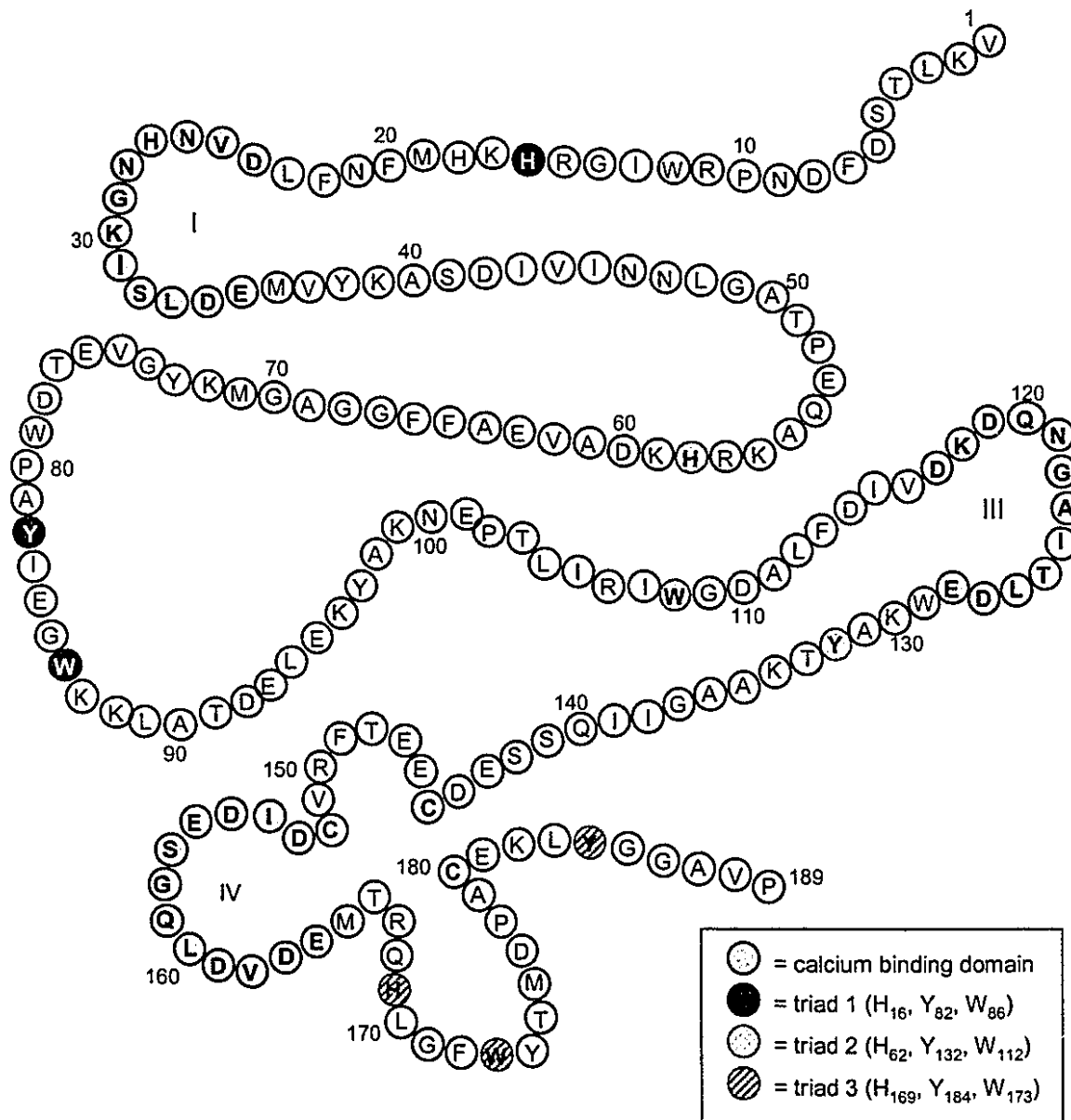


Figure 1.6. The 189 amino acid apoaequorin polypeptide. This polypeptide along with its coelenterazine chromophore make up the aequorin complex of *Aequorea victoria*. The three EF-hand domains that bind Ca^{2+} are shown. Upon Ca^{2+} binding, the complex undergoes a conformational change that allows the oxidation of coelenterazine to coelenteramide with an emission of blue light ($\lambda_{\text{max}} = 469 \text{ nm}$). The coelenterazine exists in a peroxidized form, stabilized by hydrogen bonding to three hydrophobic triads that line the cavity of the coelenterazine binding site. (Adapted from Charbonneau *et al.*, 1985; Tsuji *et al.*, 1986; Head *et al.*, 2000)

intermediate effects in domain III and basically no effect in domain IV (Tsuji *et al.*, 1986). While it is apparent that the Tyr 184 residue plays an important role in stabilizing the coelenterazine ligand, other mutational studies have determined the definite requirement of the C-terminal region, especially the terminal proline for luminescent activity (Nomura *et al.*, 1991).

Although the precise mechanism in the aequorin photoprotein remains questionable, some have speculated how the binding of Ca^{2+} may trigger the light emission reaction (Head *et al.*, 2000). The binding of Ca^{2+} to either or both of the EF-hand domains I and IV leads to a change in their relative orientations, disrupting the hydrogen bonding network of the C-terminal loop ultimately leading to the relocation of Tyr 184. This in turn, disrupts the hydrogen bonding to the peroxidized coelenterazine, which would now be free to attack the adjacent carbonyl of the C3 centre, hence initiating light emission. It was also suggested that depending on the shift of the helices during activation, the C-terminal tail might possibly open the binding cavity permitting the release of reaction products (Head *et al.*, 2000).

The efficiency of light production, or the quantum yield, for the bioluminescent reaction of aequorin has been determined to be 0.15-0.2 (Blinks *et al.*, 1976) however the quantum yield of this same chemiluminescent reaction was determined to be only 0.002. This is attributed to the ability of the apoaequorin to hold the coelenteramide molecule in a specific conformation that facilitates the generation of the singlet-excited state molecule (Teranishi *et al.*, 1995).

1.5.2.2 Recombinant Aequorin

The initial cloning and sequencing of aequorin quickly lead to the overexpression of the apoaequorin polypeptide in *E. coli* and production of recombinant aequorin following *in vitro* regeneration with pure coelenterazine and a thiol reagent (Inouye *et al.*, 1986; Prasher *et al.*, 1985). Following apoaequorin expression in *E. coli*, the polypeptide was released from the cells during cell lysis procedures (sonication, lysozyme treatment) or directly released into the culture medium through use of a fusion signal peptide coding sequence for the outer membrane protein A (Prasher *et al.*, 1985; Inouye *et al.*, 1986; 1989). Apoaequorin was then purified by acid precipitation, gel filtration and DEAE-

cellulose anion exchange chromatography, followed by regeneration with coelenterazine to generate the active aequorin complex (Inouye *et al.*, 1989; Stults *et al.*, 1992).

More recently, very high yields of recombinant aequorin have been purified by expressing apoaequorin in the periplasmic space, followed by simultaneous extraction and regeneration into active aequorin in only one step (Shimomura *et al.*, 1999). Following extraction, the regenerated aequorin was purified by anion-exchange chromatography, hydrophobic interaction chromatography and gel filtration. The regeneration of apoaequorin into aequorin prior to purification permits higher yields due to the increased stability and resistance to protease attack of the rigid aequorin molecule over the apoaequorin conformation (La *et al.*, 1982). While the aequorin complex is more resistant to protease attack, the apoaequorin polypeptide is much more stable at elevated temperatures and high salt concentrations, permitting purification at room temperature (Inouye *et al.*, 1986). Regenerated aequorin quickly loses activity above 40°C, most likely by auto-oxidation of the coelenterazine chromophore and must therefore be kept at temperatures of 0-4°C (Shimomura *et al.*, 1978; Inouye *et al.*, 1986).

1.5.2.3 Applications

The first application of aequorin was directed at the measurement of intracellular Ca^{2+} levels. The photoprotein was microinjected into the contracting muscle fibres of giant barnacles to investigate the intracellular calcium homeostasis (Ridgway *et al.*, 1967). The cloning of the apoaequorin gene led to non-invasive genetic transformation of cells, with expression of the recombinant apoaequorin and regeneration with coelenterazine *in situ*, allowing the measurement of cytosolic $[\text{Ca}^{2+}]$ to various stimuli (Inouye *et al.*, 1986; Knight *et al.*, 1991). Cloning techniques have now allowed the expression of apoaequorin and subsequent $[\text{Ca}^{2+}]$ measurement in discrete subcellular organelles such as the mitochondrion (Rizzuto *et al.*, 1992), nucleus (Brini *et al.*, 1993), endoplasmic reticulum (Kendall *et al.*, 1992), and plasma membrane (Marsault *et al.*, 1997). This protein targeting has been achieved using both minimal targeting sequences and fusion to resident organellar proteins that inherently direct efficient localization.

Aside from uses as a Ca^{2+} indicator, recombinant aequorin has been widely used as a reporter molecule in bioluminescence based assays. Aequorin can be readily

conjugated to various haptens, substrates and antibodies. Its low signal-to-noise ratio, broad range of detection sensitivity and rapid signal generation, have enabled aequorin labels to be used in both immunoassay development as well nucleic acid hybridization assays. This photoprotein also has the advantage of being active in a variety of buffers and at physiological pH (Witkowski *et al.*, 1994).

In these various assay situations, the aequorin protein acts as a quantitative label, enabling high detection sensitivity in the presence of excess Ca^{2+} . In the case of both immunoassays and nucleic acid hybridization assays, the analyte to be measured is generally immobilized on some type of solid support followed by the addition of a free aequorin-conjugate. After washing away excess label, luminescence can be measured by the addition of excess Ca^{2+} .

Immunoassays utilizing aequorin as a label have been employed for determining subnanogram quantities (fmol range) of many proteins such as glycosphingolipid (Stults *et al.*, 1992) and interleukin 6 (Rivera *et al.*, 1994), as well as attomolar ranges of human thyroid-stimulating hormone in serum (Hart *et al.*, 1993). Aequorin has also been used a label in competitive binding assays for the determination of small biomolecules such as biotin (Lewis *et al.*, 2000). Subnanogram quantities of DNA immobilized on Southern blots have been detected with biotinylated aequorin (Stults *et al.*, 1992). Microtiter-well, probe-based hybridization assays (Galvan *et al.*, 1996) as well as quantitative reverse-transcriptase PCR assays for prostate specific antigen mRNA (Verhaegen *et al.*, 1998a), have enabled target detection to attomole levels. Apoaequorin cDNA has even been employed as a label in the development of highly sensitive expression hybridization assays (White *et al.*, 1999).

Furthermore, the production of aequorin-fusion conjugates has facilitated the use of aequorin in intracellular Ca^{2+} measurements (Sala-Newby *et al.*, 1998), as well as in the development of binding assays and as labels in immunoassays. Some of these fusion proteins include the protein A-apoaequorin fusion conjugate that binds immunoglobulin G (Zenno *et al.*, 1990), and the protein C binding epitope-apoaequorin conjugate for creation of an assay for protein C (Desai *et al.*, 2001). The following work entails the production of a biotin acceptor domain-apoaequorin fusion protein for facilitating purification as well as developing a sensitive hybridization assay.

1.5.2.4 The Green Fluorescent Protein (GFP)

In vivo, the luminescence from *Aequorea victoria* is blue-green, due to the co-existence of aequorin with a second green fluorescent protein (GFP) that derives its excitation energy from the aequorin complex. As the excited state of coelenteramide falls to ground state, there is a radiationless energy transfer to the GFP, which in turn is excited and then emits light as it falls to ground state. This green light ($\lambda_{\text{ex}} = 395/475 \text{ nm}$; $\lambda_{\text{em}} = 508 \text{ nm}$) is characteristic of GFP emission rather than aequorin emission.

GFP, a 238 amino acid protein, consists of a barrel of β -sheets surrounding an α -helical heptapeptide that forms the fluorescent core (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). GFP has a high quantum yield ($\phi = 0.72\text{-}0.85$) (Cubitt *et al.*, 1995) and light emission is independent of substrates or co-factors, making it extremely useful for monitoring many biological processes such as gene expression, cell transformation and protein trafficking (Cubitt *et al.*, 1995).

1.5.3 The Luciferase of *Gaussia princeps*

A new luciferase from the copepod *Gaussia princeps* has recently been isolated from a cDNA library using expression cloning and purification from *E. coli* (Ballou *et al.*, 2000). Copepods, belonging to the subphylum *Crustacea*, are tiny crustaceans making up a large portion of plankton (ocean biomass) in the 350-1000 m depths of the oceans. Although many copepods belonging to the order *Calanoidea* produce bright extracellular bioluminescent displays (Clarke *et al.*, 1962), little is still known about the physiology of light production (Herring *et al.*, 1988; Bannister *et al.*, 1989; Bowlby *et al.*, 1991). *Gaussia princeps*, a calanoid of the family *Metriidae*, is a large copepod (10-12 mm) found mainly in the Southern California borderlands (Barnes *et al.*, 1972) as well as the south mid-Atlantic near the Cape Verde Islands and the Indian Ocean (Davis, 1949).

The *Gaussia princeps* copepod possesses luminous glands, each consisting of a single large cell discharging copious luminous secretions through a cuticular pore (Barnes *et al.*, 1972). These luminous cells consist largely of secretory vesicles contained within an endoplasmic reticulum matrix, and luminescence from the organism occurs through expulsion of these secretory vesicles from the cuticular pores. These luminous cells fluoresce when excited with ultraviolet light (Barnes *et al.*, 1972) indicating the

probable presence of a luciferin or its precursors in the cell. Furthermore, organisms withstanding starvation for weeks did not exhibit diminished luminescence, implying luciferin reactants may be provided by the organism and not the diet (Barnes *et al.*, 1972).

The presence of a second type of differentially staining intracellular organelle of the secretory vesicle was presumed to contain the luciferase (Bowlby *et al.*, 1991). The absence of any possible cofactors such as oxygen, ATP, Ca^{2+} , Mg^{2+} , Cu^{2+} in the luminous cells (Campbell, 1988), may actually prevent any luminescence from occurring in the organism. Light emission most likely occurs upon secretion into seawater, which would contain any necessary cofactors required for light emission. Alternatively, changes in permeability of the secretory vesicular membrane upon excretion may permit the luminescent reaction to occur (Bowlby *et al.*, 1991).

The actual mechanism of vesicle expulsion was determined to be unrelated to the action of muscle fibers or microfilaments, due to their absence near the cells, as well as unrelated to changes in hydrostatic pressure (Herring, 1998; Bannister *et al.*, 1989). It is now assumed that release of neurotransmitters from the neuroeffector junction synaptic vesicles in response to neural stimulation, causes changes in luminous cell membrane conductance leading to ionic changes within the cell and vesicle release to the exterior (Bowlby *et al.*, 1991).

It seems likely that luminescence in the copepod may be partially due to avoidance or escape responses since the sudden initiation of rapid swimming is often coupled with strong luminous discharges (Barnes *et al.*, 1972). To date, what is known about the luminescence from *Gaussia princeps* is that it occurs by neural activation of the luminous cell, followed by expulsion of the secretory vesicles from the cuticular pores, culminating in initiation of rapid swimming away from the luminous material (Bowlby *et al.*, 1991).

1.5.3.1 Recombinant *Gaussia* Luciferase

The recent identification, isolation and cloning of the luciferase from the copepod *Gaussia princeps* using a cDNA library (Ballou *et al.*, 2000) has now made it possible for the production of recombinant *Gaussia* luciferase from expression systems. This *Gaussia* luciferase enzyme belongs to the class of bioluminescent organisms that utilize

coelenterazine as their luciferin. At 185 amino acids in length, this is the smallest known coelenterazine-using luciferase to date, with a molecular weight of only 19.9 kDa. The enzyme is known to have a broad pH optimum with peak activity at about pH 7.7 and is exceptionally resistant to high heat exposure as well as acidic and basic conditions (Ballou *et al.*, 2000).

Like other luciferases, the *Gaussia* luciferase catalyzes the oxidation of coelenterazine in the presence of molecular oxygen forming coelenteramide and CO₂, with a simultaneous release of light as the excited coelenteramide falls to ground state. The *Gaussia* luciferase is rapidly inactivated during reaction (Ballou *et al.*, personal communication) and exhibits a flash-type emission profile. This is similar to other luciferases such as that from *Renilla reniformis*, where the bioluminescent coelenteramide product is known to act as a strong competitive inhibitor of the luciferase (Matthews *et al.*, 1977). While the *Gaussia* luciferase protein is not yet available commercially, the plasmid containing the luciferase gene from *Gaussia princeps* is now available for purchase (Nanolight Technologies). The following work involves the overexpression, purification and the first utilization of the *Gaussia* luciferase as a reporter molecule in any analytical assay.

1.6 PROTEIN ENGINEERING

1.6.1 Rational Design vs. Directed Molecular Evolution

The production of proteins and enzymes for medical, industrial and environmental applications has become prevalent today, but the ability to tailor these functions to specific applications would provide many added benefits. Traditionally, selection of proteins for a specific application, were screened from organisms that had become adapted to extreme conditions such as high temperatures or salt concentrations. This was previously done to provide a thermostable DNA polymerase for PCR, isolated from the bacterium *Thermus aquaticus* (Saiki *et al.*, 1988).

Protein engineering technology now involves the creation of novel proteins by the modification of existing ones. This approach has been made available by site-directed mutagenesis and computer-assisted modeling of the three-dimensional structure of

proteins (Cleland *et al.*, 1996). Site-directed mutagenesis based on structure-function considerations for the creation of novel proteins, uses methods with specific oligonucleotides to replace sequences with alterations that confer the desired amino acid changes (Reidhaar-Olson *et al.*, 1988). This approach has been limited by inadequate understanding of amino acid substitutions on structure and function (Encell *et al.*, 1999) and many attempts have failed due to unexpected influences of the changes. Furthermore, construction and analysis of rationally chosen site-directed mutagenesis substitutions and combinations of substitutions is labour intensive and time consuming.

Directed molecular evolution on the other hand provides the means for developing useful DNA, RNA or protein products that could not have been predicted by rational design. This technique requires no previous knowledge of three-dimensional structure or changes needed to achieve the desired end result. This engineering approach is based on random mutagenesis, following by intensive screening to identify any promising novel molecules containing desired traits. This allows molecules to be genetically altered to improve their performance or alter their function under specified conditions. The goal of applied molecular evolution is therefore to mimic the natural design process of *in vivo* evolution and speed it up by directed selection (Joyce, 1992; Stemmer, 1994a).

1.6.2 Molecular Evolution

1.6.2.1 Genetic Evolution *In Vivo*

Evolution in nature is basically defined as the consequence of genetic changes that occur within a population of organisms (Garrett *et al.*, 1995). When a genetic mutation arises, its course of evolution is determined by the probability that it will persist in the population. For genetic material to be inherited with extraordinary fidelity by future generations, the nucleotide sequences must be accurately conserved and replicated, hence the development of elaborate DNA replication and repair mechanisms that exist in many organisms. However, the long-term survival of the organism may depend on genetic variation that allows adaptations to environmental changes. An essential property of DNA in cells is therefore the ability to undergo mutations or rearrangements that can

vary the particular combination of genes present in an individual genome, as well as the timing and expression of the genes (Alberts *et al.*, 1994).

Since genetic variation in a population is fundamental for evolution to occur there are several mechanisms by which these changes may arise. Errors in replication or repair may lead to single point mutations, with about one nucleotide pair in a thousand randomly changed every 200 000 years (Alberts *et al.*, 1994). Aside from single nucleotide changes, genetic recombination causes major genome rearrangements due to gene transposition or exon shuffling, as well as gene duplication or deletion. Component parts of the genome, such as exons or regulatory elements may be transposed from one region to another, or shuffled as separate modules. This may create new proteins, those with altered functions, or even altered expression of some genes. Additionally, duplicated genes are free of any functional constraints and tend to diverge by further mutations, becoming specialized or individualized for a specific function (Alberts *et al.*, 1994).

According to the Darwinian theory of natural selection (Darwin, 1975 reprint), many variations created in the genome will be disadvantageous to the organism and will be selected against in the population. Mutations inferring some type of advantage will be passed along to progeny. The population of organisms will then begin to change as these favourable traits are expressed in more and more offspring. *In vivo* evolution is therefore a consequence of natural selection dictated by random genetic change.

1.6.2.2 Directed Evolution *In Vitro*

Inspired by natural selection and the process of Darwinian evolution *in vivo*, directed molecular evolution procedures are now being carried out *in vitro*, that allow the evolution of molecules with desired characteristics. Variation is introduced into genetic material to create a heterologous population of molecules upon which the desirable mutants are chosen through rounds of amplification and selection. Following each round, favoured molecules are selected for future rounds and this procedure is repeated until the desired end result is achieved.

Evolution, regardless of whether it is *in vivo* or *in vitro*, is always based upon rounds of selection, amplification and mutation. In nature, selection is a direct result of organisms that have survived in their environment, whereas *in vitro*, selected molecules

are those that pass certain imposed criteria. Amplification is then the process of generating copies of the genetic material, either by producing viable progeny *in vivo*, or by amplifying the population of selected molecules that have passed the imposed criteria *in vitro*. Mutation then introduces variances that allow evolution to continue both *in vivo* and *in vitro*.

1.6.2.2.1 The Origin of *In Vitro* Evolution

In vitro evolution was first demonstrated using the Q β bacteriophage, whose genetic RNA contains only four genes of which one codes for a replicase, responsible for making copies of the RNA genome. By mixing the viral RNA with its replicase and ribonucleoside triphosphates (NTPs), the replicase made copies of the RNA. Since the replicase is an error prone enzyme, mutations in the genome were inherent. The selection criterion was that of multiplication ability, and every 20 minutes the reaction mixture was transferred to a new sample containing replicase and NTPs. This was repeated 74 times with each cycle favouring the proliferation of the molecules giving rise to the most progeny before the transfer step. By the 74th transfer, the evolving RNA molecules had lost much of their sequence and the surviving molecules retained only the essential sequences required for the replicase to function (Mills *et al.*, 1967).

The major drawback of this first directed evolution system was that the replicase is a particular enzyme in that it will only replicate specific sequences. The advent of PCR and various other amplification techniques have now facilitated this aspect of molecular evolution. Although the DNA polymerase required for PCR is accurate as well, other methods have now been developed to introduce variations in the genetic material. Furthermore, the advent of high-throughput screening methods has allowed easy selections of large populations.

1.6.2.2.2 The Bias of *In Vitro* Evolution

While directed molecular evolution provides a powerful tool for the creation of altered molecules with specified characteristics, it should be noted that these variants may not necessarily be the most ideal or superior solution to the selection criteria, but rather the best solution in the evolutionary history of that particular molecule (Joyce, 1992).

Ultimately, it is the selection criteria that determine the direction of the evolution. This creates a bias in the mutant population that is dependent on all criteria set out in the evolutionary procedures. The selection of one favourable trait may delete other favourable inherent traits not selected for, as was the case with the original replicase of the Q β bacteriophage. While the rate of replication increased dramatically, the virus could no longer infect cells due to the deletion of most of its genome (Mills *et al.*, 1967; Joyce, 1992).

1.6.3 DNA Shuffling

Initially, methods for protein engineering were based on oligonucleotide-directed mutagenesis to replace short sequences with altered synthetic oligos (Reidhaar-Olson *et al.*, 1988; Stemmer *et al.*, 1992), or error prone PCR using low-fidelity polymerization conditions for random mutagenesis (Leung *et al.*, 1989). Computer simulations of evolution (genetic algorithms) have shown that recombination between individuals with a low level of point mutation was the preferred method for the evolution of complex linear sequences (Holland, 1992; Forrest, 1993). There were no simple procedures for achieving this goal until the recent advent of DNA shuffling (Stemmer, 1994a; 1994b).

The shuffling procedure as shown in Figure 1.7, involves the digestion of a gene with DNase I into a pool of random fragments that are then re-assembled into the full-length gene by repeated cycles of annealing in the presence of DNA polymerase. Based on homology, the fragments are able to prime each other allowing recombination when fragments from one copy of the gene prime on a different copy, causing a template switch. DNA shuffling combined with repeated cycles of amplification and selection has provided an efficient tool for *in vitro* recombination allowing directed molecular evolution.

Shuffling can also be used for DNA family shuffling, often called molecular breeding. This technique employs the shuffling of various genes to create a library of chimeric proteins that can then be screened for distinctive properties allowing benefits over any of the single parental genes (Cramer *et al.*, 1998; Ness *et al.*, 1999). In this way, shuffling is used as a powerful tool for recombining the existing diversity of genes and allows tailoring to specific functional parameters.

FIGURE 1.7
DNA SHUFFLING PROTOCOL

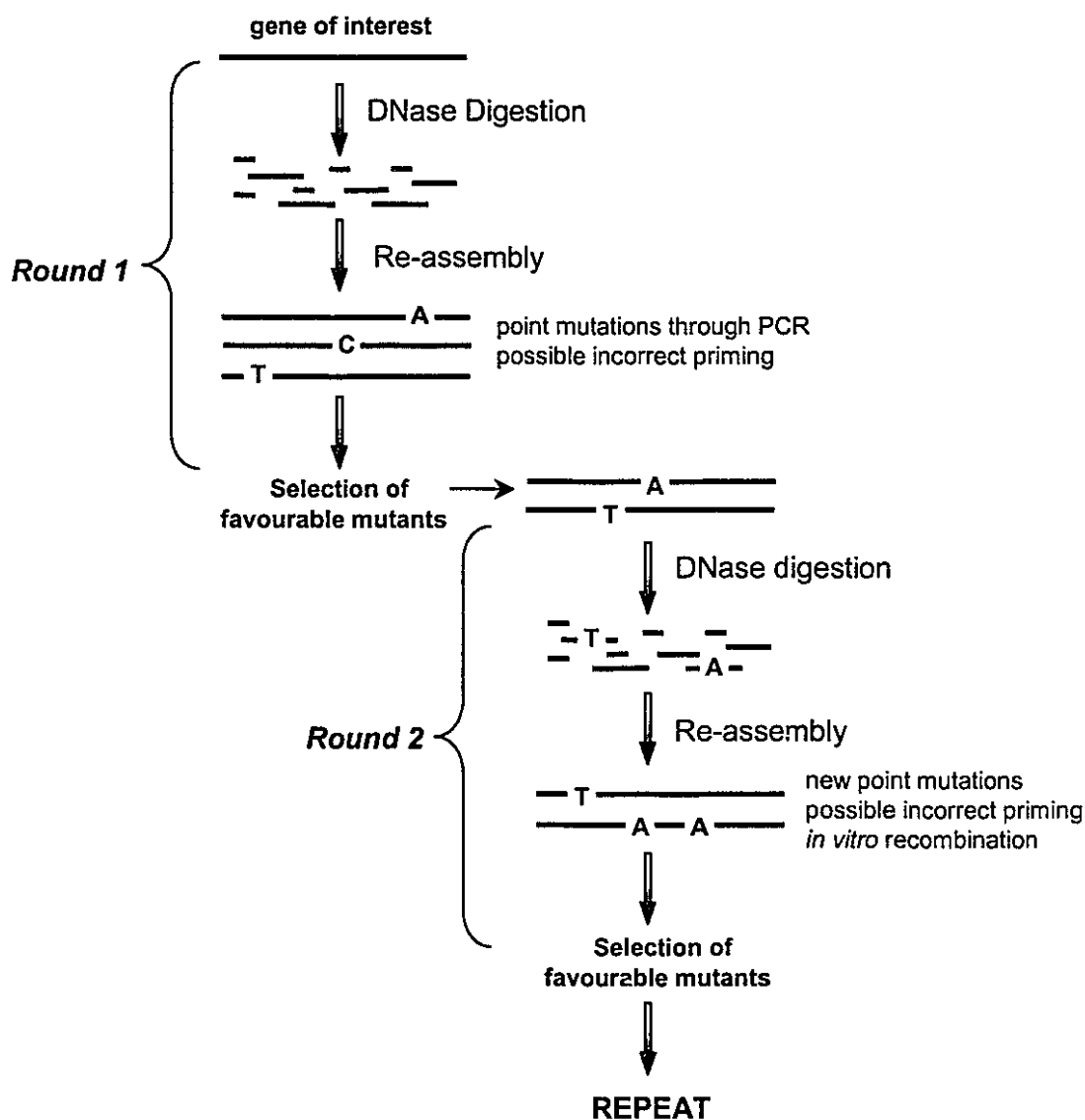


Figure 1.7. The shuffling procedure for directed molecular evolution involves the digestion of a gene with DNase I into a pool of random fragments. The fragments are then re-assembled into the full-length gene by repeated cycles of annealing in the presence of DNA polymerase. Based on homology, the fragments are able to prime each other allowing recombination when fragments from one copy of the gene prime on a different copy, causing a template switch.

1.6.4 Applications of Directed Molecular Evolution

The many applications of directed molecular evolution include drug design, novel binding proteins, novel DNA/RNA binding molecules, altered activity or stability of various proteins, and evolved enzymes with altered catalytic activity or substrate specificity. Some of the earlier applications of directed molecular evolution were aimed at drug design, such as the development of a DNA binding inhibitor to the clot-forming protein thrombin (Bock *et al.*, 1992). Random sequences of single-stranded DNA were constructed and tested for their ability to bind thrombin. Selected molecules were then amplified by PCR and the process repeated five times until a sequence was found that avidly bound the thrombin protein.

Engineering of enzymes by directed evolution has grown considerably, and enzymes have been developed with many favourable traits such as the thermostability of kanamycin nucleotidyltransferase (Liao *et al.*, 1986), the increased activity of subtilisin E in organic solvents (Chen *et al.*, 1993), and the improved activity of subtilisin BPN at low temperatures (Zhu *et al.*, 1996).

DNA shuffling procedures as a source of genetic mutation and recombination have allowed the development of novel molecules. Repeated cycles of shuffling, followed by screening or selection have proven successful for the directed evolution of single gene products such as the initial shuffled TEM-1 β -lactamase gene, resulting in mutants with a drastically higher minimum inhibitory concentration of the antibiotic cefotaxime (Stemmer, 1994b; 1994a).

Since then, DNA shuffling has been used for tailoring many systems, including increased arsenic resistance, important in the detoxification of trivalent and pentavalent arsenic ions that contaminate rivers and groundwater (Crameri *et al.*, 1997), altered substrate specificity of β -galactosidase (Zhang *et al.*, 1997), the improved protein folding of GFP (Crameri *et al.*, 1996), and the improved aldehyde resistance of β -glucuronidase for use as a gene reporter (Matsumura *et al.*, 1999).

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 CHEMICALS, BIOCHEMICALS, AND SUPPLIES

Opaque polystyrene Microlite™ 2 microtiter wells were obtained from Dynatech Laboratories Inc. (Chantilly, VA).

Sephadex® G-25 gel filtration columns (NAP™-5 and NAP™-10), alkaline phosphatase (calf intestine) and T4 DNA ligase were from Amersham-Pharmacia Biotech (Baie d'Urfe, Quebec).

Agarose, TEMED, as well as fungal proteinase K were purchased from Invitrogen Corp. (Burlington, ON).

Digoxigenin-11-dUTP (DIG-dUTP), anti-digoxigenin antibody (from sheep), and DNase I (from bovine pancreas) Grade II were from Roche Diagnostics (Laval, Quebec).

The Qiaex® II kit for purification of DNA from agarose gels and the Qiagen Plasmid Mini Kit were from Qiagen (Mississauga, ON).

Microcon®-30 concentrators were from Amicon (Beverly, MA). Ultrafree-15 centrifugal devices and Centriplus® YM-10 devices were from Millipore (Etobicoke, ON).

Spin-pure G-25 columns were from CPG Inc. (Lincoln Park, NJ).

Pfu turbo DNA polymerase was obtained from Stratagene (La Jolla, CA). Gold Label *Taq* DNA polymerase and *EcoR* I restriction enzyme were from BIO/CAN Scientific (Mississauga, ON).

SoftLink™ Soft Release avidin resin, RQ1 RNase-free DNase, low melting point (LMP) agarose-preparative grade for small fragments, Wizard® PCR Preps DNA Purification System, Wizard® Plus Maxipreps DNA Purification system, and DNA ladder (100 bp) were purchased from Promega (Madison, WI).

Coomassie brilliant blue R-250, broad range SDS-PAGE molecular weight standards, Bio-Rad protein assay dye reagent, ammonium persulfate, and 40% acrylamide/bis (37.5:1) solution were from Bio-Rad Laboratories (Mississauga, ON).

B-PER™ II bacterial protein extraction reagent, and EZ-Link Sulfo-NHS-LC-biotin were from Pierce (Rockford, IL).

Coelenterazine was obtained from Biosynth AG (Staad, Switzerland) and reconstituted in acidified methanol (0.1 mol/L HCl) that had been purged of O₂ by bubbling N₂ through the solution to make a 10 mmol/L stock solution. Working dilutions of 2.5 mmol/L were made from the 10 mmol/L stock in acidified methanol. All coelenterazine dilutions were stored at -80°C for long periods of time or at -20°C for several days.

Restriction enzymes *Kpn* I, *Bgl* II, *Not* I and *Sma* I were from MBI Fermentas (Burlington, ON). Restriction enzyme *Pst* I was from New England Biolabs (Beverly, Mass).

Equilibrated (pH>8) phenol and sodium dodecyl sulphate (SDS) were from USB (Cleveland, OH).

Nalgene™ disposable 25 mm syringe filters (0.2 µm pore size) and soda lime glass Pasteur pipettes were from Baxter Diagnostics Corp. (Toronto, ON).

Sterile, conical 15 mL and 50 mL graduated polypropylene centrifuge tubes as well as disposable universal fit pipette tips were supplied by VWR (West Chester, PA).

Recombinant streptavidin (SA), deoxycholic acid, CaCl₂, bovine serum albumin (BSA), ampicillin, Bradford protein dye reagent, lysozyme (chicken egg white), polyoxyethylene sorbitan monolaurate (Tween-20), dithiothreitol (DTT), d-biotin, ethidium bromide, and isopropyl thiogalactopyranoside (IPTG) were from Sigma-Aldrich (Oakville, ON).

General chemicals including sodium chloride, potassium chloride, magnesium chloride, sodium hydroxide, maleic acid, ethylenediaminetetra-acetic acid disodium salt (EDTA), phenylmethyl sulfonyl fluoride (PMSF), disodium hydrogen phosphate, potassium dihydrogen orthophosphate, sodium acetate, chloroform, ethanol, sodium hydrogen carbonate, trishydroxymethylaminomethane (Tris), 2-propanol, glacial acetic acid and hydrochloric acid were purchased from BDH Inc. (Toronto, ON). Chemicals not mentioned were from BDH Inc. or Sigma-Aldrich.

2.2 BACTERIAL STRAINS AND PLASMIDS

The pSVAEQN plasmid from Molecular Probes (Eugene, OR) was used as a source of the native apoaequorin gene (coding sequence shown in Appendix A) for both the *in vivo* biotinylation project described in Chapter 3 as well as the shuffling project of Chapter 4.

The pKK223-3 plasmid (GenBank Accession Number M77749) from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec) was used for general cloning and expression purposes in the construction of mutant libraries following apoaquorin shuffling procedures. The 4584 bp plasmid contained the strong *tac* promoter upstream of the multiple cloning site and the β -lactamase gene, conferring ampicillin resistance and allowing antibiotic selection.

The pGLUC plasmid used as a source of *Gaussia* luciferase gene was from Nanolight™ Technologies (Pinetop, AZ). The luciferase coding sequence is shown in Appendix G.

The PinPoint™ Xa-1 Vector from Promega contained a biotin acceptor coding sequence, allowing expression of a biotin acceptor peptide fusion protein under control of the *tac* promoter. The 3331 bp plasmid also contained the β -lactamase gene, conferring ampicillin resistance and allowing antibiotic selection.

The JM 109 *E. coli* strain (Promega) was used for general cloning purposes, over-expression of recombinant proteins and as the source of genomic DNA for extraction of the *birA* gene. This strain contains the *lacI* gene, which codes for the overproduction of the repressor protein of the *lac* operon, and hence allows induction of protein expression with IPTG.

2.3 APPARATUS AND INSTRUMENTATION

Agarose gel electrophoresis of DNA was performed using the Miniature Horizontal Gel System MLB-06 from Tyler Research Instruments (Edmonton, AB) along with the Fotoforce 500™ Power supply by Fotodyne Inc. from BIO/CAN Scientific (Mississauga, ON). Gels were viewed using the hand held Model UVG-II ShortWave UV Mineralight® Lamp from Ultra-Violet Products Inc. (San Gabriel, CA) or the Benchtop Ultraviolet Transilluminator from VWR Scientific (Mississauga, ON). The dual-vertical mini-gel unit for SDS-PAGE was from CBS Scientific (Del Mar, CA).

The AlphaImager™ 2200 Light Imaging System with AlphaEase™ software for photography of ethidium bromide stained agarose gels and Coomassie blue stained polyacrylamide gels was from Alpha Innotech Corporation (San Leandro, CA).

The Agilent 8453 UV-visible spectrophotometer for measurement of protein following Bradford dye assay (595 nm) or measurement of cell density (600 nm) was from Agilent Technologies (Mississauga, ON).

Luminescence measurements for both the *Gaussia* luciferase and aequorin reactions, were carried out using the MLX™ Microtiter® plate luminometer from Dynex Technologies (Chantilly, VA). The luminometer contained two positive displacement syringe dispensers for automated injection of triggering reagents, and was capable of performing glow endpoint, glow kinetic or flash reactions with readings taken as frequently as 10 msec (Dynex Technologies, 1996). The Revelation™ Software controlled the MLX® and collected all data.

Polymerase chain reactions (PCR) were performed using the 48-well Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer, Norwalk, CT).

The hybridization assays were performed using the Amerlite shaker/incubator from Amersham (Oakville, ON). Plate washings were carried out using the Corning ELISA Plate Washer 26300 from Corning Glass Works (Corning, NY).

The SPECTRAmax GEMINI XS dual-scanning microplate spectrofluorometer with Softmax® PRO software used for determination of DNA following PicoGreen staining was from Molecular Devices Corp. (Sunnyvale, CA).

The Eppendorf Vacufuge™ for concentration of samples was from VWR (Mississauga, ON).

Centrifugations were performed using either the J2-HS centrifuge or J-6B swinging bucket centrifuge from Beckman Instruments, Inc. (Mississauga, ON), a desktop Safety-Head Centrifuge from Canlab (Mississauga, ON) or an Eppendorf Model 5415C microcentrifuge from Baxter/Canlab, Inc. (Mississauga, ON).

Other general laboratory equipment used were as follows:

A Corning pH Meter 340 (Corning, NY) with standardized buffer solutions from BDH Inc. (Toronto, ON); a Sartorius BP61 balance; a Corning Stirrer/Hotplate; a Genie 2 Vortex (Fisher Scientific, Toronto, ON); Eppendorf pipettes (Germany); Nichiryo Model 5000 pipettes (Japan).

2.4 BUFFERS AND SOLUTIONS

2.4.1 General Buffers

- *Phosphate-Buffered Saline* (PBS buffer). Consisted of 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄ and 1.76 mmol/L KH₂PO₄, pH 7.4.
- *Sodium Phosphate Buffer 1*. Consisted of 10 mmol/L sodium dihydrogen phosphate (NaH₂PO₄), pH 6.8 and was used for purification with NAP size exclusion columns.
- *Sodium Phosphate Buffer 2*. Consisted of 100 mmol/L potassium phosphate, pH 7.0, and was used for regeneration of the monomeric avidin resin following washing with 10 % acetic acid.
- *Tris-Buffered Saline* (TBS buffer). Consisted of 25 mmol/L Tris, 2.6 mmol/L KCl, 137 mmol/L NaCl, pH 7.4
- *SET Buffer*. Consisted of 75 mmol/L NaCl, 25 mmol/L EDTA, 20 mmol/L Tris, pH 7.5.
- *Cell Lysis Buffer*. Consisted of 50 mmol/L Tris, pH 8.0, 1 mmol/L EDTA, 0.1 mol/L NaCl.
- *PMSF Stock Solution*. Consisted of 0.1 mol/L PMSF in isopropanol, stored at -20°C in aliquots.
- *Aequorin Ca²⁺ Triggering Solution*. Consisted of 0.1 mol/L CaCl₂, 0.1 mol/L Tris, pH 7.5.
- *Aequorin Regeneration Buffer*. Consisted of 4 µmol/L coelenterazine and 10 mmol/L DTT in 10 mmol/L potassium dihydrogen phosphate, pH 7.5, 0.5 mol/L NaCl, 1 mmol/L EDTA. Coelenterazine and DTT were added from frozen stocks just prior to use.
- *Coelenterazine Substrate Solution*. Consisted of various concentrations of coelenterazine (5.0 µmol/L -130 µmol/L) diluted in Buffer E. This was used as the substrate solution for bioluminescence of *Gaussia* luciferase.
- *Tris-EDTA Buffer* (TE buffer). Consisted of 10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA.
- *1x One-Phor-All Buffer* (1x OPA Buffer). Consisted of 10 mmol/L tris acetate, 10 mmol/L magnesium acetate, and 50 mmol/L potassium acetate.

- *Buffer A.* Consisted of 30 mmol/L Tris, pH 7.5, 2 mmol/L EDTA and 0.15 mol/L NaCl.
- *Buffer B.* Identical to Buffer A with the addition of 20% glycerol.
- *Buffer C.* Identical to Buffer B with the addition of 5 mmol/L biotin.
- *Buffer D.* Consisted of 10 mmol/L Tris, 10 mmol/L EGTA, 1 mol/L KCl, 10 mmol/L MgCl₂, 1 g/L BSA and 1 g/L NaN₃, pH 7.5.
- *Buffer E.* Consisted of 10 mmol/L Tris, pH 7.8, 1 mmol/L EDTA and 0.60 mol/L NaCl.
- *Buffer F.* Consisted 10 mmol/L Tris, pH 7.8, 1 mmol/L EDTA, 0.60 mol/L NaCl and 20% B-PER II Reagent (v/v).
- *Buffer G.* Identical to buffer A with the addition of 0.2 g/L BSA and 0.06 mmol/L NaN₃.
- *Buffer I.* Consisted of 30 mmol/L Tris, pH 7.5, 10 mmol/L EDTA.

2.4.2 Solutions for Bacterial Culture

- *Luri-Bertani Broth (LB broth).* Consisted of 10 g/L tryptone, 5 g/L yeast extract, 0.17 mol/L NaCl, and 2 mmol/L NaOH. Broth was sterilized by autoclaving 15 min at 121°C. Broth was stored at 4°C for several months.
- *LB Broth with Ampicillin.* Consisted of 0.1 g/L ampicillin in LB broth.
- *LB Agar Plates.* Consisted of LB broth containing 15 g/L agar. LB agar broth was sterilized by autoclaving 15 min at 121°C. After cooling, 0.1 g/L ampicillin was added and the LB agar broth was poured into plates. Solidified agar plates were stored at 4°C for several months.
- *Ampicillin Stock Solution.* Consisted of 50 mg/mL ampicillin. Solution was filter sterilized and stored at -20°C.
- *IPTG Stock Solution.* Consisted of 1 mol/L IPTG stored at -20°C in aliquots.

2.4.3 Electrophoresis Buffers

For Agarose Gel Electrophoresis:

- *Tris-Acetate-EDTA Buffer (TAE buffer).* Consisted of 40 mmol/L Tris-acetate and 1 mmol/L EDTA.

- *Gel Loading Buffer*. Consisted of 0.25% (w/v) Bromophenol Blue in 30% glycerol (v/v).
- *Ethidium Bromide Stock Solution* (EtBr solution). Consisted of 10 mg/mL EtBr and was protected from light by storing in foil.

For SDS-Polyacrylamide Gel Electrophoresis:

- *Tris-Glycine Running Buffer*. Consisted of 25 mmol/L Tris, 0.25 mol/L glycine (electrophoresis grade, pH 8.3) and 0.1% SDS (v/v).
- *Resolving Gel*. Consisted of 12% acrylamide/bis mix (37.5:1), 0.39 mol/L Tris, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED (v/v). TEMED was added last to initialize polymerization.
- *Stacking Gel*. Consisted of 5% acrylamide/bis mix (37.5:1), 0.125 mol/L Tris, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, 0.1% TEMED (v/v). TEMED was added last to initialize polymerization.
- *SDS Gel Loading Buffer*. Consisted of 25 mmol/L Tris, pH 6.8, 100 mmol/L DTT, 2% SDS (v/v), 0.1% Bromophenol Blue (w/v) and 10% glycerol. Buffer was stored in aliquots at -20°C .
- *Staining Solution*. Consisted of 2.5 g/L Coomassie Brilliant Blue R250 dissolved in 45% (v/v) methanol and 10% glacial acetic acid (v/v).
- *Destaining Solution*. Consisted of 45% (v/v) methanol and 10% glacial acetic acid (v/v).

2.4.4 Solutions for Plasmid Isolation

- *Resuspension Buffer* (buffer R). Consisted of 50 mmol/L Tris, pH 8.0, 10 mmol/L EDTA, 100 ug/mL RNase A.
- *Lysis Buffer* (buffer L). Consisted of 0.2 mol/L NaOH, 1% SDS (v/v).
- *Neutralization Buffer* (buffer N). Consisted of 3.0 mol/L potassium acetate, pH 5.5.
- *Phenol:Chloroform:Isoamyl Alcohol Mix* (Ph:CHCl₃ mix). Consisted of phenol, equilibrated to pH 7.8-8.0, chloroform, and isoamyl alcohol in a ratio of 25:24:1 (v/v/v). The mix was overlaid with Tris, pH 8.0.

2.4.5 Hybridization Assay Buffers

- *Carbonate Buffer* (for anti-DIG well coating). Consisted of 0.1 mol/L sodium hydrogen carbonate (NaHCO₃), pH 9.6. Buffer was filter sterilized before use.
- *Wash Buffer*. Consisted of 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl, 1 mL/L Tween-20 and 2 mmol/L EDTA. For *Gaussia* luciferase assays, buffer consisted of only 1 mmol/L EDTA.
- *Buffer H*. Consisted of 1% (w/v) blocking reagent (10 g/L) in 0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5 and 2 mmol/L EDTA. For *Gaussia* luciferase assays, buffer consisted of only 1 mmol/L EDTA.

2.5 OLIGONUCLEOTIDES

The following oligonucleotides were synthesized by Bio-Synthesis (Lewisville, TX) unless otherwise stated.

Oligonucleotides used as PCR primers for creation of the *birA* gene insert:

- (a) 5'-T CCC CCG GGA GGA GAT ATA CAT **ATG AAG GAT AAC ACC**, a 37-mer upstream primer introducing a *Sma* I site (bold region) with the underlined region homologous to codons 1-5 of the *birA* gene;
- (b) 5'- A TAG TTT AGC GGC CGC **TTA TTT TTC TGC ACT**, a 31-mer downstream primer introducing a *Not* I site (bold region) with the underlined region containing a stop codon and complementary to the last four codons of the *birA* gene.

Oligonucleotides used as PCR primers for creation of the apoaequorin gene inserts:

- (c) 5'-C CCG TAC CGA **GTC AAG CTT ACA TCA**, a 25-mer upstream primer introducing a *Kpn* I site (bold region) and homologous to codons 2-6 of the apoaequorin gene (underlined region);
- (d) 5'-G GAG ATC TGA **TTA GGG GAC AGC TCC**, a 25-mer downstream primer introducing a *Bgl* II site (bold region) and complementary to the last four codons of the apoaequorin gene (underlined region) with the stop codon present;

(e) 5'-AGG AAA CAG AAT TCC CGG ATG GTC AAG CTT ACA-3', a 33-mer primer introducing an *Eco* RI site (bold region) and homologous to codons 1-5 of the apoaeguorin gene (underlined region);

(f) 5'-TT TTC TGC AGC TTA GGG GAC AGC TCC ACC-3', a 29-mer containing a *Pst* I site (bold region) and used as the downstream primer.

Oligonucleotides used as PCR primers for creation the *Gaussia* luciferase gene insert were synthesized by ACGT Corp. (Toronto, ON).

(g) 5'-C CGG TAC CGA AAA CCA ACT GAA AAC, a 25-mer upstream primer introducing a *Kpn* I site (bold region) and homologous to codons 2-6 of the *Gaussia* luciferase gene (underlined region);

(h) 5'-GAG ATC TGA TTA ATC ACC ACC GGC ACC, a 27-mer downstream primer introducing a *Bgl* II site (bold region) and complementary to the last five codons of the *Gaussia* luciferase gene (underlined region) with an added stop codon present.

The following oligonucleotides were used as PCR primers for creation of the biotinylated DNA target used in all hybridization assays. The target DNA (shown in Appendix C) was a 233 bp fragment generated by RT-PCR of prostate specific antigen (PSA) mRNA (Verhaegen, 1998).

(i) 5'-(Biotin)-CTC TCG TGG CAG GGC AGT CT-3', a biotinylated 20-mer used as the upstream primer and homologous to a sequence in exon 2 of the PSA gene, allowing introduction of a 5' biotin label;

(j) 5'-GGT CGT GGC TGG AGT CAT CA-3', a 20-mer used as the downstream primer and complementary to a sequence in exon 3 of the PSA gene.

The oligonucleotide probe used in hybridization assays of amplified DNA target:

(k) 5'- ATC ACG CTT TTG TTC CTG ATG CAG-3', a 24-mer complementary to the 67-90 bp region of the DNA target. The complementary binding region is shown as a highlighted sequence in Appendix C.

2.6 GENERAL METHODS AND PROCEDURES

General molecular biology procedures repeated frequently are described below. Experiments are carried out as stated here unless otherwise specified in each chapter. Specific procedures pertaining to each project are described in the Methods sections of each appropriate chapter.

2.6.1 Polymerase Chain Reaction

2.6.1.1 PCR with *Pfu* Polymerase

PCR was carried out in a total volume of 100 μ L containing (final concentrations) 10 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.8), 0.1% Triton X-100, 2 mmol/L MgSO₄, 10 mmol/L (NH₄)₂SO₄, 0.1 g/L BSA, 0.15 mmol/L of each dNTP, 50 pmol of each primer, suitable DNA template and 2.5 units *Pfu* Turbo DNA polymerase. The appropriate DNA starting template and primers are discussed in their corresponding sections. PCR was run for 30 cycles, each consisting of denaturation (95°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 1 min) unless otherwise specified. Mixtures were then incubated at 72°C for 10 min and cooled to 4°C until further use.

2.6.1.2 PCR with *Taq* Polymerase

PCR was carried out in total volume of 100 μ L containing (final concentrations) 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2 mmol/L MgCl₂, 20 μ mol/L of each dNTP, 50 pmol of each primer, suitable DNA template and 2.5 units of Gold Label *Taq* DNA polymerase. The appropriate DNA starting template and primers are discussed in their corresponding sections. The mixtures were layered with mineral oil and placed in the Perkin-Elmer DNA thermal cycler. Primers were added to the mixture during a “hot start” protocol (D’Aquila *et al.*, 1991) once the block reached 95°C. PCR was run for 30 cycles (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) unless otherwise stated. Samples were then incubated at 72°C for 10 min and then cooled to 4°C until further use.

2.6.2 DNA Quantitation with PicoGreen

Quantitation of DNA was carried out fluorometrically by using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Briefly, a DNA sample diluted in TE buffer (50 μ L) was pipetted into a microtiter well, followed by the addition of 50 μ L of a 200 fold dilution of the PicoGreen concentrated dye in TE buffer. After 5 min incubation with shaking, the sample fluorescence was measured (excitation 480 nm, emission 520 nm) using the microplate spectrofluorometer. The concentration of sample DNA was calculated from a standard curve prepared with a lambda DNA standard supplied by Molecular Probes, Inc.

2.6.3 Bacterial Culturing

To maintain sterile techniques throughout bacterial culturing procedures, all LB broth solutions and glassware were autoclaved or filter sterilized before use. The mouths of tubes and pipettes were briefly flamed to help maintain a sterile environment.

2.6.3.1 Creation of Competent *E. Coli*

JM 109 *E. coli* were grown to mid log-phase (0.3-0.4 OD₅₅₀) in 10 mL of LB broth and pelleted at 2000 rpm for 10 min. To the cell pellet was added 5 mL of cold sterile 50 mmol/L CaCl₂ solution. The solution was vortexed and incubated on ice for 20 min followed by another centrifugation step. The cell pellet was then resuspended in 1 mL of the same fresh cold CaCl₂ solution and the cell suspension aliquoted and frozen at -80 °C until further use.

2.6.3.2 *E. Coli* Transformation with Recombinant Plasmids

Ten μ L of ligation reaction products were added to 100 μ L of competent JM 109 *E. coli* that had been thawed on ice. Following 30 min incubation on ice, the cell suspension was heat shocked at 42 °C for 50 sec, followed by another 2 min incubation on ice. After the addition of 1 mL LB broth, a 45 min recovery period was carried out at 37 °C with shaking. 100 μ L of this culture was spread on LB agar plates containing 0.1 g/L ampicillin. Plates were incubated overnight (O/N) at 37 °C.

2.6.3.3 Overexpression of Recombinant Proteins

Single colonies of transformed *E. coli* growing on LB agar plates, were inoculated in 3 mL of LB containing 0.1 g/L ampicillin and grown O/N at 30°C or 37°C. For overexpression of *E. coli* transformed with plasmids involving *in vivo* biotinylation, the broth was supplemented with 2 mg/L of biotin. Following O/N growth, the seed culture was diluted 100-fold in the same broth and grown to an absorbance of 0.8-0.9 (600 nm) at 30°C. At this point, protein synthesis was induced by addition of IPTG for 3-6 hrs. Cells were harvested by centrifugation at 2000 rpm for 25 min at 4°C and stored at -20°C until needed.

2.6.4 Plasmid Isolation from *E. coli*

Following the overnight growth of individual colonies on LB agar, single colonies were picked and grown O/N in 1.5-3 mL of LB broth containing 0.1 g/L ampicillin. Plasmid minipreparations were then prepared to isolate the recombinant DNA from the transformed *E. coli* using a procedure based on the alkali lysis method (Sambrook, 1989). The cell culture was pelleted at 12 000 g for 30 sec and the supernatant broth removed. 200 µL of buffer R and 200 µL of buffer L were added to the dry pellet. The mixture was inverted gently several times and incubated at room temperature (RT) for 5 min. 150 µL of cold buffer N was added, vortexed and incubated on ice for 5 min. This was followed by centrifugation at 12 000 g for 5 min at 4°C to pellet the cellular debris. To the supernatant containing plasmid DNA was added one volume of Ph:CHCl₃ mix. This mixture was vortexed and centrifuged at 12 000 g for 2 min at 4°C to separate the organic and aqueous layers. The plasmid DNA contained in the aqueous phase was precipitated with two volumes of 100% ethanol and again centrifuged. The resulting pellet was washed with 70% cold ethanol, centrifuged, dried and resuspended in 20-30 µL dd H₂O or 10 mmol/L Tris, pH 8.0.

2.6.5 Protein Determination by Bradford Assay

Protein mass was determined by the method of Bradford (Bradford, 1976). Briefly, the protein to be determined was diluted in a volume of 0.5 mL using buffer A (for aequorin), or buffer E (for *Gaussia luciferase*), followed by addition of 0.5 mL of

Bradford Reagent (Sigma). After 10 min incubation at RT, the absorbance was read at 595 nm. The spectrophotometer was blanked with the appropriate buffer plus the Bradford Reagent. The unknown concentration of protein was calculated from a standard curve made with standard protein solutions of BSA, ranging from 1 $\mu\text{g/mL}$ to 10 $\mu\text{g/mL}$.

2.6.6 SDS-PAGE

2.6.6.1 Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out with a discontinuous buffer system consisting of a stacking gel (pH 6.8) and a resolving gel (pH 8.8) (Ornstein, 1964; Davis, 1964), with all components containing 0.1% SDS (Laemmli, 1970). The resolving gel solution was poured between the gap of the two glass plates and immediately overlaid with isobutanol. After polymerization was complete (30 min), the overlay was washed off and the stacking gel poured directly onto the resolving gel. A Teflon comb was inserted into the stacking gel and then removed once polymerization was complete. Samples were diluted in SDS gel loading buffer, heated for 5 min at 95°C and 10-20 μL loaded in the wells. The gel was run with Tris-glycine running buffer in both the upper and lower reservoirs at approximately 100 volts. Once the samples entered the resolving gel, the voltage was increased to 190 volts and run until the bromophenol blue reached the bottom of the resolving gel.

2.6.6.2 Staining/Destaining

Polypeptides separated on the polyacrylamide gel were fixed and stained simultaneously with staining solution for 1-2 hrs at RT with gentle shaking. The excess dye was then removed by immersing the stained gel in destaining solution and microwaving on high power for approximately 5 min. A permanent photograph of the gel was then taken with the AlphaImager 2200 Light Imaging System.

2.6.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in the Miniature Horizontal Gel System. Agarose was dissolved in TAE buffer, 0.7-2% (w/v), and EtBr added to a final concentration of 10 ng/mL. Following solidification, and immersion of the agarose gel in

TAE buffer, DNA samples in gel loading buffer (10-25 μ L) were loaded into the wells and run for 1 hour at 70 V.

2.6.8 Ethanol Precipitation of DNA

DNA ethanol precipitation was based on a procedure by Sambrook (Sambrook *et al.*, 1989). 0.1 volumes of 3 mol/L sodium acetate, pH 5.0, was added to the DNA sample followed by 2 volumes of cold anhydrous ethanol. After incubation at -20°C for 15-30 min, the sample was centrifuged at 12 000 rpm for 20 min at 4°C . The resulting pellet was washed twice with 70% ethanol and air dried for 10 min. DNA pellets were resuspended in dd H_2O or TE buffer.

2.6.9 Extraction with Phenol:Chloroform

Extraction with phenol:chloroform was performed to remove proteins from nucleic acid solutions based on a method by Sambrook (Sambrook *et al.*, 1989). Briefly, one volume of Ph: CHCl_3 mix was added to the nucleic acid sample, which was vortexed to form an emulsion. Centrifugation was carried out for 15 sec at 12 000 g to separate the organic and aqueous layers. The upper aqueous phase containing the nucleic acid was removed and extracted again until no protein was visible at the interphase. An equal volume of chloroform was then added and extraction repeated. The nucleic acid was recovered by ethanol precipitation as described above in section 2.6.8.

2.6.10 Avidin Affinity Purification

The monomeric avidin resin was initially preadsorbed with free biotin (5 mmol/L) in sodium phosphate buffer 2 for 15 min to saturate any non-reversible tetrameric avidin sites that may have been present. The resin was then regenerated by washing with 8 column volumes of 10 % acetic acid, followed by washing with 8 column volumes of sodium phosphate buffer 2. When the pH reached 6.8, the wash flow was stopped for 30 min to allow time for avidin refolding. This procedure was always performed following a purification procedure to regenerate the column for future uses. Prior to purification, the column was equilibrated with a buffer compatible to the biotinylated protein to be

purified. Cell lysate volumes, binding times and washing volumes utilized for each purification procedure are specified in each appropriate chapter.

CHAPTER 3

BACTERIAL EXPRESSION OF IN VIVO BIOTINYLATED AEQUORIN FOR DIRECT APPLICATION TO BIOLUMINOMETRIC HYBRIDIZATION ASSAYS

ABSTRACT

A plasmid was constructed, suitable for bacterial expression of the *in vivo* biotinylated photoprotein aequorin. The biotin tag facilitates (i) the purification of aequorin from the crude cell extract and (ii) the direct complexation of aequorin with streptavidin for utilization as a reporter molecule in the development of highly sensitive hybridization assays, thereby avoiding the need for chemical crosslinking. The plasmid contains a biotin-acceptor coding sequence fused in-frame with the amino terminus of the apoaequorin gene. The *birA* gene, encoding biotin protein ligase (BPL), was amplified from *E. coli* genomic DNA and inserted downstream of the apoaequorin sequence. Thus, the plasmid overexpresses both a biotin acceptor peptide-apoaequorin fusion protein and the BPL, which biotinylates post-translationally the acceptor domain at a unique position. Functional aequorin is generated by incubating the crude cell lysate with coelenterazine, followed by isolation using a monomeric avidin column that allows elution with free biotin under non-denaturing conditions. The biotinylated aequorin is complexed with streptavidin and used as a reporter molecule in a hybridization assay. The assay entails immobilization of an oligonucleotide probe on microtiter wells via a digoxigenin/antidigoxigenin interaction followed by hybridization with a denatured DNA target labeled with biotin through PCR. The streptavidin-biotinylated aequorin complex is used to quantify the hybrids. Luminescence is measured in the presence of excess Ca^{2+} . The linearity of the hybridization assay extends from 80 amol of target DNA per well (with a S/B ratio of 2.1) up to 40 fmol per well. CVs are approximately 6%. A typical yield of *in vivo* biotinylated aequorin is 1.0-1.2 mg per liter of culture, which is a sufficient amount for 300 000 hybridization assays.

3.1 INTRODUCTION

Biotin is an essential cofactor of ubiquitous metabolic enzymes, the biotin carboxylases and decarboxylases (Samols *et al.*, 1988). In *E. coli*, the only biotinylated protein is the biotin carboxy carrier protein (BCCP), one of the three subunits of acetyl-CoA carboxylase, the enzyme responsible for catalyzing the carboxylation of acetyl-CoA to malonyl-CoA, the first committed step in fatty acid synthesis. The covalent attachment of biotin to BCCP is a post-translational modification catalyzed by biotin protein ligase (BPL), a 35.5 kDa monomeric enzyme encoded by the *birA* gene (Chapman-Smith *et al.*, 1999). The *in vivo* biotinylation reaction is a two-step process. First, biotin is activated by reaction with ATP to produce biotin-5'-AMP and pyrophosphate. Then, an amide linkage is formed between the carboxy group of biotin and the N⁶-amino group of a unique lysine residue in BCCP with a concomitant release of AMP (Knowles, 1989; Xu *et al.*, 1994).

The biotin-acceptor domain of a biotin-requiring enzyme is a highly conserved sequence, and BPL recognizes an Ala-Met-Lys-Met motif within the context of a folded protein. As a consequence, *in vivo* biotinylation occurs even when the ligase and its target protein are derived from different species (Cronan, 1990; Li *et al.*, 1992). The exceptional specificity of the *in vivo* biotinylation reaction allows for the exploitation of avidin-biotin technology. The high affinity ($K_d = 10^{-15}$ M) and specificity of the interaction between avidin and biotin forms the basis of purification and detection techniques (Diamandis *et al.*, 1991). In this context, biotinylated molecules are purified by affinity chromatography using columns with immobilized avidin. Alternatively, biotinylated molecules can be detected by reaction with conjugates of avidin containing a variety of labels including fluorophores, enzymes and nanoparticles.

Two remarkable structural characteristics of the biotin-acceptor domain greatly facilitate the use of the *in vivo* biotinylation reaction as an analytical tool. Firstly, studies of the three dimensional structure of the biotin acceptor domain, carried out both by x-ray crystallography and NMR (Athappilly *et al.*, 1995; Yao *et al.*, 1997; Roberts *et al.*, 1999; Waldrop *et al.*, 1994; Wilson *et al.*, 1992), have revealed that the biotinylation site is located near the tip of a tight beta-turn whose physiological role is to act as a swinging arm in the mechanism of acetyl-CoA carboxylase. As a consequence, biotin is rendered easily accessible for interaction with avidin. Secondly, the biotinylation site is located

away from both the N- and C- termini, thereby facilitating the fusion of the biotin-acceptor domain with other proteins.

Aequorin is a Ca^{2+} -dependent photoprotein consisting of the polypeptide apoaequorin, coelenterazine, and oxygen, which is attached to coelenterazine as a peroxide. When Ca^{2+} binds aequorin, it induces a conformational change that triggers the oxidation of coelenterazine by the bound oxygen to produce coelenteramide, CO_2 and light at 470 nm (Shimomura *et al.*, 1978; Head *et al.*, 2000). The apoaequorin cDNA has been cloned and sequenced (Inouye *et al.*, 1985; Prasher *et al.*, 1985). Aequorin is an excellent reporter molecule since it can be detected at the attomole level in the presence of excess Ca^{2+} (Lewis *et al.*, 1998). Chemical conjugates of recombinant aequorin have been applied to immunoassays (Sgoutas *et al.*, 1995; Deo *et al.*, 2001), enzyme assays (Deo *et al.*, 2000) and hybridization assays (Verhaegen *et al.*, 1998; Actor *et al.*, 2000).

In this work, plasmids were constructed for bacterial expression of the *in vivo* biotinylated photoprotein aequorin. The biotin tag facilitates (i) the purification of aequorin from the crude cell extract and (ii) the direct complexation of aequorin with streptavidin for utilization as a reporter molecule in the development of highly sensitive hybridization assays, thereby avoiding the need for chemical crosslinking.

3.2 EXPERIMENTAL SECTION

3.2.1 Polymerase Chain Reaction

3.2.1.1 Amplification of Apoaequorin-Coding DNA

Plasmid pSVAEQN (Molecular Probes) was used as a source of the apoaequorin gene. A 590 bp DNA fragment, containing the apoaequorin-coding sequence flanked by the *Bgl* II and *Kpn* I restriction sites, was created through PCR using the oligonucleotides (c) and (d) as upstream and downstream primers, respectively. PCR was carried out with *Pfu* Turbo DNA polymerase as described in section 2.6.1.1 of Chapter 2.

3.2.1.2 Amplification of *birA* Gene

The *birA* gene was amplified from *E. coli* genomic DNA by PCR (35 cycles with an annealing temperature of 55°C) using oligonucleotides (a) and (b) as upstream and

downstream primers, respectively. The primers were based on sequence 9667-10632 from the *E. coli* K12 genome. The *birA* gene is shown in Appendix B (GenBank accession number AE000471). PCR was carried out with *Pfu* Turbo DNA polymerase as described in section 2.6.1.1 of Chapter 2.

3.2.2 Extraction of *E. coli* Genomic DNA

Genomic DNA was isolated based on a procedure from Pospiech (Pospiech *et al.*, 1995). Briefly, *E. coli* JM 109 cells were grown O/N at 37°C in LB broth (50-mL culture). The cells were harvested by centrifugation (2000 rpm, Beckman J-6B swinging bucket centrifuge) for 30 min at 4°C and resuspended in 5 mL of SET buffer with lysozyme added to a final concentration of 1 g/L. Following a 1-hour incubation at 37°C, SDS and proteinase K were added to final concentrations of 10 g/L and 0.5 g/L, respectively. The mixture was incubated at 55°C for 2 hrs with occasional shaking, followed by the addition of 1/3 volumes of 5 mol/L NaCl (1.9 mL) and 1 volume of CHCl₃ (7.6 mL) and incubated for 30 min at RT with frequent inversion. The phases were separated by centrifugation (3000 g, 15 min) and the genomic DNA was precipitated by adding 1 volume of isopropanol to the aqueous layer. The DNA strands were resuspended in TE buffer, followed by ethanol precipitation.

3.2.3 Plasmid Construction

3.2.3.1 Construction of Plasmid pBAeq

Apoaequorin PCR products were concentrated with Microcon-30 columns and digested with 10 units *Bgl* II for 90 min at 37°C. The reaction was terminated by adding 20 mmol/L EDTA. The PinPoint Xa-1 vector (3 µg) was digested in a similar manner with 20 units *Bgl* II. After changing the buffer with Spin-pure G-25 columns, the apoaequorin gene and the vector were both digested for 90 min at 37°C with 10 and 20 units *Kpn* I, respectively. The enzyme was heat inactivated at 80°C for 20 min. The digested products were separated by agarose gel (1.2%) electrophoresis, and the appropriate fragments were purified from the gel (using the Qiaex II purification kit) and quantified fluorometrically with PicoGreen. The doubly digested purified apoaequorin and the PinPoint Xa-1 fragments were ligated (in a 25:1 molar ratio) for 12 hours at 10°C

with 11 units of T4 DNA ligase (in 1x OPA buffer), to create the plasmid pBAeq. The enzyme was then heat inactivated at 65°C for 10 min.

3.2.3.2 Construction of Plasmid pBAeq-birA

BirA amplification products were concentrated with Microcon-30 columns and digested with 20 units of *Not* I (90 min at 37°C) followed by heat inactivation of the enzyme (65°C for 20 min). The digested *birA* product was then purified from a 1% agarose gel using the Qiaex II kit. The pBAeq plasmid, isolated from a 3-mL overnight culture using the alkali lysis method (Sambrook *et al.*, 1989) followed by phenol:chloroform extraction, was linearized with 30 units of *Not* I as described above. The buffer was changed using Spin-pure G-25 columns and dephosphorylation was carried out for 30 min at 37°C with 0.05 units of alkaline phosphatase in 1x OPA buffer. The enzyme was heat inactivated at 85°C for 15 min. Both digests were quantified fluorometrically and then ligation was carried out as described above with a 15:1 molar ratio of *birA* insert to pBAeq vector. The buffer was again changed and a second digestion with 20 units of *Sma* I carried out for 2 hrs at 30°C followed by heat inactivation at 65°C for 20 min. The small fragments resulting from *Sma* I digestion were removed using Microcon-30 spin columns and recircularization was carried out for 12 hrs at 10°C with 11 units T4 DNA ligase in 1x OPA buffer, followed by enzyme inactivation at 65°C for 10 min. This created the plasmid pBAeq-birA of 4878 bp in size.

3.2.4 Selection of Recombinant Clones

Following insertion of the apoaequorin and *birA* genes into the PinPoint Xa-1 vector, competent *E. coli* cells were transformed with the recombinant plasmids (pBAeq or pBAeq-birA). Several single colonies were then chosen for plasmid minipreparations to isolate the recombinant DNA from the transformed *E. coli*. Restriction enzyme digestion was then carried out to ensure the correct clone was chosen for overexpression of biotinylated aequorin. Briefly, 5 µL of isolated plasmid were digested with 10-20 units of *Not* I, *Bgl* II and *Kpn* I (changing buffers as needed) for 2 hrs at 37°C in total volumes of 20 µL. Five µL of the resulting plasmid digest was loaded on a 1.2% agarose gel along with DNA markers (1 kb ladder) to ensure the presence and correct size of the inserts.

Details of bacterial culturing, transformation, plasmid isolation and agarose gel electrophoresis are given in section 2.6 of Chapter 2.

3.2.5 Bacterial Expression and Purification of *In Vivo* Biotinylated Aequorin

3.2.5.1 Bacterial Culture and Overexpression

Transformed JM 109 *E. coli* colonies, positive for either pBAeq or pBAeq-birA were inoculated into LB broth (3 mL) containing 0.1 g/L ampicillin and supplemented with 2 mg/L biotin. These seed cultures were grown O/N at 30°C, diluted 100 fold and again grown at 30°C until an absorbance of 0.8-0.9 (600 nm) was reached. At this point, protein synthesis was induced with 1 mmol/L IPTG for 3 hrs. Cells were harvested by centrifugation at 2000 rpm for 25 min at 4°C and pellets stored at -20°C.

3.2.5.2 Bacterial Cell Lysis

Frozen cell pellets washed twice in TBS buffer to remove excess biotin, were resuspended in cell lysis buffer, 3 mL buffer per gram (wet weight) of pellet. Lysozyme was added to a final concentration of 0.27 g/L and incubated at 4°C for 20 min with gentle shaking. Deoxycholic acid (4 mg/g cell pellet) was added and stirred at 37°C until viscous. The lysate was then supplemented with 6 mmol/L MgCl₂ and DNase I was added to a final concentration of 6.7 mg/L. Incubation at RT was carried out until the lysate was no longer viscous and the cellular debris pelleted by centrifugation (12 000 rpm, 15 min, 4°C).

3.2.5.3 Regeneration of Apoequorin

To the crude extract containing biotinylated apoequorin, 20 μmol/L coelenterazine and 5 mmol/L DTT (final concentrations) were immediately added. The generated biotinylated aequorin (BAeq) was then stored at -80°C until purification. Prior to purification, the crude cell extract containing BAeq was diluted 3 times in buffer B and incubated at -20°C for 18-24 hrs to ensure that aequorin was fully active and a plateau of luminescence was reached.

3.2.6 Purification of *In Vivo* Biotinylated Aequorin

The monomeric avidin resin was regenerated as described in section 2.6.10 of Chapter 2. Prior to use, the resin (typically 1 mL slurry per gram cell pellet) was equilibrated with buffer B. The crude cell extract was then applied directly to the slurry and binding was carried out for 60 min at RT with gentle shaking. The column was then washed with 10 mL of buffer B, followed by elution with buffer C. 1-mL fractions were collected and the aequorin was measured by diluting each fraction 100 fold in buffer B, pipetting 50 μ L into each well and dispensing 50 μ L of triggering solution from the luminometer. The luminescence was integrated for 3 sec. Fractions giving high luminescence signals were pooled, concentrated (by ultrafiltration using Centriplus YM-10) and purified twice from excess biotin by size exclusion chromatography with NAP-5 columns. The mass of purified biotinylated aequorin was determined using the Bradford assay as described in section 2.6.5 of Chapter 2 and the protein was stored immediately in aliquots at -80°C .

3.2.7 Design of Bioluminometric Hybridization Assay Using *In Vivo* Biotinylated Aequorin as a Reporter Molecule

3.2.7.1 Creation of Target DNA

The target DNA was a 233 bp fragment generated by RT-PCR of prostate-specific antigen mRNA as previously described (Verhaegen *et al.*, 1998). This product served as the PCR starting template for further creation of target DNA (Appendix C). The target was biotinylated, through PCR using the upstream primer (i) labeled with biotin at the 5' end, the downstream primer (j) and approximately 8×10^6 molecules of DNA template. PCR was carried out as described in section 2.6.1.1 using *Pfu* Turbo DNA polymerase under the conditions specified. Quantification of the target DNA was carried out fluorometrically with PicoGreen (section 2.6.2).

3.2.7.2 Labeling of Oligonucleotide Probe

The probe was tailed enzymically with DIG-dUTP using terminal deoxynucleotidyl transferase (TdT). Tailing reactions were carried out in a total volume of 20 μ L consisting of 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6),

0.25 g/L BSA, 5 mmol/L CoCl₂, 50 μmol/L DIG-dUTP, 0.5 mmol/L dATP, 25 units TdT and 100 pmol probe (k). The reactions were carried out for 60 min at 37°C. The tailed probe was purified twice from excess DIG-dUTP using NAP-5 columns and then concentrated with the Eppendorf Vacufuge. The recovery of the probe was assumed to be approximately 100% with a final concentration of 1.3 μmol/L. The tailed probe was aliquoted and stored at -20°C until future use.

3.2.7.3 Optimization of Streptavidin:Biotinylated Aequorin Complexes

Biotinylated BSA was prepared by reaction of 10 mg BSA with 1 mg NHS-LC-biotin in 1 mL PBS buffer for 2 hours, followed by the addition of 0.1% NaN₃. Opaque polystyrene microtiter wells were coated overnight at RT with 50 μL of biotinylated BSA diluted in PBS buffer to a final concentration of 5 mg/L. Likewise, wells were coated overnight at RT with 50 μL of biotinylated monoclonal antibody at a final concentration of 5 mg/L.

A constant amount of purified BAeq (50 nmol/L) was incubated with varying concentrations of streptavidin (SA) (5 nmol/L to 100 nmol/L) both diluted in buffer H for 30 min at RT. The SA-BAeq complex was then applied to wells coated with biotinylated protein that had been washed 3 times with wash solution. Following a 30-min incubation with shaking at RT, the wells were again washed 3 times and luminescence measured by injection of 50 μL triggering solution.

3.2.7.4 Preparation of Anti-DIG Coated Wells

Opaque polystyrene microtiter wells were coated, by physical adsorption, with 50 μL of 5 mg/L anti-DIG antibody diluted in 0.1 mol/L carbonate buffer, pH 9.6. The wells were covered with transparent tape and incubated O/N at RT. Wells were always coated the day prior to assaying and used immediately.

3.2.7.5 Microtiter Well-Based Hybridization Assay

Microtiter wells coated with anti-DIG antibody were washed 3 times with wash solution and 50 μL of 4 nmol/L DIG-tailed probe diluted in buffer H were added to each well. The probe was allowed to bind to the antibody for 60 min at RT, followed by

washing. Biotinylated target DNA, diluted in buffer H, was denatured at 95°C for 10 min and immediately placed on ice. A 50- μ L aliquot was added to each well and hybridization carried out at 42°C for 30 min followed by washing. The SA:BAeq complex was prepared by mixing, in a 1:2 molar ratio, 25 nmol/L SA with 50 nmol/L BAeq (both diluted in buffer H) and incubating for 30 min at RT. The complex was diluted 25 times (1 nmol/L SA:2 nmol/L BAeq) and a 50- μ L aliquot added to each well. After a 15-min incubation, the wells were washed and 50 μ L of triggering solution were dispensed into each well. The luminescence was integrated over a 3 sec period.

3.3 RESULTS AND DISCUSSION

3.3.1 Construction of Plasmids pBAeq and pBAeq-birA

The construction of plasmids for bacterial expression of *in vivo* biotinylated apoaequorin is illustrated in Figure 3.1. The apoaequorin gene was amplified using plasmid pSVAEQN as a template. Primers were designed to introduce a *Bgl* II site upstream of the gene as well as a *Kpn* I site and a stop codon downstream of the gene. The Pinpoint Xa-1 vector was used as a source of the sequence encoding the biotin-acceptor domain (from *Propionibacterium shermanii* transcarboxylase) positioned downstream of the *tac* promoter and a ribosome binding site. The structure of the biotin domain of *P. shermanii* transcarboxylase is very similar to *E. coli* acetyl-CoA carboxylase (Reddy *et al.*, 1998). The apoaequorin gene was inserted downstream of the biotin-acceptor coding sequence between the *Bgl* II and *Kpn* I sites to generate plasmid pBAeq (3899 bp). Thus, plasmid pBAeq allows expression of a biotin-acceptor domain fused to the amino terminus of apoaequorin.

The biotin protein ligase-coding gene (*birA*) was amplified from *E. coli* genomic DNA through PCR. Primers were designed to introduce a *Sma* I site and a ribosome binding site upstream of *birA* as well as a stop codon and a *Not* I site downstream of the gene. PCR generated a 1004 bp fragment. The amplification product was inserted downstream of the apoaequorin gene in plasmid pBAeq between the *Sma* I and *Not* I sites to create a new plasmid pBAeq-birA (4878 bp). Following plasmid isolation, pBAeq-birA was digested to confirm that both apoaequorin and *birA* genes were present and of

FIGURE 3.1
CONSTRUCTION OF PLASMIDS

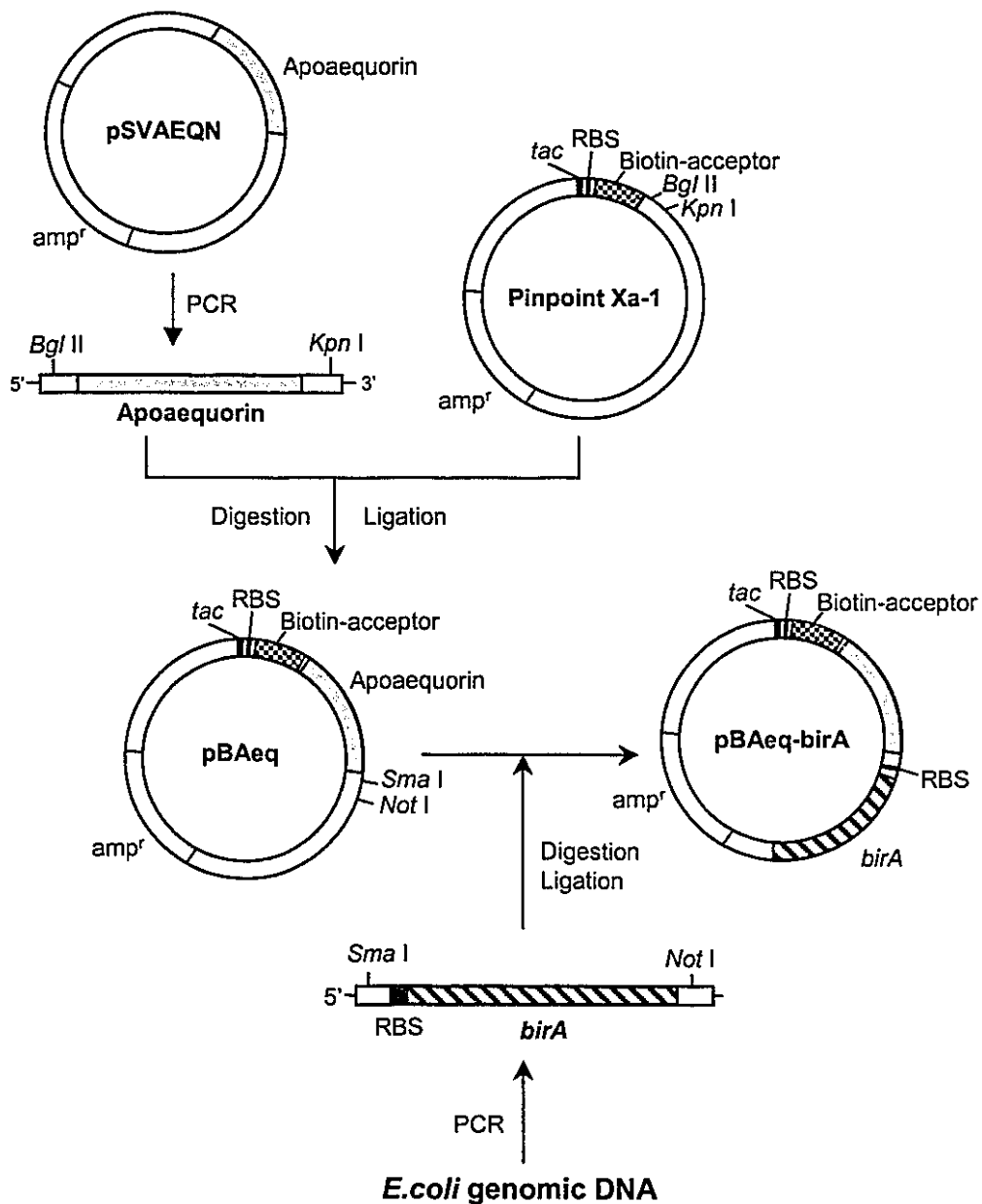


Figure 3.1. Schematic presentation of the construction of plasmids pBAeq and pBAeq-birA, for expression of in vivo biotinylated apoaequorin. Shown are relative locations of the *tac* promoter, the ribosome binding site (RBS), the restriction enzyme sites and the gene inserts. *birA* = the gene encoding the biotin protein ligase in *E. coli*; *amp^r* = the β -lactamase gene conferring ampicillin resistance.

the expected size. A 1.5% agarose gel electropherogram is shown in Figure 3.2. Thus, plasmid pBAeq-birA allows the expression of both the biotin acceptor domain-apoaequorin fusion protein and the biotin protein ligase (BPL). Both genes are under the transcriptional control of the *tac* promoter.

3.3.2 Expression and Purification of *In Vivo* Biotinylated Aequorin

The resulting expression vectors were introduced into competent JM 109 *E.coli* cells and protein synthesis was induced with IPTG. Cells were then lysed and apoaequorin in the crude cell extract was converted to active aequorin by incubation for several hours with coelenterazine in the presence of DTT. The *in vivo* BAeq was purified by affinity chromatography using monomeric avidin resin. The low dissociation constant of native tetrameric avidin renders the binding of biotinylated molecules practically irreversible. In contrast, the much lower affinity of monomeric avidin ($K_d=10^{-7}$ M) allows both the efficient binding of biotinylated molecules and subsequent elution with free biotin under non-denaturing conditions (Kohanski *et al.*, 1990).

Figure 3.3 shows a typical profile of absorbance (280 nm) and luminescence obtained during washing of the monomeric avidin resin and elution of the BAeq. The absorbance of the first fractions is high whereas the luminescence is low as soluble proteins from the cell extract are washed through the column. The column was washed until protein was undetectable. Biotinylated aequorin was then eluted with 5 mmol/L biotin. More than 90% of biotinylated aequorin was eluted in the first 3 mL of elution buffer C as indicated by the luminescence of the pooled fractions.

Initial experiments for expression of *in vivo* biotinylated aequorin were carried out using the pBAeq vector (Figure 3.1). However, only 12% of the aequorin activity applied to the resin was able to bind the avidin. The majority of aequorin activity was lost in the flow-through fraction and subsequent washes. This was attributed to the fact that the biotin acceptor domain-apoaequorin fusion protein was not biotinylated quantitatively *in vivo*. The yield of biotinylation was not improved by increasing the concentration of biotin up to 200 mg/L. It has been observed that low levels of biotinylated fusion protein may result from limiting intracellular levels of biotin protein ligase (Smith *et al.*, 1998). High expression levels of the fusion protein may also result in deficient biotinylation of

FIGURE 3.2
AGAROSE GEL ELECTROPHORETIC ANALYSIS

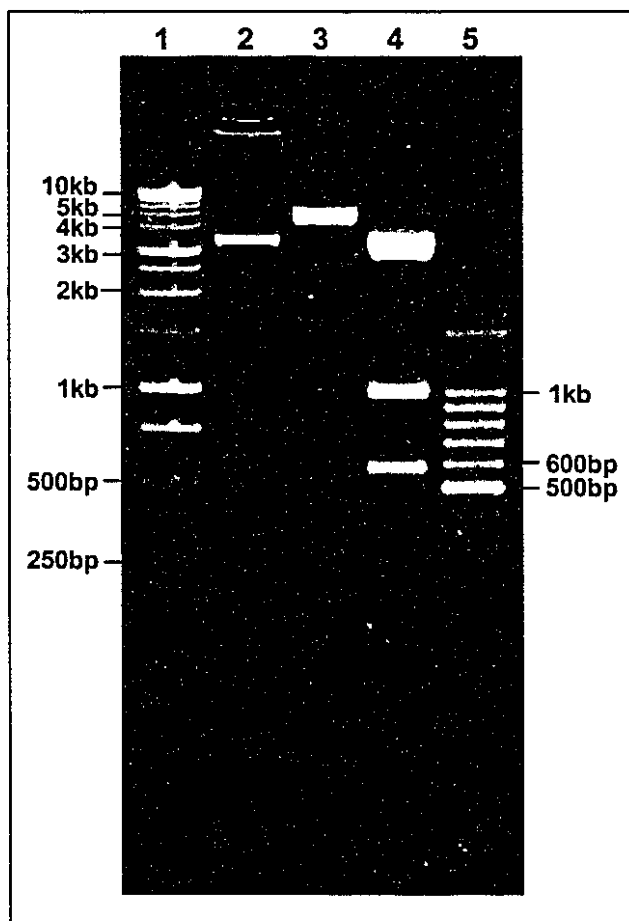


Figure 3.2. Electrophoretic analysis of plasmid pBAeq-birA. Ethidium bromide stained 1.5 % agarose gel. *Lane 1:* Molecular weight ladder. *Lane 2:* 0.25 μ g of PinPoint Xa-I vector (3331 bp) linearized with *Bgl* II. *Lane 3:* Recombinant plasmid pBAeq-birA (4878 bp) linearized with *Kpn* I. *Lane 4:* Plasmid pBAeq-birA digested with *Bgl* II, *Kpn* I, and *Not* I to excise both the apoaequorin and *birA* genes. *Lane 5:* A 100-bp DNA ladder.

FIGURE 3.3
BIOTINYLATED AEQUORIN ELUTION PROFILE

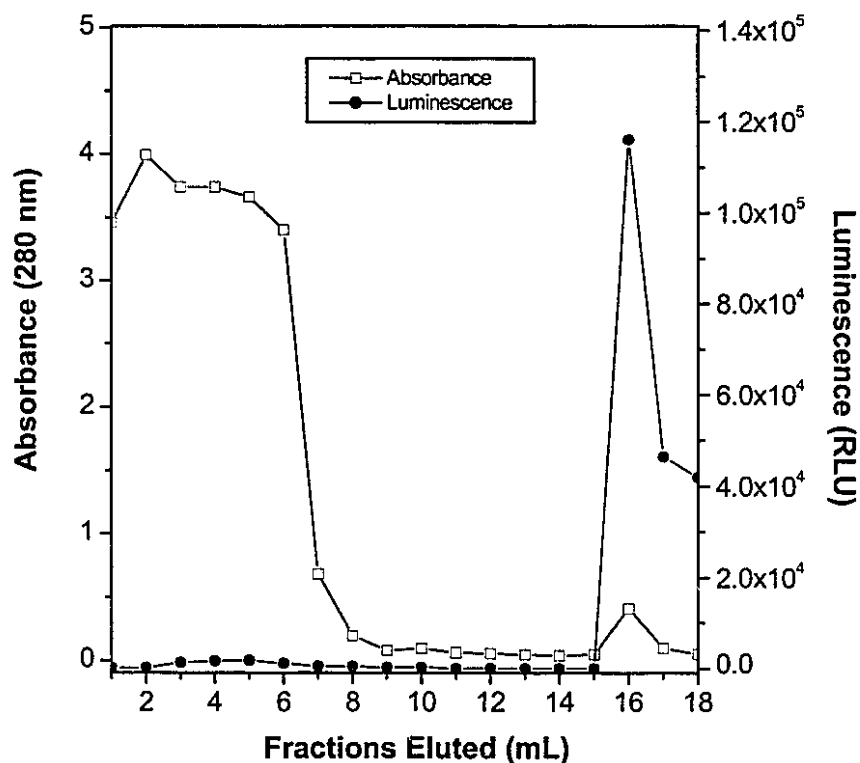


Figure 3.3. Typical profile of absorbance and luminescence obtained during the purification of *in vivo* BAeq from a crude cell extract using monomeric avidin resin. A 0.5-g cell pellet was used 1-mL fractions were collected and the absorbance was measured at 280 nm. Aequorin activity of each fraction was monitored by diluting 100-fold in buffer B, and adding 50 μ L into a well. Luminescence was measured following injection of 50 μ L of triggering solution. After 15 mL, the bound BAeq was displaced from the column by the addition of free biotin.

the endogenous BCCP subunit of acetyl-CoA carboxylase in *E. coli*, resulting in growth inhibition (Cronan Jr, 1990).

Since the *birA* gene encoding the biotin protein ligase has been cloned (Barker *et al.*, 1981a; Howard *et al.*, 1985) and the sequence is readily available, insertion of the *birA* gene into the pBAeq plasmid, downstream to the apoaequorin sequence was carried out to allow overexpression of BPL (vector pBAeq-*birA*). This would overcome any inhibitory growth effects caused by overexpression of the fusion protein as well as increase the yield of *in vivo* biotinylation of the fusion protein. Indeed, the use of vector pBAeq-*birA* allowed 85-95% of aequorin activity to be captured and eluted from the monomeric avidin column, demonstrating the efficiency of *in vivo* biotinylation.

SDS-PAGE analysis was performed to verify the size and the purity of BAeq (Figure 3.4). Following purification, a single band was observed at 36 kDa, corresponding to the sum of the 20.8 kDa apoaequorin and 15.3 kDa biotin-acceptor domain. *E. coli* contains one biotinylated protein, the BCCP subunit of acetyl-CoA carboxylase (Fall, 1979). In principle, BCCP binds to the avidin column and is co-eluted with biotinylated aequorin. However, a band corresponding to BCCP was not observed in SDS-PAGE, indicating that BCCP was present in much lower concentration than aequorin. Bradford assay of the purified protein gave approximately 0.2 mg of biotinylated aequorin per gram cell pellet, i.e., a yield of 1.0-1.2 mg per liter of culture.

It is known, that foreign proteins are often packaged in the form of cytoplasmic inclusion body granules when plasmid-encoded genes are directed to be overexpressed at high rates (Georgiou *et al.*, 1996; Strandberg *et al.*, 1991; Marston, 1986). In the present recombinant system, both biotinylated aequorin and BPL were designed to be overexpressed, almost guaranteeing some inclusion body formation. Due to the concerns with obtaining a soluble fully active aequorin, correctly refolded and regenerated after inclusion body solubilization, only the soluble fraction of biotinylated aequorin was purified.

In order to test the detectability of *in vivo* biotinylated aequorin, serial dilutions of the purified protein (from a stock whose protein concentration was determined by Bradford assay) were prepared in buffer E, 50 μ L pipetted into the well and the luminescence measured after injection of triggering solution. In Figure 3.5, the

FIGURE 3.4
SDS-PAGE ANALYSIS

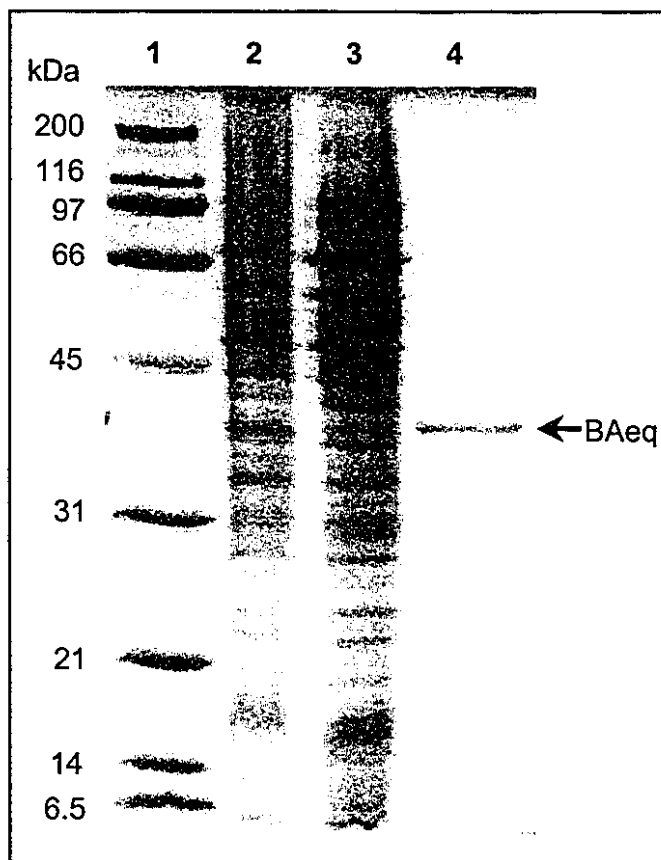


Figure 3.4. A 12% SDS-PAGE gel depicting; *Lane 1*: Broad range molecular weight markers. *Lane 2*: 5 μ L of *E. coli* cell pellet grown under conditions specified in the experimental section. *Lane 3*: 10 μ L crude cell lysate regenerated with coelenterazine prior to purification with monomeric avidin resin. *Lane 4*: 1.6 μ g of purified, concentrated *in vivo* BAeq. All procedures to obtain samples are described in the experimental section.

FIGURE 3.5
LUMINESCENCE AS A FUNCTION OF
BIOTINYLATED AEQUORIN

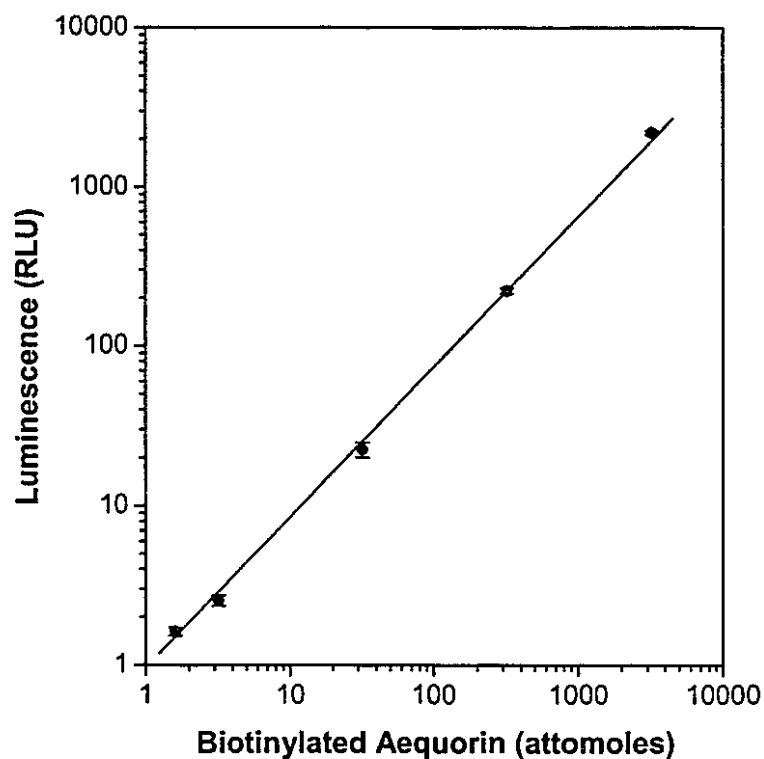


Figure 3.5. Luminescence as a function of the attomoles of *in vivo* biotinylated aequorin. Serial dilutions of biotinylated aequorin in buffer D were pipetted into microtiter wells and the luminescence was integrated for 3 sec following addition of Ca^{2+} triggering solution. Error bars correspond to plus/minus one standard deviation ($n=5$).

luminescence was plotted as a function of the mass of BAeq. As low as 1.6 attomoles could be detected with a signal-to-background ratio of 4.4. The linearity extends over three orders of magnitude. Moreover, the *in vivo* BAeq was compared to a commercial biotinylated aequorin (Molecular Probes) and found to have identical performance.

3.3.3 *In Vivo* Biotinylated Aequorin as a Label in Bioluminometric Hybridization Assays

Following purification from the crude cell extract, the elution fractions of BAeq were pooled and excess biotin was removed by size exclusion chromatography. This step was necessary prior to complexation of biotinylated aequorin with streptavidin. Optimization of biotinylated aequorin-to-streptavidin molar ratio was then carried out by incubating a constant concentration of biotinylated aequorin (50 nmol/L) with varying concentrations of streptavidin and applying the complex (SA-BAeq) to the wells. Figure 3.6 presents optimization data using (A) the DNA hybridization assay configuration described in experimental section 3.2.7.5; (B) wells coated by physical adsorption, with 50 μ L of 5 mg/L biotinylated monoclonal antibody and (C) wells coated with 50 μ L of 5 mg/L biotinylated BSA (described in experimental section 3.2.7.3). It was observed that, regardless of the protein immobilized on the wells, the optimum concentration of streptavidin is 25 nmol/L, which corresponds to a 1:2 molar ratio of streptavidin to biotinylated aequorin in the complexation reaction. In the case of excess BAeq, the four biotin binding sites on SA become fully occupied and the complex is no longer able to bind the immobilized biotinylated molecules. In the case of streptavidin excess, the free streptavidin competes with SA-BAeq complexes for binding to immobilized biotinylated molecules.

A bioluminometric hybridization assay was developed as shown in Figure 3.7, in which a biotinylated and denatured target DNA hybridizes to a DIG-labeled oligonucleotide probe bound to anti-DIG antibody-coated wells. The hybrids were quantified by addition of the SA:BAeq complex. In order to assess the performance of the hybridization assay, various dilutions of biotinylated target DNA were prepared in buffer H and analyzed as described in the experimental section. The luminescence (corrected for the background) was plotted as a function of target DNA concentration as shown in

FIGURE 3.6
OPTIMIZATION OF STREPTAVIDIN TO BIOTINYLATED AEQUORIN

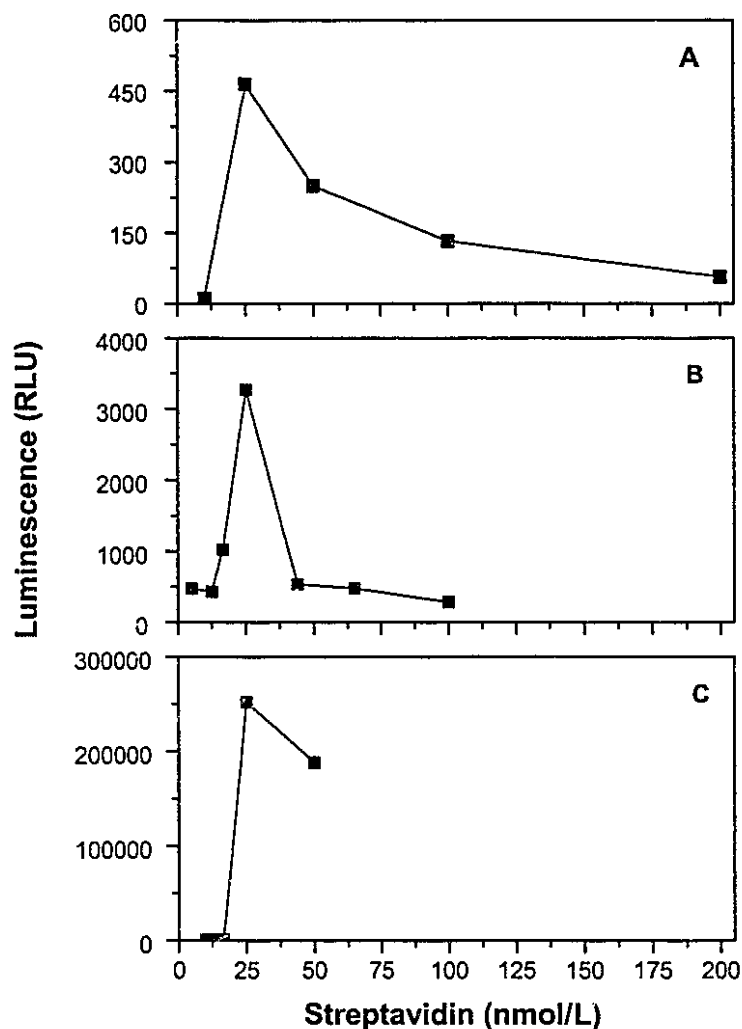


Figure 3.6 Complexation of *in vivo* biotinylated aequorin with streptavidin. Complexes were prepared by mixing 50 nmol/L BAeq with various concentrations of SA. The complexes were then tested using (A) the DNA hybridization assay configuration described in the experimental section; (B) wells coated by physical adsorption, with 50 μ L of 5 mg/L biotinylated monoclonal antibody and (C) wells coated with 50 μ L of 5 mg/L biotinylated BSA. Each experimental point represents the mean value of two assays.

FIGURE 3.7
SCHEMATIC PRESENTATION OF HYBRIDIZATION ASSAY

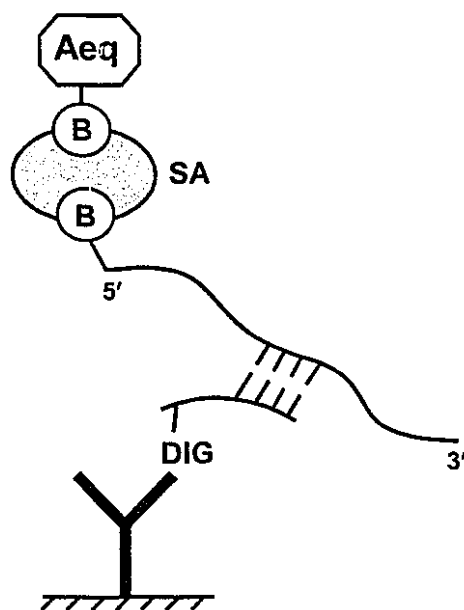


Figure 3.7. Schematic drawing of the principle of the DNA hybridization assay using recombinant *in vivo* biotinylated aequorin as a reporter molecule. Biotinylated target DNA is denatured and hybridized to a specific probe that is immobilized on microtiter wells through a DIG/anti-DIG interaction. The hybrids are determined by reacting with a SA-BAeq complex. The bound aequorin is measured by its characteristic bioluminescent reaction. DIG=digoxigenin, B=biotin, SA=streptavidin, Aeq=aequorin.

Figure 3.8. The background was defined as the luminescence obtained when no target DNA was present in the well. The linearity of the assay extends from 1.6 to 800 pmol/L. The signal-to-background ratio at 1.6 pmol/L (80 attomoles/well) was 2.2. Reproducibility studies were performed by analyzing target DNA concentrations at the low end (1.6 pmol/L), mid-range (25 pmol/L) and high end (800 pmol/L) of the assay. Coefficients of variation (CVs) were 4.7, 7.7 and 4.6%, respectively (n=5).

3.3.4 Conclusions

It was calculated that 1 L of bacterial culture provides enough biotinylated aequorin for 300 000 hybridization assays. The entire process, including culturing pBAeq-birA transformed cells, extraction of total soluble protein, and regeneration and purification of fully active biotinylated aequorin was completed in less than 2 days. The hybridization assay on wells containing immobilized probe was complete in less than 60 min including the complexation reaction. The optimized biotinylated aequorin-streptavidin complex serves as a universal reporter and may also be used as a detection reagent in numerous applications, including immunoassays when combined with biotinylated antibodies.

FIGURE 3.8
LUMINESCENCE AS A FUNCTION OF
TARGET DNA CONCENTRATION

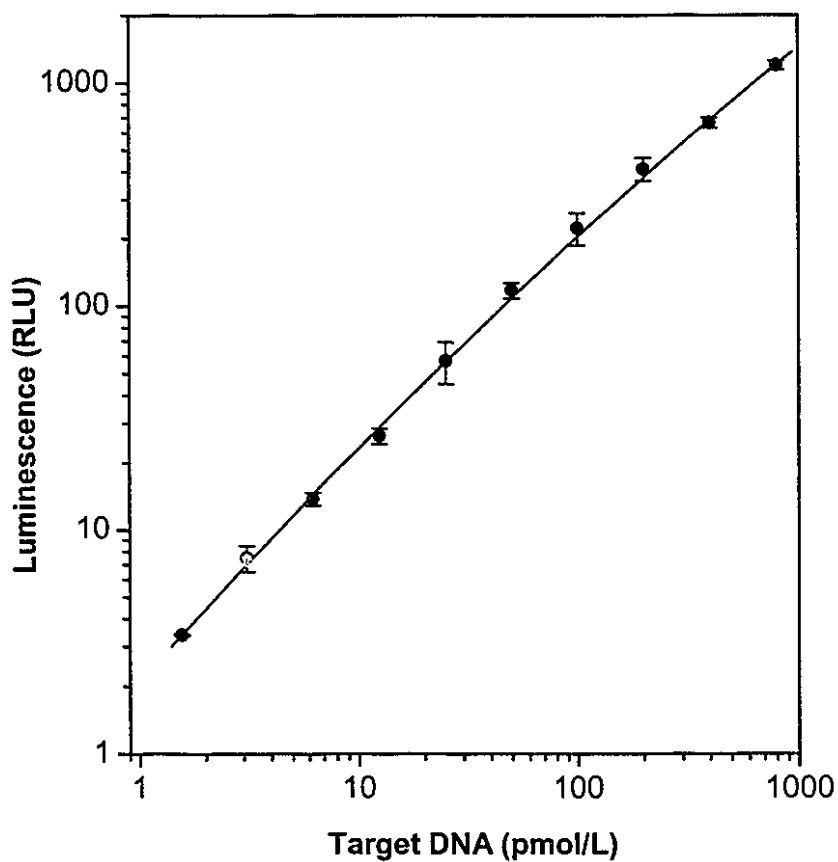


Figure 3.8. Luminescence as a function of the target DNA concentration. The hybridization assay was performed as described in the experimental section using *in vivo* biotinylated aequorin as a reporter molecule. The error bars correspond to plus/minus one standard deviation (n=5).

CHAPTER 4:
DIRECTED MOLECULAR EVOLUTION OF THE
AEQUORIN PHOTOPROTEIN

ABSTRACT

The photoprotein aequorin from the jellyfish *Aequorea victoria* has analytical applications as a reporter molecule in sensitive bioluminometric assay development as well as for intracellular Ca^{2+} measurement. Although aequorin can be detected at the attomole level, the development of more sensitive detection labels is always desirable. This work describes the production of novel aequorin photoproteins through recombinant DNA technology. The goal was to create mutant aequorin proteins with improved luminescent activity over the native aequorin complex through directed molecular evolution of the apoaequorin gene. Random mutagenesis was accomplished through DNA shuffling of the apoaequorin gene, which included repeated rounds of DNA digestion and re-assembly, cloning and expression of mutant proteins, followed by screening and selection of regenerated mutants for higher luminescent activity. The ratio of luminescent signal to the mass of total soluble protein was used as criterion for selection of mutants. Five rounds of shuffling were carried out with increasing selection criterion at each round. Initial data indicated three mutants exhibited 20-60 fold higher luminescent activities in crude samples. *In vivo* biotinylated mutant proteins were purified by avidin affinity chromatography for direct comparisons to native aequorin. Comparisons utilizing purified proteins indicated the mutant aequorins had identical luminescent activity to the native protein, and selection possibly favoured mutations allowing efficient folding of the overexpressed protein during extraction rather than higher luminescence quantum yield. It is known that heterologous proteins overexpressed in *E. coli* often form insoluble and inactive aggregates (inclusion bodies) in the cytoplasm due to misfolding. The observed improvement of the mutants was possibly attributed to better folding and therefore increased solubility of the expressed protein.

4.1 INTRODUCTION

Aequorin is a Ca^{2+} -dependent photoprotein from the hydromedusan jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). The 22 kDa aequorin complex consists of an apoaequorin polypeptide and the prosthetic chromophore coelenterazine coupled to a hydroperoxide at the C2 position (Teranishi *et al.*, 1995; Head *et al.*, 2000). Upon binding Ca^{2+} , aequorin undergoes a conformational change and coelenterazine is oxidized to coelenteramide with a simultaneous emission of blue light (469 nm) and the release of CO_2 (Shimomura *et al.*, 1978; 1962). Characteristic light emission is a direct result of the excited state of coelenteramide falling to ground state (Shimomura *et al.*, 1973).

Although the light emission from aequorin occurs as a single-turnover event, the active aequorin complex can be regenerated repeatedly and reversibly *in vitro* (Shimomura *et al.*, 1975). Following removal of the coelenteramide, the apoaequorin moiety can be regenerated into active aequorin in the presence of coelenterazine, molecular oxygen, EDTA, and a reducing agent. The initial cloning and sequencing of the 189 amino acid single chain apoaequorin polypeptide, quickly lead to overexpression of apoaequorin in *E. coli* and production of the active aequorin complex, following *in vitro* regeneration with pure coelenterazine and a thiol reagent (Inouye *et al.*, 1985; 1986; Prasher *et al.*, 1985; Charbonneau *et al.*, 1985).

Mutational studies have been performed on apoaequorin to elucidate structure-function relationships, such as the definite requirement of the C-terminal region for luminescent activity (Nomura *et al.*, 1991), the possible Ca^{2+} activation mechanism, as well as the binding site of coelenterazine and molecular oxygen (Tsuji *et al.*, 1986; Kurose *et al.*, 1989; Ohmiya *et al.*, 1992). With elucidation of the crystal structure (Head, 2000), aequorin was shown to contain four characteristic Ca^{2+} binding EF-hand helix-loop-helix motifs, arranged in pairs to form a globular protein, enclosing a hydrophobic cavity making up the coelenterazine-binding site (Head *et al.*, 2000). The hydroperoxide coupled-coelenterazine at the C2 position, is stabilized in aequorin by hydrogen bonding or close associations with three specific triads lining the cavity (Head *et al.*, 2000).

Aequorin has been widely used for measuring intracellular Ca^{2+} and its ability to be easily conjugated to various haptens, substrates and antibodies has made it useful as a reporter molecule in bioluminescence-based assays. Its high signal-to-noise ratio, broad

analytical range, rapid signal generation, and activity in a variety of buffers and at physiological pH (Witkowski *et al.*, 1994) have enabled aequorin labels to be used in both immunoassay (Stults *et al.*, 1992; Rivera *et al.*, 1994) as well as nucleic acid hybridization assays (Galvan *et al.*, 1996; Verhaegen *et al.*, 1998a; White *et al.*, 1999).

The quantum yield for the bioluminescent reaction of aequorin has been determined to be 0.15-0.2 (Blinks *et al.*, 1976), however the quantum yield of this same chemiluminescent reaction was determined to be only 0.002. This is attributed to the ability of the apoaequorin to hold the coelenteramide molecule in a specific conformation that facilitates the generation of the singlet-excited state molecule (Teranishi *et al.*, 1995). The efficiency of light emission in all the coelenterazine-luciferase systems varies, based on the type of luciferase or the coelenterazine analogue used. Luciferases utilizing the same luciferin as aequorin often exhibit higher luminescent intensity, and it has been suggested in the literature that possibly the luminescent intensity of aequorin may be enhanced by protein engineering (Inouye *et al.*, 1997).

DNA shuffling is a method for protein engineering that requires no rational design, but rather it is a directed molecular evolution approach that introduces variation, followed by selection of molecules with desired characteristics. Shuffling involves the digestion of a gene with DNase I into a pool of random fragments that are then re-assembled into the full-length gene by repeated cycles of annealing in the presence of DNA polymerase (Stemmer 1994a; 1994b). Based on homology, the fragments prime each other allowing recombination when fragments from one copy of the gene prime on a different copy, causing a template switch. DNA shuffling procedures combined with repeated cycles of amplification and selection have provided an efficient tool for *in vitro* directed molecular evolution of several proteins (Stemmer 1994b; Crameri *et al.*, 1997; Zhang *et al.*, 1997).

The aim of this project was to subject the apoaequorin gene to directed molecular evolution through successive rounds of DNA shuffling and amplification, followed by screening and selection for mutants exhibiting higher luminescent activity. Ultimately, these aequorins of higher luminescent activity would be useful as reporter molecules for analytical applications such as immunoassays, hybridization assays or *in vivo* assays for Ca^{2+} determination.

4.2 EXPERIMENTAL

4.2.1 Shuffling of the Apoaequorin Gene

4.2.1.1 Preparation of Apoaequorin DNA

The initial 603 bp apoaequorin gene subjected to the shuffling procedure in Round 1 was created through PCR using the pSVAEQN plasmid as template and oligos (e) and (f) as upstream and downstream primers, respectively. PCR was carried out with *Taq* DNA polymerase as described in section 2.6.1.2. This apoaequorin PCR product was referred to as the wildtype (WT) sequence. PCR products were purified from excess primers and dNTPs using the Wizard PCR Preps DNA Purification kit as per manufacturer's instructions.

4.2.1.2 DNase Digestion

Digestion of the apoaequorin PCR product into random fragments was carried out using 0.04 units of DNase I in a total volume of 20 μ L, containing 50 mmol/L Tris, pH 7.5, and 1 mmol/L MgCl₂. The reaction was carried out for 10 min at 37°C and the enzyme was heat inactivated at 75 °C for 10 min. The reaction mixture was loaded into low melting point agarose to separate the digested fragments. Fragment sizes varying from 40-200 bp were excised with a razor blade and purified from agarose using the Qiaex II kit. Fragments were eluted in 20 μ L of dd H₂O.

4.2.1.3 Re-assembly of the Apoaequorin Gene

The digested fragments (40-200 bp) were re-assembled through PCR containing no primers to allow the fragments to prime each other. This reaction was carried out in a total volume of 100 μ L as described in section 2.6.1.2, minus any primers and with 0.15 mmol/L of each dNTP and 20 μ L of purified digested DNA fragments. PCR was run for 40 cycles, each consisting of denaturation (95°C, 45 sec), annealing (50°C, 45 sec) and extension (72°C, 1 min). Mixtures were then incubated at 72°C for 10 min and cooled to 4°C until further use.

A second PCR amplification step including the upstream (e) and downstream (f) primers was carried out as described above, using *Taq* DNA polymerase. A 5 μ L aliquot

of a 25-fold dilution of the above described re-assembled product served as starting template in PCR.

4.2.2 Cloning and Expression of Mutant Apoaequorins

4.2.2.1 Construction of Expression Plasmids

Two Wizard-purified PCR reactions containing pools of shuffled, re-assembled apoaequorin fragments and 5 µg of plasmid pKK223-3 were subjected (separately) to *EcoR* I digestion with 12 units of enzyme in a reaction containing 90 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, and 10 mmol/L MgCl₂ for 1.5 hrs at 37°C. The enzyme was heat inactivated at 65°C for 20 min. The digested mutant apoaequorin genes were purified from a 2% agarose gel, with the Qiaex II kit, and the pKK223-3 plasmid was ethanol precipitated. The plasmid vector was dephosphorylated with 0.05 units of alkaline phosphatase in 1x OPA buffer for 30 min at 37°C, followed by heat inactivation at 85°C for 15 min. Both products were quantified using the PicoGreen Kit as described in section 2.6.2 of Chapter 2. Ligation was carried out with a 4:1 molar ratio of apoaequorin insert to pKK223-3 vector (1.8 pmol:0.45 pmol) in a total volume of 35 µL for 12 hours at 10°C with 8.25 units of T4 DNA ligase (in 1x OPA buffer). The enzyme was then heat inactivated at 65°C for 10 min. The ligation product was immediately digested with 10 units of *Pst* I for 1.5 hrs at 37°C, followed by heat inactivation of the enzyme at 85°C for 20 min. The digested fragment was purified from a 1.5 % agarose gel using the Qiaex II kit. Recircularization was carried out in a total volume of 30 µL with 5.5 units of T4 DNA ligase (in 1x OPA buffer) for 12 hours at 10°C. The enzyme was then inactivated at 65°C for 10 min. This ligation yielded the plasmid pSHAeq_m of 5176 bp size.

4.2.2.2 Expression of Mutant Apoaequorin Library

E. coli transformation was carried out as described in section 2.6.3.2 using 5 µL of the ligation reaction per 1 mL of competent cells in LB broth. All 20 µL of the ligation reaction were transformed and all 4 mL of transformed culture were plated on 20 LB agar plates (200 µL/plate). Following O/N growth, single colonies were picked and transferred to a second grid-numbered LB agar plate for quick identification of each clone. Simultaneously, the picked single colonies were grown up in 1 mL LB broth with

ampicillin, by shaking O/N at 37°C. For each shuffling round, 1000-2000 single colonies were picked for screening purposes.

4.2.3 Screening of Mutants

O/N cultures expressing mutant apoaequorins were regenerated into active aequorin by mixing 50 µL bacterial culture with 50 µL of aequorin regeneration buffer. Coelenterazine and DTT were added to the regeneration buffer directly prior to use, to prevent auto-oxidation of coelenterazine. Following 20 min incubation at RT, luminescence was measured by the addition of 50 µL Ca²⁺ triggering solution injected from the luminometer. Luminescence was integrated for 10 sec. Colonies exhibiting no luminescence were discarded and those with any luminescence activity were saved for cell lysis and mutant selection procedures.

4.2.4 Mutant Selection

4.2.4.1 Cell Lysis and Extraction of Total Soluble Protein

Cell lysis of the remaining 950 µL of overnight culture was carried out using 150 µL of B-PER II bacterial protein extraction reagent as per manufacturer's instructions. Following centrifugation the soluble protein supernatants were saved and the pellets of cellular debris were discarded. Total soluble protein extracts were kept on ice at all times and stored at -80°C.

4.2.4.2 Determination of Total Protein

The mass of total soluble protein was determined by the method of Bradford (Bradford, 1976). Briefly, 40 µL of the total soluble protein extract was diluted in a volume of 800 µL of buffer I, followed by the addition of 200 µL Bio-Rad dye reagent. After 10 min incubation at RT, absorbance was measured at 595 nm. Buffer I containing B-PER II reagent, plus the dye reagent served as the instrument blank. Unknown concentrations of total protein were calculated from a standard curve made with standard protein solutions of BSA, ranging from 0.5 µg/mL to 20 µg/mL.

4.2.4.3 Bioluminometric Assay of Aequorin

Following cell lysis and extraction of total soluble protein in B-PER II, 10 μL of each extract was diluted to 50 μL of buffer I and pipetted into microtiter wells, followed by the addition of 50 μL of regeneration buffer. After 20 min incubation at RT to allow regeneration of active aequorin, the luminescence was measured by injection of 50 μL of Ca^{2+} triggering solution from the luminometer into the microtiter wells. Luminescence was integrated for 45 sec.

4.2.4.4 Comparisons to Native Aequorin

The initial apoaequorin WT PCR product was also cloned into plasmid pKK223-3, transformed into competent JM 109 *E. coli* cells, expressed, and regenerated into active aequorin in identical procedures as those performed for the mutant population of apoaequorins. Twenty-five single colonies expressing WT apoaequorin were picked and compared in order to identify any variations in the procedures. Data from these colonies were averaged and used for comparisons to the active mutant aequorins. The selection criteria used after each round to allow for directed molecular evolution was as follows:

Shuffling Round	Selection Criteria for Following Round
WT aequorin	Ratio = 2 000 RLU/ μg total soluble protein
Round 1	Ratio > 6 000 RLU/ μg total soluble protein
Round 2	Ratio > 6 000 RLU/ μg total soluble protein
Round 3	Ratio > 15 000 RLU/ μg total soluble protein
Round 4	Ratio > 20 000 RLU/ μg total soluble protein
Round 5	Ratio > 50 000 RLU/ μg total soluble protein

4.2.5 Successive Shuffling Rounds

4.2.5.1 Creation of Target

Following the selection procedure, those colonies expressing mutant apoaequorins that met the criteria for each round were grown up O/N in 1 mL LB broth containing ampicillin. Equal cell densities of each chosen colony were then pooled and grown up O/N in 300 mL of LB broth containing ampicillin. Cells were pelleted at 5000 g for 10

min at RT. Plasmid isolation was carried out using the Wizard *Plus* Maxipreps DNA kit as per manufacturer's instructions. The column purification step was often omitted and phenol:chloroform extraction was performed instead, using a method as described in section 2.6.9, followed by ethanol precipitation (section 2.6.8)

Prior to restriction enzyme digestion to isolate the population of shuffled and selected apoaquorin genes, the plasmids were quantified with PicoGreen as described in Section 2.6.2. The isolated plasmids (10 µg) were then digested with 30 units of *Pst* I enzyme and 24 units of *EcoR* I enzyme in a total volume of 20 µL containing 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.9, 10 mmol/L MgCl₂, and 1 mmol/L DTT for 1.5 hrs at 37°C, followed by heat inactivation of the enzyme at 85°C for 20 min. The digested fragments (585 bp) were then purified from a 1.5% agarose gel using the Qiaex II kit. These purified fragments, consisting of a population of selected mutant apoaquorin genes, were then used as the starting target for the successive shuffling rounds.

4.2.5.2 Amplification, Shuffling, Screening and Selection

The selected 585 bp apoaquorin fragments were amplified through PCR as described in section 2.6.1.2 using (e) and (f) as upstream and downstream primers, respectively, with 3 µL of purified, digested target from above. Three PCR reactions were set up, pooled and purified from excess primers and dNTPs using the Wizard PCR Preps Purification kit as per manufacturer's instructions. This 603 bp PCR product was then subjected to the shuffling procedure consisting of DNase digestion and re-assembly as described above. Identical cloning and expression procedures, as well as screening and selection procedures were also performed as described above.

After the completion of each round, the selection criteria were followed as stated in section 4.2.4.4. The amplification, shuffling, screening and selection procedures were repeated five times in a similar manner.

4.2.5.3 DNA Sequencing of Mutants

Three mutant apoaquorin genes (#436, #1026, #1768) selected after the completion of round 5, were sequenced by ACGT Corp (Toronto, ON) using the LI-COR DNA Sequencing System. This system is based on the enzymatic method of Sanger

(Sanger *et al.*, 1977) and uses a Thermo Sequenase that allows cycling with fluorescent-labelled primers incorporated at the 5' terminus to create various lengths of fragments using the four dideoxynucleoside triphosphates.

The colonies expressing these selected apoaquorins were grown up O/N in 9 mL of LB broth containing ampicillin and plasmids (pSHAeq_{m436}, pSHAeq_{m1026}, pSHAeq_{m1786}) were isolated using the Qiagen Plasmid Mini Kit.

4.2.6 Purification of Selected Mutant Apoaquorins

4.2.6.1 Creation of Mutant Apoaquorin Inserts

The mutant apoaquorin genes from #436 and #1026 (#1768 was omitted since it was found to be the same clone as #1026) were prepared as follows. Single colonies expressing these apoaquorins were picked and grown O/N in 3 mL LB broth containing ampicillin. Plasmid isolation was carried out as described in section 2.6.4. 10 µL of these plasmid preparations were digested with 12 units of *EcoR* I and 20 units of *Pst* I in a total volume of 20 µL containing 90 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, and 10 mmol/L MgCl₂. 10 µL of the digests were then separated on a 1.5% agarose gel. The 585 bp fragments were excised, purified using the Qiaex II Kit as per manufacturer's directions and resuspended in 30 µL of dd H₂O.

PCR reactions were set up to introduce *Bgl* II and *Kpn* I sites for insertion into the PinPoint Xa-1 vector containing the *birA* gene. PCR reactions were set up as described in section 2.6.1.1 with *Pfu* polymerase using primers (c) and (d) as upstream and downstream primers, respectively. Digestion of this 590 bp PCR product was carried out first with 10 units of *Bgl* II in buffer containing 20 mmol/L Tris, pH 8.8, 10 mmol/L MgCl₂, 100 mmol/L KCl, 10 mmol/L NH₄(SO₄)₂, 0.1 % Triton, and 0.1 g/L BSA, for 1 hr at 37°C. The buffer was then removed by ethanol precipitation (section 2.6.8). A second digestion was then performed with 10 units of *Kpn* I in a total volume of 30 µL containing 10 mmol/L Tris, pH 7.5, 10 mmol/L MgCl₂, 0.02% Triton X-100, and 0.1 g/L BSA, for 1 hr at 37°C, followed by phenol:chloroform extraction and ethanol precipitation. Digestions yielded fragments of 580 bp, which were quantified with PicoGreen as described in section 2.6.2.

4.2.6.2 Creation of PinPoint-birA fragment

Plasmid pBAeq-birA constructed in section 3.2.3.2 was used as a source of the biotin acceptor-coding domain from the PinPoint Xa-1 vector and the *birA* gene encoding the biotin protein ligase. Ligation of the *birA* gene isolated from genomic *E. coli* DNA into the PinPoint Xa-1 vector is described fully in sections 3.2.1.2, 3.2.2 and 3.2.3.2 of Chapter 3. The PinPoint vector containing the *birA* gene was obtained from a typical plasmid isolation procedure (section 2.6.4) and digested with 10 units of *Kpn* I in a total volume of 35 μ L containing 10 mmol/L Tris, pH 7.5, 10 mmol/L MgCl₂, 0.02 % Triton X-100, and 0.1 g/L BSA, for 1 hr at 37°C followed by buffer exchange with a Spin-pure G-25 column. Digestion with 10 units of *Bgl* II was then carried out in total volume of 40 μ L containing 50 mmol/L Tris, pH 7.5, 10 mmol/L MgCl₂, 100 mmol/L NaCl and 0.1 g/L BSA, for 1 hr at 37°C. The digested fragments were separated on a 1.5% agarose gel, and fragments of 4298 bp were excised and purified with the Qiaex II kit. Vector fragments were quantified with PicoGreen prior to ligation reactions.

4.2.6.3 Construction of pBAeq_m Plasmids

The purified and quantified doubly digested mutant apoaequorin gene inserts (m₄₃₆, m₁₀₂₆) and PinPoint-birA vector fragments were ligated in a 4:1 molar ratio of insert to vector (115 fmol:29 fmol), for 12 hrs at 10°C with 11 units of T4 DNA ligase (in 1x OPA buffer), to create the plasmids pBAeq_{m436} and pBAeq_{m1026}. The enzyme was then heat inactivated at 65°C for 10 min.

4.2.6.4 Selection of Recombinant Clones

Following insertion of the selected apoaequorin and *birA* genes into the PinPoint Xa-1 vector, competent *E. coli* cells were transformed with the recombinant plasmids (pBAeq_{m436} and pBAeq_{m1026}). Several single colonies were then chosen for plasmid minipreparations to isolate the recombinant DNA. Subsequently, restriction enzyme digestion was performed, as described in section 3.2.4 of Chapter 3, to ensure the correct clone was chosen for overexpression of biotinylated mutant aequorin. All details of general bacterial culturing, transformation, plasmid isolation and agarose gel electrophoresis are given in section 2.6 of Chapter 2.

4.2.6.5 Expression of *In Vivo* Biotinylated Apoaequorins and Regeneration of Active Aequorins

Single colonies transformed with the mutant apoaequorins from either pBAeq_{m436} or pBAeq_{m1026}, or the WT apoaequorin from pBAeq-birA (from section 3.2.3.2) were induced to overexpress the recombinant *in vivo* biotinylated aequorins as described in section 2.6.3.3. Cell lysis and extraction of soluble protein was carried out using the B-PER II extraction reagent as per manufacturer's instructions. Regeneration of apoaequorin into active aequorin with coelenterazine and DTT was performed exactly as described in section 3.2.5.3 of Chapter 3.

4.2.6.6 Purification with Avidin Resin

Prior to use, the resin (typically 1 mL slurry per 0.5 g cell pellet) was equilibrated with buffer B, following regeneration as described in section 2.6.10. The crude cell extract containing regenerated aequorin was then applied directly to the slurry and binding was carried out in batch method for 60 min at RT with gentle shaking. The slurry was centrifuged at 500 g for 2 min to pellet the resin, and the supernatant containing unbound soluble protein was removed. The resin was transferred to a small column and washed with 11 mL of buffer B, followed by elution with buffer C. 1-mL fractions were collected and aequorin was measured by diluting each fraction 100 fold in buffer B, pipetting 50 μ L into each well and dispensing 50 μ L of triggering solution from the luminometer. The luminescence was integrated for 30 s. Fractions giving high luminescence signals were pooled and the mass of purified biotinylated aequorin was determined using the Bradford assay as described in section 2.6.5. Purified aequorin proteins were immediately stored at -80°C.

4.2.6.7 Bioluminometric Assay of Purified Aequorins

Serial dilutions of purified biotinylated mutant aequorins, WT aequorin and a commercial aequorin were prepared in buffer D (from stocks whose protein concentration was determined by Bradford assay) and 50 μ L were pipetted into microtiter wells. Luminescence was initiated by the injection of 50 μ L Ca²⁺ triggering solution from the luminometer. Luminescence was integrated for 30 sec.

4.3 RESULTS AND DISCUSSION

4.3.1 DNA Shuffling of the Apoaequorin Gene

The WT apoaequorin gene (see Appendix A) originally isolated from the commercial plasmid pSVAEQN using PCR was subjected to DNA shuffling as originally described by Stemmer (Stemmer, 1994a; 1994b). PCR was first carried out with *Taq* DNA polymerase, an enzyme of low fidelity that has an error rate of about 1 in 125 000 bases (Stratagene product insert). This created a 603 bp apoaequorin gene that was to be subjected to the shuffling procedure. Prior to DNase digestion, it was shown to be crucial to purify the PCR product from excess primers (Stemmer, 1994a) that would have otherwise interfered in the shuffling process. The apoaequorin gene was then digested with DNase I, creating a pool of various fragment sizes. The digested fragments were separated by size in low melting point agarose and fragments varying in size from 40-200 bp were excised and purified from the gel.

The pool of random fragments was re-assembled into full-length genes by repeated cycles of annealing and extension in the presence of DNA polymerase. This was basically carried out as a PCR reaction with no primers, but with an increase in dNTP concentration. The DNA re-assembly procedure was actually an inverse chain reaction, since the number of start sites and the number of molecules decreases over time, unlike PCR, which allows exponential amplification. Recombination occurs during this re-assembly as fragments prime each other based on homology, and fragments from one gene copy prime a different gene copy, causing a template switch. By sequencing random clones, it was originally shown that DNA shuffling by means of DNase digestion and re-assembly produces a point mutagenesis rate of 0.7%, however this rate of point mutagenesis may depend on the size of the fragments used in re-assembly (Stemmer, 1994a; 1994b). Depending on the goal of the shuffling procedure, various fragment sizes are chosen for re-assembly.

Following re-assembly, the products were amplified through PCR to create a library of point-mutated and *in vitro* recombined apoaequorin genes. The PCR step also introduced *EcoR* I and *Pst* I terminal restriction enzyme sites for subsequent cloning purposes. The overall shuffling and cloning scheme is depicted in Figure 4.1. The 603 bp

FIGURE 4.1
SHUFFLING AND CLONING SCHEME

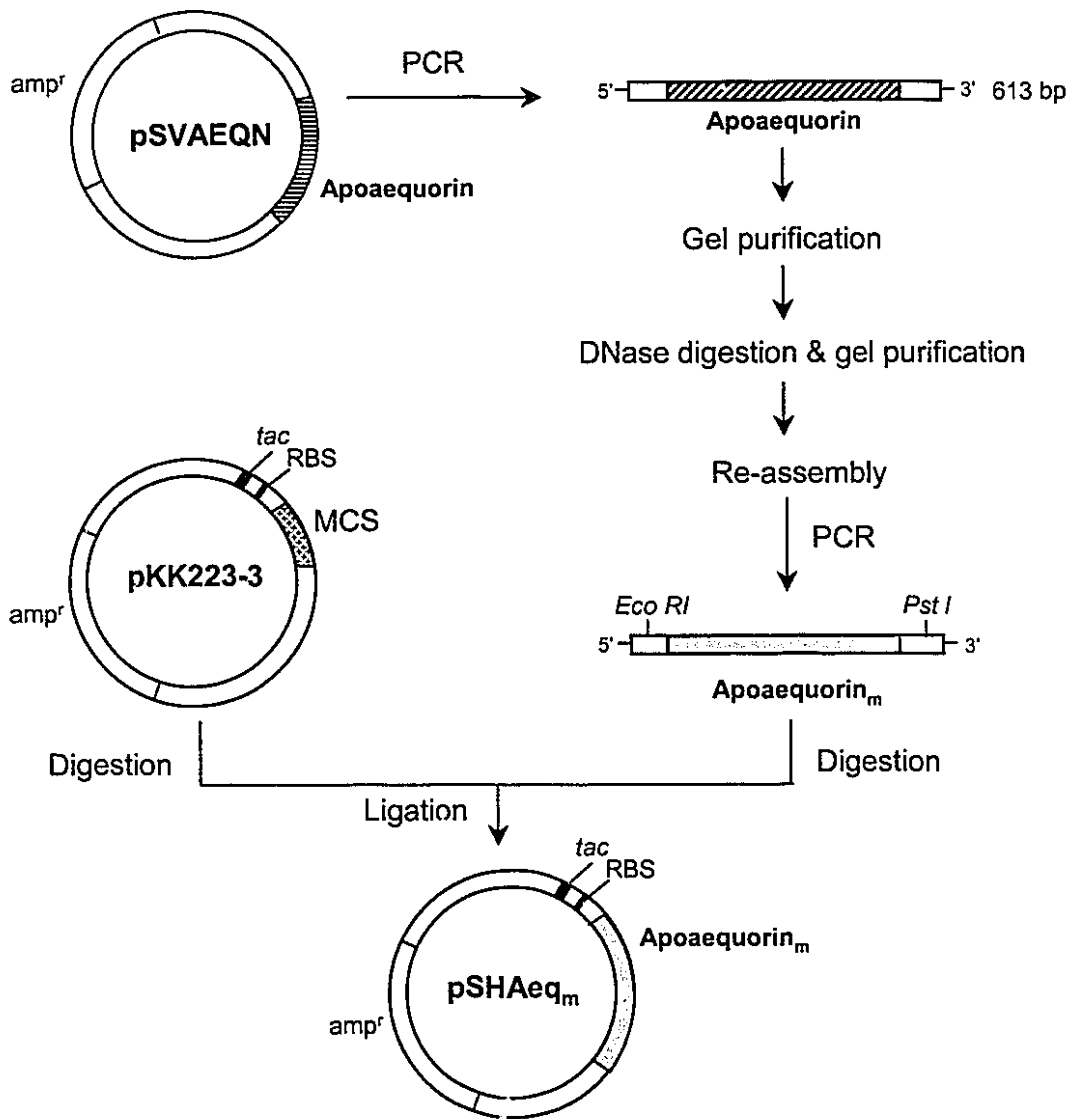


Figure 4.2. The overall scheme for shuffling of the apoaequorin gene and construction of the plasmid pSHAeq_m for expression of the library of point-mutated and *in vitro* recombined apoaequorin genes. *tac* = tac promoter; RBS = ribosome binding site; amp^r = β-lactamase gene conferring ampicillin resistance; apoaequorin_m = shuffled apoaequorin gene

apoaequorin gene, the DNase digested fragments, and the re-assembled full-length apoaequorin gene, are shown as agarose gel electrophoresis products in Figure 4.2.

4.3.2 Cloning, Expression, and Screening of the Shuffled Apoaequorin Library

The pool of point-mutated and *in vitro* recombined apoaequorin genes, were cloned into the expression vector pKK223-3 as shown in Figure 4.1 to create a pool of pSHAeq_m plasmids of 5147 bp. JM 109 *E. coli* were transformed with the entire population of pSHAeq_m plasmids and plated over 20 LB agar plates to create thousands of colonies expressing the shuffled apoaequorin genes. Each round entailed the screening of 1000-2000 of these single colonies.

Screening consisted of growing each isolated colony in a small 1 mL volume of LB broth containing ampicillin, O/N at 37°C. Since coelenterazine is permeable to the cell membrane, any expressed apoaequorin was regenerated directly in culture by the addition of coelenterazine and DTT in regeneration buffer. Following a 20 min regeneration period at RT, Ca²⁺ triggering solution was added to measure any luminescence resulting from active aequorin.

While many colonies exhibited no luminescence, most likely due to detrimental mutations in the apoaequorin gene, a certain percentage in each round exhibited luminescence. Colonies expressing apoaequorin that could be regenerated into some form of active aequorin were chosen for selection procedures. The only criteria required following screening was that the regenerated aequorin exhibit luminescence activity.

The following table displays the percentage of identified luminescent colonies following screening of each shuffled round. There appears to be no apparent pattern to the number of colonies expressing apoaequorin that could be readily regenerated into active aequorin following each round. This was a result of the random nature of the mutations arising in each round, maintaining heterogeneity in the population. While the parental genes chosen for each round were themselves a result of selection and amplification, each round gave rise to new mutations, some beneficial and others detrimental.

FIGURE 4.2
AGAROSE GEL ELECTROPHORETIC ANALYSIS

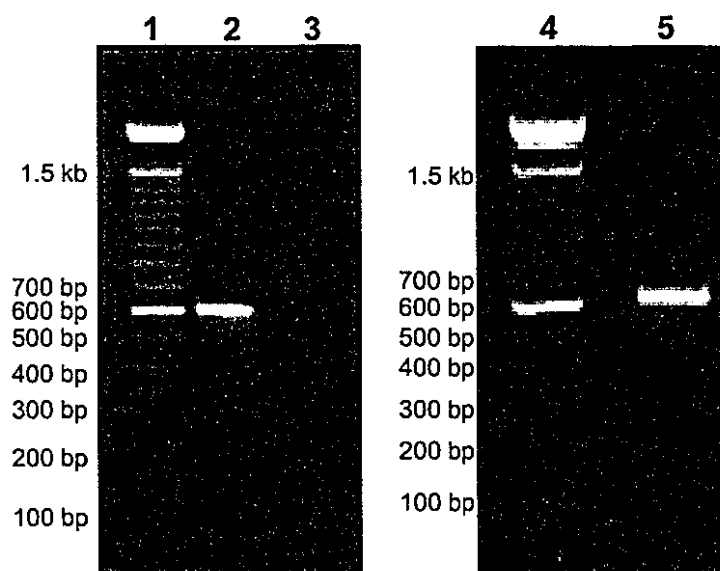


Figure 4.2. An ethidium bromide stained 1.5% agarose gel depicting the electrophoretic analysis of DNA fragments associated with shuffling of the apoaequorin gene. *Lane 1:* 100 bp molecular weight ladder. *Lane 2:* Initial 603 bp apoaequorin gene prior to shuffling. *Lane 3:* DNA fragments of varying sizes resulting from the DNase digestion of the apoaequorin gene. *Lane 4:* 100 bp molecular weight ladder. *Lane 5:* The re-assembled full-length shuffled apoaequorin gene following DNase digestion, re-assembly and PCR procedures.

Shuffled Round	Percentage of Luminescent Colonies
Round 1	31.5 %
Round 2	41.2 %
Round 3	14.7 %
Round 4	47.6 %
Round 5	35.3 %

4.3.3 Selection and Comparisons to WT Aequorin

Following screening procedures, colonies exhibiting luminescence upon regeneration with coelenterazine were subjected to cell lysis and extraction of total soluble protein. This was carried out using the B-PER II extraction reagent, a non-ionic, Tris-based detergent, especially designed for the extraction of soluble recombinant proteins from *E. coli*. Following the extraction step, the total soluble protein retrieved from the cell pellet was measured by the Bradford method. The apoaequorin expressed from each colony was also regenerated into active aequorin by the addition of coelenterazine and DTT and then measured for luminescent activity by injecting Ca^{2+} triggering solution. Each of these luminescent mutants could now be compared to each other based on their specific activity ratio of luminescence to total mass of soluble protein (RLU/ μg total soluble protein).

The WT apoaequorin gene was also subjected to the same cloning and expression procedures as the shuffled genes, and gave an average ratio of about 2000 RLU/ μg total soluble protein. Although more than 25 WT colonies were subjected to cell lysis and protein extraction, with most displaying this characteristic ratio of 2000 RLU/ μg total soluble protein, there were some outliers displaying either low luminescence or up to 5000 RLU/ μg total soluble protein. Since the cells were not at equal cell density during these experiments, nor were they induced to overexpress the recombinant protein, variations in procedures, varying expression levels among the clones or alternatively, random mutations introduced during PCR, may have accounted for these differences.

The specific activity ratios for each mutant were compared to the WT value following each round, and those meeting the selection criteria set out in section 4.2.4.4 were chosen for shuffling purposes of the subsequent round. The selected colonies

expressing the shuffled apoaequorin genes were pooled and grown up for plasmid isolation. The apoaequorin fragments were then excised from the population of plasmids through restriction enzyme digestion and subjected to the shuffling procedure again to create a new library of point-mutated and *in vitro* recombined apoaequorin genes.

In this manner, the shuffled genes were directed towards higher luminescent signals at each round. A flowchart of the complete screening and selection protocol is outlined in Figure 4.3. A summary of the specific activity ratios obtained following each shuffling round is shown in Figure 4.4. After screening 7000-10 000 mutants in total, there was a noticeable trend towards higher specific activity ratios as the rounds progressed and advantageous mutations were selected for according to specific criteria.

4.3.3.1 Mutants # 436, # 1026 and # 1768

Following selection of round 5, three mutants (#436, #1026 and #1768) exhibited luminescence resulting in ratios greater than 50 000 RLU/ μ g total soluble protein. As shown in Figure 4.5.A, initial data indicated approximately a 70-fold increase in luminescence activity for mutant #436 and approximately a 45-fold increase in luminescence activity for mutants #1026 and #1768 under the specified conditions. These mutants were selected for further study. Experiments were repeated under identical conditions at equal cell density or under induced conditions with IPTG to allow for variations in expression, as shown in Figure 4.5.B and 4.5.C, respectively.

All three mutant apoaequorin genes as well as the WT apoaequorin gene were sequenced in both the forward and reverse directions. Complete DNA sequences of the WT apoaequorin gene and the three mutant apoaequorin genes are shown in Appendices A, D, E, and F. All three mutant genes were identified as having 5 base changes, leading to amino acid changes. The apoaequorin gene of mutant #436 had an additional mutation resulting in a synonymous codon, leading to no alteration of the amino acid sequence. Identical base mutations in the apoaequorin genes of mutants #1026 and #1768 indicated they were actually the same clone (see Appendix E and F). Inferred amino acid changes are shown below.

FIGURE 4.3
SCREENING AND SELECTION PROTOCOL

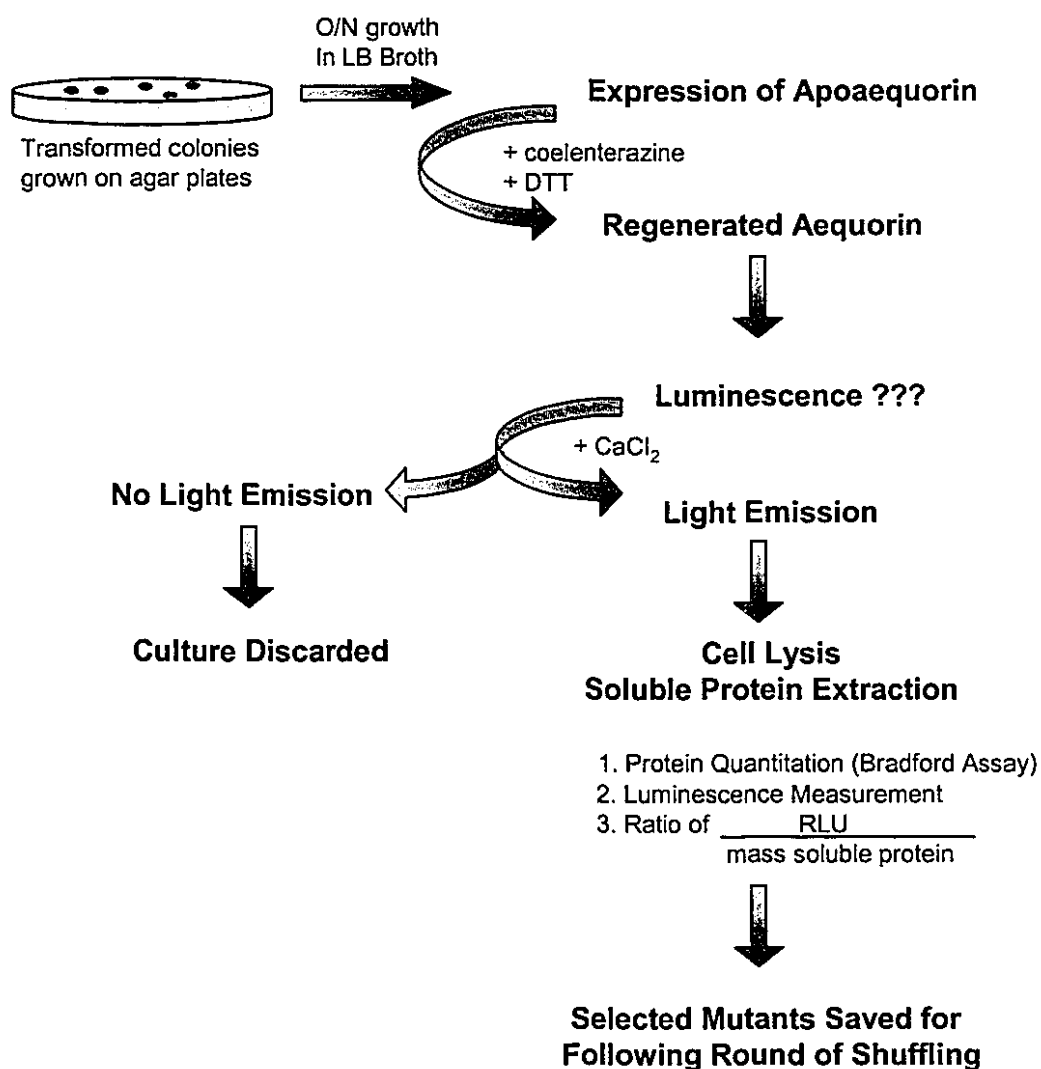


Figure 4.3. The overall scheme for screening and selection of the shuffled apoaequorin genes with desired characteristics. The sole criterion for screening was the regeneration of some form of active aequorin displaying luminescence. Selection entailed cell lysis, and measurement of luminescence as well as protein determination. Mutants passing selection criteria at each round were shuffled in the following round.

FIGURE 4.4
SUMMARY OF SELECTION DATA

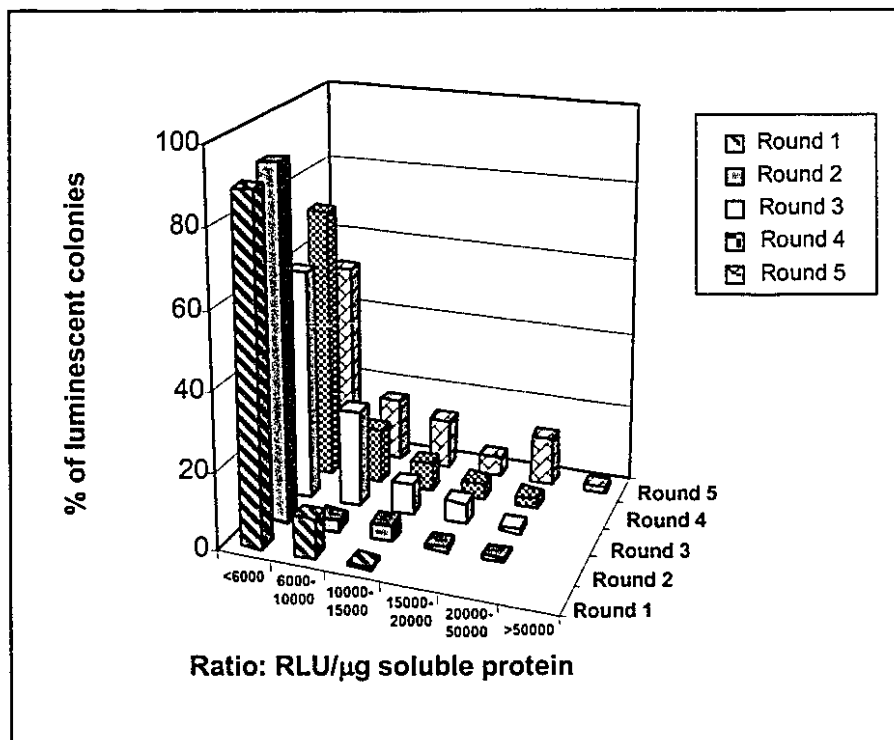


Figure 4.4. Summary of all data from selection procedures of all five rounds. The percentage of luminescent colonies exhibiting a specific activity ratio in terms of RLU/ μ g total soluble protein is shown for each round. Trends towards higher ratios are seen as the rounds progress.

FIGURE 4.5
LUMINESCENCE OF SELECTED AEQUORINS

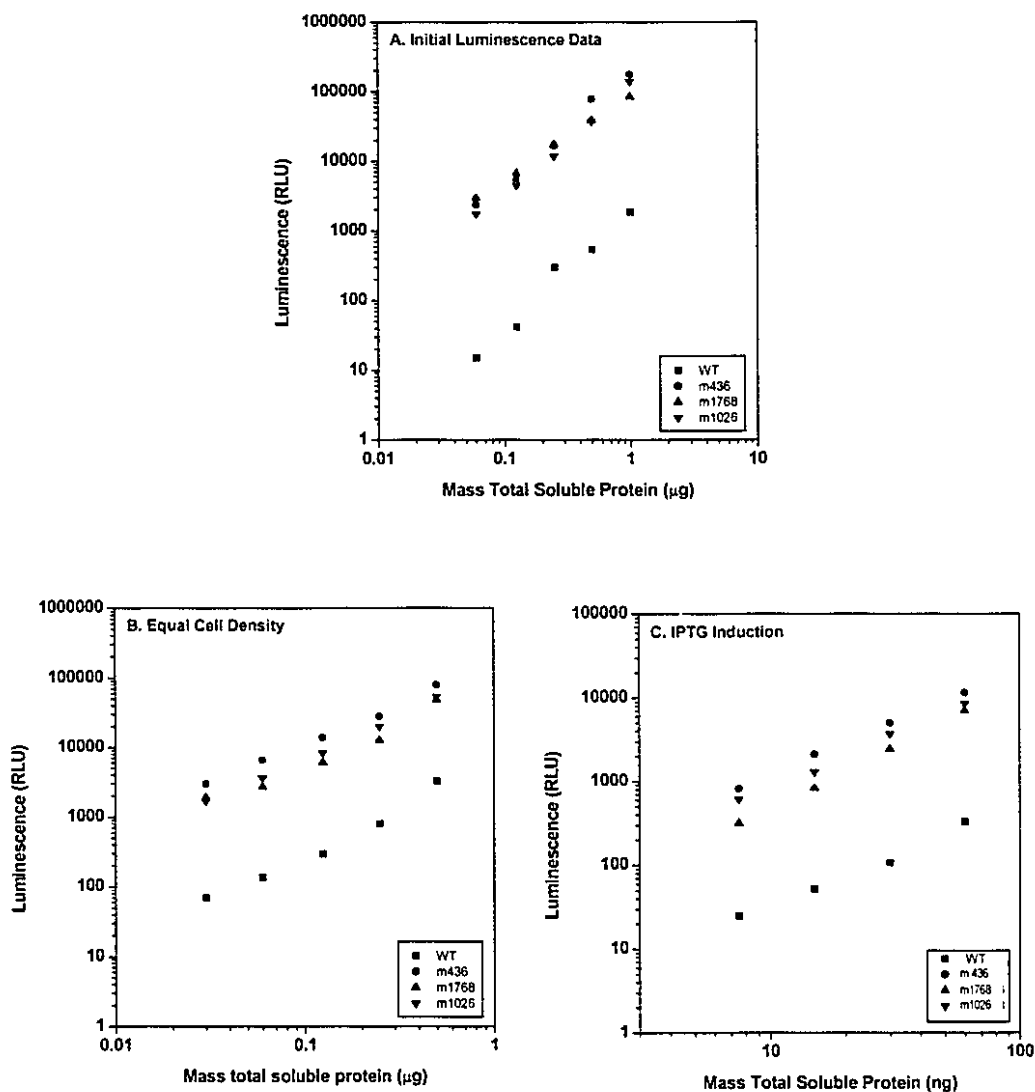


Figure 4.5. Following selection of round 5, three mutants (#436, #1026 and #1768) exhibited luminescence resulting in ratios greater than 50 000 RLU/ μg total soluble protein. Figure 4.5.A depicts initial data obtained following selection of round 5. Experiments were repeated at equal cell density (4.5.B) and under induced conditions with IPTG (4.5.C) to allow for variations in expression.

WT Aequorin	Mutant # 436	Mutant # 1026	Mutant # 1768
Gly ₁₄	-	Arg ₁₄	Arg ₁₄
Asn ₂₈	Asp ₂₈	-	-
Ala ₆₉	Val ₆₉	Val ₆₉	Val ₆₉
Ala ₉₀	Val ₉₀	Val ₉₀	Val ₉₀
Ser ₁₅₇	Asn ₁₅₇	Asn ₁₅₇	Asn ₁₅₇
Gln ₁₅₉	Arg ₁₅₉	Arg ₁₅₉	Arg ₁₅₉

4.3.4 Purification of Mutant Aequorins

In order to further study the luminescent activity of the selected mutant aequorins and make any direct comparisons to WT aequorin, mutants #436 and #1026 were purified from crude cell lysates. This was achieved by the creation of recombinant *in vivo* biotinylated mutant aequorins for purification purposes. Mutant apoaequorin genes were cloned into the PinPoint vector as described in detail in Chapter 3 to create a biotin-acceptor domain fusion tag at the N-terminus of the apoaequorin gene. The *birA* gene was also cloned downstream of the apoaequorin-fusion sequence, to create one transcriptional unit, with each gene relying on the presence of an upstream ribosome binding site for translation. Following overexpression of the two genes under control of the induced *tac* promoter, the biotin protein ligase encoded by the *birA* gene, biotinylated a specific lysine residue present on the biotin-acceptor domain of the apoaequorin fusion product (Chapman-Smith *et al.*, 1990; Reddy *et al.*, 1998).

Following construction of pBAeq_m plasmids, transformation into competent JM 109 *E. coli*, and identification of correct clones, single colonies were grown up and induced to overexpress the *in vivo* biotinylated apoproteins of mutant #436, mutant #1026, or WT aequorin. Cell pellets were harvested and lysed using the B-PER II extraction reagent. Regenerated biotinylated aequorins were then purified from crude lysates by affinity chromatography on a monomeric avidin resin, followed by elution with excess free biotin. Eluted fractions showing high luminescence, were pooled and the mass of purified aequorin was determined by Bradford assay.

4.3.4.1 Direct Comparisons to WT Aequorin

For direct comparisons of the luminescent activity of the two purified mutant aequorins to the WT aequorin as well as to a commercial biotinylated aequorin, the luminescence was plotted as a function of the attomoles of aequorin, as shown in Figure 4.6. Luminescence data from purified aequorin indicated no increase in activity of the purified Aeq_{m436} or Aeq_{m1026}. In fact, all four aequorins, including the commercial, the WT, and the two mutant aequorins, were found to have identical performance, with as low as approximately one attomole detected and linearity extending over three orders of magnitude.

While there was apparently no increase in the quantum yield of the luminescent reaction from the mutant aequorins, there was definitely higher luminescence observed in the original crude lysates containing the aequorin regenerated from the shuffled apoaequorin genes. Comparisons were made between the masses of total purified biotinylated aequorin from cell pellets (of equal mass), expressing either the mutant or WT aequorins. It was found that the mass of soluble biotinylated aequorin extracted from mutant #436 was about 6-fold higher than that of the WT aequorin, whereas #1026 yielded a mass that was 2-fold higher than that of the WT aequorin. While this does not completely explain the large increases in luminescence initially observed, it indicates that the higher luminescence may have been due to factors associated with the expression of the recombinant apoaequorin, such as variations in expression or the folding of aequorin.

It is known that recombinant proteins overexpressed in *E. coli* often undergo precipitation in the cell and formation of insoluble, inactive inclusion bodies (Georgiou *et al.*, 1996; Strandbeg *et al.*, 1991; Marston, 1986). The regeneration of active aequorin with coelenterazine would not be possible if the majority of expressed apoaequorin existed in the form of these granular inclusion bodies. The amino acid changes observed following the shuffling rounds might have aided the apoaequorin in correct folding, allowing a higher yield of soluble apoaequorin following extraction. Mutant #436 always exhibited higher luminescence than WT aequorin or even that of mutant #1026, as well as allowing higher yields of extracted soluble biotinylated aequorin. Possibly this could be attributed to the Asn₁₄ to Asp₁₄ mutation, since this is the only mutation not present in mutant #1026.

FIGURE 4.6
LUMINESCENCE AS A FUNCTION OF PURIFIED AEQUORIN

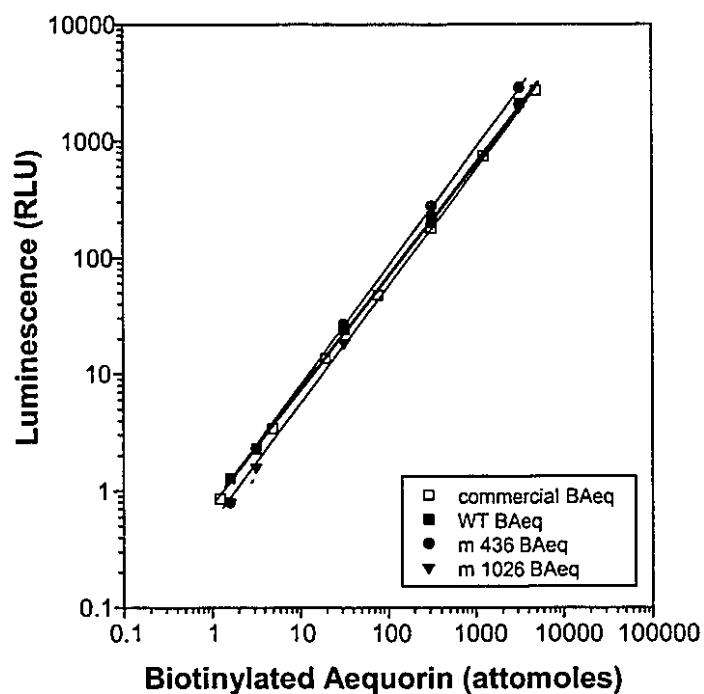


Figure 4.6. Luminescence plotted as a function of the attomoles of various biotinylated aequorins, including WT aequorin, mutant #436 and mutant #1026, as well as a commercial aequoim. Serial dilutions in buffer D (50 μ L) were pipetted into microtiter wells and luminescence integrated for 10 sec following injection of Ca²⁺ triggering solution (50 μ L). Each experimental point represents the mean value of two assays.

Increases in solubility were also found to be the case when GFP was subjected to DNA shuffling with selection based on improved whole cell fluorescence (Crameri *et al.*, 1996). Even though GFP has a quantum yield of 0.7-0.8 (Ward *et al.*, 1982), the goal was to improve the whole cell fluorescence for use as a reporter in cells. While a 45-fold increase in total cell fluorescence was reported, it was attributed to improved folding rather than improved quantum yield.

Because directed molecular evolution is biased based on the selection procedure, the resulting products are always dependent on this selection process. The selection of desired aequorin characteristics obtained from shuffling of the apoaequorin gene would have been dependent on the expression system and conditions used, as well as the extraction and regeneration procedures used for obtaining active aequorin. In the case of apoaequorin, the extraction was always performed in the presence of the B-PER II reagent, which may have affected the solubility of the apoaequorin and driven selection in the direction of creating more soluble protein rather than increasing the luminescent activity. Furthermore, regeneration was only carried out for 20 min with a constant concentration of coelenterazine, which may have also created a selection bias towards these criteria.

4.3.5 Conclusions

In conclusion, novel aequorin photoproteins with higher luminescent activity were not created by directed molecular evolution, but rather an increase in soluble apoaequorin allowed higher activity upon extraction from crude lysates. These aequorin mutants may be more useful in assays requiring *in vivo* expression of aequorin, such as determination of intracellular Ca^{2+} using apoaequorin cDNA expressed in the cytoplasm or subcellular organelles. Aequorin mutants that fold more efficiently may perform better in assays of this type.

CHAPTER 5**RECOMBINANT *GAUSSIA* LUCIFERASE. OVEREXPRESSION,
PURIFICATION AND ANALYTICAL APPLICATION OF A NEW REPORTER
FOR DNA HYBRIDIZATION****ABSTRACT**

Gaussia luciferase (GLuc), the enzyme responsible for the bioluminescent reaction of the marine copepod *Gaussia princeps*, has recently been cloned as the smallest known coelenterazine-using luciferase with a molecular weight of 19.9 kDa. GLuc catalyzes the oxidative decarboxylation of coelenterazine to produce the excited state of coelenteramide, which upon relaxation to the ground state emits blue light (470 nm). Presently, there are no analytical studies using GLuc as a bioluminescent reporter. Starting with GLuc cDNA, a plasmid encoding a biotin acceptor peptide-GLuc fusion protein was engineered, which allowed overexpression of *in vivo* biotinylated *Gaussia* luciferase in *E. coli*, and purification by avidin affinity chromatography. Besides facilitating purification, the *in vivo* biotinylation enabled subsequent complexation of GLuc with streptavidin thereby avoiding chemical conjugation procedures. The light emission of purified GLuc was studied at various coelenterazine concentrations to establish the detectability of the new reporter. Detectability of the bioluminescent reaction of GLuc was found to reach attomole levels. Furthermore, the complexation of biotinylated GLuc with streptavidin was used as a detection reagent in a microtiter well-based, bioluminometric DNA hybridization assay.

5.1 INTRODUCTION

Bio- and chemiluminescence, the emission of light from chemically generated excited states, find rapidly expanding and diverse analytical applications in areas where the determination of analytes in low concentrations is required (Ziegler *et al.*, 2000). The principal advantage of bio(chemi)luminometric assays is their superior detectability to conventional spectrophotometric and fluorometric ones. Because bioluminescence and chemiluminescence do not require the use of excitation light, problems arising from background excitation radiation, fluorescence from other components of the sample and photobleaching, are eliminated.

Major areas of analytical applications of BL and CL include: (i) Development of nonradioactive detection systems for nucleic acid hybridization assays (Christopoulos, 1999). In one such category of assays, an enzyme is used as a label, whose activity is determined by using a chemiluminogenic substrate such as alkaline phosphatase along with a dioxetane derivative. Alternatively, a chemiluminescent compound such as an acridinium ester may be used as a label. (ii) Immunoassays and protein blotting (Kricka, 2000). (iii) Luciferase-encoding cDNAs are used as reporter genes for spatial and temporal monitoring of gene expression and for studying the strength and developmental regulation of promoters/enhancers (Bronstein *et al.*, 1994; Srikantha *et al.*, 1996). (iv) Development of light-emitting biosensors consisting of whole cells (bacteria) as the transducer for direct determination of e.g. pollutants in environmental samples (Lewis *et al.*, 1998; Ramanathan *et al.*, 1997). (v) Protein-protein interaction assays carried out in living cells and based on bioluminescence resonance energy transfer (Xu *et al.*, 1999). This rapid growth of applications has stimulated research activity for investigation and exploitation of new bioluminescent systems.

Gaussia princeps (see Figure 5.1) is a bioluminescent marine copepod (body length 10 mm) living in 350-1000 m ocean depths (Barnes *et al.*, 1972; Latz *et al.*, 1990; Bowlby *et al.*, 1991). Bioluminescence originates as a secretion from 30 glands, located in the antennae, cephalothorax, thorax and abdomen, in response to mechanical, electrical or light stimuli. The release of a luminous blue bolus is generally accompanied by rapid swimming, which displaces the copepod away from the bolus. *Gaussia princeps* has the ability to control both the number of luminous glands and the strength of discharge from

FIGURE 5.1
GAUSSIA PRINCEPS ORGANISM



Figure 5.1. *Gaussia princeps* is a bioluminescent marine copepod of 10-12 mm in length, living in ocean depths of 350-1000 m. Bioluminescence originates as a secretion from glands, located in the antennae, cephalothorax, thorax and abdomen, in response to mechanical, electrical or light stimuli. The release of a luminous blue bolus is accompanied by rapid swimming, which displaces the copepod away from the bolus.

individual glands, thus varying the emission pattern from quick, bright flashes to a fixed pattern of discrete points glowing long after the animal has departed. Bioluminescence in *G. princeps* probably serves as a defense mechanism. The flashes startle and blind a dark-adapted predator, while the glowing decoy provides a spatially defined target to hold the predator's attention away from the escaping copepod (Barnes, 1972; Latz, 1990; Bowlby, 1991a; 1991b).

While studies on light emission from the whole organism under various stimuli have been carried out for a long time, the cloning of *Gaussia* luciferase, the enzyme responsible for the bioluminescent reaction, was accomplished very recently (Bryan *et al.*, 2001). GLuc, a single polypeptide chain consisting of 185 amino acids (19.9 kDa), catalyzes the oxidative decarboxylation of coelenterazine to produce the excited state of coelenteramide, which upon relaxation to the ground state emits blue light (470 nm) (Ballou *et al.*, 2000; Bryan *et al.*, 2001). It has been reported that GLuc transfected into mammalian cells gives high levels of light emission (Ballou *et al.*, 2000).

The objective of this work was to carry out the first quantitative analytical study of *Gaussia* luciferase and examine its potential as a new reporter molecule for DNA hybridization. It is well known that luciferases are inactivated upon conjugation to other biomolecules, such as DNA probes or antibodies (Kricka, 1988), limiting their application to DNA hybridization assays. To avoid inactivation problems, a system was designed that allows production of *in vivo* biotinylated Gluc (BGLuc). A vector was constructed that drives the overexpression of a) a biotin acceptor peptide-GLuc fusion protein and b) the biotin protein ligase.

BPL catalyzes the covalent attachment of a single biotin to the fusion protein. Purification can then be accomplished by affinity chromatography using monomeric avidin resin. Besides facilitating purification, the *in vivo* biotinylation enables subsequent complexation of GLuc with SA thereby avoiding chemical conjugation procedures. The light emission of purified BGLuc was studied at various coelenterazine concentrations. The relationship between luminescence and the concentration of GLuc was investigated in order to establish the detectability of the new reporter. Subsequently, the complexation of SA with BGLuc was studied and the complexes used for determination of hybrids in a microtiter well-based bioluminometric DNA hybridization assay.

5.2 EXPERIMENTAL

5.2.1 Amplification of *Gaussia* Luciferase-Coding DNA

Polymerase chain reaction was carried out as described in section 2.6.1.1, using *Pfu* polymerase. The plasmid pGLuc was used as a source of the *Gaussia princeps* luciferase gene (see Appendix G). A 526 bp DNA fragment, containing the GLuc-coding sequence flanked by *Bgl* II and *Kpn* I restriction sites, was created through PCR using the oligos (c) and (d) as upstream and downstream primers, respectively.

5.2.2 Amplification of *birA* Gene from *E. coli* Genomic DNA

Extraction of *E. coli* genomic DNA and amplification of the *birA* gene to introduce *Not* I and *Sma* I restriction sites were carried out exactly as described in section 3.2.2 and section 3.2.1.2 of Chapter 3, respectively.

5.2.3 Construction of Plasmid pBGLuc-*birA*

BirA amplification products were concentrated with Microcon-30 columns and digested with 20 units of *Not* I (90 min at 37°C) followed by heat inactivation of the enzyme (65°C for 20 min). The digested *birA* PCR product was then purified from a 1% agarose gel using the Qiaex II kit. The PinPoint Xa-1 (pXa) vector (3 µg) was linearized with 30 units of *Not* I as described above. The buffer was changed using Spin-pure G-25 columns and dephosphorylation was carried out for 30 min at 37°C with 0.05 units of alkaline phosphatase in 10 mmol/L Tris acetate, 10 mmol/L magnesium acetate, and 50 mmol/L potassium acetate, pH 7.5. The enzyme was heat inactivated at 85°C for 15 min. Both digests were quantified fluorometrically with PicoGreen and then ligation was carried out for 12 hours at 10°C with 11 units T4 DNA ligase using a 15:1 molar ratio of *birA* insert to PinPoint Xa-1 vector. The enzyme was then inactivated at 65°C for 10 min. The buffer was changed and a second digestion with 20 units of *Sma* I carried out for 2 hours at 30°C followed by heat inactivation at 65°C for 20 min. The small *Sma* I digested fragments were removed using a Microcon-30 spin column and recircularization was carried out as above creating the plasmid pXa-*birA*.

The GLuc PCR products were then purified and concentrated with the Wizard PCR Preps DNA Purification system and subsequently digested with 10 units of *Kpn* I for

90 min at 37°C. Spin-pure G-25 columns were used to exchange the buffer followed by digestion with 10 units of *Bgl* II for 90 min at 37°C. The recombinant pXa-birA plasmid was similarly digested with both *Bgl* II and *Kpn* I.

The digested products were separated by agarose gel (1.2%) electrophoresis, and the appropriate fragments were purified from the gel using the Qiaex II purification kit, and quantified fluorometrically with PicoGreen. The doubly digested purified GLuc coding sequence and the pXa-birA fragments were ligated (in a 4:1 molar ratio) as above to create the plasmid pBGLuc-birA.

5.2.4 Bacterial Expression and Purification of *In Vivo* Biotinylated *Gaussia* Luciferase (BGLuc)

5.2.4.1 Bacterial Culture and Cell Lysis

E. coli strain JM 109 transformed with the pBGLuc-birA was inoculated into LB broth (3 mL) containing 0.1 g/L ampicillin and supplemented with 2 mg/L biotin. This seed culture was grown overnight at 30°C, diluted 100 fold and again grown at 30°C until an absorbance of 0.8-0.9 (at 600 nm) was reached. At this point protein synthesis was induced with 1 mmol/L IPTG for 6 hours. Cells were harvested by centrifugation at 2000 rpm for 25 min at 4°C and stored at -20°C.

Frozen cell pellets were washed twice in TBS buffer (25 mmol/L Tris, 2.6 mmol/L KCl, 137 mmol/L NaCl, pH 7.4) to remove excess biotin. Cell lysis and soluble protein extraction were carried out using the B-PER II extraction reagent as per manufacturer's instructions. Briefly, the cell pellet was resuspended in B-PER II Reagent (4 mL/gram wet cell pellet) with PMSF included at a final concentration of 0.7 mmol/L. After gentle shaking for 15 minutes the cellular debris was pelleted (15000 RPM for 15 minutes, Beckman JA20 rotor) and the supernatant containing soluble proteins removed for further purification.

5.2.4.2 Purification of *In Vivo* Biotinylated *Gaussia* Luciferase

The monomeric avidin resin was regenerated as described in section 2.6.10. Prior to use, the resin (typically 1 mL slurry per gram cell pellet) was equilibrated with buffer F. The crude cell extract diluted 5 times in buffer E was then applied directly to the slurry

and binding was allowed to occur for 60 min at room temperature with gentle shaking. After spinning at 1000 g for 2 min, (Beckman, swinging bucket Model J6-B) the crude supernatant was removed and the avidin resin containing bound BGLuc protein was transferred to a small column. This column was washed with 10 mL of buffer F, followed by elution with 5 mmol/L biotin in buffer F. 1-mL fractions were collected and the BGLuc activity was measured by diluting each fraction 600 fold in buffer G, pipetting 5 μ L into each well containing 45 μ L of buffer G and dispensing 25 μ L of 0.01 mmol/L coelenterazine diluted in buffer E from the luminometer. The luminescence was integrated for 15 sec.

Fractions giving high luminescence signals were pooled, concentrated (by ultrafiltration using an Ultrafree-15 Centrifugal Device) and purified three times from excess biotin by size exclusion chromatography with NAP-5 columns, followed by final concentration using the Eppendorf Vacufuge. Final preparations of the purified BGLuc enzyme were present in Buffer E containing 10% B-PER II Reagent and supplemented with 10% glycerol. The mass of purified biotinylated GLuc was determined using the Bradford assay and aliquots were then stored immediately at -20°C .

5.2.5 Bioluminometric Assay of *Gaussia* Luciferase

Assays for luminescence activity of *Gaussia* luciferase were carried out by diluting the purified BGLuc in buffer H and pipetting 50 μ L into a microtiter well. The coelenterazine substrate solution of varying concentrations (5 μ mol/L-130 μ mol/L) was diluted in buffer E and 50 μ L injected into the well. Luminescence was integrated for 20 sec.

5.2.6 Bioluminometric Hybridization Assay Using *In Vivo* Biotinylated *Gaussia* Luciferase as a Reporter Molecule

The oligo probe (g) was tailed enzymically with DIG-dUTP and concentrated to a final concentration of 2.0 μ mol/L as described in section 3.2.6.2 of Chapter 3. The 233 bp fragment used as the target DNA in the hybridization assay was previously described in section 3.2.6.1 of Chapter 3.

Opaque polystyrene microtiter wells were coated (O/N at RT) with 50 μL of 5 mg/L anti-digoxigenin antibody diluted in 0.1 mol/L carbonate buffer, pH 9.6. The wells were washed 3 times with wash solution and 50 μL of 4 nmol/L DIG-tailed probe diluted in buffer H were added into each well. The probe was allowed to bind to the antibody for 60 min at room temperature, followed by washing. Biotinylated target DNA, diluted in buffer H, was denatured at 95°C for 10 min and immediately placed on ice. A 10- μL aliquot was added to each well containing 40 μL of buffer H and hybridization was carried out at 42°C for 30 min followed by washing. A complex of streptavidin with BGLuc was prepared by mixing 20 nmol/L biotinylated GLuc with 10 nmol/L SA (both diluted in buffer H) and incubating for 30 min at room temperature. The complex was diluted 5 times in buffer D and a 50- μL aliquot was added to each well. After a 30-min incubation, the wells were washed and 50 μL of 20 $\mu\text{mol/L}$ coelenterazine substrate (diluted in Buffer E) were dispensed into each well. The luminescence was integrated over a 15 sec period.

5.3 RESULTS AND DISCUSSION

5.3.1 Construction of Plasmid pBGLuc-birA

The plasmid pBGLuc-birA drives the overexpression, in *E. coli*, of both the biotin protein ligase (the *birA* gene product) and a fusion protein consisting of the biotin-acceptor peptide genetically fused to the amino-terminus of *Gaussia* luciferase. The biotin protein ligase then catalyzes the *in vivo* biotinylation of the biotin acceptor peptide at a unique site, thus producing a biotinylated GLuc fusion protein. This facilitates (a) the purification of BGLuc, in a single step, from the crude cellular extract by affinity chromatography using monomeric avidin resin and (b) the complexation of purified BGLuc with streptavidin for direct use in bioluminometric hybridization assays, thus avoiding chemical conjugation.

The construction of a plasmid for bacterial expression of both *in vivo* biotinylated luciferase from *Gaussia princeps* as well as the biotin protein ligase is illustrated in Figure 5.2. Both, the GLuc and *birA* genes are under transcriptional control of the *tac* promoter.

FIGURE 5.2
PLASMID CONSTRUCTION

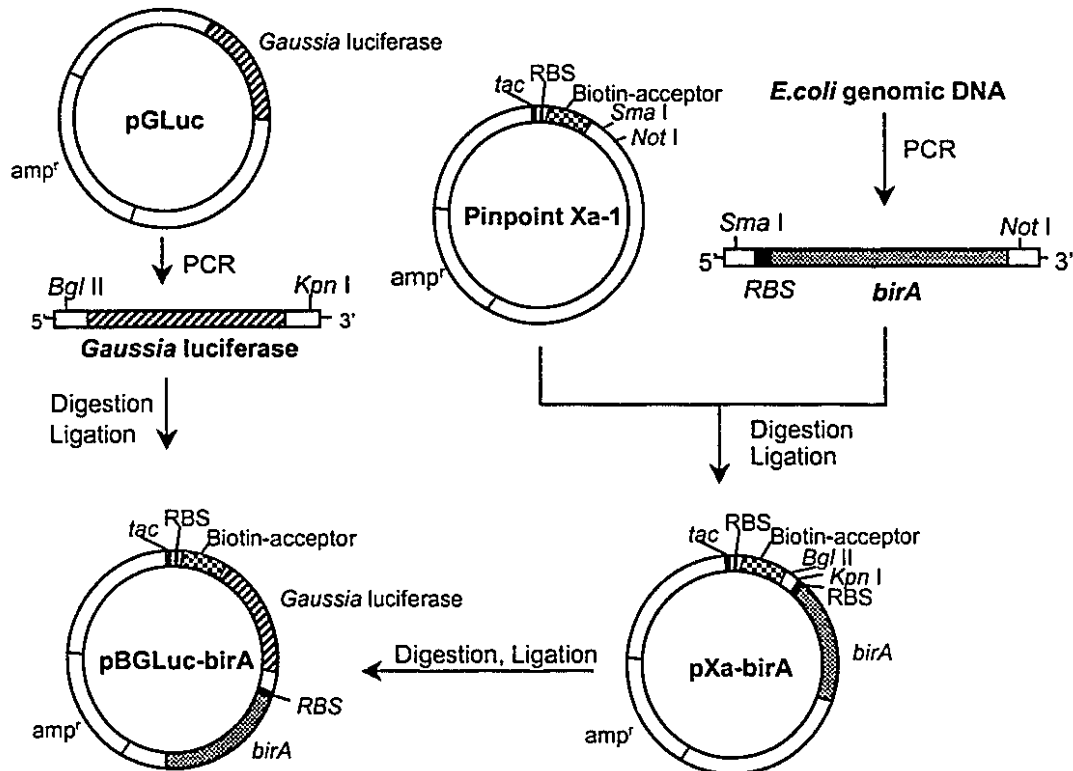


Figure 5.2. A schematic presentation of the construction of plasmid pBGLuc-birA for expression of in vivo biotinylated *Gaussia luciferase*. Relative locations of the *tac* promoter, the ribosome binding site (RBS), the restriction enzyme sites and the gene inserts are indicated. *birA* = gene encoding the biotin protein ligase in *E. coli*; *amp^r* = β -lactamase gene conferring ampicillin resistance

The PinPoint Xa-1 vector was used as a source of the sequence encoding the biotin-acceptor domain (from *Propionibacterium shermanii* transcarboxylase) positioned downstream of the *tac* promoter and a ribosome binding site, thus allowing expression of a biotin-acceptor domain fused to the amino terminus of a recombinant protein. The structure of the biotin domain of *P. shermanii* transcarboxylase is very similar to that of *E. coli* acetyl-CoA carboxylase, which is the physiological substrate of biotin protein ligase (Reddy *et al.*, 1998). Consequently, the biotin acceptor domain of *P. shermanii* is biotinylated from expressed BPL.

The biotin protein ligase-coding gene (*birA*) was amplified from *E. coli* genomic DNA through PCR using primers that introduced a *Sma* I site and a ribosome binding site upstream of *birA*, as well as a stop codon and a *Not* I site downstream of the gene. The 1004 bp PCR amplification product was inserted between the *Sma* I and *Not* I sites of the Pinpoint Xa-1 vector to create the plasmid pXa-*birA* (4298 bp) capable of expressing biotin protein ligase.

The *Gaussia* luciferase gene was amplified using plasmid pGLuc as a template with primers designed to introduce a *Bgl* II site upstream of the gene as well as a *Kpn* I site and a stop codon downstream of the gene. The luciferase gene was inserted downstream of the biotin-acceptor coding sequence between the *Bgl* II and *Kpn* I sites and upstream of the *birA* gene. This generated the 4815 bp plasmid pBGLuc-*birA*. Following plasmid isolation, pBGLuc-*birA* was digested to confirm the presence and size of both the *Gaussia* luciferase and *birA* genes. A 1.2% agarose gel electropherogram is shown in Figure. 5.3.

5.3.2 Expression and Purification of *In vivo* Biotinylated *Gaussia* Luciferase

The plasmid pBGLuc-*birA* was introduced into competent JM 109 *E. coli* cells and protein synthesis was induced with IPTG. Cells were then lysed and soluble protein extracted using the B-PER II reagent. The crude extract was applied to monomeric avidin resin in batch form. The supernatant containing unbound protein was removed and the avidin resin transferred to a column for further washing followed by elution of the biotinylated protein. Monomeric avidin has a much lower affinity for biotin ($K_d=10^{-7}$ M) than the native tetrameric avidin ($K_d=10^{-15}$ M), thus allowing both the binding of

FIGURE 5.3
AGAROSE GEL ELECTROPHORETIC ANALYSIS

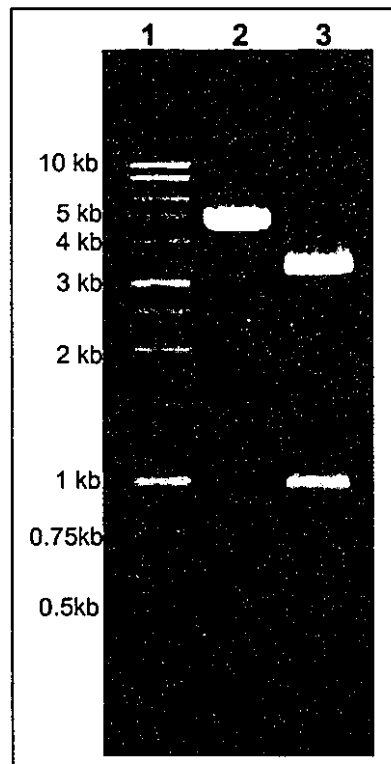


Figure 5.3. A 1.2% ethidium bromide stained agarose gel depicting the electrophoretic analysis of plasmid pBGLuc-birA. *Lane 1:* Molecular weight ladder. *Lane 2:* Recombinant plasmid pBGLuc-birA (4815 bp) linearized with *Not* I. *Lane 3:* Plasmid pBGLuc-birA digested with *Bgl* II, *Kpn* I, and *Not* I to excise both the *Gaussia* luciferase (517 bp) and *birA* (992 bp) genes.

biotinylated molecules and the subsequent elution with free biotin under mild, non-denaturing conditions (Kohanski *et al.*, 1990).

A typical profile of the protein mass (as determined by Bradford assay) and the luminescence obtained during washing of the monomeric avidin resin and elution of the BGLuc are shown in Figure 5.4. The protein mass of the first fraction (corresponding to the removed supernatant) is high whereas the luminescence is low. The total protein then decreases to undetectable levels as soluble proteins from the cell extract are washed through the column. Subsequently, biotinylated *Gaussia* luciferase is eluted using buffer F supplemented with 5 mmol/L biotin. Most of the GLuc activity is eluted in the first 3-4 mL of elution buffer and pooled for further concentration.

Following the affinity chromatography purification step, approximately 40% of the luciferase activity (as determined by the luminescence signal) was recovered in the pooled fractions. The remaining activity was removed with the supernatant and passed through the column during washing. Supplementing the growth media with increasing concentrations of biotin (up to 10 mg/L) and prolonging the induction time resulted in no improvement in capture of the fusion protein on the avidin resin. Conditions of BGLuc purification were mimicked and repeated with a different biotinylated protein (aequorin). It was found that these conditions did not compromise the avidin-biotin binding required for the affinity purification procedure.

The expression system was designed such that the *in vivo* biotinylation reaction does not rely only on the catalytic activity of the endogenous BPL of the host. Plasmid pBGLuc-birA drives the overexpression of BPL along with GLuc (in a single mRNA molecule). It has been shown that about 90% biotinylation is attained by combined use of the *birA* expression and biotin supplemented growth media (Smith *et al.*, 1998). Consequently, the loss of a fraction of GLuc during purification was not attributed to low BPL activity, but rather to the fact that, in this fraction, the folding of the fusion protein either rendered the biotinylation site inaccessible to BPL or it resulted in a limited accessibility of the biotin moiety to the avidin resin.

In order to test if the presence of B-PER II reagent in the cellular extract affected the binding of biotinylated protein to monomeric avidin resin, the cell extract was diluted 5 to 50-fold in buffer E prior to mixing with the resin. There was no improvement of

FIGURE 5.4
BIOTINYLATED GAUSSIA LUCIFERASE ELUTION PROFILE

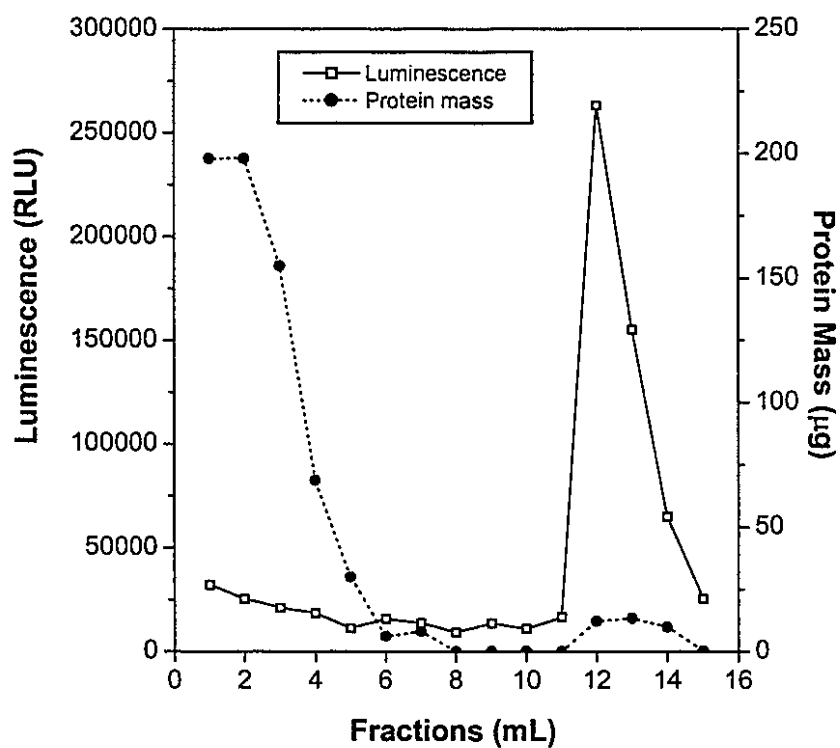


Figure 5.4. A typical profile of protein mass and luminescence obtained during purification of *in vivo* biotinylated Gaussia luciferase from crude cell extract using monomeric avidin resin. A 1-gram cell pellet was used and 1-mL fractions were collected. The protein mass was determined by Bradford assay and luciferase activity monitored by diluting each fraction 600-fold in buffer G and adding 50 µL to the well. Following the injection of 50 µL coelenterazine substrate, the luminescence was integrated for 15 sec.

BGLuc binding with decreasing B-PER concentrations. It should be noted, however, that the addition of B-PER to the elution buffer contributed to the stability of purified BGLuc. When BGLuc was eluted directly in buffer E (containing no B-PER), the luminescence activity was lost rapidly whereas elution in buffer F (buffer E plus B-PER) resulted in no decrease in luminescence even after 3 weeks at 4°C (and much longer at -20°C).

Bradford assay of the purified protein, following affinity purification, concentration by ultrafiltration and biotin removal by size exclusion chromatography (repeated 3 times), gave approximately 90-115 µg of BGLuc per gram of cell pellet. This corresponds to a yield of 0.55-0.69 mg of purified protein per litre of bacterial culture.

Heterologous proteins in *E. coli* are often packaged in the form of inactive aggregates (inclusion bodies) when plasmid-encoded genes are overexpressed at high rates (Georgiou *et al.*, 1996; Strandberg *et al.*, 1991; Marston, 1986). Since both recombinant GLuc and BPL were overexpressed, inclusion body formation was unavoidable. Only the soluble fraction of BGLuc was purified in this work.

SDS-PAGE analysis was performed to verify the size and purity of the *in vivo* biotinylated *Gaussia* luciferase (Figure 5.5). Following purification, a single band was observed at 33.9 kDa, corresponding to the sum of the 18.8 kDa *Gaussia* luciferase and the 15.1 kDa biotin-acceptor domain. *E. coli* contains only one biotinylated protein, the biotin carboxy carrier protein, one of the three subunits of acetyl-CoA carboxylase (Fall *et al.*, 1979), which also binds avidin and would have been co-eluted with the BGLuc. However, no band corresponding to BCCP was observed in SDS-PAGE, indicating that BCCP was present at a much lower concentration than *Gaussia* luciferase.

5.3.3 Bioluminometric Determination of *Gaussia* Luciferase

Data pertaining to the time course of light emission from the *in vivo* biotinylated *Gaussia* luciferase-catalyzed reaction, at various coelenterazine concentrations, is presented in Figure 5.6. The light emission peaked at 1 sec and then decreased rapidly with a half-life dependent on coelenterazine concentration (flash type emission). The decrease was attributed to the inhibition of GLuc from the reaction product (coelenteramide), as was previously shown with other luciferases (Matthews *et al.*, 1977). The light emission was integrated for 20 sec, for all subsequent studies.

FIGURE 5.5
SDS-PAGE ANALYSIS

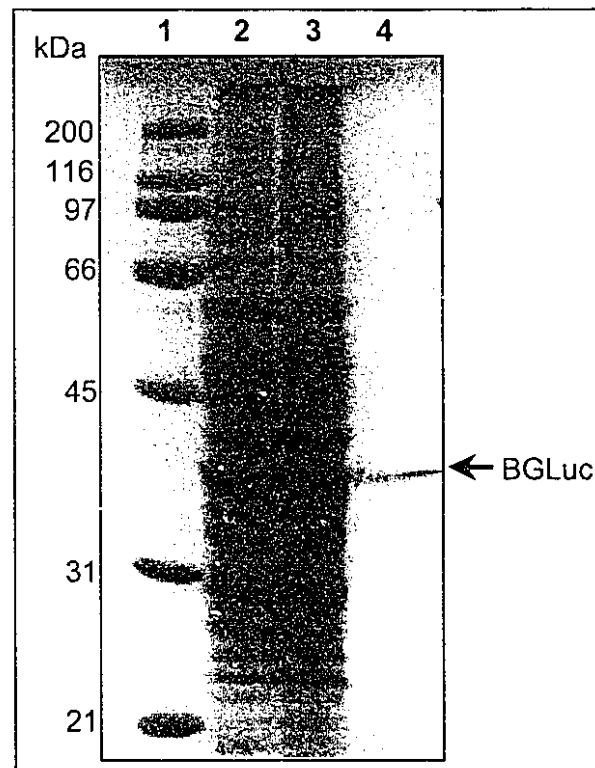


Figure 5.5. A 12% SDS-PAGE gel depicting: *Lane 1:* Broad range molecular weight markers. *Lane 2:* 10 μ L of crude lysate resulting from B-PER II cell lysis. *Lane 3:* 10 μ L of total soluble protein fraction prior to purification with monomeric avidin resin. *Lane 4:* \sim 2 μ g purified, concentrated *in vivo* biotinylated *Gaussia* luciferase. All procedures to obtain samples are described in the experimental section.

FIGURE 5.6
LIGHT EMISSION PROFILE OF *GAUSSIA* LUCIFERASE

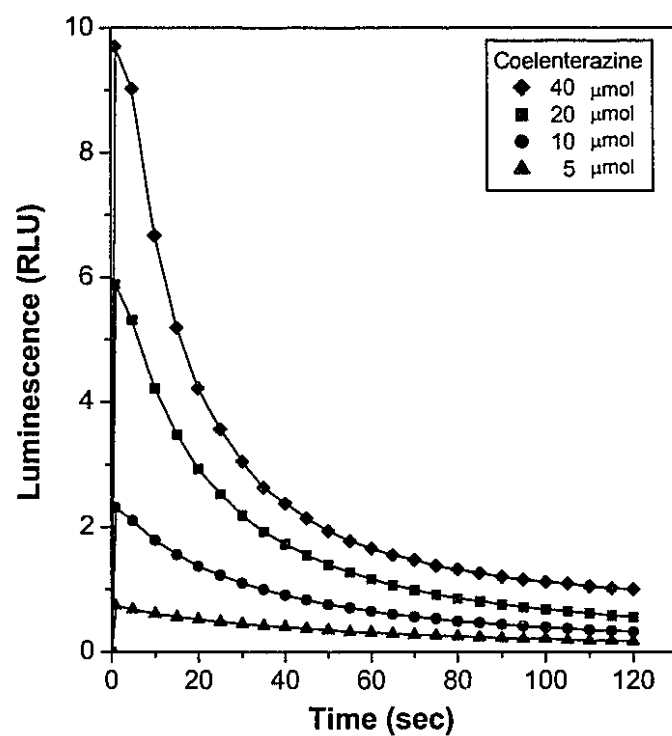


Figure 5.6. Light emission profile for *Gaussia* luciferase at various concentrations of coelenterazine substrate. Data is shown for 400 attomoles of BGLuc (50 μ L) diluted in Buffer H, followed by injection of 50 μ L coelenterazine diluted in buffer E. Initial light emission peaks at 1 sec and rapidly decays. Both peak height and total light production increase with substrate concentration.

The effect of coelenterazine concentration on the activity of BGLuc was studied in the range of 5-130 $\mu\text{mol/L}$ and results are presented in Figure 5.7. While the luminescence increased with higher coelenterazine concentrations, the background also increased accordingly. The background is defined as the luminescence obtained with all reagents present except the luciferase. As a result, the signal-to-background ratio is maintained relatively constant over the entire range of coelenterazine concentrations studied, with a slight enhancement at 20 $\mu\text{mol/L}$. It has been reported previously that coelenterazine in aqueous solutions exhibits a low level luminescence, which has been attributed to auto-oxidation (Taranishi *et al.*, 1997).

In order to assess the detectability of *in vivo* BGLuc, serial dilutions of the purified protein (from a stock whose protein concentration was determined by Bradford assay) were prepared in buffer H, 50 μL were pipetted into the well and the luminescence was measured after injecting 50 μL of 20 $\mu\text{mol/L}$ coelenterazine solution. In Figure 5.8, the luminescence (corrected for the background) is plotted as a function of the amount of biotinylated *Gaussia* luciferase. As low as one attomole of *Gaussia* luciferase can be detected with a signal-to-background ratio of 2.0. Moreover, the linearity extends over five orders of magnitude. This experiment was repeated five times over the period of one month, using different stock coelenterazine solutions, and the standard deviations of the signals are presented as error bars in Figure 5.8.

5.3.4 *In Vivo* Biotinylated *Gaussia* Luciferase as a Reporter in Bioluminometric DNA Hybridization Assays

A hybridization assay was developed in which a biotinylated, denatured target DNA was hybridized to an oligonucleotide probe immobilized in microtiter wells through a digoxigenin-antidigoxigenin interaction. The hybrids were then quantified by adding a streptavidin-biotinylated *Gaussia* luciferase complex and using coelenterazine as the substrate.

The removal of excess biotin following purification of the BGLuc from the avidin resin was crucial prior to complexation with streptavidin. Optimization of the biotinylated *Gaussia* luciferase-to-streptavidin molar ratio was then carried out by incubating a constant concentration of BGLuc (4 nmol/L) with varying concentrations of streptavidin

FIGURE 5.7
EFFECT OF COELENTERAZINE CONCENTRATION

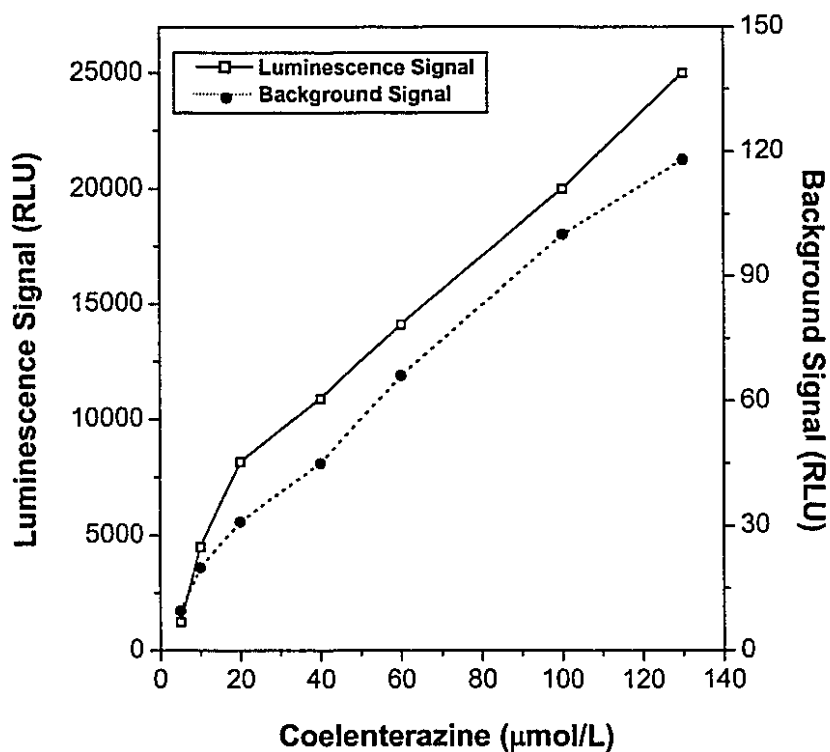


Figure 5.7. The luminescence signal and background signal of biotinylated *Gaussia* luciferase are shown as a function of coelenterazine concentration. Data is shown for 400 attomoles of BGLuc (50 μL) diluted in buffer H, followed by injection of coelenterazine (50 μL) diluted in buffer E. The background is determined by injection of substrate into a well containing only buffer H. Luminescence signal and background both increase with increasing concentrations of coelenterazine.

FIGURE 5.8
LUMINESCENCE AS A FUNCTION OF BIOTINYLATED
GAUSSIA LUCIFERASE

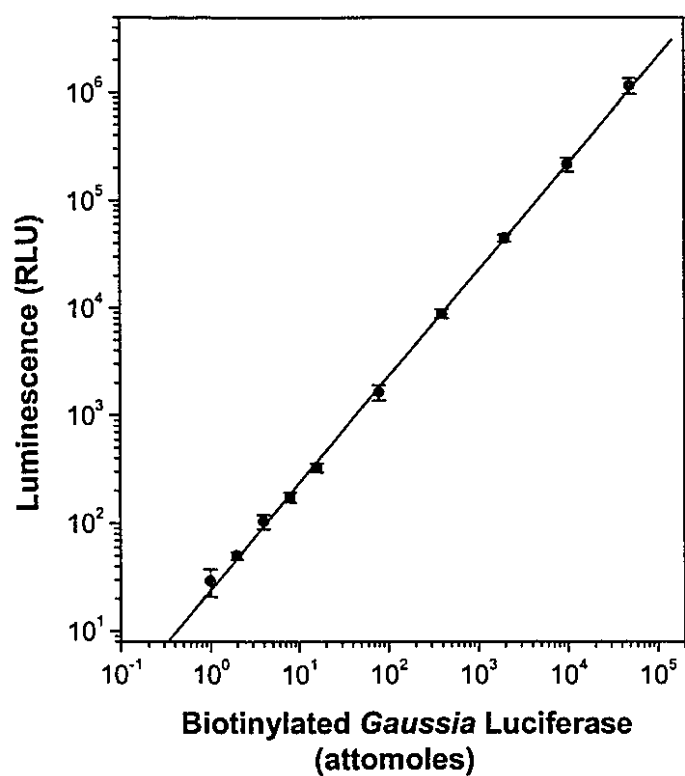


Figure 5.8. Luminescence as a function of the attomoles of *in vivo* biotinylated *Gaussia* luciferase. Serial dilutions of BGLuc in buffer H (50 μ L) were pipetted into microtiter wells and the luminescence was integrated for 20 sec following injection of 50 μ L coelenterazine substrate (20 μ mol/L) (n=5).

(1 to 16 nmol/L), and the (SA-BGLuc) complex applied to the wells. As shown in Figure 5.9, maximum luminescence occurred at a 1:2 molar ratio of SA to BGLuc. If biotinylated GLuc was in excess, the four biotin-binding sites on streptavidin became saturated and the SA-BGLuc complex was no longer able to bind the immobilized biotinylated hybrids. If streptavidin was in excess, then free SA competed with the SA-BGLuc complex for binding to biotinylated hybrids on the well.

In order to assess the overall performance of the hybridization assay, various dilutions of biotinylated target DNA were prepared in buffer H and analyzed as described in the experimental section. In Figure 5.10, the luminescence (corrected for the background) was plotted as a function of target DNA concentration. The background was defined as the luminescence obtained when no target DNA was present in the well. The linearity of the assay extended from 1.6 to 800 pmol/L. The signal-to-background ratio at 1.6 pmol/L (80 attomoles/well) was 1.4. The assay was repeated over a 4-day period and the signals averaged ($n=5$). These day-to-day reproducibility studies revealed CVs of approximately 10%.

5.3.5 Conclusions

It was calculated that 1 L of bacterial culture provided enough biotinylated *Gaussia* luciferase for 150 000 hybridization assays. The entire process, including culturing pBGLuc-birA transformed cells, extraction of total soluble protein, and purification of active biotinylated *Gaussia* luciferase was completed in less than 2 days. The hybridization assay on wells containing immobilized probe was complete in less than 60 min including the complexation reaction.

Gaussia luciferase activity was measured rapidly and easily with high sensitivity and a wide linear range. It should be noted that the detectability of GLuc may be further enhanced by suppressing the auto-oxidation (auto-luminescence) of coelenterazine, thus allowing higher substrate concentrations to be used. Work in this direction is currently being performed. Finally, the biotinylated GLuc-streptavidin complex may also be used as a detection reagent in numerous applications including immunoassays when combined with specific biotinylated antibodies.

FIGURE 5.9
OPTIMIZATION OF COMPLEXATION OF STREPTAVIDIN TO
BIOTINYLATED *GAUSSIA* LUCIFERASE

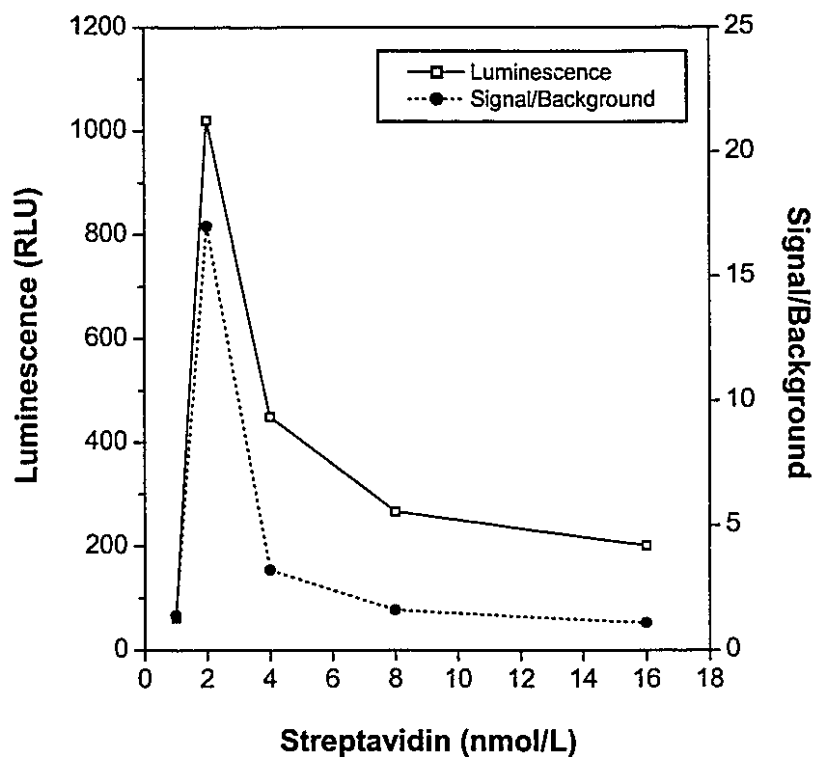


Figure 5.9. Complexation of *in vivo* biotinylated *Gaussia* luciferase with streptavidin. The luminescence signal and signal-to-background ratios of complexes were plotted as a function of the SA concentration. Complexes were prepared by mixing 4 nmol/L BGLuc with various concentrations of SA (1 nmol/L to 16 nmol/L). The complexes were then tested for optimum binding to immobilized biotinylated hybrids in an assay as described in the experimental section.

FIGURE 5.10
LUMINESCENCE AS A FUNCTION OF TARGET
DNA CONCENTRATION

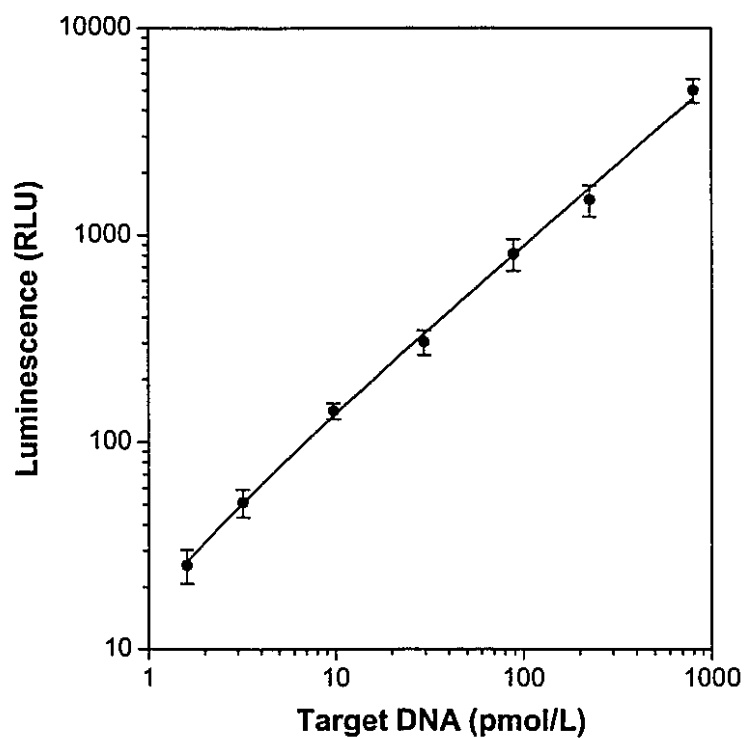


Figure 5.10. Luminescence as a function of the target DNA concentration. The hybridization assay was performed as described in the experimental section using *in vivo* biotinylated *Gaussia* luciferase as a reporter molecule. The error bars correspond to plus/minus one standard deviation (n=5).

GENERAL CONCLUSIONS

The goals of the previous research entailed the engineering, overexpression, purification and analytical applications of various bioluminescent reporters. General plasmids were constructed that allowed overexpression of the *in vivo* biotinylated photoprotein aequorin as well as the luciferase of *Gaussia princeps*. Each plasmid also contained the *birA* gene that codes for the biotin protein ligase, which is responsible for *in vivo* biotinylation at a specific Lys residue. *In vivo* biotinylation allowed both, purification of the overexpressed fusion proteins, as well as complexation with streptavidin for analytical applications. Model bioluminometric hybridization assays were developed using these universal reporters.

The entire process, including culturing of transformed cells, extraction of total soluble protein, and purification of fully active bioluminescent proteins was complete in less than 2 days. The hybridization assays on wells containing immobilized probe were complete in less than 60 min including the complexation with streptavidin. Both the biotinylated aequorin-streptavidin complex as well as the biotinylated luciferase-streptavidin complex may be used as universal reporter molecules, including such applications as detection reagents in immunoassays.

The first luminescence studies with the luciferase of *Gaussia princeps* were performed. Detectability of this new reporter reached attomole levels and it was found that increasing coelenterazine concentrations increased the luminescence. Furthermore, since most luciferases are inactivated upon conjugation, the *in vivo* biotinylation provided an alternative means to chemical conjugation and easy complexation with streptavidin.

Shuffling of the apoaequorin gene was also carried out in an attempt to increase the luminescence activity of this photoprotein and possibly use any desirable mutant aequorins as reporter molecules in sensitive hybridization assays. Following shuffling procedures, screening, selection and final purification of the mutants, it was found that all mutants and wild type aequorin had identical performance. Higher soluble yields of these mutants were obtained following purification procedures, and it was concluded that selection possibly favoured mutations allowing improved folding leading to increased solubility of the mutants.

APPENDIX A
Coding Sequence of Apoaequorin Gene

5'-1 ATG GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA AAA TGG ATT
43 GGACGA CAC AAG CAT ATG TTC AAT TTT CTT GAT GTC AAT CAC
85 AAT GGAAGA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCT
127 GAT ATT GTT ATA AAC AAT CTT GGAGCA ACA CCT GAA CAA GCC
169 AAA CGA CAC AAA GAC GCT GTA GAA GCT TTC TTT GGA GGA GCT
211 GGAATG AAA TAT GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC
253 GAA GGATGG AAA AGA CTG GCT ACC GAA GAA TTG GAA AGG TAT
295 TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA
337 TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT GGA GCT ATT ACA
379 CTC GAT GAA TGG AAA GCA TAT ACC AAA TCT GCT GGCATC ATC
421 CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT
463 ATT GAT GAA AGT GGA CAG CTC GAT GTT GAT GAG ATG ACA AGA
505 CAA CAT TTA GGATTT TGG TAC ACC ATG GAT CCT GCT TGC GAA
547 AAG CTC TAC GGT GGA GCT GTC CCA TAA -3'

Apoaequorin gene initially isolated from plasmid pSVAEQN through PCR for creation of the biotin-acceptor domain fusion protein (BAeq) in Chapter 3. This is considered the wildtype (WT) sequence and was also subjected to shuffling procedures for directed molecular evolution in Chapter 4. Sequencing was performed by ACGT Corp. (Toronto, ON).

APPENDIX B
Coding Sequence of *E. coli birA* Gene

```

5'-1 ATGAAGGATA ACACCGTGCC ACTGAAATTG ATTGCCCTGT TAGCGAACGG
  51 TGAATTTCACT CTGGCGAGCA GTTGGGTGAA ACGCTGGGAA TGAGCCGGG
 101 CGGCTATTAA TAAACACATT CAGACACTGC GTGACTGGGG CGTTGATGTC
 151 TTTACCGTTC CGGGTAAAGG ATACAGCCTG CCTGAGCCTA TCCAGTTACT
 201 TAATGCTAAA CAGATATTGG GTCAGCTGGA TGGCGGTAGT GTAGCCGTGC
 251 TGCCAGTGAT TGA CTCCACG AATCAGTACC TTCTTGATCG TATCGGAGAG
 301 CTTAAATCGG GCGATGCTTG CATTGCAGAA TACCAGCAGG CTGGCCGTGG
 351 TCGCCGGGGT CGGAAATGGT TTTGCGCTTT TGGCGCAAAC TTATATTTGT
 401 CGATGTTCTG GCGTCTGGAA CAAGGCCCGG CGGCGGCGAT TGGTTTAAGT
 451 CTGGTTATCG GTATCGTGAT GGCGGAAGTA TTACGCAAGC TGGGTGCAGA
 501 TAAAGTTCGT GTTAAATGGC CTAATGACCT CTATCTGCAG GATCGCAAGC
 551 TGGCAGGCAT TCTGGTGGAG CTGACTGGCA AACTGGCGA TCGGCGCAA
 601 ATAGTCATTG GAGCCGGGAT CAACATGGCA ATGCGCCGTG TTGAAGAGAG
 651 TGTCGTTAAT CAGGGGTGGA TCACGCTGCA GGAAGCGGGG ATCAATCTCG
 701 ATCGTAATAC GTTGGCGGCC ATGCTAATAC GTGAATTACG TGCTGCGTTG
 751 GAACTCTTCG AACAAGAAGG ATTGGCACCT TATCTGTGCG GCTGGGAAAA
 801 GCTGGATAAT TTTATTAATC GCCCAGTGAA ACTTATCATT GGTGATAAAG
 851 AAATATTTGG CATTTACGCG GGAATAGACA AACAGGGGGC TTTATTACTT
 901 GAGCAGGATG GAATAATAAA ACCCTGGATG GCGCGTGAAA TATCCCTGCG
 951 TAGTGCAGAAAATAA -3'

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BirA sequence 9667-10632 from the *E. coli* K12 genome coding for the biotin protein ligase. GenBank Accession Number AE000471.

APPENDIX C
Sequence of Target DNA

5'- CTC TCG TGG CAG GGCAGT CTG CGGCGGTGT TCT GGT GCA CCC
CCA GTG GGT CCT CAC AGC TGC CCA **CTG CAT CAG GAA CAA AAG CGT**
GAT CTT GCT GGGTCG GCA CAG CCT GTT TCA TCC TGA AGA CAC AGG
CCA GGT ATT TCA GGT CAG CCA CAG CTT CCC ACA CCC GCT CTA CGA
TAT GAG CCT CCT GAA GAA TCG ATT CCT CAG GCC AGG TGA TGA CTC
CAG CCA CGA CC -3'

Target sequence of DNA used for hybridization assays was a 233 bp fragment of prostate-specific antigen mRNA generated by RT-PCR as previously described (Verhaegen *et al.*, 1998). Additional target DNA was created through PCR using primers (i) and (j). The region of complementary probe binding is highlighted.

APPENDIX D

Gene Sequence of Mutant Apoaequorin #436

5'-1 ATG GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA AAA TGG ATC
 43 GGACGA CAC AAG CAT ATG TTC AAT TTT CTT GAT GTC AAT CAC
 85 GAT GGAAGA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCT
 127 GAT ATT GTT ATA AAC AAT CTT GGAGCA ACA CCT GAA CAA GCC
 169 AAA CGA CAC AAA GAC GCT GTA GAA GCT TTC TTT GGAGGA GTT
 211 GGAATG AAA TAT GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC
 253 GAA GGATGG AAA AGA CTG GTT ACC GAA GAA TTG GAA AGG TAT
 295 TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA
 337 TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT GGA GCT ATT ACA
 379 CTC GAT GAA TGG AAA GCA TAT ACC AAA TCT GCT GGCATC ATC
 421 CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT
 463 ATT GAT GAA AAT GGACGGCTC GAT GTT GAT GAGATG ACA AGA
 505 CAA CAT TTA GGATTT TGG TAC ACC ATG GAT CCT GCT TGC GAA
 547 AAG CTC TAC GGT GGAGCT GTC CCA TAA -3'

Apoaequorin coding sequence of mutant #436 following 5 rounds of DNA shuffling. Point mutations varying from the WT sequence are shown in bold and underlined. Sequencing performed by ACGT Corp. (Toronto, ON).

APPENDIX E

Gene Sequence of Mutant Apoaequorin #1026

5'-1 ATG GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA AAA TGG ATT
43 AGA CGA CAC AAG CAT ATG TTC AAT TTT CTT GAT GTC AAT CAC
85 AAT GGA AGA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCT
127 GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC
163 AAA CGA CAC AAA GAC GCT GTA GAA GCT TTC TTT GGA GGA GTT
211 GGA ATG AAA TAT GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC
253 GAA GGA TGG AAA AGA CTG GTT ACC GAA GAA TTG GAA AGG TAT
295 TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA
337 TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT GGAGCT ATT ACA
379 CTC GAT GAA TGG AAA GCA TAT ACC AAA TCT GCT GGCATC ATC
421 CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT
463 ATT GAT GAA AAT GGA CGG CTC GAT GTT GAT GAGATG ACA AGA
505 CAA CAT TTA GGATTT TGG TAC ACC ATG GAT CCT GCT TGC GAA
547 AAG CTC TAC GGT GGA GCT GTC CCA TAA -3'

Apoaequorin coding sequence of mutant #1026 following 5 rounds of DNA shuffling. Point mutations varying from the WT sequence are shown in bold and underlined. Sequencing performed by ACGT Corp. (Toronto, ON).

APPENDIX F

Gene Sequence of Mutant Apoaequorin #1768

5'-1 ATG GTC AAG CTT ACA TCA GAC TTC GAC AAC CCA AAA TGG ATT
 43 AGA CGA CAC AAG CAT ATG TTC AAT TTT CTT GAT GTC AAT CAC
 85 AAT GGA AGA ATC TCT CTT GAC GAG ATG GTC TAC AAG GCG TCT
 127 GAT ATT GTT ATA AAC AAT CTT GGA GCA ACA CCT GAA CAA GCC
 169 AAA CGA CAC AAA GAC GCT GTA GAA GCT TTC TTT GGA GGA GTT
 211 GGA ATG AAA TAT GGT GTA GAA ACT GAA TGG CCT GAA TAC ATC
 253 GAA GGA TGG AAA AGA CTG GTT ACC GAA GAA TTG GAA AGG TAT
 295 TCA AAA AAC CAA ATC ACA CTT ATT CGT TTA TGG GGT GAT GCA
 337 TTG TTC GAT ATC ATT GAC AAA GAC CAA AAT GGA GCT ATT ACA
 379 CTC GAT GAA TGG AAA GCA TAT ACC AAA TCT GCT GGCATC ATC
 421 CAA TCG TCA GAA GAT TGC GAG GAA ACA TTC AGA GTG TGC GAT
 463 ATT GAT GAA AAT GGA CGG CTC GAT GTT GAT GAG ATG ACA AGA
 505 CAA CAT TTA GGATTT TGG TAC ACC ATG GAT CCT GCT TGC GAA
 547 AAG CTC TAC GGT GGA GCT GTC CCA TAA -3'

Apoaequorin coding sequence of mutant #1768 following 5 rounds of DNA shuffling. Point mutations varying from the WT sequence are shown in bold and underlined. Sequencing performed by ACGT Corp. (Toronto, ON).

APPENDIX G

Luciferase Gene of the *Gaussia princeps*

5'-1 GGTACTCAAAG TATCTTCTG GCAGGGAAAA TGGGAGTGAA AGTTCTTTTT
 51 GCCCTTATTT GTATTGCTGT GGCCGAGGCC AAACCAACTG AAAACAATGA
 101 AGATTCAAC ATTGTAGCTG TAGCTAGCAA CTTTGCTACA ACGGATCTCG
 151 ATGCTGACCG TGGTAAATTG CCCGGAAAAA AATTACCACT TGAGGTACTC
 201 AAAGAAATGG AAGCCAATGC TAGGAAAGCT GGCTGCACTA GGGGATGTCT
 251 GATATGCCTG TCACACATCA AGTGTACACC CAAAATGAAG AAGTTTATCC
 301 CAGGAAGATG CCACACCTAT GAAGGAGACA AAGAAAGTGC ACAGGGAGGA
 351 ATAGGAGAGG CTATTGTTGA CATTCCTGAA ATTCCTGGGT TTAAGGATTI
 401 GGAACCCATG GAACAATTCA TTGCACAAGT TGACCTATGT GTAGACTGCA
 451 CAACTGGATG CCTCAAAGGT CTTGCCAATT GCAATGTTCT GATTTACTCA
 501 AGAAATGGCT GCCACAAAG ATGTGCAACT TTTGCTAGCA AAATTCAAGG
 551 CCAAGTGGAC AAAATAAAGG GTGCCGGTGG TGATTAATCC TAATAGAATA
 601 CTGCATAACT GGATGATGAT ATACTAGCTT ATTGCTCATA AAATGGCCAT
 651 TTTTTGTAAC AAATCGAGTC TATGTAATTC AAAATACCTA ATTAATTGTT
 701 AATACATATG TAATTCCTAT AAATATAATT TATGCAATCC AAAAAAAAAA
 751 AAAAAAAAA -3'

Complete cDNA sequence of luciferase gene isolated from *Gaussia princeps*. GenBank Accession Number AY015993. The coding sequence derived from plasmid pGLUC (Nanolight Technologies) through PCR for creation of the biotin-acceptor domain fusion protein is underlined.

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- Chemistry (59-110/131)
 - Biochemistry (59-261)
 - Intermediary Metabolism (59-360/361)

PUBLICATIONS

- Verhaegen M, Christopoulos TK. (2002) **Recombinant *Gaussia* Luciferase. Overexpression, Purification, and Analytical Application of a New Reporter for DNA Hybridization.** (submitted)
- Verhaegen M, Christopoulos TK. (2002) **Bacterial Expression of In Vivo Biotinylated Aequorin and Application to Bioluminometric Hybridization Assays.** *Anal. Biochem.* (accepted)
- Verhaegen M, Christopoulos TK. (1998) **Quantitative Polymerase Chain Reaction Based on a Dual-Analyte Chemiluminescent Hybridization Assay for Target DNA and Internal Standard.** *Anal. Chem.* **70**: 4120-4125.
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ABSTRACT POSTER PRESENTATIONS

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- Al-Anouti F, Verhaegen M, Annanvoranich S. **The Expression of Ribozyme , Antisense RNA and RNA Interference in *Toxoplasma gondii*.** Symposium of RNA Biology, RNA Target, and Tools, North Carolina, USA, Oct 2001. Poster.
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