GREEN FLUORESCENT PROTEIN

Properties, Applications, and Protocols

SECOND EDITION

Edited by
Martin Chalfie
Steven R. Kain

WILEY-INTERSCIENCE
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METHODS OF
BIOCHEMICAL ANALYSIS

Volume 47

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FIRST EDITION PREFACE

Now it is such a bizarrely improbable coincidence that anything so mind-bogglingly useful could have evolved purely by chance that some thinkers have chosen to see it as a final and clinching proof of the nonexistence of God.

Douglas Adams, Hitchhikers Guide to the Galaxy

In 1955, Davenport and Nicol reported that the light-producing cells of the jellyfish *Aequorea victoria* fluoresced green when animals were irradiated with long-wave ultraviolet. Five years later, Shimomura et al. (1962) described a protein extract from this jellyfish that could produce this fluorescence. Independently, Morin and Hastings (1971) found the same protein a few years later. This protein, now called the Green Fluorescent Protein (GFP), was studied for many years in virtual obscurity. However, with the cloning and expression of *A. victoria* GFP (Prasher et al., 1992; Chalfie et al., 1994), interest in this protein has grown enormously. To steal a phrase from a recent movie, GFP has gone from "zero to hero." As of January, 1998 at least 500 scientific publications have been published with the term "GFP" in their titles or abstracts. In the last 3 years hundreds of people have used GFP to mark proteins, cells, and organisms in a wide range of prokaryotic and eukaryotic species. They have used GFP to investigate fundamental questions in cell biology, developmental biology, neurobiology, and ecology. The interest in GFP goes beyond its utility as a biological marker. The protein is intrinsically intriguing, and investigators have sought to understand its structure, fluorescent properties, and biochemistry. This increased interest in GFP, serves as an important reminder of the usefulness of studying the biology of organisms that are not among the chosen "model" systems.

The usefulness of GFP as a biological marker derives from the finding that the protein's fluorescence requires no other cofactor: The fluorophore forms from the cyclization of the peptide backbone. This feature makes the molecule a virtually unobtrusive indicator of protein position in cells. Indeed, use of GFP as a tag suggests that the protein does not alter the normal function or localization of the fusion partner. Because permeabilization for substrate entry and fixation are not needed to localize GFP, proteins, organelles, and cells marked with this protein can be examined in living tissue. This ability to examine processes in living cells has permitted biologists to study the dynamics of cellular and developmental processes in intact tissues.

In addition to the broad impact of GFP technology on basic research, several companies have also incorporated this important reporter into more applied efforts such as high throughput drug screening, evaluation of viral vectors for human gene therapy, biological pest control, and monitoring genetically altered microbes in the environment. Most notable on this list are applications for GFP in drug discovery, here the potential for real time kinetics, ease of use, and cost savings provided by this reporter are leading to the replacement of other markers such as firefly luciferase and β-galactosidase. As the development of GFP technology continues to expand, the instrument companies are introducing new and better instruments for detecting GFP fluorescent. Finally, two U.S. patents have issued (as of July, 1997) on GFP and its variants, with many more certain to appear in the next few years.

As editors we find ourselves in the exciting, yet frustrating, position of producing a book that, in some aspects, will be out of date as it is published. The excitement comes from seeing the wealth of information being discovered about GFP and the many uses that people are finding for this molecule. The frustration results from the same source: New applications and information about GFP are published weekly, and no book on this subject can remain current. For example, as we write this preface, two papers have appeared on single molecule fluorescence of GFP (Dickson et al., 1997; Pierce et al., 1997), three on modifying GFP to measure calcium levels (Miyawaki et al., 1997; Persechini et al., 1997; Romoser et al., 1997), two on conditions that make GFP fluoresce red (Elowitz et al., 1997; Sawin and Nurse, 1997), and one on converting GFP to a voltage indicator (Siegel and Isacoff, 1997). We feel, however, that the contents of this volume serve as an important foundation for strategies that utilize GFP, and should guide the reader in using the marker in his or her system. We are in a period of rapid development of GFP as a tool for the biological sciences as people adapt the molecule for use in different organisms, generate variants with altered properties, and discover new ways that the protein can be used.

Despite the intrinsic incompleteness of this enterprise, we have asked our colleagues to summarize the state of GFP research and they have done an admirable job. We are grateful that so many of the initial investigators that pioneered the study and use of GFP consented to write chapters for this volume. We have organized this book into four sections. We start with two introductory chapters by Osamu Shimomura on the discovery of GFP and by Woody Hastings and James Morin on bioluminescence and biofluorescence in nature.

The second section describes the biochemistry and molecular biology of GFP. Bill Ward has written a very useful description of the biochemistry of GFP, pointing out both the gaps in our knowledge and the importance of physical chemical criteria for evaluating new variants of GFP. George Phillips then discusses the structure of GFP and implications of this structure for its function as a fluorescent molecule. In the last chapter in this section, Roger Tsien and Douglas Prasher describe many of these variants, their uses, and how they were derived.

The third section documents various biological applications of GFP. The people we asked to contribute these chapters are the major developers of GFP in the various organisms described. As described above these chapter are incomplete in that new information and application are developing at a very rapid rate. Nonetheless, each of these chapters provides insights into how GFP is being applied to particular species. We urge readers not to look only at the organism they love best, since approaches used for one organism may prove important when applied to others. For example, the use of species-specific codon usage, presumably by allowing greater production of protein, has been very important for GFP expression in mammalian cells. Also Andy Fire has found that GFP (and β-galactosidase) expression is elevated in the nematode *Caenorhabditis elegans* when artificial introns are interspersed in the cDNA sequence. Both of these observations may be important for those considering optimizing GFP expression in their organisms. Finally, we asked the contributors in the third section to provide protocols on the purification of GFP and its application in various organisms and Bill Ward to contribute information on purifying GFP. Sharyn Endow and David Piston have admirably taken on the formidable task of collecting, editing, and adding to this material for the fourth section of this book. In particular, they have provided outstanding protocols for visualizing and recording GFP fluorescence.

We are just beginning to learn about and use GFP, and, as always, many questions remain. Much still needs to be learned about the chemistry of fluorophore formation and

the role of the protein structure in this formation. Additional variants are needed. In particular, variants with spectra that do not significantly overlap with those of existing variants would be very useful. Such variants could be used in multiple labeling experiments, but they may have an even greater potential. Specifically, the use of fluorescence resonance energy transfer between two fluorescent proteins would enable the generation of a system analogous to the yeast two-hybrid system (Fields and Song, 1989) to look at protein: protein interactions. The advantage of such a system is that it would not require transcription as a readout of the interaction, and could therefore be used anywhere in the cell (e.g., cytosol, plasma membrane, mitochondria). Morever, suitably marked molecules would allow the testing of protein interactions in situ in a variety of organisms. Finally, as we learn more about the properties of these protein, we need to take advantage of this information to optimize GFP fluorescence intensity, excitation and emission spectra, and protein and message stability for different uses. In the next few years, we will undoubtedly see many more uses for this protein. The future does look bright for GFP.

The editors of a book have, perhaps, the easiest jobs; everyone contributes to an effort that they get the credit for. As this was the first volume that either of us had edited, we are particularly grateful for all the help that we have been given. Foremost we wish to thank the contributors who graciously consented to write chapters and then put up with our requests for rewrites and for "just a little more information" with great good humor. We are indebted to David Ades and Kaaren Janssen for starting us on this endeavor. We will get even. Finally, we are most obligated to Colette Bean, our editor at John Wiley, for showing us the ropes, keeping us on schedule, and getting us over the anxieties of producing this volume.

Martin Chalfie
Steven Kain

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PREFACE

In the preface to the first edition of this book seven years ago, we predicted that we were just beginning to see the usefulness of GFP. Although we expected many new uses for this molecule, we are amazed at the extent to which GFP, it derivatives, and similar fluorescent proteins have been used in biology today. A very approximate estimate of the general usefulness of these proteins can be seen in the number of journal articles that have citations to them. In 2004 roughly 50%, 35%, 60%, and 20% of the articles in *Cell*, *Development*, *Journal of Cell Biology*, and *Neuron*, respectively, mentioned or used these proteins (values were obtained by searching journal web site for articles with the words GFP, CFP, YFP or dsRed and then estimating the total number of articles published in the year). The fluorescent proteins are not only a general tool in basic biological research; they have also been used extensively by industry. As one unusual example, a group of entrepreneurs in the San Francisco Bay Area are pursuing a venture called Canary, Inc. which uses GFP as the basis for detecting landmines and other unexploded remnants of war. Another indicator of the growing use and importance of GFP is that this year saw the publication of the first book about GFP for the general public (Zimmer, 2005).

The last seven years have also seen the introduction of many new fluorescent proteins and derivatives. Perhaps the most striking change in the field has been the discovery of the coral fluorescent proteins (Matz et al., 1999). These proteins not only provide a wealth of new colors, but also demonstrate that these types of proteins exist in a wide range of organisms.

People continue to modify the fluorescent proteins and discover interesting new properties and uses. As the first edition was coming to press, we noted the GFP-based calcium sensors had just been developed. Now many more derivatives have been produced. One intriguing discovery was made by Ghosh et al., 2000. They split GFP into two separate polypeptioles. Coexpression of these proteins alone did not yield any fluorescence. Remarkably however, fluorescence could be reconstituted when covalently linked interacting protein domains brought the two parts of GFP together. This discovery has already led to an alternative to fluorescence resonance energy transfer (FRET) as a way of examining protein-protein interactions and a combinatorial method of labeling cells.

These and other advances rendered the first edition of this book considerably out-of-date. In the hope of bringing these more recent discoveries to the attention of a general audience, we were persuaded to edit a second edition of this book. As we noted in the preface to the first edition, trying to evaluate the field of fluorescent proteins is very difficult, because it is a moving target. The field is changing all the time. This dynamic feature of the field reflects its strength, but also means that reviews will always be incomplete. For that reason, we are delighted that so many of our previous authors were gracious enough to consider updating and rewriting their contributions. In addition, we are delighted to include as new authors, researchers who have done so much to move the field in new directions.

As for the first editions, our editors at Wiley have been particularly supportive and diligent. These people include Luna Han, who first convinced us that this enterprise was

worthwhile, and Darla Henderson and Danielle Lacourciere, who saw this second edition to completion. We are grateful for their help.

Finally, we once again look forward to being astonished by even newer uses for this remarkable collection of proteins. Judging by the past, the future continues to look bright.

Martin Chalfie
Steven Kain

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DISCOVERY OF GREEN FLUORESCENT PROTEIN

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1.1 DISCOVERY OF GFP

It was early July in 1961. Dr. Frank Johnson and I were studying the bioluminescence of the jellyfish *Aequorea aequorea* (see Section 1.4 concerning the species name) at the Friday Harbor Laboratories of the University of Washington, located on a small island near Victoria, British Columbia, Canada. Since early morning of that day, we were trying to develop a practical method to isolate the light-emitting matter of the jellyfish, a substance later named "aequorin" (cf. Shimomura et al., 1962; Shimomura, 1995a), of which we had found the basic principle of solubilization and extraction the day before. In the course of our experiments, however, I became deeply annoyed and also puzzled when I realized that the light emitted from the extract was clearly blue, contrary to our expectation of green light identical to the luminescence of live specimens.

A mature specimen of *A. aequorea* looks like a transparent, hemispherical umbrella, with its mouth at the underside of the body (Fig. 1.1, top). Average mature specimens measure 7–10 cm in diameter. Due to the high transparency of the body, the jellyfish can function as a magnifier lens when the mouth is fully open. The light organs, consisting of about 200 tiny granules, are distributed evenly along the edge of the umbrella, making a full circle. Soaking a specimen of the jellyfish in a dilute potassium chloride (KCl) solution in a darkroom causes the light organs to luminesce, exhibiting a ring of bright green light in the darkness (Fig. 1.1, bottom). If a specimen is soaked in distilled water, a green ring is first observed, which gradually changes into blue with the progress of the cytolysis of cells. Under an ultraviolet light, a specimen of fresh jellyfish exhibits a ring of brilliant green fluorescence, similar to the luminescence caused by KCl.

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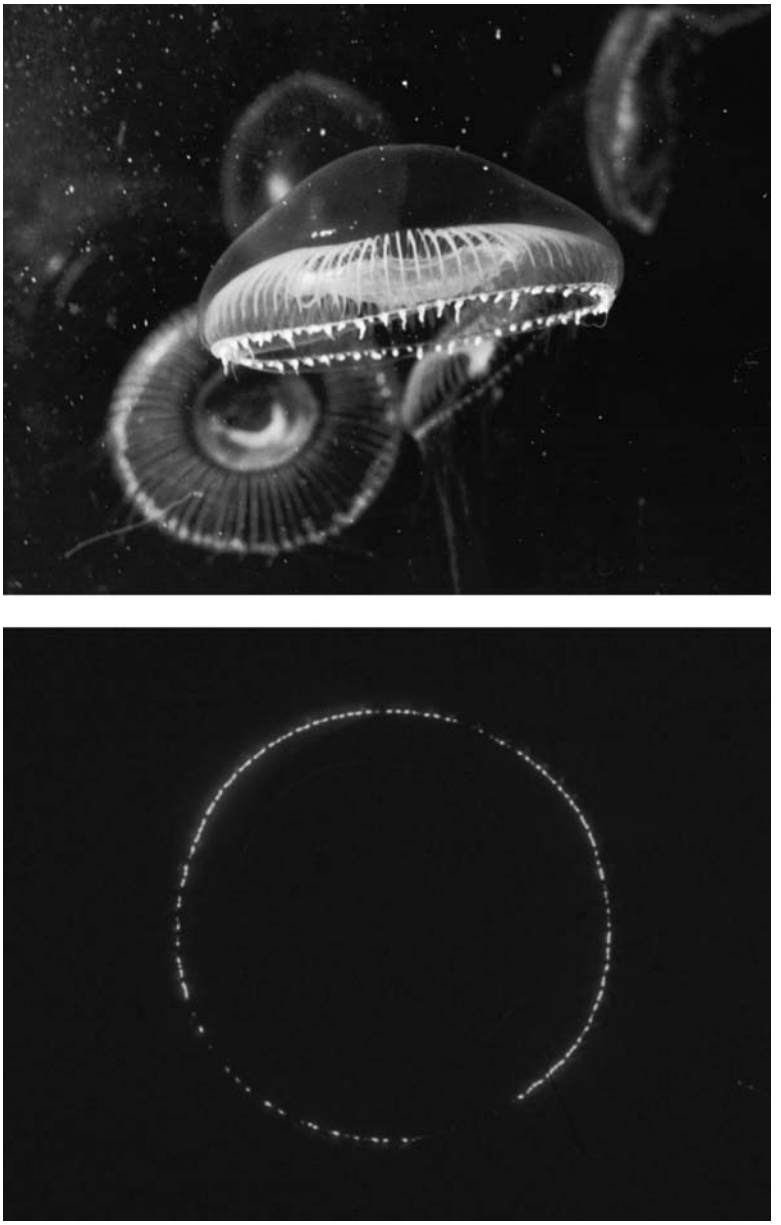

Figure 1.1. Mid-summer specimens of *Aequorea aequorea* photographed in natural environment (**top**) and in seawater supplemented with KCl in darkroom (**bottom**), both at the University of Washington's Friday Harbor Laboratories. See color insert.

The margin of the umbrella containing the light organs can be cut off with a pair of scissors, yielding a 2- to 3-mm-wide strip called the "ring." When the rings obtained from 20–30 jellyfish were squeezed through a rayon gauze, a dimly luminescent, turbid liquid called the "squeezate" is obtained. The granules of light organs in the squeezate can be collected by filtration or centrifugation. When the granules are mixed with dilute neutral

buffer solutions, they are cytolyzed and emit light. When mixed with a pH 4.0 buffer, however, the granules are cytolyzed without light emission, preserving the light-emitting activity in the solution. After the removal of cell debris by centrifugation, the pH 4.0 cell-free solution can be luminesced by the addition of a neutral buffer solution containing Ca^{2+}. These are the outline of the procedure we were doing on that day in July 1961, and I saw that the luminescence of the neutralized solution was blue, contrary to our expectation. I doubled, then tripled, the number of the jellyfish used in each experiment in order to make the final luminescence stronger and clearer, but these efforts only helped to confirm my observation. My question concerning the seeming discrepancy remained in my consciousness, until we found an explanation more than 10 years later.

After returning to Princeton University with the jellyfish extracts, we purified the light-emitting substance. The substance obtained was a protein capable of emitting light in the presence of Ca^{2+}; the protein was named aequorin. During the purification of aequorin, we noticed the existence of a green fluorescent protein in the jellyfish extract. Upon column chromatography, a green fluorescent band moved closely together with the band of aequorin on a Sephadex G-100 column and moved ahead of the aequorin band on a DEAE-cellulose column. Although the presence of a green fluorescent substance in the light organs was previously known (Davenport and Nicol, 1955), it was the first time that the substance was isolated and recognized to be a protein. Our observation was mentioned in our first full article on the purification and characterization of aequorin (Shimomura et al., 1962), in a footnote, as follows:

> A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has also been isolated from the squeezates. No indications of a luminescent reaction of this substance could be detected.

The first measurements of the luminescence spectrum of aequorin and the fluorescence spectrum of the green protein were reported quickly (Johnson et al., 1962). The luminescence spectrum of aequorin was broad, with a peak at 460 nm. The fluorescence spectrum of the green protein was sharp, with a peak at 508 nm. Apparently, the light organs of the jellyfish contain these two proteins, aequorin and the green protein, of which the former emits blue light in the presence of Ca^{2+} and the latter emits green fluorescence when excited. The green protein was later called green fluorescent protein, GFP (Hastings and Morin, 1969). One average-sized specimen contains 20–30 μg of aequorin (Shimomura and Johnson, 1979), and each of its about 200 light organs contains approximately 0.1 μg of aequorin and 0.02 μg of GFP (Morise et al., 1974; Cutler, 1995).

How can a protein, aequorin, luminesce just by the addition of Ca^{2+}, even in the absence of oxygen? Why is the luminescence of a live jellyfish green, while aequorin emits blue light? Regarding the first question, it seems clear that the luminescence is produced by an intramolecular chemical reaction of aequorin triggered by Ca^{2+}. Thus, we would need to understand the mechanism of this intramolecular reaction, which was a formidable task to accomplish at the time. To answer the second question, it would be necessary to consider two possibilities: (1) a filtering effect by the green protein or something else that shifts the emission maximum of aequorin luminescence to longer wavelength and (2) an energy transfer from aequorin molecules to the green protein by a certain mechanism. Considering that the fluorescent protein was created by nature presumably under some selective pressure, the possibility of an energy transfer would be more likely. In those days, however, we were not concerned with the details of the energy-transfer mechanism;

we merely assumed that the green protein absorbed the blue light of aequorin, and then reemitted the absorbed energy as green light (i.e., an energy transfer by the trivial mechanism). We deferred the studies of these subjects for the next 5 years, because of various reasons.

Aequorin is an unusual protein that contains an energy-producing source for light emission inside the molecule, resembling a luciferin in this respect. However, it seemed inappropriate to designate aequorin a luciferin because of its heat-labile and nondiffusible nature. In 1965, we discovered the second example of a bioluminescent protein that contains the energy source of luminescence in the molecule from the marine tubeworm *Chaetopterus*, and we proposed to use the general term "photoprotein" to refer to this type of protein (Shimomura and Johnson, 1966). Thus, a photoprotein is a naturally occurring bioluminescent protein that is capable of emitting light in proportion to the amount of the protein (Shimomura, 1984). The term is now widely used, and many different kinds of photoprotein are presently known—for example, Ca^{2+}-sensitive photoproteins from coelenterates (aequorin, obelin, mnemiopsin) and protozoa (thalassicolin); superoxide-activated photoproteins from scaleworm (polynoidin) and the clam *Pholas* (pholasin); and an ATP-activated photoprotein from a Sequoia millipede *Luminodesmus* (Shimomura, 1984).

1.2 ISOLATION AND PROPERTIES OF THE GREEN FLUORESCENT PROTEIN

Ridgway and Ashley (1967) reported the first successful application of aequorin bioluminescence. They microinjected aequorin into barnacle muscle single fibers, and they monitored the concentration changes of Ca^{2+} that occur during muscle contraction. The study clearly demonstrated the usefulness and importance of aequorin in the studies of intracellular calcium, causing a rush of requests for this photoprotein. For the efficient and productive use of aequorin, detailed knowledge on the properties of aequorin and the mechanism of light emission became necessary. Thus, we decided to try to solve the chemical mechanism of aequorin luminescence, an intramolecular reaction. It seemed to be an exceedingly difficult, almost unachievable undertaking at the time. After several years of strenuous efforts, however, we had the luck to be able to uncover a large part of the intramolecular chemistry involved in the Ca^{2+} triggered luminescence of aequorin, including the chemical structure of the functional moiety "coelenterazine" in the protein and also the means to regenerate spent aequorin into the original, active aequorin (Shimomura and Johnson, 1969, 1972, 1973, 1975).

During the same period, green fluorescent proteins similar to *Aequorea* GFP were found in a number of other bioluminescent coelenterates (Hastings and Morin, 1969; Morin and Hastings, 1971a,b; Wampler et al., 1971, 1973; Cormier et al., 1973, 1974; Morin, 1974); those green fluorescent proteins apparently function as the light emitter of *in vivo* bioluminescence, as in the case of *Aequorea*. Green fluorescent protein was not found in the jellyfish of Scyphozoa (such as *Pelagia* and *Periphylla*) and Ctenophora (such as *Mnemiopsis* and *Beroë*). The following genera of bioluminescent coelenterates contain GFP:

Class Hydrozoa
- The jellyfish *Aequorea*
- The jellyfish *Mitrocoma* (synonym *Halistaura*)
- The hydroid *Obelia*
- The jellyfish *Phialidium* (hydroid *Clytia*)

Class Anthozoa
Acanthoptilum
The sea cactus *Cavernularia*
The sea pansy *Renilla*
The sea pen *Ptilosarcus* and *Pennatula*
Stylatula

Concerning the mechanism of energy transfer from the excited state of photoprotein molecule to GFP molecule, Morin and Hastings (1971b) suggested for the first time that the mechanism of coelenterate bioluminescence possibly involves the Förster-type radiationless energy transfer.

To clarify the mechanism of energy transfer involved in the emission of green light from the jellyfish *Aequorea*, we isolated and purified the green fluorescent protein from the jellyfish, and then we studied its properties in detail (Morise et al., 1974). The purified *Aequorea* GFP was easily crystallized by decreasing the ionic strength of the solvent (Fig. 1.2). We investigated the energy transfer from aequorin molecule to GFP molecule during the Ca^{2+}-triggered luminescence reaction of aequorin, under two sets of conditions: one with high concentrations of GFP (1.7–5.5 mg/ml) and the other with relatively low concentrations of GFP (0.15–1.1 mg/ml).

In the presence of the high concentrations of GFP, apparently an energy transfer by the trivial (radiative) mechanism takes place, at least to some extent. Namely, the light emitted from aequorin (emission λ_{max} 460 nm) is absorbed by GFP (λ_{max} 400 and 480 nm),

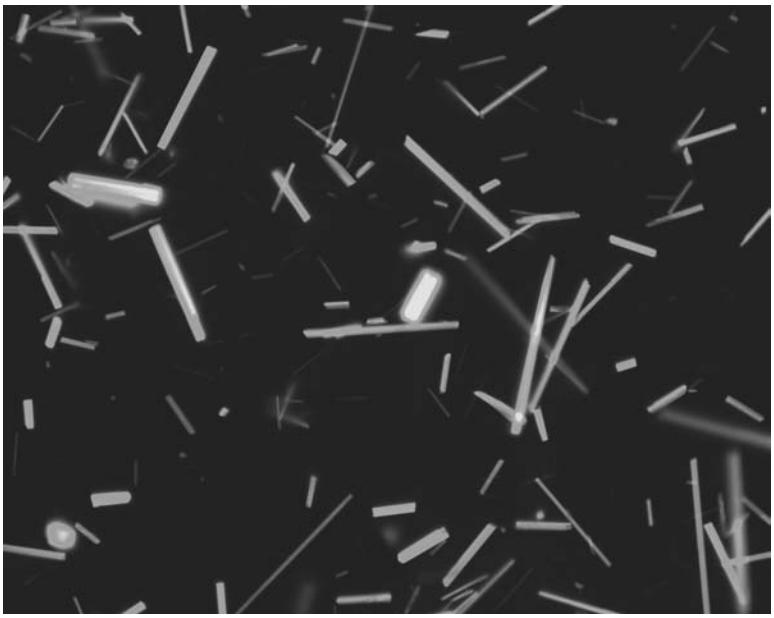

Figure 1.2. The fluorescence photomicrograph of the crystals of *Aequorea* GFP formed in a low ionic strength aqueous solution, by dialysis against pure water. The fluorescence of GFP crystal is strongly anisotropic (Inoué et al., 2002). The view field shown is about 0.5 mm wide. Photograph by Dr. Shinya Inoué.

followed by reemission of the absorbed energy from GFP as fluorescence (emission λ_{max} 509 nm). In this mechanism, the extent of energy transfer and the spectral shape of emitted light are dependent on the GFP concentration. It is clear, however, that GFP cannot absorb all the light emitted from aequorin, because the luminescence emission of aequorin extends to about 600 nm on the red side of wavelength whereas GFP can absorb light up to only about 510 nm. Therefore, a complete energy transfer by the trivial mechanism is clearly impossible. In any event, a very high concentration of GFP (with a very high level of absorbance) is required to obtain a significant extent of energy transfer by the trivial mechanism. Under such a condition, the self-absorption of GFP would strongly affect the spectral shape of the fluorescence emitted from GFP, in two ways: (1) a very steep decrease in the light intensities below 510 nm and (2) a red shift of the fluorescence peak position. The actual luminescence spectrum should be the sum of the aequorin luminescence unabsorbed by GFP and the GFP fluorescence distorted by self-absorption; it would be unthinkable that such a spectrum coincides with the true, undistorted spectrum of GFP fluorescence or with the luminescence from the live *Aequorea*.

When aequorin was luminesced with Ca^{2+} in a low ionic strength buffer (10 mM sodium phosphate) in the presence of relatively low concentrations of GFP (about 0.15 mg/ml), the emission spectrum of aequorin was little affected by GFP. However, when a small amount of fine particles of diethylaminoethyl (DEAE) cellulose or DEAE Sephadex (anion exchangers) was mixed in advance to the same solution, the Ca^{2+}-triggered luminescence of the clouded mixture became spectrally identical with the *in vivo* bioluminescence of *Aequorea*, indicating the occurrence of an efficient energy transfer from the aequorin light emitter to GFP. It should be pointed out that the amounts of aequorin and GFP, as well as the volume used, were kept equal in the aforementioned experiments (i.e., the overall concentrations and the absorbance values were unchanged); the only difference was the DEAE material added in the latter experiment.

The interpretation of the above-mentioned finding is as follows. Under the conditions used, the DEAE cellulose particles had co-adsorbed GFP and aequorin by anion exchange mechanism, greatly increasing the local concentrations of the two proteins around the particles. The co-adsorption perhaps made the distance between the GFP molecules and the aequorin molecules sufficiently short (roughly 30 Å) to make the Förster-type (radiationless) energy transfer workable. Thus, the result observed was the green light that spectrally matches with the *in vivo* luminescence and the fluorescence emission of GFP. Because the radiationless process is not significantly influenced by the concentration of GFP and does not require a very high concentration of GFP, the energy transfer can take place without being significantly affected by the absorbance of GFP. On the basis of the above experiments and discussion, the energy transfer involved in the emission of green light from live *Aequorea* is considered to be mostly, if not entirely, a radiationless process.

The quantum yield of the Ca^{2+}-triggered aequorin luminescence is approximately 0.16 at 23–24°C (Shimomura and Johnson, 1970; Shimomura, 1986), and that of aequorin co-adsorbed with GFP is the same as that of aequorin alone (Morise et al., 1974).

In a live specimen of *Aequorea*, each light organ ($0.4 \times 0.2 \times 0.1$ mm) is packed with photogenic cells (average size 10 μm), and each photogenic cell is again densely packed with fine particles (diameter 0.5 μm), according to Davenport and Nicol (1955). It is believed that these particles contain high concentrations of aequorin and GFP. In the particles, aequorin molecules and GFP molecules must be very closely and tightly arranged, if they are not directly bound to each other, to allow an efficient energy transfer by a radiationless process. In fact, the concentration of aequorin and GFP in the photogenic cells are previously estimated to be 5% each, or 10% altogether, of the weight of the cells

(Morise et al., 1974). In a more recent estimate, the concentration of GFP was estimated at 2.5% (Cutler, 1995).

Another kind of green fluorescent protein, the GFP of the sea pansy *Renilla*, was purified and physicochemically characterized (Ward and Cormier, 1979). There are substantial differences between the bioluminescence systems of *Aequorea* and *Renilla*, though in both systems the light energy is provided by the oxidation of coelenterazine. The *in vivo* bioluminescence reaction of *Renilla* requires coelenterazine (the luciferin), *Renilla* luciferase, *Renilla* GFP, and molecular oxygen, whereas that of *Aequorea* requires only aequorin, Ca^{2+}, and *Aequorea* GFP. The fluorescence emission peak of *Renilla* GFP (509 nm) is identical to that of *Aequorea* GFP, but its absorption spectrum (λ_{max} 498 nm) is markedly different from that of *Aequorea* GFP (λ_{max} 400 nm and 480 nm).

Addition of coelenterazine to a solution containing *Renilla* luciferase results in the emission of blue light. However, when *Renilla* GFP has been added to the luciferase solution before the addition of coelenterazine, green luminescence is emitted with a threefold increase in the quantum yield, clearly indicating the occurrence of radiationless energy transfer (Ward and Cormier, 1979). Thus, in the case of *Renilla*, there must be a sufficiently strong binding affinity between the molecules of luciferase and GFP, to make the distance between the chromophores sufficiently short for the energy transfer by radiationless process. It appears that the affinity between *Renilla* luciferase and *Renilla* GFP is much greater than that between aequorin and *Aequorea* GFP.

The fluorescence quantum yields of *Aequorea* GFP and *Renilla* GFP are nearly equal in a range of 0.7–0.8 (Morise et al., 1974; Kurian et al., 1994; Chapter 4, this volume). However, *Renilla* GFP significantly increases the quantum yield of bioluminescence, but *Aequorea* GFP does not, as noted earlier. The difference must come mainly from the difference in the fluorescence quantum yields of coelenterazine light-emitters in the two systems, on the basis of the following discussion.

The quantum yield of bioluminescence, Q_{bl}, can be expressed, in a practical way, as the product of the yield of the excited state generated, E, and the fluorescence quantum yield of the light emitter, Q_f. Thus, $Q_{bl} = EQ_f$, where the values of Q_{bl}, E, and Q_f cannot exceed 1. In the Ca^{2+}-triggered light emission of aequorin, the photoprotein is decomposed into apoaequorin, coelenteramide, and carbon dioxide, wherein apoaequorin, coelenteramide, and calcium ions form a complex called the blue fluorescent protein "BFP" (Shimomura and Johnson, 1970). The aequorin luminescence is emitted from BFP or, more precisely, from the amide anion of coelenteramide in excited state (i.e., coelenterazine light emitter) bound to apoaequorin in BFP (Hori et al., 1973; Shimomura, 1995b). The quantum yield Q_{bl} of aequorin luminescence is 0.16 as already noted. The fluorescence quantum yield Q_f of BFP measured a few seconds after the light emission was 0.12, but this value is considered to be inaccurate on the basis that BFP is a dissociable equilibrium complex and also that the conformation of apoaequorin changes after the light-emitting reaction (Morise et al., 1974). At present, there seems to be no way to measure an accurate value of Q_f at the moment of light emission in the case of aequorin luminescence. However, because the Q_{bl} of aequorin luminescence and the Q_{bl} of the luminescence of aequorin coadsorbed with GFP are equal (0.16), the Q_f of BFP at the moment of light emission should be equal to that of GFP (0.7–0.8) when the energy transfer from BFP to GFP is 100%.

In the luciferase-catalyzed *Renilla* bioluminescence, quantum yield Q_{bl} is increased three times by the addition of *Renilla* GFP, and the fluorescence quantum yield Q_f of *Renilla* GFP is 0.7–0.8 as already noted. The bioluminescence quantum yield Q_{bl} in the absence of GFP was reported at 0.055 (Matthews et al., 1977) and 0.1 (Inouye and

Shimomura, 1997). Assuming the energy transfer at 100%, the fluorescence quantum yield Q_f of the coelenterazine light-emitter of the *Renilla* system is calculated to be approximately 0.15–0.27, which is significantly lower than the Q_f value for the coelenterazine light emitter in BFP (0.7–0.8).

Regarding the nature of the chromophore, it is believed that the GFPs of *Aequorea*, *Renilla*, and many other coelenterates contain an identical chromophore (Ward and Cormier, 1978; Ward et al., 1980); the only exception presently known is the GFP of the jellyfish *Phialidium* that shows a blue-shifted fluorescence emission peak at 497 nm (Levine and Ward, 1982).

1.3 DISCOVERY OF THE STRUCTURE OF GFP CHROMOPHORE

In 1979, I was interested in the chemical structure of the chromophore of *Aequorea* GFP, which had never been studied before. From a papain digest of heat-denatured GFP, I isolated a small peptide containing the chromophore. I synthesized a model compound of the chromophore. Based on the resemblance between this model compound and the chromophore of the peptide, I was able to deduce the structure of the GFP chromophore to be the structure **D** in Fig. 1.3 (Shimomura, 1979). It might look as though I were very lucky in my guesswork, because the data obtained from the peptide were clearly insufficient to elucidate the structure of the chromophore. In fact, several people questioned me as to how I could guess the imidazolone structure. The truth is that I was certainly lucky, but not only in my guesswork.

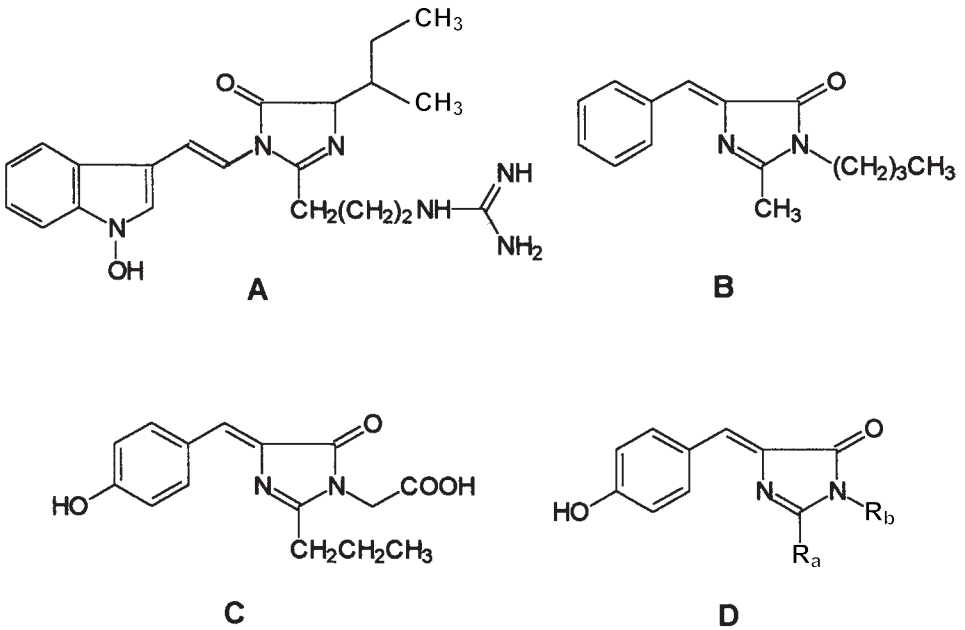

Figure 1.3. (**A**) A tentative structure of *Cypridina* luciferin proposed in 1959. (**B**) One of the model compounds synthesized to test the feasibility of the structure **A**. (**C**) A model compound of GFP chromophore synthesized. (**D**) The chromophore of GFP proposed in 1979. Both R_a and R_b are peptide residues.

In the period of the late 1950s, I was studying the structure of the luciferin of the ostracod *Cypridina* at the laboratory of Professor Y. Hirata, Nagoya University. The techniques for structure determination available at the time were not as sophisticated as at present. The modern techniques that would produce clear-cut information, such as nuclear magnetic resonance (NMR), high-resolution mass spectroscopy, and high-performance liquid chromatography (HPLC), were not available. In an early stage of our study on *Cypridina* luciferin, we arrived at a tentative structure that contained an imidazolone ring, **A** (Hirata et al., 1959). To test the absorption spectrum of this tentative structure, we synthesized various imidazolone compounds that contained one double bond conjugated with the imidazolone ring (Shimomura and Eguchi, 1960), although the results eventually showed that structure **A** was incorrect. Compound **B** was one of the imidazolones synthesized at that time. When I obtained the chromophore-bearing peptide from *Aequorea* GFP in 1979, I immediately noticed a close resemblance in spectroscopic and other properties between the chromophore-bearing peptide obtained from GFP and the imidazolone compound **B**, which was synthesized some 20 years before. A small difference found in the wavelength of the absorption peak was thought to be the effect of a phenolic OH, based on the evidence that acid hydrolysis of the peptide yielded *p*-hydroxybenzaldehyde. I synthesized a new model compound **C**. The spectroscopic properties of compound **C** were in satisfactory agreement with those of the peptide. Thus, structure **D** was proposed as the chromophore of GFP (Shimomura, 1979). The chromophore structure was confirmed later to be correct, although the side chains were different (Cody et al., 1993).

I learned in 1979 that W. W. Ward of Rutgers University, the pioneer of the isolation of the photosensitive ctenophore photoproteins (Ward and Seliger, 1974a,b), had been working on *Aequorea* GFP in addition to *Renilla* GFP. I thought my role was over and decided to discontinue my work on GFP. Since then, the work on *Aequorea* GFP by Ward and others has steadily progressed, finally developing into the successful cloning of GFP (Prasher et al., 1992), a memorable event that established the basis of using GFP. The cloning was soon followed by the expression of GFP in living organisms by Chalfie et al. (1994) that triggered the explosive popularity of GFP and made the foundation of the present volume.

1.4 A NOTE ON THE SPECIES NAME OF THE JELLYFISH FROM WHICH AEQUORIN AND GFP WERE ISOLATED

This brief discussion concerning the names of *Aequorea* species is included here in consideration of the problems and confusions induced by the recent common use of the species name *Aequorea victoria* in place of *Aequorea aequorea* (and *Aequorea forskalea*). The species names *A. aequorea* (Forskal, 1775) and *A. forskalea* (Peron and Lesueur, 1809) are synonymous, and both names have been commonly used; the decision of priority between them appears to be a matter of opinion. The species *A. aequorea* is highly variable in both form and color (Mayer, 1910) and is distributed very widely—Mediterranean; Atlantic coasts, from Norway to South Africa and Cape Cod to Florida; northeastern Pacific; east coast of Australia; and Iranian Gulf (Kramp, 1968). According to Mayer (1910), *A. victoria* (Murbach and Shearer, 1902) from the northeastern Pacific is probably a variety of *A. aequorea*. He stated "I cannot distinguish this medusa from *Aequorea forskalea* of the Atlantic and Mediterranean. Were it described from the Atlantic I would not hesitate to designate it *A. forskalea*." Mayer's opinion has been overwhelmingly accepted until recently (Russell, 1953; Kramp, 1965, 1968); thus the jellyfish we collected

in the Friday Harbor area have been called *A. aequorea*. The situation changed, however, after Arai and Brinckmann-Voss (1980) reported their conclusion to separate *A. victoria* from the species *A. aequorea*, based on their study of about 40 specimens collected from more than 10 different areas around Vancouver Island (Friday Harbor included). Their reasons were that *A. victoria* has much more regularly serrated mouth lobes and a much larger (thus, taller), almost hemispherical lens in the stomach region, when compared with *A. aequorea* from the Mediterranean.

It is not clear in the Arai and Brinckmann-Voss article why the conclusion to separate *A. victoria* from *A. aequorea* was made on the basis of the comparison between the former (from British Columbia and Puget Sound) and the latter from *only* the Mediterranean; their use of only a few specimens per study area, collected probably on a single occasion, brings about another problem. It has been well documented that a wide intraspecific variation of *A. aequorea* by geography exists (Mayer, 1910; Russell, 1953; Kramp, 1959, 1965). The Mediterranean form of *A. aequorea* is only one of many variations of this species. Therefore, the difference between *A. victoria* and *A. aequorea* cannot be fully determined by the comparison between the former and the latter from only the Mediterranean; to determine the difference, *A. victoria* should be compared with various other forms of *A. aequorea*. It seems particularly intriguing to compare *A. victoria* with the varieties of *A. aequorea* from the northern and western Atlantic. In our record, we collected a large number of *A. aequorea* at Woods Hole, MA, in the summer of 1987, when there was a strong easterly wind; those medusae appeared to be indistinguishable from the average specimens of *Aequorea* obtained at Friday Harbor in both form and the composition of aequorin isoforms.

Most of the several million specimens of *Aequorea* used for biochemical research had been collected around Friday Harbor, where the specimens were extremely abundant at least until 1988 (since then, they virtually disappeared from the area for unknown reason). If all those medusae were a single species of *A. victoria*, as implied by Arai and Brinckmann-Voss (1980), it seems that *A. victoria* must have a very wide variation, like *A. aequorea*. We have collected over 1 million specimens of *Aequorea* in the vicinity of Friday Harbor in 17 summers between 1961 and 1988. All the specimens were mature (>7 cm in diameter), and they were collected, handled, and excised individually. More than several times during our operation, we observed pronounced changes in the form of the jellyfish collected. The jellyfish can drift far and widely by current, tide, and wind in groups, and the changes that we observed usually lasted for only a few days but occasionally continued for several weeks. The bell height of the medusae were sometimes markedly higher than usual relative to the diameter (thus taller than hemispherical), and sometimes much flatter and saucer-like. In one of these occasions, we thought that the jellyfish we had collected were a wrong species because they were too flat; we suspended our operation until we had an assurance by a jellyfish expert in the lab that they were indeed a variety of *A. aequorea* (as known then). If all those medusae at Friday Harbor are the variations of *A. victoria*, the situation would be very confusing. Because both species have very wide variations without any clear difference between them, distinction of the two species would be extremely difficult. A detailed discussion on the species name of *Aequorea* is given by Claudia E. Mills (2003).

Despite the high intraspecific variability that causes confusions, the species names *A. aequorea* and *A. forskalea* have been accepted and used by the majority of researchers for the period of at least 60 years until 1980. To avoid further confusion, and with reference to discussion in other chapters of this volume, the name *A. victoria* should be considered as a synonym, not as the name of a separate species, until the difference between *A.*

victoria and *A. aequorea* (or *A. forskalea*) is firmly established on genetic basis. Until that time, the species name *A. aequorea* (or *A. forskalea*) should have priority.

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