DISCOVERY OF GREEN FLUORESCENT PROTEIN, GFP

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by

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PROLOGUE

I discovered the green fluorescent protein GFP from the jellyfish *Aequorea aequorea* in 1961 as a byproduct of the Ca-sensitive photoprotein aequorin (Shimomura *et al.*, 1962; Johnson *et al.*, 1962), and identified its chromophore in 1979 (Shimomura, 1979). GFP was a beautiful protein but it remained useless for the next 30 years after the discovery.

My story begins in 1945, the year the city of Nagasaki was destroyed by an atomic bomb and World War II ended. At that time I was a 16-year old high school student, and I was working at a factory about 15 km northeast of Nagasaki. I watched the B-29 that carried the atomic bomb heading toward Nagasaki, then soon I was exposed to a blinding bright flash and a strong pressure wave that were caused by a gigantic explosion. I was lucky to survive the war. In the mess after the war, however, I could not find any school to attend. I idled for 2 years, and then I learned that the pharmacy school of Nagasaki Medical College, which had been completely destroyed by the atomic bomb, was going to open a temporary campus near my home. I applied to the pharmacy school and was accepted. Although I didn't have any interest in pharmacy, it was the only way that I could have some education.

After graduating from the pharmacy school, I worked as a teaching assistant at the same school, which was reorganized as a part of Nagasaki University. My boss Professor Shungo Yasunaga was a gentle and very kind person. In 1955, when I had worked for four years on the job, he arranged for me a paid leave of absence for one year, and he sent me to Nagoya University, to study at the laboratory of Professor Yoshimasa Hirata.

CYPRIDINA LUCIFERIN

The research subject that Professor Hirata gave me was the bioluminescence of the crustacean ostracod *Cypridina hilgendorfii*. *Cypridina* emits blue light when its luciferin is oxidized in the presence of an enzyme luciferase and molecular oxygen (Fig. 1). The luciferin had been studied for many years

at Newton Harvey's laboratory at Princeton University (Harvey, 1952), but it had never been completely purified, due to its extreme instability. Prof. Hirata wanted to determine the structure of the luciferin of *Cypridina*, and he asked me to purify the luciferin and to crystallize it, because crystallization was the only way to confirm the purity of substances at the time.

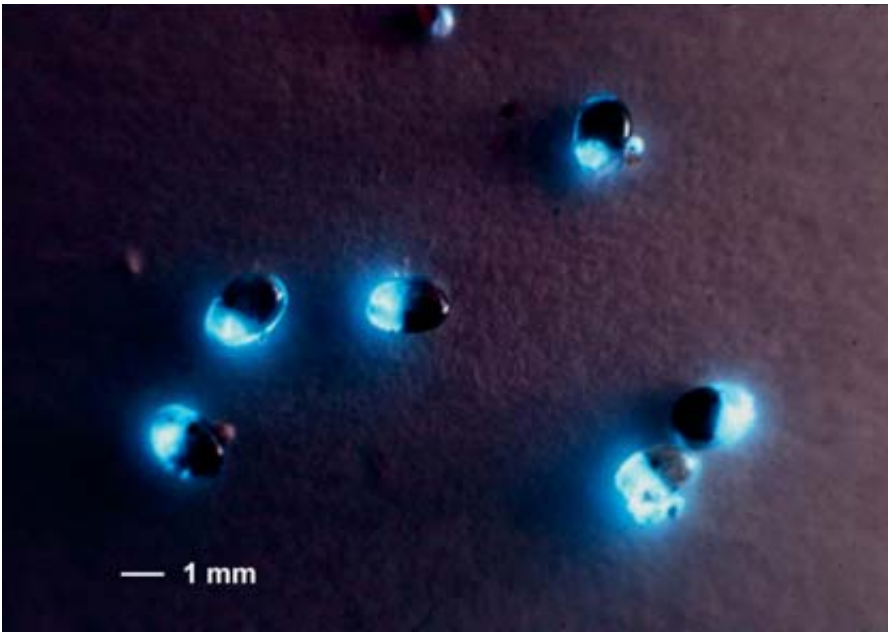

Figure 1. The ostracod *Cypridina hilgendorfii* freshly caught, and placed on a dark surface.

Using 500 g of dried *Cypridina* (about 2.5 kg before drying), I began the extraction and purification of luciferin in an atmosphere of purified hydrogen using a large specially made Soxhlet apparatus (Fig. 2). After 5 days of day-and-night work, 500 g of dried *Cypridina* yielded about 2 mg of luciferin after purification. I tried to crystallize the purified luciferin, but all my efforts ended up with amorphous precipitates, and any leftover luciferin became useless by oxidation by the next morning. So I had to repeat the extraction and purification again and again. I worked very hard, and tried every method of crystallization that I could think of, without success. Ten months later, however, I finally found that the luciferin could be crystallized in a highly unusual solvent (Shimomura *et al.*, 1957). The solvent I found was a high concentration of hydrochloric acid. Using the crystallized luciferin, we were able to determine the chemical structures of the luciferin and its oxidation products (Kishi *et al.*, 1966; Fig. 3). Those data became essential later in the study of aequorin.

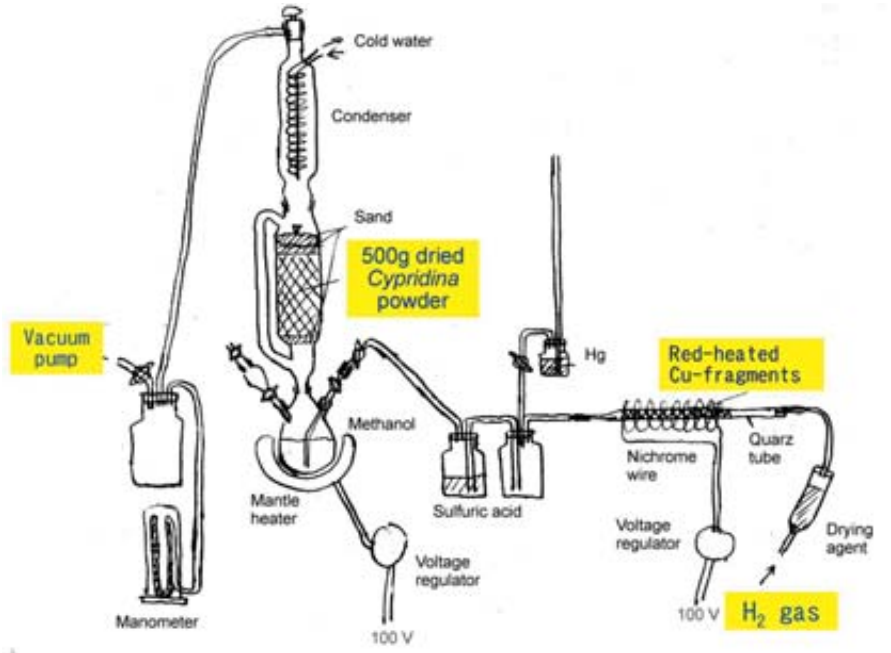

Figure 2. The apparatus used for extracting *Cypridina* luciferin.

In 1959, Professor Frank Johnson invited me to work at his Princeton laboratory owing to my success in *Cypridina* work. In September 1960, shortly after my arrival at Princeton, Dr. Johnson asked me if I would be interested in studying the bioluminescence of the jellyfish *Aequorea*. I was strongly impressed by his description of the brilliant luminescence and the abundance of the jellyfish at Friday Harbor in the state of Washington. I agreed to study the jellyfish.

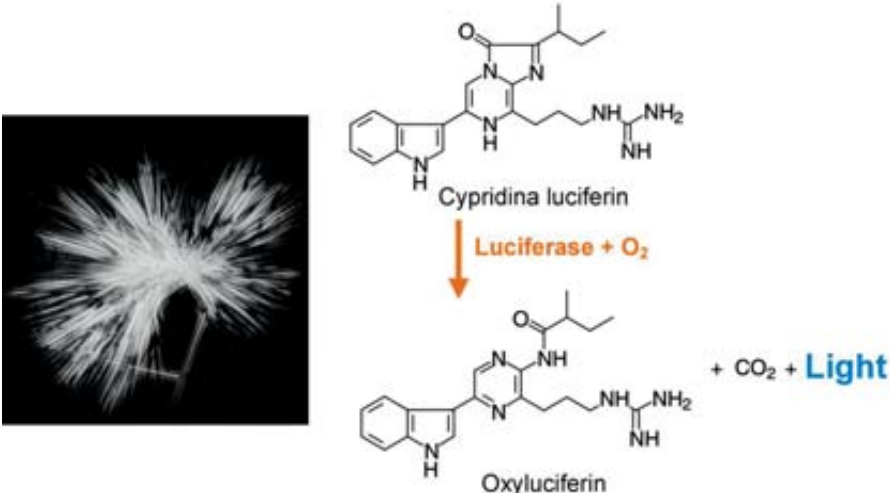

Figure 3. Crystals of *Cypridina* luciferin (left), and the chemical structures of *Cypridina* luciferin and its luminescence reaction product, oxyluciferin (right).

THE JELLYFISH *AEQUOREA* AND THE PHOTOPROTEIN AEQUORIN

Early in the summer of 1961, we traveled from Princeton, NJ, to Friday Harbor, WA, driving 5,000 kilometers. Friday Harbor was a quiet, peaceful small village at the time (Fig. 4). The jellyfish were abundant in the water (Fig. 5). At the University of Washington laboratory there, we carefully scooped up the jellyfish one by one using a shallow dip net. The light organs of *Aequorea aequorea* (Fig. 6) are located along the edge of the umbrella, which we called a ring. The ring could be cut off with a pair of scissors, eliminating most of the unnecessary body part.

Figure 4. Friday Harbor, 1961. The University of Washington Laboratory is located on the opposite shore of the bay, at left center in the picture. By 1980, about one third of the bay in the foreground had been turned into a marina.

At the time, it was a common belief that the light of all bioluminescent organisms was produced by the reaction of luciferin and luciferase. Therefore, we tried to extract luciferin and luciferase from the rings of the jellyfish. We tried every method we could think of, but all our efforts failed. After only a few days of work, we ran out of ideas.

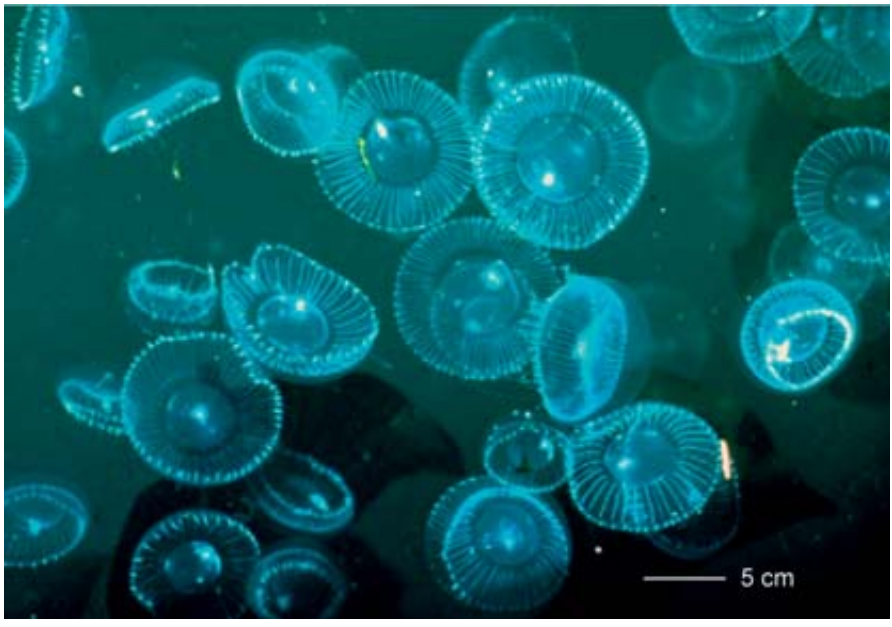

Figure 5. The jellyfish *Aequorea aequorea* in nature.

I was convinced that the cause of our failure was the luciferin-luciferase hypothesis that dominated our mind. I suggested to Dr. Johnson that we forget the idea of extracting luciferin and luciferase and, instead, try to extract a luminescent substance whatever it might be. However, I was unable to convince him. Because of the disagreement on experimental method, I started to work alone at one side of a table, while, on the other side, Dr. Johnson and his assistant continued their efforts to extract a luciferin. It was an awkward, uncomfortable situation.

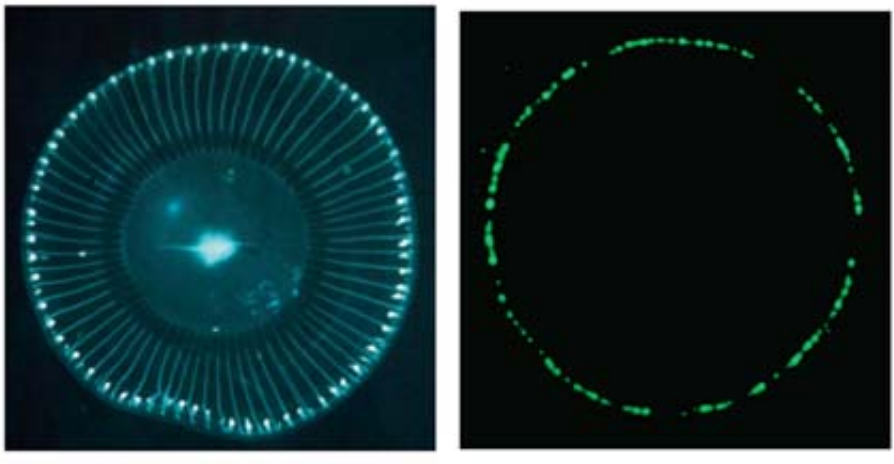

Figure 6. Top view of a specimen of *Aequorea aequorea* in daylight at sea (left), and when stimulated in a darkroom (right).

Since the emission of light means the consumption (loss) of active bioluminescent substance, the extraction of bioluminescent substances from light organs must be performed under a condition that reversibly inhibits the luminescence reaction. Therefore, I tried to reversibly inhibit luminescence with various kinds of inhibitors of enzymes and proteins. I tried very hard, but nothing worked. I spent the next several days soul-searching, trying to find out something missing in my experiments and in my thought. I thought day and night. I often took a rowboat out to the middle of the bay to avoid interference by people. One afternoon, an idea suddenly struck me on the boat. It was a very simple idea: "Luminescence reaction probably involves a protein. If so, luminescence might be reversibly inhibited at a certain pH."

I immediately went back to the lab and tested the luminescence of light organs at various pHs. I clearly saw luminescence at pH 7, 6 and 5, but not at pH 4. I ground the light organs in a pH 4 buffer, and then filtered the mixture. The cell-free filtrate was nearly dark. But it regained luminescence when it was neutralized with sodium bicarbonate. The experiment showed that I could extract the luminescence substance, at least in principle (Fig. 7).

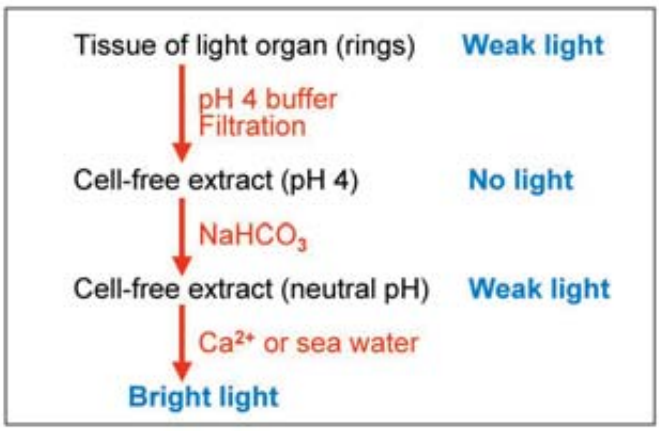

Figure 7. The process that revealed Ca^{2+} to be the activator of luminescence reaction.

But a big surprise came the next moment. When I threw the extract into a sink, the inside of the sink lit up with a bright blue flash. The overflow of an aquarium was flowing into the sink, so I figured out that seawater had caused the luminescence. Because the composition of seawater is known, I easily found out that Ca^{2+} activated the luminescence. The discovery of Ca^{2+} as the activator suggested that the luminescence material could be extracted utilizing the Ca-chelator EDTA, and we devised an extraction method of the luminescent substance (Fig. 8).

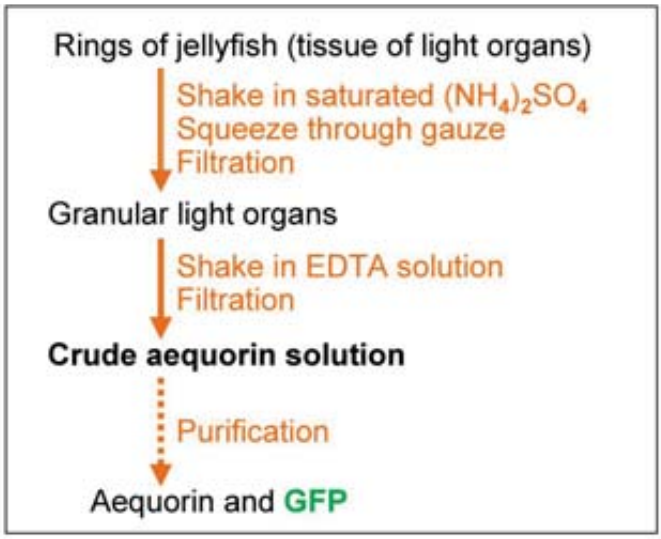

Figure 8. Procedure for extraction of aequorin and GFP.

During the rest of the summer of 1961, we extracted the luminescent substance from about 10,000 jellyfish. After returning to Princeton, we purified the luminescent substance and obtained a few milligrams of purified protein. The protein emitted blue light in the presence of a trace of Ca^{2+}. We named the protein aequorin (Shimomura *et al.*, 1962). Aequorin was the first example of photoproteins discovered (Shimomura, 1985). During the purification of aequorin, we found another protein that exhibited a bright green fluorescence. It was only in a trace amount, but we purified this protein too, and called it "green protein." The protein was renamed "green fluorescent protein" by Morin and Hastings (1971).

We wanted to understand the mechanism of the aequorin bioluminescence reaction; because it became clear in 1967 that aequorin was highly useful and important as a calcium probe in biological studies (Ridgway and Ashley, 1967). First, we tried to isolate the light-emitting chromophore of aequorin. However, there was no way to extract the native chromophore (Shimomura and Johnson, 1969; Shimomura *et al.*, 1974), because any attempt to extract the chromophore always resulted in an intramolecular reaction of aequorin that triggered the emission of light, destroying the original chromophore. Indeed, the secret of light emission of *Aequorea* was well protected.

We nevertheless found that a fluorescent compound was formed when aequorin was denatured with urea in the presence of 2-mercaptoethanol (Shimomura and Johnson, 1969). We named this fluorescent compound AF-350, based on its absorption maximum at 350 nm. We decided to determine the structure of AF-350. However, to obtain the 1 mg of AF-350 needed for a single experiment toward the structural study of this compound, about 150 mg of purified aequorin was needed, and that meant we had to collect and extract at least 50,000 jellyfish. Considering that we probably would need several milligrams of AF-350, the structure determination was a huge undertaking for us.

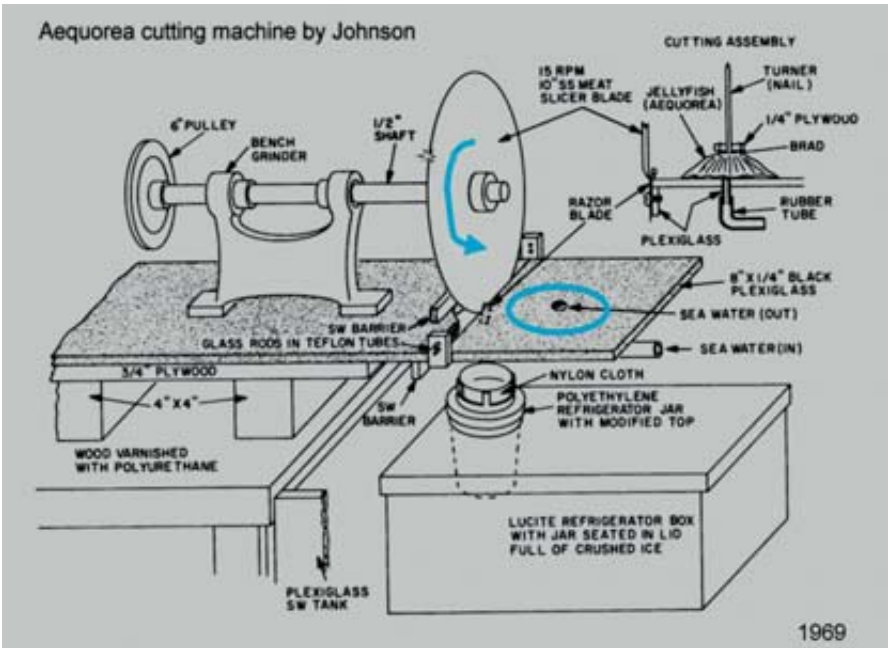

Figure 9. The jellyfish ring cutting machine constructed by Frank H. Johnson in 1969. A specimen is placed on the black Plexiglas platform and rotated to spread the edge of the umbrella. While rotating, the specimen is pushed toward the rotating blade (10-inch meat cutting blade) to cut off a 2–3 mm wide strip containing the light organs. The strip drops into a container below.

In processing a large number of jellyfish to obtain a sufficient amount of AF-350, we found that cutting rings with a pair of scissors was too slow. To speed up the process, Dr. Johnson constructed a jellyfish cutting machine (Fig. 9), which enabled one person to cut more than 600 rings per hour, or 10 times more than by hand.

Figure 10. Left: Jellyfish collectors of the summer of 1974. From left, my wife Akemi, Dr. Chang, myself, Mrs. Chang, Mrs. Johnson, Dr. Johnson, Debby (a helper). At lower right are my children, Tsutomu and Sachi. We collected 30–40 bucketfuls of jellyfish each day. Right: My family collecting jellyfish.

We started to collect jellyfish at 6 AM, and a part of our group began to cut off the rings at 8 AM. We spent all afternoon extracting aequorin from the rings. Then, we collected more jellyfish in the evening, 7 PM to 9 PM, for the next day (Figs. 10). Our laboratory looked like a jellyfish factory (Fig. 11), and was filled with the jellyfish smell.

Figure 11. Scenes of jellyfish cutting by two assistants (left), and the extraction of aequorin (right).

After five years of hard work, we determined the chemical structure of AF-350 in 1972 (Shimomura and Johnson, 1972). The result was surprising. The structure of AF-350 contained the skeleton of a 2-aminopyrazine (Fig. 12) that was previously found in the oxidation products of *Cypridina* luciferin, although the side chains are different. This finding suggested a close relationship between the luminescence systems of *Aequorea* and *Cypridina*. Based on that information, we were able to determine the structure of the chromophore of aequorin to be coelenterazine (Fig. 12). Eventually we elucidated that the luminescence reaction of aequorin takes place as shown in Fig. 13.

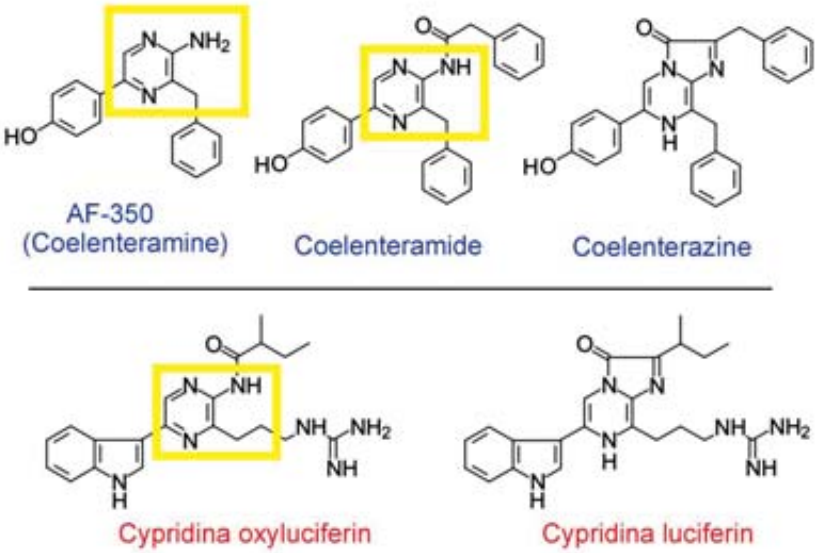

Figure 12. The chemical structures of AF-350 (coelenteramine), coelenteramide (a product of luminescence reaction of aequorin) and coelenterazine, compared with those of *Cypridina* oxyluciferin and luciferin.

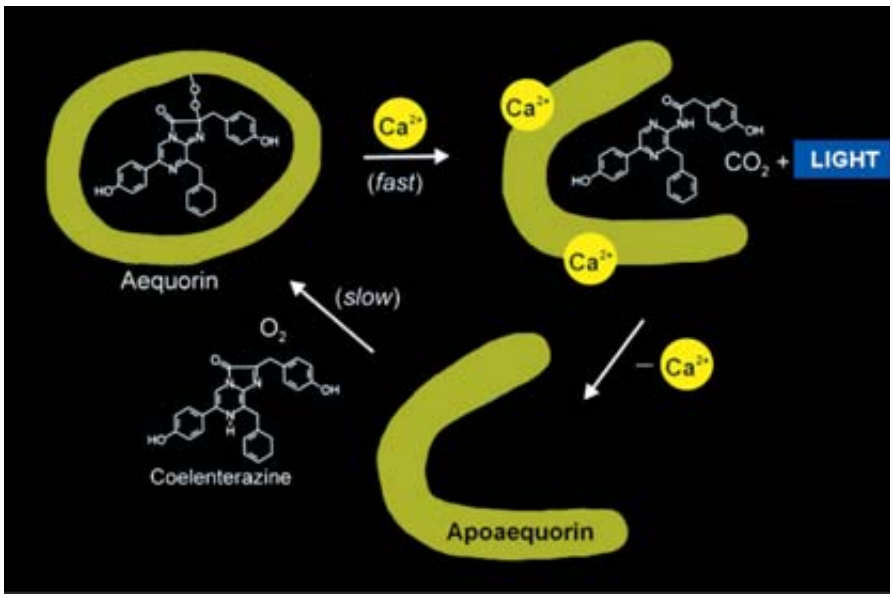

Figure 13. The luminescence and regeneration of aequorin. The photoprotein aequorin binds with two Ca^{2+} ions (Shimomura, 1995; Shimomura and Inouye, 1996), and decomposes into coelenteramide, CO_2 and apoaequorin accompanied by the emission of light (emission maximum at 465 nm). Apoaequorin can be regenerated into the original aequorin by incubation with coelenterazine in the presence of oxygen.

In a live specimen of *Aequorea*, the light organs contain GFP in addition to aequorin, and the energy of the blue light produced by the aequorin molecule is transferred to the GFP molecule, and GFP emits green light (Morise *et al.*, 1974).

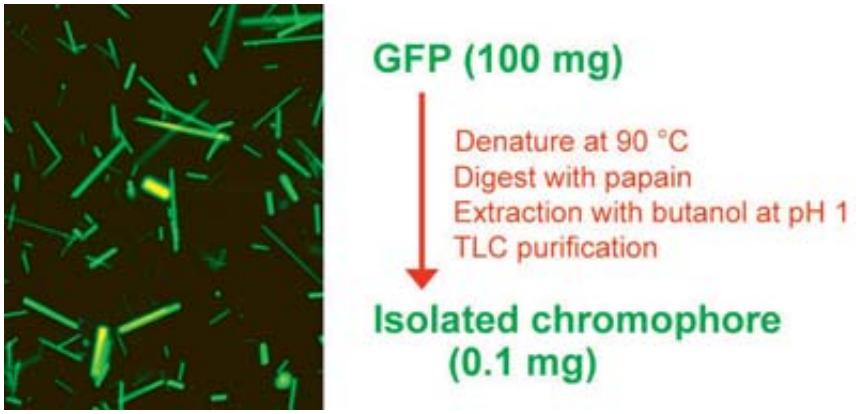

Figure 14. Crystals of GFP (left; photo by Dr. Shinya Inoué), and the process of isolating the chromophore of GFP (right).

Although GFP is highly visible and easily crystallizable (Fig. 14, left), the yield of GFP from the jellyfish was extremely low, much lower than that of aequorin. Therefore, to study GFP, we had to accumulate GFP little by little for many years while we studied the chemistry of aequorin luminescence. The amount of GFP we accumulated reached a sufficient amount to study this protein in 1979. Thus, we tried to find out the nature of the GFP chromophore by a series of experiments, using 100 mg of the protein in one experiment (Fig. 14, right).

We first cut the molecule of GFP into small pieces of peptide by enzymic digestion. We isolated and purified the peptide that contained the chromophore, and then analyzed the structure of the chromophore. I was surprised when I measured the absorption spectrum of the peptide. The spectrum was nearly identical to that of a compound that I had synthesized in my study of *Cypridina* luciferin 20 years earlier. Based on the spectral resemblance and some other properties, I could quickly identify the chromophore structure of GFP (Shimomura, 1979).

What I found is illustrated in Fig. 15. Fluorescent proteins are usually a complex of a protein and a fluorescent compound (as shown at upper left). However, GFP was a very special fluorescent protein that contained a fluorescent chromophore within the protein molecule (as shown at upper right). The lower part of the figure shows further details of GFP. GFP is a single chain of peptide containing more than 200 amino acid residues. The chromophore was formed from 3 amino acid residues in the peptide chain, by dehydration and dehydrogenation reactions. This finding was extremely

important because it showed that the chromophore is a part of the peptide chain, and thus it opened the possibility of cloning GFP. The chromophore structure was later confirmed by Cody *et al.* (1993).

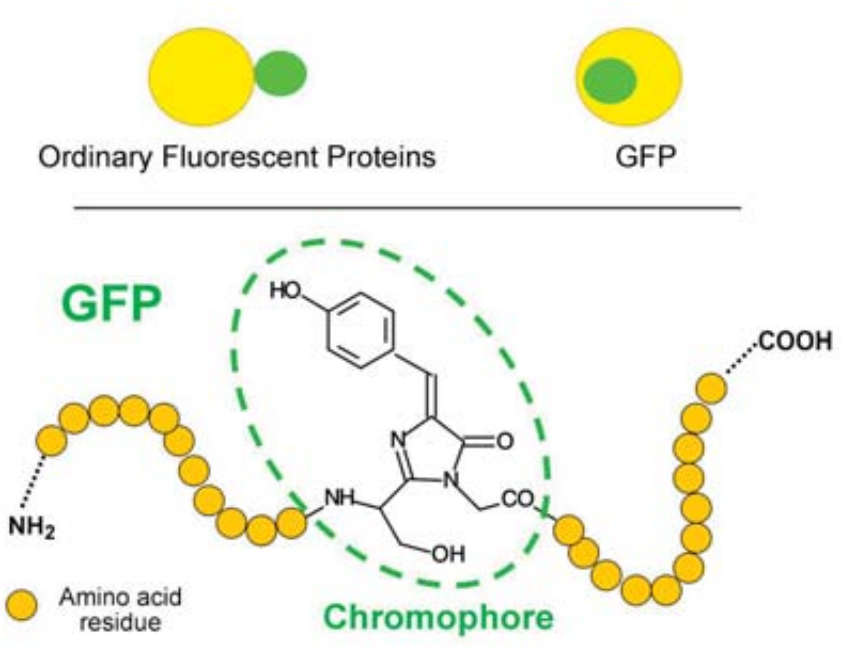

Figure 15. The structure of the chromophore in the molecules of GFP.

When I found the chromophore of GFP in 1979, I thought I had done all I could do with GFP, and decided to terminate my work on GFP in order to concentrate my efforts in the study of bioluminescence, my lifework. Then a mysterious thing happened. The population of *Aequorea* in the Friday Harbor area drastically decreased after 1990, thus making it practically impossible to prepare any new samples of natural aequorin or GFP. Fortunately, however, aequorin had been cloned by Inouye *et al.* (1985; 1986) and Prasher *et al.* (1985), and GFP was cloned by Prasher *et al.* (1992), thus making the natural proteins unessential. In 1994, GFP was successfully expressed in living organisms by Chalfie *et al.*, and it was further developed into its present prosperous state by Roger Tsien.

Now GFP and its homologues are indispensable in biomedical research, due to the fact that these proteins self-contain a fluorescent chromophore in their peptide chains and they can be expressed in living bodies. The identification of the fluorescent chromophore, however, depended on the GFP that had been accumulated for many years in our study of aequorin. Without the study of aequorin, the chromophore of GFP would have remained unknown and the flourishing of fluorescent proteins would not have occurred.

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Portrait photo of Osamu Shimomura by photographer Ulla Montan.