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Expression of Apoaequorin Complementary DNA in *Escherichia coli*†

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ABSTRACT: Aequorin is a photoprotein that reacts with Ca^{2+} to yield light, CO_2, and a blue fluorescent protein. The blue fluorescent protein is dissociable into apoaequorin and a small organic molecule, coelenteramide. Cloning and sequence analyses of the cDNA for apoaequorin show that it is made up of 189 amino acid residues and contains three Ca^{2+}-binding sites. Various expression plasmids for apoaequorin cDNA were constructed and expressed in *Escherichia coli*. One plasmid, piQ5, was found to give excellent expression in *E. coli* D1210 and BNN103. Best expression was obtained when the growth temperature was shifted from 37 to 42 °C immediately after the addition of inducer (isopropyl β-D-thiogalactopyranoside) to the culture medium. Aequorin was regenerated from the expressed apoaequorin by incubating with coelenterazine, 2-mercaptoethanol, and ethylenediaminetetraacetic acid. The results show that large amounts of apoaequorin may be prepared by the procedure described.

Aequorin is a chromophore-containing protein present in the outer margin of the umbrella of the jellyfish, *Aequorea victoria* (Shimomura et al., 1962, 1963). When mixed with a trace amount of Ca^{2+}, aequorin undergoes an intramolecular oxidation reaction, yielding as products light (λ_{max} = 470 nm), CO_2, and a blue fluorescent protein [Shimomura et al., 1962, 1963, 1974; for a review, see Johnson and Shimomura (1978)]. Because aequorin has a high specificity for Ca^{2+}, it has been used as a biological indicator of Ca^{2+} (Blinks et al., 1978). The blue fluorescent protein may be dissociated into apoaequorin (apoprotein) and coelenteramide by gel filtration or by treatment with acid or ether (Shimomura & Johnson, 1976). Coelenteramide, the product of the oxidation of the chromophore coelenterazine, serves as the emitter in the reaction (Shimomura & Johnson, 1973). Aequorin may be regenerated from apoaequorin by incubation with coelenterazine, 2-mercaptoethanol (2-ME),[1] and ethylenediaminetetraacetic acid (EDTA) (Shimomura & Johnson, 1975).

Recently, the cDNA for apoaequorin has been cloned and the primary structure of the protein deduced from the nucleotide sequence (Inouye et al., 1985). The primary structure has also been determined by sequencing the protein (Charbonneau et al., 1985). Apoaequorin is composed of 189 amino acid residues, has a molecular weight of 21 400, and has three EF hand structures that are characteristic of Ca^{2+}-binding sites. Electron paramagnetic resonance and proton nuclear magnetic resonance investigations have also been carried out on aequorin in order to understand the molecular mechanism of the light-emitting reaction (Kemple et al., 1984; Ray et al., 1985). Although crystal structure studies have not yet been performed on aequorin, recently several Ca^{2+}-binding proteins have undergone such studies (Herzberg & James, 1985a,b; Babu et al., 1985; Sundralingam et al., 1985). In order to carry out analogous studies on aequorin, and even to develop better methods for using aequorin in Ca^{2+} assay, relatively larger amounts of aequorin are necessary than are presently available. Site-directed mutagenesis studies also require amino acid substitutions to be made and the modified protein produced in sufficient amounts for further study. In order to meet these requirements, a study of the expression of cDNA for apoaequorin in *Escherichia coli* was undertaken. This paper describes procedures for obtaining good expression of the cDNA and for regenerating aequorin from apoaequorin.

Materials and Methods

Bacterial Strains and Plasmids. The *E. coli* strains used were JM83 (Vieira & Messing, 1982), D1210 carrying *lac*I^q and *lac*^{Y} (deBoer et al., 1983), and a protease-deficient strain BNN103 (Young & Davis, 1983). The plasmids employed were pUC9 (Vieira & Messing, 1982) and pUC9-1 and pUC9-2 (Hanna et al., 1984). Plasmid pDR540 (Russell & Bennet, 1982) was used as a source of the *tac* promoter and was obtained from P-L Biochemicals.

Enzymes and Chemicals. All restriction endonucleases, *E. coli* T_4 DNA ligase, Klenow enzyme (from DNA polymerase I), and T_4 polynucleotide kinase were purchased from Takara

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); 2-ME, 2-mercaptoethanol; IPTG, isopropyl β-D-thiogalactopyranoside; bp, base pair(s).

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Shuzo, Kyoto, Japan. Synthetic deoxyoligonucleotide linkers AR, 5′-G-A-T-C-G-A-T-G-G-T-C-A, and AQ, 5′-A-G-C-T-T-G-A-C-C-A-T-C, were synthesized by the phosphoramidite method with the Applied Biosystems (Foster City, CA) Model 380A DNA synthesizer. 5-Bromo-4-chloro-3-indolyl β-D-galactoside, isopropyl β-D-thiogalactopyranoside (IPTG), 2-mercaptoethanol (2-ME), tris(hydroxymethyl)aminomethane (Tris), and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) were purchased from Wako Pure Chemicals, Osaka, Japan. Ethylenediaminetetraacetic acid (disodium salt; EDTA) was obtained from Nakarai Chemicals, Kyoto, and ampicillin (sodium salt) from Meiji Seika, Tokyo, Japan. Coelenterazine [2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one] (M_r 423) was prepared by chemical synthesis (Inoue et al., 1975). All other chemicals used were of the highest grade available.

Bacterial Cell Growth. Transformed *E. coli* strains carrying the recombinant plasmid were grown in 10 mL of LB medium, with glucose omitted, the pH was adjusted to 7.5 (Bertani, 1951), and the medium was supplemented with 50 μg of ampicillin/mL. Incubation was carried out in an incubator/shaker at 37 °C. When the density of the culture medium reached 50–100 Klett units (~2 h; Klett colorimeter reading with red filter no. 66; 30 Klett units = ~2 × 10^8 cells/mL), IPTG was added to a final concentration of 1 mM, and the incubation was continued at 37 or 42 °C for various time periods. For comparison of the activities of extracts of *E. coli* D1210 carrying the various expression plasmids with *lac* and *tac* promoters, the incubation was continued at 37 °C for 2 h. For the growth–activity experiments with piQ5/BNN103 and piQ5/D1210, the incubation was continued at 37 or 42 °C for up to 6 h. For preparation of extracts used in regeneration experiments, the incubation was continued at 42 °C for 4 h.

Preparation of Extract and Regeneration of Aequorin. The cells were harvested by centrifugation, washed with 10 mL of M9 salt (Maniatis et al., 1982), and resuspended in 5 mL of 30 mM Tris-HCl, pH 7.60/10 mM EDTA. Cell disruption was achieved by sonication (4 × 15 s, in an ice bath) with a Branson Model 200 Sonifier. After centrifugation at 12000*g* for 10 min in a Hitachi Model RP20 refrigerated centrifuge, the supernatant, containing the expressed apoaequorin and hereafter called the "extract", was stored at −70 °C. Aequorin was regenerated from apoaequorin by adding 10 μL of 2-ME and 6 μg of coelenterazine (1 μg/μL, in absolute methyl alcohol) (unless otherwise specified) to 1.0 mL of the extract and allowing the mixture to stand for 2 h in an ice bath (Shimomura & Johnson, 1975).

Assay for Aequorin Activity. The above mixture (in a 20-mL scintillation glass vial) was placed in a Mitchell–Hastings photometer (Mitchell & Hastings, 1971) and injected with 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60. The initial maximal light intensity was recorded with Soltec Model S-4201 strip-chart recorder. Conversion of the initial light intensity to quanta per second as a measure of aequorin activity was made by calibrating the photometer with a carbon-14 light standard (Hastings & Weber, 1963).

Results

Construction of Apoaequorin cDNA Expression Plasmids. The *Pst*I/*Eco*RI or *Hin*dIII/*Eco*RI fragment of apoaequorin cDNA in pAQ440 (Inouye et al., 1985) was introduced into expression plasmid pUC9 or its derivatives with *lac* promoter (piQ9-PE, piQ9-1PE, piQ9-2PE, piQ9-HE, piQ9-2HE) (Figure 1). Expression plasmid piQ5 with *tac* promoter was also constructed. Figure 2 presents an outline of the scheme

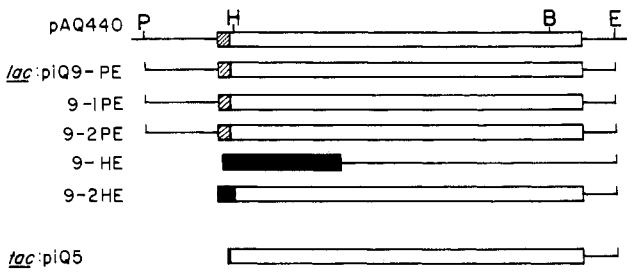

FIGURE 1: Construction of apoaequorin cDNA expression plasmids. For plasmids piQ9-PE, piQ9-1PE, and piQ9-2PE, the *Pst*I/*Eco*RI fragment of aequorin cDNA in pAQ440 was inserted into the *Pst*I/*Eco*RI digest of pUC9, pUC9-1, and pUC9-2, respectively. For plasmids piQ9-HE and piQ9-2HE, the *Hin*dIII/*Eco*RI fragment of aequorin cDNA in pAQ440 was inserted into the *Hin*dIII/*Eco*RI digest of pUC9 and pUC9-2, respectively. For plasmid piQ5, see Figure 2 and text for details. P, *Pst*I; H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; thin horizontal line, noncoding region of the cDNA; open box, apoaequorin coding region; diagonal-striped box, uncharacterized peptide region; filled box, region coding for a non-apoaequorin peptide.

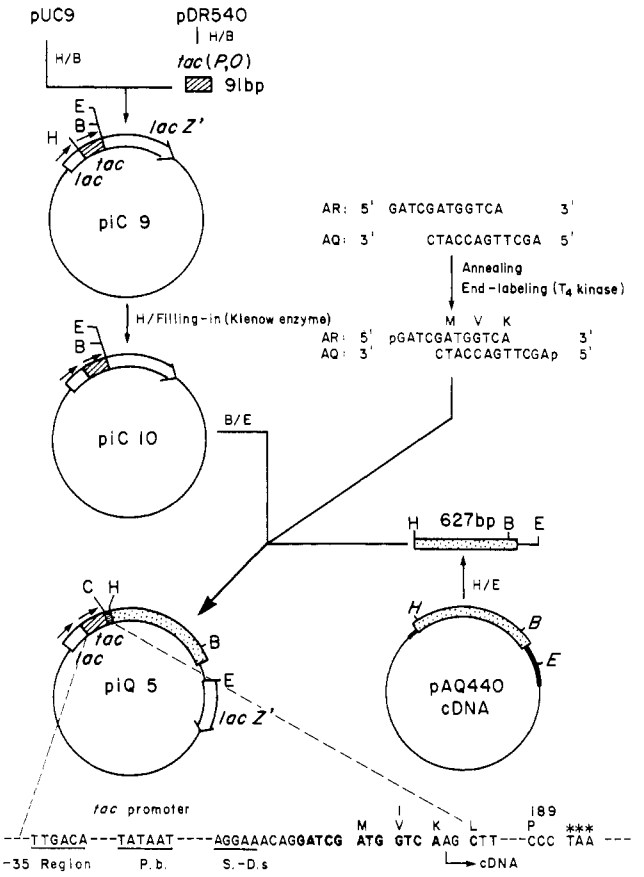

FIGURE 2: Scheme used in constructing piQ5 expression plasmid. The area marked with diagonal stripes represents the *tac* promoter, the lightly stippled region the apoaequorin cDNA, and the heavily stippled portion the synthetic deoxyoligonucleotide linker. H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; C, *Cla*I; P, O, *lac* promoter, operator; P. b., Pribnow box; S.-D. s, Shine-Dalgarno sequence.

used to construct piQ5. The *tac* promoter was isolated as a *Hin*dIII/*Bam*HI fragment from the plasmid pDR540 (Russell & Bennett, 1982) and inserted into the *Hin*dIII/*Bam*HI site of pUC9 to give piC9. piC10 was then formed from piC9 by *Hin*dIII digestion and then by filling-in with the Klenow enzyme. The apoaequorin cDNA was isolated from pAQ440 (Inouye et al., 1985) by treatment with *Hin*dIII/*Eco*RI and inserted into the *Bam*HI/*Eco*RI site of piC10 by using the synthetic oligonucleotide linker AR/AQ. The resultant plasmid (piQ5) was expected to have the structure shown in Figure 2. The DNA sequence of the constructed plasmids was

Table I: Aequorin Activity in Extracts of *E. coli* D1210 Carrying Various cDNA Expression Plasmids

pro-moter	plasmid	amino acid sequence[a]	activity[b] expt I	activity[b] expt II
		1 189		
lac	piQ9-PE	MTSKNYSVKLTS.....AVP*	1.77	3.26
	piQ9-1PE	MTSKNYSVKLTS.....AVP*		0.72
	piQ9-2PE	MTSKNYSVKLTS.....AVP*		0.94
		1 56		
	piQ9-HE	MTMITPS-LHQT....KLM*		0
		2 189		
	piQ9-2HE	MTMITPSSKLTS.....AVP*	2.51	4.37
		1 189		
tac	piQ5	MVKLTS.....AVP*	3.25	7.00

[a] An asterisk represents the termination signal. [b] Extracts prepared and assayed for activity as described under Materials and Methods; activity expressed in quanta per second × 10^{10}.

confirmed by DNA sequencing according to a modified dideoxynucleic acid sequencing method (Hattori & Sakaki, 1986).

Expression of Apoaequorin cDNA Using lac and tac Promoters. The activity of apoaequorin produced by these expression plasmids is shown in Table I. Except for plasmid piQ9-HE, which was made up of an improper reading frame and produced a fused, non-apoaequorin peptide, all of the plasmids gave some expression of activity. Even though *lac* gave reasonably good activity (piQ9-2HE), the best activity was obtained with piQ5, which employed the *tac* promoter and produced an apoaequorin polypeptide with a methionine at the N-terminus. We previously reported that the aequorin cDNA clone AQ440 contains a sequence coding for a seven-member leader peptide (M-T-S-K-Q-Y-S-) (Inouye et al., 1985), but the present results indicate that the inclusion of this sequence is not necessary for expression of activity.

Expression of Apoaequorin in E. coli Carrying Plasmid piQ5. The growth curves for piQ5/BNN103 and piQ5/D1210 are shown in Figure 3, with corresponding activities for aequorin in quanta per second. Apoaequorin was not found in the growth medium but was released only upon sonication of the cells, following which it could be readily assayed for aequorin activity. For an unknown reason, at 37 °C, the addition of IPTG to the medium did not significantly increase the level of apoaequorin in piQ5/BNN103, in comparison to a second culture without added IPTG, but it did produce a pronounced increase in apoaequorin in piQ5/D1210. This increase roughly paralleled the growth curve. The greatest stimulation was obtained when a temperature shift was made from 37 to 42 °C, immediately after the addition of IPTG, whereupon both piQ5/BNN103 and piQ5/D1210 reached their highest level of aequorin activity. The growth curve in the last heat-induction experiment with piQ5/D1210 was checked separately by adding [^{35}S]methionine (Amersham) and IPTG simultaneously to the culture medium after 2 h of incubation. After the temperature shift, the [^{35}S]methionine incorporation curve assumed the same shape as the growth curve in the previous experiment and was flat between 4 and 8 h of incubation.

Effect of pH on the Regeneration of Aequorin. Figure 4a shows the time course for the regeneration of aequorin at pH 7.4 and 8.5. The regeneration curves indicate that, even though regeneration started off more slowly at pH 8.5, the regenerations reached the same level of aequorin activity after 2 h and remained the same at 2.5 h (data not shown). The pH–activity curve in Figure 4b shows that the optimum pH for regeneration is between 7.3 and 7.6 when measured after 60

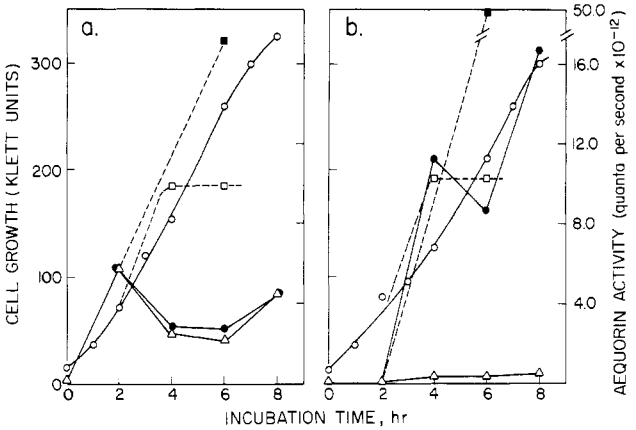

FIGURE 3: Growth curves of *E. coli* BNN103 and D1210 containing plasmid piQ5 and measured aequorin activity with time of incubation: (a) piQ5/BNN103 and (b) piQ5/D1210. Each *E. coli* strain was grown overnight at 37 °C in 10 mL of LB medium supplemented with 50 μg of ampicillin/mL. Two-milliliter aliquots of the overnight culture were transferred to two 500-mL flasks, each containing 200 mL of LB medium supplemented with 50 μg of ampicillin/mL. After incubation for 2 h at 37 °C, IPTG (1 mM) was added to one of the culture media but not the other. Immediately, a 30-mL aliquot was removed from the IPTG flask and incubated at 42 °C. The original flasks continued to be incubated at 37 °C. For +IPTG/37 °C: ○, growth; ●, activity. For +IPTG/42 °C: □, growth; ■, activity. For −IPTG/37 °C: △, activity. All incubations were carried out with vigorous shaking. At prescribed time intervals, 30-mL aliquots were removed from each flask and centrifuged. The harvested cells were washed with 10 mL of M9 salt and assayed for aequorin activity as described under Materials and Methods.

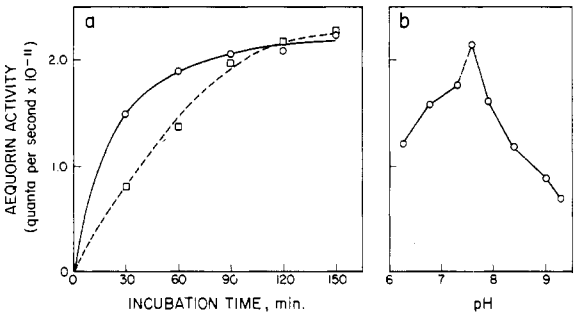

FIGURE 4: Effect of pH on regeneration of aequorin. (a) Time course of aequorin regeneration at pH 7.4 (○) and 8.5 (□). Composition of regeneration mixture: 10 μL of piQ5/D1210 42 °C extract, 1.0 mL of 20 mM Tris-HCl buffer/10 mM EDTA, 10 μL of 2-ME, and 5 μg of coelenterazine (1 μg/μL, in absolute methyl alcohol), incubated in an ice bath. Assay for aequorin: 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60, injected into regeneration mixture. (b) Optimum pH of aequorin regeneration. Composition of regeneration mixture: 10 μL of piQ5/D1210 42 °C extract, 1.0 mL of 100 mM PIPES (pH 6.3, 6.8, and 7.3) or Tris-HCl (pH 7.6, 7.9, 8.4, 9.0, and 9.3) buffer/2.5 mM EDTA, 10 μL of 2-ME, and 5 μg of coelenterazine (1 μg/μL, in absolute methyl alcohol); regeneration was for 60 min in an ice bath. Assay for aequorin: 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60, injected into regeneration mixture.

min of incubation. When the activities were measured after 2 h of incubation, they were virtually the same, averaging 2.2 × 10^{11} quanta/s, which is close to the value reached by the pH 7.6 mixture after 60 min, indicating that the pH affects the rate of regeneration but not the final activities.

Effect of Coelenterazine and 2-Mercaptoethanol. Figure 5 shows that 1–2 μg (2.3–4.6 μM) of coelenterazine is sufficient to regenerate aequorin to a maximum level in 1.0 mL of the piQ5/BNN103 42 °C extract. Higher concentrations of coelenterazine did not result in further increases in aequorin activity. Inasmuch as the aequorin activity of the piQ5/D1210 42 °C extract is usually greater by a factor of 3–30 than that of the piQ5/BNN103 42 °C extract, the use of 5–6 μg of

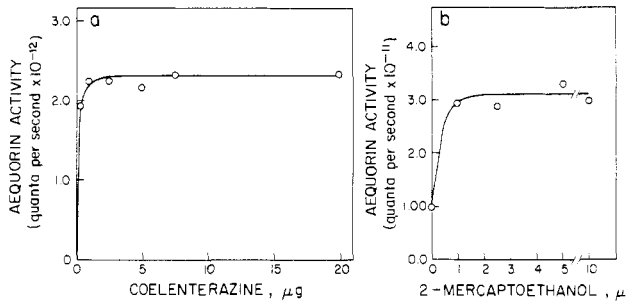

FIGURE 5: Effect of coelenterazine and 2-mercaptoethanol concentration on regeneration of aequorin. (a) Coelenterazine. Composition of regeneration mixture: 1.0 mL of piQ5/BNN103 42 °C extract, 10 μL of 2-ME, and 0.3, 1.0, 2.5, 7.5, or 20.0 μg of added coelenterazine (1 μg/μL, in absolute methyl alcohol), incubated in an ice bath for 2 h. Assay for aequorin: 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60, injected into regeneration mixture. (b) 2-Mercaptoethanol. Composition of regeneration mixture: 10 μL of piQ5/D1210 42 °C extract, 1.0 mL of 50 mM Tris-HCl, pH 8.06/10 mM EDTA, 5 μg of coelenterazine (1 μg/μL, in absolute methyl alcohol), and 0, 1.0, 2.5, 5.0, or 10.0 μL of added 2-ME, incubated in an ice bath for 2 h. Assay for aequorin: 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60, injected into regeneration mixture.

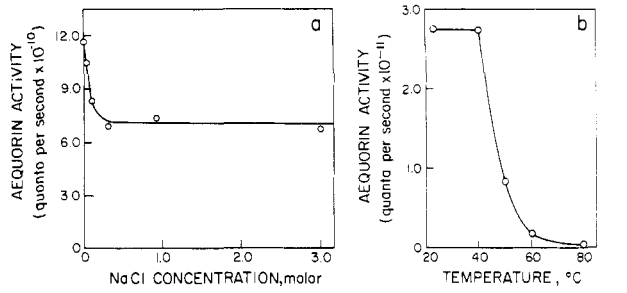

FIGURE 6: Effect of NaCl and temperature on regeneration of aequorin. (a) Effect of NaCl concentration. Composition of regeneration mixture: 1.0 mL of 100 mM Tris-HCl, pH 8.35/2.5 mM EDTA, containing 0, 0.03, 0.10, 0.30, 0.90, or 3.0 M NaCl plus 10 μL of piQ5/D1210 42 °C extract, 10 μL of 2-ME, and 5 μg of coelenterazine (1 μg/μL, in absolute methyl alcohol), incubated for 60 min in an ice bath. Assay for aequorin: 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60, injected into regeneration mixture. (b) Effect of temperature. These experiments were carried out as follows: 9.75 mL of 50 mM Tris-HCl, pH 8.06/10 mM EDTA, 100 μL of piQ5/D1210 42 °C extract, 100 μL of 2-ME, and 50 μg of coelenterazine (1 μg/μL, in absolute methyl alcohol) were incubated in an ice bath for 2 h; then 1.0-mL aliquots were removed and placed in separate water baths for 10 min at 23, 40, 50, 60, and 80 °C. Subsequently, all five aliquots were cooled in an ice bath for 15 min and then assayed for aequorin by injecting 1.5 mL of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.60, into the mixture.

coelenterazine was considered adequate for regenerating aequorin in the rest of the study. Figure 5b shows that 1 μL (14.3 mM) of 2-ME is sufficient to obtain maximum regeneration of aequorin activity from 10 μL of the piQ5/D1210 42 °C extract. Increasing the concentration of 2-ME did not cause a further increase in aequorin activity.

Effects of NaCl and Temperature. Increasing the concentration of NaCl (from 0 to 0.3 M) in the 60-min regeneration mixtures first caused a drop in aequorin activity and then a leveling off of activity between 0.3 and 3.0 M (Figure 6a). Although the activity dropped by about 35% from the initial value, the drop was not directly proportional to NaCl concentration. However, when the activities of the 0.03–0.90 M NaCl mixtures were assayed after 2 h, they were practically the same, averaging 10.27 quanta/s, indicating that, at these concentrations, NaCl affects the regeneration reaction but not final aequorin activity. At 3.0 M, the activity (6.9×10^{10} quanta/s) was nearly identical with the value at 60 min of incubation, suggesting that NaCl affects either the light-emitting or regeneration reaction, or both. Figure 6b shows the effect of incubating regenerated aequorin for 10 min at 23, 40, 50, 60, and 80 °C and cooling the solutions in an ice bath, followed by assay for aequorin. The results show a sharp drop in aequorin activity above 40 °C and an almost complete loss of activity at 80 °C. Apoaequorin was also tested for heat stability by exposing 10 μL of the piQ5/D1210 42 °C extract in 1.0 mL of 50 mM Tris-HCl, pH 8.06/10 mM EDTA for 10 min at 23, 40, 50, 60, and 80 °C and cooling rapidly in an ice bath. The aequorin was then regenerated by incubating with 2-ME and coelenterazine for 2 h. When the solutions were assayed for aequorin activity, it was found that the aequorin activity was essentially the same for all temperatures (data not shown). Thus, apoaequorin appears to be relatively stable up to a temperature of 80 °C.

Discussion

Among the many recombinants studied, the most favorable expression was obtained with plasmid piQ5, which employed the *tac* promoter and produced an apoaequorin polypeptide with a methionine at the N-terminus (Figures 1 and 2 and Table I). The best expression occurred when the incubation temperature for piQ5/D1210 and piQ5/BNN103 was shifted to 42 °C, after an initial incubation period at 37 °C (Figure 3). Here, also, a difference showed up in the two hosts in that D1210 gave better expression by a factor of 3–30 over that of BNN103. However, piQ5/D1210 and piQ5/BNN103 always gave excellent expression and did not require the addition of a protease inhibitor, such as phenylmethanesulfonyl fluoride, in the sonication medium. Supernatants obtained by centrifuging extracts of the disrupted cells remained stable for many months when kept frozen at −70 °C and could be defrosted and refrozen many times without loss of activity.

The observed stability of apoaequorin at temperatures up to 80 °C and at high concentrations of NaCl (Figure 6a) provides a basis for purifying apoaequorin at room temperature by conventional salting-out and chromatographic techniques. Since aequorin may be regenerated and assayed at high NaCl concentrations (Figure 6a), apoaequorin may thus be assayed directly on aliquots removed from column fractions following NaCl gradient elution chromatography with (diethylaminoethyl)cellulose. However, regenerated aequorin quickly loses activity at temperatures above 40 °C (Figure 6b), presumably by oxidation of the coelenterazine, and present methods for the purification of aequorin from *Aequorea* have the disadvantage that low temperatures of 0–4 °C are required to prevent loss of activity during successive gel filtration and ion-exchange chromatographies (Johnson & Shimoura, 1978; Blinks et al., 1978). High stability of apoaequorin at elevated temperatures has also been reported by Shimomura and Shimomura (1981).

It has been estimated that 5300 specimens of *Aequorea* contain 70–100 mg of aequorin (Shimomura, personal communication). Although determination of the precise number of apoaequorin molecules produced per bacterial cell must await further study, we observed that 1.0 mL of the piQ5/D1210 42 °C extract (equivalent to $\sim 1.29 \times 10^9$ cells/mL) triggered by an excess of Ca^{2+} emitted a flash of light lasting for 2–3 s with a peak intensity of $\sim 50 \times 10^{12}$ quanta/s (Figure 3b), whereas Johnson and Shimomura (1978) reported an emission of 4.3×10^{15} quanta from 1 mg of pure native aequorin at peak wavelength of 470 nm with a quantum yield of 0.15. Assuming that the total quanta emitted are 50×10^{12} and since one quantum is produced per aequorin molecule, the number of apoaequorin molecules synthesized would be ap-

proximately 3.9×10^4/cell. This value would be increased by a factor of 6.7 if the quantum yield were taken into account. Thus, provided coelenterazine is available, recombinant DNA techniques may outweigh the enormous task associated with collecting and processing large numbers of *Aequorea* and purifying the highly unstable aequorin (Johnson & Shimomura, 1978; Blinks et al., 1978).

The cloning and expression of cDNA for apoaequorin have been described in another recent paper (Prasher et al., 1985). The authors isolated a clone (pAEQ1) that yielded active extracts of apoaequorin, but the reported activity of the regenerated aequorin appears to be substantially less than those observed in the present study. Since the coding sequence for pAEQ1 was not given, it is not possible to compare the clones from the two studies. One reason for the lower activity may be due to the use of a β-lactamase promoter and another to the use of a different host. Also, there appears to be a significant difference in the regeneration times of 6 and 2 h observed between the two studies. The reason for this is not clear, but it may be due to a difference in 2-ME or coelenterazine concentration, since the observed regeneration time was also 6 h when these authors used spent native aequorin as a control. These regeneration times may be compared with a regeneration time of 3 h and an aequorin yield of 90% previously reported by Shimomura and Johnson (1975).

The effect of pH also provides a possibly interesting insight into the regeneration and bioluminescence of aequorin in the photocyte of the animal. The pH–activity curve for the regeneration of aequorin shows a broad peak, extending from pH 6.3 to pH 9.3, with a maximum at around pH 7.3–7.6 (Figure 4b), whereas the light yield of the Ca^{2+}-triggered aequorin reaction has been found essentially independent of pH between pH 6.0 and pH 8.5 (Johnson & Shimomura, 1978; Blinks et al., 1978). A recycling process therefore conceivably could be accommodated directly in the photocyte within the pH range of 6.3–8.5. Another interesting aspect of the regeneration problem is the finding by Prasher et al. (1985) of an absolute requirement for 2-ME and coelenterazine in the regeneration of aequorin, whereas the present results show an absolute requirement for coelenterazine but not for 2-ME (Figure 5b). In the absence of 2-ME, some aequorin was always regenerated upon the addition of coelenterazine. It remains for future studies to determine what role 2-ME plays in the regeneration of aequorin.

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