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Site-specific mutagenesis of the calcium-binding photoprotein aequorin

(bioluminescence/oxygenase/oligonucleotide-directed mutagenesis/protein modification)

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ABSTRACT **The luminescent protein aequorin from the jellyfish *Aequoria victoria* emits light by an intramolecular reaction in the presence of a trace amount of Ca^{2+}. In order to understand the mechanism of the reaction, a study of structure–function relationships was undertaken with respect to modifying certain of its amino acid residues. This was done by carrying out oligonucleotide-directed site-specific mutagenesis of apoaequorin cDNA and expressing the mutagenized cDNA in *Escherichia coli*. Amino acid substitutions were made at the three Ca^{2+}-binding sites, the three cysteines, and a histidine in one of the hydrophobic regions. Subsequent assay of the modified aequorin showed that the Ca^{2+}-binding sites, the cysteines, and probably the histidine all play a role in the bioluminescence reaction of aequorin.**

The luminescent protein aequorin from the umbrella of the jellyfish *Aequorea victoria* emits light when mixed with a trace amount of Ca^{2+} (1, 2). The binding of Ca^{2+} to aequorin results in an intramolecular reaction in which coelenterazine, an imidazopyrazine compound bound noncovalently to the protein, is oxidized to coelenteramide, yielding light (λ_{max} = 470 nm), CO_2, and a blue fluorescent protein (3, 4). The excited state of coelenteramide bound to the protein is the emitter in the reaction (5). Because the reaction takes place in the absence of molecular oxygen, it is presumed that the oxygen is bound in some manner to the protein, possibly in the form of a hydroperoxide to an amino acid residue in the active center of the molecule, together with coelenterazine. The blue fluorescent protein may be dissociated into apoaequorin and coelenteramide by gel filtration or by treatment with either ether or acid (5). Aequorin may be regenerated by incubating apoaequorin with coelenterazine, dissolved oxygen, 2-mercaptoethanol, and EDTA (6).

Recently, cloning and sequence analysis of the cDNA for apoaequorin (7) and direct sequencing of the protein (8) have revealed that aequorin is composed of 189 amino acid residues and has a molecular weight of 21,400, with three EF-hand structures characteristic of Ca^{2+}-binding sites and three hydrophobic regions. The amino acid sequences of the Ca^{2+}-binding sites of aequorin, bovine calmodulin, parvalbumin, and troponin C show strong homologies. Further, the distance between the second and third EF-hand structures in aequorin and between the third and fourth EF-hand structures in bovine calmodulin is conserved, suggesting that both proteins have a common evolutionary origin. Cloning of the cDNA for apoaequorin has made it possible to study the relationship between structure and function by modifying amino acid residues in the protein by site-specific mutagenesis and by comparing the activities of the expressed proteins. This paper describes the results of such a study in which amino acid substitutions were made at the three Ca^{2+}-binding sites, at the three cysteines, and at histidine in the hydrophobic region.

MATERIALS AND METHODS

Enzymes and Chemicals. All restriction endonucleases, *Escherichia coli* T4 DNA ligase, Klenow enzyme of DNA polymerase 1, and T4 polynucleotide kinase were purchased from Takara Shuzo (Kyoto, Japan). Tris, 2-mercaptoethanol, and isopropyl-β-D-thiogalactopyranoside were obtained from Wako Pure Chemicals (Osaka, Japan). Ampicillin (sodium salt) was purchased from Meiji Seika (Tokyo), and disodium EDTA was from Nakarai Chemicals (Kyoto, Japan). Coelenterazine, 2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one, was prepared by chemical synthesis (9). All other chemicals were of the highest grade available.

Bacterial Strains and Plasmids. The *E. coli* strains used were JM83 (10) and D1210 carrying $lacI^q$ and lac^Y (11). The recombinant plasmid used was piQ9-2HE, which on being expressed in *E. coli* gives the highly active apoaequorin possessing the amino acid sequence: Met-Thr-Met-Ile-Thr-Pro-Ser-Ser-Lys-Leu-Thr-Ser-...-Ala-Val-Pro (12), where Lys is residue 2 and Pro is residue 189.

Site-Specific Mutagenesis. The oligonucleotides used were synthesized by the phosphoramidite method using an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer. By using the oligonucleotides as "mutagens," site-specific mutagenesis was carried out by the method of Morinaga *et al.* (13). The mutagenized closed, circular, double-stranded DNA plasmid was then used to transform *E. coli* JM83 (14). Clones carrying the mutant plasmid were identified by the technique of colony hybridization (15), with the synthetic oligonucleotides as probes. The mutant plasmid was isolated after retransformation, and the nucleotide sequence was confirmed by DNA sequencing by a modified dideoxynucleic acid sequencing method (16).

Growth of Bacteria and Preparation of Cell Extract. Ten milliliters of LB medium containing 50 μg of ampicillin/ml (14) was inoculated with 100 μl of an overnight culture of the transformed *E. coli* D1210 carrying the recombinant plasmid. The medium was incubated at 37°C for 2 hr (cell density = 60–70 Klett colorimeter units with red filter 66; 30 Klett units ≈ 2×10^8 cells per ml), and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The medium was incubated for an additional 2 hr (cell density ≈ 200–210 Klett units) and was centrifuged at 12,000 × *g* for 5 min at 4°C. The cells were resuspended in 2 ml of M9 salt (14) with a Vortex mixer and centrifuged at 12,000 × *g* for 5 min at 4°C. The cells then were resuspended in 2.5 ml of 30 mM Tris·HCl, pH 7.60/30 mM EDTA with a Vortex mixer. Cell

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disruption was carried out with a Branson model 200 sonifier (20 sec three times) with the cell holder immersed in an ice bath. The suspension was centrifuged at 12,000 × *g* for 10 min at 4°C, and the pellet was discarded. The supernatant was diluted with 5.0 ml of 30 mM Tris·HCl (pH 7.60) to give an EDTA concentration of 10 mM, and this cell extract was stored at −70°C.

Regeneration of Aequorin Activity. Regeneration was carried out by pipetting measured volumes of cell extract, 30 mM Tris·HCl, pH 7.60/10 mM EDTA, 2-mercaptoethanol (except when noted otherwise), and coelenterazine into a 1.5-ml Eppendorf tube. After the tube was stoppered and shaken vigorously, the mixture was allowed to incubate in an ice bath for 2 hr.

Assay for Luminescence Activity with Ca^{2+}. The content of the Eppendorf tube was transferred to a 20-ml scintillation glass vial, and the vial was placed in the holder of the photomultiplier compartment of a Mitchell–Hastings photometer (17). The mixture was injected with 1.5 ml of 30 mM $CaCl_2$/30 mM Tris·HCl, pH 7.60, and the initial maximal light intensity was recorded with a Soltec (Sun Valley, CA) model S-4201 strip-chart recorder. A carbon-14 light standard (18) was used to calibrate the photometer, and light intensity, converted to quanta per sec, served as a measure of activity.

RESULTS AND DISCUSSION

Table 1 lists the oligonucleotides that were synthesized and the corresponding amino acid substitutions that were made by using the oligonucleotides as mutagens.

Ca^{2+}-Binding Sites. The triggering of light emission from aequorin by Ca^{2+} appears to be due to a conformational change in the molecule caused by the binding of Ca^{2+} to the protein. The conformational change presumably converts a region of the protein molecule into a catalytically active site, in essence transforming the molecule into an oxygenase. Noncovalently bound coelenterazine is then oxidized by internally bound molecular oxygen, catalyzed by the site (Fig. 1). Except for its amino acid sequence, little is known about the role that the Ca^{2+}-binding sites play in aequorin light emission. Other studies have shown that EF-hand structures of Ca^{2+}-binding proteins consist of 12-amino acid residues arranged sequentially in a loop, with residues at positions 1, 3, 5, 7, 9, and 12 in the loop coordinating to Ca^{2+} (19, 20). The cation is coordinated by the oxygen atoms of these residues. Aequorin contains three Ca^{2+}-binding sites (Fig. 1) whose amino acid sequences are homologous with three of the four Ca^{2+}-binding sites of calmodulin (7, 8). Sequence alignment of the amino acid residues in the Ca^{2+}-binding loops of parvalbumin (21), troponin C (22, 23), calmodulin (24), intestinal Ca^{2+}-binding protein (25), sarcoplasmic Ca^{2+}-binding protein (26), and aequorin (7, 8) shows that the glycine at position 6 is highly conserved. This is believed to allow the side chain of the amino acid residue at position 5 and the main-chain carbonyl oxygen atom of the amino acid residue at position 7 to coordinate to Ca^{2+} (20).

Because glycine at position 6 is conserved so highly in the Ca^{2+}-binding loops of virtually all Ca^{2+}-binding proteins, this amino acid was chosen as the first residue for site-specific mutagenesis. Table 2 shows the result of substituting arginine for glycine at position 6 in sites I, II, and III. Aequorin lost all of its activity when arginine was substituted for glycine at position 29 and half of its activity when the same substitution was made at position 122, indicating that these two sites are essential for activity. Unexpectedly, no loss of activity was detected when arginine was substituted for glycine at position 158. This result indicates that either the binding of Ca^{2+} to site III is unessential for light emission or arginine did not modify the site sufficiently to affect Ca^{2+} binding. Interestingly, site III contains amino acid residues that are highly conserved in EF-hand structures, and for this reason it is difficult to believe that the site would be unessential for aequorin activity.

Possible O_2-Binding Site. Aequorin is not known to contain a metal. However, the fact that light emission can be triggered from aequorin by Ca^{2+} in a vessel completely evacuated of air and the fact that aequorin cannot be regenerated from apoaequorin in the absence of molecular oxygen (1, 6) suggest that molecular oxygen binds to an amino acid residue. Also, relatively little is known about the binding of coelenterazine. Coelenterazine is retained by aequorin during purification by gel filtration (4, 27) and, therefore, is not easily diffusible; it is not extracted by organic solvents (3), indicating that it, too, is strongly bound within the molecule. Since the triggering of bioluminescence by Ca^{2+} is instantaneous, oxygen must be in close proximity to coelenterazine because of mechanistic constraints.

Previously, it has been found that coelenterazine and its analogues produce a brilliant blue chemiluminescence when dissolved in the hydrophobic solvent dimethyl sulfoxide or *N,N*-dimethylformamide (28, 29). This indicates that the catalytic site is hydrophobic. By assuming that molecular oxygen and coelenterazine are bound to a common hydrophobic region of aequorin, two schemes are possible: (*i*) molecular oxygen is bound to an amino acid residue, and coelenterazine in turn is bound to it, or (*ii*) both are bound separately to different amino acids. Even though the mutagenesis technique would not distinguish between the two schemes, an attempt was made to identify the residue to which molecular oxygen might bind. Two residues were considered likely candidates: histidine-58 and tryptophan-108, both of which occur in an hydrophobic region (alanine-55

Table 1. Synthetic oligonucleotides used for site-specific mutagenesis

Name	Oligonucleotide	Amino acid substitution	Function
G1R	ACCACAAT*CGAAAAATC	Gly-29 → Arg	EF-hand(I)
G2R	ATCAAAAT*CGAGCCATT	Gly-122 → Arg	EF-hand(II)
G3R	TGAAAGT*CGACAACTCG	Gly-158 → Arg	EF-hand(III)
C1S	CAGAAGATT*CCGAGGAA	Cys-145 → Ser	−SH group
C1R	CAGAAGAT*CGCGAGGAA	Cys-145 → Arg	−SH group
C2S	CAGAGTGT*CCGATATTG	Cys-152 → Ser	−SH group
C3S	TCCTGCTT*CCGAAAAGC	Cys-180 → Ser	−SH group
HF	CCAAACGA**TTCAAAGAT	His-58 → Phe	Active site(?)

*Position of nucleotide substitution.

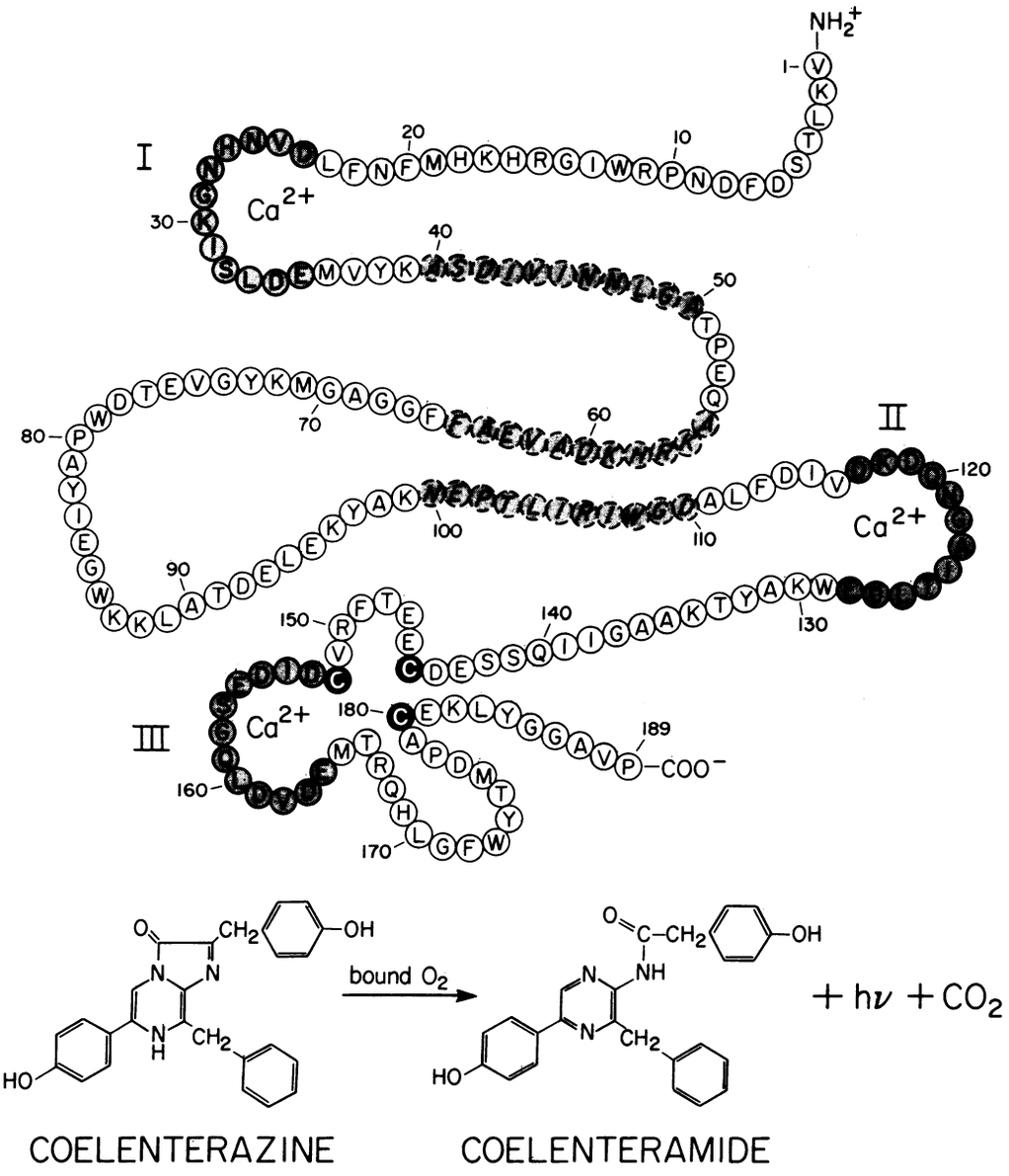

FIG. 1. Primary structure of apoaequorin (7) and the Ca^{2+}-triggered bioluminescence reaction of aequorin (4). The sequence is arranged to show the Ca^{2+}-binding sites (heavily stippled), hydrophobic regions (broken circle, lightly stippled), and cysteines (white lettering on dark background).

to phenylalanine-65; asparagine-100 to aspartic acid-110) of aequorin (7). Histidine-58 was selected for mutagenesis over tryptophan-108 because, besides being able to bind molecular oxygen to its positively charged nitrogen to form an hydroperoxide, histidine-58 is flanked by lysine-56, arginine-57, and lysine-59, one of which could coordinate a positive nitrogen to the negatively charged hydroxyl group of the coelenterazine phenyl side chain (Fig. 1) rather than to the hydroxyl group of the coelenterazine benzyl side chain, which is not essential for regenerating aequorin activity (3, 30); tryptophan-108 is flanked by only arginine at position 106. In this connection, it should be noted that, depending on the folding of the protein, molecular oxygen and coelenterazine could also be attached to different portions of the polypeptide chain so that, upon Ca^{2+} activation, they are brought into proper orientation for the light-emitting reaction to take place. Table 2 shows that when histidine-58 was replaced with phenylalanine, aequorin lost its activity completely. Results of this nature require further studies to confirm that the loss of activity is due to a specific interference with the binding of a particular ligand and not to a change in the tertiary structure of the protein.

Sulfhydryl Groups. Cysteine is relatively rare among the Ca^{2+}-binding proteins, one residue being found in parvalbumin (21) and in chicken skeletal muscle troponin C (23) but none in rabbit skeletal muscle troponin C (22), bovine uterus troponin C-like protein (31), bovine calmodulin (24), sea anemone calmodulin (32), *Tetrahymena* calmodulin (33), rat testis Ca^{2+}-dependent regulator protein (37), bovine intestinal Ca^{2+}-binding protein (25), and sandworm sarcoplasmic Ca^{2+}-binding protein (26). Yet, aequorin contains three cysteines (Fig. 1). For these reasons and the fact that 2-mercaptoethanol is required for full regeneration of aequorin activity, it is assumed that cysteine serves some function in the aequorin bioluminescence and regeneration reactions. Table 2 gives the activities of aequorin after replacement of cysteine with serine at positions 145, 152, and 180. The great loss in activity that occurred following substitution at position 180 suggests that this cysteine plays an essential role in aequorin bioluminescence, followed by

Table 2. Luminescence activity of apoaequorin and modified apoaequorin after regeneration with coelenterazine, dissolved oxygen, 2-mercaptoethanol, and EDTA

Exp.	Name	Without $HSCH_2CH_2OH$		With $HSCH_2CH_2OH$	
		Quanta × 10^{-8}/sec	Relative activity	Quanta × 10^{-8}/sec	Relative activity
1*	Aequorin	—	—	38.9	100
	G1R	—	—	0	0
	G2R	—	—	19.2	49
	G3R	—	—	37.9	97
	HF	—	—	0	0
2†	Aequorin	1.64	100	22.9	100
	C1S	1.31	80	15.4	67
	C1R	0.55	33	11.0	48
	C2S	2.01	123	13.6	59
	C3S	3.60	220	6.8	29

*Regeneration mixture: 500 μl of cell extract from D1210, 500 μl of 30 mM Tris·HCl, pH 7.60/10 mM EDTA, 5 μl of 2-mercaptoethanol, and 5 μg of coelenterazine (1 μg/μl of absolute methyl alcohol).
†Regeneration mixture: 250 μl of cell extract from D1210, 250 μl of 30 mM Tris·HCl, pH 7.60/10 mM EDTA, 5 μl of 2-mercaptoethanol, and 5 μg of coelenterazine (1 μg/μl of absolute methyl alcohol).

cysteines 152 and 145. However, a modified apoaequorin possessing the C-terminal sequence: ...Thr^{175}-Met-Asp-Arg-Ser-Cys^{180}-Leu-Arg-Lys-Ala-Leu-Arg-Trp-Ser-Cys-Pro^{190}-Leu-Arg-Ser-Ser-Thr-Val-Val-Met-His-Pro^{200}-Arg-Lys-Met-Met^{204} showed no activity in spite of the presence of cysteine at position 180, indicating that the presence of cysteine at this position itself is not sufficient for activity. Thus, both cysteine-180 and the C-terminal region are required for full aequorin activity. Further, complete loss of activity was never observed, and this rules out an -SH group as a sole binding site for molecular oxygen. Interestingly, when 2-mercaptoethanol was omitted from the regeneration mixture (Table 2), aequorin was still regenerated, but at a lower level. Substituting serine at positions 145 and 152 gave activities corresponding to 80% and 123%, respectively, of the control value, but substituting serine at position 180 gave an impressive 220% of the control that was half of the activity of the 2-mercaptoethanol-regenerated aequorin.

The Ca^{2+}-triggered aequorin reaction involves only a single turnover of aequorin, after which aequorin is regenerated for the next light emission. However, it has been noticed that native aequorin emits a weak, spontaneous, Ca^{2+}-independent luminescence with a progressive change from a rigid, fully active form to an unfolded, inactive form (34). On the other hand, apoaequorin in the presence of coelenterazine, $CaCl_2$, and 2-mercaptoethanol acts as an oxygenase, catalyzing the oxidation of coelenterazine with a weak, long-lasting light emission (6). Treatment of native aequorin with *p*-chloromercuribenzoic acid (1) or *N*-ethylmaleimide (3, 35) results in complete loss of Ca^{2+}-triggered activity, and the effect of *N*-ethylmaleimide is accompanied by a prolonged, weak light emission. Kemple *et al.* (36) have found that -SH modification causes a structural change in the protein molecule that makes coelenterazine easily dissociable. These observations suggest that the integrity of the -SH groups is important in stabilizing the aequorin molecule and that the oxidation and reduction of these groups are implicated in the bioluminescence and regeneration reactions. This model of the aequorin molecule is not incompatible with the present results. The previous report (3, 35) that *N*-ethylmaleimide completely inactivates aequorin and the fact that C1S, C2S, and C3S regenerated with 2-mercaptoethanol (Table 2) are partially active with Ca^{2+} indicate that all three cysteines are necessary for full aequorin activity. Further, the fact that C1S, C2S, and C3S regenerated in the absence of 2-mercaptoethanol (Table 2) are active with Ca^{2+} suggest that at least two -SH groups are required for aequorin activity. The relatively high activity achieved by aequorin regenerated in the absence of 2-mercaptoethanol cannot be explained without further study, but it may be due to decreased possibilities for -SH modification, allowing for the binding of coelenterazine and molecular oxygen to take place at the active site but in an unstabilized form.

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