Thermostable Mutants of the Photoprotein Aequorin Obtained by *in Vitro Evolution*

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Keisuke Tsuzuki, Ludovic Tricoire, Olivier Courjean, Nathalie Gibelin, Jean Rossier, and Bertrand Lambolez

From the Laboratoire de Neurobiologie et Diversité Cellulaire, CNRS UMR 7637, Ecole Supérieure de Physique et de Chimie Industrielles, 75005 Paris, France

Aequorin is a photoprotein that emits light upon binding calcium. Aequorin mutants showing increased intensity or slow decay of bioluminescence were isolated by *in vitro* evolution combining DNA shuffling and functional screening in bacteria. Luminescence decay mutants were isolated at the first round of screening and carried mutations located in EF-hand calcium binding sites or their vicinity. During *in vitro* evolution, the luminescence intensity of the population of mutants increased with the frequency of effective mutations whereas the frequency of other amino acid substitutions remained roughly stable. Luminescence intensity mutations neighbored the His-16 or His-169 coelenterazine binding residues or were located in the first EF-hand. None of the selected mutants exhibited an increase in photon yield when examined in a cell-free assay. However, we observed that two mutants, Q168R and L170I, exhibited an increase of the photoprotein lifetime at 37 °C that may underlie their high luminescence intensity in bacteria. Further analysis of Q168R and L170I mutations showed that they increased aequorin thermostability. Conversely, examination of luminescence decay mutants revealed that the F149S substitution decreased aequorin thermostability. Finally, screening of a library of random Gln-168 and Leu-170 mutants confirmed the involvement of both positions in thermostability and indicated that optimal thermostability was conferred by Q168R and L170I mutations selected through *in vitro* evolution. Our results suggest that Phe-149 and Gln-168 residues participate in stabilization of the coelenterazine peroxide and the triggering of photon emission by linking the third EF-hand to Trp-129 and His-169 coelenterazine binding residues.

The photoprotein aequorin, isolated from the jellyfish *Aequorea victoria*, is a bioluminescent complex formed with the protein apoequorin and the prosthetic factor coelenterazine that emits light upon calcium binding (1, 2). Aequorin belongs to the family of calcium-dependent luciferase/luciferin systems (3) and has been extensively used as a Ca\(^{2+}\) sensor in living cells (4) as a luminescent label in binding assays (5). The 189-amino acid polypeptide apoequorin contains three EF-hand Ca\(^{2+}\) binding sites located close to the N terminus (EF1) and the C terminus (EF2, -3 pair) of the protein (6, 7). As in other luciferase/luciferin systems (3, 8) aequorin bioluminescence involves the formation of an oxygenated reaction intermediate. In the case of aequorin, this is a slow process (9) resulting in the formation of a coelenterazine peroxide (10) that is stabilized in the hydrophobic core of the protein through interactions with several amino acid residues (11). The binding of Ca\(^{2+}\) leads to the fast release of a photon with a quantum yield of 0.23 at 25 °C because of the final oxidation of coelenterazine (1). Aequorin bioluminescence thus occurs as a flash in which intensity and exponential decay kinetics depend on calcium concentration (12, 13). Site-directed mutagenesis (14–19) and crystal structure (11, 20) have suggested differential roles of the three EFs and identified key residues involved in aequorin bioluminescence.

Bioluminescence provides a simple readout for mutant selection, and early studies have demonstrated the usefulness of random mutagenesis to investigate specific luciferase properties (21, 22). Nonetheless, relatively few reports have used this approach, and none of them deals with calcium-dependent photoproteins (21–27). Here, we used a random mutagenesis and *in vitro* evolution approach based on DNA shuffling (28) to isolate functional aequorin mutants with a simple bacterial screening system. This resulted in the selection of aequorin mutants characterized by an increased luminescence in bacterial screening (Bright mutants) or by slow decay kinetics of bioluminescence (SloDK mutants). Most of the Bright and SloDK mutants selected exhibited modifications of Ca\(^{2+}\) sensitivity and/or decay kinetics that will be described elsewhere. In the present study we investigated the stability of the coelenterazine-apequorin complex formed by Bright and SloDK mutants. Our results provide insights into mechanisms that stabilize the apoequorin-coelenterazine complex and enable calcium-dependent photon emission.

**MATERIALS AND METHODS**

*Random Mutagenesis and in Vitro Evolution of Aequorin—Random mutagenesis by DNA shuffling was performed essentially as described previously (28). Our WT \(^{\text{apoaequorin}}\) (GenBank\(^{TM}\) accession number AY601106, kind gift from Dr. T. Pozzan) differs from the natural gene product by 10 additional amino acids at the N terminus. However, we retained the sequence numbering of the natural gene product (6). Three µg of the WT apoequorin cDNA (coding sequence, 600 bp) was digested with DNase I (1 ng/µl) at 25 °C for 7 min. DNA fragments between 50 and 300 bp were purified from a 2% agarose gel using the Qiagen gel extraction kit (Qiagen GmbH, Hilden, Germany), and 1 µg was used in a primerless PCR (35 cycles: 94 °C, 30 s; 45 °C, 30 s; 72 °C, 30 s) for DNA shuffling. A 2.5-µl aliquot of the shuffling reaction was used in a new PCR (20 cycles: 94 °C, 30 s; 58 °C, 30 s, 72 °C, 40 s) with sense primer UpAeq 5′-CGGTTACGCCATGCTTTATGATGTTCTCT-GAT-3′ and antisense primer LoAeq 5′-TGGAAATTCTTAGGGGACAGCTCCAC-3′ to obtain a pool of mutant full-length cDNA (start and stop codons underlined). After digestion by KpnI and EcoRI, mutant apoequorin cDNAs were inserted into the bacterial expression vector pET28a (+) H11001/EBI Data Bank with accession number(s) AY601106.

To whom correspondence should be addressed: Neurobiologie des Processus Adaptifs, CNRS UMR 7102, Université Pierre et Marie Curie, 9 quai St Bernard, 75005 Paris, France. Tel.: 33-1-44-27-25-09; Fax: 33-1-44-27-25-84; E-mail: bertrand.lambolez@snv.jussieu.fr.

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plasmid pPD16 (29) under control of the Plac promoter and used to transform the XLI *Escherichia coli* strain (Stratagene, La Jolla, CA). Bacteria were grown overnight at 37 °C on an LB agar plate containing ampicillin, 100 μg/ml. Next, single colonies were transferred to 96-well plates either manually or using a Qbot automated colony picker (Genetix, New Milton, UK). Every second plate contained only 95 mutant colonies plus 1 colony of WT aequorin as a control. Colonies were grown overnight at 37 °C in 200 μl of a medium supplemented with ampicillin containing (in g/liter): bacto-tryptone, 26; bacto yeast extract, 10; NaCl, 5; K2HPO4, 0.27; KH2PO4, 7.16; sodium citrate, 2; MgSO4·7H2O, 0.1; (NH4)2SO4, 0.9; glycerol, 50. Finally, 96-well plate replicas were grown for 4–6 h at 37 °C in 50 μl of LB ampicillin medium. Aequorin was then reconstituted by adding 50 μl of a solution containing 100 mM Tris (pH 8), 90 mM NaCl, 10 mM EDTA, 5 μM coelenterazine. A heat shock (55 °C, 30 min) was applied to aequorin-containing bacteria before light intensity was measured.

**Functional Analysis of Apoaequorin Mutants**—The KpnI-EcoRI full-length coding sequences of WT and mutant apoaequorins were subcloned into the pRSETc expression vector (Invitrogen). This resulted in the addition of 40 amino acids at the N terminus, including a His6 tag for purification. Cell-free expression of apoprotein was performed using the Rapid Translation System (RTS, Roche Diagnostics). The reaction proceeded for 24 h at 30 °C, with stirring at 135 rpm, using 10 μg of recombinant pRSETc plasmid. The product of the reaction was then diluted 1:1 in glycerol, and this working stock was stored at −20 °C. Relative amounts of WT and mutant apoaequorins in RTS reaction products were determined by immuno-Western blot analysis using an ECL Western blotting analysis system (Amersham Biosciences) according to the manufacturer’s instructions. Relative maximum photon yield of WT and mutant aequorins was determined by normalizing the integral of the light emitted at saturating Ca2+ concentration to relative amounts of the corresponding apoproteins.

Aequorin was reconstituted for 1 h at 4 °C in the presence of 10 mM 1,4-dithiothreitol, 50 mM Tris (pH 8), 5 mM EDTA, and 5 μM coelenterazine and then diluted 20 times into Tris (pH 8, 50 mM) and EDTA (5 mM) to minimize luminescence background because of unincorporated coelenterazine. Fifty μl of this solution (corresponding to 0.2 μl of apoaequorin working stock) was used per well for functional analysis performed in 96-well plates with a PhL microplate luminometer.

For investigation of aequorin lifetime at 37 °C, all aliquots were initially stored at 4 °C and sequentially transferred at 37 °C so as to stop all incubations simultaneously. For thermostability at different temperatures, aliquots were incubated at 25, 37, 42, 48, 54, or 60 °C for 30 min. For recovery from thermal inactivation, undiluted aequorin aliquots were incubated at 60 °C for 30 min. Then, aequorin was reconstituted for 1 h at 4 °C by adding 10 mM 1,4-dithiothreitol and 5 μM coelenterazine. These aliquots were then diluted as described above, and their activity was compared with that of control aliquots kept at 4 °C during the heat shock and reconstitution process.
Following equilibration at room temperature for 15 min, aequorin activity was measured at 22 °C by injecting 100 μL of a solution containing 50 mM Tris (pH 8), 15 mM CaCl₂, and 5 mM EDTA. Luminescence data were collected with 0.1 s integration time. In our PhL luminometer, injection of 100 μL of solution required 280 ms, and recording started 365 ms after the beginning of injection during the decay phase after luminescence onset (~10 ms, see Ref. 12). Hence, bioluminescence intensities were measured at the start of the recording unless otherwise stated. Each value represents the mean of at least two experiments performed in triplicate. Results are expressed as mean ± S.E.

Some of the lifetime and thermostability experiments presented in this study for RTS-expressed apoaequorins were confirmed with purified proteins that yielded the same results (WT, K17R, and Q168R). Purified apoaequorins were obtained from recombinant pRSETc plasmids expressed in the BL21DE3(pLys) strain of E. coli, using the Xpress purification kit (Invitrogen) with a His₆ affinity Probond column (Invitrogen) according to the manufacturer’s instructions. Purified proteins were used as described above for RTS-expressed apoaequorin.

Local Environments of Residues at Amino Acid Positions 149 and 168—Distances between atoms were obtained from the crystal structure of WT aequorin (Protein Data Bank code 1EJ3, see Ref. 11) or from the predicted three-dimensional structures of the Q168R and F149S aequorin mutants. Predictions were performed by homology modeling through the Geno3D server (see Ref. 30) based on spatial restraints in the crystal structure of WT aequorin.

RESULTS

Isolation of Bright and SloDK Aequorin Mutants—A library of aequorin mutants was generated by DNA shuffling and expressed in E. coli (see “Materials and Methods”). The mean number of nucleotide mutations was 1 per molecule, as determined by sequencing 16 randomly picked clones (TABLE ONE). In this sample, the occurrence of A→G or T→C substitutions was highest, whereas C→G or G→C substitutions were not found. The absence of these latter substitutions in apoaequorin mutants selected through functional screening confirmed their low occurrence in the library. The distribution of these nucleotide substitutions spanned most of the aequorin sequence (Fig. 1C). No nucleotide deletion or addition was observed in any clone sequenced in this study.
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TABLE TWO

Relative bioluminescence in bacteria of Bright mutants compared with that of WT aequorin

| Bright mutation | $L_{0-4}$ activity/WT |
|-----------------|------------------------|
| K17R           | 13 ± 2 (n = 21)        |
| V25A           | 10 ± 2 (n = 3)         |
| N26D           | 13 ± 2 (n = 16)        |
| H27N           | 18 ± 3 (n = 5)         |
| Q168R          | 11 ± 3 (n = 6)         |
| L170I          | 16 ± 4 (n = 4)         |
| K17R + N26D    | 14 ± 2 (n = 8)         |
| K17R + Q168R   | 5 ± 9 (n = 22)         |
| K17R + L170I   | 8 ± 2 (n = 8)          |
| V25I + Q168R   | 20 ± 8 (n = 3)         |
| V25I + L170I   | 24 ± 3 (n = 4)         |
| N26D + Q168R   | 8 ± 1 (n = 16)         |
| H27N + Q168R   | 13 ± 5 (n = 4)         |
| K17R + N26D + Q168R | 14 ± 3 (n = 8) |
| K17R + H27N + Q168R | 11 ± 2 (n = 12)    |
| KR + HN + QR + LI | 14 ± 3 (n = 4)       |

After reconstitution with coelenterazine, 15,853 clones were screened in a luminometer by applying a Ca$^{2+}$-Triton solution onto intact bacteria (see "Materials and Methods"). The light emitted during the first 4 s after injection ($L_{0-4}$) and during the 4 following s ($L_{4-8}$) was measured. As aequorin flash bioluminescence follows an exponential decay, the $L_{4-8}/L_{0-4}$ ratio is an index of decay kinetics. Screening is illustrated in Fig. 1A for a sample of 1146 clones. This sample population exhibited lower activity ($640 ± 30$ RLU) and slower kinetics ($L_{4-8}/L_{0-4} = 0.39 ± 0.01$) than WT ($1433 ± 417$ RLU and $0.10 ± 0.01$, respectively, n = 6). Given a mean number of one mutation per molecule, this indicates that a large proportion of amino acid substitutions affects aequorin bioluminescence and its kinetics. Mutants showing a high $L_{0-4}$ activity as compared with WT aequorin were selected and termed Bright. Mutants with a high $L_{4-8}/L_{0-4}$ value as compared with WT, i.e. with slow decay kinetics, were termed SloDK.

Selected SloDK mutants showed strikingly slower decay kinetics than WT (not shown). The 20 SloDK mutants showing the highest $L_{4-8}/L_{0-4}$ and $L_{0-4}$ values were sequenced. Clones contained a single substitution that consisted of the E35G, V44A, D117G, E128G, F149S, or D153G mutation, located in EFs or their vicinity (Fig. 1). Four of six types of SloDK mutations consisted of an Asp or Glu to Gly substitution at the first 4 s after injection ($L_{0-4}$) and during the 4 following s ($L_{4-8}$). Screening aimed at segregating Bright mutants by WT aequorin among selected Bright clones (n = 2 of 14 clones sequenced). This prompted us to perform two additional rounds of DNA shuffling and screening aimed at segregating Bright mutants by in vitro evolution.

The 40 Bright mutants showing the highest $L_{0-4}$ activity were used for the next round of shuffling-screening (see "Materials and Methods"). Two additional rounds were performed, with 19,100 and 17,859 clones screened in the second and third rounds, respectively. Fig. 1B shows that two mutations, K17R and Q168R, selected during the in vitro evolution process, eventually combined and spread to be present in 33 of 34 Bright clones selected at the third round. The mean activity of the population increased concomitantly with the relative occurrence of the K17R and Q168R mutations. Consistently, the highest activity was found in the double K17R/Q168R Bright mutant that showed a 25 ± 9-fold higher activity than WT in our bacterial system (TABLE TWO), indicating that successful adaptation of aequorin to bacterial expression occurred during in vitro evolution. Other Bright mutations obtained were V25A, V25I, N26D, H27N, and L170I. TABLE TWO describes their relative activities versus WT aequorin, either as single mutants or in combinations found in selected mutants. All Bright mutations were observed in at least two independently selected clones, with V25A showing the lowest occurrence (n = 2). The mean number of Bright mutations per molecule increased during in vitro evolution to reach 2.26 at the third round (TABLE ONE), with K17R and N26D remaining as the only single Bright mutations in third round mutants selected. In contrast with N26D and H27N that persisted throughout the in vitro evolution process, the V25A and V25I mutations disappeared at the third round suggesting a lower efficiency, at least in combination with other mutations. The increasing number of silent mutations found in selected clones suggests that further mutations were introduced at each round of shuffling (TABLE ONE). Nonetheless, the frequency of other-than-Bright amino acid substitutions remained roughly stable (TABLE ONE), showing that the mutational load was eliminated during in vitro evolution, presumably because of deleterious effects on bioluminescence.

Bright mutations of aequorin were all found in EF1 or close to coelenterazine binding residues (Fig. 1C). Indeed, the K17R mutation was flanked by His-16 and Met-19, which are coelenterazine binding residues. Similarly, the Q168R and L170I mutations were located within an $\alpha$-helix containing a cluster of residues (Met-165, Thr-166, His-169, and Thr-173) that bind coelenterazine (11). The K17R, N26D, Q168R, and L170I substitutions affected residues conserved among members of the calcium-activated photoprotein family (31–33). In contrast with SloDK mutants, no amino acid substitution expected to decrease EF affinity was observed in the Bright mutants. The distribution of other-than-Bright amino acid substitutions found in selected Bright mutants delineated mutation-free regions of aequorin (Fig. 1C) in which modification may be deleterious to bioluminescence. In particular, none of the coelenterazine binding residues (11) or the essential Cys-145, Cys-152, or Cys-180 residues (18) were affected. Indeed, only 13 positions were affected by other-than-Bright substitutions, much less than the 23 silent mutations that distributed more evenly across the sequence. However, the absence of mutation in some parts of the sequence (e.g. between amino acid positions 95 and 115) suggests that amino acid substitution at all possible aequorin positions have not been explored in this study.

Thermostable Shuffled Mutants—The functional properties of Bright mutants were next examined with the aim of identifying changes that may explain their increased luminescence in bacteria. Detailed characterizations of K17R, N26D, Q168R, and L170I were carried out in cell-free assays on RTS-expressed proteins (see "Materials and Methods"). None of these mutants showed a higher maximum photon yield than WT (not shown). Given large number of clones (n = 52,992) screened during in vitro evolution, this suggests that the quantum yield of WT aequorin cannot be increased by single amino acid substitutions.

We first noticed that, in bacteria, the Bright mutants Q168R and L170I showed an extended lifetime at 37 °C as compared with WT (not shown), which could explain their selection by in vitro evolution. This property was confirmed in the cell-free assay (Fig. 2A). This was more prominent for the Q168R mutation, and the effects of each mutation were additive in the double Q168R/L170I mutant that retained ~50% of its activity after 72 h at
37 °C (4 h for WT). Free energies deduced from inactivation curves of photoproteins at 37 °C were 98.7 ± 0.2, 99.9 ± 0.1, 103.6 ± 0.2, and 105.2 ± 0.3 kJ/mol for WT, L170I, Q168R, and Q168R/L170I aequorins, respectively. The K17R mutant showed a lifetime at 37 °C identical to that of WT aequorin. Thermostability was next investigated by applying a 30-min heat shock at different temperatures. Consistent with their extended lifetime, the Q168R and L170I mutants were resistant to higher temperatures than WT (Fig. 2B). This effect was more pronounced for Q168R, and the effects of each mutation were additive in the double Q168R/L170I mutant, which retained 77 ± 1% of its activity after a 54 °C heat shock (Fig. 2B). Temperatures for 50% inactivation (T_{50%}) interpolated from thermostability curves of WT and mutant aequorins are given in TABLE THREE. The thermostabilities of the K17R and N26D mutants were identical to that of WT aequorin. The same lifetime and thermostability experiments performed on purified WT, K17R, and Q168R aequorins (see "Materials and Methods") yielded similar results (not shown).

The increased stability conferred by the Q168R and L170I mutations presumably resulted from stabilization of the local aequorin structure surrounding His-169 and other C-terminal coelenterazine binding residues. We thus anticipated that some SloDK mutations, which may affect the rigidity of the protein scaffold (e.g. Asp/Glu to Gly mutations in EF1–3), may alter aequorin thermostability. Strikingly, the largest effects on thermostability were observed for SloDK mutations of the EF3 domain (Fig. 2C). Indeed, the F149S mutant showed decreased thermostability, whereas D153G substantially increased aequorin thermostability. In contrast, EF1 mutations had little or no effect (V44A and E35G, respectively), and both EF2 mutations (D117G and E128G) moderately increased aequorin thermostability (Fig. 2C). T_{50%} values obtained for all SloDK mutants are summarized in TABLE THREE.

Following thermal inactivation of photoproteins for 30 min at 60 °C, the bioluminescence of WT aequorin and of the F149S, Q168R, and L170I mutants could be restored to almost control levels by reconstitution with coelenterazine (see "Materials and Methods"). Indeed, heat-inactivated WT, F149S, Q168R, L170I, and Q168R/L170I aequorins recovered 91 ± 4, 104 ± 2, 93 ± 4, 89 ± 4, and 100 ± 8% of their activity after reconstitution, respectively. This observation, together with the smooth temperature dependence of the heat inactivation curve (Fig. 2B and C), suggests that bioluminescence inactivation did not result from apoprotein denaturation.
The characterization of the sensitivity of bioluminescent responses of the mutants to Ca\(^{2+}\) concentration will be reported elsewhere. This revealed that N26D exhibited a higher sensitivity to Ca\(^{2+}\) than WT aequorin, which could explain its Bright phenotype in bacterial cells. In contrast, no change was observed for K17R, whereas Q168R and L170I showed a lower Ca\(^{2+}\) affinity than WT aequorin.

Random Substitutions of Gln-168 and Leu-170 Residues—The above results suggest that the EF3 domain is critically involved in both bioluminescence thermostability and decay kinetics. Structural correlates of these properties were further investigated at the key 168QHL170 spot. Indeed, these amino acids are at the interface between EF3 calcium binding residues and coelenterazine and may thus constitute an essential trigger of photon emission. This spot was explored using a library of random Gln-168 and Leu-170 mutants expressed in E. coli (see “Materials and Methods”). Analysis of 500 clones showed that the mean $L_{0.4}$ activity was reduced to 5% of WT, indicating that most Gln-168 or Leu-170 substitutions disrupt aequorin function, consistent with a direct role of 168QHL170 in bioluminescence. Subsequently, 3840 clones were screened for resistance to a 30-min heat shock at 55 °C (see “Materials and Methods”) and for decay kinetics of light emission as described for shuffled mutants. Among 191 heat-resistant clones, 29 were selected that showed a range of decay kinetics similar to that of the population of 191 clones. Results pertaining to calcium sensitivity and decay kinetics of selected clones will be reported elsewhere. Amino acids found at position 168 were Arg, Ser, Lys, or Ala and were Ile, Val, Phe, Met, or Leu at position 170. Both Bright mutations Q168R and L170I were isolated using this procedure, and the WT Gln-168 residue was never found in thermostable mutants, confirming this position as critical for thermostability. Only hydrophobic residues were observed at position 170. Mutants at the 168QHL170 triplet are designated by their amino acid sequence (e.g., RHL is the double Q168R/L170I mutant).

Thermostability was characterized in the cell-free assay on RTS-expressed proteins as described above. $T_{50\%}$ values, investigated for four mutants, were 57 °C for RHV and 51.5 °C for AHV, RHM, and KHV (TABLE THREE). Comparison between AHV, KHV, and RHV indicates that at position 168, the highest thermostability was conferred by the Gln→Arg substitution. The increased thermostability observed following the order RHM < RHL < RHV = RHI indicates that at position 170, Ile or Val conferred optimal thermostability. These results confirm the importance of both amino acid positions 168 and 170 in aequorin stability. They further indicate that maximal stability was reached with the RHI mutant that combines the Q168R and L170I mutations selected through in vitro evolution.

**DISCUSSION**

Screening a library of random mutants is a means to explore an entire protein sequence to identify amino acids involved in specific functional properties without a priori knowledge of its structure-activity relationships (34, 35). In the present study, this allowed identification of key residues throughout the aequorin sequence and assessment of the functional relevance of conservative substitutions (e.g., V44A and L170I). The subsequent use of site-directed random mutagenesis proved useful in exploring amino acid diversity at the Gln-168 and Leu-170 spots. Mutations selected appeared to preserve the overall aequorin structure and function. Importantly, none of the residues reportedly involved in the coelenterazine binding and oxidation process (11, 18) were affected in selected mutants, confirming their absolute requirement for aequorin bioluminescence.

Random Mutagenesis of Aequorin—As reported for other proteins (28, 36–41), DNA shuffling efficiently introduced mutations that spanned the entire aequorin sequence and recombined these mutations during the in vitro evolution process. Several observations indicate that a relatively limited proportion of all functional apoaequorin amino acid substitutions has been explored in this study, despite the large number of mutants screened and the small size of apoaequorin. Indeed, the high occurrence of A→G or T→C and the absence of C→G or G→C substitutions in shuffled mutants indicate a bias in the mutation rate affecting both the type of amino acid substitution and its distribution along the sequence. This bias presumably resulted from Taq polymerase and DNase I base preference (42–44). Additionally, no mutation was found in the region between amino acid positions 95 and 115. This may result from the relatively large fragments selected after DNase I treatment (300 bp, see “Materials and Methods”). Indeed, the complete cDNA sequences that are subsequently screened are expected to originate preferentially from the largest fragments during primerless PCR. This is also a likely explanation for the low mean number of mutations in the first round library (one per molecule). Although this low mutation rate favored the isolation of key amino acid substitutions, it did not allow exploration of the combinatorial effect of multiple mutations. Despite these limitations, the present study identifies many amino acids throughout the aequorin sequence that are involved in various functional aspects of bioluminescence, thereby confirming the robustness of DNA shuffling and its usefulness in the study of structure-activity relationships. It is noteworthy that, although generated by different random mutagenesis techniques, most mutations of other luciferases affecting stability, decay kinetics, and/or binding affinity result from single amino acid changes (23–27).

In Vitro Evolution of Bright Mutants—The presence of WT aequorin in clones selected in the first round revealed that screening for Bright mutants was performed at low stringency. This presumably resulted from variability in bacterial growth and from the use of the Plac promoter, in which induction starts after exponential growth. The selection pressure was nonetheless sufficient to induce evolution of the mutant population as evidenced by the spread and recombination of Bright mutations, the increase of population activity, and the low level of other-than-Bright mutations.

Strikingly, Bright mutations were distributed exclusively in the N-terminal and C-terminal peptides that are in closest proximity to coelenterazine, in particular for K17R, Q168R, and L170I, which flank the coelenterazine binding residues His-16 and His-169 (11). In contrast, SloDK mutations were all found in the sequence located between EF1 and EF3. Bright mutations maintained throughout the third round affected residues conserved in other photoproteins (31–33), except for H27N. Although no distinctive functional property could be subsequently identified for K17R, its dominance throughout in vitro evolution and its position close to His-16 point toward an important role for Lys-17 in aequorin bioluminescence. Two types of functional adaptation to selection in bacteria could be identified in Bright mutants. One is an increase in Ca\(^{2+}\) affinity (N26D, not shown) that likely favors maximal luminescence discharge in intact bacterial cells, where Ca\(^{2+}\) influx upon activation is presumably limited. On the other hand, the selection of the Q168R and L170I mutants probably relates to their increased stability resulting in an extended lifetime in bacteria. Indeed, WT aequorin appears sensitive to the intracellular environment, as its lifetime in eukaryotic cells greatly depends on the subcellular compartment (45). Because none of the Bright mutants were screened for their specific adaptive property, higher calcium sensitivity or thermostability of aequorin may be achieved through other mutations.

Interestingly, in the photoproteins obelin, clytin, and mitrocin, Val-25 is replaced by Ile (as in our V25I Bright mutant), and His-27 is replaced by Gly or Ser (31–33). The observation that obelin shows lower

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sensitivity to inhibition by Mg$^{2+}$ and faster decay kinetics than WT aequorin (46) suggests that similar properties of the V25A, V25I, and H27N Bright mutants may be responsible for their increased $I_{0-4}$ activity in bacteria. Indeed, a lower sensitivity to Mg$^{2+}$ is expected to enhance the aequorin peak response to Ca$^{2+}$ (46). Similarly, faster decay kinetics result in an increased luminescence peak amplitude provided that the maximum photon yield does not change (12), as has indeed been demonstrated for obelin (46).

Aequorin is a small photoprotein specialized in the calcium-dependent emission of light. For all mutants of the present study, identified gain of function was associated with loss of function that consisted in modification of stability, apparent Ca$^{2+}$ affinity, or decay kinetics. This indicates that the
flexibility of the aequorin protein space allows gain of function through the interplay between these three functional properties. In contrast, no mutant presenting an increased maximum photon yield was isolated. This suggests that WT apoaequorin is optimized for maximal bioluminescence quantum yield, which would thus be essentially limited by the fluorescence quantum yield of the prosthetic light-emitter coelenterazine (3). This may relate to the energetic cost of coelenterazine and its dietary requirement for Aequorea bioluminescence (47).

Thermostability—Among residues that interact with coelenterazine (11), a large number are present in the C-terminal peptide. These residues are under the direct influence of EF3 conformational changes and surround Gln-168 and Leu-170, the mutations of which influence thermostability. In particular, His-169 and Tyr-184 are essential to bioluminescence yield, which would thus be essentially limited by the fluorescence quantum efficiency. Indeed, the positions of SloDK mutations in apoaequorin suggest that each of the three EF domains of photoproteins may efficiently bind Ca$^{2+}$ and trigger bioluminescence.

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