Supporting Text

Materials and Methods

Luminescence assays using a fast-mixing stopped-flow apparatus. WT and mutant aequorins were reconstituted as described in the main text (10 μM EDTA) and diluted in a decalcified Tris (pH 8)/50 mM solution (final EDTA, 0.6 μM). Decalcified Tris solutions were prepared with a column packed with chelex-100 resin (Bio-Rad) according to the manufacturer's instructions. Decay kinetics of bioluminescence were recorded in a SF 300 stopped-flow device (Biologic, Grenoble, France) equipped with a photomultiplier. Light emission was initiated in the stopped-flow apparatus by mixing 60 μl of the photoprotein solution with 140 μl of a Tris (pH 8)/50 mM solution containing variable $CaCl_2$ concentrations. Data were acquired at 100 Hz. Each value represents the mean of two experiments with at least four shots per $[Ca^{2+}]$. Results are expressed as mean ± SEM. Complete data are available by e-mail on request.

A model of aequorin calcium sensitivity. The following equations describe the reaction schemes in Fig. 5*B* and derive from Hill and MWC formalisms (1, 2). Experimental ratios of Σ_S/Σ_T were fitted to the following theoretical expression using the ORIGIN 5.0 software (Microcal, Northampton, MA).

$$\Sigma_S / \Sigma_T = \frac{K^n + \left[Ca^{2+}\right]^n}{K^n\left(1 + K_{SF1}\right) + \left(1 + K_{SF2}\right)\left[Ca^{2+}\right]^n}$$

K is the apparent dissociation constant of Ca^{2+} binding, n is the corresponding Hill coefficient, and K_{SF1} and K_{SF2} are interconversion constants between the slow (S) and fast (F) emitting forms at low (K_{SF1}) and high (K_{SF2}) $[Ca^{2+}]$. These equilibrium constants are defined by the following equations:

$$K^n = \frac{[SAeqCa_i][Ca]^n}{[SAeqCa_{(i+n)}]}\ ;\ K_{SF1} = \frac{[FAeqCa_i]}{[SAeqCa_i]}\ ;\ K_{SF2} = \frac{[FAeqCa_{(i+n)}]}{[SAeqCa_{(i+n)}]}$$

Experimental τ_s values were fitted to:

$$\tau_S = \tau_0 \frac{K_0^m + \left[Ca^{2+}\right]^m}{\left[Ca^{2+}\right]^m}$$

where K_0 is the apparent dissociation constant of Ca^{2+} binding, *m* is the corresponding Hill coefficient, and t_0 is the time constant at saturating $[Ca^{2+}]$.

Determination of theoretical initial maximum intensity. Theoretical initial amplitudes of the fast and slow components were calculated from Σ_S/Σ_T, Σ_F/Σ_T, and τ_F and τ_S values derived from the best fit of the model with experimental data, according to equations:

$$I_{fast} = \frac{\Sigma_F / \Sigma_T}{\tau_F} \text{ and } I_{slow} = \frac{\Sigma_S / \Sigma_T}{\tau_S}$$

Theoretical initial activity (*I*) was calculated from corrected initial amplitudes (I^*_{fast} and I^*_{slow}) to take into account the lag of 365 ms between the beginning of Ca^{2+} injection and activity measurement in our experimental setup, according to equation:

$$I = I^*_{fast} + I^*_{slow} = I_{fast}\ e^{-\frac{0.365}{\tau_S}} + I_{slow}\ e^{-\frac{0.365}{\tau_F}}$$

Results

Screening random Q^{168} and L^{170} aequorin mutants for decay kinetics. Construction of a library of random Q^{168} and L^{170} mutants and expression in *Escherichia coli* has been described in a previous report (3). Bioluminescence was analyzed in a luminometer by

applying a Ca^{2+}-triton solution onto intact bacteria (3). Analysis of 500 clones showed that the mean bioluminescence intensity was reduced to 5.2% of WT, indicating that most Q^{168} or L^{170} substitutions disrupt aequorin function, consistently with a direct role of QHL[168-170] residues in bioluminescence. Subsequently, 3,840 clones were screened for both resistance to a 30 min heat shock at 55°C and decay kinetics of light emission. Characterization of thermostability of the mutants has been reported (3). The light emitted during the first 2 sec after injection ($L_{0\text{-}2}$) and during the 2 sec following ($L_{2\text{-}4}$) was measured. As aequorin flash bioluminescence follows an exponential decay, the $L_{2\text{-}4}/L_{0\text{-}4}$ ratio is an index of decay kinetics. Among 191 heat-resistant clones, 29 were selected for sequence analysis that showed a range of kinetics (Fig. 8 *Left*) similar to that of the population of 191 clones. Mutants at the $QHL^{[168\text{-}170]}$ triplet are designated by amino acid sequence (e.g., the AHV is the double Q^{168}A and L^{170}V mutant). Amino acids found at position 168 were R, S, K, or A and were I, V, F, M, or L at position 170. Only hydrophobic residues were detected at position 170. Both positions influenced kinetics with, from slow to fast: A, K, and R at position 168 and I, V, F, and L at position 170 (Fig. 8 *Right*). The fast RHL mutant corresponds to the Bright Q^{168}R whose decay kinetics were similar to WT aequorin. Characterizations of the AHV mutant that presented the slowest decay kinetics and of the RHI mutant whose kinetics were intermediate between AHV and RHL are described in main text.

1. Hill, A. V. (1910) *J. Physiol. London* **40,** 4-7.

2. Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol.* **12:88-118.**

3. Tsuzuki, K., Tricoire, L., Courjean, O., Gibelin, N., Rossier, J. & Lambolez, B. (2005) *J. Biol. Chem.* **280,** 34324-34331.