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Quenching of Biotinylated Aequorin Bioluminescence by Dye-Labeled Avidin Conjugates: Application to Homogeneous Bioluminescence Resonance Energy Transfer Assays

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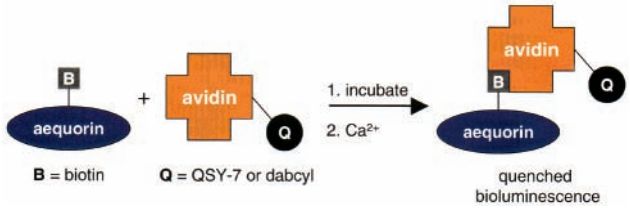
ABSTRACT



Avidin conjugates containing the covalently attached dyes QSY-7 and dabcyl were prepared and shown to quench the bioluminescence of biotinylated aequorin. Quenching efficiency was shown to be dependent on both the label-to-avidin ratio and the concentration of the avidin conjugate. These properties were exploited to develop a homogeneous bioluminescence resonance energy transfer (BRET) assay for biotin.

Recent exploitation of bioluminescent photoproteins and luciferases has led to numerous biotechnological applications, revolutionizing research into cellular, molecular biological, and applied processes.[1,2] One such calcium-activated photoprotein, aequorin, has been used extensively due to the relatively high quantum yield of the bioluminescent reaction and its negligible background signal. Such properties have permitted the in vitro detection of aequorin down to attomole levels and have led to the widespread use of aequorin as an intracellular calcium indicator.[3−5] With the advent of recombinant DNA techniques and the ability to generate substantial quantities of recombinant material, the utility of the photoprotein has been further extended to that of an exquisitely sensitive label for use in binding assays.[6−15]

Aequorin was originally isolated from the jellyfish *Aequorea victoria* and consists of an ∼22 kDa apoprotein and

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a noncovalently bound coelenterazine hydroperoxide derivative.[16] Binding of Ca^{2+} by aequorin triggers a conformational change of the photoprotein, resulting in the rapid oxidation of coelenterazine to coelenteramide with concomitant generation of CO_2 and a flash of blue light ($\leq$10s, $\lambda_{max,em}$ ~469 nm).[17] In vivo, bioluminescence in *Aequorea victoria* actually involves two associated protein components: aequorin and an ~27 kDa green fluorescent protein (GFP). Calcium triggers generation of the excited coelenteramide complex, which, in turn, decays to the ground state via a radiationless energy transfer to the associated green fluorescent protein. The resulting absorbed energy is subsequently emitted as green light ($\lambda_{max,em}$ ~509 nm) from GFP (Figure 1).

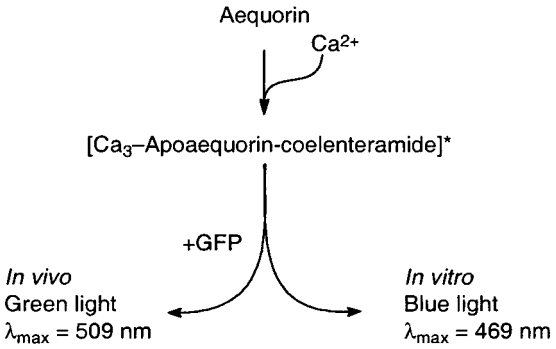

Figure 1. Bioluminescence pathways of aequorin in vivo and in vitro.

Naturally occurring energy transfer processes between bioluminescent energy donors and fluorescent acceptor proteins have been recently exploited for investigations into both in vitro and in vivo protein−protein interactions.[18,19] The methods developed require genetic fusions of one candidate protein to *Renilla* luciferase and a second protein of interest to an enhanced red-shifted GFP (YFP). Such protein−protein interaction assays have been referred to as bioluminescent resonance energy transfer (BRET) assays.[18,19]

We envisioned an alternative BRET format useful for the general development of homogeneous competitive binding assays. In this format bioluminescence from an analyte-labeled photoprotein is transferred to a dye conjugated to the complementary analyte binding partner. The dye conjugated is selected on the basis of the ability to quench the bioluminescent emission. To demonstrate this homogeneous BRET concept, we developed a model competitive binding assay for biotin employing biotinylated aequorin and dye-labeled avidin conjugates.

Biotinylated aequorin was prepared in a manner analogous to a previously described procedure.[15] Briefly, recombinant apoaequorin was purified by a combination of ion-exchange and size exclusion chromatographies from cultures of *E. coli* BL21(DE3)pLysS transformed with a pET-derived apoaequorin construct. The active photoprotein was generated by incubation of the purified apoaequorin with a 10-fold molar excess of coelenterazine overnight at 4 °C in 30 mM Tris, 2 mM EDTA, 5 mM DTT, pH 7.6 (storage buffer). The resulting aequorin was dialyzed into 100 mM $NaHCO_3$, 200 mM NaCl, and 0.5 mM EDTA, pH 8.6 and biotinylated using biotin active ester **1a** (Figure 2). Excess labeling reagent was

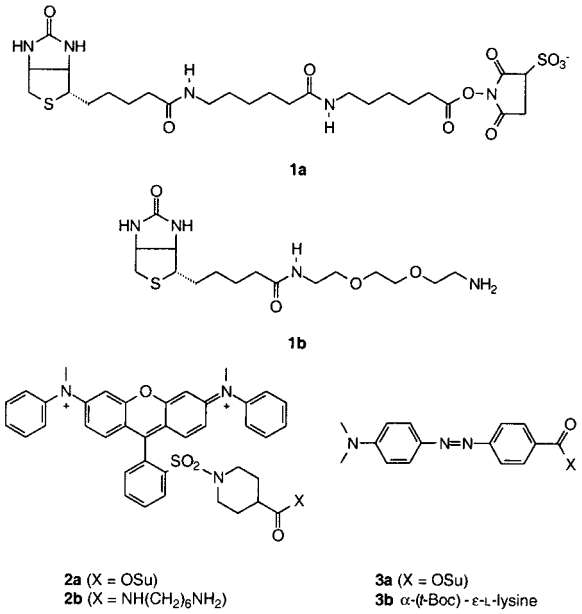

Figure 2. Structures of the biotin (**1**), QSY-7 (**2**), and dabcyl (**3**) derivatives used in this study.

subsequently removed by exhaustive dialysis against storage buffer. Electrospray ionization mass spectrometry analysis indicated an average biotin-to-aequorin ratio of 0.8.

Labeled avidin conjugates containing the dyes QSY-7 ($\lambda_{max\ vis}$ ~560 nm) and dabcyl ($\lambda_{max\ vis}$ ~470 nm) were prepared as potential quenchers of aequorin bioluminescence (Figure 2). The QSY-7 and dabcyl labels were chosen on the basis of their previous use in resonance energy transfer applications and their spectral overlap with the emission profile of aequorin bioluminescence.[20−22] Specifically, NHS esters of QSY-7 (**2a**) or dabcyl (**3a**) dissolved in DMF were added to avidin in 100 mM sodium borate, pH 8.6 (1 mg/mL) such that the final concentration of DMF did not exceed 5% (v/v). The resulting solutions were allowed to stand at ambient temperature in the dark for 2 h after which insoluble material was pelleted. The supernatant containing the con-

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jugate was recovered and purified using a desalting column (MWCO = 6 kDa) equilibrated with 100 mM sodium borate, pH 8.6 to remove unconjugated label. Purified conjugates showed no evidence of precipitation or loss of activity upon prolonged storage in the dark at 4 °C. The ratio of label to avidin was determined using a previously described UV−visible spectroscopy based method.[23] For this UV−visible characterization the hydrolytically stable dyes **2b** and **3b** (Figure 2) were used as model compounds to obtain extinction coefficients in 100 mM sodium borate, pH 8.6. For **2b**, ϵ(282) and ϵ(560) values were determined to be 15000 and 76000, respectively. For **3b**, ϵ(282) and ϵ(453) values were determined to be 7800 and 21000, respectively. The dye-to-avidin ratios for conjugates determined by the UV−visible method are shown in Table 1.

Table 1

conjugate	label	dye-to-avidin ratio
4a	QSY-7	0.92
4b	QSY-7	1.7
4c	QSY-7	2.4
4d	QSY-7	2.9
4e	QSY-7	3.3
4f	QSY-7	3.6
5a	dabcyl	0.51
5b	dabcyl	0.65
5c	dabcyl	0.65
5d	dabcyl	0.87
5e	dabcyl	0.94
5f	dabcyl	1.1

Avidin conjugates with varying ratios of dye-to-protein were employed to assess the ability of QSY-7 and dabcyl to quench aequorin bioluminescence. Thus, biotinylated aequorin (4 nM) was incubated for 1 h with an excess of each labeled avidin conjugate (20 nM) in assay buffer (50 mM Tris, 150 mM NaCl, 2 mM ETDA, 0.1 mg/mL BSA, pH 7.6). Subsequent triggering of 100 μL of each mixture with 100 μL of 50 mM Tris, 100 mM $CaCl_2$, pH 7.6 in a microplate luminometer (MicroLumat Plus, PerkinElmer) provided a bioluminescent response from which the total integrated relative light units (RLU) over a 9 s window were determined. Plots of % bioluminescence quenching vs the label-to-avidin ratios for both the QSY-7 and dabcyl conjugate series are shown in Figure 3. Importantly, the addition of avidin (20 nM, label-to-avidin ratio = 0) did not result in any significant decrease in the bioluminescence. Figure 3 clearly shows an increase in aequorin bioluminescence quenching efficiency with increasing label-to-avidin ratios. The maximum bioluminescence quenching achieved with the avidin conjugates containing the highest QSY-7 and dabcyl-to-protein ratios were 52% and 27% for **4f** and **5f**, respectively. Attempts to achieve additional quenching by increasing the ratio of label-to-avidin were not successful due to the insolubility of conjugates containing levels of dye greater than that shown in Table 1.

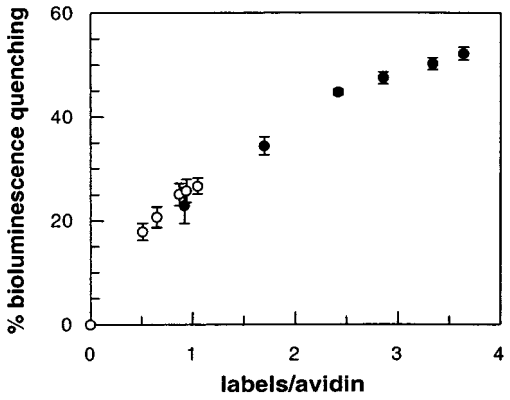

Figure 3. Plot of % bioluminescence quenching vs label-to-avidin ratio: QSY-7−avidin series (●); dabcyl−avidin series (○). The zero label-to-avidin ratio response represents the bioluminescent signal obtained from biotinylated aequorin in the presence of unlabeled avidin. Data points represent the average of triplicate values.

The dependence of biotinylated aequorin bioluminescence on the ratio of QSY-7- or dabcyl-to-avidin is consistent with quenching occurring by way of a BRET mechanism. Interestingly, Daunert et al. reported that binding of avidin to biotinylated aequorin resulted in up to a 30% decrease in bioluminescence. This property was exploited to develop the first example of a homogeneous aequorin-based assay. A putative conformational change in aequorin resulting from protein−protein contact was proposed to give rise to this phenomenon.[5,24] In our study, no such decrease in emission was observed in the biotinylated aequorin−avidin complex, thereby excluding a conformational change as a reason for decreased bioluminescence.

The concentration dependence of QSY-7− and dabcyl−avidin conjugate quenching on aequorin bioluminescence was evaluated using conjugates **4e** and **5e**, respectively. Specifically, biotinylated aequorin (4 nM) in assay buffer was mixed with several concentrations of each dye-labeled avidin conjugate (0.005−10 nM), and the solutions were allowed to incubate for 1 h. Identical solutions with biotinylated aequorin and unlabeled avidin were also generated in a separate series to serve as a control. The resulting bioluminescent response was obtained using 100 μL of each mixture as described above. Plots of % bioluminescence quenching vs avidin quencher conjugate concentrations are shown in Figure 4. The data shows that aequorin bioluminescence is inhibited by avidin quencher conjugates **4e** and **5e** in a concentration-dependent manner. The lowest concentrations tested exhibited minimal quenching of aequorin bioluminescence while the highest concentrations tested exhibited maximum quenching values of 51% and 28% for the QSY-7−and dabcyl−avidin conjugates, respectively. These results

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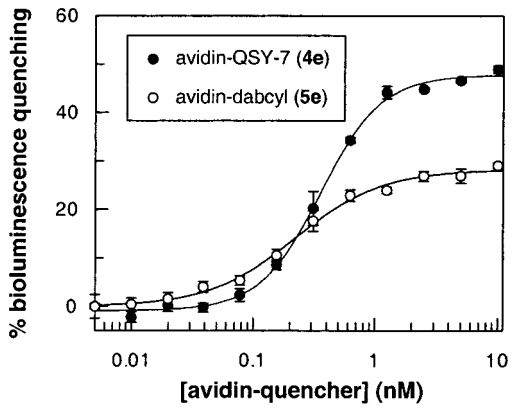

Figure 4. Plot of % bioluminescence quenching vs avidin−quencher conjugate concentration. Data points represent the average of triplicate values. Curves represent the best nonlinear fit of the data using a four-parameter logistic.

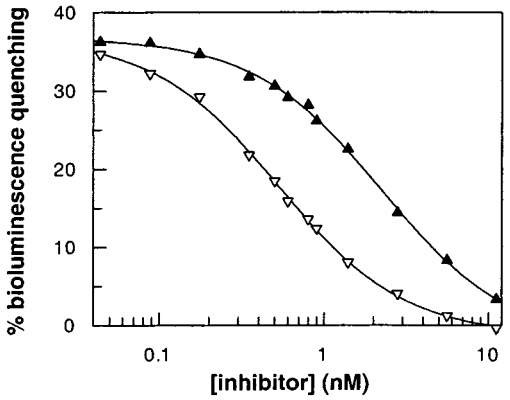

Figure 5. Dose−response curves generated for water-soluble biotin derivative **1b** (△) and biotinylated BSA (▲) inhibition of QSY-7−avidin quenching of aequorin bioluminescence. Data points represent the average of triplicate values. Curves represent the best nonlinear fit of the data using a four-parameter logistic.

are consistent with the values observed in Figure 3. In contrast, aequorin bioluminescence values over the entire unlabeled avidin concentration range did not vary more than ±3% of native aequorin (data not shown).

Dose−response curves were generated for both a water-soluble biotin derivative (**1b**, Pierce, Figure 1) and a biotinylated macromolecule (biotinylated BSA, Pierce). To maximize the dynamic range of the assay, fixed concentrations of QSY-7−avidin conjugate **4e** and dabcyl−avidin conjugate **5e** corresponding to ∼35% and ∼25% quenching of aequorin bioluminescence, respectively, were chosen (vide supra Figure 4). Specifically, a range of concentrations of each biotin inhibitor (0.04−11.2 nM) in assay buffer was mixed with biotinylated aequorin (4 nM). The mixtures were subsequently added to solutions of the QSY-7- or dabcyl-labeled avidin conjugates (0.7 nM final concentrations). The solutions were incubated for 1 h, and 100 μL of each mixture was triggered as described above. A plot of % bioluminescence quenching vs inhibitor concentration is shown in Figure 5 for the QSY-7−avidin system. The dabcyl−avidin system provided similar results although with a lesser dynamic range (data not shown). As expected, aequorin bioluminescence quenching due to association of the QSY-7−avidin conjugate decreases with increasing concentrations of the biotin analyte.

The dose−response curves shown in Figure 5 demonstrate the feasibility of homogeneous in vitro assays for a wide molecular weight range of analytes based on the quenching of bioluminescence with synthetic dyes. In general, this concept displays the simplicity of homogeneous assay formats such as fluorescence polarization immunoassays (FPIA) and the high sensitivity associated with bioluminescence. While this specific example was based on the quenching of biotinylated aequorin by avidin labeled with QSY-7 or dabcyl, the approach is quite general. For example, biotin−avidin could be replaced with a hapten-antibody pair and/or other quencher dyes could be used. Furthermore, the use of site-specific conjugation to decrease the distance between the bioluminescent donor and quencher or more water-soluble dyes to prevent conjugate insolubility could be used to improve quenching efficiency. Work along these lines is currently in progress.

Supporting Information Available: Electrospray analysis of the biotinylated aequorin construct. This material is available free of charge via the Internet at http://pubs.acs.org.

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