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Intermolecular Quenching of Edans/Dabcyl Donor–Acceptor FRET Pair †

Cátia D. F. Martins, M. Manuela M. Raposo and Susana P. G. Costa *

Centre of Chemistry, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; catiadf_martins@hotmail.com (C.D.F.M.); mfox@quimica.uminho.pt (M.M.M.R.)

* Correspondence: spc@quimica.uminho.pt

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Abstract: The intermolecular quenching between 5-(2′-aminoethyl)aminonaphthalene sulfonic acid (Edans) and 4-[[4′-(*N*,*N*-dimethylamino)phenyl]diazenyl]benzoic acid (Dabcyl) was studied by photometric and fluorimetric measurements at pH 7.5 in phosphate buffer. The spectral properties of the Edans/Dabcyl donor–acceptor pair were determined and Dabcyl exhibited an intense absorption band at 463 nm, contributing to the quenching efficiency. We also found the primary requirement for fluorescence resonance energy transfer (FRET), the excellent overlap between the fluorescence emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule, resulting in efficient energy transfer. The quenching mechanism was studied using the Stern–Volmer plot, confirming that this FRET pair was involved in a dynamic quenching process.

Keywords: FRET; donor–acceptor pair; Edans; Dabcyl

1. Introduction

Fluorescence resonance energy transfer (FRET) is one of the most sensitive techniques for monitoring biochemical events. The donor–acceptor pair 5-(2′-aminoethyl)aminonaphthalene sulfonic acid (Edans) and 4-[[4′-(*N*,*N*-dimethylamino)phenyl]diazenyl]benzoic acid (Dabcyl) has excellent spectral overlap between the fluorescence emission of the former and the absorption of the latter, resulting in efficient energy transfer [1]. Strategies incorporating this donor–acceptor pair have been successfully applied to fluorescence-based assays of HIV-1 protease [2], human neutrophil elastase [3], human cytomegalovirus protease [4], and hepatitis C virus protease [5].

The use of a FRET strategy is of particular importance considering our current interest in the design of a formulation able to respond to internal and external stimuli to locally release a cocktail of immunostimulating and chemotherapeutic drugs against colorectal cancer, using a fluorescence reporting system to monitor in real time the response to the treatment. For this, we have synthesised and fully characterized a specific short sequence for granzyme B (GzmB), a serine protease found in the cytoplasmic granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, that plays an important role in biochemical events leading to cell death. GzmB mediates target cell apoptosis when released by CTL or NK cells, representing one of the two dominant mechanisms by which T cells mediate cancer cell death [6,7]. Several short peptides have been reported as GzmB substrates, such as IEPD, IETD and AAD [8,9]. Therefore, our specific tetrapeptide was obtained by microwave assisted solid phase peptide synthesis and coupled to Edans and Dabcyl at its N- and C-termini, in order to make a proof of concept regarding the feasibility of using this FRET pair in the monitoring of GzmB activity in the presence of the tetrapeptide probe by fluorescence techniques.

In this communication, considering this fluorescent probe of peptidic nature, the mechanism of quenching of the FRET pair Edans/Dabcyl was studied by performing photometric and fluorimetric measurements at pH 7.5 in phosphate buffer.

2. Materials and Methods

2.1. General

All reagents were purchased from Sigma-Aldrich and used as received. UV-visible absorption spectra were obtained using a Shimadzu UV/2501PC spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany) and fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer (HORIBA Europe GmbH, Darmstadt, Germany) in quartz cuvettes. Solutions were made with deionised water and phosphate buffer at pH 7.5 was prepared by mixing appropriate volumes of aqueous solutions of NaH_2PO_4 (0.1 M) and Na_2HPO_4 (0.1 M).

2.2. Spectral Properties of Edans–Dabcyl Pair

The spectroscopic characterization of Edans and Dabcyl was carried out by UV-vis absorption spectroscopy of 1×10^{-5} M solutions at pH 7.5 in phosphate buffer of each compound. The fluorescence spectrum of Edans was obtained by excitation at the wavelength of maximum absorption, with a 5 nm slit.

2.3. Fluorescence Quenching of Edans by Dabcyl

Fluorescence quenching measurements were obtained at an excitation wavelength of 342 nm. The fluorescence intensity of the Edans solution (5×10^{-6} M) in the absence of quencher was measured first and incremental amounts of Dabcyl solution (1×10^{-3} M) in phosphate buffer were then added and a measurement taken after each addition. The resulting data was analyzed using the Stern–Volmer equation described below.

Explanation of Data Analysis: Stern–Volmer Approach

The quenching mechanism was studied using the Stern–Volmer Equation (1) [10]:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q] \quad (1)$$

The Stern–Volmer equation allows easy experimental determination of the quenching rate constant, K_q. If the emission intensity in the absence of the quencher and then in the presence of incremental amounts of the quencher is measured, and the resulting ratio of emission intensities (F_0/F) is plotted as a function of quencher concentration [Q], the resulting graph (called a Stern–Volmer plot) will have an intercept of 1 and a slope called the Stern–Volmer constant, K_{SV}. K_{SV} is the product of the lifetime in the absence of quencher, τ_0, and the quenching rate constant, K_q. Knowing the slope and the natural radiative lifetime allows for easy calculation of the quenching rate constant.

3. Results and Discussion

3.1. Spectral Properties of Edans–Dabcyl Pair

Spectral properties of Edans and Dabcyl were carried out in phosphate buffer solutions at pH 7.5. In these conditions, Edans fluoresces at 496 nm whereas Dabcyl shows an intense absorption band at 463 nm ($\log \varepsilon = 4.37$), contributing to the quenching efficiency. The Stokes shift of the Edans was relatively large, of 154 nm, as usual for efficient fluorophores (Table 1). Through the study of the absorption and emission of Edans/Dabcyl, it was found the primary requirement for FRET, the excellent overlap between the fluorescence spectrum of the donor molecule (Edans) and the absorption spectrum of the acceptor molecule (Dabcyl) as illustrated below (Figure 1).

Table 1. UV-visible absorption and fluorescence data for Edans and Dabcyl in 1 × 10^{-5} M solutions at pH 7.5 in phosphate buffer.

Compound	UV-vis		Fluorescence	
	λ_{max} (nm)	log ε	λ_{emi} (nm)	Stokes Shift (nm)
Edans	342	3.57	496	154
Dabcyl	463	4.37	-	-

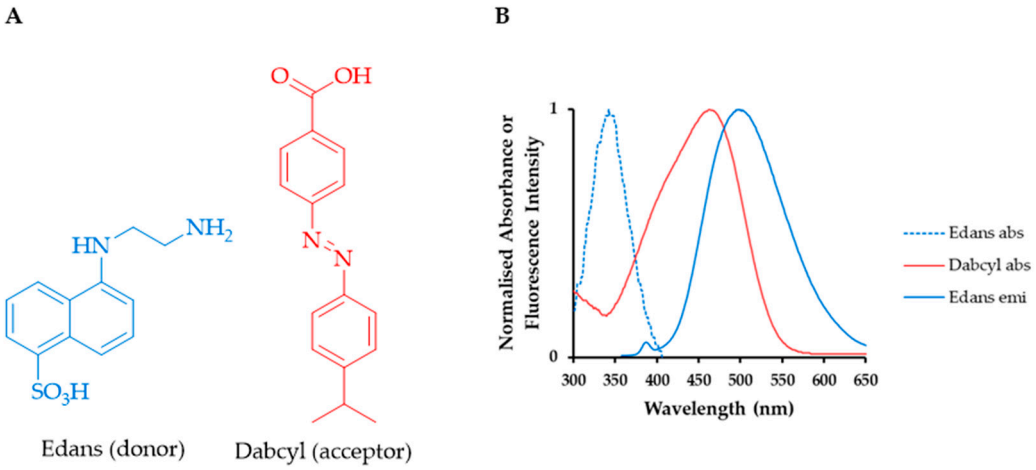

Figure 1. (**A**) Structures of the donor–acceptor pair Edans/Dabcyl. (**B**) The absorption and fluorescence spectra of Edans, and the absorption spectrum of Dabcyl.

3.2. Fluorescence Quenching of Edans by Dabcyl

The addition of a Dabcyl solution (1 × 10^{-3} M) to a Edans solution (5 × 10^{-6} M) resulted in the concomitant quenching of Edans fluorescence as illustrated in Figure 2A. A slight bathochromic shift was also observed, dependent on the concentration that may be related to the formation of charge transfer complexes between Edans and Dabcyl. The formation of the Edans–Dabcyl complex may indeed be induced by hydrogen bonds or by π-π interactions.

The Stern-Volmer plot of the fluorescence quenching of Edans by Dabcyl, namely the plot of the ratio of emission intensities (F_0/F) as a function of quencher concentration [Q], is shown in Figure 2B. It was found a linear variation and the slope gives the Stern–Volmer constant K_{SV}. Knowing the slope and the fluorescence lifetime of Edans, it allows for easy calculation of the quenching rate constant (K_q). The fluorescence lifetime of Edans is reported to be 13 ns [11]. According to $K_{SV} = K_q \tau_0$, some parameters from the Stern–Volmer plot were determined and are listed in Table 2. The results indicate that, given the linearity, the pair Edans/Dabcyl is involved in a dynamic quenching process.

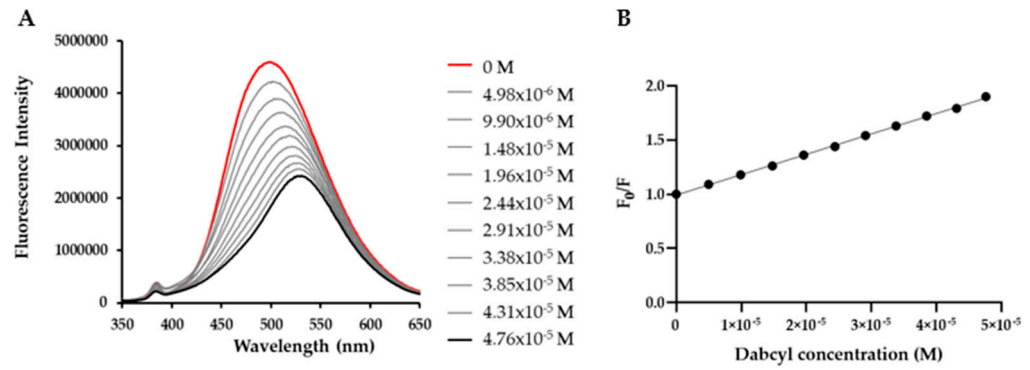

Figure 2. (**A**) Fluorescence quenching of Edans by Dabcyl after addition of Dabcyl (1 × 10^{-3} M) to Edans (5 × 10^{-6} M) solution. (**B**) Stern Volmer plots of the Edans/Dabcyl interaction (F_0—initial fluorescence intensity, F—fluorescence intensity at a given Dabcyl concentration).

Table 2. Stern-Volmer parameters for the Edans/Dabcyl interaction.

Stern–Volmer Equation	Correlation Coefficient (r)	K_{SV} (M^{-1})	K_q (M^{-1} s^{-1})
$F_0/F = 0.99 + 1.88 \times 10^4[Q]$	0.9995	1.88×10^4	1.45×10^{12}

3. Conclusions

The Stern-Volmer parameters and the mechanism of quenching of a widely-used donor–acceptor pair was studied. It was found the excellent spectral properties of the FRET pair Edans/Dabcyl between the Edans emission and Dabcyl absorption along with the high extinction coefficient of Dabcyl. Our studies on Edans interactions with Dabcyl using the Stern–Volmer plot have shown that this FRET pair was involved in a dynamic quenching process.

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Conflicts of Interest: The authors declare no conflict of interest.

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