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Misconceptions over Förster resonance energy transfer between proteins and ANS/bis-ANS: Direct excitation dominates dye fluorescence

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ABSTRACT

Our aim was to disprove the widespread misconception that Förster resonance energy transfer (FRET) is the only explanation for observing fluorescence from ANS (8-anilino-1-naphthalenesulfonic acid) and bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt) following excitation at 280 nm in the presence of protein. From ultraviolet (UV) absorption spectra and fluorescence emission spectra of bis-ANS and ANS in buffer and ethanol, direct excitation at 280 nm was found to be the dominant mechanism for the resulting dye fluorescence. Furthermore, Tyr/Trp quenching studies were performed for solutions of *N*-acetyl-L-tryptophanamide, heat-stressed immunoglobulin G (IgG), and bovine serum albumin (BSA) by monitoring changes in steady state fluorescence spectra and time-resolved fluorescence decays as a function of dye concentration. Stronger quenching of the intrinsic BSA and IgG fluorescence in steady state than in time-resolved fluorescence by bis-ANS and ANS pointed toward static quenching being the dominant mechanism in addition to dynamic quenching and/or FRET. In conclusion, one should consider the role of direct excitation of ANS and bis-ANS at 280 nm to ensure a proper interpretation of fluorescence signals resulting from dye–protein interactions. When ANS or bis-ANS is to be used for protein characterization, we recommend selectively exciting the dyes at the higher absorption wavelength maximum (370 or 385 nm, respectively).

ANS (8-anilino-1-naphthalenesulfonic acid)[1] and bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt) are fluorescent dyes that have been employed extensively for the characterization of protein aggregates, fibrils, folding intermediates, and structural variants [1]. In addition to this, the characterization of hydrophobic pockets or surfaces on native proteins is a common application (e.g., ANS for human/bovine serum albumin [HSA/BSA] [2,3] or apomyoglobin [4,5]). Recently, they have gained in popularity with the advancement of high-throughput screening as a tool to aid development of therapeutic protein formulations [6,7]. The emission spectra of bis-ANS and ANS are sensitive to the polarity of their environment. An increase in quantum yield and blue shift of the emission maximum is measured in hydrophobic environments because of inhibition of the essentially nonfluorescent twisted intramolecular charge transfer (TICT) state in nonpolar environments [8,9]. Because protein unfolding/misfolding and aggregation often lead to an exposure of hydrophobic parts, bis-ANS and ANS can be used for sensitive detection of structurally changed and aggregated protein.

A number of authors have published on the use of ANS and bis-ANS for Förster resonance energy transfer (FRET) studies to study interaction and binding behavior of the dyes with proteins [3,10], to determine binding constants [3,11], to measure the distance between Trp and bound dye [12], and to gain insight into the conformation of proteins [13]. In these studies, the simultaneous reduction in intensity of protein fluorescence and the corresponding increase in dye fluorescence (at ~490 nm) has been cited as evidence for FRET from Tyr/Trp to bound dye molecules following excitation at wavelengths between 275 and 295 nm [3,10–12,14]. An important requirement for FRET to occur is that there is overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. Although Tyr/Trp and ANS/bis-ANS clearly meet this requirement, care should be taken before evoking FRET as an explanation each time one observes this behavior. Already in 1998, it was published by Vekshin that the intrinsic fluorescence of BSA excited at 286 nm is also quenched by dyes whose absorption spectra do not overlap with the emission spectrum of BSA; under these circumstances, FRET is impossible [15]. In addition, a huge contribution of direct excitation at 286 nm to the total dye fluorescence in BSA–ANS solution was mentioned [15].

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[1] *Abbreviations used:* ANS, 8-anilino-1-naphthalenesulfonic acid; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt; HSA, human serum albumin; BSA, bovine serum albumin; TICT, twisted intramolecular charge transfer; FRET, Förster resonance energy transfer; IgG, immunoglobulin G; NATA, *N*-acetyl-L-tryptophanamide; UV, ultraviolet; PMT, photomultiplier tube; IRF, instrumental response function; GLSA, global least square analysis; NTSVD, non-negative singular value decomposition; Orange G, 7-hydroxy-8-(phenylazo)-1,3-naphthalenedisulfonate.

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Moreover, it has been stated in several publications that FRET is the only possible explanation for observing dye fluorescence after excitation at 280 to 295 nm because free ANS and bis-ANS are nonemissive when irradiated at the wavelengths used to excite the intrinsic protein fluorophores [16–18]. A brief glance at Fig. 2 reveals that this statement is incorrect: bis-ANS and ANS can be directly excited at a wavelength of 280 nm (this is explained in detail later). This finding encouraged us to conduct the current study, which aimed to address the common misconception that FRET is the only explanation for observing fluorescence from ANS and bis-ANS following excitation at 280 nm in the presence of protein. A monoclonal antibody, immunoglobulin G (IgG), was chosen as a model protein because it represents an important class of therapeutic proteins and (Bis-)ANS was previously shown to be very useful for studying its aggregation behavior on heat stress [19,20]. *N*-Acetyl-L-tryptophanamide (NATA) and BSA, two well-studied model compounds, were included to support the findings for heat-stressed IgG. We show that direct excitation is the dominant mechanism for the observed dye fluorescence after excitation at 280 nm. Moreover, we demonstrate that the decrease in Tyr/Trp fluorescence intensity in the presence of ANS or bis-ANS can be attributed mainly to static quenching in addition to dynamic quenching and/or FRET.

Materials and methods

Materials

Bis-ANS and ANS were used as stock solutions of 500 μM (bis-ANS) and 2000 μM (ANS) in 99.9% ethanol (Biosolve, Valkenswaard, Netherlands). The dye content was determined by ultraviolet (UV) spectroscopy after dilution with ethanol to an absorbance between 0.1 and 1.0 and using a molar extinction coefficient of 21,200 M^{-1} cm^{-1} for bis-ANS at 397 nm and 4950 M^{-1} cm^{-1} for ANS at 350 nm [1]. A humanized IgG_1 monoclonal antibody (MW = 150 kDa, isoelectric region 9–10) in 100 mM phosphate buffer (pH 7.2) was used. The protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of 1.49 for a 1-mg/ml IgG solution at a pathlength of 1 cm. To create protein species that interact with the dyes, 1.0 mg/ml IgG formulation was heat-stressed for 10 min at 80 °C as described recently [19]. NATA (Sigma, Steinheim, Germany) was dissolved in 100 mM phosphate buffer (pH 7.2) at a concentration of 6.3 μg/ml (25 μM). The NATA concentration was determined from the UV absorbance at 280 nm using a molar extinction coefficient of 5630 M^{-1} cm^{-1} [21]. BSA (>96% purity, Sigma) was formulated at a concentration of 1.0 mg/ml in 100 mM phosphate buffer (pH 7.2), and the concentration was determined using an extinction coefficient at 280 nm of 0.668 for a 1-mg/ml BSA solution at a pathlength of 1 cm.

UV absorption spectroscopy

UV absorption spectra were measured with an Agilent 8453 UV–Vis spectrometer (Agilent, Waldbronn, Germany). Dye stock solution (10 μl) was added to 990 μl of solvent to obtain a final concentration of 5 μM bis-ANS and 20 μM ANS. The samples were measured in half-micro quartz cuvettes (Hellma, Muellheim, Germany) with a pathlength of 10 mm. UV absorbance was recorded for the wavelength range from 250 to 500 nm using an integration time of 1 s and steps of 1 nm. The spectra were corrected for the absorbance of the solvent.

Steady state fluorescence spectroscopy

Steady state fluorescence was measured using a Tecan M1000 plate reader (Tecan Benelux, Giessen, Netherlands) with 96-well plates (Greiner), a sample volume of 100 μl per well (n = 3), a gain of 120 (heat-stressed IgG) or 150 (NATA or BSA), and a Z-position of 19,500 μm. To measure the fluorescence spectra of ANS and bis-ANS in water and ethanol, the same solutions as for UV spectroscopy were used. Furthermore, heat-stressed IgG was measured at a concentration of 1.0 mg/ml in the presence of 5 μM bis-ANS and 20 μM ANS. The samples were excited at 280 and 370 nm (ANS) or 385 nm (bis-ANS), and the emission spectra were recorded from 300 or 400 nm, respectively, to 600 nm with slits of 5 μm.

Quenching studies

Steady state fluorescence quenching

Steady state fluorescence quenching studies with bis-ANS and ANS were performed using a Tecan M1000 plate reader (Tecan Benelux) with 96-well plates (Greiner) and a sample volume of 100 μl per well. IgG was measured at a concentration of 1.0 mg/ml (6.6 μM), NATA at a concentration of 6.3 μg/ml (25 μM), and BSA at a concentration of 1.0 mg/ml (15 μM). The samples were excited at 280 nm, and the emission spectrum was recorded from 330 to 360 nm with a slit of 5 nm. The Z-position was set to 19,500 μm, and a gain of 125 was used. Under these conditions, the inner filter effect was found to be negligible. To obtain F_0/F for the Stern–Volmer plots, the fluorescence intensity in the peak maximum was averaged from three to five wells per dye concentration without dye (F_0) and after the addition of dye (F).

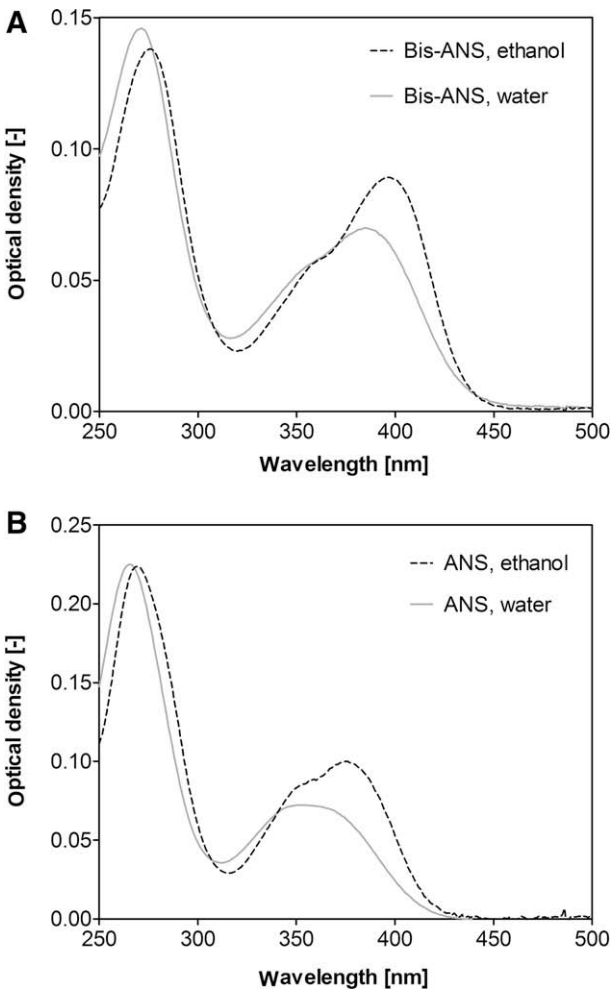

Fig. 1. UV absorption spectra of 5 μM bis-ANS (A) and 20 μM ANS (B) in ethanol and water.