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Notes & Tips

Guanidinium chloride-induced spectral perturbations of 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid confound interpretation of data on molten globule states

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ABSTRACT

We describe limitations in the use of 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) to examine unfolding intermediates associated with guanidinium chloride (GuHCl)-induced protein denaturation. Several studies have used alterations in fluorescence emission of bis-ANS to quantify the population of "molten globule" states. Our findings indicate that the observed changes in bis-ANS spectroscopic properties could originate from the interactions of bis-ANS and GuHCl and the aggregation of the dye at higher GuHCl concentrations. We posit that in the absence of additional complementary structural or spectroscopic measurements, the use of bis-ANS emission alone to monitor protein conformations can be misleading.

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The naphthalene-based fluorescent probe, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS),[4] first introduced by Rosen and Weber in 1969 [1], is an environmentally sensitive dye that is commonly used to monitor "molten globule" states [2]. The fluorescence quantum yield of bis-ANS increases from 0.004 to 0.8 on moving from aqueous environments into the hydrophobic interior of proteins [1], making it a sensitive indicator of protein unfolding intermediates. Chaotropic chemicals such as urea and guanidinium chloride (GuHCl) are commonly used to achieve protein unfolding, thereby populating molten globule-like conformations that display altered solvent accessibility of the hydrophobic protein interior.

Although bis-ANS has been used extensively to characterize GuHCl-induced unfolding of proteins (see Table S1 in Supplementary material), the effects of this denaturant on the spectral properties of the probe are not well understood. A wide range of bis-ANS concentrations, varying from 1.5 μM to 1 mM, have been used for studies on proteins of different molecular weights (10–60 kDa), and several excitation wavelengths (280–395 nm) have been used. The data from such experiments are usually interpreted either after normalizing the bis-ANS intensity at each GuHCl concentration to the initial intensity in the absence of the denaturant or by monitoring the emission peak wavelength shift (Table S1). An increase in bis-ANS emission intensity as a function of GuHCl is usually interpreted as indicative of a greater solvent accessibility of the protein interior and the formation of an intermediate conformational state.

In this investigation, we characterized the perturbations of the ground and excited state spectral properties of bis-ANS in the presence of GuHCl. We found that the spectral properties of bis-ANS display dramatic dependence on the probe as well as the GuHCl concentrations. Both the ground and singlet excited states of bis-ANS are quite sensitive to even low concentrations (~0.25 M) of GuHCl. For bis-ANS concentrations greater than 8 μM, these spectral perturbations in the integrated intensity of the free probe appear to be strikingly similar to some of the denaturation curves reported previously for protein studies (Fig. 4 in Ref. [3], Fig. 9 in Ref. [4], and Fig. 9 in Ref. [5]). Accordingly, we posit that in the absence of additional complementary measurements (e.g., circular dichroism), the use of bis-ANS intensity alone to monitor protein conformations can be misleading. The endogenous spectral perturbations of the bis-ANS fluorescence emission by GuHCl are so extensive that under certain conditions they can mask the effects of the denaturant on the protein, thereby confounding the interpretation of solvent accessibility or "compactness" of intermediate protein structures. In addition, bis-ANS has limited solubility and precipitates at higher GuHCl concentrations. The above observations suggest that some of the published inferences about the population of molten globule states that have been derived from

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[4] *Abbreviations used:* bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; GuHCl, guanidinium chloride; UV–Vis, ultraviolet–visible; FWHM, full-width half-maximum.

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experiments using bis-ANS and GuHCl combinations may have been misinterpreted.

Bis-ANS (Invitrogen, Eugene, OR, USA) was reconstituted in deionized water to a stock concentration of 3.2 mM (extinction coefficient of 16,790 cm^2 $mmol^{-1}$ at 385 nm [6]). GuHCl (99% pure) was obtained from Sigma–Aldrich and reconstituted to 8 M stock using deionized water. A matrix of 12×8 1.5-ml Plastibrand PMMA (polymethyl methacrylate) cuvettes (Perfector Scientific, cat. no. 9008) was prepared from respective stocks covering a GuHCl concentration range from 0 to 5 M and bis-ANS from 0 to 50 μM in 100 mM Tris buffer (pH 7.4, Fisher Scientific, cat. no. BP152-1, impurities Cu, Pb < 1 ppm, reconstituted with deionized water, and filtered through 0.2-μm membrane) containing 200 mM NaCl. The absorption spectrum (300–500 nm) of each sample (10 mm pathlength) was measured using a Beckman Coulter DU 800 ultraviolet–visible (UV–Vis) spectrophotometer blanked against a deionized water-filled cuvette.

Steady-state fluorescence spectroscopy was performed using ISS PC1 and ISS K2 instruments (ISS, Champaign, IL, USA). The excitation wavelength was set to 385 nm with 16-nm slits (full-width half-maximum [FWHM]). Emission spectra were measured from 430 to 650 nm using 16-nm slits (FWHM) and 1-nm steps. To correct for the Wood's anomaly, a polarizer oriented at 0° was inserted into the emission path. To account for background scattering from UV clear disposable cuvettes, the signal from a water-filled cuvette was subtracted at every wavelength. Inner filter effects were negligible for concentrations up to 50 μM bis-ANS (no GuHCl). The fluorescence of GuHCl and Tris alone was negligible. Manufacturer-supplied corrections for grating efficiency and PMT (photomultiplier tube) response were applied to obtain corrected emission spectra.

Time-resolved measurements were obtained using an ISS K2 fluorometer. Excitation was accomplished using a 370-nm LED (light-emitting diode). Emission of bis-ANS was observed through a 435-nm longpass filter (Melles Griot, cat. no. 03FCG461). In the multifrequency phase and modulation technique, the intensity of the exciting light is modulated, the phase shift and relative modulation of the emitted light (with respect to the excitation) are determined, and lifetimes are then calculated according to well-known equations [7,8]. The measured phase and modulation values were analyzed as a sum of exponentials by using a nonlinear least squares procedure implemented using ISS Vinci software. The goodness of the fit to a particular model was judged by the value of the reduced chi-square [9].

We determined the effect of increasing GuHCl concentrations on the integrated bis-ANS emission intensity (Fig. 1A) at varying concentrations of bis-ANS. The fluorescence intensity of bis-ANS emission was markedly affected by the GuHCl concentration (Fig. 1A). This effect is independent of excitation wavelength (295 or 370 nm) (data not shown). For bis-ANS concentrations higher than 5 μM, the integrated fluorescence intensity increased 5- to 10-fold as GuHCl concentrations were increased up to 1.5 M. Interestingly, as the GuHCl concentration was increased further from 1.5 to 5 M, the integrated fluorescence intensity returned back to the levels observed in the absence of GuHCl. The observed spectral changes are quite similar to those reported previously during equilibrium unfolding in multiple published studies (Fig. 4 in Ref. [3], Fig. 9 in Ref. [4], and Fig. 9 in Ref. [5]). Our findings call into question the inferences of partially unfolded protein states drawn in these publications from experiments using bis-ANS and GuHCl.

The addition of GuHCl resulted in a nonmonotonic shift in the peak wavelength of the bis-ANS fluorescence spectrum (Fig. 1B). In the absence of GuHCl, the spectral emission maximum of bis-ANS was observed at 535 nm. Even at a bis-ANS concentration of 5 μM, where perturbations in the integrated emission intensity were not apparent, a significant blue shift of 18 nm was observed in the emission peak in the presence of GuHCl. At higher concentrations of bis-ANS and GuHCl, the emission peak showed a further blue shift to 500 nm. Similar to the concentration dependence observed in the integrated intensity, the shift in emission maximum was also restored to 535 nm as the GuHCl concentration was increased to 5 M.

To determine whether the addition of GuHCl induced alterations in the ground state properties of bis-ANS, we collected absorption spectra of the same samples that were used to generate Fig. 1A and B. In the absence of GuHCl, the bis-ANS absorption spectrum maximum was at 385 nm. As the GuHCl concentration was increased to 3 M, the absorption maxima displayed a significant red shift ($\Delta\lambda_{max}$ = 15 nm) (Fig. 1C). A subsequent increase in GuHCl concentration restored the absorption maximum back to 385 nm. Concomitant measurements of absorption spectra (Fig. 1C, inset) revealed that the extinction coefficient did not display as dramatic a dependence on GuHCl concentration as did the fluorescence quantum yield or absorption maximum.

Consistent with a previous report [10], we observed turbidity in the bis-ANS solution when GuHCl was added. Accordingly, we examined whether the soluble fraction of bis-ANS alone in GuHCl elicited perturbations in spectral properties. The solutions containing 40 μM bis-ANS, 100 mM Tris (pH 7.4), 200 mM NaCl, and varying concentrations of GuHCl were loaded into a Sorvall 100SE ultracentrifuge and spun using a Ti41 swinging bucket rotor at $2 \times 10^5 g$ and 25 °C for 1 h. The concentration of bis-ANS in the supernatant solution was measured by absorbance as described above. After a subsequent spin (2 h at $2 \times 10^5 g$), the measured supernatant concentration remained unaltered (within 2%). In the tubes with GuHCl, a yellow pellet was formed at the bottom. The fraction of bis-ANS that stayed in the supernatant displayed altered emission properties (data not shown). The known interactions of sulfonate-bearing compounds with guanidinium cation [11–13] could partly account for the observed effects on ground and excited states of bis-ANS. However, the extent of the spectral perturbations (Fig. 1A–C) depends nonmonotonically on the guanidine concentration. An absence of a clear sigmoidal dose–response curve suggests complex molecular interactions between bis-ANS and guanidinium cation. Additional studies are required to elucidate the exact mechanisms of bis-ANS and GuHCl interaction.

Finally, the observation that integrated intensities at 0 and 5 M GuHCl appeared to be similar led us to examine the excited state lifetimes of the fluorophore under the two solution conditions. Fig. 1D displays the nonmonotonic heterogeneity observed in the bis-ANS fluorescence lifetimes. In the absence of GuHCl, bis-ANS possesses predominantly a very short lifetime (τ_1 = 0.14 ns, α_1 = 5.5; τ_2 = 1.5 ns, α_2 = 0.09). As the GuHCl concentration was increased, there was a significant shift in the lifetimes of two components along with their relative contributions to the total emission intensity (Fig. 1D). Interestingly, we observed that at approximately 5 M GuHCl, whereas the integrated intensity appeared to have come back to the initial conditions (Fig. 1A), the fluorophore clearly exhibited perturbed photophysical properties. Usually, the drop of fluorescent intensity of bis-ANS at high concentrations of GuHCl is interpreted as complete protein denaturation and release of unperturbed bis-ANS into the solution. The combination of absorption and emission data further highlights the complexities in interpreting bis-ANS response to unfolding with GuHCl.

In conclusion, the bis-ANS fluorescent observables, such as the integrated fluorescent intensity, excited state lifetimes, and position of the fluorescence emission spectrum maximum, are perturbed on interaction with GuHCl. These perturbations are dependent on the concentrations of bis-ANS and GuHCl. Increasing concentrations of GuHCl not only induce excited state perturbations in bis-ANS emission but also affect its ground state properties. These findings raise questions about the inferences of

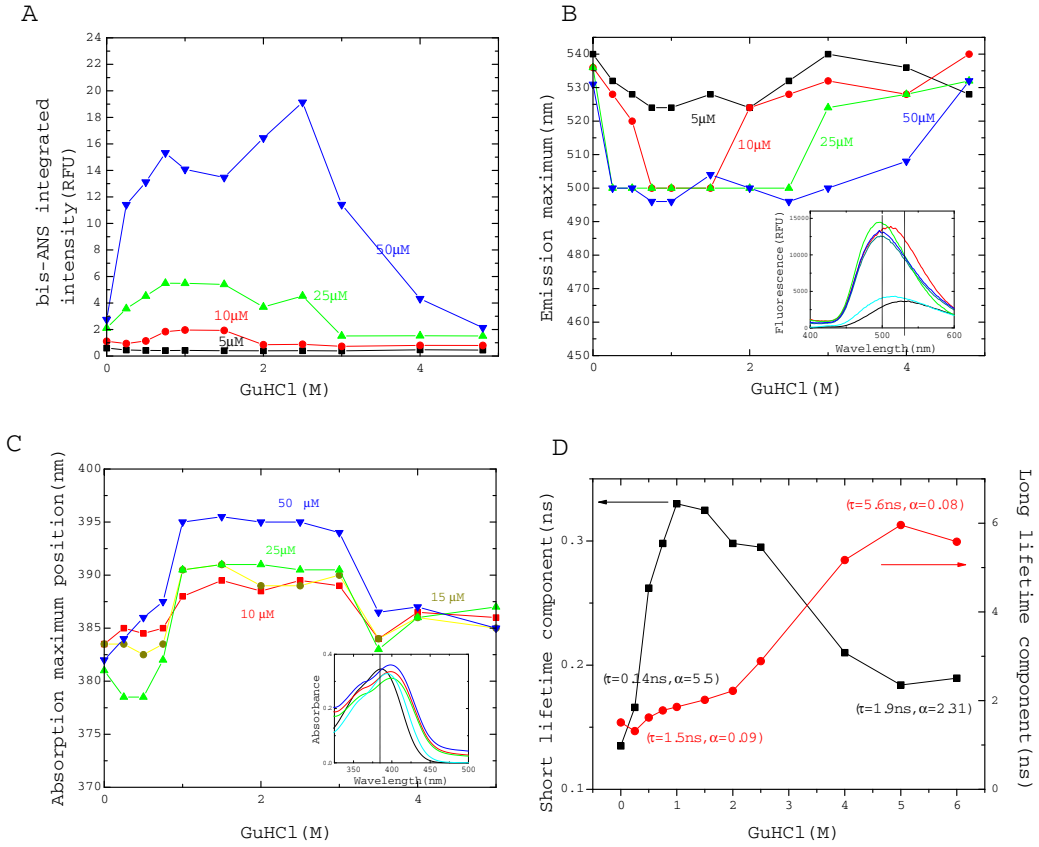

Fig.1. Change of observed bis-ANS parameters at varying GuHCl concentrations. (A) Change in integrated fluorescent intensity of various concentrations of bis-ANS at graded concentrations of GuHCl: ▼, 50 μM bis-ANS; ▲, 25 μM bis-ANS; ●, 10 μM bis-ANS; ■, 5 μM bis-ANS. RFU, relative fluorescence units. (B) Shift in fluorescent maximum as GuHCl concentrations are increased: ■, 5 μM bis-ANS; ●, 10 μM bis-ANS; ▲, 20 μM bis-ANS; ▼, 50 μM bis-ANS. The inset shows the fluorescent spectra for 50 μM bis-ANS for different GuHCl concentrations. (C) Shift of absorption maximum with increasing GuHCl concentrations: ■, 10 μM bis-ANS; ●, 15 μM bis-ANS; ▲, 25 μM bis-ANS; ▼, 50 μM bis-ANS. The inset shows the actual absorbance spectra for 50 μM bis-ANS for different GuHCl concentrations. (D) Dependence of two detected lifetime components on GuHCl concentration. The concentration of bis-ANS is 25 μM. The black curve (■) indicates shorter components (left *y* axis), and the red curve (●) indicates longer components (right *y* axis). Lifetime (τ) and preexponential factor (α) are shown for two extreme points for both lifetime components. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein folding intermediates in published studies that used bis-ANS as a sole conformational probe and GuHCl as a denaturing agent; these inferences should be reexamined and verified using other methodological approaches. Our findings indicate that the observed changes in bis-ANS spectroscopic properties in the presence of increasing concentrations GuHCl, on which these inferences were based in these previous publications, could partly or wholly be related to interactions of bis-ANS and GuHCl and the aggregation of the dye at higher GuHCl concentrations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.04.022.

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