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Photophysics of ANS. I. Protein–ANS complexes: Intestinal fatty acid binding protein and single-*trp* mutants

Elena Klimtchuk, Sergei Venyaminov, Elizabeth Kurian ✠, William Wessels, William Kirk *, Franklyn G. Prendergast

Mayo Clinic College of Medicine, Rochester Minn. 55902, USA

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Abstract

We continue investigations into the physical chemistry of intestinal fatty acid binding protein, I-FABP, and its interaction with ANS and other ligands [*cf* references [Kirk, W., E. Kurian, and F. Prendergast. 1996. Characterization of the sources of protein-ligand affinity: 1-sulfonato-8-anilinonaphthalene binding to intestinal fatty acid binding protein. Biophys. J. 70: 69–83., Kurian, E., W. Kirk, and F. Prendergast. 1996. Affinity of fatty acid for rRat intestinal fatty acid binding protein: Further examination. Biochemistry. 35:3865–74]. The photophysics of the *wt* protein is compared with that in two mutants which lack respectively one or the other of two *trp* moieties, one of which, *trp* 82, is located near the binding region for the polar head group of ligands. These studies afford a look into how the fluorescence of the *wt* protein is established, that is, as an almost direct sum of the fluorescence of the two individual *trp* residues, and how this fluorescence is quenched upon binding to ANS. Though we have access to all the relevant spectroscopic and geometric information necessary to specify in detail the Foerster–Dexter energy transfer model, the quenching process is not explicable in terms of very-weak coupling, as is usually assumed in fluorescence studies in protein systems, but in terms of a stronger effect which goes beyond the simple very-weak dipole:dipole formalism. The quenching of *trp* emission by bound ANS is not as great as that anticipated by ordinary resonance energy transfer, neither is the quenching observed in the reduced lifetimes of the *trp* emission upon ANS binding as great as that observed in steady-state intensity. However the observed steady-state quenching is explicable in terms derived from the lifetime measurements, together with observed spectral band shifts, by the exciton coupling model we invoke here.
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Keywords: 1,8 ANS; Non-Foerster energy transfer; Exciton coupling

We have extended our studies of the interaction of intestinal fatty acid binding protein (I-FABP) and ligands, especially 1,8 ANS (1-sulfo, 8-phenylaminonaphthalene). The purpose of this paper is to investigate the specific involvement of *trp* residues in the thermodynamics of I-FABP-ligand interactions, with a long-range view to attempting to understand some of the photophysical characteristics we have already observed in the wild type (*wt*) protein with ANS [1]. To that end, we employ specific *trp*-to-*tyr* mutants of I-FABP, and compare our results to those of our previous study with *wt* I-FABP. Since the *wt* protein has *trp* situated at two positions, we substitute alternatively one or the other *trp* with *tyr* residues, and as a control, substitute both *trp* residues with *tyr*. Single-*trp* mutants should simplify the photophysics with ANS, we reason. We shall refer to the specific *trp*-to-*tyr* mutants employed herein by the common usage WxxY, where the 'xx' gives the canonical residue position in the *wt* protein sequence.

It has been hypothesized that *trp*-82, which is closely situated near a 'cuff' of aromatic residues at the polar-head-group end of the fatty acid binding cavity, may be crucial in establishing the folding pathway of the *wt* protein [2]. In addition, this same *trp* makes a hydrogen-bond to a highly-conserved, and, at least on the time-scale of molecular dynamics simulations, essentially immobile structural water molecule [3–5]. Thus, the folding and stability, as well as ligand-binding affinity of the W82Y mutant, is of some interest in its own right.

Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; ANS, 8-anilino1-sulfonaphthalene; I-FABP, rat intestinal fatty acid binding protein; uv, ultraviolet; FTIR, Fourier Transform infrared spectroscopy.

* Corresponding author.
E-mail address: kirk.william@mayo.edu (W. Kirk).
✠ Deceased.

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ANS is a very widely used fluorescence probe, yet, despite this popularity, significant questions remain concerning its binding selectivity for hydrophobic 'patches' as well as the precise origin of its enhanced quantum yield when bound to such regions. We already observed fluorescence energy transfer from *trp* to ANS in the *wt* protein, and we now have access to structural data from a recent NMR study of the solution structure of I-FABP complexed with ANS, performed in this laboratory [6]. This latter study yields a distribution of distances and orientations of the ANS naphthalene moiety with respect to the *trp* residues of the *wt* protein, hence we have all the information we need to deduce quantitative predictions of energy transfer efficiencies, which we can then compare to direct measurements of quenching via steady-state or time-resolved (lifetime) techniques. We specifically address the following questions in this contribution: 1) Do the mutants fold like the *wt* protein, and do they bind ANS and oleic acid similarly to the *wt* protein? These are structural and functional 'control' questions, necessarily underlying any use of mutant proteins to address structure/function questions about *wt* proteins; 2) Are the *wt* fluorescence properties explicable in terms of contributions from each *trp* e.g. are the two residues additive?; 3) Is the observed quenching of *trp* by ANS explicable in terms of dipole–dipole energy transfer alone, or are other mechanisms (due, say, to the near proximity of *trp* 82 in the binding pocket) operative? We report here the CD and FTIR spectra and secondary-structure fittings for W82Y, W6Y, and the double mutant W6YW82Y, as well as thermal denaturation temperatures (providing a measure of the 'stability') for these proteins. We report on assays for the ligand-binding affinity of the mutant proteins, demonstrating their functional similarity with *wt*. We also report on fluorescence lifetime measurements on these mutant proteins, either in the *apo* form or as bound 1,8 ANS, as well as the steady-state fluorescence spectra in these systems.

In the second and later contributions (seq. II, III, IV, V.), we utilize the information presented here, supplemented by additional studies, to address more specifically the photophysics of ANS itself (and comparable compounds), namely the spectral properties in solution and as bound to I-FABP, the nature of the underlying transitions (in II,) a quantitative reconstruction of the unusually strong near uv CD as shown in this paper (in III), and an analysis of the fluorescence decay characteristics of ANS in solvents and its probable mechanisms (in IV, and, with respect to protein-bound ANS in V). In these studies we attempt to quantitatively fit, and even predict, spectral and rate parameters using recent theories of solvent-controlled dynamics in electron transfer reactions, and include a theoretical model of our own to attack the problem of Gaussian-distributed emission lifetimes.

1. Materials and methods — experimental rationale

E. coli strain MG 1655 containing the pMON-IFABP vector and the singly-mutated pMON-IFABP vectors were kindly provided by the Frieden lab. at Washington Univ. Sch. Med. (St. Louis). The double mutant W6Y-W82Y was prepared by digesting the single mutant plasmids with pPum1 and Xba1 restriction enzymes (Sigma, St. Louis.) and then ligating. An automated Sequencer analyzed the double mutant DNA to demonstrate the sequence conformity with the expected result. Wild type I-FABP as well as the three mutants were expressed and purified as described [1,4]. No impurities were found on SDS PAGE gels or via TOF MS (as performed by an in-house facility — data not shown).

Protein concentrations were obtained by quantitative nitrogen determinations [7], based upon ashing protein samples with boiling $HClO_4$, followed by direct photometric analysis of ammonia evolved by the indophenol blue method. Nitrogen content calculated from the sequence data then yielded, via comparison with the uv-absorption spectrum, the following molar absorptivities at 280 nm: *wt*: 18,700 [1]; *W6Y* 13360; *W82Y* 13520; *W6YW82Y* 10500 M^{-1} cm^{-1}. The errors are roughly ±5%. 8-Anilinonapthalene-1-sulfonate ammonium salt was from Fluka (Ronkonkoma NY), $\varepsilon_{350}=4995\pm10$ in neutral water. Stock solutions of oleic acid (Avanti Polar Lipids, Birmingham Ala) were prepared as described [8], and all other reagents were commercially supplied high purity grade. We employed 10 mM potassium phosphate buffer, pH 7, for all studies reported here.

Circular dichroism (CD) measurements in the far uv region were performed on a JASCO J-710 spectropolarimeter with an RTE-111 Neslab circulating water bath for temperature control. Measurements were conducted in a 233 μm path length quartz cell at protein concentrations ~ 50 μM over the temperature range from 5–80 °C. Four methods of secondary structure calculation were employed for the data interpretation.

We discuss below our more detailed investigations of relative binding affinity for the mutant proteins *vs* the wild type. Here we wish to describe experiments conducted in an attempt to corroborate these results with CD measurements. There being a strong signal evident in the near uv CD of wild type protein upon interaction with ANS (*vide infra*), it is possible to calculate the dissociation constant K values from these data. The technique employed is essentially similar to that used earlier [1,8,9] with fluorescence, whereby now the ellipticity of ANS at a given wavelength obtained per mole of protein is ascertained, as a function of added ANS concentration, or θ_{tota}.

$$\theta_{tota} = (\theta_{compl}-\theta_{apo})(nA + P + K - \{(nA-P+K)^2 + 4PK\}^2)/2P + \theta_{apo}. \quad (1)$$

θ_{compl} is the ellipticity that would be obtained for the 1:1 ANS/I-FABP complex at saturation, and θ_{apo} is the ellipticity of the uncomplexed protein. The ANS dissociation constant 'K' obtained here, and the stoichiometric factor 'n', which effectively absorbs variations in the ANS concentration between identical runs, is to be compared with that obtained from techniques described below. 'P' and 'A' refer to the protein and ANS concentrations.

It should be noted that this analysis is based on two single-point measurements, and hence subject to a bit more 'noise' than those discussed below, which are based on integrated fluorescence spectra.

Steady-state fluorescence spectra, as well as uv–vis. absorption spectra were performed as described (1). Briefly, the