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Photophysics of ANS V. decay modes of ANS in proteins: The IFABP–ANS complex

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Abstract

The fluorescence properties of ANS as bound to proteins are treated. Several points of view concerning the origin of these properties are reviewed and synthesized into one framework. On proteins where the quantum yield (QY) is appreciable, as in organic solvents, the preferred conformation of ANS is often with the phenyl ring nearly (65°–85°) orthogonal to the naphthalene. The major consequence of this geometry is water exclusion from the critical zone of ANS at which the largest amount of solvent dipolar relaxation originates. This, in turn, leads to a depression of the rate of electron transfer to the surroundings, together with other effects, as noted in the literature and in our lab. Alternative quenching pathways for ANS on the protein *vs* in water are also elucidated.
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Keywords: ANS; Fluorescence quenching; Static quenching; Nonadiabatic effects, protein-ANS interaction; Predissociation

1. Introduction

Why is ANS apparently so extremely sensitive to the presence of water? Time and again it has been employed as a reporter in relatively hydrophobic pockets of proteins, or on membranes [1], becoming strongly luminescent only upon binding to such surfaces. In our own studies [2–4] in addition to our series on ANS Photophysics referred to herein as (*I*), (*II*), (*III*), and (*IV*) with I-FABP this was the expectation and the result.

First, one can ask if water acts in fact as a causative agent in quenching ANS fluorescence, or if the effect is an epiphenomenon with respect to some other cause, like rate and extent of dipolar relaxation, for instance, which just happens to correlate with water content? And if the effect is indeed due to the actual presence of water itself, something which will surely excite the interest of protein chemists, could ANS decay kinetics be a way of 'titrating' how much water is available?

There is a plenitude of explanations of ANS decay characteristics in the literature. Various groups have each their salient observations. It should be clear that, given the continued controversy, each signal observation supports distinct interpretations of just how ANS quenching happens. Is it possible to include all the signal observations into one quantitative mechanism?, or if some observations must be left out of account, can they at least be satisfactorily explained?

We first summarize herein these signal observations and then the most important 'schools of thought' explaining the decay properties of ANS. We hope to systematize thereby the observations and models in the literature. There is, in fact, a vast literature on the subject. The lack of consensus is remarkable for such a well-studied molecule. We introduce a number of outstanding controversies below, and strive to sift out experimental and theoretical observations that may allow us to formulate a consistent account of ANS decay characteristics, especially when brought together with our own results as detailed here and in our previous accounts (*I–IV*).

In this contribution, we also produce experimental results with fluorescence lifetime analyses of ANS on the protein I-FABP. It was because of the results we obtained from these experiments that it became necessary to consider how it is that distributed decays arise in the first place, and led us to consider the model of electron-transfer-to-solvent we presented in (*IV*).

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On the protein I-FABP, and on Apomyoglobin, however, we obtained results which are not easily interpreted in terms of the model of a polaron[1] induced in a homogeneous solvent, except perhaps as concerns minor components of the decay in I-FABP. Indeed, we think an important decay component on I-FABP is *inhomogeneously* broadened instead.

We will also confront, in this paper, the differences between solution and protein-bound ANS complexes in efficiencies of *static quenching*. This occurs whenever the calculated Einstein spontaneous radiation constant (*cf.* [2]) $k_{tad,calc}$ exceeds the actual value of the observed $k_{rad,obs}$=Q.Y./<τ> (the quantum yield divided by the average lifetime). In simple terms, static quenching represents is a changes in 1) relative occupancy in some normal coordinates or *vibrational state* during the excited state into modes different from those populated directly *via* Franck–Condon displacements during the absorption process (which displacements map 1:1 back during the emission process onto ground-state coordinates); or 2) changes in occupancy of *electronic* state during the excited state lifetime. In solution, static quenching seems to delimit a maximal quantum yield of about 40% for ANS. But this limit may differ on proteins. Our task in this contribution will be thus more descriptive, and we shall attempt to tie together a number of 'loose ends' from previous studies.

Overview — The most significant observations with respect to ANS quenching process can be summarized under a small number of select headings: i) the value of the (logarithm of the) nonradiative decay rate, lnk_{nonrad}, is a linear function of Stokes' shift [4,6–9], the larger the Stokes shift (the mean energy difference between the absorption and emission envelopes) the lower the quantum yield, and presumably the faster the fluorescence decay time — appreciably less than can be explained by a decreased spontaneous emission rate, or k_{rad}, expected because of the increased mean emission wavelength, alone. Generally, the Stokes' shift is supposed to arise from dipolar relaxation of the environment about the excited chromophore [10,11]. In more detailed investigations of these effects, it is found that k_{nonrad} and Kosower's "*Z*" value are linearly correlated in dioxane water mixtures, but that there is a break between two linear ranges of plots (*Z* increases with increasing water content). Moreover upon *N*-methyl substitution, the same plots had a smaller slope 'break' in them than with the original N–H aniline. In addition, the QY was uniformly lower in the *N*-methyl analogs. The same group also found that Hammett's σ values for substituted benzoic acids correlated with the emission energy of substituted ANS derivatives (phenyl substituents). Similarly, upon titration of ethanol:water solutions with water, a large nonlinear increase in k_{nonrad} of ANS is observed [12]. ii) a solvent isotope effect of 2.6 (for 2,6 ANS) and 2.5 (for 1,8 ANS) lifetime ratios in H_2O *vs* D_2O is found [2,13]. iii) Kurian obtained [3], and we report here, nmr results in organic solvents, a phenyl ring conformation in which the phenyl is 'high', disposed at an angle from 60° to ~75° to the naphthalene ring. Yet in water, the same angle is measurably 'lower', ~30 to 45°

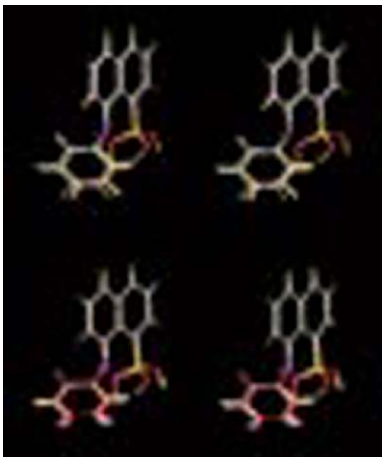

Fig. 1. Stereo picture of ANS ring conformers as determined by nmr spectroscopy (3); above in D_2O; below in acetoritrile and dioxane.

(Fig. 1). iv) Seliskar and Brand [9] found that emission from ANS in glycerol water mixtures continuously red-shifts at longer emission times. We reported similar experiments in (*IV*), although there, two emitting species were resolved by us. We discuss below the rather different set of most relevant findings, as we believe, when ANS is bound to proteins.

The various positions taken by investigators on the dominant *mechanisms* of ANS quenching based on the above observations can also be summarized: α) two-state emission, with differential occupation of a putative high emissive state (thought to be a charge-transfer, or CT state) *vs* a putatively low emissive states dependent on environment; *b*) electron transfer (e.t.) quenching. *c*) influence of other environmental or geometric factors, generally related to increased intersystem crossing; *d*) specific solvation effects (e.g. direct effect of solvent water). Generally this is envisioned to take place in the context of enhanced internal conversion.

1.1. Critique of 2-state emission

This is the Kosower group's interpretation [6–8]. In Ref. [4] we already pointed out how in *amino*naphthalene sulfonate (AmNS) and anilinonaphthalene (AN) the predictions of this model are disconfirmed, inasmuch as AmNS cannot possess the putative CT state, yet shifts much like ANS and has nearly the same maximal QY as ANS, while AN shifts very little and has a high QY throughout titration with I-FABP. While the existence of a CT state was found (in *II*), and while it is possible that some mixing may occur with the locally excited naphthalene state (such as was found in the CD spectrum of ANS in the naphthalene region in *III*), we expect this mixing to be manifest in the ground-to-excited state transition brought about by vibronic, or Herzberg–Teller coupling.[2] The reason for this is again, that

[1] "The electron creates in its neighborhood a distortion of the crystal lattice... This electron, together with its distorted environment, has been called a *polaron*" — Ref. [5], p. 310.

[2] One can perturb a transition matrix element with a more allowed transition, without noticeably perturbing the final state. The difference is due to the fact that Hertzberg–Teller borrowing follows through the *slope* along some nuclear coordinate of the coupling interaction between the two states, i.e.<$m|\partial H/\partial Q|f$>, rather than the coupling matrix element itself <$m|H|f$> ('*m*' refers to some 'intermediate' state, and '*f*' the final state for the transition moment <$i|\mu|f$>=<$i|\mu_0|f$>+<$i|\mu|m$>.<$m|\partial H/\partial Q|f$>/$\Delta E_{mf}$ ($I|Q|F$)— cf. Fischer, [15]).

AN displays much less the CD effect of ANS in the 330–360 nm region (*III*), and that we found a difference in the CT-to-aniline-excited-state transition moment between AN and ANS (*II*) such that the presence of the sulfonate seems to tip the transition moment out of the C1′phenyl-*N*-C1 naphthalene plane. Thus SO_3^- tips a possibly perturbing transition moment towards itself. This putative rovibronic coupling might also explain the well-known, but as far as we are aware, yet unexplained finding, that the ANS extinction coefficient (and hence k_{rad}) *increases* with temperature.

Their results suggesting a 'break' in the plots of Kosower's "*Z*" value with emission energy in a series of dioxane:water mixtures are also less telling than they supposed. Dioxane is suspected to have two kinds of quite specific associations with water molecules a 1:1 water:dioxane complex (probably with a boat conformation for dioxane), and a 2:1 complex (more chair-like-*cf* Boettcher and Bourdewik, Ref. [14]). So that, if 'free' water species are important to the dipolar relaxation (and, coincidentally, quenching) process, there is good reason to expect a 'break' in a plot of fluorescent energy *vs* polarity, somewhere between the mole fraction 1:1 and 2:1 water:dioxane (from the data presented by these authors, the break would seem to appear at roughly 58% mole fraction water— *cf* [7,8]). As for their correlation of emission energy with Hammett substituent effects, it is important to be careful about just what this means. A molecular absorption event can be looked on as the electromagnetic field doing work on a system of charges. The fixed charges of the system are positive (nuclei) and the mobile charges (especially for aromatics) are electrons. This work induces a separation of charge. Increasing the electron density, almost anywhere in the aromatic system, *decreases* the work necessary to perform the same separation of charge. The Hammett substituent values correlate the pK_a of a substituted benzoic acid to the fluorescence emission energy, or, in other words, the work performed on the electromagnetic field *by* the system of charges. The stronger the correlation, the more charge, presumably, is separated in the absorption, or recombined upon emission. That these effects occur with substitution in the *phenyl* ring may only mean that charge density is contributed through the anilino nitrogen (and possibly C1′) into the naphthyl L_a excited state charge distribution, which, naturally enough, is mostly altered in the *naphthyl* moiety— not presumptively that the phenyl actually is a *locus* of electron density changes in the excited state. Indeed the charges of the CT state on aniline we found to be concentrated just at those two positions (*II*). Their interpretation of *N*-methyl substitution results are also less than conclusive, since the presence of the methyl group may inhibit formation of the high phenyl conformer and 'inner salt' formation in nonpolar solvents (methyl substitution could also promote electron transfer by decreasing the ionization potential and lowering the barrier energy).

1.2. Critique of e.t. to solvent quenching

The suggestion by Sadkowski and Fleming [16] that the sulfo serves to create a preexisting electron trap in water-rich solvents— is still open, even if the data of Ebbeson and Ghiron [25] suggest that the quenching is not a simple electron ejection. These latter authors found that typical solvated electron absorbance following ANS excitation was *biphotonic*. In their interpretation, electron ejection is subsequent to absorption from some excited state, and not directly from the production of that state. We do not believe that the evidence presented by Ebbeson and Ghiron [17], however impressive in itself, is necessarily telling, since the only thing they can strictly deduce from it is that the production of transient absorption spectra *from solvated electrons* follows a power law with '*n*' roughly equal to 2. The reason that that does not exclude electron transfer to solvent as a decay mode has to do with the distinction between adiabatic and nonadiabatic electron transfer as we briefly survey:

Let H_{da} be the interaction energy of the electron between the donor and acceptor energy surfaces. If h^-/H_{da} is small compared with t_{pol}, the time required for the solvent to repolarize in response to the electron motion, then the electron responds to an essentially 'frozen' dielectric, and we have adiabatic electron transfer. If the situation is reversed, the electron must await the chance recurrence of near equality in the energies of the reactant and product states. Thus, by far the most likely event is for the acceptor state energy to resemble fully hydrated electron, with a nearly full amount of energy available to decrease the large effective barrier to e.t. characteristic of nonadiabatic transfers — those with large differences in diabatic 'slopes' at least. To reiterate, the crucial parameter of the total electrochemical free energy change between electron donor and acceptor states *includes* (in general) the energy gained by completely solvating the electron in the nonadiabatic case, but includes something different– the solvation energy of the successor state– for the adiabatic case. Since the successor state of adiabatic e.t. to solvent is the polaron in solvent, the second photon must have sufficient energy to dissociate *that* bound state.

1.3. Critique of ring conformation effects

Certain correlations were observed with ring conformation *vs* solvent (*cf. II*, also [3]). The rings of ANS tended to assume nearly orthogonal conformations in dioxane:water (2:1) solvent, or even in acetonitrile (Fig. 1). In water however, the rings assumed a more 'open' conformation (~50° *vs* ~80°). A similar, 'high exo' conformation was observed on I-FABP, and indeed, on MurA (12). The near orthogonal conformation on the proteins would seem to be conducive to restricting the access of water to the sulfonate, anilino nitrogen, C1′ phenyl, and 'bay region' of naphthalene, when bound to the protein. A similar conformation is also observed in chymotrypsin (18). For both proteins, while water is restricted from this particular region, ANS is nonetheless bound on the surface— i.e. with one face of ANS exposed to solvent. Yet it is *the crucial region* (bay region of naphthalene on the anilino side) from which water can be excluded, allowing, in the transition density, the 7 possibility of an 'inner-salt' to be formed, if some CT character is mixed into the transition. The phenyl group could be considered as acting in this way as a 'gate' to water. With enough water present, that is, with a chain of dipolar-relaxing H-bonded molecules extending perhaps into the 2nd and 3rd coordination spheres, the 'high' (near orthogonal) conformer is no longer energetically

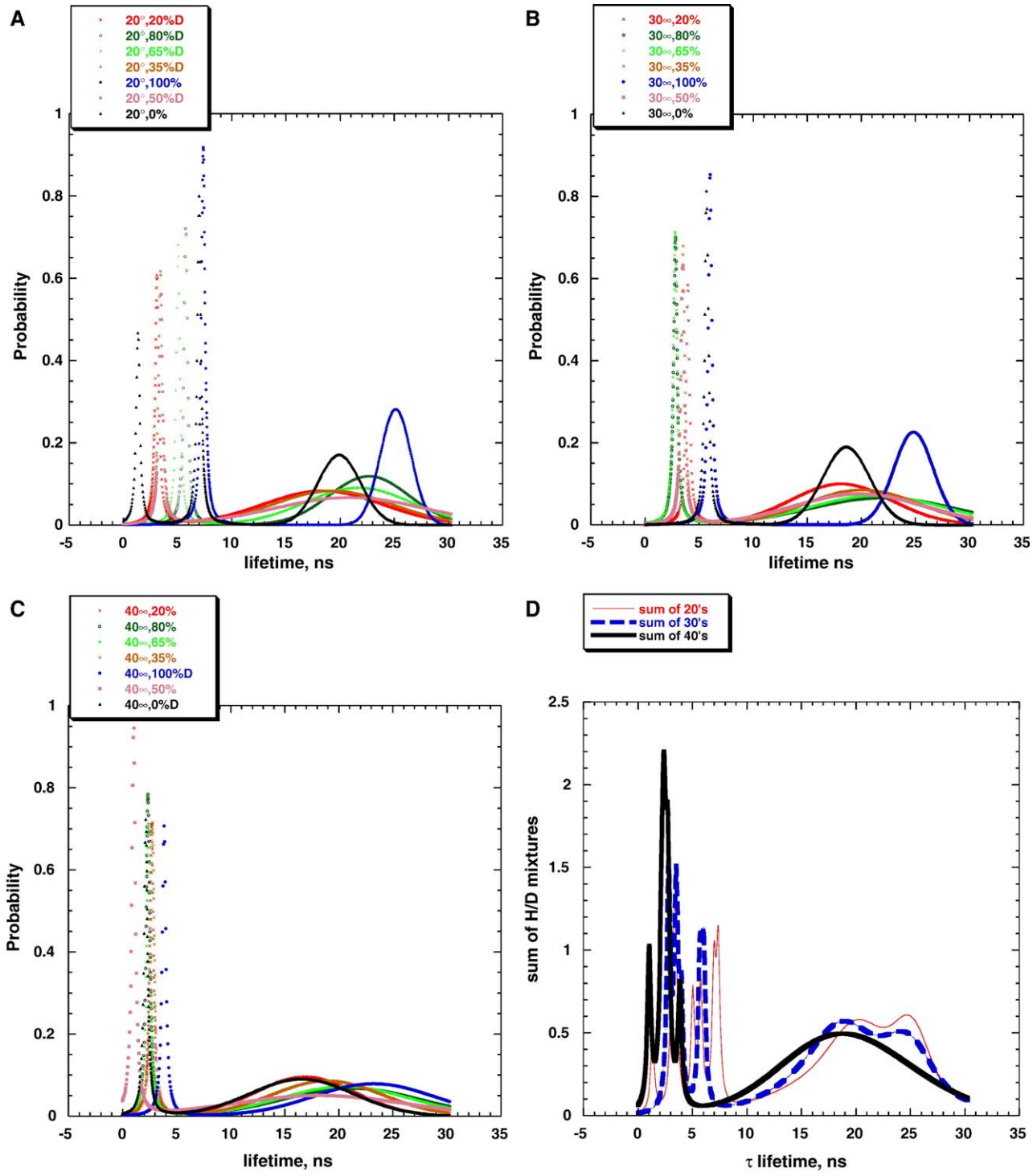

Fig. 2. Fluorescence decay distributions for A) 20 °C, B) 30 °C and C) 40 °C, for the following isotopic buffer concentrations: 100% *D*, 80% *D*, 65% *D*, 50% *D*, 35% *D*, 20% *D*, and 0% *D*. D) plot of the same data (from A, B, C) wherein all the compositions are summed up for 20°, 30°, and 40 °C.

favorable in the watery solvent, and the sulfonate and nitrogen can be more exposed (*cf.* [19]). Consequently, the Stokes shift will also become greater, because of this 'opening up'.

1.4. Critique of intersystem crossing mechanisms

There is scant evidence for increased intersystem crossing, even if that is a dominant mechanism in other amino naphthalenes. In pure ethanol, the k_{isc} does rival that of the radiative rate k_{rad}, while in water it appears to be negligible. Nonetheless, the energy gap between the excited (emissive) state and the triplet is very small in water, and is $\sim$2000 cm^{-1} larger in ethanol. Intersystem crossing is enhanced when there is a rapid spatial gradient in electron density e.g. a large potential gradient at an atom ($1/r\ \partial V/\partial r$, *cf.* [20]). This localization is probably abetted by the absence of water or exchangeable

protons in the solvent, or on the amino nitrogen, which otherwise would tend to neutralize any charge buildup. The greater delocalization of charge generally also implies a larger Stokes shift— since a greater volume and dielectric constant will contribute to the reorganization energy (Marcus, 24, see also below). The reorganization energy is given roughly by $(1/kT)\left[\int \mathbf{E}_{in} \bullet \boldsymbol{\chi} \bullet \mathbf{E}_{in} - \mathbf{E}_{out} \bullet \boldsymbol{\chi} \bullet \mathbf{E}_{out} d^3\mathbf{r}\right]^2$, where the electric field due to the sources is $\mathbf{E}$ (for the 'in' vs 'out' states for electron transfer), and $\boldsymbol{\chi}$ is a dielectric susceptibility tensor. The same total number of source field lines would be involved in any solvent for the same transition, but the dielectric susceptibility of water is greater than for a nonpolar substance, and the volume to be integrated, as we said, in a delocalized electron density, is much greater.

Taken together, the aforementioned facts imply that, as observed, ANS should display a larger spin–orbit coupling interaction, due to charge localization, and hence a higher intersystem crossing rate in aprotic solvents than in protic solvents, while in protic solvents, and especially in water the Stokes' shift is greater than in nonpolar solvents. Given that the rate of intersystem crossing is less in water while the triplet/excited singlet state energy gap is simultaneously significantly lower, on account of the larger Stokes' shift, then presumably the energy surface for ANS in water could rather easily cross the triplet energy surface. This may be responsible for the static quenching observed in water: i.e. the triplet yield would be greater than can be explained by the intersystem crossing *rate*, which is near zero, because the excited state simply branches onto a state already substantially mixed with triplet. For that matter, the triplet yield in ethanol is about 10× what it is in water [17], despite the fact that we cannot recover a measurable rate contribution (*IV*) in ethanol, and the energy gap between singlet and triplet surfaces is larger.[3] As we saw (see note 2), the *rate* of a vibronically enhanced transition from one state to another may not be closely related to the *direct* matrix element between the two states. Much as noted by Ebbeson and Ghiron [17], in the end, the major indication pointing to electron transfer quenching is the strong correlation of Stokes shift with nonradiative decay rate. This *should* be because the barrier to e.t. process is decreased by the reorganization energy λ, which, at least for charge-transfer (including charge transfer to solvent) transitions, is directly related to the Stokes shift. That the Kosower lab. and others [9,12,19] showed a correlation of k_{nonrad} with dielectric relaxation rates in alcohols, and that Robinson, Thistlewaite et al., [21] showed that the quenching process in water has nearly zero activation enthalpy, both suggest an *adiabatic* electron transfer, since the adiabatic rate is partially determined by $\tau_{diel}^{-1}(IV)$ and because nonadiabatic rates are accompanied by a gap jumping which resembles a significant energy barrier [22].

2. Materials and methods

The steady-state fluorescence and time-resolved fluorescence apparatus were as previously described (*I* and *II*).

Reagents were as previously described (*I*). Apomyoglobin was produced from Horse Myoglobin according to the method of Rothgeb and Gurd [23].

Perdeutero, ^{13}C ANS was prepared by custom synthesis by Molecular Probes (now Invitrogen). NMR COSY and NOSY spectra in acetonitrile, dioxane and as bound to IFABP were conducted as described (3).

3. Results and discussion

It was the results shown in Fig. 2 for ANS bound to I-FABP which led us to consider the origin of distributed lifetimes. It is clear from the figure that the long-time component possesses a fairly large width, even greater in lifetime than is found in ethanol. We investigated this distribution as a function of '*D*' *vs*'*H*' content of the buffer, as described in the caption. One sees that between 30 and 40 °C there is a kind of 'break' in that the long lifetime component in D_2O, which is as long as the k_{rad}^{-1}, i.e. as long as it possibly could be, is at 40 °C, now susceptible to another rate process, rather as if the protein structure now admits of some exchange (say, with water) that was not possible below 40 °C. This is in fact mirrored in an effect we observed with isothermal titration calorimetry of ANS binding in I-FABP (2). In that study, the $\Delta H°$ of the reaction as a function of temperature of reaction also had a 'break' at roughly the same place. The effect is also apparent in the lifetime distribution in H_2O. The choice of *H*/*D* mixtures employed in Fig. 2 was such that we have fluorescence samples of atom fraction '*D*' symmetrically disposed about 1:1. So, in Fig. 2d, we have plotted the same data by summing up all the data at each temperature, because of the symmetry of our solution '*D*' fractions, this 'summing' procedure is equivalent to what would be seen at each temperature *if solvent H/D exchange took place infinitely slowly* in an experiment with precisely 50% '*D*'. The plot bears a close resemblance to a spectrum. Instead of the 'decay spectrum' being completely 'washed out' and uninformative after summation, the '*H*' and '*D*' peaks are in fact quite prominent for 20 °C and 30 °C. The only way this can happen, we argue, is if this decay channel is coupled to *one* proton (deuteron). If e.g. *two* indistinguishable protons were involved, there would be *three* peaks of relative ratio 1:2:1, or again, two *distinct* hydrogen-sites would yield 4 peaks of ratios *x*:*x* and *y*:*y* in size, with $x \approx y$. If *many* protons were involved the effect would indeed be averaged away by summing over the various atom-ratio solvents, something like what appears in the 40 °C decay 'spectrum'. The fact that the decay in D_2O is essentially the radiative lifetime, while in H_2O it is rather less than that, implies that some nonradiative decay rate of $\approx 1 \times 107\ s^{-1}$ has a fairly significant isotope effect, i.e. one that effectively abolishes nonradiative decay in the '*D*' case ($<10^6\ s^{-1}$). Moreover, this decay also displays a fairly considerable activation energy (*cf.* 20 °C *vs* 30° in pure H_2O). Therefore, this decay process might well be a *non*adiabatic decay which can effectively discriminate against '*D*' (because of the presence of Frank–Condon factors in the rate). This putative process might involve N–H(*D*) bond scission, for example, because it must represent a *single* proton. At the same time, however, there is obvious sensitivity to the degree of 'proton disorder' in the neighborhood of the affected bond(s). That is, it is apparent

[3] This result is probably due to the fact that the excited state is much longer lasting (>20×) in ethanol, and it has more of a chance to either branch onto the triplet surface, or suffer a transition to that surface at some small, but nonzero rate.

that the distribution is widest where the composition is most uncertain, i.e. at 50% '*D*'. The lifetime width in this buffer is greater than the summed widths of the pure '*H*' and '*D*' buffers—in fact, it is appreciably so for practically all the mixed-isotope buffers. Since we considered the origin of decay distributions in light of adiabatic e.t. events in homogeneous solvents, the conclusion here in the protein, is that the observed width of the lifetime distribution is in fact due to inhomogeneity effects. For 100% D_2O, this would be inhomogeneity in the k_{rad} and must be due to slight changes in the effective field the chromophore 'sees' in different side-chain conformers of I-FABP, or differential occupancy of ANS conformers (ring angle), etc. This would further imply that the mixed 11 solvents are indeed more disordered than the pure solvents, and explain the very large lifetime widths and the pattern observed (i.e. mixed solvents' widths greater than pure, and 50% greatest of all).

At 40 °C, some new decay process has become possible for the pure '*D*' solvent. As we argued earlier, the ΔCp evidence [2] suggests that this change has to do with the protein structure itself, and may represent a water exchanging into the binding site (*cf.* [3]) within the excited state lifetime. If the protein responds on a number of time scales to the charge distribution in the excited state, one can imagine that the long-lifetime component is unable to cross some barrier to another emissive state characterized by a shorter lifetime, but that the reverse process, at least at the lower temperatures, does occur within the lifetime of the species. In such a case the long lifetime decay rate is still given by its intrinsic rate, but the shorter lifetime-component rate is given by k_2+k_x, that is, the sum of its intrinsic decay rate and the rate of barrier-crossing.[4] Then, about 40 °C, the transition between the two species becomes more reversible, so that the slower species can also now 'supply' the faster. The additional decay modes (<5 ns) shown in Fig. 2 might represent our more typical adiaba`tic e.t. with additional water molecules (or possibly protein residues as acceptors) present. It is tempting to suggest that a single water molecule can enter– attracted by the field induced by the excited-state charge distribution– near the naphthyl–aniline junction region to quench the longer lived species by a new mechanism (as in the adiabatic polaron mechanisms of *IV*), and that at the lower temperatures this introduction of water is effectively irreversible during the fluorescence lifetime, but that at 40 °C the protein is more 'open' and the water can drift in or out of this region. Indeed, in the nmr results of Kurian [3], there is room for one water molecule to coordinate to the sulfonate, acting as an intermediary between *arg* 126 and the sulfonate. Since such specific water molecule was not actually identified *per se* in nmr experiments, it is probable that it may transiently occupy this site, with exchange times long on the fluorescence time scale but nonetheless short on the nmr time scale.

Why doesn't the width of the lifetime-distribution in mixed isotopic composition solvents or at higher temperature, if it truly represents increasing 'disorder' of the protons, essentially vanish, as we supposed in the case of dioxane, because of Anderson localization? [24,25] We have to keep in mind the difference between the disorder of each individual system and that of an ensemble of systems. Our claim is that, in fact, the environment in each individual protein binding cavity is highly structured–highly ordered– even on the 20+ ns time scale. But over the whole set of protein-molecules there is a great deal of variation in the mentioned cases, compared with lower temperatures and pure isotopic waters. It is essentially a distinction between homogeneous disorder (in dioxane or 1:1 EtOH–H_2O), and inhomogeneous disorder. The faster decay component(s) are however, single exponentials, so these components may very well be from 'localized' polarons.

Table 1 summarizes a great deal of the information we gained from studies of ANS in solvents together with the proteins I-FABP and Apomyoglobin. Other protein studies have been conducted by many laboratories (*cf.* [26–28]). Most of these, however have not concluded QY determinations, and thus are not in a position to compare k_{rad} with the calculated k_{rad} from an accurate spectrum of *bound* ANS, as we do in the Table; additionally many studies have titrated protein *into* ANS, rather than the reverse, which procedure, we feel, jeopardizes the utility of the spectral information they have obtained, because of the possibility of nonspecific binding detergent-like effects of ANS [4,29].

In Table 1, there are certain entries which concern *static quenching*. We see that ANS and AmNS have $k_{rad,obs} \sim 1/3\ k_{rad,}$

Table 1

System	Q.Y.	$<\tau>$ (10^{-9} s.)	τ_{Long}	$k_{rad,calc}$ (10^7 s^{-1})	$k_{rad,obs}$*
ANS in H_2O	0.0025	0.27	–	2.4	0.93
ANS in D_2O	0.0054	0.67	–	2.4	0.81
ANS.*H* dioxane/pure	0.57	12.6	–	7.5	4.5
ANS.*D* dioxane/pure	0.59	13.2	–	7.5	4.5
ANS dioxane/H_2O	0.43	10.6	–	8.2	4.1
ANS dioxane/D_2O	0.55	13.9	–	8.2	4.0
ANS MeCN/H_2O	N.D.	7.37	7.60		
ANS MeCN/D_2O	N.D.	9.78	10.02		
ANS.I-FABP/H_2O	0.39	12.1	18.6	4.6	3.2
ANS.I-FABP/D_2O	0.44	13.9	24.85	4.6	3.2
ANS.ApoMb/D_2O	0.75	14.7	–	4.9	5.1
ANS.ApoMb/H_2O	0.65	N.D.			N.D.
AmNS H_2O	0.015	2.05	–	3.1	0.73
AnN H_2O	0.11	15.76	20.49	6.5	0.83

*Q.Y. stands for quantum yield, determined as in Kirk et al. [2], units for the longest component lifetime, τ_{Long}, are the same as those for$<\tau>$, the averaged fluorescence decay time, obtained by integrating the decay function: $\int x(\tau)\exp(-t/\tau)dt=<\tau>$. The units for the observed radiative decay rate, $k_{rad,obs}$ (=Q.Y./$<\tau>$), are the same as for $k_{rad,calc}$, the radiative decay rate calculated as in Kirk, et al. [2], via a Stricker–Berg relationship using the absorption and fluorescence spectra. The dioxane/H_2O mixtures refer to 65 mol% dioxane+water, which has an emission spectrum resembling that of protein-bound ANS (Fig. 1). Similarly, the MeCN/H_2O mixtures, which are 30:1 MeCN:H_2O v:v, emit like ANS in FABP. Apomyoglobin (ApoMb) was prepared in this laboratory from horse myoglobin as discussed in the text and measured in 0.08 M lactate buffer pD 4.6 (pH=4.1) with 10× protein concentration at an ANS concentration of 5 μM. AmNS refers to 8-amino-naphthalene-1-sulfonate. AnN refers to 1-anilinonaphthalene.

[4] If k_{x1} is the exchange rate constant from the short to the long decay species, and k_{x2} from long to short (so that we assume $k_{x2}\rightarrow 0$) then the two lifetimes are $k_1+\gamma_1+\gamma_2/2$, and $k_2+k_{x1}+k_{x2}-\gamma_1-\gamma_2/2$, where γ_1 is (k_2-k_1) $k_{x2}/(k_2-k_1+k_{x1})$, and $+\gamma_2=k_{x2}\ 2/(k_2-k_1+k_{x1})$; The values of the eigenfunctions (the components) are $A_1(0)$; and $k_{x1}A_1(0)+(k_2-k_1-k_{x1}+\gamma_1+\gamma_2/2)A_2$ (0); where $A_1(0)$ is the fraction of excited molecules in the long emitting state at time 0, and $A_2(0)$ that for the shorter species.

$_{calc}$, and $k_{rad,obs}$ for AnN is about one eighth of that calculated. In dioxane, the ratio is better for ANS than in water, while, as one might expect *a priori*, with the enhanced rigidity of the bound state (and in the case of ApoMb, the probable absence of water, as can be suspected from the blue-shift of the emission and high QY, *cf.* both Fig. 1 and Table 1) in *protein complexes*, this ratio is significantly closer to 1.0. These observations suggest that it would be worthwhile to keep in mind the possibility of distinct mechanisms for this static quenching in nonpolar *vs* in aqueous environments.

Our early studies of the correlations of phenyl ring position and polar/nonpolar solvent composition suggested to us that the emissive geometry of the excited state might differ from the ground state geometry, and this effect would be more pronounced in water than in nonpolar solvent. Specifically, a twisted emissive geometry with some contribution from the CT state would resemble the ground state geometry in the nonpolar solvent, and would explain why the k_{rad} in these solvents is closer to that calculated from the absorption/emission spectra (*cf.* Table 1) than is the case with water. This hypothesis would also explain why such static quenching nearly disappears when ANS is bound tightly to protein. Nonetheless, while some 'twist' may in fact occur (*cf II*, *III*) we believe the our results with AmNS *dis*confirms our original hypothesis — there is no phenyl to twist, and yet the discrepancy between $k_{rad,calc}$ and $k_{rad,obs}$ persists. We now believe that this effect has more to do with the presence or absence of a nearby triplet state, i.e. it has to do more with *electronic* state mixing than with emissive *vs* nonemissive *nuclear* geometries.

The importance of the sulfo group was discussed, as we mentioned in the Introduction, by Sadkowski and Fleming [16], and is also clear in the comparison with 1-AN (Table 1). The presence of the sulfo seems to increase the dynamic quenching from the data reported there. While the presence of a solvating water at the '1' position has a slight electrostatic *de*stabilizing effect on the CT state energy (*cf. II*), the presence of a sulfo group there instead may be stabilizing. If borrowing from the CT-like transition is important for the naphthalene ^1L_a transition in 1,8 ANS, relative to non-sulfonated analogs, and more so in nonpolar than polar solvents (not unexpectedly—because of charge compensation/dissipation in the latter), it is possible that vibronic coupling terms could be the means whereby this occurs, manifesting as Herzberg–Teller effects in the transition matrix element of the naphthalene, as we discussed in the Introduction. This coupling through the sulfo group, then, would indirectly (because of larger effects in water-poor environments) be responsible for the CD activity of the naphthalene group – the borrowing from the large CD signal of the CT-like absorption – in the more tightly complexed (presumably because of less residual water) FABP protein sites, which effect disappears in less affine protein complexes (as we observed in *I* and *III*).

There seems also to be a low-probability SO_3^- dissociation channel in nonaqueous mentioned by some authors (e.g. 9)—but we think this particular channel is more likely to be coupled to a proton transfer channel that, in highly nonaqueous conditions, can appear as a predissociation– static quenching– channel. In these solvents, one can hypothesize that at least part of this process involves proton transfer to sulfonate. That would be the electronic deactivation process. But it leaves SO_3H in a vibrationally excited mode, which may then proceed to cleavage (in nonaquous solvent an *uncharged* group is more likely a leaving group than a charged one). On the protein, this channel may devolve into the 'pure' H° atom dissociation (nonadiabatic) rate, because the sulfonate would be coordinated by groups, as it seems often to be the case, on the protein. This channel is what we believe we see in the small contribution to the long lifetime component on IFABP in Fig. 2, only observable with '*H*', not '*D*' substitution, and due to one single proton site (presumably N–H (*D*) bond scission).

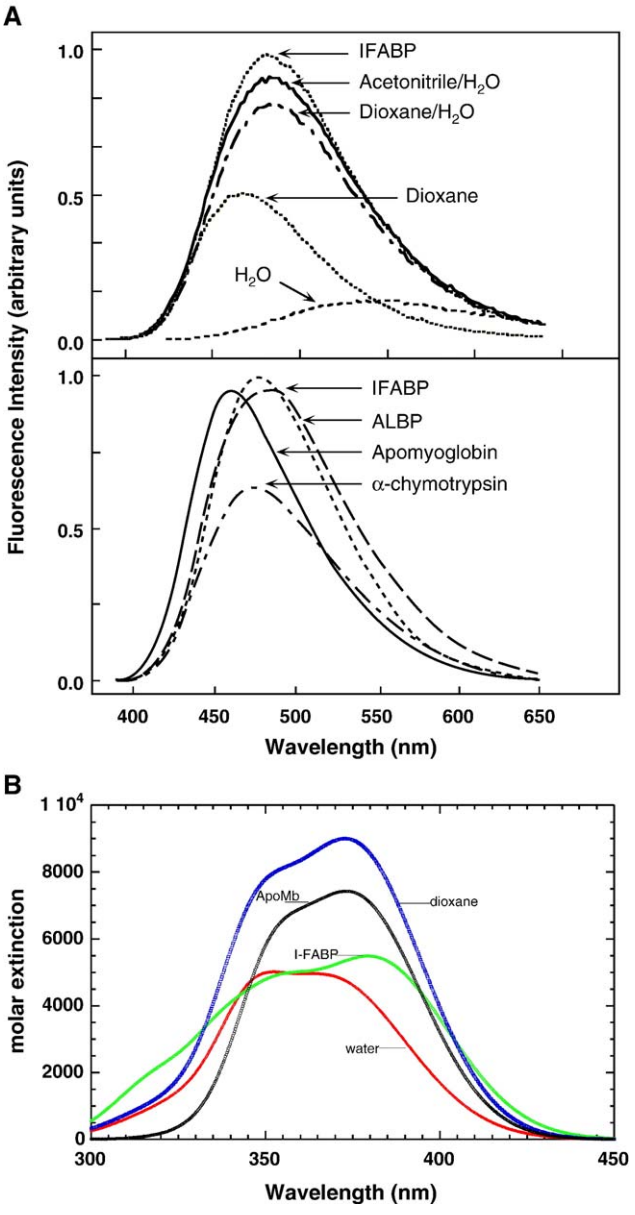

Fig. 3. A. Representative fluorescence spectra of ANS in a variety of solvents (A), and on some proteins (B), as given in the legend. ALBP=adipocyte-lipid binding protein. Acetonitrile/H_2O is 30:1 acetonitrile:water v:v, and dioxane/H_2O is 65 mol% dioxane. The concentrations are only approximately the same for each set: ~2 μM in solvents (~20 μM for water), and ~5 μM in the proteins. B. Molar absorptivity of ANS in various conditions.

It is difficult to make comparisons between nonpolar *vs* polar solvents, on the one hand, and protein systems on the other, but we will summarize here what we think the most important contributions to ANS photophysics are, and how well these extrapolate to protein studies.

1) The correlation of small Stokes shift with high QY/long lifetime, and the further correlation with 'high' phenyl conformation. The induction of the high phenyl conformer was imagined to be due to exclusion of water, which was responsible for the Stokes' shift and QY. On proteins this correlation would be expected to vanish, since water can be excluded and particular ring conformations induced quite independently. Nonetheless in IFABP, MurA and chymotrypsin, a relatively high QY and a high phenyl conformation are observed. This may be adventitious, since the binding sites are on the surface of the MurA and chymotrypsin, and in a water filled channel in I-FABP. Exclusion of water would have to be accomplished by the phenyl 'gate'. The presence of a chain of H-bonded waters would otherwise produce an efficient dipolar relaxation. Most proteins seem indeed to emit with peaks between 450 and 490 nm. Fig. 3 [1,18,26,27], but this may again be due to the amphipathicity of ANS, binding in (or inducing) hydrophobic patches of otherwise solvent accessible surfaces, as long as the critical 'bay region' – encompassing the sulfonate, the proximal bridge carbon of naphthalene, the nitrogen, and the C1′ of phenyl – are all disposed in a water-excluding environment.
   In ApoMb, however, ANS is supposed to bind in the heme pocket. One expects the rings to be relatively flat, and there is little solvent exposure. It is also possible that, as in glycerol water mixtures, a relatively small Stokes' shift could occur with a low QY, depending on the local polarizability, which, if there were a nearby, but isolated water molecule would be relatively low, and the local reorganization energy. This is possibly what is observed in enolpyruvyl shikimate 3-phosphate synthase, which emits at ~460 nm, or possibly α-lactalbumin/Ca^{2+}. [26,27].
2) In addition to the correlation of Stokes' shift and electron transfer barrier, we also find the larger the Stokes' shift, the smaller the singlet–triplet energy gap. Charge being compensated in aqueous solutions depresses the spin-orbit coupling matrix element H_{S-O}. In *non*aqueous solvents, the charge buildup *not* being compensated, there is a larger spin–orbit coupling. Because both the energy gap and the H_{S-O} are smaller in water, the excited state in water *branches* to the triplet.
3) Uncompensated charge from the sulfonate can induce borrowing from a CT band. It is possible that it also increases H_{S-O}. It may also participate in predissociation pathways in nonaqueous solvents, and thus be responsible for an additional static quenching channel in these cases. It is not impossible that all three effects are intimately related. The predissociation/rate may appear in proteins as a N–H scission process. The emission of enolpyruvyl shikimate 3-phosphate synthase is very broad, which may indicate significant involvement of the predissociation mechanism.

A last point of some general interest in protein chemistry: Kurian [3] observed that many close contacts in the I-FABP-oleic acid complex were contributed by aromatic groups, while, in contradistinction, in the ANS complex, it seemed that aliphatic residues made the most significant contacts. This general pattern was also observed in MurA [26] —aliphatic residues making significant contacts with the aryl portions of ANS. While this result runs counter to most current thinking (*cf.* Klebe, [30]), it may reflect the actual situation typical of both *binding proteins* (which must be able to both bind and release ligand), and in *allosteric effector binding sites* of proteins (where cellular physiological state must be capable of modulating ligand-induced effector function). In these two cases, optimal contacts, in terms of the maximum negative free energy of binding, are neither requisite nor consistent with optimal function.

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References

[1] J. Skavik, Anilinonaphthalene sulfonate as a probe of membrane structure and function, Biochim. Biophys. Acta 94 (1982) 1–25.
[2] W. Kirk, E. Kurian, F. Prendergast, Characterization of the sources of protein-ligand affinity: 1-sulfonato-8-anilinonaphthalene binding to intestinal fatty acid binding protein, Biophys. J. 70 (1996) 69–83.
[3] E. Kurian, Solution Structure of Intestinal Fatty Acid Binding Protein complexed with 1-Anilinonaphthalene-8-sulfonate: Implications for Ligand Binding, Thesis, Mayo Graduate School of Medicine, Rochester Minn. (1998).
[4] W. Kirk, The binding of 1,8 ANS congeners to I-FABP and comparison of some hypotheses about ANS' spectral sensitivity to environment, Biochim. Biophys. Acta 1748 (2005) 84–93.
[5] R. Feynman, A. Hibbs, Quantum Mechanics and Path Integrals, McGraw–Hill, N.Y., 1965.
[6] E. Kosower, H. Dodiuk, H. Kanety, Intramolecular donor–acceptor systems. 4. Solvent effects on radiative and nonradiative processes for the charge-transfer states of *N*-Arylaminonaphthalenesulfonates, J.A.C.S. 100 (1978) 4179–4188.
[7] E. Kosower, Intramolecular donor–acceptor systems. 9. Photophysics of Phenylamino-naphthalenesulfonates: a paradigm for excited-state intramolecular charge transfer, Acc. Chem. Res. 15 (1982) 259–266.
[8] E. Kosower, H. Kanety, Intramolecular donor–acceptor systems. 10. Multiple fluorescences from 8-(Phenylamino)-1-naphthalenesulfonates, J.A.C.S. 105 (1983) 6236–6243.
[9] C. Seliskar, L. Brand, Electronic spectra of 2-Aminonaphthalene-6-sulfonate and related molecules. I., and I, J.A.C.S. 93 (1971) 5405–5420.
[10] K. Schweizer, D. Chandler, Quantum theory of solvent effects on electronic spectra: predictions of the exact solution of the mean spherical model, J. Chem. Phys. 78 (1983) 4118–4125.
[11] X. Song, D. Chandler, R. Marcus, Gaussian field model of dielectric solvation properties, J. Phys. Chem. 100 (1996) 11954–11959.
[12] G. Robinson, R. Robbins, G. Fleming, J. Morris, A. Knight, R. Morrison, Picosecond studies of the fluorescence probe molecule 8-Anilino-1-naphthalenesulfonic Acid, J.A.C.S. 100 (1978) 7145–7150.

[13] T. Foester, K. Rokos, A deuterium isotope solvent effect on fluorescence, Chem. Phys. Lett. 1 (1967) 279–280.
[14] C. Boettcher, P. Bordewijk, Theory of Electric Polarization, vol. II, Elsevier, Amsterdam, 1978.
[15] G. Fischer, Vibronic Coupling, Acad. Pr., N. Y., 1981.
[16] P. Sadkowski, G. Fleming, The influence of solvent–solute interaction on radiationless processes: excited state dynamics of 1,8-Anilinonaphthalene sulphonate and related molecules, Chem. Phys. 54 (1980) 79–89.
[17] T. Ebbeson, C. Ghiron, Role of specific solvation in the fluorescence sensitivity of 1,8 ANS to water, J. Phys. Chem. 93 (1989) 7139–7143.
[18] L. Weber, A. Tulinsky, J. Johnson, M. El-Bayoumi, Expression of functionality of α-chymotrypsin. The structure of a fluorescent probe-α-chymotrypsin complex and the nature of its pH dependence, Biochemistry 18 (1979) 1297–1303.
[19] R. DeToma, J. Easter, L. Brand, Dynamic interactions of fluorescence probes with the solvent environment, J.A.C.S. 98 (1976) 5001–5007.
[20] J. Michl, Spin–orbit coupling in biradicals. The 2-electrons-in-2-orbitals model revisited, J.A.C.S. 118 (1996) 3568–3579.
[21] G. Robinson, P. Thistlewaite, J. Lee, Molecular aspects of ionic hydration reactions, J. Phys. Chem. 90 (1986) 4224–4233.
[22] R. Marcus, Relation between charge transfer absorption and fluorescence spectra and the inverted region, J. Phys. Chem. 93 (1989) 3078–3086.
[23] T. Rothgeb, F. Gurd, Physical methods for the study of myoglobin, Methods Enzymol. 52 (1978) 473–486.
[24] P. Anderson, Absence of diffusion in certain random lattices, Phys. Rev. 109 (1958) 1492–1499.
[25] J. Froehlich, T. Spencer, Absence of diffusion in the anderson tight binding model for large disorder or low energy, Comm. Math. Phys. 89 (1983) 151–184.
[26] E. Schoenbrunn, S. Eschenburg, K. Luger, W. Kabsch, N. Amrhein, Structural basis for the interaction of the fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS) with the antibiotic target MurA, Proc. Natl. Acad. Sci. 97 (2000) 6345–6349.
[27] S. Gaikwad, M. Gurjur, M. Khan, *Artocarpus hirsute* lectin. Differential modes of chemical and thermal denaturation, Eur. J. Biochem. 269 (2002) 1413–1417.
[28] M. Svensson, A. Håkansson, A. Mossburg, S. Linse, C. Svenborg, Conversion of αlactalbumin to a protein inducing apoptosis, P.N.A.S. 97 (2000) 4221–4226.
[29] D. Matulis, R. Lovrien, 1-anilino-8-napthalene sulfonate anion–protein binding depends primarily on ion-pair formation, Biophys. J. 74 (1998) 422–429.
[30] G. Klebe, H. Boehm, Energetic and entropic factors determining binding affinity in protein–ligand complexes, Period. Biol. 100 (Supplement 2) (1998) 77–83.