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Interaction of α-Chymotrypsin with the Fluorescent Probe 1-Anilinonaphthalene-8-sulfonate in Solution†

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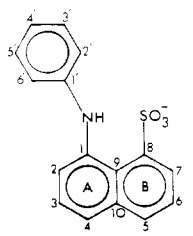
ABSTRACT: The binding of the fluorescence probe 1-anilinonaphthalene-8-sulfonate (Ans) to α-chymotrypsin (α-CHT) at pH 3.6 is accompanied by a dramatic enhancement of Ans fluorescence and a shift of the emission maximum to shorter wavelengths. Our study reveals that one Ans molecule binds to α-CHT at a site different from either the active site of α-CHT or the 2-*p*-toluidinylnaphthalene-6-sulfonate binding site. The binding constant of Ans is about the same (10^4 M^{-1}) at pH 3.6 and 6.4. Nanosecond fluorescence depolarization data indicate that Ans is rigidly bound to α-CHT. The fluorescence enhancement due to binding of Ans to α-CHT at low pH could be due to binding either to a hydrophobic site or to a site where local dipoles do not relax during the excited-state lifetime of Ans. As the pH is increased, fluorescence intensity of the Ans–α-CHT complex decreases appreciably, and the emission maximum shifts to longer wavelengths. The fluorescence decay curves exhibit a corresponding sensitivity to pH. The pH effect on the fluorescence of Ans–α-CHT can be interpreted in terms of a pH-dependent equilibrium between α-CHT conformers differing in the degree of mobility of polar residues and water molecules at the Ans binding site or structural changes in the Ans binding site.

1-Anilinonaphthalene-8-sulfonate (Ans) (I) has been used



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extensively as an extrinsic fluorescent probe of biological macromolecules and membranes (Brand & Gohlke, 1972). In constrast to the weak green fluorescence of Ans in aqueous solutions, an intense blue fluorescence is exhibited when Ans binds in proteins and membranes. Fluorescence spectra, fluorescence polarization, quantum yields, and decay times of Ans are sensitive to the microenvironment of the Ans probe. Such sensitivity has made Ans a popular probe to monitor structural changes in proteins and membranes. The dependence of the emission properties of Ans on the environment derives from an increase in its permanent dipole moment as a result of excitation and the subsequent relaxation of the dipoles of the environment to achieve an equilibrium excited-state configuration. This leads to a red shift of the fluorescence emission maximum and a decrease in the fluorescence yield in polar media. Thus, the polarity of the environment can be measured if the relaxation of the medium occurs during the lifetime of the excited state. However, if the dipoles of the environment are restricted in motion as in a protein molecule or in a viscous medium, relaxation may not be complete during the lifetime of the excited state, giving rise to an intense fluorescence at shorter wavelengths similar to Ans in nonpolar media. It is clear, therefore, that caution must be exercised in deriving conclusions regarding the characteristics of protein sites from fluorescent-probe studies.

Since Ans is used extensively to monitor conformation changes in proteins and to give an indication of the degree of polarity of specific protein sites, it becomes of vital importance to correlate the structural features of the Ans binding site with changes in the fluorescence properties of Ans as a result of binding or other factors such as pH change. This prompted us to a detailed study of the Ans interaction with α-chymotrypsin (α-CHT) using fluorescence and X-ray crystallographic techniques. In this paper, we report fluorescence studies of Ans interaction with α-CHT in solutions under various conditions. In the next paper, X-ray crystallographic results and fluorescence properties of Ans–α-CHT crystals under various conditions are presented. The structural details of the Ans binding site are discussed, and a mechanism of fluorescence enhancement at low pH (3.6) and of fluorescence quenching at higher pH (6.6) is presented. Our results clearly suggest that fluorescence spectral shifts and intensity changes due to binding of a fluorescent probe to a protein molecule can be misleading with regard to the polarity of the binding site.

Experimental Section

Materials. α-CHT, three-times recrystallized and with an activity of 54–61 units/mg, and tosylated α-CHT were obtained from Worthington Biochemical Corp. and were used without further purification. We obtained β-phenylpropionic acid (βPP) from Fluka, *N*-formyltryptophan from International Chemical and Nuclear Corp., proflavin (3,6-di-

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aminoacridine) from Aldrich Chemical Corp., *N*-acetyl-L-tyrosine ethyl ester (ATEE) from Sigma Biochemical Corp., and Ans (ammonium salt) from Sigma Biochemical Co.

Methods. Absorption spectra were measured on a Cary 15 recording spectrophotometer, and fluorescence spectra were measured on an Aminco-Keirs spectrophosphorimeter modified with an EML 978 IR phototube for lower dark current, higher gain, and better red response.

The pH–fluorescence data were obtained by use of buffer solutions made for each pH value. Below pH 5.0, a Walpole's acetate buffer was used and above this pH a Sorenson's phosphate buffer was used. Similar pH–fluorescence curves were obtained in aqueous solutions where the pH was adjusted by titrations with acetic acid. Relative fluorescence intensities were measured at the emission maximum.

In the titration studies of α-CHT with Ans, the fluorescence intensities were corrected (McClure & Edleman, 1967) for self-absorption of the incident light by use of the relationship $I_c = I_o[2.303\epsilon_{350}D_0/(1 - 10\epsilon_{350}D_0)]$, where D_0 is the total dye concentration, ϵ_{350} is the molar extinction coefficient of Ans at the exciting wavelength 350 nm (5000 M^{-1} cm^{-1}), and I_c and I_o refer to the corrected and observed intensities of fluorescence, respectively. Self-absorption of the emitted light was negligible at wavelengths above 430 nm. Absorption of the protein was always less than 0.1 *A* units at the wavelength of Ans excitation and emission and therefore did not interfere with the relative fluorescence yields.

Assays of chymotryptic activity were conducted in 0.067 M phosphate buffer at pH 7.0. The hydrolysis of 5×10^{-4} M ATEE with 2×10^{-7} M α-CHT was followed by measurement of the decrease of absorption of ATEE at 237 nm (Schwert & Takenaka, 1955) in the presence and absence of Ans.

The nanosecond fluorescence decay curves were obtained by use of a single-proton-counting, time-resolved spectrophotometer. This instrument consists mainly of a nanosecond pulser, Ortec 9352, a time to amplitude converter, Ortec 475, a Nuclear Data 100 multichannel analyzer, an Amprex DUVP/03 single-photon-counting phototube, and a nanosecond flash lamp. Excitation was through a CS 7-51 Corning absorption filter, and emission was monitored at 475 nm through a Bausch & Lomb monochromator. The parallel and perpendicular components of the fluorescence decay curves were obtained by use of polaroid plastic sheets mounted on the cell holders. The fluorescence lifetimes were determined in 0.067 M buffers, 10^{-4} M in protein and 5×10^{-5} M in Ans, by observation of the emission at 475 nm. Depolarization studies were conducted under the same conditions. Deconvolution of the lamp and data curves was performed by use of a deconvolution analysis developed by Ware (Ware et al., 1973), and the data were fitted by a nonlinear least-squares analysis by use of KINFIT (Dye & Nicely, 1971).

Results

Ans–α-CHT Fluorescence at Different pH Values. Below pH 4.0, Ans–α-CHT solutions exhibit an intense blue-green fluorescence. As the pH is raised the fluorescence intensity dramatically drops and a very weak emission is observed at pH 7.0. Successive spectral shifts to lower energies also occur with increasing pH as shown in Figure 1. As the pH of the Ans-α-CHT solution is raised from 3.6 to 7.0, the fluorescence intensity is decreased by 50-fold, and the emission maximum is red-shifted by about 1100 cm^{-1}. The fluorescence of Ans in solution alone is insensitive to changes in this pH range. The pH profile of Ans–α-CHT fluorescence intensity is shown in Figure 2.

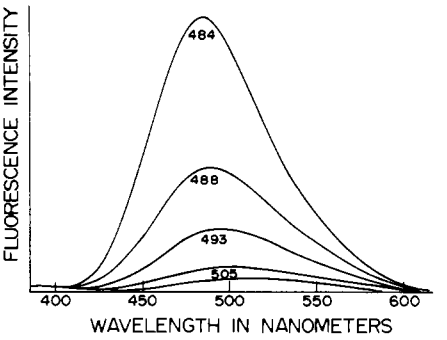

FIGURE 1: Fluorescence spectra of 10^{-4} M α-CHT in the presence of 2×10^{-5} M Ans at various pH values: 2.4, 3.6, 4.75, 7.0, and 8.0. Successive red shifts of the emission maxima occur as the pH value is increased. Since the spectra were recorded at various sensitivities, relative intensities are not reported.

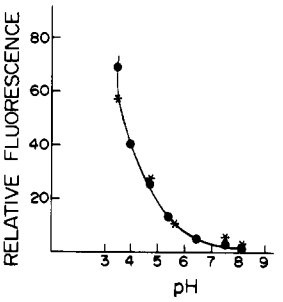

FIGURE 2: Relative fluorescence intensity of 2×10^{-5} M Ans in the presence of 10^{-4} M α-CHT (●) and tosylated α-CHT (*); fluorescence intensities were measured at the emission maxima.

Ans Binding Site. Ans complexes of tosylated α-CHT, in which the active site is blocked with toluenesulfonyl fluoride, show the same fluorescence–pH profile as the native Ans–α-CHT complex (Figure 2). Similar results were obtained for α-CHT in the presence of 0.01 M βPP which is a competitive inhibitor of α-CHT activity. These studies show that the fluorescence and thus the binding affinity of Ans is unaffected when the active site of α-CHT is blocked. The effect of Ans binding on the catalytic activity of α-CHT was monitored by measurement of the rate of decrease in absorption of ATEE at 237 nm. In the presence of Ans concentrations as high as 4×10^{-4} M, the catalytic activity of α-CHT remained the same. Our conclusion is that Ans does not bind at the active site of α-CHT since such binding would inhibit its catalytic activity.

Proflavin is known to bind to the active site of α-CHT and to inhibit its activity. Upon binding, its absorption maximum is shifted from 444 to 465 nm (Bernhard et al., 1966). The amount of bound proflavin can be measured by changes in its absorption maximum. By use of this technique, the binding constant for many substrates and substrate analogues of α-CHT have been determined (Brandt et al., 1967). However, no change in the absorption maximum was observed with the addition of concentrations of Ans as high as 2×10^{-4} M. These results suggest that Ans does not affect the binding of proflavin to α-CHT and further indicate that Ans does not bind to the active site.

Affinity of α-CHT for Ans. After determining that Ans binds to a site other than the active site of α-CHT, we determined the equilibrium constant of Ans binding to α-CHT at both low (3.6) and high pH (6.4). The results of titrations of α-CHT with Ans are shown in Figure 3. The protein concentration was 5×10^{-5} M while Ans concentration was varied between 10^{-5} and 2.5×10^{-4} M. A plot of the intensity of fluorescence (I_F) vs. I_F/[Ans] gave lines with slopes cor-

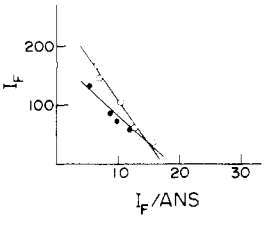

FIGURE 3: Plots of I_F vs. I_F/[Ans], where $I_{F's}$ are relative fluorescence intensities of variable concentrations of Ans (1×10^{-5}–2.5×10^{-4} M) in the presence of 5×10^{-5} M α-CHT at pH 3.6 (○) and pH 6.4 (●). The fluorescence intensities for the pH 6.4 titration have been increased 10-fold.

responding to the respective dissociation constant of the complex, K_D. The value of K_D for the Ans–α–CHT complex is 1.4×10^{-4} and 1.0×10^{-4} M for pH 3.6 and 6.4, respectively. Such a small difference in the equilibrium binding constant for Ans suggests that Ans binds with essentially equal affinity to α-CHT at pH 3.6 and 6.4. Thus, the fluorescence changes observed with pH are not due to changes in the amount of bound Ans but rather due to the quenching of bound Ans fluorescence.

Number of Ans Binding Sites. The number of Ans binding sites on α-CHT was determined according to Christian & Janetzko (1971)

$$\frac{1}{d} = \frac{1}{nK_A(1-x)D} + \frac{1}{n}$$

where d is the number of moles of bound Ans per mole of protein, n is the number of binding sites per protein molecule, K_A is the association constant, D is the total Ans concentration, and x is the fraction of Ans bound.

The above assumes that once a ligand is bound it does not influence the binding of succeeding ligands and that all binding sites are identical in character.

For ligands like Ans, which fluoresce very intensely when bound and are virtually nonfluorescent when free in aqueous solution, the fraction of bound ligand can be written as $x = (F_p/F_{max})D$, where F_p is the fluorescence intensity of Ans in the presence of protein and F_{max} is the fluorescence intensity at protein concentrations where all the dye is bound. It is assumed that the fluorescence is a linear function of ligand or protein concentration.

To obtain F_{max}, a solution with a fixed dye concentration was titrated with increasing concentration of protein at pH 3.6 in 0.067 M phosphate buffer; a plot of the reciprocal fluorescence intensity vs. the reciprocal protein concentration gave a straight line intercepting the ordinate at $1/F_{max}$.

Titrations of a fixed protein concentration (5×10^{-5} M) with increasing concentrations of dye (5×10^{-6}–7×10^{-5} M) were conducted at pH 3.6 in 0.067 M phosphate buffer. A plot of $1/d$ vs. $1/[(1-x)D]$, shown in Figure 4, gives values of both n and K_A: the intercept on the $1/d$ axis is $1/n$ and the slope is $1/K_An$. Our results gave a value of 1.1 for the number of Ans molecules bound per α-CHT molecule.

Fluorescence Decay of Ans–α-CHT. The fluorescence decay curves of Ans–α-CHT in solution at pH 3.6 and pH 7.0 are shown in Figure 5. The emission was monitored at 475 nm. The large observed change in the fluorescence decay curves corresponds to the decrease in fluorescence intensity with increasing pH. At pH 3.6, the fluorescence decay curve corresponds to a major 12-ns component, while at pH 7.0 the fluorescence decay is dominated by a very short-lived component in addition to the 12-ns one.

Similar changes in the fluorescence decay curves with pH were observed for Ans–tosylated α-CHT solutions. The

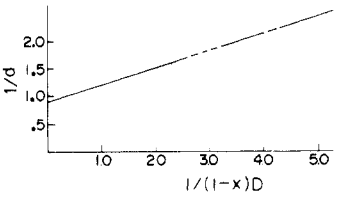

FIGURE 4: A plot of $1/d$ vs. $1/[(1-x)D]$, where d is the number of moles of bound Ans per mole of protein, x is the fraction of Ans bound, and D is the total Ans concentration.

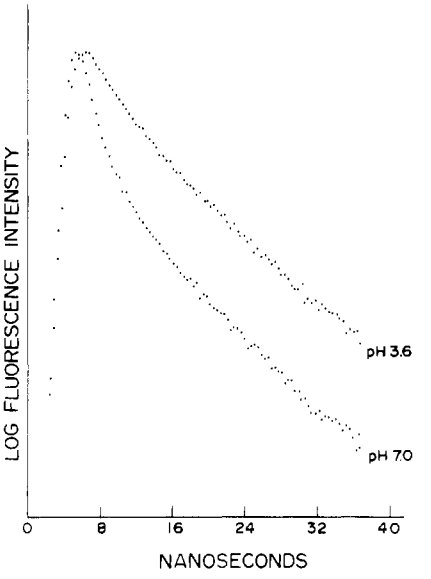

FIGURE 5: Fluorescence decay curves of Ans–α-CHT solutions at pH 3.6 and 7.0.

presence of competitive inhibitors such as βPP or *N*-formyltryptophan did not affect the fluorescence decay characteristics of Ans–α-CHT solutions, indicating further that Ans does not bind to the active site of α-CHT.

Fluorescence Depolarization. Using the nanosecond decay curves of the parallel and perpendicular components of Ans–α-CHT emission and assuming that α-CHT is approximately spherical in shape, we calculated the anisotropy of the emission of Ans bound to α-CHT. For a rotating sphere, the slope of such a plot is equal to $-3/\rho$, where ρ is the rotational relaxation time of the bound dye. If the dye is bound rigidly to the protein such that it rotates with the protein as a protein–dye complex, then the rotational relaxation time will be that for the protein–dye complex. If the dye undergoes independent rotation during the lifetime of its excited state, the rotational relaxation time would be much less than that of the protein. The rotational relaxation time obtained from our data at pH 7.0, 4.75, and 3.6 is 45 ns which compares well with a value of 49 ns obtained by Haugland & Stryer (1967) for α-CHT to which a fluorescent anthraniloyl group was covalently bound at the active site. These data indicate that Ans is strongly bound to α-CHT over the 3.6–7.0 pH range such that it rotates as a protein–dye complex. Data at other pH values also indicate that Ans is rigidly bound.

Discussion

Our data indicate that *one molecule of Ans binds to α-CHT at a site distinct from the active site* since Ans binding does not affect the catalytic activity of α-CHT. Substrate analogues or tosylation of serine-195, both of which inhibit α-CHT activity, does not affect Ans binding. The large fluorescence enhancement at low pH does not appear to be altered by dimerization of α-CHT, since identical fluorescence–pH

profiles are observed for native and tosylated α-CHT, the latter not dimerizing at low pH (Horbett & Teller, 1973).

The Ans binding site is also different from the binding site of 2-p-toluidinylnaphthalene-6-sulfonate (TNS). The fluorescence–pH profile of the TNS–α-CHT complex is different from that of the Ans–α-CHT complex. In the former case, the fluorescence intensity exhibits a peak at pH 7.8 and the fluorescence maximum shifts to longer wavelengths (15-nm shift) as the pH is increased from 2.5 to 7.8 (McClure & Edelman, 1967). In the same study, TNS was shown to cause a marked inhibition of the initial velocity of α-CHT-catalyzed hydrolysis of ATEE, but it is likely that the interaction between TNS and ATEE is noncompetitive, and it was concluded that TNS does not bind in the active site of α-CHT but is bound at a second spatially distinct site.

Small molecules of the form 3-(*n*-alkanoyl)-O-benzoate have been reported to bind to α-CHT at a hydrophobic site other than the active site and to inhibit its activity by inducing conformation changes which make the active site less effective for enzymic action (Smith & Hansch, 1973). The more hydrophobic the molecule of this series, the greater the observed inhibition. As a class, neutral molecules are found to be poor inhibitors compared to charged inhibitors. These studies suggest that the inhibition is not competitive, that it is controlled from a hydrophobic site separate from the active site, and that the binding of charged inhibitors at such a control site is accompanied by an electrostatic interaction with the enzyme, resulting in a conformational change unfavorable to chymotryptic activity. Since TNS may be regarded as a charged hydrophobic molecule and since it was shown that it also inhibits chymotryptic activity, it appears that TNS might be binding to the site reported by Smith and Hansch. On the other hand, other auxiliary sites are also possible (East & Trowbridge, 1973; Tulinsky et al., 1978). From our study, Ans binds at a site other than the active site or this hydrophobic site.

Fluorescence Enhancement of Ans Due to α-CHT Binding at Low pH. One possible mechanism that may be considered to account for the fluorescence enhancement of Ans when it binds to α-CHT at low pH values is the alteration of Ans geometry (Penzer, 1972). NMR and fluorescence spectral measurements under various conditions led Penzer to believe that the blue shift of the emission maximum and the fluorescence enhancement are due to an Ans conformation where the phenyl group and the napthalene ring are coplanar. According to this, Ans can exist in different conformations, the coplanar one with an intense fluorescence at 460 nm and noncoplanar conformations that weakly emit at 520 nm. The NMR results also show that there is anomalous behavior of the proton signals of the H(2), H(2′), and H(6′) protons in water and methanol solutions. Since the deshielding effects are more pronounced in alcohol, it is assumed that the rings of Ans are more nearly coplanar in this solvent as the result of a stronger interaction between the anilino hydrogen and the sulfonate group.

Four X-ray crystallographic conformations of Ans have been reported to date: two as a triclinic ammonium Ans hemihydrate salt (Cody & Hazel, 1977a), one as a monoclinic ammonium Ans monohydrate (Weber, 1978), and one as a triclinic hexaaquomagnesium bis(1-anilinonaphthalene-8-sulfonate) hexahydrate (Cody & Hazel, 1977b). In only one of these (Cody & Hazel, 1977a) does the hybridization of the anilino nitrogen atom appear to vary from nearly sp^2 geometry. A comparison of the known conformations (Weber, 1978) shows that as the plane through atoms C(1′), N, and C(1) approaches coplanarity with that of the naphthyl, the plane through atoms C(6′), C(1′), and N becomes more noncoplanar with respect to the naphthalene moiety. This is not in agreement with the conclusion drawn from NMR measurements where it was assumed that hydrogen bonding leads to a more coplanar conformation (Penzer, 1972). In the monoclinic form, the Ans exhibits a nonlinear intramolecular hydrogen bond, as do all other conformations. This is consistent with the assertion that it is the peri-group geometry which determines such hydrogen bonding and not coplanarity of the aromatic rings. Finally, it should be noted that the X-ray crystallographic and NMR data pertain only to ground-state conformations.

A detailed study of the dependence of fluorescence energy and quantum yield on a solvent polarity parameter for various Ans derivatives (Kosower et al., 1975) has revealed that two emitting states are involved in fluorescence: a nonplanar, singlet state, S_{np}, and a planar, intramolecular charge-transfer state, S_{ct}. The relative population of the two states depends on the solvent polarity and viscosity as well as steric hindrance to rotation of the *N*-aryl group with respect to the naphthalene ring. Emission energies plotted against solvent polarity indicate that the emission energies have a low sensitivity to solvent polarity change in the nonpolar range and a high sensitivity in the polar range. The large slope in the polar range characterizes the emission as charge transfer. Thus, in a polar medium the emitting state is predominantly S_{ct} while, in a relatively nonpolar medium, the emitting state is predominantly S_{np}. Fluorescence in polar media is quenched via an efficient internal conversion process: $S_{ct} \rightsquigarrow S_0$, where S_0 is the ground state. In viscous polar media, solvent relaxation is decreased, giving rise to a predominantly S_{np} emission. However, it is important to note here that a sterically hindered 2,6-Me_2Ans derivative exhibits a quantum yield plot similar to that of other Ans derivatives, indicating that both S_{np} and S_{ct} are active as emitting states in mobile polar solvents. Inspection of the four crystalline Ans conformations has shown that there is a significant decrease in each of the C(1)–N and C(1′)–N bond lengths relative to their expected single-bond values (Weber, 1978), indicating that π-orbital overlap exists between the phenyl and naphthyl ring system through the anilino nitrogen, independent of the observed conformation or apparent nitrogen hybridizations. Thus, it is misleading to use planarity to label the two emitting states, and the charge-transfer character of the emitting state should be used instead. It is perhaps more meaningful to refer to S_{np} as a nonpolar state relative to S_{ct}.

Penzer's data of Ans fluorescence in aqueous solutions in the presence of $MgCl_2$ can be accounted for in terms of two different environments of Ans: Ans surrounded with mobile water molecules emitting weakly at 520 nm corresponding to a S_{ct} emitting state and Ans surrounded with ordered water molecules that do not relax during the lifetime of the excited singlet state of Ans and exhibiting emission corresponding to a S_{np} state. The Ans fluorescence spectrum in a given medium can be considered as a composite spectrum of two emitting states; their relative contribution to the emission intensity is governed by the degree of relaxation of the solvent dipoles surrounding the Ans molecules. Time-dependent spectral shifts have been observed (Chakrabarti & Ware, 1971) and interpreted in terms of dipole reorientation of the solvent around the electronically excited molecule.

The fluorescence enhancement of Ans when it binds to α-CHT suggests that the emission arises predominantly from a S_{np} state in contrast to the situation in aqueous solution when a weak red-shifted emission arises from a S_{ct} state. This may

indicate a nonpolar hydrophobic binding site; however, it may also indicate that the binding site consists of polar residues where water molecules do not relax during the excited-state lifetime. Evidence for such a possibility will be discussed in the next paper.

Ans–α-CHT Fluorescence at Different pH Values. The affinity studies show that Ans binds to α-CHT with the same apparent affinity at low and neutral pH. Thus, the observed fluorescence changes with pH are due to the quenching of bound Ans at higher pH values. The fluorescence depolarization data clearly show that Ans is rigidly bound in α-CHT in the 3.6–7.0 pH range.

Proteins can exist in various conformations depending on the pH, and α-CHT has been shown to undergo structural changes in crystals due to pH changes (Mavridis et al., 1974). In the pH range of 1.0–10.00, seven pH conformers exist, corresponding to distinct structural stabilities. The degree of order to solvent molecules at the Ans binding site might change as the pH of the solution is increased. Thus, the rate of relaxation of water molecules during the Ans excited-state lifetime could be enhanced as the pH is increased, giving rise to the observed red shift and fluorescence quenching. On the other hand, the change in fluorescence with pH could be due to protein structural changes in the Ans binding site.

The short-lived component of the Ans fluorescence decay at pH 7.0 could correspond to emission from Ans–α-CHT complexes in which relaxation of the polar solvent molecules occurs during the lifetime of the excited state. The persistence of the 12-ns component at high pH may indicate the presence of an equilibrium between various α-CHT conformers differing in mobility of water molecules at the Ans binding site. These different conformers could give rise to different Ans emission maxima and different fluorescence lifetimes. At any given pH, the emission spectrum has to be considered as a composite emission spectrum of various conformers with the low pH conformer contributing to the emission more than in its stoichiometric proportion.

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