FLUORESCENCE EMISSION AND STOPPED-FLOW KINETIC STUDIES OF THE ACID EXPANSION OF BOVINE SERUM ALBUMIN

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SUMMARY

The tryptophan fluorescence of bovine serum albumin shifts to shorter wavelengths as the protein expands on reducing the pH from 7 to 2. This indicates that the acid expansion of the protein does not result in an open network but a conformation which retains globular regions. The absorption and fluorescence changes accompanying the acid expansion occur largely within the dead time of the stopped-flow, but the binding site for 1,8 anilinonaphthalene sulfonic acid is lost much more slowly. This suggests that the ligand binding site is on a globular region rather than in a crevice between globular regions which breaks up when the protein expands.

The acid expansion of bovine serum albumin (BSA*) occurs at pH's below 3.2, is readily reversible, and is electrostatic in origin (i.e. ionic strength-dependent). The conformation of the protein at pH's between 4.5 and 3.2 ("F form") is not grossly different from the neutral pH ("N") form. (1) During the transition to lower pH's relatively large absorbance changes occur (2).

Previous work (3,4) has suggested that the expansion involves separation of two or three globular regions ("domains") which remain globular and are connected by the polypeptide chain, rather than formation of an open structure, all parts of which are accessible to the solvent. It has also been suggested (4) that the binding site for such ligands as 1,8 anilinonaphthalene sulfonic acid (ANS*) is located between segments that separate at low pH's, thus causing loss of the binding site. We have determined the effect of pH and ionic strength on the fluorescence emission spectrum of BSA and measured the kinetics of the acid expansion of the protein by monitoring the absorbance of BSA and the fluorescence of adsorbed ANS. This is to help elucidate the mechanism of the expansion and reformation, and to test the above suggestions.

*Abbreviations: BSA, bovine serum albumin; ANS, 1,8 anilinonaphthalene sulfonic acid.

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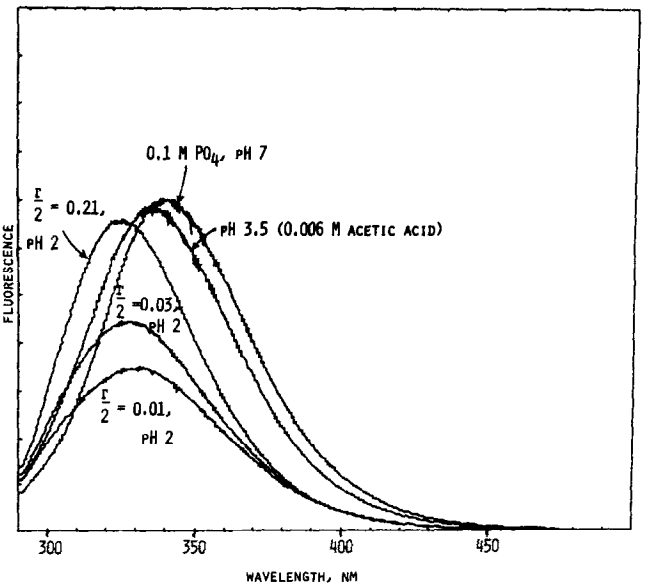

Figure 1. Fluorescence emission spectra of bovine serum albumin ($5 \cdot 10^{-6}$ M) at various pH's and ionic strengths. Data are averages of four replicate scans. The temperature was 5° with excitation at 280 nm (6.7 nm excitation and emission slit widths).

MATERIALS AND METHODS

Crystallized, lyophilized bovine serum albumin was obtained from Sigma Chemical Co. ANS was obtained from Eastman Kodak and recrystallized twice from hot water as the magnesium salt. Other chemicals were reagent grade; doubly distilled water was used.

The stopped-flow apparatus, fluorimeter and spectrophotometer are computer-controlled (5,6). All signal analysis is done by the computer, which permits numerical data manipulation and data averaging (5,6). pH measurements were made with a Corning Model 12 pH meter. Values given for initial and final pH's were checked with the initial solutions and the reacted solutions from the stopped-flow.

RESULTS AND DISCUSSION

The acid pH expansion of BSA is accompanied by a shift of the protein tryptophan fluorescence to shorter wavelengths and by quenching (Figure 1), compared to the "N" and "F" forms. This is not the effect that one would expect with unfolding of a peptide chain, but, in fact, just the opposite. It suggests that the tryptophan residues in the acid expanded protein are less exposed to solvent than at the higher pH's and, since the quenching is largely removed by increasing ionic strength, that the quenching is due

to interactions with other amino acid residues. On the other hand, the tyrosyl fluorescence, as revealed by the shoulders at 305 nm, appears to be greater at pH 2 and 3.5 than at pH 7 and a higher apparent fluorescence yield is again found with increased ionic strength.

Overall these data suggest that the tryptophan residues are involved in a region of the protein which undergoes a transition toward a more compact structure during the acid expansion. The effects of low pH on the tyrosyl residues are a general but not complete removal of quenching interactions. These data are consistent with the model of acid expansion wherein the protein expands its conformation into distinct globular regions separated by interconnecting peptide chain.

Williams and Foster (2) showed, and we have confirmed, that nearly all the absorbance change in the protein produced by conversion of BSA from the "N" form to the acid expanded conformation occurs on reducing the pH from 3.5 to 2 (Table I). On raising the ionic strength of BSA at pH 2 from 0.01 to 0.11, the overall size of the acid expanded protein is reduced (1). This is also accompanied by a significant absorbance change. Evidently, conversion of the protein from the "N" to the "F" form does not strongly perturb the tyrosines and tryptophans, but the acid expansion does, and the magnitude of the perturbation is related to the increase in effective size of the protein.

In the stopped-flow apparatus, we observed very small changes in protein absorbance at 287 nm (Table I). This was true whether the stopped-flow experiments were carried out at 20° or at 5°; at most, 6% of the total absorbance difference was observable. Clearly, most of the absorbance difference occurs within the dead time of the stopped-flow (less than 3 msec.). Measurements of protein fluorescence in the stopped-flow gave similar results (J. M. Odom and J. M. Brewer, unpublished observations). Summers and McPhie (7) showed that pH-induced unfolding of several other proteins was often characterized by large changes within the dead time of their stopped-flow apparatus.

There is a single high affinity binding site for ANS on BSA, and the ANS

TABLE I

Absorbance Difference Spectral Changes in $4 \cdot 10^{-5}$ M BSA Observed Using the Stopped-Flow Spectrophotometer at 5°

INITIAL CONDITION:		FINAL CONDITION:		ΔOD_{287}:	
pH	Ionic Strength	pH	Ionic Strength	Observed:	Expected:
2	0.01	7	0.01	0.005	0.480
2	0.21	7	0.21	0.006	0.350
2	0.01	2	0.11	0.003	0.130
2	0.01	3.5	0.01	0.010	0.428
2	0.01	4.5	0.01	0.007	0.491
7	0.001	2	0.03	0.024	0.420
7	0.20	2	0.23	0.003	0.350

"Observed" absorbance differences were obtained using the stopped-flow spectrophotometer.

"Expected" differences are the magnitudes of the absorbance difference spectral changes obtained using a computer-controlled scanning double beam spectrophotometer (6).

fluorescence quantum yield increases 140-fold on binding at this site (8). ANS appears to bind to BSA more weakly at pH 2 than at pH 7, since the observable increase in its fluorescence at pH 2, and 0.25 μM BSA and ANS (final concentrations) is about one thirty-fifth the increase at pH 7 (not shown). Also, raising the pH of an ANS-BSA solution from 2 to 5 or 7 produces a second-order increase in ANS fluorescence. (The affinity of ANS for the protein appears higher at 0.2 ionic strength, where the protein is more compact, since the fluorescence increase on mixing 10 μM ANS with 10 μM BSA is twelve times that at 0.01 ionic strength.)

On reducing the pH of an ANS-BSA solution from 7 to 2 at ionic strengths ranging from 0.01 to 0.21, we do not get a single first order reaction, al-

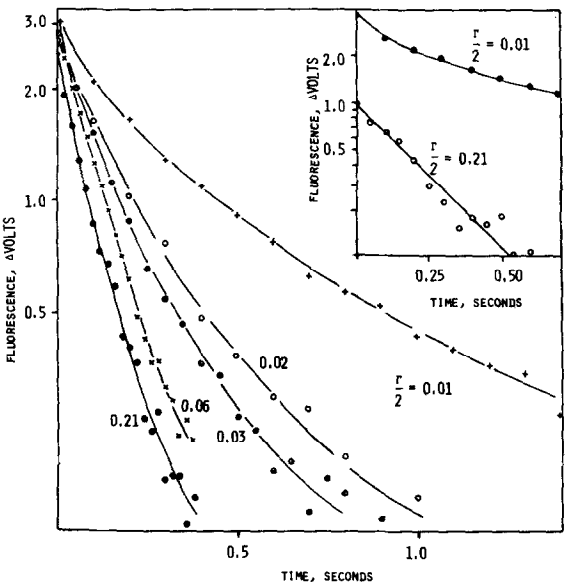

Figure 2. Kinetics of ANS fluorescence of $5 \cdot 10^{-7}$M (initial concentrations) protein and ANS upon reducing the pH from 7.5 to 2.3 at various ionic strengths. The numbers on the figure are the ionic strengths (NaCl) at which the reaction was measured. Measurements at 5°. Excitation was at 362 nm with a 6 nm slit width; emitted light was isolated using a Corning 3-72 glass color filter.

Inset. Effect of pH reduction of $5 \cdot 10^{-7}$M (initial concentrations) protein and ANS from pH 3.5 (0.006 M acetic acid) to pH 2.3 at 0.01 and 0.21 ionic strength (upper and lower curves, respectively). The pH was lowered by reaction of the protein-ANS solution with 0.02 M HCl. Reaction at 5°.

though essentially only one binding site is partially occupied, but kinetics that suggest a series of first order reactions, extending out to 10-15 seconds at 0.01 ionic strength (Figure 2). At higher ionic strengths, the bulk of the reaction occurs within a much shorter time span. The overall rate of the reactions increases about a factor of ten with a six-fold increase in ionic strength. This indicates that electrostatic factors are involved in maintenance of this ANS binding site.

Tanford (1) and later Vijai and Foster (9) showed that 40 or so of the carboxyl groups in BSA are "inaccessible" to titration at neutral pH's. These presumably are interacting with positively charged groups within the protein;

the interactions help hold the protein in a compact conformation. At pH's within the "F" region (4.5-3.2) the carboxyl groups become titratable. However, it appears that other electrostatic interactions are involved in maintenance of the ANS binding site, since an effect of ionic strength is still seen when the initial pH of the BSA-ANS complex is at 3.5 instead of 7 (inset to Figure 2).

Control experiments showed we are seeing the entire fluorescence change occurring, in sharp contrast to the results obtained by monitoring protein absorbance. Other control experiments showed that the ANS does not stabilize BSA to changes in pH: we still find very small absorbance changes at 287 and 296 nm, with kinetics similar to those seen without ANS. Also, if ANS is added along with sufficient acid to reduce the pH of the protein to 2, the added ANS apparently binds, then comes off in a fashion similar to when the pH of a BSA-ANS complex is lowered from 7 to 2. The protein is still largely in the pH 7 form as far as the ANS binding site is concerned although the bulk of tyrosine and tryptophan absorbance changes have occurred already.

The most likely reason for the complex kinetics obtained is that ANS dissociates at different rates from each of a sequential series of protein conformations. It is possible, however, that some reactions involve changes in the ANS binding site that merely reduce the fluorescence yield of the ligand without affecting the extent of binding.

The rate of the putative ANS release is strongly temperature-dependent, being much faster at 20° than at 5°.

Weber and Young (4) suggested that the ANS binding site was formed by the junction of two of the globular regions of BSA, and that the site was lost when the regions separated. Our data indicate that the ANS binding site must be on one of the globular regions, since the globular regions appear to separate within the dead time of the stopped-flow and the ANS binding site persists for much longer than that. Evidently the ANS binding site is lost as a result of the adaptation of the separated globular regions to their new environment.

The acid expansion of BSA is clearly a complex process involving a series

of consecutive reactions, consistent with the highly cooperative nature of protein structure, and indeed a consequence of it.

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