Biophysical Chemistry 28 (1987) 77–88
Elsevier

BPC 01184

Fluorescence lifetime and spectral study of the acid expansion of bovine serum albumin

John M. Brewer, Phillippe Bastiaens and John Lee

Department of Biochemistry, School of Chemical Sciences, University of Georgia, Athens, GA 30602, U.S.A.

Received 9 March 1987
Revised manuscript received 6 July 1987
Accepted 7 July 1987

Bovine serum albumin; Fluorescence lifetime; Tryptophan fluorescence; Fluorescence anisotropy decay; pH effect; Protein conformation

The fluorescence lifetimes of the tryptophan residues of bovine serum albumin were measured in the native and acid-expanded conformation. A three-exponential process is required to fit the fluorescence decay data. The results are interpreted empirically in terms of two emitting species. The emission at longer wavelength (360 nm) has slower rates of decay than that at shorter wavelength (325 nm). For both emitting species the average lifetime decreases when the N-F transition occurs and shortens further when the protein expands. Rotational correlation times, derived from the decay of the fluorescence anisotropy of the tryptophan residues, suggest that longer emission wavelengths are associated with somewhat shorter correlation times. There is no certain indication of any independent motion of the tryptophans in any conformation, although some very fast process, perhaps Raman scattering, appears to occur. On acid expansion the long correlation times decrease to around 10 ns in the fully expanded form. Static quenching experiments using I^- or acrylamide suggest a greater average exposure of the tryptophans when the protein is most greatly expanded. This is despite the fact that the fluorescence emission maximum shifts to shorter wavelength under these conditions. Also, there is no difference in accessibility to quenching between the longer and shorter wavelength emissions.

1. Introduction

A variety of physical and chemical evidence suggests that bovine serum albumin molecules consist of three globular domains held together at neutral pH by ionic or electrostatic interactions [1–4]. When these interactions are disrupted, either by low pH values [3,4] or by the addition of moderate concentrations of some lanthanides [5], the protein expands as the domains separate. The extent of this expansion is inversely dependent on ionic strength [3,4].

One of the two tryptophans is near the C-terminal end of domain I while the other is close to the N-terminal of domain II [2]. Changes in the fluorescence emission spectrum correlated with the acid expansion suggest that the environment of the two tryptophans in the protein also changes but in opposite fashion to that expected from the character of the overall conformational changes [6]. The spectrum of the protein at pH 7 suggests that the tryptophans are in a relatively polar environment (emission maximum 344 nm). At pH 2 and low ionic strength (0.01), the emission is strongly quenched and shifted to shorter wavelengths (emission maximum 340 nm). This suggests a more hydrophobic environment [6]. The effect was interpreted as the result of a conformational change subsequent to domain separation. Increasing the ionic strength to 0.11 increases the fluorescence yield by a factor of nearly two and the emission maximum shifts further to 336 nm.

Correspondence address: J.M. Brewer, Department of Biochemistry, School of Chemical Sciences, University of Georgia, Athens, GA 30602, U.S.A.

Knowledge of the mechanism of the large changes in fluorescence observed would expand our knowledge of the effects of domain interactions on protein conformation. A complete picture of the fluorescence properties requires knowledge of fluorescence lifetimes. This coupled with static quenching studies can provide a better idea of the accessibility of the environment of the tryptophans to the solvent in each conformation of the protein [7]. Additionally, the fluorescence anisotropy decay of the tryptophan residues also yields rotational correlation times for the protein in each of its structural forms.

In this work we present high-resolution measurements of the fluorescence lifetimes of the tryptophan residues for bovine serum albumin in several of its conformations. We show that the fluorescence decay is complex, consisting of three exponential components, and that the parameters are wavelength-dependent. The results can be rationalized in terms of two tryptophan environments in this protein emitting with characteristic properties.

2. Materials and methods

Bovine serum albumin and defatted bovine serum albumin were purchased from Sigma (fraction V, lot nos. 47C-0433 and 45F-0064) (type F, lot no. 1015-7260). For some of the measurements the protein was purified using HPLC (LKB model 2150) and a molecular exclusion resin (TSK-G3000 SWG). The chromatographed protein appeared to be monodisperse on rechromatography on an analytical scale column. The unchromatographed protein had 21% dimer and 7% tetramer, but this is not expected to alter our interpretations as the acid expansions of oligomeric serum albumins differ little from that in the monomer [8] save for a small effect on the midpoint pH.

Acrylamide from Eastman Kodak was twice recrystallized from benzene. 8-Anilino-1-naphthalenesulfonic acid (ANS) was also obtained from Eastman Kodak and twice recrystallized as the magnesium salt from hot water. 4-Morpholineethanesulfonic acid (Mes) was purchased from Calbiochem-Boehringer, Lutetium(III) chloride was obtained from Alfa. Other reagents were from J.T. Baker. Water was filtered and deionized (Continental Deionized Water).

Measurements of pH were carried out using an Orion model 701A Ionalyzer or a Radiometer PHM82 standard pH meter, both with combined (single-probe) electrodes. Absorbances were measured using a Bausch and Lomb Spectronic 200 spectrophotometer equipped with a digital readout. Static fluorescence measurements were made with an SLM 8000C spectrofluorometer.

For the time-dependent fluorescence measurements, excitation was from a laser system. The mode-locked output of a Spectra-Physics 8 W Nd/YAG laser was frequency doubled to 530 nm and used to pump synchronously a dye laser containing the dye rhodamine 6G. The tuned output, adjustable around 600 nm, was intracavity-dumped by a Bragg-cell device (Spectra-Physics model 344S) driven at 390 MHz, which converted the mode-locked oscillation into single pulses of nominal width 10 ps, at a repetition rate of 800 kHz. The average power of these pulses was less than 15 mW. These pulses were then frequency doubled to 300 nm, their polarization returned to the vertical with a Babinet-Soleil compensator, and then passed through color filters (Corning CS 7-54) to remove any leak-through of the 600 nm radiation. These pulses were then impinged on the sample contained in an Applied Photophysics (London, U.K.) PS-60 optical unit. On the excitation side of the sample was a Glan-Thompson prism fixed at the vertical position. The cuvet containing the sample was in a thermostatted housing. The fluorescence emission, viewed at 90° to the excitation, was passed first through a Glan-Thompson prism which could be oriented either parallel or perpendicular to the polarization direction of the excitation pulses. Before entering the emission monochromator the radiation was passed through color filters. For prompt measurement this was a Corning CS 7-54. For 325 nm emission this was a CS 7-54 and Schott WG-320, and for the 360 nm measurements a Corning CS 7-59 was employed. It was essential to use these color filters to minimize stray light interference in the emission decay measurements, because it was necessary to use quite wide slit widths for the emission mono-

chromator (usually 5 mm, 23 nm). Quantitation of stray light interference at the detecting wavelength was verified using solutions of glycogen having the same amount of scattered light signal as the sample signal at 300 nm (see section 3).

The fluorescence signal was detected usually with a three-stage microchannel plate (MCP) photomultiplier (Hammamatsu 2287U) operating at −3300 V. The output was sent to a 44 dB amplifier (Hewlett-Packard 8447F) and then to a constant-fraction discriminator (Tennelec 455) modified for the fast output of the MCP as recommended by Tennelec (Oak Ridge, TN). In earlier experiments the detector used was a photomultiplier (Phillips 2020) operating at −2750 V and its output was sent directly to a constant-fraction discriminator (Ortec 473A). The output of the constant-fraction discriminator was used to start a time-to-amplitude converter (Applied Photophysics/Ortec 457) and stopped by a pulse synchronized with the cavity dumper, from the cavity dumper driver (Spectra-Physics 454). This is called the 'reverse' single-photon-counting mode. The time-converted-to-amplitude events were accumulated on a multichannel analyzer. The emission events were accumulated with the emission polarizer alternately in the parallel (I_v) or perpendicular (I_h) positions, for a 10 s period each, and the counts stored in corresponding halves of the MCA buffer. Measurements were continued until at least 10^6 counts were accumulated in each half. At the end of the experiment, the data were transferred to a computer for analysis (DEC 11/73). As the count rates from the converter were always below 4 kHz, no corrections for pulse pile-up were necessary.

We used commercial software (Applied Photophics) for the analysis of fluorescence and anisotropy decays. These were based on the routines described in ref. 9. The procedure is standard, where the fluorescence decay is deconvoluted from the instrumental response function, the 'prompt' pulse, and then fitted to a sum of exponential functions. An acceptable fit requires that the reduced χ^2_R be minimized and close to unity, and that the Durbin-Watson parameter be greater than a certain value which depends on the number of parameters assumed in the model [9].

The time-dependent fluorescence intensity is given by:

$$I(t) = I_v(t) + 2GI_h(t).$$

where the factor G is the ratio of transmission of vertically to horizontally polarized light through the detection system. The G factor is evaluated for each emission wavelength by measuring the ratio I_v/I_h for the fluorescence from a small molecule (1-cyanonaphthalene in hexane) which, following about 1 ns from the excitation pulse, should be completely depolarized [9].

Alternatively, the value of G at various emission wavelengths and slit widths was determined by subsequent excitation of the protein solutions with horizontally polarized light without altering any other condition. Values of G obtained by the two methods were the same and were independent of emission monochromator slit width, although not of the glass color filter used.

The time dependent anisotropy is calculated from:

$$r(t) = [I_v(t) - GI_h(t)]/I(t).$$

Both the fluorescence and anisotropy are deconvolved and fitted to the exponential decay functions using the same least-squares procedure. The prompt curve was determined before and after each sample measurement. Its peak position usually changed at a rate of several ps/min and so a drift correction was included in the analysis procedure.

Superimposed on the decay curves was usually a small oscillation, the source being identified from its frequency (approx. 400 MHz) as the cavity dumper driver. It revealed itself in the residual or autocorrelation plots after fitting a calculated function. It had the effect of distorting the statistical parameters but not the fitted parameters. It was removed from the raw data by a Fourier transform routine, where a few channels corresponding to the cavity dumper frequency of 390 MHz were linearized, followed by back-transformation and least-square analysis as described above.

Time calibration of the multichannel analyzer channels was performed by measuring the shift in prompt maximum on inserting calibrated delay

lines (Ortec 425A) between the constant-fraction discriminator and the time-to-pulse-height converter. The fluorescence decay of standard samples known to give a single-exponential fluorescence decay was measured and found to yield literature values [9].

The resolution of single-photon-counting systems such as this is usually taken to be about 10% of the full-width at half-maximum (FWHM) of the prompt function. The FWHM was 170 ps for the MCP detector and 500 ps for the 2020 photomultiplier.

Samples were made up the day of excitation. Control experiments showed that the fluorescence properties of stock solutions (made up at 30–50 mg/ml) of protein did not change with time and that the changes in fluorescence properties with pH observed were largely reversible. This rules out the possibility that any effects observed were due to denaturation or proteolysis [10].

3. Results

3.1. Effect of protein conformation on fluorescence lifetimes

The fluorescence and anisotropy decay plots are not shown because they are very similar to ones previously published [11,12] and these papers can be referred to for analytical procedures as well. The results are collected in table 1. Under all conditions the fluorescence decay of the two tryptophans in bovine serum albumin must be fitted by a minimum of three exponential components. Table 1 also gives the average decay time and the statistical parameters. For an acceptable fit the reduced ψ^2 must be as close as possible to 1.00; the Durbin-Watson parameter for a three-component fit must be greater than 1.80 [9,11]. For one condition, 360 nm emission, pH 6.9 (row 5 in table 1), eight separate samples were measured to determine the precision of the measurements. The percent standard deviations are: for parameter 1 (i.e., α_1 or τ_1), $\pm 20\%$; parameter 2, $\pm 5\%$; parameter 3, $\pm 3\%$; average τ, $\pm 4\%$.

Table 1 shows that in all the conformations of bovine serum albumin, the average lifetime is longer for the emission measured at 360 nm than at 325 nm. A similar effect has been observed before for other multi-tryptophan-containing proteins, e.g., the lac repressor protein [13]. The differences arise from changes in both amplitudes and values of each component. We can interpret this effect in terms of two classes of emission, one at the shorter wavelength with a shorter average lifetime, and the other at longer wavelength with a longer lifetime.

While it is tempting to ascribe each class to a particular tryptophan residue, it should be noted that the lifetimes of the single tryptophan in human serum albumin and apolipoproteins I and II also increase with emission wavelength [14,15]. Hence, we will describe the emission properties in terms of classes or environments.

We rechecked our measurements for the chromatographed protein in 0.05 M phosphate, pH 7.0, using G factors obtained by altering the direction of polarization of the exciting light from vertical to horizontal but without disturbing the protein solution or making any other change.

We confirmed the trends in the data presented in table 1 (results not shown) although the average lifetimes were 5.0 ns at 325 nm (23 nm slits) and 5.8 ns at 360 nm (23 nm slits), about 16% longer than the values we obtained previously (table 1).

Again, we were unable to fit the 325 nm data using two-exponential functions. For example, χ^2 and Durbin-Watson parameters for three measurements (23, 19 and 14 nm slit widths) at 325 nm averaged 1.09 and 1.88, respectively, for three-component fits, but 1.61 and 1.27 for two-component fits. At 325 nm, a component with a short (< 1 ns) lifetime makes a contribution that is too great to be ignored.

The 360 nm data, however, could be fitted fairly well using two components. The χ^2 and Durbin-Watson parameters averaged 1.16 and 1.91, respectively, for the three sets of data fitted using three components and 1.19 and 1.85 using two. We conclude that a fast process, possibly a tryptophan environment which is influenced by some rapid environmental quenching process, is responsible for a significant part of the shorter wavelength tryptophan emission in this protein at pH 7.0. The data in table 1 suggest that this

Table 1

Fluorescence lifetime parameters of different conformations of bovine serum albumin as a function of emission wavelength (nm)

All solutions contained 30 μM HPLC-chromatographed bovine serum albumin. The 'native' sample contained 0.05 M sodium phosphate, pH 6.9. The 'moderately expanded' sample contained 0.0125 M HCl and 0.10 M NaCl; the 'highly expanded' samples contained 0.0125 M HCl only. The 'neutralized' values were obtained using samples that originally contained 0.0125 M HCl or 0.0125 M HCl plus 0.10 M NaCl; following fluorescence and anisotropy decay measurements, 0.05–0.10 ml of 1 M sodium phosphate, pH 7.0, was added (0.05 M final concentrations) and after mixing the fluorescence parameters of the proteins were measured again. Final pH values are given. Excitation was at 300 nm. The emission band pass was 23 nm. The temperature was maintained at 22° C. τ is expressed in ns; $\Sigma\alpha_i = 100$. Average lifetimes were calculated from fitted fluorescence decay parameters using the relation $\tau_{ave} = \Sigma_n \alpha_n \tau_n / \Sigma_n \alpha_n$. DW, Durbin-Watson parameter.

Conformation	(pH)	λ_{em}	α_1	τ_1	α_2	τ_2	α_3	τ_3	τ_{ave}	χ^2_R	DW
Native	(6.9)	325 [a]	48	0.24	21	1.9	31	6.3	2.5	1.20	2.00
	(6.9)	325	22	0.44	24	2.8	54	6.5	4.3	1.00	1.89
		340	16	0.45	20	2.7	64	6.5	4.9	1.20	1.97
		360	12	0.53	21	3.2	67	6.7	5.2	1.10	1.93
	(6.9)	360 [b]	24	0.48	27	3.1	49	7.2	4.8	<1.23	>1.83
Moderately expanded	(1.8)	325	29	0.70	35	2.6	36	5.3	3.0	0.96	1.82
		360	25	0.45	41	2.5	33	5.7	3.0	1.13	1.80
	(2.3)	325	29	0.40	37	2.2	34	5.2	2.7	1.15	2.04
		340	28	0.50	39	2.5	33	5.6	3.0	1.06	2.01
		360	23	0.80	37	2.7	40	5.6	3.4	1.03	1.87
(Neutralized)	(6.1)	360	8	1.1	24	4.3	68	6.8	5.7	1.10	1.83
Highly expanded	(1.9)	325	30	0.32	40	1.8	30	4.7	2.2	1.10	1.84
		360	33	0.60	46	2.5	21	5.4	2.5	1.19	1.92
	(2.4)	325	40	0.34	38	1.7	22	5.0	1.9	–	–
		340	22	0.42	42	1.7	27	5.3	2.2	1.19	1.93
		360	29	0.67	44	2.5	27	5.4	2.8	1.09	1.83
(Neutralized)	(6.3)	340	14	1.1	30	4.1	56	6.9	5.2	1.01	1.93
	(6.3)	360	9	1.0	25	4.2	66	6.8	5.6	1.18	1.86

[a] One sample at 165 μM (~1.1 *A* at 300 nm) (13.8 nm slits).
[b] Eight samples at 165 μm (average values).

process is also important at acid pH.

The major effect presented, however, is a marked shortening of the average lifetime at low pH. This shortening is largely reversible. There may be a small degree of hysteresis. We did not investigate whether formation of polymers occurred on neutralization. There is no evidence of laser degradation of the samples, as the lifetime parameters obtained after excitation at several wavelengths are not significantly different from those obtained upon a first excitation.

Average lifetimes of tryptophan fluorescence in unchromatographed bovine serum albumin in solutions covering pH values from 2 to 8 are presented in fig. 1. The qualitative effects of acid pH and ionic strength observed were the same. The data resemble a titration curve with an inflection at pH 3.9, which in turn is the approximate midpoint pH for the N-F transition of the protein (pH 3.7, corrected for the oligomer content) [8,16]. These data suggest that the shortened average lifetime observed at lower pH values is a result of the conformational changes. It is not due to proton quenching of the tryptophans because in fig. 1 and table 1 we can see that changes in pH, e.g., from 1.8 to 2.3, moderately expanded, do not affect the individual lifetimes.

It is known that some lanthanides, especially lutetium at moderate concentrations, produce a conformational change in the protein at near neutral pH values which resembles the N-F transition [5]. The effect of 0.02–0.06 M lutetium on the

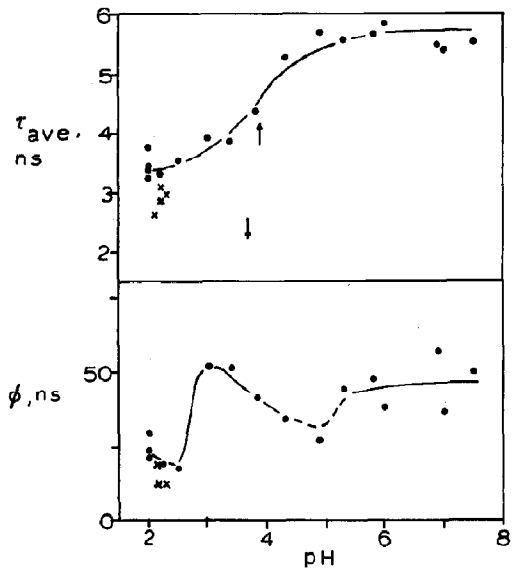

Fig. 1. Dependence on pH of average tryptophan fluorescence lifetime (upper curve) or rotational correlation time (lower curve) in bovine serum albumin. The solutions contained 30 μM unchromatographed protein in solutions buffered with HCl, NaCl-HCl, acetate, Mes, phosphate or Tris. The ionic strengths were 0.05–0.15 except for those samples denoted by (x), which were at 0.01–0.015 ionic strength. The temperature was maintained at 22°C. Excitation was at 300–303 nm and emission was at 340 nm with a 35 nm slit width. The arrow pointing upwards denotes the estimated midpoint of the curve; the arrow pointing downwards is the midpoint of the N-F transition, corrected for the oligomer content [8].

calculated lifetimes was identical to that of low pH values (data not shown). The midpoint of the effect was at approx. 30 mM, similar to that described in ref. 5. (~ 25 mM). We conclude that the effect on the calculated average lifetimes described is the result of an effect on the conformation of the protein.

Examination of the effect of ionic strength on the fluorescence decay of tryptophans in bovine serum albumin, at 0.01 and 0.11 ionic strength, showed a smaller effect (table 1). The acid expansion itself has less effect on lifetime than does the N-F transition. The effect of ionic strength on the lifetime seems to be less than that on the fluorescence yield. It is difficult to correct for differences in measurement conditions, particularly excitation and emission bandwidths, but we believe that the further expansion of the protein at low pH values and ionic strengths due to electrostatic factors does not affect the lifetime as much as the quantum yield. This suggests that the differences in fluorescence emission due to ionic strength are partly due to a complex quenching at low ionic strengths which is relieved at higher values.

Acid expansion of bovine serum albumin produces a genuine shift in emission to lower wavelengths of one of the tryptophan environments, since the emission intensity at shorter wavelengths is greater at low pH than at neutral pH [6]. However, acid expansion must also involve a preferential quenching of the longer-lived, longer wavelength emitting tryptophan environments. The increase in fluorescence at 0.11 ionic strength relative to 0.01 ionic strength is interpreted as a result of reduced complex and collisional quenching of the shorter wavelength, shorter lived environments, so that the emission wavelength shifts to shorter values, however, the fluorescence lifetime actually increases somewhat.

3.2. Steady-state quenching of serum albumin at low and neutral pH values

Differences in emission wavelength presumably reflect differences in polarity about each residue and these have been shown to exhibit different accessibilities to quenchers [7,17]. The emission spectrum of the enzyme was measured at pH 7, at pH 2 and 0.01 ionic strength and at pH 2 and 0.11 ionic strength in the presence of 0.02–0.20 M acrylamide. The effect of 0.02–0.20 M KI was also measured, although at a constant KCl plus KI concentration of 0.20 M. The unquenched spectrum was divided by each of the quenched ones in turn and some of these data are plotted in fig. 2. Stern-Volmer plots were made of these data, taken at 320 and 380 nm (fig. 3) (the 350 nm data are not shown for the sake of illustrating the effects more strongly).

The scatter of the data is due to the fact that they are from two relatively highly resolved (2 nm bandwidth) spectra and in addition are from wavelengths (320 and 380 nm) where the emission intensities are low.

The iodide quenching data at pH 7 show a

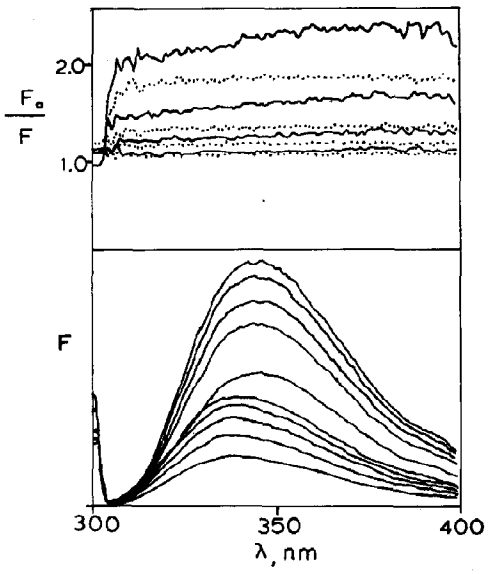

Fig. 2. Effect of 0.02–0.2 M acrylamide on uncorrected fluorescence (F) of bovine serum albumin at pH 7 (upper five curves of lower panel) and pH 2 (lower five curves) at 0.015 ionic strength (where the acid expansion is greatest). The upper panel shows relative fluorescence, unquenched divided by quenched, as a function of wavelength at pH 7 (broken lines) and at pH 2. The greater F_0/F values at pH 2 indicate greater apparent accessibility of tryptophan(s) to the quencher acrylamide. Data are from the lower panel and increasing acrylamide results in progressively lower fluorescence (F) and higher values of F_0/F. The excitation wavelength was 300 nm; excitation and emission slit widths were 2 nm. The temperature was maintained at 22°C. Samples contained 30 μM protein. Other solvent conditions were as described in the legend to fig. 1.

downward curvature toward the abscissa (iodide concentration axis). The data suggest two environments with very different accessibilities. The two environments are not dramatically different in emission wavelength. At pH 2, there is no clear distinction with respect to emission wavelength as far as iodide is concerned; the major effect is that the difference in apparent accessibility seen at pH 7 is no longer so obvious.

Human serum albumin, which has a single tryptophan, also shows downward-curving plots. Lehrer and Leavis [18] attributed this to binding of the iodide by that protein. We would expect binding of the quencher near the tryptophan(s) to produce upward-curving plots.

Acrylamide produces quenching with qualitative differences from the effect of iodide. At pH 7, there is little difference between quenching of the fluorescence at 320 nm and that at 380 nm, but a greater differentiation occurs at pH 2. At 0.01 ionic strength, the overall apparent accessibility appears to be greater, although it is possible that the data actually show an upswing, suggestive of complex formation between acrylamide and the protein. The main effect of the higher ionic strength at pH 2 (more compact structure) is to make both tryptophan environments less accessible to a nearly equal extent.

Despite the differences between iodide and acrylamide quenching, the apparent quenching constants are similar. If a linear quenching response is assumed (i.e., collisional deactivation only) and average lifetimes are used from table 1, average quenching constants can be calculated and are listed in table 2. These values were calculated from the 320 and 380 nm emission data. The quenching constant for low molecular weight indole derivatives is 7×10^9 M^{-1} s^{-1} [7,14,17]. The values we obtained are lower, indicating the existence of significant barriers to access of these quenchers. From the difference in effect of the two quenchers, it appears that an electrostatic barrier exists to quenching of one of the environments at pH 7, possibly a barrier involving an ionized carboxyl group.

Very similar values of the quenching constant were obtained with human serum albumin in its native conformation (0.5×10^9 M^{-1} s^{-1}) [17] to those we calculated for the bovine protein. An 8 nm shift to shorter emission maximum in human serum albumin at pH 2.5 (0.01 M formic acid) is accompanied by a doubling in acrylamide quenching constant [19], nearly the same as that observed for bovine serum albumin.

The most significant conclusion, however, is that we cannot distinguish between the two emitting environments on the basis of quenching constants. While the lifetimes used are extrapolated values, and since the bandwidths employed in lifetime measurements (23 nm) are much greater than those used for emission measurements (2 nm), the increase in quenching at longer wavelengths must at least partly matched by the in-

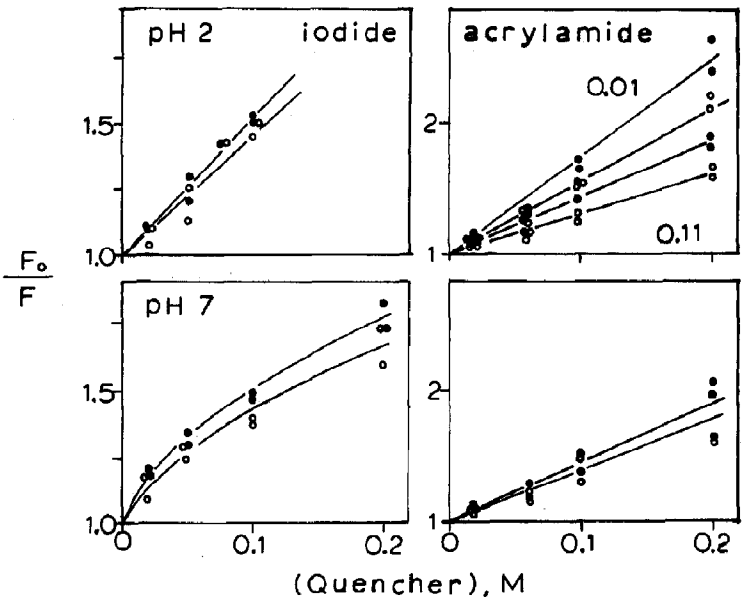

Fig. 3. Stern-Volmer plots of quenching data. Conditions were as described in the legend to fig. 2. Data were taken from two experiments each at 320, 350 and 380 nm but only the 320 nm (○) and 380 nm (●) values are presented. Iodide quenching data are presented in the two left-hand graphs and acrylamide quenching data in the lower two right-hand graphs. Acid pH data are represented by the two upper graphs and pH 7 data by the two lower ones.

Table 2

Apparent second-order quenching constants of tryptophan fluorescence in bovine serum albumin in several conformations

The average lifetimes used in these calculations were: (pH 7), 3.95 and 5.65 ns at 320 and 380 nm, respectively; pH 2 (0.11 or 0.20), 2.60 and 3.15 ns; pH 2 (0.01), 1.90 and 2.50 ns. These were extrapolated from the data in table 1. The two values of the quenching constant at pH 7 with iodide were calculated from the initial and final slopes of the appropriate lines in fig. 3. Other values were also calculated from the slopes of the other Stern-Volmer plots in fig. 3.

Quencher	pH (ionic strength)	Conformation	Average quenching constant ($\times 10^{-9}$) ($M^{-1}\,s^{-1}$) at emission wavelength (nm)	
			320	380
Iodide	7	native	0.61, 1.60	0.50, 1.40
	2 (0.20)	moderately expanded	1.80	1.70
Acrylamide	7	native	0.51	0.43
	2 (0.11)	moderately expanded	1.20	1.40
	2 (0.01)	highly expanded	3.00	3.00

crease in lifetime. The shifts in apparent environmental polarity about the tryptophans do not involve major changes in accessibility to the quenchers. Some changes in apparent accessibility which are observed such as that produced by low pH and 0.11 ionic strength run opposite to those predicted. However, Maliwal and Lakowicz [14] observed the same effect with human serum albumin. The single tryptophan of this protein emits at shorter wavelengths in its F conformation, but shows a higher oxygen quenching constant.

It is possible that the upward slope of the F_0/F lines is a result of the short-lifetime component (table 1) being relatively unaffected by the quencher because its lifetime is so short. This would also explain why the average apparent quenching constants do not vary with emission wavelength.

3.3. Tryptophan fluorescence anisotropy decay

If the overall bovine serum albumin molecule gains rotational freedom of motion at low pH

values [1,3], as measurements of steady-state anisotropies of dye-protein conjugates indicate, is this reflected in the freedom of motion of the tryptophan residues? Table 3 lists the rotational correlation times for the tryptophan residues calculated from the decay functions of the emission anisotropy. Unlike the fluorescence lifetime behavior in table 1, the anisotropy decay is simpler, requiring a two-exponential function for a fit, but the longer correlation time obtained is also dependent on emission wavelength. The analytical procedure used is not statistically rigorous [9] so the χ^2 values are not listed and the fits are made to minimize the value of χ^2_R. For a two-exponential fit it is required that the Durbin-Watson parameter be greater than 1.75.

The chromatographed protein in its native conformation (pH 7) gave the longest correlation times, 35.6 ± 1.4 ns (all 325 nm emission data) (some data not shown; see below) and 31.3 ± 2.6 ns from 360 nm emission (all values, some not shown; see below). Shorter values (~ 22 ns) are obtained for moderately expanded (pH 1.8 and 2.3 at 0.11 ionic strength) and even shorter values (averaging 11.5 ns) are found at pH 1.9 and 2.4 and 0.01 ionic strength (highly expanded protein). These results are qualitatively and quantitatively consistent with expectations [1,3]: the acid pH (0.01 ionic strength), longer correlation times approach one-third of their value at neutral pH. This is in agreement with the hypothetical structure of the protein (three linked domains of nearly equal molecular weight). It also reinforces the picture of domains as being nearly independent, structurally, of the degree of domain interaction. The overall structure of the domains is rigid at neutral pH and remains so on expansion of the bovine serum albumin molecule.

The data also persistently suggested either significant movement of the tryptophan residues independently of the rest of the protein, with a correlation time averaging about 70 ps and about twice the amplitude of the 30 ns component or a scattering artifact.

However, calculated limiting anisotropy (r_0) values were over 0.4 and the emission wavelength dependence of these values suggested a scattering artifact. We measured the decays at 325, 340 and 360 nm, then the scattering at 300 nm of a solution of chromatographed bovine serum albumin. We then measured the signal decay at 325, 340 and 360 nm of a solution of glycogen which produced the same scattering signal (at 300 nm) as the protein solution. The scattering data at 325, 340 and 360 nm were subtracted from the decay data provided by the protein solution at those wavelengths. When this was done, we were left with a long anisotropy decay, together with a very fast (< 10 ps) initial signal. The major exponential decay was as described in table 3 with a 325 nm correlation time of 36.7 ns and a 360 nm correlation time of 29.7 ns. The origin of the very fast initial signal is not known. The value of r_0 for a sample of protein at pH 7 and 22°C was 0.31, near that of 0.29 from corrected fluorescence-polarization plots of protein solutions (data not

Table 3

Decay of the tryptophan residues' emission anisotropy in different conformations of bovine serum albumin

These values were obtained from the same data as were the lifetime parameters in table 1. A two-component fit was needed, but the shorter component (< 1 ns) is not listed as it is considered to be partly artifactual (see text). DW, Durbin-Watson parameter.

Conformation	(pH)	λ_{em}	ϕ (ns)	DW
Native	(6.9)	325	34.2	1.93
		340	31.0	2.05
		360	28.8	2.03
	(6.9) [a]	360	28.1	(>1.7)
		325 [b]	36.4	1.81
Moderately expanded	(2.3)	325	22.7	1.77
		340	22.3	1.76
		360	19.6	1.71
	(1.8)	325	24.5	1.88
		360	20.5	2.18
(Neutralized)	(6.1)	360	31.7	1.87
Highly expanded	(2.4)	325	13.5	1.93
		340	9.8	2.03
		360	14.0	1.72
	(1.9)	325	11.7	2.06
		360	8.3	1.99
(Neutralized)	(6.3)	340	31.8	1.76
	(6.3)	360	31.4	2.08

[a] Eight samples at 165 μM (~ 1.1 A at 300 nm).
[b] One sample at 165 μM.

shown). The broader bandwidth of excitation would probably account for the remaining discrepancy. We must conclude that the tryptophans in this protein appear to be rigidly held at pH 7, and since the initial decays of the acid pH data gave identical results to the largely artifactual initial pH 7 data, we must conclude further that the tryptophans do no 'loosen' at acid pH values.

In human serum albumin, no rotational freedom of the single tryptophan was observed at low temperatures (8°C), but significant motion was found at 43°C [19]. Van Hoek et al. [20] reported a 6 ns segmental motion but nothing shorter in bovine and human serum albumin. They later [21] decided that the 6 ns motion in human albumin was a deconvolution artifact, originating possibly from the oscillatory component in the cavity dumper driver mentioned above. The literature value of 34 ns [14] for the correlation time of monomeric bovine serum albumin at pH 7 is in excellent agreement with the average value (all emission wavelengths) of 33.4 ± 2.1 ns.

We conclude the tryptophans are rigidly held within the bovine serum albumin molecule, independently of the degree of domain association and extent of quenching. This is again consistent with the concept of domain structure: considerable independence of the microscopic environment from degree of domain association. The sampling of several environments by individual tryptophan residues indicated by the lifetime data must be too fast for our methods of measurement.

The longer correlation times also show a trend to lower values at longer emission wavelengths. This is interpreted as the result of the two environments emitting at somewhat different wavelengths and being fixed at different angles, relative to the rotational axes of the molecule [12,22] (see below).

We also measured the pH dependence of correlation times of unchromatographed bovine serum albumin (fig. 1). The average correlation time (values above pH 4.9) is 46 ± 8 ns. After correction for the observed content of oligomers, we obtain a value of 27 ± 5 ns, in fair agreement with a value in the literature of 34 ns [14] and with the average of our values (e.g., table 3). The correlation times may fall below pH 5, suggestive of a loosening of the structure, but they return to the neutral pH correlation time at pH values near 3, possibly indicating a predominating rigid swelling of the molecule undergoing the N-F transition. Such a swelling has been demonstrated in human serum albumin [14] and shown by steady-state polarization techniques in the bovine protein [23]. The acid expansion itself, as opposed to the N-F transition, is correlated with a sharp drop in correlation times (pH < 3).

There was no significant effect of lutetium on the correlation time at pH 5.3 (data not shown), in agreement with the interpretation [5] that the lanthanide effects the N-F transition. The molecule may expand, but if it does, it loses rigidity to the same extent. These data agree well with measurements using dye-protein conjugates [1,24].

The average correlation times in fig. 1 were obtained using the 2020 photomultiplier which is slower than the microchannel plate photomultiplier. We found only a single correlation time using the 2020 photomultiplier.

3.4. Fluorescence and anisotropy decays of ANS bound to serum albumin

ANS binds at several sites on bovine serum albumin, and binding at some of these sites is accompanied by a marked increase in ANS fluorescence [1]. The tryptophan fluorescence of the protein is quenched, apparently because of energy transfer to bound ANS [24]. We measured the effect of bound ANS on tryptophan lifetimes as well as ANS lifetimes and anisotropies.

The presence of a 1:1 molar ratio of ANS reduced the average tryptophan lifetime by only 15 and 18% in two experiments. This is far less than the reduction in tryptophan steady-state fluorescence by that amount of ligand under our conditions (90 and 75% in two experiments). We have no explanation for this finding; ANS appears to 'sample' all tryptophan environments about equally. We were unable to observe any lag in the ANS fluorescence rise on excitation at 302 nm, suggesting that energy transfer [24] is very rapid [12]. The major ANS lifetime was 18.6 ± 1.0 ns (five samples), in agreement with literature values [7]. About 19% of the ANS fluorescence had a shorter lifetime, viz., 4.6 ns. The longest correla-

tion time for chromatographed protein from ANS anisotropy decay was 39 ± 3 ns (six samples). A separate set of three measurements calculated using G factors from horizontally polarized excitation gave 40.0 ± 2.1 ns, in excellent agreement. These values are higher than those of tryptophan anisotropy decay. This again may reflect a difference in orientation of ANS relative to the axes of the protein compared with the tryptophan environments. Interestingly, in the presence of 60 mM Lu^{3+} (at pH 4.7), at which the protein has undergone the N-F transition [5], the ANS fluorescence showed the same fluorescence lifetimes but the correlation times were 0.6 and 46 ns in this case, also suggestive of a swollen molecule [14,23]. The ANS fluorescence yield remained high. The loss of ANS fluorescence in acid [6] is apparently due to proton quenching or to the expansion per se, not to the N-F transition conformational changes.

4. Discussion

We excite our samples at 300 nm in order to avoid excitation of tyrosine or of tryptophan into the ^1L_b state. Comparatively large changes in tryptophan absorption occur during these conformational changes [4,6], absorbance changes that are significant at 300 nm. However, the fluorescence emission spectra observed in the quenching experiments are very similar to the spectra obtained by excitation at 280 nm [6].

Although our microchannel plate photomultiplier data fitted three-component decay curves well, the protein contains only two tryptophans. Even free tryptophan shows two lifetimes [25] and there are other proteins which exhibit more lifetimes than the number of tryptophans they possess [12,14,15]. We believe that the tryptophans in this protein exhibit a spectrum of lifetimes, and hence the measured lifetimes are functions of instrument resolution as well as detection wavelength. Our average lifetime for the native conformation is shorter than the value in the literature (6.3 ns) [26], perhaps for this reason.

The source of heterogeneity in tryptophan lifetimes is not certain [25]. A variety of interactions can affect lifetimes, interactions which could reasonably be expected to exist in proteins. Generally, tryptophan in native proteins seems to have longer lifetimes than in denatured proteins. Bovine serum albumin in 6 M guanidine hydrochloride with 50 mM phosphate, pH 7, has an apparent average lifetime similar to those obtained at acid pH values (data not shown). It seems that the conformational change brings the tryptophans into proximity with dynamic quenching groups.

The picture we obtain from lifetimes is different from that provided by static quenching. Some of the shifts in emission maxima are not consistent with the rate constants for quenching [7,27] or the similarity of the acid-expanded and guanidine hydrochloride-denatured lifetimes. It is also possible that complex quenching may be a factor in some of the acrylamide results, however, the data obtained with iodide show the same effect: greater Stern-Volmer quenching constants along with tryptophan fluorescence at shorter wavelengths.

While we can rationalize much of our data in terms of two environments, we do not assign either to a particular residue. The interactions between the domains appear to produce different effects on the environments but these are not obvious or simple. They do not seem to involve significant restrictions on the mobility of the tryptophans, unless such restrictions quench, consistent with domain structure. Examinations of the fluorescence behavior of proteins at the picosecond level has provided new and interesting results, but much more work remains before we can interpret these results in terms of detailed protein structure.

Acknowledgements

This work was supported by The National Science Foundation (PCM-8312669) and The National Institutes of Health (RR02389 and GM28139).

References

1 G. Weber and L.B. Young, J. Biol. Chem. 239 (1964) 1415.
2 J.R. Brown, Fed. Proc. 35 (1976) 2141.

3 C. Tanford, Physical chemistry of Macromolecules (Wiley, New York, 1961).
4 J.F. Foster, The plasma proteins, ed. F.W. Putnam (Academic Press, New York, 1960) vol. 1, p. 179.
5 F. Shahid, J.E. Gomez, E.R. Birnbaum and D.W. Darnall, J. Biol. Chem. 257 (1982) 5618.
6 J.M. Brewer, R.J. DeSa and J.E. Wampler, Biochem. Biophys. Res. Commun. 76 (1977) 572.
7 J.R. Lakowicz, Principles of fluorescence spectroscopy (Plenum Press, New York, 1983).
8 M.M. Mir and M.A. Qasim, Biochem. Int. 13 (1986) 487.
9 D.V. O'Connor and D. Phillips, Time-correlated single-photon counting (Academic Press, London, 1984).
10 W.D. Wilson and J.F. Foster, Biochemistry 10 (1971) 1772.
11 A.J.W.G. Visser, T. Ykema, A. van Hoek, D.J. O'Kane and J. Lee, Biochemistry 24 (1986) 1489.
12 T. Kulinski, A.J.W.G. Visser, D.J. O'Kane and J. Lee, Biochemistry 26 (1987) 540.
13 J.C. Brochon, P. Wahl, M. Charlier, J.C. Maurizot and C. Helene, Biochem. Biophys. Res. Commun. 79 (1977) 1261.
14 B.P. Maliwal and J.R. Lakowicz, Biophys. Chem. 19 (1984) 337.
15 B.P. Maliwal, A.D. Cardin, R.L. Jackson and J.R. Lakowicz, Arch. Biochem. Biophys. 236 (1985) 370.
16 J. Steinhardt and J.A. Reynolds, Multiple equilibria in proteins (Academic Press, New York, 1969).
17 M.R. Eftink and C.A. Ghiron, Biochemistry 15 (1976) 672.
18 S.S. Lehrer and P.C. Leavis, Methods Enzymol. 49 (1978) 222.
19 I. Munro, I. Pecht and L. Stryer, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 56.
20 A. van Hoek, J. Vervoort and A.J.W.G. Visser, J. Biochem. Biophys. Methods 7 (1983) 243.
21 K. Vos, A. van Hoek and A.J.W.G. Visser, Eur. J. Biochem. 165 (1987) 55.
22 M.D. Barkley, A.A. Kowalczyk and L. Brand, J. Chem. Phys. 75 (1981) 3581.
23 M. Sogami, S. Era, S. Nagaoka and H. Inouye, Int. J. Peptide Protein Res. 19 (1982) 263.
24 E. Daniel and G. Weber, Biochemistry 5 (1966) 1893.
25 J.M. Beechem and L. Brand, Annu. Rev. Biochem. 54 (1985) 43.
26 A. Grinvald and I.Z. Steinberg, Biochim. Biophys. Acta 427 (1976) 663.
27 B. van Duuren, Chem. Rev. 63 (1963) 325.