Phosphorescence and Optically Detected Magnetic Resonance Study of the Tryptophan Residue in Human Serum Albumin†

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ABSTRACT: The phosphorescence and optically detected magnetic resonance (ODMR) of the lone tryptophan residue in the protein human serum albumin (HSA) were studied. This residue shows a red-shifted phosphorescence and *D–E* triplet zero-field splitting frequency, which suggests that it is buried in a hydrophobic region of the protein, based on trends in triplet state properties of tryptophan residues in peptides and proteins, which have recently been observed by other workers. This conclusion is in agreement with the results of previous fluorescence quenching studies of HSA with hydrophobic quenchers. Also, the ODMR line width is consistent with the line widths usually obtained from buried tryptophans, and the ODMR line shifts and broadens when the protein is heat denatured with urea, in qualitative agreement with results observed with several other proteins. Phosphorescence wavelengths, decay times, and zero-field splittings are also studied as a function of binding of halides and acetate to HSA. The tryptophan triplet lifetime is shortened when bromide and iodide bind, apparently due to the external heavy atom effect; chloride and acetate have no effect on the phosphorescence lifetime. ODMR and phosphorescence measurements suggest that the degree of solvent exposure of the tryptophan does not change appreciably upon the binding of these anions.

In recent years, the technique of optically detected magnetic resonance (ODMR)[1] has emerged as a useful probe of protein structure (Kwiram, 1982). In the ODMR technique, the phosphorescence of a triplet chromophore is observed while pumping the microwave frequency transitions between the magnetic sublevels of the triplet state. In applications of ODMR to proteins and peptides, the chromophore is usually tryptophan or tyrosine. As is well-known (McGlynn et al., 1969), the magnetic dipole interaction of the two unpaired electron spins in the triplet state gives rise to an energy splitting even in zero-applied magnetic field. Since the sublevels in general have different radiative probabilities, application of microwave radiation coupling any two of the sublevels can cause a change in phosphorescence intensity. With ODMR techniques (Kwiram, 1972), the zero-field splitting frequencies and the lifetimes of the individual sublevels can be measured.

In several recent studies, the groups of Kwiram and Maki have shown that ODMR may be used to characterize the environment of tryptophan residues in proteins and peptides (Zuclich et al., 1973; von Schütz et al., 1974; Ugurbil et al., 1977; Deranleau et al., 1978; Rousslang et al., 1978; Ross et al., 1977). In particular, the trends in triplet state properties of tryptophan vs. degree of exposure to the polar solvent have been summarized recently by Rousslang et al. (1979) and by Hershberger et al. (1980). It has been found that tryptophans that are inaccessible to polar solvents generally have red-shifted phosphorescence and *D–E* zero-field transition frequencies, relative to solvent-exposed tryptophans. Also, the ODMR line widths tend to become smaller as the tryptophan residues become less solvent accessible. This latter trend can be rationalized qualitatively with the expectation that the environment of a tryptophan residue would be more homogeneous if that residue were located within a hydrophobic pocket of a protein, instead of being exposed to a variety of solvent conformations. These correlations have in most cases been established by comparison with X-ray crystal structure data, although in recent work (Ross et al., 1980), it was possible to make inferences about the structure of the catalytic subunit (C subunit) of protein kinase on the basis of ODMR data alone; there is no X-ray crystal structure model for this protein at the present time.

In agreement with the ODMR "model" described above, it is observed that ODMR lines often shift and broaden when the protein under study is denatured by the addition of urea (Ross et al., 1980). The zero-field splitting frequencies and line widths often approach those seen for solvent-exposed tryptophan residues. Very recent experiments have also revealed, however, a number of exceptions to the model. For example, the values of zero-field splittings and phosphorescence wavelengths obtained in ODMR studies of RNase T_1 and phospholipase A_2 suggest that the tryptophan residue in each protein is exposed to the polar solvent (1:1 ethylene glycol–aqueous buffer), while the narrow phosphorescence and ODMR line widths give evidence for a more homogeneous environment than would be expected from a solvent-exposed residue (Hershberger et al., 1980). Similarly, the phosphorescence of C subunit is relatively red shifted, suggesting that the tryptophan residues are not exposed to the solvent, while the zero-field parameter *E* for one class of tryptophan residues in this protein falls into the category of exposed residues (Ross et al., 1980). These exceptions to the ODMR model are at least partially explained in terms of local interactions with specific residues within the protein, which lead to anomalous values of the magnetic and/or optical transition energies.

To further test the ODMR model, we examine the protein, human serum albumin (HSA), by ODMR and phosphores-

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[1] Abbreviations: ODMR, optically detected magnetic resonance; HSA, human serum albumin; EGB, ethylene glycol–phosphate buffer, 1:1 (v/v); RNase, ribonuclease; C subunit, catalytic subunit of protein kinase; BzNiCo, aqueous filter solution containing 5 mM sodium benzoate, 0.5 M $NiCl_2$, and 0.5 M $CoCl_2$; Tris, tris(hydroxymethyl)aminomethane.

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cence techniques in this study. Fluorescence quenching experiments (Eftink et al., 1977; Eftink & Ghiron, 1977) with hydrophobic quenchers have provided strong evidence that the lone tryptophan in this protein is buried in a region that is not exposed to polar solvents. Our results qualitatively agree with the predictions of the ODMR model described above; the D–E zero-field splitting, ODMR line width, and phosphorescence 0,0 wavelength of tryptophan in this protein fall within or near the range of values typical for tryptophans that are not accessible to polar solvents, based on other work. Moreover, denaturation with heat and 8 M urea affects the phosphorescence and ODMR in a manner consistent with the model.

In the second part of this study, we have investigated the effect of anion binding on the triplet state properties of the tryptophan residue in HSA. Anion binding is one of the principal biological functions of this carrier protein, and it is of interest to examine conformational changes of the protein, as monitored by zero-field splitting frequencies, phosphorescence wavelengths, and lifetimes, as affected by anion binding. Triplet state properties of tryptophan may be affected in at least three ways: (1) Direct interactions with heavy atoms within the bound anion, e.g., bromide or iodide, may shorten the phosphorescence lifetime by means of the external heavy atom effect (Kasha, 1952). (2) Anion binding may alter the protein configuration around the tryptophan so as to decrease the external heavy atom interaction with sulfur-containing amino acids; this can also lead to changes in phosphorescence lifetimes (King & Miller, 1976). (3) Anion binding may induce a change in protein configuration such that the degree of solvent exposure of the tryptophan residue is altered. This would be expected to produce changes in phosphorescence wavelengths and zero-field splittings, as indicated above. Our results, which are discussed in detail below, show that only subtle changes in zero-field parameters and phosphorescence lifetimes occur at low values of the binding parameter $\bar{\nu}$, where $\bar{\nu}$ represents the average number of anions bound per molecule of protein. At higher values of $\bar{\nu}$, the phosphorescence lifetimes are markedly shortened by binding with bromide and iodide; this is most probably a direct manifestation of the external heavy atom perturbation of the tryptophan chromophore as caused by these ions.

Materials and Methods

Human serum albumin (HSA), fraction V, essentially fatty acid free, and lysozyme, grade I, were purchased from Sigma Chemical Co. L(−)-Tryptophan was obtained from Aldrich Chemical Co. Ultrapure urea was purchased from Schwarz/Mann Biochemicals, and ethylene glycol was obtained from Fisher Scientific Co. These reagents were used without further purification.

The solvent used for the ODMR and phosphorescence measurements on HSA was a 1:1 (v/v) mixture of ethylene glycol and aqueous sodium phosphate buffer. The pH of the aqueous buffer was adjusted to 7.4 at room temperature with phosphoric acid, and ethylene glycol was then added; the final concentration of the buffer was 0.05 M. A 1:1 (v/v) mixture of ethylene glycol and aqueous Tris buffer (Trizma base, reagent grade, Sigma Chemical Co.) was used for the experiments with free tryptophan and lysozyme. These solvents yield good-quality optical glasses for low-temperature spectroscopy. The sample concentrations were 2.5×10^{-4} M for the HSA ODMR experiments, 7.5×10^{-5} M for the HSA phosphorescence decay measurements, and 10^{-3} and 5×10^{-4} M for the experiments with lysozyme and tryptophan, respectively.

Samples were cooled in quartz tubes (3-mm inner diameter) to 2 K in a liquid-helium cryostat for ODMR measurements. Samples were excited by light from a 100-W Hg lamp whose output was passed through a Corning 7-54 filter plus an aqueous $NiSO_4$ solution (excitation band-pass ~240–350 nm). Phosphorescence emission was monitored at a variety of wavelengths between 400 and 460 nm with a 0.75-m Jarrell Ash monochromator (slit width = 2500 μm, band-pass = ~5 nm) and detected with a Bailey Centronics Q4283B photomultiplier tube. Phosphorescence was isolated from fluorescence and scattered light by a double blade chopper whose frequency (33 Hz) was used as the reference signal for a PAR Model 126 lock-in amplifier. In the ODMR experiments, the emission monochromator was set to the 0,0 phosphorescence maximum (between 405 and 415 nm, depending on the sample). The microwave source was a Hewlett-Packard 8690A sweeper with an 8699B plug-in. A Hughes 1403 H 1–2 GHz 1-W amplifier (attenuated to 100 mW) was used to enhance the ODMR signals. Slow passage ODMR spectra were obtained by recording the phosphorescence signal on a Tracor Northern 570A signal averager, whose sweep was synchronized with the frequency sweep of the microwave source. Sweep rates were typically 10 MHz/s or less whenever careful measurements of ODMR line widths were required. Typically 100–150 sweeps were needed to obtain HSA tryptophan ODMR signals with acceptable signal to noise ratios. A Hewlett-Packard Model 540 B transfer oscillator and a 5382A frequency counter were used for microwave frequency measurements.

As noted previously by Ross et al. (1977), the phosphorescence lifetime of tryptophan is such that the above described sweep rates do not allow a steady state to be attained among the sublevel populations as one passes through the ODMR line. Thus, the apparent peak positions depend on whether the sweeps are made toward increasing or decreasing frequency. Accordingly, we report the average of the two observed peak frequencies, obtained with sweeps in the two directions. It should be noted, however, that with the sweep rate cited above the apparent peak positions for the two directions agreed fairly well (within 15–30 MHz), and we are thus reasonably confident of our reported line width values under these conditions. In the case of native HSA, the sweep rates were typically 8 MHz/s, and the peak positions obtained with forward and reverse sweeps agreed to within less than 5 MHz.

Phosphorescence decay times were measured at ~77 K with the same cryostat as was employed for the ODMR experiments. The samples were immersed in liquid nitrogen for these experiments. For phosphorescence decays, the excitation filter was the Corning 7-54 filter plus an aqueous solution containing 5 mM sodium benzoate, 0.5 M $NiCl_2$, and 0.5 M $CoCl_2$ (BzNiCo). The band-pass of this filter was approximately 300–355 nm, and the purpose of the filter was to minimize the excitation of tyrosine residues in the HSA (Hicks et al., 1978) and thus to simplify the analysis of the phosphorescence decay. This aspect is discussed in more detail below.

The decays were obtained by a double shutter arrangement. The shutter in the excitation path was opened for 17 s, allowing a quasi steady state to be attained in the tryptophan-excited triplet population. This shutter was then closed (closing time ~5 ms), and the shutter in the phosphorescence observation path was opened; the latter shutter was kept open for 16 s. Typically, 32 decay curves were accumulated on our signal averager in this way for each sample run. A sum of two exponential functions $A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$ was fit

Table I: Phosphorescence and ODMR Properties of HSA, Tryptophan, and Lysozyme

sample	phosphorescence, λ (nm) 0,0	0,1	D-E (GHz)	$\nu_{1/2}$-$(D$-$E)^a$ (MHz)
HSA				
EGB[b]	412.5	439.5	1.653 ± 0.003	90
EGB + urea, 85–90 °C[c]	410.6	436.5	1.688 ± 0.008	110
EGB + urea, 100 °C[d]	409.0	435.7	1.710 ± 0.010	130
EGB + urea, 100 °C, stored[e]	410.6	437.0	1.681 ± 0.008	120
tryptophan, EGB[f]	406	434	1.779 ± 0.020	165
lysozyme, EGB[f]	415	444	1.526 ± 0.020	80

[a] Full width at half-maximum of the D-E transition. [b] EGB, 1:1 ethylene glycol-phosphate buffer solvent; see text. [c] Denatured in 8 M urea and heated to 85–90 °C for 1 h. [d] Denatured in 8 M urea and heated to 100 °C for 1 h. [e] Denatured in 8 M urea, heated to 100 °C for 1 h, and stored for 1 week. [f] Tris buffer used instead of phosphate.

to these data after subtraction of the measured base line by a generalized least-squares fitting program. As indicated in more detail below, this gave significantly better fits than a single exponential alone.

Results and Discussion

Phosphorescence and ODMR of HSA. The trends in phosphorescence and ODMR properties of tryptophan with solvent exposure are nicely summarized in the recent paper by Hershberger et al. (1980): (a) The 0,0 phosphorescence maximum for solvent-exposed tryptophans usually occurs in the range 405–409 nm, as compared to 410–415 nm for tryptophans buried in hydrophobic regions. (b) The D-E transition occurs at higher frequencies ("blue shifted") for solvent-exposed tryptophans, i.e., 1.7–1.8 GHz, as compared to 1.5–1.7 GHz for buried tryptophans. [It should be noted that both D and E appear to change with solvent exposure, and thus with phosphorescence 0,0 maxima. A better correlation is obtained between D-E values and the 0,0 maxima than with D or E independently (Hershberger et al., 1980). Thus, we quote D-E values in Table I.] (c) Narrower lines are generally observed for the ODMR transitions of buried tryptophans (40–80 MHz for the D-E transition) than for solvent-exposed tryptophans (100–165 MHz).

Phosphorescence and ODMR data obtained in this work are given in Table I. HSA is found to display phosphorescence and ODMR maxima at 412.5 nm and 1.653 GHz, respectively. Thus, on the basis of these data, it falls into the category of tryptophans buried in hydrophobic regions. Also, the D-E line width of 90 MHz is at the upper edge of the range of line widths observed for buried tryptophans. We should add that the $2E$ ODMR frequency, 2.600 ± 0.020 GHz (not shown in Table I), was also measured and falls among the values obtained for buried tryptophans (Hershberger et al., 1980).

The assignment of the HSA tryptophan to a buried region of the protein agrees with the conclusions of the earlier fluorescence quenching studies (Eftink & Ghiron, 1977; Eftink et al., 1977). Also, in qualitative agreement with previous work, the D-E transition is shifted by several tens of megahertz to higher frequency by denaturation with heat and urea (Figure 1). Roussland et al. (1978) noted a blue shift of about 98 MHz upon denaturation of lysozyme. Also, the phosphorescence 0,0 shifts toward the blue when the protein is denaturated (Figure 2), as has been seen with several other proteins [Ross et al. (1980) and references cited therein].

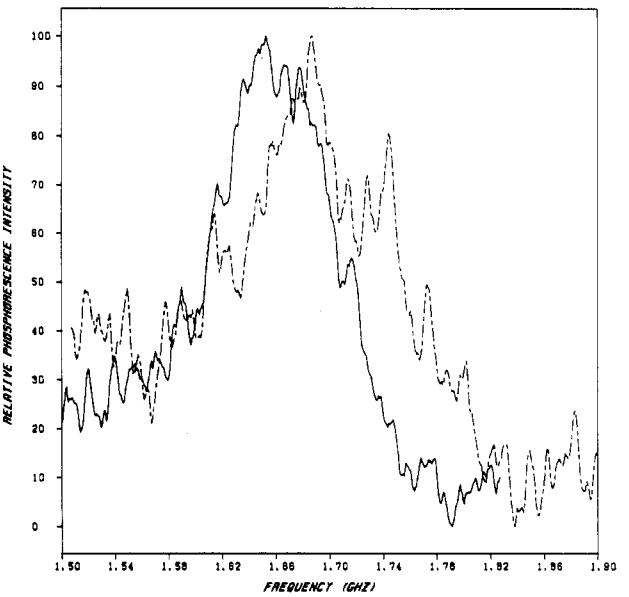

FIGURE 1: ODMR of native (solid line) and denatured (broken line) HSA at 2 K. HSA was denatured with 8 M urea and heating to 85–90 °C for 1 h. Zero on the ordinate corresponds to phosphorescence intensity in the absence of microwaves; both scans are from left to right.

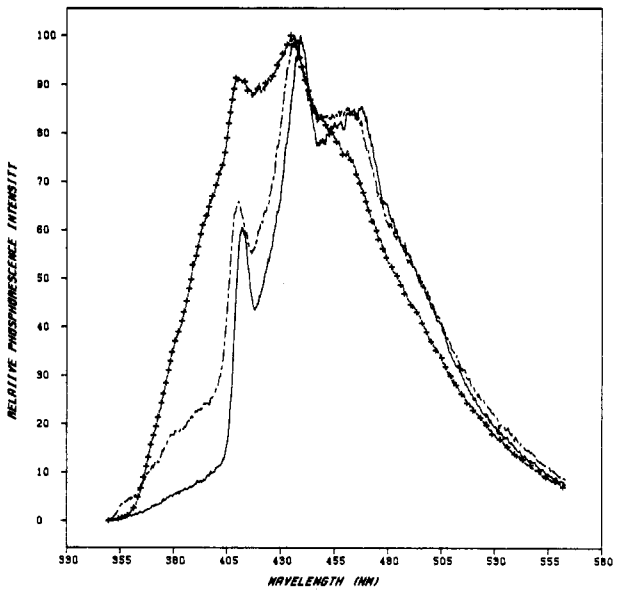

FIGURE 2: Phosphorescence at 77 K of native (solid line) and denatured HSA. (Broken line) Sample denatured with 8 M urea and heating to 85–90 °C for 1 h. (Crosses) Sample denatured with 8 M urea and heating to 100 °C for 1 h. The spectra have been normalized to the same maximum intensity; actual relative intensity was 1.0 (native):2.0 (denatured at 85–90 °C):3.4 (denatured at 100 °C).

As indicated by the data in Table I and Figures 1 and 2, the phosphorescence and ODMR of the "denatured" protein depend on the severity of heating during the denaturation. A sample subjected to heating at 85–90 °C for 1 h suffered less of a phosphorescence and ODMR shift than one that was kept at 100 °C for the same period. Also the apparent broadening was less for the sample that was given milder treatment. Interestingly, the phosphorescence and ODMR of the sample exposed to 100 °C became very similar to the spectra obtained from the sample exposed to the lower temperatures, after the former sample was stored at room temperature for 1 week, suggesting partial renaturation under these conditions.

The ODMR line width is seen to increase upon denaturation (Figure 1), as has been observed with several other proteins,

although ODMR line narrowing may occur in some cases (Ross et al., 1980). One expects that in a protein with a single tryptophan such as HSA, the degree of heterogeneity of environment as sensed by the ODMR will increase with denaturation, and this appears to be the case here. The apparent high-frequency shoulder in the denatured spectra is reproducible and probably reflects the heterogeneity of conformations in the denatured protein. Similar structured spectra have been observed in the denaturation of lysozyme (Rousslang et al., 1978).

While the phosphorescence appears to broaden with denaturation, it is very difficult to ascribe this broadening to the tryptophan emission alone, since tyrosine phosphorescence makes a dominant contribution to the denatured spectrum and greatly distorts the apparent phosphorescence line width of the tryptophan. It is known that under these conditions of excitation ($\lambda < 300$ nm) tyrosine contributes to the phosphorescence of HSA (Longworth, 1968). This contribution is evident in the emission shoulder below 400 nm (Figure 2). Moreover, denaturation often greatly enhances the phosphorescence yield of tyrosine residues, due to a "turning off" of quenching interactions that were in effect in the native protein (Longworth, 1961, 1968; Maki & Zuclich, 1975). In HSA, which contains 18 tyrosines, this enhancement is appreciable.

We note that the *D–E* line width we obtain for native HSA is at the upper range of values obtained for other buried tryptophans. One explanation for this somewhat large ODMR line width could be the larger optical bandwidth of our excitation system, as compared to other workers. For example, Ross et al. (1977) excite their samples through a monochromator with either a 1.5- or 3-nm band-pass and observe phosphorescence using a 3-nm band-pass. von Schütz et al. (1974) also employ a monochromator for excitation. By contrast, although our phosphorescence detection system has a bandwidth (~5 nm) that is roughly comparable to those of other workers, we excite through filters with a band-pass of about 110 nm.

It is certainly conceivable that the broadness of our excitation band-pass could lead to a broadening of the ODMR lines. In regard to this point and in order to calibrate our measurements against those of other workers, we examined the phosphorescence and ODMR of tryptophan and lysozyme. Tryptophan itself is obviously solvent exposed, and we find values (Table I) for the phosphorescence 0,0 and *D–E* frequency that are within the ranges given above for solvent-exposed tryptophan residues. The ODMR line width is also relatively large. For comparison, Zuclich et al. (1973) find values of 406.1 nm, 1.736 GHz, and 165 MHz for the phosphorescence 0,0, *D–E* frequency, and line width, respectively, for free tryptophan. By contrast, most of the phosphorescence of native lysozyme is due to a buried tryptophan (Rousslang et al., 1979), and the phosphorescence and ODMR parameters given in Table I are consistent with the trends mentioned earlier. For comparison, Hershberger et al. (1980) list 415 nm, 1.54 GHz, and 80 MHz for the 0,0 wavelength, *D–E* frequency, and line width, respectively, while Ross et al. (1980) report 413.8 nm, 1.571 GHz, and 83 MHz for these parameters. Thus, our results for these samples agree fairly well with those of other groups, and in particular, the ODMR line widths that we observe are not significantly larger. Of course it should be realized that the degree of heterogeneity in the excited triplet properties of tryptophan varies strongly with the protein studied, and narrower ODMR lines might well be obtained in our case with narrow-band excitation of HSA.

The broad ODMR line width, which we see for HSA, may simply reflect a greater heterogeneity in environment in the native protein than is usually obtained with buried tryptophans. Large ODMR line widths for solvent-exposed tryptophans can be assumed to result from heterogeneity in the geometry of solvation by polar solvents (Hershberger et al., 1980). By contrast, buried tryptophans are expected to see a more homogeneous environment. However, the temperature dependence of the quenching of HSA fluorescence by acrylamide suggests that HSA is a very loosely organized protein, containing poorly packed domains with many holes for smaller molecules (Eftink & Ghiron, 1977). This characterization is also consistent with the known difficulty in crystallizing this protein. Thus, we may conclude that the observed tryptophan line width in HSA is merely a result of a large heterogeneity of conformations of the frozen protein.

Effects of Anion Binding of Phosphorescence and ODMR of Tryptophan in HSA. It is well-known that serum albumins bind a wide variety of molecular species. One of the physiological functions of the albumin molecule is that of binding and transport of fatty acids, lecithins, bilirubin, and many hydrophobic drugs. In this work we investigated the effects of binding of chloride, bromide, iodide, and acetate, added in the form of sodium salts, on the phosphorescence and ODMR of the tryptophan residue in HSA. As mentioned earlier, we expected to see effects due either to the direct interaction of the anions with the tryptophan chromophore by means of the external heavy atom effect or to conformational changes in the protein as induced by the binding.

As noted above, the phosphorescence emission spectrum of native HSA, when excited through the $NiSO_4$ + 7-54 filter, contains a substantial contribution from tyrosine emission. Tyrosine emits to the blue of tryptophan and is the major source of the emission at wavelengths less than 400 nm (Figure 2). This was further verified by measuring the phosphorescence decay at 370 nm; a 2.2 s long component was seen, which is within the range of decay times observed from tyrosine residues in proteins (Maki & Zuclich, 1975). By contrast, the major component of the phosphorescence at 412 nm has a lifetime of 5.8 s and is due to tryptophan. Lifetimes of 5.8 and 6.56 s for free tryptophan in aqueous media have been reported by Nag-Chaudhuri & Augenstein (1964) and Longworth (1971), respectively. A lifetime of 6.7 s was found at 77 K in 50% aqueous ethylene glycol (King & Miller, 1976). *Protein* phosphorescence often decays as a single exponential at 77 K, and lifetimes are usually within the range 5.5–6.5 s (Maki & Zuclich, 1975). The somewhat shorter lifetimes observed for tryptophan in proteins are thought to be due at least in part to external heavy atom interactions with sulfur-containing groups in the protein (King & Miller, 1976).

To simplify the analysis of the phosphorescence decays in this study, we sought to minimize the excitation of tyrosine phosphorescence by passing the exciting light through the BzNiCo + 7-54 filter (see Materials and Methods). This filter cuts off below 300 nm, and thus excites the red edge of the indole chromophore absorption, in a region where tyrosine absorbs very weakly (Wetlaufer, 1962). Excitation of tyrosine is thus greatly reduced (Hicks et al., 1978; Longworth, 1981; Purkey & Galley, 1970).

Unfortunately, use of this filter reduces the excitation of tryptophan also, and the emission spectrum is now more susceptible to phosphorescent impurities in the solvent. Such an impurity was present in the ethylene glycol that we used. Its emission, which peaked around 465 nm, significantly reduced the resolution of the tryptophan peaks in the phos-

Table II: Phosphorescence Decay Times of HSA Tryptophan[a]

salt concn	wavelength monitored (nm) 412	438	460
nothing added	5.75 (0.76)	5.69 (0.65)	5.34 (0.54)
0.006 M NaCl	5.72 (0.76)	5.69 (0.63)	5.46 (0.52)
0.050 M NaCl	5.68 (0.75)	5.62 (0.60)	5.38 (0.49)
0.50 M NaCl	5.63 (0.75)	5.61 (0.62)	5.36 (0.50)
0.006 M NaBr	5.62 (0.71)	5.56 (0.56)	5.23 (0.44)
0.022 M NaBr	5.45 (0.69)	5.37 (0.53)	5.04 (0.42)
0.50 M NaBr	4.63 (0.64)	4.54 (0.51)	4.21 (0.40)
0.006 M NaI	5.29 (0.59)	5.27 (0.49)	4.90 (0.39)
0.50 M NaI	3.17 (0.36)	3.05 (0.34)	2.69 (0.31)
0.006 M NaOAc	5.74 (0.75)	5.67 (0.63)	5.46 (0.51)
0.0625 M NaOAc	5.71 (0.74)	5.63 (0.62)	5.41 (0.51)
0.50 M NaOAc	5.65 (0.74)	5.63 (0.61)	5.36 (0.48)
0.006 M NaI + 0.50 M NaOAc	5.35 (0.62)	5.24 (0.49)	4.92 (0.39)

[a] Decay times are in seconds; the fraction of initial intensity due to the long-lived component is in parentheses alongside each lifetime.

phorescence spectrum when excited under these conditions (not shown). Efforts to remove the impurity by vacuum distillation were unsuccessful. Fortunately, however, the lifetime of the phosphorescence from this impurity (0.4–0.5 s) was well separated from the lifetime of the tryptophan so that the lifetime of the HSA tryptophan could be determined by biexponential fitting of the phosphorescence decay, as described above. Thus, we report only the long component of the decay in Table II, which contains the observed lifetimes monitored at three wavelengths as a function of anion binding.

It was found that the lifetime and preexponential factors obtained with freshly prepared mixtures of HSA solutions and various salt solutions showed a large amount of scatter and nonreproducibility. However, if the mixed solutions were stored for 1 week in a refrigerator (~4 °C) and then frozen for phosphorescence decay measurements, the reproducibility improved significantly. The limits of error for our lifetimes are about 0.1 s.

We now discuss the lifetime changes observed with the addition of various anions (Table II). Basically there are two qualitative trends. The lifetime shortens significantly with the addition of bromide and iodide, with the amount of the shortening increasing with added ion. By contrast, the lifetime remains nearly constant with added chloride and acetate. On the basis of the arguments presented below, we conclude that the lifetime shortening seen with bromide and iodide is simply a result of a direct interaction with the tryptophan chromophore by means of the external heavy atom effect.

The binding constants for all of these anions have been determined by nuclear magnetic resonance techniques by Norne et al. (1975). These workers found two classes of binding sites with greatly different binding constants. One can easily calculate the number of bound anions per HSA molecule, assuming that the binding sites are independent, using the equation $1/\bar{\nu} = 1/(nKc_f) + 1/n$, where n is the maximum number of anions which can be accomodated at the binding site, c_f is the concentration of unbound anion, K is the equilibrium binding constant for a given anion, and $\bar{\nu}$ is the average number of anions bound per protein molecule (Lindman et al., 1972). It has been found that n_1 for the site with the higher binding constant (the "high-affinity" site) is between 8 and 10 while n_2 for the low-affinity site is about 50, for detergents binding to HSA (Lindman et al., 1972). One may apply the above equation to the two binding sites, using the K values measured by Norne et al. (1975) for the various anions. The values obtained when the anion concentrations are 0.006 and 0.50 M are listed in Table III. We note that, although n may be different for the anions studied here than for the detergents investigated by Lindman et al. (1972), the fractional occupation numbers ($\bar{\nu}/n$) should be the same, assuming the validity of the above equation. We cite these values at this point merely to point out that a good number of anions are indeed bound to the protein at these anion concentrations.

Table III: Fractional Occupancy of Anion Binding Sites in HSA[a]

	$\bar{\nu}/n$, high-affinity site 0.006 M	0.50 M	$\bar{\nu}/n$, low-affinity site 0.006 M	0.50 M
Cl^-	0.375–0.706 (3.4–6.4)	0.980–0.995 (8.8–9.0)	0.00567 (0.28)	0.322 (16)
Br^-	0.580–0.847 (5.2–7.6)	0.991–0.998 (8.9–9.0)	0.0261 (1.3)	0.691 (35)
I^-	0.824–0.949 (7.4–8.5)	0.997–0.999 (9.0)	0.0539 (2.7)	0.826 (41)
OAc^-	0.324–0.658 (2.9–5.9)	0.976–0.994 (8.8–8.9)	0.00960 (0.48)	0.447 (22)

[a] $\bar{\nu}$ values (in parentheses) are calculated in each case above by assuming the number of high-affinity sites is 9 and the number of low-affinity sites is 50. The range of values given for the high-affinity sites reflects the uncertainty in K as determined by Norne et al. (1975).

One could argue that the observed lifetime changes with bromide and iodide might be due to conformational changes in the protein, which alter the external heavy atom effects with sulfur-containing residues within the protein. Since, however, the lifetime is remarkably constant upon addition of acetate and chloride, this factor does not appear to be very important.

Furthermore, on the basis of the values of $\bar{\nu}/n$ presented in Table III, we can say that binding to the high-affinity sites does not seem to account for very much of the lifetime change. For example, for iodide, increasing the concentration from 0.006 to 0.50 M produces a 40% shortening of the lifetime. The fractional occupation of the high-affinity sites is concomitantly increased by only a factor of 1.2, however. By contrast, the occupancy of the low-affinity site increases by a factor of 15.

In order to investigate the possibility that binding of anionic ligands at high (0.50 M) concentration might lead to a large change in the tryptophan lifetime by promoting a conformational change in the protein, which results in moving the tryptophan residue closer to the anions bound at the high-affinity site, we did the following experiment. The lifetime of HSA with 0.006 M iodide was compared to that for HSA with 0.006 M iodide and 0.50 M acetate. Since iodide binds nearly 10 times more tightly to the high-affinity site than does acetate, we may assume that iodide would remain bound to the high-affinity site after addition of the acetate. If such a conformational change as described above were in fact occurring one would then expect to see a decrease in the tryptophan lifetime in the sample containing both types of anions, compared to the sample containing only the iodide. By contrast, the lifetime was practically unaffected by the addition of acetate (Table II, last entry).

From the above, one may infer that the observed changes in tryptophan triplet lifetime in HSA are due primarily to binding at the low-affinity site. Furthermore, since acetate ion, even at 0.50 M concentration, has no effect upon the lifetime, this effect must be due to the binding of heavy atoms to the low-affinity site and is not explainable as a "salt effect" which, for example, changes the protein conformation in such a way as to increase intramolecular quenching by sulfur-containing amino acids in the protein.

Thus, one concludes that the ions that bind to the high-affinity sites must be too far from the tryptophan residue to exert a heavy atom effect. It is in fact known from singlet energy transfer experiments that the first four fatty acid anions that bind to HSA are located at distances between 19 and 24 Å from the tryptophan (Berde et al., 1979). The external heavy atom effect is known to be a short-range effect (McGlynn et al., 1969; Giachino & Kearns, 1970) and may not be operative beyond the distance at which charge clouds on two chromophores overlap, e.g., 13–15 Å.

Finally, we note that the added anions did not produce a measureable change in solvent exposure of the tryptophan residue, at least in terms of the ODMR model referred to in the above sections. The wavelength of the phosphorescence 0,0 maximum was constant to within 1 nm as ions were added. Also, rough ODMR measurements made with fast (200 MHz/s) sweeps in both directions showed that D–E for the 0.006 and 0.50 M chloride and iodide samples were all within 30 MHz of the frequency measured for HSA with no added anions.

Summary and Conclusions

The wavelength of the phosphorescence 0,0 maximum and the D–E zero-field splitting and ODMR line width of the triplet state of the lone tryptophan in human serum albumin place this residue in the class of tryptophan residues that are buried in hydrophobic regions of proteins, and this is consistent with the conclusions of fluorescence quenching experiments with hydrophobic quenchers (Eftink & Ghiron, 1977; Eftink et al., 1977). Moreover the ODMR shifts and broadens on denaturation in a manner consistent with that observed by other workers (Ross et al., 1980) for several other proteins. Additional support for the general validity of the "ODMR model" of the groups of Kwiram (Rousslang et al., 1979) and Maki (Hershberger et al., 1980) is thus obtained.

The phosphorescence decay of HSA at 77 K was also studied as a function of added halide ions and acetate. It was found that bromide and iodide ions cause a noticeable shortening of the lifetime, but only at concentrations such that the low-affinity sites are at least half occupied. Chloride and acetate have no effect on the phosphorescence lifetime; this tends to exclude binding-induced conformational changes as a cause of the lifetime shortening. The effect of bromide and iodide is thus attributed to direct external heavy atom quenching interactions with the tryptophan. ODMR and phosphorescence wavelength measurements provide evidence that the degree of solvent exposure of the tryptophan does not change appreciably upon anion binding.

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