groove as suggested from the crystal structure of the unliganded protein. Hydrophobic interactions between the incoming bases and suitably placed aromatic rings appear to be an attractive way of stabilizing nucleotide–protein complexes. In the gene 5 protein complexes with the longer oligonucleotides, this interaction appears to become actual intercalation of the ring which probably also characterizes the DNA complexes. The crystal structure of ribonuclease T_1, specific for guanylic acid, shows the ring of Tyr-45 at the active center of this enzyme to be stacked over the six-membered ring of guanine at 3.5 Å (Heinemann & Saenger, 1982). Whether intercalation as observed in the gene 5 protein–nucleotide complexes forms a general feature of the binding of helix-destabilizing proteins to single-stranded DNA requires exploration of other examples by NMR techniques.

Acknowledgments

We thank Dr. Gary Brayer and Professor Alex McPherson for supplying us with their most recent fitting of the electron density map of gene 5 protein prior to publication.

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Heterogeneity of Albumin As Detected by Its Interactions with Deuteroporphyrin IX†

Gregory A. Moehring, Amy H. Chu, Leon Kurlansik, and Taffy J. Williams*

ABSTRACT: The interaction of deuteroporphyrin IX with human serum albumin was examined by absorption and fluorescence methods. Changes in the porphyrin fluorescence polarization were utilized in the determination of the association constant of deuteroporphyrin IX binding to albumin. The results were consistent with a model where human albumin exists in at least two conformers, with 37% having an association constant of 4.85×10^8 M^{-1} and 63% a value of 1.47 $\times 10^7$ M^{-1}. Analysis of the temperature dependence of binding yielded enthalpy terms found to be of opposite sign, high affinity having a value of $\Delta H = -4.77$ kcal/mol and the lower affinity having a value of $\Delta H = 2.70$ kcal/mol. The comparison of thermodynamic values, spectral shifts, and iodide quench curves suggests that the porphyrin binding site is hydrophobic in character and the bound porphyrin has limited access to at least some of the ions in solution.

In order to better understand the role of human serum albumin (HSA) in the in vivo clearance of porphyrins and the relationship of albumin to other serum proteins involved in the in vivo clearance of porphyrins, many studies have been reported that attempt to characterize the interactions between porphyrins and HSA (Muller-Eberhard & Morgan, 1975; Beaven et al., 1974; Lamola et al., 1981; Morgan et al., 1980; Reddi et al., 1981; Rosenfeld & Surgenor, 1950; Adams & Berman, 1980; Little & Neilands, 1960). Most of these investigations have utilized absorption and fluorescence spectroscopy measurements and the method of Halfman & Nishida

† From the Naval Medical Research Institute, Bethesda, Maryland 20814. *Received October 26, 1982; revised manuscript received March 4, 1983.* This research was supported by the Naval Medical Research and Development Command, Research Work Unit No. MF 58524013.1044.

(1972) for the determinations of the association constants (the method of Halfman and Nishida is useful for obtaining equilibrium constants when one cannot obtain the optical maxima normally used to calculate the free and bound ligand). Most investigators agree that their data are consistent with a model where HSA contains one high-affinity porphyrin binding site, with reported association constants ranging from 3.6×10^6 M^{-1} (Reddi et al., 1981) to 3.2×10^9 M^{-1} (Lamola et al., 1981), depending on the porphryin studied. However, because of the concentration ranges and methods of measurements (Muller-Eberhard & Morgan, 1975; Beaven et al., 1974; Lamola et al., 1981; Morgan et al., 1980; Reddi et al., 1981; Rosenfeld & Surgenor, 1950; Adams & Berman, 1980; Little & Neilands, 1960), it is doubtful whether previous investigators could have detected multiple affinities for the tight site had the albumin acted as a heterogeneous population.

The method of Halfman and Nishida, when used with optical techniques, requires the assumption that protein is homogeneous and/or at least behaves as a homogeneous species with respect to the measured values. Yet, albumin has been shown to be physically heterogeneous by several methods, as described in a recent review (Foster, 1977). Some of the sources of heterogeneity may arise from acid and base transitions, variations in disulfide linkages, and polymerization of the monomeric units of the protein. Since previous porphyrin–albumin binding studies do not appear to have considered the possibilities of a heterogeneous protein, we have explored heterogeneity of the high-affinity porphyrin binding site as the reason for the broad range of reported values.

In this study, the changes in the deuteroporphyrin fluorescence properties were examined and used to characterize the binding of deuteroporphyrin to albumin. When the porphyrin was titrated with albumin, a maximal change in the flluorescence polarization could be obtained and then used to produce binding curves. The results were consistent with a model where human albumin exists in at least two stable conformers; one conformer has a primary porphyrin binding site with a 30-fold greater affinity for porphyrin than the primary binding site on the other conformer. Other characteristics of the porphyrin binding site have been examined to provide additional information about this site.

Materials and Methods

Materials. Human serum albumin, purified by affinity chromatography, and fraction V albumin were purchased from Calbiochem-Behring Corp. Fatty acid free albumin was purchased from Sigma Chemical Co., and pure monomer human albumin was obtained as a gift from Dr. D. Millar of the Naval Medical Research Institute, Bethesda, MD. Protein concentrations were determined from the absorbance of a solution at 279 nm = 3.517×10^4 M^{-1} cm^{-1} (Peters, 1975).

Deuteroporphyrin dimethyl ester was synthesized by the method of Caughey et al. (1966) and converted to the free acid (Smith, 1975). Purity was found to be greater than 99% as determined from TLC[1] in several solvent systems, NMR, HPLC, and several types of mass spectral analysis. Deuteroporphyrin concentrations were determined from absorbance measurements, E(398 nm) = 433 mM in 0.1 M HCl (Rimington, 1960).

Buffer (PBS), unless otherwise stated, was sodium phosphate (0.01 M) with sodium chloride (0.15 M), pH 7.4. The temperature for all experiments, unless stated otherwise, was 25 °C. All solutions were treated with Chelex-100 (Bio-Rad) to remove contaminating metals.

Methods. Absorption measurements were made on a Varian Cary-219 interfaced to a Digital PDP-11/34 computer for signal averaging and spectral accumulation. Fluorescence measurements were made on an SLM-4800A spectrofluorometer equipped with 9817 QA red-sensitive phototubes. All fluorescence spectra were corrected for lamp intensities and phototube responses. Polarization measurements were made in the T-format, so that the horizontal and vertical components of the emission could be observed simultaneously (Weber & Bablouzian, 1966). Fluorescence lifetimes were performed by the phase-modulation method with the polarizer oriented at the "magic angle", and glycogen-scatterer solution was used as a reference (Spencer & Weber, 1970). Since the time response of the photomultiplier is known to be dependent upon wavelength (Muller et al., 1965), lifetimes were also checked by varying the excitation wavelength and by using other fluorescent compounds, with known lifetimes and emissions closer to that of the porphyrin, as a reference rather than glycogen. All three methods produced the same lifetimes for deuteroporphyrin and the deuteroporphyrin–HSA complex.

Two independent methods were used to obtain binding curves: (1) a premixed solution of porphyrin and albumin was diluted into a cuvette, or (2) aliquots of stock solutions of ligand and protein were combined in the cuvette. Binding curves obtained by each of these methods provided the same result. Porphyrins are known to undergo photooxidation (Wasser & Fuhrhop, 1973) and to catalyze photooxidations of other compounds such as tryptophan (Rossi et al., 1981). Also, deuteroporphyrin may become absorbed to the walls of glass and plastic tubes and cuvettes. For these reasons, all solutions used for titrations were prepared fresh daily, and concentrations and spectral characteristics of the solutions were checked periodically. Each point of a titration curve represents a new sample, and any serial titration points were no more than four additions over 5–10 minutes. All intensities and/or polarization measurements were made in a stirred cuvette with each sample being exposed to light no longer than 15 min, during which time no detectable absorbance or fluorescence spectral changes were observed for the porphyrin–HSA complex.

The maximal polarization change was obtained by titration of a constant ligand concentration with protein. By use of the maxima, minima, and values of titration points, free ligand (L_F) and bound ligand (L_B) were obtained. Numerical values of the equilibrium constants and fractions of conformers (f_1, α, f_2) of albumin were obtained from a computer fit of eq 1:

$$r = \frac{L_B}{P_T} = \frac{K_1 L_F}{1 + K_1 L_F} f_1 + \frac{K_2 L_F}{1 + K_2 L_F}(1 - f_1) \qquad (1)$$

where K_1 and K_2 represent independent association constants for two conformers of a single protein. Equation 1 was derived by assuming the original protein sample behaves as a two-component system, both independent and stable, with respect to binding ligand.

Quench constants for iodide quenching of a deuteroporphyrin fluorescence were obtained from fluorescence changes with respect to iodide concentration. Both static and dynamic quench was observed in nonstirred solutions with prolonged light exposure. Experiments later proved that the contribution of the static quenching could be eliminated by stirring the solutions and minimizing light exposures to less than 10 min per curve for such quench measurements. Dynamic quench could then be determined directly by using a

[1] Abbreviations: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

Table I: Absorption Characteristics of Deuteroporphyrin IX

	peak 1	peak 2	peak 3	peak 4	peak 5
buffer[a]	390[b]	497	532	555	606
	(13.6)[c]	(0.89)	(0.63)	(0.54)	(0.28)
ethanol	393	494	526	563	618
	(17.4)	(1.4)	(0.83)	(0.64)	(0.40)
octanol	398	496	526	567	620
	(18.3)	(1.6)	(0.97)	(0.82)	(0.52)
toluene	400	497	528	566	621
	(15.2)	(1.4)	(0.80)	(0.64)	(0.42)
pyridine	400	496	528	566	620
	(18.4)	(1.5)	(0.82)	(0.62)	(0.39)
complex	402	499	533	569	618
	(14.4)	(1.2)	(0.73)	(0.56)	(0.33)

[a] Sodium phosphate (0.01 M) and NaCl (0.15 M), pH 7.4. [b] Wavelength in nanometers. [c] Molar absorptivity $\times 10^{-4}$.

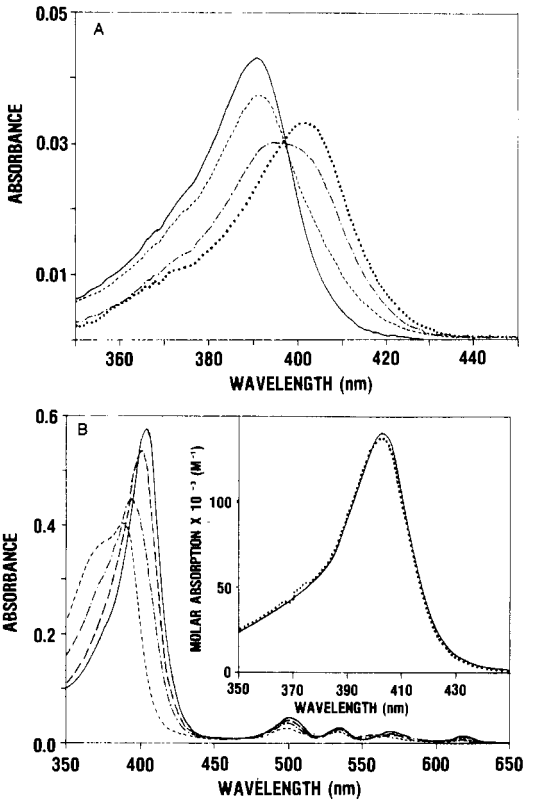

FIGURE 1: (A) Absorbance spectra of 50 nM deuteroporphyrin IX, in PBS, titrated with HSA: (—) 0, (---) 20, (- - -) 40, and (···) 150 nM HSA. (B) Absorbance spectra of 40 μM deuteroporphyrin IX, in PBS, titrated with HSA: (---) 0, (- - -) 1, (---) 2, and (—) 25 μM HSA. The inset is the molar absorptivity for the Soret band of complex at (···) 50 nM porphyrin/150 nM HSA and at (—) 4 μM porphyrin/25 μM HSA.

modified form of the Stern–Volmer relationship (Vaughan & Weber, 1970)

$$\frac{F_0}{F} = 1 + K[I] \qquad (2)$$

where F is the fluorescence intensity at different iodide concentrations and F_0 is the fluorescence in the absence of quencher. The quench constant may then be converted to appropriate units by multiplying K times the rate constant for fluorescence decay of the porphyrins without iodide.

Results

Absorption Spectral Characteristics. Comparisons of absorption spectral characteristics for deuteroporphyrin in various solvents (Table I) demonstrate spectral shifts toward the red as the solvent becomes more hydrophobic. Titration of ligand with HSA also produces spectral shifts toward the red (Figure 1A and Table I), similar to those obtained on solvent changes from water to toluene or pyridine, while the area under the absorption curve remains constant.

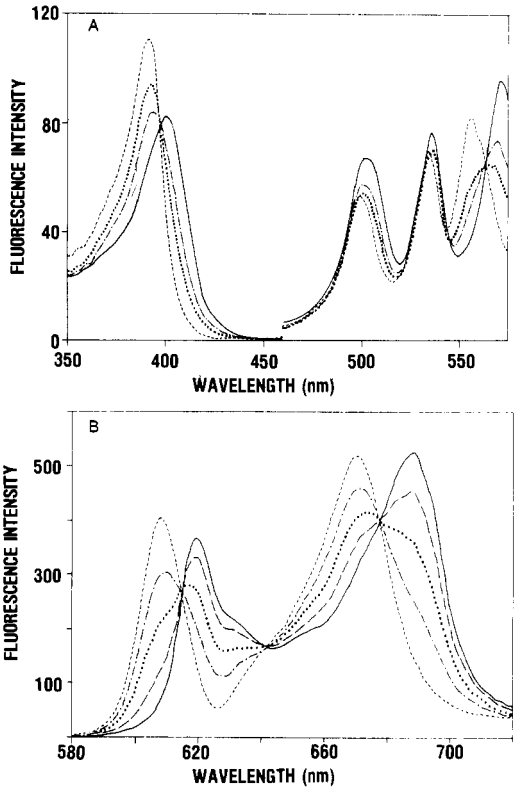

FIGURE 2: (A) Fluorescence excitation spectra of 50 nM deuteroporphyrin IX, in PBS, titrated with HSA: (- - -) 0 nM HSA (···) 40 nM HSA, (---) 80 nM HSA, and (—) 1 μM HSA. (B) Fluorescence emission spectra of 50 nM deuteroporphyrin IX, in PBS, titrated with HSA: (- - -) 0 nM HSA, (---) 23 nM HSA, (···) 47 nM HSA, (– –) 58 nM HSA, and (—) 1 μM HSA.

Deuteroporphyrin is known to form dimer and larger aggregates at concentrations above 4 μM (White, 1978). Such aggregates are apparent in the absorption spectral properties of the ligand in Figure 1B by the lower intensity and a shoulder on the Soret band. Addition of HSA to aggregated deuteroporphyrin changes the spectral characteristics from those of the aggregated form to that of the monomer. At concentrations where porphyrin is essentially all monomer (below 100 nm), an isosbestic point at 397 nm is observed when porphyrin is titrated with HSA. Comparisons of the molar absorptivities of the Soret bands demonstrated that the complex formed at low ligand concentrations was the same as that obtained at higher concentrations of ligand and protein (Figure 1B). This suggests that the monomeric ligand is bound to HSA at both high and low ligand concentrations.

Fluorescent Properties. Complexation of deuteroporphyrin with HSA was paralleled by red spectral shifts in both the excitation and emission spectra (Figure 2). The most dramatic changes in wavelength for the excitation spectra of deuteroporphyrin (Figure 2A) occurred in the Soret region (392 nm) and the excitation peak at 556 nm, showing shifts of 10 and 14 nm, respectively, matching those peaks and shifts obtained by absorption methods. For the emission spectra (Figure 2B), both peaks were found to exhibit dramatic spectral shifts. The band at 607 nm shifted to 619 nm upon the complexation, and the more intense band at 670 nm shifted to 689 nm. While spectral characteristics were found to be

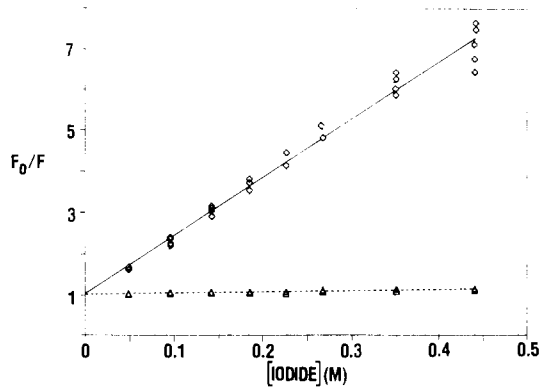

FIGURE 3: Stern–Volmer quenching plots for deuteroporphyrin IX. F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. Excitation wavelengths were 392 nm for 500 nM free porphyrin (◇) and 402 nm for complex 500 nM porphyrin–25 μM HSA (△). A 550-nm cutoff filter was used for each curve. The line through the points represents the least-squares fit of the data.

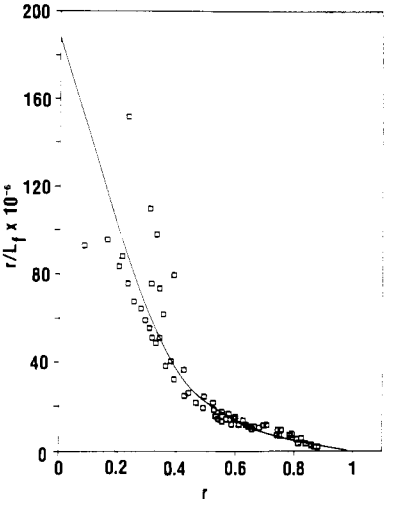

FIGURE 4: Scatchard plot for the binding of deuteroporphyrin IX to HSA as determined by fluorescence polarization measurements. The solid line was obtained from a computer fit of the data by using eq 1.

altered, fluorescence lifetimes were much less sensitive to such changes.

When deuteroporphyrin was titrated with HSA, two isosbestic points were clearly observed in the excitation spectra (397 and 563 nm). Three isoemissive points were observed in the emission spectra (615, 642, and 680 nm). As evidenced by the identical isosbestic points in the absorbance spectra (Figure 1), both free ligand and the complex have identical extinction coefficients at 397 nm. Relative values of the quantum yield were calculated from the areas obtained upon integration of the emission spectra acquired while exciting at the 397-nm isosbestic wavelength. A calculated 5% drop in the quantum yield was found to occur on complexation of deuteroporphyrin with HSA. This value is in excellent agreement with a 6% drop in the quantum yield calculated from independently determined phase-modulation lifetimes of the free ligand (15.9 ± 0.6 ns) and the ligand–protein complex at a variety of ratios (15.0 ± 0.7 ns). In each of these measurements, the fluorescence lifetime was found to be homogeneous, i.e., only a single lifetime.

Fluorescence Quench by Iodide. The iodide quenching of deuteroporphyrin (Figure 3) was analyzed by eq 2. Both the complex and free ligand were found to be quenched, but the quenching of the free ligand was greater than the quenching observed for the complex. Quench constants determined by analysis of the data provided $K_{free} = 13.18 \pm 0.54$ M^{-1} and $K_{complex} = 0.17 \pm 0.03$ M^{-1}. By use of the independently measured values of the fluorescence lifetimes, quenching rate constants were calculated to be $k_{free} = 8.24 \times 10^8$ M^{-1} s^{-1} and $k_{complex} = 1.27 \times 10^7$ M^{-1} at pH 7.4.

Association Constants. The maximal change in the polarization (ΔP_m) was determined at an excitation wavelength of 397 nm (isobestic point) and with 550-nm cutoff filters on the emission sides (total emission). A single plateau value of $\Delta P_m = 0.075 \pm 0.005$ was obtained by either titrating ligand with protein or by successively increasing the concentrations of premixed solutions containing both species at known constant ratios. An identical maximal value was obtained if a monochromator (615 nm) was used to select the emission wavelength. This change in the polarization reflects the change in the rate of rotation of the free ligand to that of a ligand bound to protein.

The polarization for equilibrium mixtures of ligand and protein reflects the quantities of bound and free ligand. By use of $\Delta P_m = 0.075$ value and equilibrium polarization values

Table II: Temperature Dependence of the Association of Deuteroporphyrin IX and Human Serum Albumin

temp (°C)	$K_1 \times 10^{-8}$ (M^{-1})	$K_2 \times 10^{-7}$ (M^{-1})	f_1 [a]
37	3.27 ± 0.95	1.84 ± 0.37	0.34 ± 0.05
25	4.85 ± 1.0	1.47 ± 0.36	0.37 ± 0.04
16	5.62 ± 0.89	1.28 ± 0.30	0.30 ± 0.04
6.8	7.79 ± 1.2	1.15 ± 0.14	0.29 ± 0.04

ΔH_1 [b] = −4.77 ± 0.30 kcal/mol $\Delta H_2 = 2.70 \pm 0.18$ kcal/mol
$\Delta S_1 = 23.63 \pm 1.0$ eu $\Delta S_2 = 41.91 \pm 0.62$ eu

[a] f_1 is the fractional population of the high-affinity form.
[b] Thermodynamic values were obtained by a linear least-squares fit of the data in the form of $R \ln K$ vs. T^{-1}. The standard deviations for the thermodynamic values reflect the analysis of the equilibrium constants of pooled data. However, the 95% confidence regions about ΔH and ΔS may be larger. This is so in part because only four points are used in the regression and in part because of the large covariance between ΔH and ΔS. Estimates based on extremes of a 95% confidence ellipse suggest that 95% confidence limits may be as much as 6 times the standard deviation.

obtained from titrations of constant ligand, constant protein, and dilution of equimolar mixtures of ligand and protein, a Scatchard plot was constructed (Figure 4). The biphasic nature of this plot and intercepts suggests one binding site and multiple association constants, i.e., a heterogeneous population of protein capable of binding only one ligand with two different affinities. The values obtained from the fit of eq 1 (Table II) to the raw data obtained at 25 °C were consistent with a heterogeneous model containing at least two major binding populations of protein, with 37% of the protein having a 33-fold greater affinity for the porphyrin. Human serum albumin purified by affinity chromatography, defatted HSA, and pure monomer HSA all provided the same results.

The demonstration of heterogeneity in the HSA suggested a possibility of more than a single polarization maximum. The two methods used in the determination of ΔP_m were designed to isolate a polarization maximum for the ligand bound only in the high-affinity site and compare that value to the case when the porphyrin was bound in both types of sites on HSA. In one type of experiment, the concentration of ligand (10–500 nM) was kept constant, while the concentration of HSA was increased incrementally to a final value of 15 μM. Alternatively, the ratio of HSA/ligand (2, 5, and 10) was allowed to

remain constant and the concentration of both species increased until the final HSA concentration was 4, 10, and 20 μM, respectively. The same polarization maximum was obtained when the concentration reached levels where all of the ligand was bound. This result is not unreasonable considering the fluorescence lifetime of the bound porphyrin is homogeneous and the rotational properties of the two forms of HSA are most likely the same. While the individual populations of HSA may have different ΔP_m values, no evidence could be obtained to support this idea, and the binding constants and fractional populations were not significantly altered by a curve-fit model containing multiple values of ΔP_m.

Mathematical models were developed based on the work of Weber (1952), and the existence of secondary binding (two or more binding sites on one protein) was explored, as a possible explanation of the nonlinear Scatchard plot. Such models were considered to produce siignificantly poorer fits to the data than those obtained with eq 1 for the following reasons. The sum of errors squared was found to be 5-fold larger for the secondary binding site model. Further, the errors for the fit of this model were of a clear, systematic character as opposed to the smaller random errors for the fit of the heterogeneous protein model to the data. Finally, while reasonable standard deviations were obtained for both binding constants in the heterogeneous protein model, the standard deviations obtained for the estimate of the weaker affinity site in the secondary binding site model were large enough to make the constant indistinguishable from zero. Secondary binding was detectable at higher concentrations of ligand and protein but was found to be negligible under the conditions employed to observe the primary binding sites. This secondary association constant was estimated to be less than 5×10^5 M^{-1}, at least 30-fold lower than that of the weaker primary site.

The two populations of protein were tested for their ability to interconvert over time. If the two forms were able to interconvert, the presence of equimolar ligand should increase the high affinity from a 37% contribution at equilibrium in the absence of ligand to nearly 100%. Equimolar mixtures incubated at 4 or 25 °C from 1 to 4 days were found to produce no change in the fractions of the high- and low-affinity forms of the protein. This result suggests that the two forms of HSA do not exist in a state of interconvertible equilibrium but are independent and stable forms of the protein.

Since the pH 7.4 used in these studies lies between both the N–F and N–B transitions (Foster, 1977), binding curves at pH 6.5 and pH 8.5 were obtained. At both pH values the fractions of the two forms were the same as at pH 7.4, while the association constants for both forms were found to be lower. The inability to affect the quantity of the population of each species by pH suggests that the heterogeneity is not the reported acid or base transitions.

Thermodynamic Constants. The two association constants obtained for the binding of deuteroporphyrin to HSA were examined as a function of temperature. Plots of $R \ln K$ vs. T^{-1} (Figure 5) and linear regression analysis provided values of the enthalpy and entropy for each form (Table II). Both binding sites were found to have equally high positive entropy contributions. In contrast, the enthalpy values are lower and of opposing sign, the higher affinity site having a value of –4.77 kcal/mol and the lower affinity site having a positive value of 2.70 kcal/mol. The ratio of the higher and lower affinity populations of protein were found to be essentially temperature independent.

Discussion

Porphyrin fluorescence intensity measurements are more

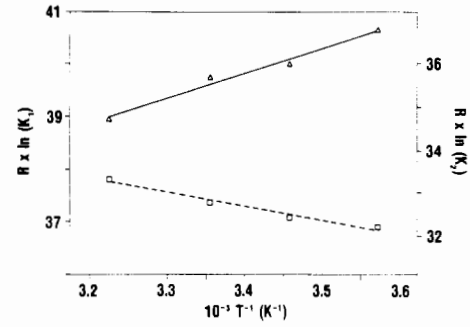

FIGURE 5: Effect of temperature on the binding of deuteroporphyrin IX to HSA. Equilibrium constants were determined in PBS buffer at temperatures indicated. The lines through the high-affinity constants, (△) and low-affinity constants, (□) represent the least-squares fits of the data. The left axis refers to the high-affinity site, and the right axis refers to the low affinity site.

sensitive than HSA fluorescence intensity in characterizing the interactions between these two species. This is a result of the 200-nm separation between the porphyrin excitation and porphyrin emission wavelengths, which helps eliminate background due to scatter and/or Raman bands, as well as the fact that the fluorescence intensity of the porphyrin is not quenched on complexation with HSA. In this study, concentration levels as low as 0.5 nM porphyrin were detected. Previously reported binding studies, performed by monitoring porphyrin fluorescence on protoporphyrin IX (Lamola et al., 1981), revealed a value in the 10^9 M^{-1} range for the association constant, while a value in the 10^7 M^{-1} range was reported for the same ligand in a prior study (Reddi et al., 1981). The methods used and the concentration ranges employed in these two studies would have made it difficult to detect the presence of two constants for saturation of the primary site(s), especially when the constants were separated by 100-fold. Also, the ligand which was used, protoporphyrin IX, is known to photooxidize to hematoporphyrin (Cox & Whitten, 1982), adding additional complications to such measurements.

Initial attempts to obtain a binding constant for deuteroporphyrin using fluorescence intensity measurements were complicated by the inability to obtain the maximal change in the fluorescence intensity. Analyses of intensity measurements (unpublished results) were suggestive of multiple binding constants on the HSA before saturation of the primary site. For this reason, polarization measurements used in this study were found to have an added advantage over intensity measurements, i.e., that polarization is proportional to the free and bound ligand through the entire concentration range employed. Equations may be derived from basic work (Weber, 1952; Deranleau et al., 1980) that allow for calculation of free and bound forms of ligand and contributions to the polarization for multiple site models. Characterizing the binding of deuteroporphyrin to HSA through a large concentration range, 0.5–1000 nM, was found to produce data that are best explained by a model where HSA may be represented by at least two conformers with a 33-fold difference in affinity for deuteroporphyrin.

In this study, deuteroporphyrin IX was selected as a ligand rather than protoporhyrin IX because of its greater stability in the presence of oxygen and light. The identical values of the relative quantum yields obtained from lifetime measurements and spectral integration methods indicate that nonfluorescent porphyrin–protein complexes are not formed. The optical isosbestic points observed on titrations of ligand with protein are suggestive of only two optically distinguishable forms, free and bound. Other optical forms were not de-

tectable until experimental concentrations of ligand are sufficient to cause aggregation.

Both absorbance and fluorescence studies of the binding of the deuteroporphyrin to HSA yielded changes similar to those obtained when free ligand is transferred from aqueous media to organic media. The analysis of the temperature dependence of the two binding constants yielded a large positive value for the entropy, suggestive of strong hydrophobic interactions between the ligand and protein (Tanford, 1973). These results, together with the reduced rate of fluorescence quenching of the complex by iodide ion, are suggestive of a hydrophobic binding site on the protein with limited access to some aqueous ions.

The ability to detect the HSA heterogeneity with this ligand comes primarily from the analysis of binding curves. Experiments performed with HSA monomer–dimer, HSA that contains fatty acid, or HSA from alternate sources all yielded the same result, a fact that suggests that protein impurity is not the source of the heterogeneity. The true nature of the heterogeneity is unknown at this time; however, numerous types of heterogeneity have been documented in the literature (Foster, 1977). On the basis of the known types of microheterogeneity, several experiments were conducted. Failure of pH to affect the ratio of the "two" populations ruled out the N–F and N–B transitions. Temperature or prolonged incubation of porphyrin with HSA did not affect the ratios of the forms, which suggests that these protein forms are not in an equilibrium that may be altered by any of the experimental conditions varied in this study.

Registry No. Deuteroporphyrin IX, 448-65-7.

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