Binding of the Triton X Series of Nonionic Surfactants to Bovine Serum Albumin†

Wayne W. Sukow,‡ Howard E. Sandberg,§ Edwin A. Lewis,¶ Delbert J. Eatough, and Lee D. Hansen*

ABSTRACT: The binding of a series of Triton X nonionic surfactants (NIS) to bovine serum albumin (BSA) has been studied by equilibrium dialysis and titration calorimetry. At pH 7.0, Triton X molecules bind to two classes of sites, the first 2 molecules binding with positive cooperativity to high-affinity sites followed by the binding of ~15 additional molecules to lower affinity, thermodynamically identical, and independent sites. The strength of the binding decreases as the number of oxyethylene units is increased in the surfactants Triton X-114, X-100, X-102, and X-165. Calorimetric measurements show the enthalpy change for the NIS–BSA interaction to be small and endothermic. Increasing the hydrophilic oxyethylene chain length results in a more endothermic enthalpy change and a smaller association constant. Electron spin resonance studies of Triton X binding to BSA, covalently spin-labeled with *N*-(2,2,6,6-tetramethylpiperidinyl-1-oxy)maleimide, indicated that the protein conformation in the vicinity of the labeled sulfhydryl was insensitive to NIS binding from dilute monomeric solutions. Calorimetric experiments near the critical micelle concentration indicate, however, that the protein probably undergoes a conformational change associated with the population of the lower affinity NIS binding sites.

Previous studies have shown that membrane-bound proteins as well as globular proteins bind nonionic surfactant molecules (Srinivasan et al., 1972; Bachorik & Dietrich, 1972; Heller & Villemez, 1972; Bradford et al., 1964; Eldefrawi et al., 1972; Cuatrecasas, 1972; Takeda & Hizukuri, 1972; Dowben & Koehler, 1961; Koehler & Dowben, 1961; Helenius & Simons, 1972). Generally the earlier studies have been done at concentrations of the surfactant above the critical micelle concentration (cmc). However, Helenius & Soderland (1973) and Makino et al. (1973) have observed the binding of nonionic surfactants (NIS) from monomeric solutions to membrane and serum proteins.

In this communication we report the binding behavior of four members from the Triton X series of nonionic surfactants (see Figure 1) to bovine serum albumin (BSA) at pH 7.0. The experimental binding isotherms obtained from equilibrium dialysis were fitted by a nonlinear least-squares computer program for a stepwise equilibrium model (Fletcher et al., 1970). In addition, calorimetric titrations provided a direct measure of the value of ΔH for the binding and served as a test of the proposed reaction stoichiometry. The final or best values of the association constants were required not only to fit the experimental binding isotherms but also to calculate the cmc and to predict the shape of the thermogram from the calorimetric titrations. A critical point to be made here is that the protein concentration in the calorimetric experiments was ~15 times greater than in the dialysis experiments. These rigorous constraints on the association constants were invaluable in selecting the appropriate binding model. The model for the binding which describes both the calorimetric and the equilibrium dialysis measurements is one where the first 2 ligands bind cooperatively to high-affinity sites and ~15 additional ligands bind at lower affinity, thermodynamically identical, and independent sites. The thermodynamic parameters for formation of this complex indicate a large entropic contribution to the binding which is consistent with a predominantly hydrophobic interaction.

Experimental Section

Materials

Crystalline BSA was purchased from Sigma Chemical Co. (Lot A-4378) and Miles Laboratories (Pentex Lot No. 22 and 24). The nonionic surfactants Triton X-114, X-100, X-102, and X-165 were purchased from Sigma Chemical Co. (prepared by Rohm and Haas, Philadelphia) and used without further purification. Dialysis membranes used in all equilibrium dialysis work were Visking tubing from Union Carbide Corp.

Methods

Equilibrium Dialysis Measurements. The membranes used in the equilibrium dialysis cells were prepared by the method of McPhie (1971). Subsequent to this treatment, they were soaked for 24 h in pH 7.0 phosphate buffer, I = 0.05 M, to leach out UV-absorbing impurities. Shallow three-chambered dialysis cells, fabricated from polycarbonate plastic, provided a large surface area for the diffusion of solutes across the membranes. The distal and proximal sections of the dialysis cells had a nominal volume of 5 mL, and the center section had a nominal volume of 10 mL. The assembly of the cells with membranes in place was done quickly to avoid drying of the membranes, which will change their porosity. The center section received 10 mL of the buffered surfactant solution. Initial surfactant concentrations were selected to give equilibrium free monomer concentrations ranging from 10^{-6} M to concentrations approaching the cmc (typically ~3.5×10^{-4} M). The distal section received 5 mL of a 0.1% BSA solution in pH 7.0 phosphate buffer. This protein concentration is low enough to avoid concentration effects (Ray et al., 1966). The proximal section was filled with 5 mL of buffer. The assembled and loaded cells were brought to equilibrium in a thermostated water bath held at a predetermined temperature,

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‡ Present address: Department of Physics, University of Wisconsin, River Falls, WI 54022.

§ Present address: Department of Physiology and Biophysics, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.

¶ On leave from the Chemistry Department, University of Alabama, University, AL 35486.

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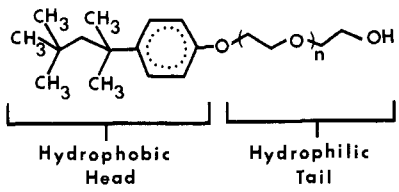

FIGURE 1: Chemical structure for Triton X showing both hydrophobic and hydrophilic regions.

±0.1 °C. The dialysis cells were tumbled 28 times/minute during the approach to equilibrium to provide the desired mixing. This mixing caused no foaming of the protein solution. Time course measurements indicated that equilibrium was established within 36–48 h. At equilibrium, surfactant concentrations were determined by recording the UV difference and absorption spectra with a Cary 14 spectrophotometer using either a 0.1–0.2 or a 1–2 slide-wire. All spectral measurements were made by using quartz cuvettes with a 1-cm path and were recorded at predetermined constant temperatures by using thermostated cuvette holders. For the UV difference spectra the two cuvettes in the reference beam contained (1) surfactant solution from the "buffer only" side of the sample cell and (2) buffered BSA solution from a control cell where no surfactant was present. The two cuvettes in the sample beam contained (3) BSA and NIS from the sample cell and (4) buffer solution from the control cell. Surfactant concentrations were then calculated from the absorbance readings by using an absorptivity of 1.33×10^3 M^{-1} cm^{-1} at 275.5 nm (Kelly & Greenwald, 1958; Griffith, 1957).

Spectra were routinely recorded in the wavelength from 350 to 240 nm. Since the protein and nonionic surfactants are colorless at 350 nm, any change in absorbance at this wavelength is due to aggregation of the protein with its resultant scattering or it indicates the presence of other chromophores. In either case it provides a convenient monitor of spurious effects. Scattering, which causes a sloping base line, was corrected by using the Morton–Stubbs procedure (Bauman, 1962). The BSA concentration was determined by using an extinction coefficient of $E_{1cm}^{1\%} = 6.67$ at 279 nm (Sober, 1970).

Binding isotherms were determined by using the 5 times recrystallized commercial preparations of BSA without further purification. Initial work with Triton X-100 binding to both native BSA and defatted mercaptalbumin (Foster & Hagenmaier, 1971) indicated that removal of the residual fatty acids in the commercial preparations did not alter the binding characteristics of the protein. This is in agreement with the results of others (Gallagher & Steinhardt, 1969). Since the defatted mercaptalbumin, which is a highly purified albumin, showed binding characteristics with regard to the NIS which were indistinguishable from the native BSA, all subsequent data for binding isotherms were obtained by using native BSA. This avoids complications with the irreversible changes introduced by the defatting procedure (Sogami & Foster, 1968).

The association constants for NIS binding to bovine serum albumin were calculated by minimizing an error function $E(\bar{\nu},\bar{\nu}_{exptl})$, where $\bar{\nu}$ is the average number of ligands bound as predicted by the binding model and $\bar{\nu}_{exptl}$ is the experimental value for the average number of ligands bound. [The computer program for fitting the data to the equilibrium binding model was supplied by J. Douglas Ashbrook (1973), National Institutes of Health, Bethesda, MD, private communication.]

Calorimetric Measurements. The calorimetric measurements were made by using a TRONAC 450 isoperibol titration calorimeter employing a 3-mL reaction vessel. This titration

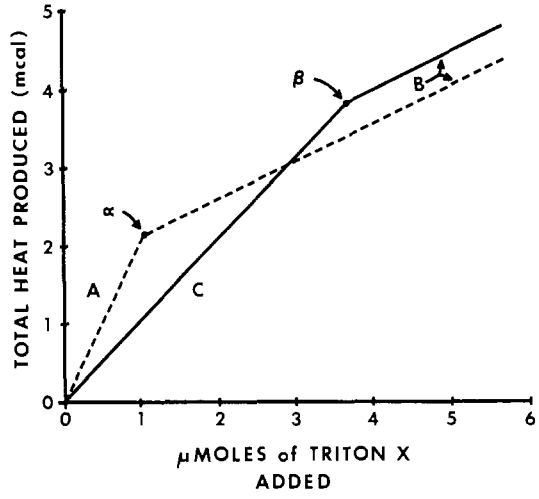

FIGURE 2: Thermograms for titrations with Triton X-100 in phosphate buffer. Region A corresponds to the dilution of concentrated micelles (in the titrant) to give monomers. Region B corresponds to the dilution of concentrated micelles to give dilute micelles. Region C corresponds to the dilution of concentrated micelles to give monomers plus the reaction of monomers with BSA. Therefore, the ΔH value for reaction of monomers with BSA is equal to $\Delta H_C - \Delta H_A$ where the ΔH values are equal to the slopes of the various regions. The moles of Triton bound to BSA can be obtained directly from the difference in the end points of region A and region C, i.e., α and β. α is the cmc in the absence of BSA, and β is the cmc in the presence of 0.227 mM BSA.

calorimeter (Hansen et al., 1974) and the calculation methods (Hansen et al., 1975) have been described in detail previously. A calorimetric titration consisted of titrating 0.23 mL of 2.8×10^{-2} M Triton into 2.69 mL of 1.6% BSA solution. The titration was done continuously over a 400-s period, and the temperature was measured every 4 s. Three or four replicate runs were made for each system. Titrations were carried out in two buffers, phosphate (pH 7.0, $I = 0.05$ M) and Hepes (pH 7.0, $I = 0.05$ M), in order to determine whether ionizable groups on the protein were involved in NIS binding. In different experiments the buffered NIS titrant was either diluted into the same buffer or added to bovine serum albumin in the same buffer. Particular care was taken to match the pH of titrant and titrate.

Since the plots of total heat vs. titrant added (thermograms) consisted of straight or only slightly curved lines up to the cmc end point for most of the systems studied, it was assumed that the stepwise ΔH values were equal and thus only an average ΔH value was calculated. In fact the only way a thermogram could show only a linear dependence of heat on titrant volume for reaction conditions under which competing and incomplete reactions were occurring would be for the assumed equality of the ΔH_i values to be true within the accuracy of the measurements.

Correction for the heat of dilution of the titrant was made by using data obtained by titrating the Triton X into an aliquot of the same buffer used for the titrations of BSA. The reactions assumed to occur are given in the legend of Figure 2. The association constants obtained from the dialysis experiments were used to calculate the molar enthalpy changes from the measured heat changes. The cmc of the surfactant and the average number of Triton molecules bound to a BSA molecule at the cmc were obtained directly from the thermograms as shown in Figure 2.

Electron Spin Resonance Measurements. ESR spectra were recorded by using a Varian E-9 ESR spectrometer with quartz flat cells. Spectra were recorded at ambient temperatures. The spin-label employed was *N*-(2,2,6,6-tetramethylpiperidinyl-1-oxy)maleimide, which was covalently bound to

Table I: Critical Micelle Concentrations of Triton X Surfactants in Phosphate Buffer at pH 7.0 and Ionic Strength = 0.05 M

Triton surfactant	mean degree of polymerization	cmc (mM)	
		dialysis	calorimetry
X-114	7.5	0.32 ± 0.02	*a*
X-100	9.5	0.39 ± 0.02	0.39 ± 0.09
X-102	12.5	0.44 ± 0.02	0.51 ± 0.12
X-165	16	0.51 ± 0.02	ND[b]

[a] These data were complicated by the appearance of a second micellar phase at the high concentration of NIS employed as the titrant in the calorimetric study. [b] ND, not determined.

Table II: Equilibrium Binding of Triton X at the Critical Micelle Concentration to Bovine Serum Albumin[a]

surfactant	$\bar{\nu}$ at cmc	
	dialysis	calorimetry
Triton X-114	5.5 ± 0.2	
Triton X-100	4.7 ± 0.2	4.2 ± 0.2
Triton X-102	4.3 ± 0.2	3.5 ± 0.2
Triton X-165	3.7 ± 0.2	ND[b]

[a] Determined from dialysis and calorimetric measurements at 25 °C in phosphate buffer at pH 7.0 and ionic strength = 0.05 M. [b] ND, not determined.

the free sulfhydryl of mercaptoalbumin.

Selection of Binding Model. For the general stepwise equilibrium model (Edsall & Wyman, 1958; Tanford, 1961; Klotz, 1953) for formation of PL_n from P and L, association constants can be obtained by fitting the binding isotherm data to a polynomial of the form given by

$$\bar{\nu} = \frac{\sum_{i=1}^{n} i\kappa_i[L]^i}{1 + \sum_{i=1}^{n} \kappa_i[L]^i} \quad (1)$$

where $\bar{\nu}$ is the average number of ligands bound, [L] is the concentration of free ligand, and κ_i is generally equated with the thermodynamically definable equilibrium constant K_i. Any binding isotherm may be fitted to eq 1 as long as the polynomial contains enough terms. However, for eq 1 to be valid in the thermodynamic sense (i.e., each $\kappa_i = K_i$), certain criteria must be met. These criteria are that (1) each species from PL to PL_n must exist in solution at a significant concentration at some point on the binding isotherm and (2) both P and L must not undergo any other reaction. Examples of conditions which lead to an inequality of the κ_i and K_i values are (1) a highly cooperative binding sequence so that one or more PL_i do not exist in significant concentration at any point in the isotherm, (2) P has more than or less than n binding sites, and (3) P or L undergoes self-association or reaction with another solute. If any of these conditions exists, it will be reflected in the κ_i value obtained from eq 1 being thermodynamically invalid since some or all of the κ_i values will be dependent on the total concentration of P or of L. The only proof that the κ_i values obtained by fitting eq 1 are true association constants is to show that they do not vary with the total concentration of P or of L. This criterion of thermodynamic validity must apply to any function (whether similar to eq 1 or not) derived from an assumed set of reactions.

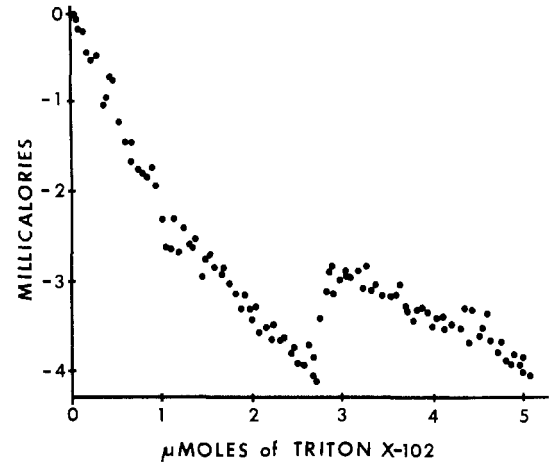

FIGURE 3: Typical experimental thermogram. Titration shown is of BSA in phosphate buffer with Triton X-102.

In this study values of κ_i and n, the maximum number of ligands bound, were calculated from the dialysis data at a BSA concentration of 0.1% and at Triton concentrations from $\sim 10^{-6}$ M to the cmc. A model which is embodied in eq 1 must be assumed in order to do this calculation. Such a model was deemed to be untrue unless it was capable of correctly predicting the cmc end points (point β in Figure 2) in the calorimetric titration curve at a BSA concentration of 1.6%.

Results

Values of the experimentally determined cmc of each of the surfactants used in this study are given in Table I.

A typical experimental thermogram is given in Figure 3 while combined and smoothed thermograms for the titration of BSA with Triton X-114, X-100, and X-102 in both phosphate and Hepes buffers are shown in Figure 4.

Experimental values of the average number of Triton X ligands bound to BSA at the cmc are given in Table II.

The binding isotherms shown in Figure 5 are plotted according to the method of McGhee & Von Hippel (1974). The solid curves shown in Figure 5 were calculated from the association constants given in Table III, which were obtained by assuming 2 cooperative sites and 15 thermodynamically identical and independent sites as the binding model. Other models, e.g., assuming four or more consecutive binding sites, can be fit equally well to these isotherms (concentration of BSA = 0.1%), but the resulting association constants predict

Table III: Thermodynamic Values for Triton X Binding to Bovine Serum Albumin[a]

surfactant	log (K_1K_2)	$-\overline{\Delta G}_{12}$ (kcal/mol)[b]	log K_s	$-\Delta G_s$ (kcal/mol)	$\overline{\Delta H}_{12}$ (kcal/mol)	$\overline{\Delta S}_{12}$ [cal/(mol K)][c]	ΔS_s [cal/(mol K)]
Triton X-114	8.10	5.5	3.00	4.1	0.0 ± 0.5	18.5	13.8
Triton X-100	7.87	5.3	2.83	3.8	+1.4 ± 0.5	22.6	19.5
Triton X-102	7.36	5.0	2.46	3.3	+2.8 ± 0.5	26.2	20.2
Triton X-165	7.28	4.9	2.25	3.1			

[a] Determined at 25 °C in phosphate buffer at pH 7.0 and ionic strength = 0.05 M. n = 15 for the thermodynamically identical and independent sites. Values of the Gibbs free energy are calculated from the association constants. The enthalpy changes are obtained from the calorimetric measurements. [b] Average ΔG for first two ligands binding calculated by $\Delta G_{12} = [-RT \ln (K_1K_2)]/2$. [c] Calculated from the average $\overline{\Delta G}_{12}$ value for the first two Triton molecules bound and the average enthalpy change, ΔH_{12}.

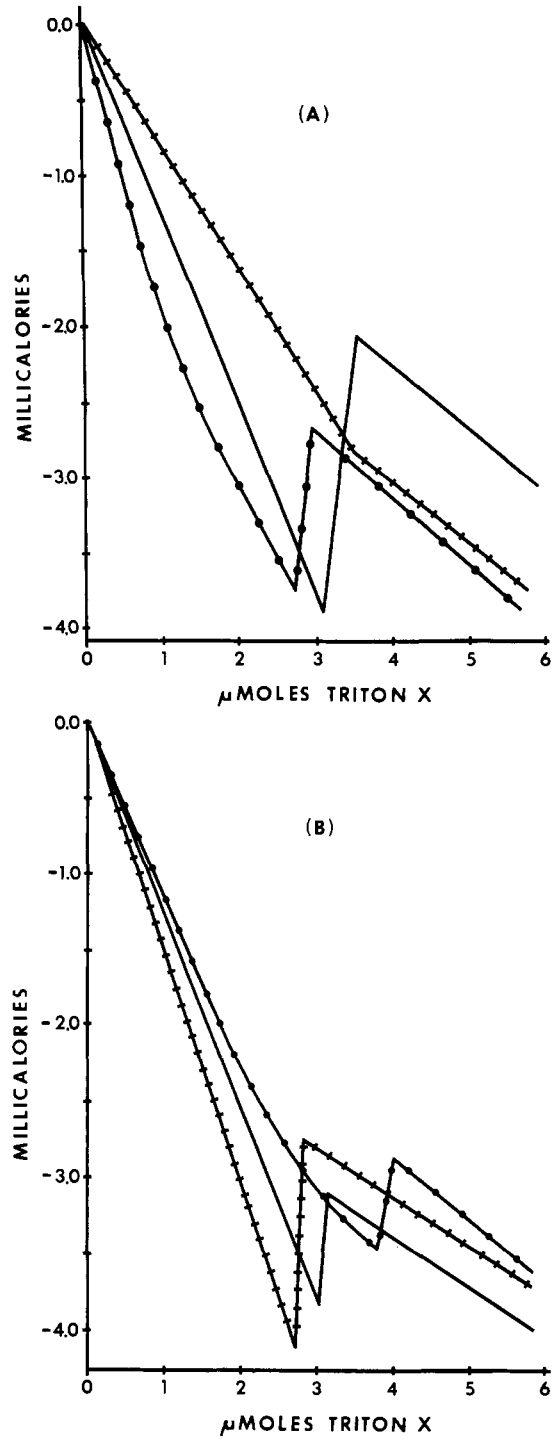

FIGURE 4: Smoothed thermograms for titration of bovine serum albumin in either phosphate (A) or Hepes (B) buffer (pH 7.0, I = 0.05 M) with Triton X-114 (—), Triton X-100 (+), and Triton X-102 (●). The BSA conformational change is indicated by the sharp exothermic heat burst in all but the X-100–phosphate buffer thermogram.

a cmc in the calorimetric titration of BSA (1.6%) which is too large by more than an order of magnitude. Such consecutive binding site models also suggest that the thermograms should be highly curved prior to the cmc, while only slight curvature was seen and then only in the case of Triton X-102. The association constants given in Table III predict monomer concentrations at the cmc end points of the thermograms shown in Figure 5 of 0.33 and 0.49 mM for Triton X-100 and X-102, respectively. These values are in good agreement with the cmc values determined independently (see Table I) and

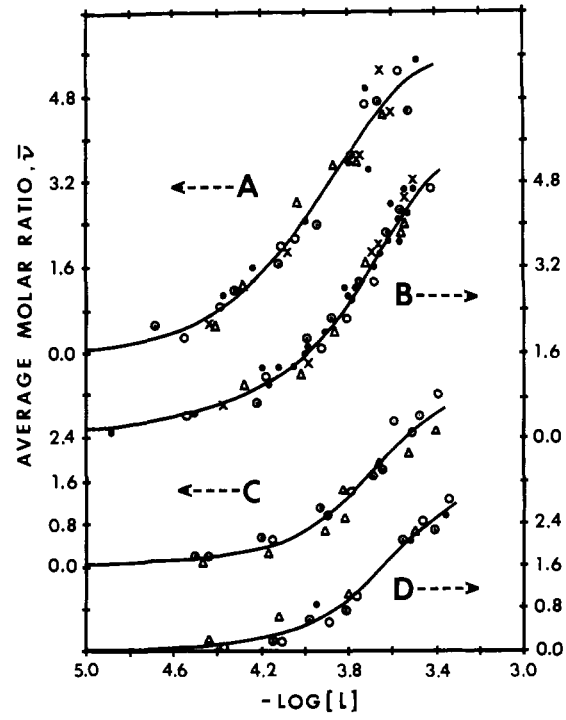

FIGURE 5: Logarithmic plot of isotherms for (A) Triton X-114, (B) Triton X-100, (C) Triton X-102, and (D) Triton X-165 binding to bovine serum albumin. All measurements were made in phosphate buffer, pH 7.0, I = 0.05 M. Temperatures are (○) 4.0, (△) 10.1, (●) 16.2, (×) 22.5, and (⊙) 29 °C.

serve as the justification for the model chosen.

Discussion

The most significant result of this study is probably the definition of the binding model of two cooperative sites and a set of weaker binding sites which are thermodynamically identical and independent. This complex binding model invites criticism. If the model was selected only on the basis of reproducing the binding isotherm, good condition numbers, and a low sum of squares (the latter two are mathematical criteria), it would be suspect. However, the fact that this model also reproduces the thermograms and predicts the cmc in the calorimetric measurements is a sensitive test of its validity. The real significance of this agreement between the dialysis and calorimetric experiments is that it proves the calculated association constants to be independent of both ligand and protein concentration. Excellent agreement was found between the experimental cmc determined from calorimetric measurements at a BSA concentration of 1.6% and the cmc calculated by using the association constants for the binding model which are taken from the equilibrium dialysis measurements at a BSA concentration of 0.1%. Binding models which are pure stepwise equilibrium models can reproduce the binding isotherms and satisfy the mathematical criteria but cannot describe the data as a function of BSA concentration. A model which includes only noninteracting sites cannot fit the binding isotherms because of the presence of cooperativity.

The total number of binding sites available on the BSA as determined in this study (17) is only approximate. For both the calorimetric and dialysis measurements the maximum number of surfactant molecules actually bound is determined by the cmc of the particular Triton X. The low cmc critically restricts the populating of all available sites, and one simply cannot saturate enough sites to place a high degree of confidence in extrapolation of a Scatchard plot to high free

concentrations of the Triton which would define the number of sites. Also, the cmc for an NIS is not very sensitive to salt concentration, which means one cannot experimentally saturate the Triton X binding sites on the protein under any conditions. Hence, the maximum number of NIS binding sites was selected on the basis of two criteria. The first was a qualitative criterion that the total number of binding sites must be the same for each of the different Triton molecules. The second and quantitative criterion was that the number chosen must fit both the equilibrium binding isotherms and the calorimetric titration curves. The total number of binding sites thus arrived at is 17 ± 7.

Our experimental value of $\bar{\nu}$ for Triton X-100 at the cmc may be compared with that reported by Makino et al. (1973) at pH 9.2 using tritium-tagged Triton X-100. Their value of 2.8, which is slightly smaller, may reflect a difference in BSA conformation between pH 9.2 and 7.0. Other studies in this laboratory indicate that the maximum number bound does vary as pH is changed. Their binding isotherm shows no indication of saturation, which is in agreement with our results.

Another important feature of these association constants, and consequently the binding model, is the appearance of positive cooperativity between the first two molecules bound. Evaluation of the Hill coefficients for Triton X-114 and X-100 binding to BSA yields values greater than 1.5. These values provide definite evidence for the positive cooperativity in this binding. In an earlier communication it was shown that the binding of Triton X-100 to BSA at pH 7.0 and 16.2 °C was not described by a model with only noninteracting sites (Sukow & Sandberg, 1974). Similarly, the binding data for Triton X-114, X-102, and X-165 to BSA obtained in this study cannot be described by such a model.

Values of K_1 and K_2 determined in this study are not reported separately since they fluctuated wildly from one set of data to another while their product remained constant and because mole ratio calculations show there are so few molecules of BSA with only one Triton X bound that the equations for K_1 are indeterminate. Thus, the positive cooperativity between the first two Triton X molecules bound is total. In eq 1 this is equivalent to starting both sums at $i = 2$.

A model with 3 cooperative sites and 14 independent and thermodynamically identical sites was also tested. This model gave a somewhat poorer fit to both the binding isotherms and the thermograms but was within the experimental error. Models with more than three cooperative sites definitely did not fit the data.

In an earlier study Makino et al. (1973) described the binding of Triton X-100 to BSA with four consecutive binding sites. That they found neither the cooperativity nor the additional low-affinity sites can probably be attributed to the limited data in their study, since they did not vary the BSA concentration. However, it may reflect a real difference in the protein molecule at pH 9.2 (their study) and at pH 7.0 (this study).

Preliminary studies using Triton X homopolymers indicate that the assumed model and the data derived from it are not affected significantly by the polydispersity of the commercial surfactant preparations used here.

No consistent differences outside of the estimated uncertainties were observed for the thermograms obtained in Hepes buffer and in phosphate buffer. This indicates that no significant ionization or protonation of the protein occurs on binding Triton X molecules.

A striking feature of all the thermograms except that for X-100 in phosphate buffer is the very sharp endothermic transition that occurs as the free NIS concentration in the reaction vessel nears the cmc. The most reasonable interpretation of this heat effect is that the protein is undergoing some conformational change as the lower affinity NIS binding sites become populated. The argument that this endothermic region could be due to some surfactant reaction or micellar phase transition is ruled out by the fact that the enthalpy change per mole of surfactant would be extremely large (ca. +17 kcal/mol). However, the enthalpy change of this transition is only 3.5 ± 1.0 kcal/mol of protein. The proposed conformational change thus could result from breaking only one hydrogen bond. That the protein conformational change takes place near the cmc is probably fortuitous. In fact, this transition appears to be extremely sensitive to surfactant concentration, and if it were possible to achieve higher free monomer concentrations of Triton X-100 in phosphate buffer, we would expect to see the same protein conformational changes as observed under all the other conditions.

The thermograms in Figure 2 show no evidence for any conformational change other than the one near the cmc. Association of Triton X-114, X-100, X-102, and X-165 with BSA also causes no change in the ESR spectrum of the maleimide spin-label at pH 7.0. The bound spin-label has an ESR spectrum characteristic of a strongly immobilized spin-label molecule in both the absence and presence of NIS up to the cmc. The ESR spectrum recorded in the absence of NIS is in agreement with that reported by Griffith & McConnell (1966). The absence of conformational change near the maleimide binding site is in agreement with the absence of change in optical rotation as Triton X-100 binds (Makino et al., 1973).

Values of thermodynamic quantities associated with the binding of Triton X to BSA are given in Table III. The free energies of binding are negative for all Triton X binding sites on BSA. For the two cooperative sites the magnitude of the average free energy per site decreases in a regular fashion from 5.5_0 kcal/mol for Triton X-114 to 4.9_5 kcal/mol for Triton X-165. The independent and thermodynamically identical sites also show a regular decrease from 4.1 kcal/mol for Triton X-114 to 3.1 kcal/mol for Triton X-165. For both types of sites the free energy associated with the binding agrees well with the values reported for other neutral ligands binding to BSA, as summarized by Steinhardt & Reynolds (1969). The regular decrease in $-\Delta G$, for all sites, occurs as the number of ethoxy units increases. A plot of ΔG vs. the mean degree of polymerization of the ethoxy chain indicates a change in the free energy of binding equal to 0.12 kcal/mol for each additional ethoxy unit. This contrasts with the alkyl sulfates where binding increases as the number of carbon atoms in the alkyl portion increases. For the Triton X, the decrease in binding as the mean degree of polymerization increases may indicate there are steric problems as the ethoxy chain becomes more bulky. The ethoxy chain is transformed from a zigzag (extended) to a meander (compact) configuration above a degree of polymerization of 9 (Rosch, 1967). Therefore, starting with Triton X-100, one would expect that a significant mole fraction in the commercial preparations would have the compact conformation.

The calorimetric measurements show that the enthalpy change associated with binding is zero or positive and that it becomes increasingly more endothermic as the degree of polymerization of the ethoxy chain is increased on going from Triton X-114 to X-100 to X-102 (see Table III). That ΔH is small or endothermic indicates that the binding is primarily hydrophobic in nature, as has been observed for other neutral

ligands binding to BSA (Steinhardt & Reynolds, 1969). The average ΔS values for the first two NIS molecules bound at the cooperative sites are listed in Table III for Triton X-114, X-100, and X-102. In all cases the smallest value for ΔS occurs for that Triton X which has the smallest degree of polymerization. The regular increase in the entropy change associated with the binding of NIS to albumin as the number of ethoxy units increases exhibits the typical (ΔH, ΔS) compensation effect commonly seen in water. As the longer ethoxy chains are removed from the aqueous environment, more dipole–dipole interactions are disrupted as evidenced by the increasingly positive ΔH. Concomitantly larger numbers of waters of hydration are released from the longer ethoxy chains with a more positive energy change resulting. In the binding of Triton X molecules to BSA there is an inexact compensation in these two effects with ΔH changing more rapidly than ΔS, and thus the free energy of binding is decreased somewhat with increasing chain length.

In view of the observed positive cooperativity in the albumin–NIS binding, a multiple site which can accommodate two (or three) Triton molecules is suggested, perhaps as a hydrophobic pocket in the protein. Serum albumin is a ubiquitous carrier protein which transports fatty acids and tryptophan in vivo (Scow & Chernick, 1970; Feinstrom & Wurtman, 1974). The pickup and release of these ligands could be enhanced by positive cooperativity as was seen here for Triton X binding.

The magnitude of the association constants for the cooperative binding of the Tritons employed in this study is inversely proportional to the degree of polymerization of the oxyethylene chain. Umbreit & Stominger (1973) have reported that solubilization of D-alanine carboxypeptidase from *Bacillus subtilus* membrane is also dependent on the number of oxyethylene units in the Triton X used. In their study, Triton X-114 and X-100 were more efficient than Triton X-102 and X-165. In our studies this would correspond to the surfactants with the largest association constants for binding to BSA. Perhaps in general those surfactants with the largest association constants will be the most effective solubilization agents. It is tempting to conclude that Triton X-114 should be the most efficient in solubilizing membrane-bound proteins.

The presence of several lower affinity and thermodynamically identical sites on bovine serum albumin suggests that the Triton X molecules bound to these sites are widely separated on the protein surface. The thermodynamic parameters indicate that binding of NIS at these sites is again predominantly hydrophobic in nature. The Triton molecules with higher degrees of polymerization again exhibit an increasingly less favorable enthalpy change for binding as the oxyethylene chain is lengthened. That fewer X-102 molecules are bound to BSA at the cmc than X-114 molecules at its lower cmc is explained by the more endothermic ΔH for X-102. The surface of BSA must have a number of hydrophobic openings, cracks, or crevices, which can accommodate the bulky (1,1,3,3-tetramethylbutyl)phenyl head group of the Triton X molecule.

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