EVIDENCE FOR COOPERATIVE BINDING OF TRITON X-100 TO BOVINE SERUM ALBUMIN

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1. Introduction

A common goal in the biophysical and biochemical characterization of individual membrane proteins in aqueous solution is the separation of a protein from the macromolecular aggregate defined as the membrane. Surfactants are frequently used to achieve this objective. There are three types of surfactants: anionic, cationic and nonionic. The interaction of sodium dodecyl sulfate (SDS) and sodium deoxycholate, which are examples of anionic surfactants, with proteins has been characterized thermodynamically and physically for their effect on protein conformation [1–7]. When the concentration of monomeric SDS exceeds a threshold of 0.5 mM, proteins bind with a cooperative mechanism between 1.1 and 2.2 g of SDS per g of protein and the protein conformation becomes rodlike within the constraints of cystine bridges. In contrast to SDS, deoxycholate (DOC) binds up to 0.6 g per g lipophilic protein. Although DOC does not appear to cause conformational changes, one is restricted to alkaline pH regions to maintain the anionic form of DOC [8]. Physical studies of the interaction between cationic surfactants and proteins are fewer in number than for anionic surfactants and usually show that the tertiary structure is altered as large numbers of cationic molecules bind to the protein [9].

The binding of Triton X-100 to proteins is apparently very selective and favors lipophilic proteins [5,7,10]. Many membrane-bound proteins have been isolated with biological activity in the presence of nonionic surfactants; selected examples include acetylcholine receptor [11], cytochrome b_5 reductase [12], acetylcholinesterase [13–15], and insulin receptor protein [16]. Fundamental questions concerning the mechanism by which nonionic surfactants bind to proteins remain to be answered.

In this paper we report our study of the binding of Triton X-100 to bovine serum albumin (BSA) which is a protein with a known affinity for amphiphiles. Binding isotherms were measured for Triton X-100 and several other members of the Triton X series. Scatchard plots are non-linear and suggest that the binding is cooperative. The most efficient binding occurs with Triton X-114 and suggests that the choice of surfactant must match the type of binding site. Thus the separation of membrane-bound proteins may be improved with Triton X-114.

2. Materials and methods

BSA and Triton X concentrations were measured spectrophotometrically with a Cary 14 spectrophotometer using thermostated cell holders and either 0 to 2 or 0 to 0.2 absorbance slidewires. For the Triton X phenoxychromophore, $a_{275\ nm} = 1.33 \times 10^3\ M^{-1}\ cm^{-1}$ [17]. For BSA, $A^{280}_{1\%} = 6.6$ [18]. A three chambered dialysis cell was fabricated from polycarbonate. At time zero, the distal section contained 5 ml of 0.1% BSA in buffer,

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the center contained 10 ml of Triton-X concentrations ranging from 10^{-6} M to twice the critical micelle concentration (CMC = 3.2×10^{-4} M for Triton X-100), and the proximal section contained 5 ml of the buffer. Visking cellulose membranes No. 27 (Union Carbide Corporation, Chicago, Illinois, USA) which were prepared by the method of McPhie to remove soluble UV absorbing substances [19] separated the three sections. BSA was obtained from Pentex as crystallized bovine albumin (Lot # 24) and was used [1] without further purification or [2] to prepare defatted mercaptalbumin by the method of Hagenmaier and Foster [20]. The dialysis cells were tumbled at 28 rpm in a constant temperature water bath until equilibrium was established. Typically, a dialysis time of 48 hr was required to reach equilibrium.

When a macromolecule has only independent and identical binding sites, the experimental binding data is described by a Scatchard model with one class of binding sites [21]. For this case, the Scatchard plot is defined by eq. 1,

$$\frac{\bar{v}}{c_{free}} = (n-\bar{v})K \qquad (1)$$

where $\bar{v}$ is the average molar ratio of ligands bound per macromolecule c_{free} is the molar concentration of unbound ligand in equilibrium with the complex, n is the total number of binding sites on the macromolecule and K is the association constant. The parameter n is evaluated by extrapolation of the linear plot of equation 1 to the intercepts on either the abscissa or ordinate.

In order to verify that an experimental system is adequately described by the Scatchard model, experimental data points must span the range from $0 \leq \bar{v} \leq n$. This extrapolation is potentially erroneous if limited data is used to define the Scatchard plot [22].

3. Results and discussion

A Scatchard plot for the binding of Triton X-100 to BSA at pH 7.0 and 16.2°C is shown in fig. 1. The experimental data do not distinguish between defatted and crystallized BSA and suggest that the binding

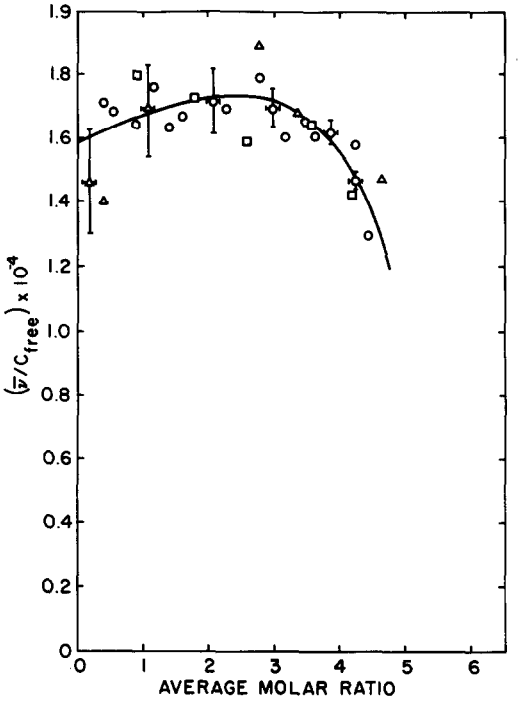

Fig. 1. A Scatchard plot for the binding of Triton X-100 to BSA. All measurements were made with pH 7.0 phosphate buffer with I = 0.05 and at 16.2 ± 0.1°C. (○, □) native BSA. (△) defatted mercaptalbumin. Representative error bars are root mean square errors using 9 measurements.

sites for Triton X-100 are not blocked by residual fatty acids bound to the crystallized BSA. As c_{free} approaches the critical micelle concentration, the maximum number of Triton X-100 molecules bound approaches 6. The striking aspect of the Scatchard plot for the binding of Triton X-100 by BSA is its convex curvature. A non-linear Scatchard plot is evidence for cooperativity [22].

The convex curvature in a Scatchard plot is observed in the binding of substrates to enzymes [23–25], of optically active dyes to BSA [26] and of divalent metal ions to tRNA [27]. In all of these cases this convex curvature is interpreted as resulting from the cooperative nature of the binding. By inference, the binding of Triton X-100 by BSA also involves cooperativity. Evaluation of the Hill coefficient for the Triton X-100–BSA system yields a value of 1.9 which is evidence for positive coopera-

tivity. This cooperativity in the binding of Triton X-100 to BSA is in contrast to the binding of other neutral and anionic amphiphiles by this protein [5, 28–31].

A logarithmic plot for the binding of Triton X-100 to BSA at pH 9.2 has been reported but Scatchard plots are not given. Also at pH 9.2, competitive binding studies between ring-tagged ^{3}H Triton X-100 and ^{14}C-deoxycholate were attempted. Unfortunately the low specific activity of the tritium label prevented accurate measurements [5]. These studies are of particular interest because the deoxycholate ligand binding experimentally fits a Scatchard plot. Direct comparison between the binding studies at pH 7.0 and pH 9.2 is complicated by structural changes in the molecule. These changes have been observed in our laboratory with maleimide spin-labeled BSA and are similar to the changes reported for the N–F transition [32]. We find that the number of alkyl groups of Triton X-100 bound to BSA depends on the pH.

The presence of cooperativity in the binding of *d*-phenyl-(*p*-(*p*-dimethylaminobenzeneazo)-benzoyl-amino)-acetic acid to BSA and the absence of cooperativity in the binding of the *l*-isomer suggests that steric effects can be important factors in the mechanism of cooperative binding to BSA [26]. For proteins with subunits, cooperative binding of ligands to subunits has been described by the Monod–Wyman–Changeux model [33] and the Koshland–Nemethy–Filmer model [34]. Although BSA contains a single polypeptide chain, peptide cleavage experiments indicate that three sequential regions may exist with internal cross-linking by cystine bridges but without cross-linking between regions [35]. The number of bound ligands is greater than the number of potential subunits. A requirement of the MWC model is the conformational change in the allosteric subunits. Another possible mechanism for cooperative binding is the pocket model. In the pocket model, more than one ligand is bound per pocket. The cooperativity may arise by interactions between the protein and nonionic surfactant or between the adjacent surfactant molecules. The absence of cooperativity with competitive anionic or cationic amphiphiles is accounted for by electrostatic repulsion between these amphiphiles which may restrict the number per pocket to one.

Conformational changes in BSA have been observed as a function of bound ligands for anionic or cationic surfactants [36–38]. Electron paramagnetic resonance spectra from the covalent attachment of *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide to BSA, at pH 7.0, do not show a conformational change as a function of bound Triton X-100. The large conformational change associated with the N–F transition of BSA is sensed by this spin-probe. Hence we conclude that the binding of Triton X-100 to BSA does not cause a large conformational change at pH 7.0. Unfortunately we cannot exclude the possibility of local conformational changes near the surfactant binding sites, yet far from the sulfhydryl site to which the spin probe is attached.

The Scatchard plot shown in fig. 1 is unable to describe multiple equilibria which involve a cooperative interaction between neighboring ligands or the incorporation of a ligand into the binding site for a second ligand. If the Triton X-100 binding site on BSA can accommodate multiple ligands then the interactions between the polyoxyethylene head groups may be appreciable. The average molar ratio of Triton molecules bound per BSA molecule depends on the average degree of polymerization of the polyoxyethylene chain (Sukow and Sandberg, manuscript in preparation). The conformation of the polyoxyethylene chain also depends on the degree of polymerization. Triton X-100 exists in a zig-zag conformation with a calculated diameter of 0.25 nm in contrast to longer chain lengths which exist in the meander configuration with a calculated diameter of 0.4 nm [39]. The smaller number of bound ligands for the higher degrees of polymerization is consistent with a binding site which can accommodate multiple ligands. The importance of the polyoxyethylene chain length has also been shown in the solubilization of D-alanine carboxypeptidase from *B. subtilis* membrane [40]. Although the evidence is not conclusive, our results support the view that the structure of the polyoxyethylene chain plays an important role in determining the binding behavior to proteins and interactions between these extended moieties may be the cause of the cooperative behavior.

The interpretation of results based on data using commercial preparations of Triton X surfactants is

Table 1
Comparison of $\bar{\upsilon}$ at $c_{free} = 4 \times 10^{-5}$ M for several members of the Triton X series and of calculated mole percent content of specified oligomers.

Triton surfactant	$\bar{\ell}$	$\bar{\upsilon}$	Mole percent of 7-mer	Mole percent of 16-mer
X-114	∿7.5	0.80	16	0.2
X-100	∿9.5	0.60	11	1.4
X-102	∿12.5	0.17	3	6
X-165	16	0.18	0.5	10

$\bar{\ell}$ is the average degree of polymerization for the nonionic surfactant. $\bar{\upsilon}$ is the average molar ratio of surfactant bound to BSA. The mole percent of the $(x + 1)$mer is given by

$$\left(\frac{e^{-m}m^{x}}{x!}\right) 100 \text{ where } m = \bar{\ell} - 1.$$

complicated by the fact that they are heterogeneous oligomers of *p*-(1,1,3,3-tetramethylbutyl) phenoxy-polyoxyethylene ethanol. Several different average degrees of polymerization are available in the Triton X series and have been used to isolate proteins from macromolecular aggregates such as biological membranes. A comparison of $\bar{\upsilon}$ for members of the Triton X series at a free concentration of 4×10^{-5} M is given in table 1. The free concentration was selected to yield a maximum $\bar{\upsilon}$ of approximately one, because it is suggested from data in fig. 1 that cooperative binding occurs at low average molar binding ratios. Clearly Triton X-114 with an average degree of polymerization of approximately 7.5 binds most efficiently to BSA. Theoretically, the mole percent of homogeneous oligomers is calculated from a Poisson distribution [41]. The calculated mole percent of a 7-mer and 16-mer for $\bar{\ell}$–7.5, 9.5, 12.5, and 16 is also tabulated in table 1. The ratio of 16-mer to 7-mer is 0.012, 0.13, 2, 20 for Triton X-114, 100, 102, 165 respectively. Note that $\bar{\upsilon}$ is the same for X-102 and X-165 and supports the hypothesis that oligomers in the meander configuration are bound to the same extent. It is unlikely that the cooperativity is a result of this heterogeneity. If each specific oligomer had a different association constant and bound to BSA at independent and identical sites, the summation of these Scatchard model curves would not yield a cooperative curve as shown in fig. 1.

It has been suggested that commercial preparations of Triton X-100 may contain as much as 5% of a di-2,4-(1,1,3,3-tetramethylbutyl) phenoxy group in place of the *p*-(1,1,3,3 tetramethylbutyl)-phenoxy group (5). At the lowest total concentrations of Triton X-100 shown on fig. 1, 25% of the Triton X-100 is bound to the BSA. Thus the cooperativity cannot be explained by the presence of these different alkyl substituents.

It is informative to consider the binding of Triton X-100 to BSA at $\bar{\upsilon}$ values greater than 2. A Scatchard plot for $\bar{\upsilon} > 2$ is shown in fig. 2. It is evident that this restricted range of data is fitted by a straight line which results in the conclusion that the binding sites are identical and independent. Furthermore, extrapolation as $\bar{\upsilon}/c_{free} \rightarrow 0$ yields a value of 10 for the total number of these independent and identical sites. This value is in good agreement with the number of sites in the high-energy class for binding of other ligands to BSA [1,30,31,42]. In addition the average association constant of these sites is 3×10^{3} M^{-1}, again in agreement with that for octanol binding to BSA.

The preceding analysis shows that use of a restricted range of data gives results which one would interpret as being in good agreement with values

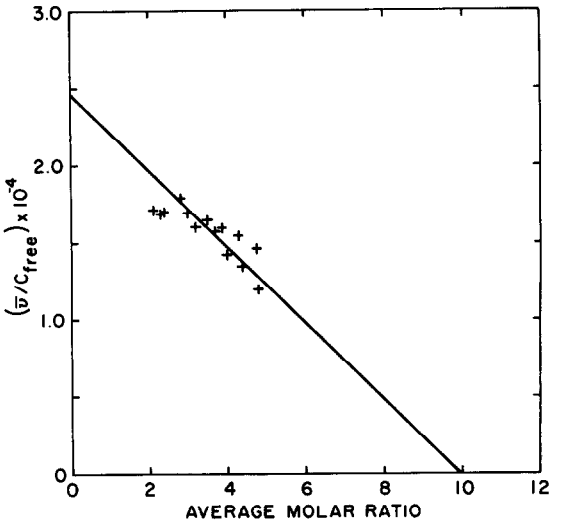

Fig. 2. A Scatchard plot for the binding of Triton X-100 to BSA using a truncated data set. All measurements were made with pH 7.0 phosphate buffer I = 0.05 and at 16.2 ± 0.1°C. Data is truncated to $\bar{\upsilon} > 2$.

reported in the literature for similar systems. However, the interpretation of the nature of the binding is not the same. From the restricted range of data one concludes that the binding sites are independent and identical, yet more complete data reveal cooperativity among them. This difference in interpretation of the binding is significant and points out the necessity for obtaining data at low $\bar{\upsilon}$ values where the effect of cooperative binding is most readily observed. A review of the literature shows that often data are not obtained for low $\bar{\upsilon}$ values.

4. Conclusion

Scatchard plots for the binding of Triton X-100 and other members of the Triton X series of nonionic surfactants to BSA are non-linear and suggest cooperative binding. Evidence for the cooperativity is obtained at low extents of binding, $\bar{\upsilon} < 2$. If the experimental data are limited to values of $\bar{\upsilon} \geqslant 2$, the Scatchard plots are linear in agreement with the reported binding of neutral and anionic molecules to BSA. Triton X-114 binds more efficiently to BSA and may increase the yield in the solubilization of proteins from macromolecular aggregates such as membranes.

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