  Bull. Chem. Soc. Jpn., 55, 3085—3088 (1982)

Interaction between Hydrophilic Proteins and Nonionic Detergents Studied by Surface Tension Measurements

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(Received January 14, 1982)

The surface tensions of the systems of hexa(oxyethylene) dodecyl ether ($C_{12}E_6$) *vs.* lysozyme (Lyz) or bovine serum albumin (BSA) were measured for constructing the binding isotherms, since the surface tension method is more convenient for the systems of nonionic detergents and proteins than is equilibrium dialysis. The obtained binding isotherms depend on the protein concentration; this effect is much more remarkable for Lyz–$C_{12}E_6$ systems than for BSA–$C_{12}E_6$ ones. The binding isotherms of BSA–$C_{12}E_6$ systems obey the Scatchard equation, and the maximum number and estimated free energy for $C_{12}E_6$ binding to BSA are comparable to those for the system of Triton X-100 and BSA determined by equilibrium dialysis. The agreement suggests that the surface tension method is sound and has advantages over equilibrium dialysis for the estimation of the number of detergent molecules bound to protein. In the systems of Lyz–$C_{12}E_6$, the binding isotherms follow the Freundlich-type equation rather than the Scatchard equation. The remarkable protein concentration dependence of the binding isotherms and this result suggest the occurrence of protein aggregation.

Nonionic detergents such as Triton X-100 (poly-(oxyethylene) (9—10) *p*-(1,1,3,3-tetramethylbuthyl)-phenyl ether) have been widely used to solubilize membranes and to purify membrane proteins.[1,2] That is, they can often extract proteins from membranes without disruption of native conformation or loss of biological activity, in contrast to common synthetic ionic detergents, notably sodium dodecyl sulfate (SDS), which ordinarily solubilize lipid-associated proteins in a denatured and inactive form.

The interaction of proteins with ionic detergents has been a subject of intensive investigation. However, the interaction of nonionic detergents with proteins has not been investigated widely and the information so far gained is as follows.[1,3-5] The lipophilic proteins bind cooperatively large amounts of nonionic detergents at their critical micelle concentrations (cmc's), whereas a number of hydrophilic proteins bind few or no nonionic detergents. It seems that nonionic detergents can bind only to hydrophilic proteins possessing hydrophobic surface patch. For example, ovalbumin does not bind Triton X-100 at all within the concentration range studied (10^{-5}—10^{-4} mol dm^{-3}). Bovine serum albumin (BSA), possessing a hydrophobic surface patch, binds Triton X-100 by the maximum number of four per protein in a Langmuir-type binding, and no gross structural change occurs in BSA by binding of Triton X-100.

The interaction between detergents and proteins may be attributed to the binding of detergents to proteins, *i.e.*, to complex formation, and may be expressed directly and quantitatively by the binding isotherms. The amount of detergents bound to proteins is usually determined by means of equilibrium dialysis. However, nonionic detergents such as Triton X-100 do not penetrate the dialysis membrane fast enough for dialysis experiments.[1,3] On the contrary, the surface tension can be measured more easily and reflects sensitively the changes of dissolved state in bulk.

In this report, we have measured the surface tension of aqueous solutions of hexa (oxyethylene) dodecyl ether ($C_{12}E_6$) *vs.* lysozyme (Lyz) or bovine serum albumin (BSA), and constructed the binding isotherms by applying the surface tension data to the equations derived for determining the number of detergent molecules bound to a protein.

Experimental

Materials. Hen egg-white Lyz was supplied from Miles Biochemicals (six times recrystallized; activity: above 51000 units/mg; homogeneity: above 97%). BSA was also supplied from Miles Biochemicals (monomer standard protein powder; purity: above 99%). $C_{12}E_6$ was supplied from Nikkō Chemicals Co., Ltd. The high purity (above 99.5%) was confirmed by gas chromatography.

Surface Tension Measurements. The aqueous solutions of mixed BSA– or Lyz–$C_{12}E_6$ were free from buffer and salts. The pH's of the solutions were 5.6. The surface tension measurements for the solution were performed at 298.15 ± 0.01 K by the Wilhelmy method with a 24 mm × 24 mm glass plate and a Kyowa CBVP Surface Tension-Meter A-3. Before the measurement the plate was cleaned by heating it in a "chromic acid mixture" for more than six hours and was washed thoroughly with three-times-distilled water.

Results and Discussion

In Figs. 1 and 2 are shown the plots of surface tension *vs.* detergent concentration at constant protein concentration for BAS– and Lyz–$C_{12}E_6$ systems, respectively. Each plot exhibits a sharp break at each cmc and the surface tension remains constant above the cmc. The values of the constant surface tensions for all the systems are identical regardless of the protein concentration. Next, we will derive the equations for determining the amount of nonionic detergents bound to proteins from these surface tension data.

It can be supposed that protein-nonionic detergent complexes are surface inactive, since the hydrophobic areas of protein and detergent are almost hidden by the complex formation. Then the following Langmuir equation may be valid for the adsorption equilibrium of nonionic detergent monomers at the air/water interface from the aqueous solution of protein and detergent, in the same way as in the case of the adsorption of detergent alone:[6-9]

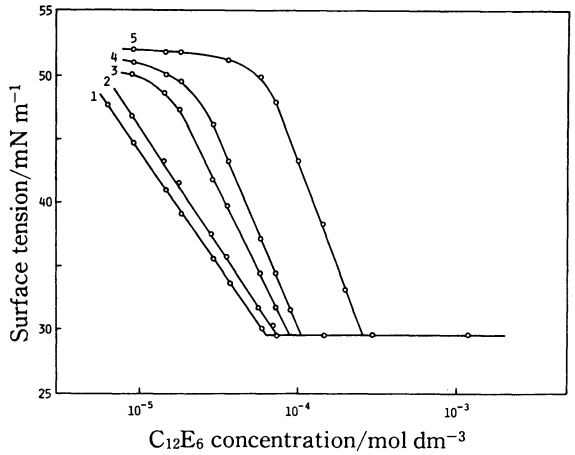

Fig. 1. Plots of surface tension *vs.* logarithm of $C_{12}E_6$ concentration at various BSA concentrations for BSA–$C_{12}E_6$ systems at 298.15 K.
BSA concentration: (1) $C_{12}E_6$ alone, (2) 1×10^{-6}, (3) 5×10^{-6}, (4) 1×10^{-5}, (5) 5×10^{-5} mol dm^{-3}.

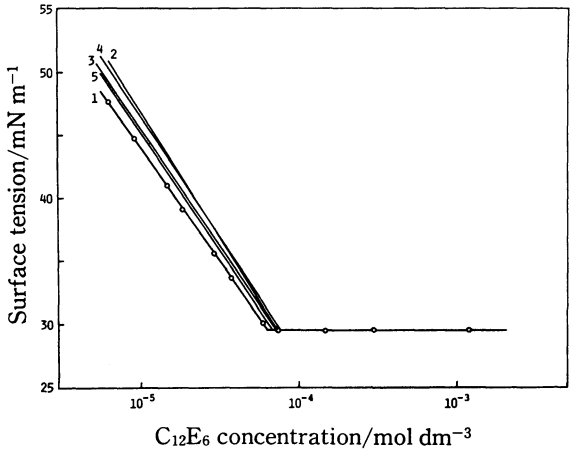

Fig. 2. Plots of surface tension *vs.* logarithm of $C_{12}E_6$ concentration at various Lyz concentrations for Lyz–$C_{12}E_6$ systems at 298.15 K.
Lyz concentration: (1) $C_{12}E_6$ alone, (2) 1×10^{-6}, (3) 2×10^{-6}, (4) 1.25×10^{-5}, (5) 1×10^{-4} mol dm^{-3}.

$$\Gamma_s = \frac{K_s(a-\Gamma_p f)C_s^f}{1+K_sC_s^f}, \tag{1}$$

where Γ_s: the amount of adsorbed detergent at the air/water interface,
Γ_p: the amount of adsorbed protein,
f: the factor for converting Γ_p to Γ_s-basis, defined as the ratio of the maximum adsorption of detergent to that of protein,
a: the maximum adsorption of detergent,
C_s^f: the concentration of detergent monomer,
K_s: the equilibrium constant.

It is also known that the Langmuir equation applies empirically to the adsorption of protein at the low concentration,[10,11] and thus we may write for the protein-component:

$$\Gamma_p f = \frac{K_p(a-\Gamma_s)C_p^f}{1+K_pC_p^f}, \quad \Gamma_p^o f = \frac{K_p a C_p^o}{1+K_pC_p^o}, \tag{2a,2b}$$

where the subscript o denotes the quantity for the adsorption in the aqueous solutions of the protein alone, and the subscript p refers to protein. If the protein concentration is low enough so that $K_pC_p^f \approx K_pC_p^o \ll 1$ (Henry-type adsorption), we have from Eqs. 2a and 2b:

$$\Gamma_p f = \Gamma_p^o f + K_p(\Gamma_s-a)(C_p^o-C_p^f) - K_pC_p^o\Gamma_s. \tag{3}$$

In the case of the adsorption of nonionic detergent ($C_{12}E_6$) alone, the value of Γ_s is nearly equal to that of a in the concentration range greater than 1×10^{-5} mol dm^{-3}; this is the result of the direct measurement of the adsorption of $C_{12}E_6$ by a radiotracer technique.[12] Then, in the case of the adsorption from protein-detergent mixed solutions, the magnitude of the second term is expected to be much less than those of the others terms in Eq. 3 at the $C_{12}E_6$ concentration studied and we have:

$$\Gamma_p f \simeq \Gamma_p^o f - K_pC_p^o\Gamma_s = \Gamma_p^o f - \frac{\Gamma_p^o f}{a}\Gamma_s. \tag{4}$$

Irrespective of the concentration range where Eq. 4 is valid, Eq. 4 may be regarded as a working hypothesis for the mixed adsorption of detergent and protein. That is, Eq. 4 gives a relation between Γ_p and Γ_s, and means that the value of Γ_p decreases linearly with that of Γ_s. By introducing Eq. 4 into Eq. 1, we obtain

$$\Gamma_s = \frac{K_s a(a-\Gamma_p^o f)C_s^f}{a+K_s(a-\Gamma_p^o f)C_s^f}. \tag{5}$$

Next, we consider the Gibbs adsorption equation under the conditions of constant protein concentration, Eq. 6, since the concentrations of detergent and protein are low in the present case:[13]

$$\left(\frac{\partial\gamma}{\partial\mu_s}\right)_{T,\mu_p} \simeq \left(\frac{\partial\gamma}{\partial\ln C_s^f}\right)_{T,c_p} = -RT\Gamma_s, \tag{6}$$

where γ refers to the surface tension of the aqueous solution containing protein and detergent, and μ is the chemical potential. By integration of Eq. 6 after introduction of Eq. 5, we have

$$\gamma = -aRT\ln[a+K_s(a-\Gamma_p^o f)C_s^f] + \gamma_p + aRT\ln a, \tag{7}$$

where γ_p refers to the surface tension of the protein solution. In the case of detergent alone,

$$\gamma_s = -aRT\ln[1+K_sC_s^o] + \gamma_{H_2O}, \tag{8}$$

where γ_{H_2O} refers to the surface tension of pure water.

Now we assume: at the concentration (cmc′) where the surface tension γ begins to stay constant, detergent monomers begin to form micelles by themselves and the concentration of monomers is identical with the cmc of detergent alone (cmc°). In addition, as seen from Figs. 1 and 2, we take into account the experimental observation that the surface tensions at the cmc′ and cmc° are identical ($\gamma_{cmc'}=\gamma_{cmc^o}\equiv\gamma_{cmc}$). Then, we have by Eqs. 7 and 8:

$$\gamma_{cmc} = -aRT\ln[1+K_s\,cmc^o] + \gamma_{H_2O}, \tag{9}$$

$$\Gamma_p^o f = \frac{a(1+K_s\,cmc^o)\left\{1-\exp\left(-\frac{\gamma_{H_2O}-\gamma_p}{aRT}\right)\right\}}{K_s\,cmc^o}. \tag{10}$$

By applying Eqs. 7, 9, and 10 to the surface tension data shown in Figs. 1 and 2, we can obtain the mean number of detergent molecules bound to proteins, $\bar{\nu}$; $\bar{\nu}=$

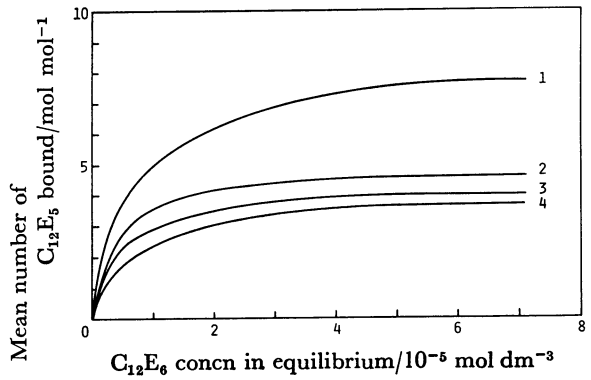

Fig. 3. Binding isotherms at various BSA concentrations for BSA–$C_{12}E_6$ systems at 298.15 K.
BSA concentration: (1) 1×10^{-6}, (2) 5×10^{-6}, (3) 1×10^{-5}, (4) 5×10^{-5} mol dm^{-3}.

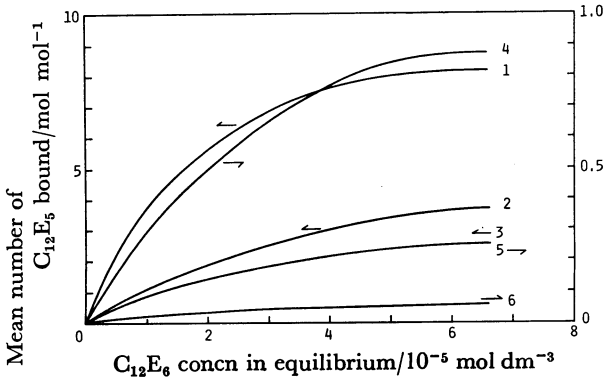

Fig. 4. Binding isotherms at various Lyz concentrations for Lyz–$C_{12}E_6$ systems at 298.15 K.
Lyz concentration: (1) 1×10^{-6}, (2) 2×10^{-6}, (3) 5×10^{-6}, (4) 1.25×10^{-5}, (5) 5×10^{-5}, (6) 1×10^{-4} mol dm^{-3}.

$(C_s-C_s^f)/C_p$, where C_s denotes the total detergent concentration. The process used to obtain the value of $\bar{\nu}$ is as follows. The value of a may be obtained from the slope of the surface tension *vs.* logarithm of concentration plot below the cmc of $C_{12}E_6$ solution (Fig. 1 or 2) by applying the Gibbs adsorption equation. Next, the value of K_s can be calculated by introducing the values of a, cmc° and γ_{H_2O} into Eq. 9 and then that of $\Gamma_p^\circ f$ can be calculated by the use of Eq. 10. Finally, it is possible to calculate the value of C_s^f by introducing the obtained values of K_s and $\Gamma_p^\circ f$ into Eq. 7 and thus we obtain the value of $\bar{\nu}$.

Figures 3 and 4 show the binding isotherms for BSA– and Lyz–$C_{12}E_6$ systems, respectively, at various protein concentrations. The confidence limit of protein concentration for estimating the value of $\bar{\nu}$ primarily depends on the value of cmc of detergent alone (cmc°) since the value of cmc° is the maximum concentration of detergent in unbound state. In this work, the confidence range of protein concentration is estimated to be from 10^{-6} to 10^{-4} mol dm^{-3}. It is realized from Figs. 3 and 4 that the bound number explicitly depends upon the protein concentration and decreases with the increase of concentration. The tendency of the concentration dependence is much more remarkable for Lyz–$C_{12}E_6$ than for BSA–$C_{12}E_6$ systems. In BSA–$C_{12}E_6$ systems, the bound number approaches a constant value with an increase in BSA concentration, in other words, the binding isotherms approximately coincide with one another at higher BSA concentrations. In this case, the maximum number of $C_{12}E_6$ bound is about four moles per mole of BSA, which agrees with that for Triton X-100 and BSA systems determined by equilibrium dialysis.[5] The dependence of the bound numbers on protein concentrations was also found for the systems of SDS *vs.* membrane protein, ferricytochrome *c*,[14] which exhibits a similar behavior to that of hydrophilic protein for the interaction with detergents.[15]

It is known that ionic detergents (at lower concentractions) and nonionic detergents bind to the hydrophobic regions of proteins primarily with hydrophobic bonds.[1,5,16] It may be anticipated that the hydrophobic regions of proteins, which are detergent-binding sites, come into contact and are effectively removed by protein-protein interactions within the protein aggregates; this aggregation effect may be enhanced by the increase in protein concentration.[1] In fact, Lyz is known to aggregate in solution, depending on the pH.[17] In addition, protein aggregation may also be favored sterically by detergent binding by way of the flexible hydrocarbon chain. The concentration dependence of the bound number is considered to be somehow due to the fact that detergent-binding sites become effectively hidden.

Just as the systems of BSA and Triton X-100, which were studied by equilibrium dialysis, the binding isotherms for BSA–$C_{12}E_6$ systems obey the Scatchard equation:[18]

$$\bar{\nu}/C_s^f = k_0 n - k_0\bar{\nu}, \qquad (11)$$

where k_0 denotes the intrinsic binding constant and n the maximum number of binding sites. The estimated values of k_0 and the free energies ΔG for $C_{12}E_6$ binding to BSA are listed in Table 1, with BSA concentrations. The mean value of ΔG, -7.2 ± 0.2 kcal mol^{-1}, can be made comparable to the value for the systems of Triton X-100 and BSA, -5.9 kcal mol^{-1}, by taking into account the difference of hydrophobic chain of $C_{12}E_6$ and of Triton X-100.[19] This agreement and the agreement of the maximum number of detergent molecules bound suggest that the surface tension method is not only sound but very advantageous for the estimation of the number of detergent molecules bound to proteins, since the surface tension measurements are more easily performed than the equilibrium dialysis

Table 1. Intrinsic binding constants k_0 and free energies ΔG for $C_{12}E_6$ binding to BSA

BSA concentration / mol dm^{-3}	k_0 / dm^3 mol^{-1}	ΔG / kcal mol^{-1} a)
1×10^{-5}	1.7×10^5	-7.2 ± 0.2
5×10^{-6}	2.3×10^5	-7.4 ± 0.2
1×10^{-5}	2.2×10^5	-7.3 ± 0.2
5×10^{-5}	1.6×10^5	-7.1 ± 0.2

a) 1 cal=4.184 J.

experiments. To establish the advantage of the surface tension method is one of the objectives of this work. The detergent binding for BSA–$C_{12}E_6$ systems means the statistical binding by hydrophobic bonding. However, the binding isotherms for Lyz–$C_{12}E_6$ systems obey the following Freundlich-type equation rather than the Scatchard equation:

$$\bar{\nu}/n = K(C_s^f)^a, \quad 0 \leq a \leq 1. \tag{12}$$

Equation 12 has been derived by assuming an exponential distribution of binding energies, *i.e.*, the heterogeneity of binding sites. This heterogeneity may be caused by the protein aggregation, which is more remarkable for Lyz. In the present case, the protein aggregate consists of several monomers at the most on the average. They may assume various arrangements, since the aggregating force including steric factors is rather weak compared to that for ordinary micelles of detergents. In addition, it is considered that the hydrophobic areas of protein are not completely hidden by the aggregation and thus the residual areas of the aggregate exhibit different geometries owing to the distribution of arrangements and sizes of aggregate. This may reflect the heterogeneity of binding sites. Thus, binding isotherms must be viewed with some caution, as it is probable that detergent-binding sites are effectively hidden by protein-protein interaction.

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