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Reduced lysozyme in solution and its interaction with non-ionic surfactants

Hayahito Nishiyama and Hiroshi Maeda

Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812 (Japan)

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Abstract

Reduced lysozyme at pH 2.5 bound poly(oxyethylene) alkylethers in two steps and the maximum bound amount Q_{max} of the surfactant reached as large as 0.5–0.7 mole per mole amino acid residue in the cooperative binding step. Binding isotherms were well superimposed when surfactant concentrations were normalized by respective values of the critical micelle concentration, *cmc*. In terms of the onset concentrations of the cooperative binding C^*, hydrophobicity of reduced lysozyme was quantitatively defined as RT ln (cmc/C^*) which amounted to 670 J per mole surfactant and was unique to the protein irrespective of the kind of surfactant. Q_{max} could be used as another measure of the hydrophobicity of the protein. The binding isotherms were evaluated by two methods: equilibrium dialysis and surface tension. Their results were consistent with each other and rather complementary. Reduced lysozymes were molecularly dispersed at pH below 2.5 in 0.01 M NaCl but aggregation took place as pH increased. The aggregates could not be dissociated on dilution nor by the addition of nonionic surfactants but by lowering pH. The irreversible nature of the aggregation was reasonably interpreted with a model based on the 'entangled' arrangement of the β-sheets, which could account for the irreversible aggregation of unfolded proteins in general.

Keywords: Reduced lysozyme; Non-ionic surfactants; Binding isotherm; Equilibrium dialysis; Osmotic pressure; Irreversible aggregation

1. Introduction

Characterization of the unfolded state of globular proteins is important to evaluate the stability of their native state, as well as to elucidate the mechanism of the chain folding process. Contribution from the hydrophobic interaction to the stability of a globular protein is expected to be closely related to the hydrophobicity in the unfolded state, which can be assessed in several ways. On the basis of a given amino acid sequence various hydrophobic indices have been proposed [1,2]. However, it is not yet clear which one plays a crucial role, the total hydrophobicity or its average per residue or some clustering of hydrophobic residues along the polypeptide chain. Hence, an experimental approach to the problem is required. Reduced lysozyme has been shown to unfold in the absence of any denaturant and its hydrophobicity is expected not to be high accordingly. Binding of sodium dodecylsulfate on reduced lysozyme has been studied [3,4]. Non-ionic

Correspondence to: H. Maeda, Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812, Japan.

surfactants are known to be much less effective than ionic surfactants as protein denaturants [5]. The interaction of non-ionic surfactants with proteins has been studied on bovine serum albumin [6], histones [7], and some other proteins [8].

Recently, it has been shown that non-ionic surfactant binds to unfolded lysozymes through hydrophobic interaction and induces a conformational change [9]. A measure was proposed for the hydrophobicity in terms of the onset concentration of the cooperative binding. In the present study, the interaction of reduced lysozyme with three non-ionic surfactants was examined with equilibrium dialysis and surface tension. Since the dispersed state of unfolded proteins is an unexplored area, osmotic pressures of solutions of reduced lysozyme were measured under the presence and absence of the surfactants. Aggregation of native lysozyme was also examined.

2. Experimental

Chicken egg-white lysozyme (Sigma, four times recrystallized) was recrystallized twice according to a reported method [10]. Purity of the sample and the complete reduction of disulfide bonds were confirmed with the same procedures as used previously [9]. Oxidation of sulfhydryl groups did not take place after four days incubation in the dialysis experiments. Protein concentration C_p expressed in residue molarity was kept constant at 1 mM throughout the binding and circular dichroism (CD) study and it was determined on the basis of its UV absorbance at 280 nm, i.e. $A_{280} = 25.25$ [11]. For native lysozyme, a value of the extinction coefficient of $\epsilon_{280} = 3.85 \times 10^4\ M^{-1}\ cm^{-1}$ was used [12]. As a solvent, 10 mM NaCl (pH 2.5) was used in the binding measurements. For osmotic pressure and CD measurements, solutions of different values of pH in 10 mM NaCl were used. In some cases, 10 mM glycine buffer was used. Hexa(oxyethylene)decyl ether ($C_{10}E_6$), hexa(oxyethylene)dodecyl ether ($C_{12}E_6$), and octa(oxyethylene)dodecyl ether ($C_{12}E_8$) were purchased from Nikko Chemicals and used without further purification.

Surface tension was measured by the Wilhelmy method with a platinum plate by using a Kyowa CBVP A-3 surface tension meter. Osmotic pressure was measured with a Knauer Electronic Membrane Osmometer with a membrane (Y1244: 5 nm) at 33°C. Solutions of different concentrations were dialyzed against solvent for two to four days before measurement. Circular dichroism (CD) spectra were measured with a Jasco J-600C spectrodichrograph using cells of 2 mm light paths.

Equilibrium dialysis was carried out as previously reported [9]. A dialysis bag containing a solution of about 5 ml [reduced lysozyme (1 mM) + a surfactant (concentration C_{in})] was dipped in a polyethylene bottle filled with about 40 ml of the surfactant solution of concentration C_{out}. The bottles were stored in a thermostat (25.0 ± 0.1°C) for four days with occasional shaking. Four days proved long enough to attain binding equilibrium under the variety of conditions encountered in the present study. Concentration of the surfactant in the outer solution, C_f, was determined from surface tension measurements, as described previously [9]. The bound amount Q, defined as mole surfactant per mole amino acid residue, was calculated according to eq. (1), which states the conservation of mass with respect to the surfactant.

$$C_{out}V_{out} + C_{in}V_{in} = C_f(V'_{out} + V'_{in}) + QC_pV'_{in} \quad (1)$$

Here, $V_{out}(V'_{out})$ and $V_{in}(V'_{in})$ denote, respectively, the volumes of the outer and the inner solutions before (after) dialysis. Protein concentration C_p of the solution after equilibrium dialysis was determined from UV absorption at 280 nm. The main source of error associated with Q originated from C_f, as is evident from eq. (1).

3. Results

3.1 Evaluation of the bound amount from surface tension measurements

Surface tension of $C_{12}E_6$ solutions in the presence of 1 mM reduced lysozyme at 25°C and pH 2.5 is shown in Fig. 1 together with the result in

the absence of the protein (a solid line). According to the Gibbs equation, the dependence of the surface tension γ on the surfactant concentration C is given as eq. (2) with $d\mu_D = RT\ d\ln C$.

$$-d\gamma = \left(\Gamma_D + \Gamma_p \frac{d\mu_p}{d\mu_D}\right) d\mu_D \qquad (2)$$

Here Γ and μ denote the surface excess and the chemical potential, respectively and subscripts D and p refer to the surfactant and the protein, respectively. At low surfactant concentration C, γ is lower in the presence of the protein than at higher C, which indicates that the protein itself is surface active. For native proteins such as lysozyme and bovine serum albumin, this has not been observed [13]. As C increases, protein–surfactant complexes are formed and they counteract the surface tension lowering in a dual way: by consumption of both surfactant and free protein and by the formation of surface inactive complexes. When C decreases further, γ decreases almost linearly with log C, but the slope is less steep than that in the absence of the protein. The latter corresponds to the maximum surface excess.

A smaller (in magnitude) slope indicated that the second term between the parentheses of eq. (2), which is negative since $d\mu_p/d\mu_D < 0$, was significant. However, since the difference was small, we tentatively assumed it to be negligible. The bound amount Q, then, can be calculated from eq. (3).

$$Q = (C - C_f)/C_p \qquad (3)$$

Here C_f is given by the concentration where the reference curve ($C_p = 0$) indicates the same γ value as the actual solution of C and C_p. This method in terms of eq. (3) is a simplified version of a more sophisticated approach [13].

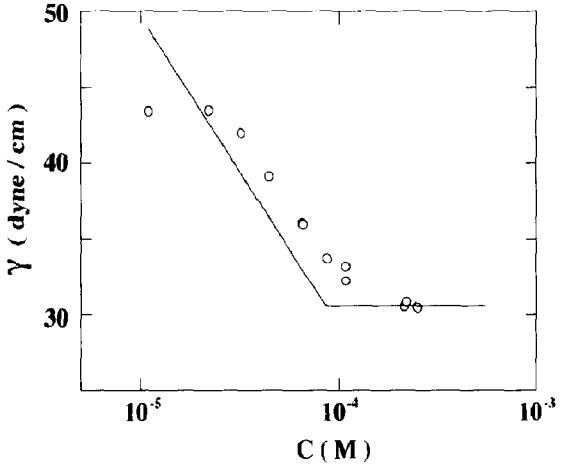

Fig. 1. Surface tension γ of $C_{12}E_6$ solutions in the presence of 1 mM reduced lysozyme. C: $C_{12}E_6$ concentration. A solid line represents the results in the absence of the protein.

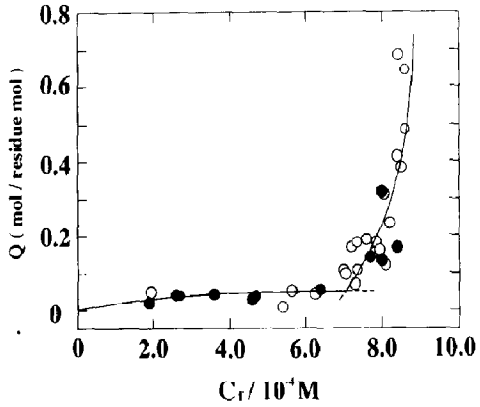

Fig. 2. Binding isotherm of $C_{10}E_6$ at 25°C. Open (filled) symbols represent the results from equilibrium dialysis (surface tension) method.

3.2 Binding isotherms

The binding isotherm of $C_{10}E_6$ is given in Fig. 2 and obtained from two methods: equilibrium dialysis and surface tension. Agreement between the results from both methods is good. This shows that the approximations employed in the surface tension method are reasonable and that equilibrium dialysis as performed properly. It was found that the two methods were complementary; the surface tension method performed in the low binding region while equilibrium dialysis did so in the high binding region.

In Fig. 3, the binding isotherm of $C_{12}E_6$ is shown. The filled circles refer to the data obtained from surface tension measurement in the present study, while the open circles refer to the previous data from equilibrium dialysis [9].

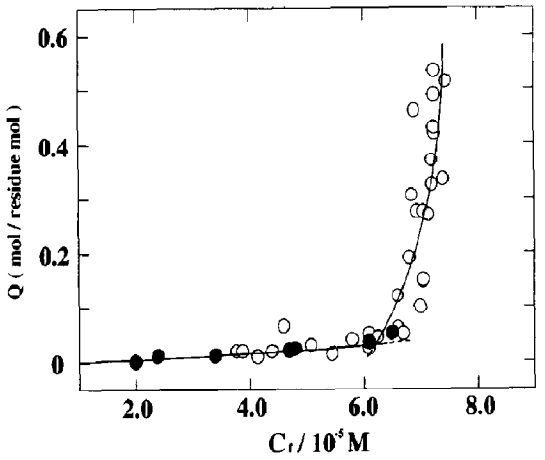

Fig. 3. Binding isotherm of $C_{12}E_6$ at 25°C. Open and filled symbols refer to the previous data from the equilibrium dialysis and the present data from the surface tension, respectively.

In Fig. 4, binding isotherm of $C_{12}E_8$ obtained from equilibrium dialysis is shown. For a series of solutions in which the initial surfactant concentration were higher than the critical micelle concentration *cmc*, the results are represented by the open circles. In the range of Q smaller than about 0.2, they collapsed onto the filled circles of the differently prepared solutions. In the range of Q larger than 0.2, values of Q did not correlate with the initial concentrations. These results indicate that micelles dissociated readily in the time

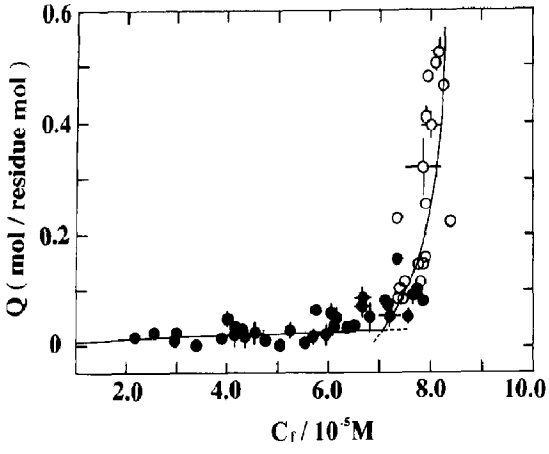

Fig. 4. Binding isotherm of $C_{12}E_8$ at 25°C obtained from the equilibrium dialysis. Open (filled) symbols refer to the data where the initial surfactant concentrations of the inner solutions were higher (lower) than the *cmc*.

scale of the present experiments. Similar results were obtained on other two surfactants (not shown).

The binding isotherms are characterized with two regions just as found on $C_{12}E_6$ previously [9]. The first weak binding step was analyzed in terms of the Langmuir type of isotherm.

$$Q = bC_f/(1 + aC_f) \tag{4}$$

while the second strongly cooperative step was analyzed by means of the Freundlich type of isotherm.

$$Q = A'C_f^n \tag{5}$$

In terms of the onset concentration C^* for the second cooperative step, we have proposed a measure of the hydrophobicity of a given protein by $RT\ \ln(cmc/C^*)$ [9]. In the present study, we define C^* as the point of intersection of two regimes. First, we define a set of A' and n from eq. (5) by the least squares method based on the data corresponding to high Q values. Best fits were found for the data corresponding to C_f higher than about 0.8 *cmc*: 6.5×10^{-5} M, 7.5×10^{-5} M and 7.5×10^{-4} M for $C_{12}E_6$, $C_{12}E_8$ and $C_{10}E_6$, respectively. Then, we define a set of a and b from eq. (4) by taking the least squares fit of the remaining data. In this way C^* can be determined uniquely and unambiguously from a given isotherm. In Figs. 2–4, the fitted curves are indicated. They deviate from experimental points definitely in the Freundlich regime. This is partly because the absolute error in C_f is not the same for each point since it was evaluated from log C_f. The deviation suggests, on the other hand, the data in the cooperative binding step is described with eq. (5) only approximately. Note that eqs. (4) and (5) were used in the present study just to represent analytically the two parts of the isotherm and hence are of no further significance. Binding isotherms of the three surfactants can be superimposed if their free concentrations are normalized by their respective *cmc* values, as shown in Fig. 5. The scaling works well and the isotherm of the cooperative binding step can be expressed as

$$Q = A(C_f/cmc)^n \tag{6}$$

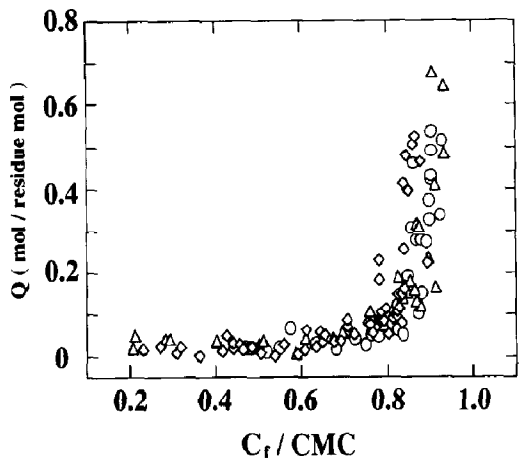

Fig. 5. A universal binding isotherm for the three surfactants at 25°C and pH 2.5. Concentrations are normalized by respective cmc values. (○) $C_{12}E_6$, (△) $C_{12}E_8$ and (◇) $C_{10}E_6$.

Values of A and n were 2.03 and 14.9, respectively.

The free energy of transfer ΔG^0 per mole of surfactant from the micelle-like domain within the protein–surfactant complex to its micelles can be defined as

$$\Delta G^0 = RT \ln(cmc/C_f^*) \quad (7)$$

An average value of 669 ± 128 J mol^{-1} (160 ± 33 cal mol^{-1}) was determined common to the three surfactants used. In this way, a unique measure for the hydrophobicity of reduced lysozyme is obtained irrespective of the kind of surfactant. Results of the analysis are summarized in Table 1.

According to the Freundlich isotherm, the maximum bound amount has little significance. Experimentally, however, Q_{max} did not vary very much but converged within a certain range for three surfactants: 0.5 for $C_{12}E_6$ and $C_{12}E_8$ and 0.7 for $C_{10}E_6$. The convergent value (0.5–0.7) could serve as another measure for the hydrophobicity of the protein.

3.3 Dispersed state of reduced lysozyme at low pH

Characterizations of reduced lysozyme in solution have been carried out [11,14,15] but its dispersed state is not yet well understood. Aggregation of reduced lysozymes at pH higher than 2.5 was found when osmotic pressures of the solutions were measured, as shown by the filled symbols in Fig. 6. These solutions were prepared by dilution from rather concentrated stock solutions (5–10 g/L: 45–90 mM) at the same pH and dialyzed against the solvent for two to four days. Aggregation number increased with pH as follows: 1 (no aggregates) at pH 2.29, 1.1 at pH 2.80, and 3.6 at pH 3.54. Second virial coefficients B_2 of these solutions were negligibly small, not only for a solution free from aggregation, but also for those containing aggregates. The results are puzzling. For the latter solutions (pH 2.80 and 3.54), it implies that they are ideal solutions of aggregates. In other words, the aggregates do not dissociate on dilution. For the former solution (pH 2.29), a significant B_2 is expected because the protein carries about 27 net charges and the ionic strength of the medium is low. Indeed, apo-cyto-

Table 1

Summary of binding isotherms at 25°C

Parameter	Reduced lysozyme		
	$C_{12}E_6$	$C_{12}E_8$	$C_{10}E_6$
cmc	$(8.0 \pm 0.5) \times 10^{-5}$ *M*	$(9.4 \times 0.4) \times 10^{-5}$ *M*	$(9.2 \pm 0.2) \times 10^{-4}$ *M*
C^*	$(6.2 \sim 6.3) \times 10^{-5}$ *M*	$(7.0 \sim 7.1) \times 10^{-5}$ *M*	$(6.9 \sim 7.0) \times 10^{-4}$ *M*
ΔG^0	(100 ~ 190) cal mol^{-1}	(140 ~ 200) cal mol^{-1}	(140 ~ 190) cal mol^{-1}
	(430 ~ 780) J mol^{-1}	(590 ~ 830) J mol^{-1}	(620 ~ 770) J mol^{-1}
A'	1.3×10^{63}	1.6×10^{76}	1.3×10^{32}
n	15	19	11
a	-8.5×10^3	1.9×10^4	4.8×10^3
b	2.9×10^2	8.0×10^2	2.9×10^2

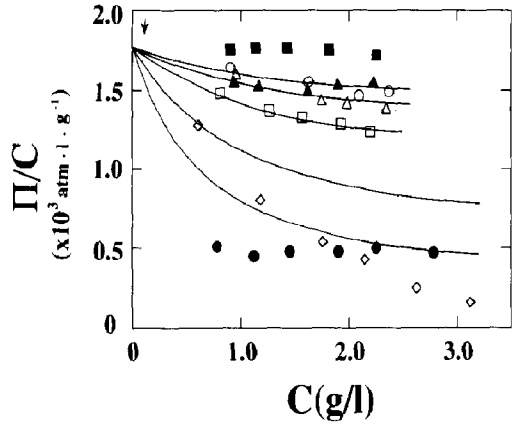

Fig. 6. Osmotic pressures of reduced lysozyme solutions at 33°C. Filled symbols refer to the solutions prepared by dilution at a constant pH from concentrated stock solutions. pH: 2.29 (■), 2.80 (▲) and 3.54 (●). Open symbols refer to the solutions under association equilibrium. pH: 2.46 (○), 2.94 (△), 3.28 (□) and 3.95 (◇). Upper three curves are drawn according to eq. (8). Lower two curves are drawn according to the infinite isodesmic model with the association constants of $1.2\times10^4\ M^{-1}$ (upper) and $4.7\times10^4\ M^{-1}$ (lower). An arrow represents the condition under which binding isotherms were obtained.

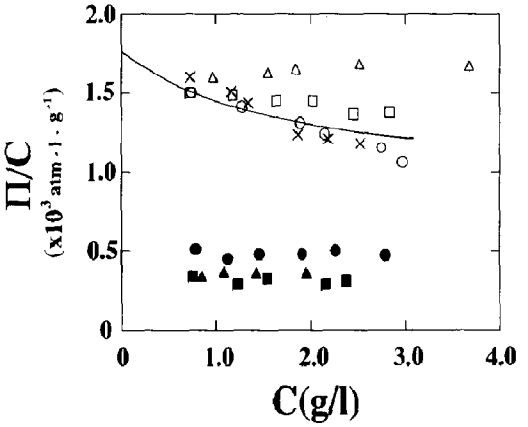

Fig. 7. Osmotic pressures of solutions of reduced and native lysozyme in the presence of surfactants at 33°C. Native lysozyme (pH 7.1–7.3): without surfactant (○), $C_{12}E_6$ (10^{-5} M): 6.2 (×) and 6.6 (△), $C_{10}E_6$ (10^{-4} M): 6.1 (□). A solid curve is drawn according to the infinite isodesmic model with the association constant of $3.46\times10^3\ M^{-1}$. Reduced lysozyme with $C_{12}E_0$ (10^{-5} M): 0 (●, pH 3.54), 6.6 (▲, pH 3.82) and 6.9 (■, pH 3.4).

chrome c at pH 3.1 showed a positive B_2 (8.34×10^{-6} mol L g^{-2}) under similar solvent conditions [16].

When solutions of different concentrations were prepared first at pH 2.2, where no aggregation took place, and then were brought to final values of pH, the extent of aggregation regularly decreased with concentration as shown in Fig. 6 by the open symbols. The concentration dependence of the osmotic pressure Π can be well described, except for that at pH 3.95, in terms of dimerization (eq. 8).

$$\Pi/cRT = \frac{1}{M_n} = \frac{4Kc/M_1 - 1 + \sqrt{1 + 8Kc/M_1}}{8Kc} \tag{8}$$

Here, K, c, and M_1 represent, respectively, the dimerization constant (in molarity scale), protein concentration in g/L and monomer molecular weight. Upper three solid curves are drawn according to eq. (8). Dimerization constants K and K_2 (in mole fraction scale) and the corresponding standard free energy changes (per mole of dimer) are given in Table 2. For a solution at pH 3.95, results of the analysis in terms of an isodesmic infinite association are shown (association constant 1.2×10^4–$4.7\times10^4\ M^{-1}$). Deviation at high concentrations is significant, which suggests that aggregates become more stable at higher concentrations. An n-mer–monomer equilibrium could not explain the results in the range of n between 11–20.

Note that under the conditions where the binding isotherms were obtained (C_p = 1 mM, 10 mM NaCl, pH 2.5: indicated by an arrow in Fig.

Table 2

Assumed dimerization constant of reduced lysozyme at 33°C

pH	K (M^{-1})	K_2 [a]	$\Delta G'$ (kcal mol^{-1})
2.46	1.73×10^3	9.60×10^4	−6.98
2.94	3.25×10^3	1.80×10^5	−7.36
3.28	8.49×10^3	4.71×10^5	−7.95

[a] K_2 is evaluated on mole fraction basis.
[b] $\Delta G' = -RT \ln K_2$.

6), practically no aggregation took place. Changes in CD and fluorescence spectra of reduced lysozyme associated with the surfactant binding found in the previous study [9] now can be interpreted in terms of the conformational change of isolated polypeptide chains. This was suggested in [9] but could not be unambiguously concluded.

Effects of non-ionic surfactants were examined on both native lysozyme at neutral pH and reduced lysozyme at acidic pH. As shown in Fig. 7, aggregates of native lysozyme dissociated on dilution. The presence of non-ionic surfactants effectively blocked the aggregation although the effect critically depended on the surfactant concentration: for $C_{12}E_6$ 6.2×10^{-5} M was ineffective, but 6.6×10^{-5} M was effective. Thus native lysozymes weakly aggregate through hydrophobic interaction. A curve drawn according to the isodesmic infinite association model (association constant 3.46×10^3 M^{-1}) is shown in Fig. 7, which describes the experimental data rather well. In the case of reduced lysozyme, on the other hand, the addition of $C_{12}E_6$ did not induce the dissociation of the aggregates.

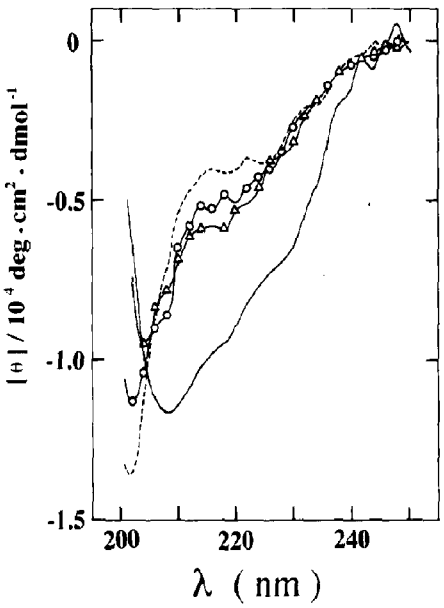

Fig. 8. Effect of the way of preparing the solution on the CD spectra of reduced lysozyme. Solution A (pH 4.0, ○): solution B (pH 3.8, △). For comparison. CD spectra of reduced lysozyme at pH 2.5 (- - - - -) and native lysozyme at pH 7.0 (———) are shown.

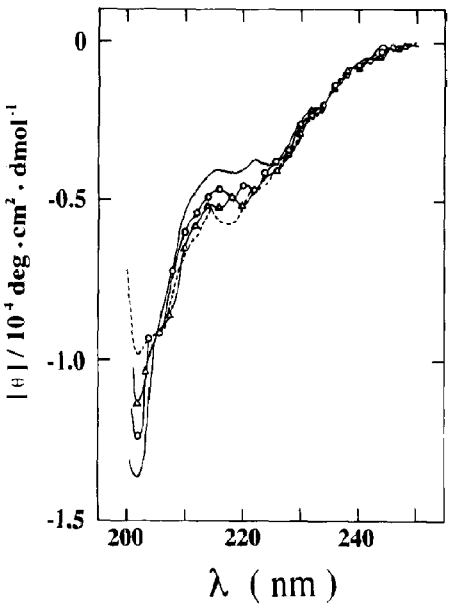

Fig. 9. CD spectra of reduced lysozyme at different pH. pH: 2.22 (———), 3.49 (○), 3.99 (△) and 5.09 (- - - - -).

We prepared two solutions of similar compositions ($C_p = 1$ mM, pH 3.8–4.0) by two different ways: solution A from unaggregated state (pH 2.2) by adding NaOH, solution B by dilution of a concentrated solution ($C_p = 45$ mM, pH 3.5). Small but significant differences between the CD spectra of the two solutions shown in Fig. 8 is consistent with the expectation that the β-sheet content is greater for solution B than solution A. However, it is not easy to assess the β-sheet content quantitatively from CD spectra in the present case. The small difference suggests that the irreversible behavior will disappear considerably at such a low concentrations of 1 mM. Figure 9 shows the CD spectra of solutions ($C_p \sim$ 1 mM) at various pH. The spectra indicate that conformation of reduced lysozyme changes with pH.

4. Discussion

4.1 The maximum bound amount Q_{max} and mode of binding of surfactants

It should be noted that Q_{max} need to be evaluated on the basis of level-off values which can

only be obtained at concentrations above *cmc*, i.e. where neither the equilibrium dialysis nor the surface tension method applies. We are therefore obliged to adopt the experimentally found maximum values as Q_{max}. On weight basis, values of Q_{max} (g/g protein) range from 2.0–2.2, 2.4–2.7 and 2.5–2.7 for $C_{12}E_6$, $C_{12}E_8$ and $C_{10}E_6$, respectively. These values are comparable with or greater than the maximum bound amount of sodium dodecylsulfate (SDS) to unfolded proteins (g/g protein): i.e. 1.4 [3,17], 1.1–2.2 [18], and 1.2–1.5 [19], respectively.

The maximum numbers of bound surfactants per lysozyme (129 residues) are about 65 for $C_{12}E_6$ and $C_{12}E_8$ and 90 for $C_{10}E_6$, while the micelle aggregation numbers m are 400 ($C_{12}E_6$) [20], 123 ($C_{12}E_8$) [21] and 73 ($C_{10}E_6$) [20]. Convergent Q_{max} values are in contrast with divergent m values. This suggests that the mechanism controlling Q_{max} considerably differs from that based on the geometric packing consideration which affects the shape and size of micelles [22]. Comparison between Q_{max} and m also indicates that there is only one surfactant cluster on the polypeptide chain and hence the binding most likely takes place in the all-or-none manner. When the hydropathy indices of Kyte and Doolittle [1] are averaged over ten (or fifteen) successive residues, there are four (or three) hydrophobic clusters along the polypeptide chain. Of them, the region consisting of 21 residues, ^{75}Leu to ^{95}Ala, is likely the site for the cooperative binding *.

4.2 Applicability of the present approach to other proteins

The applicability of the present approach is limited to those proteins which unfold, by proper chemical modifications, in the medium where the solvent condition is least altered from that favoring their native folded state. The interaction of the parent protein with solvent should be least perturbed by the modifications. Reduction of disulfide bonds and the depletion of prosthetic groups are believed to satisfy this requirement. Unfolded proteins may aggregate depending on pH but this is not serious if the binding of surfactants prevents the aggregation.

It is appropriate to compare the present result with that on apo-cytochrome c–$C_{12}E_6$ interaction [16]: $\Delta G^0 = 301$ J mol^{-1} (72 cal mol^{-1}) and $Q_{max} = 0.25$. For reduced lysozyme and apo-cytochrome c, the total Kyte–Doolittle hydropathy indices [1] are -60.9 and -93.8 and its averages are -0.47 and -0.90, respectively. (The index is more positive if more hydrophobic.) Our measure (ΔG^0 or Q_{max}) not only defines uniquely the hydrophobicity of a given protein but also it is consistent with the Kyte–Doolittle index. Our results, however, are still insufficient to judge which one plays an important role in the interaction with non-ionic surfactants: the total hydrophobicity or its average or some clustering of hydrophobic residues.

4.3 A model to account for the irreversible aggregation

The characteristics of the aggregation of reduced lysozymes are summarized as (1) irreversible, (2) the hydrophobic interaction is not of first importance, and (3) β-sheet content is not high. These can be accounted for by introducing an idea of a particular arrangement of the β-sheet, which we tentatively name 'entangled' arrangement. A β-sheet in this arrangement consists of strands where most or many adjacent strands are not connected directly in contrast to the meander type β-sheet where adjacent strands are directly connected through tight bends or loops. The rate of dissociation of a polypeptide chain from an aggregate of 'entangled' arrangement, schematically depicted in Fig. 10(a) is expected to be very small. At high concentrations, various types of aggregates are formed: some of them are rather simple as shown in Fig. 10(b) but some are rather complex as shown in Fig. 10(a). Simple aggregates dissociate on dilution while complexed ones do not. Beyond some extent of dilution most simple aggregates disappear and we have an ideal solution of complex aggregates. The

* This region includes ^{87}Asp which is considered to be uncharged at pH 2.5.

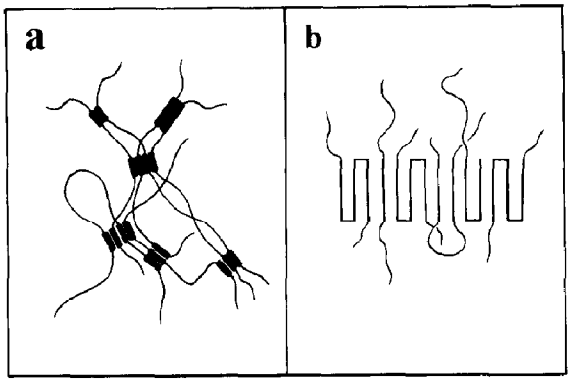

Fig. 10. Schematic drawing of two examples of β-sheet aggregates. (a) 'entanglement' arrangement and (b) a simple aggregate consisting of meander types and extended chains. (See text).

solution has lower entropy and enthalpy but higher free energy than the equilibrium solution of the same composition. As pH decreases, the increased net charge enhances the dissociation rate. The 'entangled' arrangement is expected to account for the irreversible aggregation of unfolded proteins in general such as encountered in the inclusion bodies.

It should be pointed out that exactly the same irreversible behavior has been observed for the aggregation of extended polypeptide chains to form β-sheets [23,24]. The slow or blocked dissociation has been proved to be responsible for the irreversible behavior in this case [24].

It is pertinent to refer to a possible contribution of this 'entangled' arrangement to the stability of the β-sheet in the native proteins. Consider, for example, two arrangements consisting of two equivalent β-sheets; one is of 'entangled' arrangement and the other is composed of two meander type β-sheets connected by a short chain. When one of the two β-sheets in both arrangements is disintegrated by any chance, the corresponding entropy gain is much smaller for the 'entangled' arrangement than the other one. In other words, the intermediate state in the former is much less favorable than in the latter. This introduces a cooperativity for the change from the intact state to the fully disordered random coil and the stability of the intact state in 'entangled' arrangement increases as a result of this cooperativity. It is thus reasonable that the 'entangled' arrangement of β-sheets is frequently found in proteins such as concanavalin A [25] and immunoglobulin [26].

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