The Binding of Sodium Dodecyl Sulfate to Lysozyme in Aqueous Solutions

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The binding mode of sodium dodecyl sulfate to lysozyme and the accompanying structural change of lysozyme by binding have been investigated by means of the binding isotherm, the precipitation curve, and the CD spectra in pure water, NaCl, and borate buffer solutions. The precipitation phenomena could be explained in terms of the neutralization of the net charge of lysozyme due to dodecyl sulfate-ion binding. The analysis of the binding isotherms by the use of the BET equation gave the site number of the first layer corresponding to the positively charged residues at the pH studied. The conformational change from the β-structure to the α-helix has been observed in the second-layer binding. The environmental change of the side-chain residues has also been observed.

It has been well known that the denaturation of protein is caused or influenced by many factors, such as heat, the pH, and the addition of inorganic or organic substances.[1] Above all, it is interesting that most ionic surfactants behave uniquely as strong denaturants for proteins.[1-3] The addition of a surfactant to a protein solution causes a drastic change in the conformation of protein. Even in a very low surfactant concentration (far below the critical micelle concentration, CMC), the protein is denatured by surfactant molecules. For instance, the extent of the conformational change of serum albumin increases with the surfactant concentration, but it reaches a certain limit at a concentration close to 10^{-5} mol dm^{-3}.[4]

With respect to the hen egg-white lysozyme, an X-ray study revealed that the lysozyme is a rigid and stable enzyme.[5] In the range of physiological pH values, the lysozyme does not show any detectable change in its structure up to 77 °C, and at the physiological temperature no detectable change in the structure was observed with a pH change from 1.2 to 11.3. The stability of lysozyme has been attributed to the four disulfide bonds besides hydrogen bonds and hydrophobic interactions among the 129 amino-acid residues. Thus, it is supposed that the lysozyme molecule may be comparatively resistant to the addition of a surfactant.

The precipitation and the inactivation of the enzyme occurred when a surfactant mixture of sodium dodecyl sulfate (SDS) and sodium tetradecyl sulfate (STS) was added to the lysozyme. The mixing ratio of SDS to STS was 80 to 20.[6] In the past, these phenomena were interpreted in terms of the weight-mixing ratio of surfactant to protein. However, these experimental results should be interpreted with reference to binding isotherms.

In a study of the binding of SDS to various proteins including lysozyme, it was found that as much SDS bound to a protein as the weight of the protein itself,[7] and it was suggested, from the solubilizing ability of the SDS–protein complex for water-insoluble dye, that SDS formed a so-called hemimicelle containing a polypeptide as a core. We are interested in what type of hemimicelle is formed in the lysozyme–SDS system. Fortunately, the structure of lysozyme has been well defined, so that lysozyme may give some detailed information about the interaction.

In this work, the binding of SDS to lysozyme, and the precipitation and structural change upon binding are investigated as a function of the concentrations of SDS and the added NaCl, and the pH by means of circular dichroism (CD) measurement and potentiometry.

Experimental

Materials. Hen egg-white lysozyme (6 times recrystallized) was purchased from the Seikagaku Kogyo Co., Ltd., and was used without further purification. The SDS was synthesized from the purified 1-dodecanol according to the literature.[8] The product was purified by recrystallizing it three times from ethanol. The CMC of the SDS was determined by electric-conductivity measurements. The value of 8.28×10^{-3} mol dm^{-3} at 25 °C was consistent with that in the literature.[9] There was no minimum in the plot of the surface tension *vs.* the concentration. All the inorganic salts (Nakarai Chemical Co., Ltd.) were of a special grade and were used without further purification.

Precipitation Measurements. An aqueous solution of lysozyme and one of the SDS solution were mixed in a test tube, and then water was added to make a 25-ml solution. Throughout the series of experiments, the concentration of lysozyme in the test tube was kept constant. After the samples had been allowed to stand at 25 °C for over 20 h, the precipitates were separated through a membrane filter (pore size, 0.1 μm; Toyo Roshi, Co., Ltd.) The concentration of lysozyme in the filtrate was determined spectrophotometrically at 280 nm (Model 323 of Hitachi Mfg. Co.) using the molar extinction coefficient of 36500 for lysozyme.[10] The amount of protein precipitated was evaluated by comparing the absorbance of the filtrate with that of the reference solution without SDS.

Binding Isotherms. The binding isotherms of SDS to lysozyme were constructed by the determination of the equilibrium SDS concentration by means of potentiometry.[11] The electromotive force (emf) of the cell with a liquid membrane was measured by means of a digital multimeter(Takeda Riken TR 6856). The temperature of the solution in the cell was kept within 25 ± 1 °C using a thermostated water bath. The corresponding calibration curves of emf for SDS in pure water, NaCl, and buffer solutions showed a good linearity and gave a slope consistent with the Nernstian response (59.4 ± 0.5 mV per decade of change in SDS at 25 °C). After the mixed solutions of lysozyme and SDS had been allowed to stand for 1 d at 25 °C, the emf was measured. The concentration of lysozyme in the sample solution was constant (2×10^{-5} mol dm^{-3}) throughout the series of experiments. The coexistence of lysozyme in the solution has been reasonably assumed not to affect the response for the DS^- ion, and so the

change of emf should be ascribable specifically to the change in the free-DS^--ion concentration in solution.

The average number of SDS molecules ($\bar{\nu}$) bound to a lysozyme molecule can be given as:

$$\bar{\nu} = (C_t - C_e)/C_l, \quad (1)$$

where C_t is the total SDS concentration, C_e is the equilibrium SDS concentration, as determined from the curve of emf *vs.* the concentration, and C_l is the lysozyme concentration.

Circular Dichroism (CD). The CD spectra in the ranges of 240—200 nm and 320—270 nm were measured by the use of a spectropolarimeter (JASCO-A40) under nitrogen flush. Calibration was made with a D-10-camphorsulfonic acid solution. The path length of the cell used was 10 mm. The temperature was controlled at 25±0.1 °C by circulating thermostated water through the cell.

The CD spectra were obtained by accumulating 4 times for 240—200 nm and 8 times for 320—270 nm, with a microproccessor equipped with a spectropolarimeter, and their mean values were recorded. The data were expressed in terms of the mean residue ellipiticity.

All samples were equilibrated in a water bath at 25 °C for 1 d, and then the CD spectra were measured. The concentrations of lysozyme were 1.2×10^{-6} mol dm^{-3} and 2.7×10^{-5} mol dm^{-3} in the ranges of 240—200 nm and 320—270 nm respectively.

Results

Figure 1 shows the binding isotherm of SDS to lysozyme as well as the precipitation curve at two lysozyme concentration in pure water. The $\bar{\nu}$ was calculated according to Eq. 1. In the lower equilibrium concentration (4×10^{-5}—9×10^{-4} mol dm^{-3} SDS), the $\bar{\nu}$ is about 10, while in the range higher than 9×10^{-4} mol dm^{-3} the $\bar{\nu}$ gradually rises to about 20, and then it reaches a certain limit of measurement, *i.e.*, the upper limit of the linearity in the calibration curve of emf.

The shape of the precipitation curve of lysozyme depends on the lysozyme concentration when the total concentration of SDS, C_t, is taken as the abscissa. However, the data can be replotted against the equilibrium SDS concentration, C_e, which is calculated by using the binding isotherm. As is shown in Fig. 1, the precipitation curves at two different lysozyme concentrations coincide with each other when C_e at equilibrium is taken as the abscissa. It can be seen from Fig. 1 that the amount of the precipitate increases parallel with the binding, and thereafter it is kept constant (*i.e.*, the lysozyme is almost completely precipitated) when $\bar{\nu}$ is about 10. In the range of the binding number of 10, the lysozyme keeps being precipitated almost completely. With an increase in $\bar{\nu}$ from 10 to 20, the precipitation curve goes down to zero.

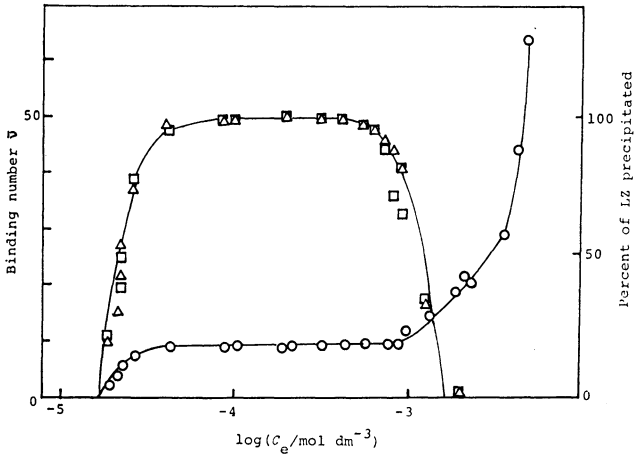

Fig. 1. The binding isotherm of SDS (○) and precipitation curve of lysozyme (LZ) in pure water (pH 5.8) at 25 °C. △: Lysozyme concentration 3.76×10^{-5} mol dm^{-3}, □: lysozyme concentration 2.19×10^{-5} mol dm^{-3}.

For the NaCl solution system whose ionic strength is 0.1, as may be seen in Fig. 2, the binding isotherm begins to rise at $C_e=1\times10^{-4}$ mol dm^{-3}. The region where the binding number is kept at 10 is very narrow as compared with Fig. 1. After reaching a $\bar{\nu}$ value of about 13, the binding isotherm is characterized by a sharp uprise. The precipitation (nearly 100%) is seen in the same manner as in a pure water system when $\bar{\nu}$ is also about 10. With an increase in $\bar{\nu}$ from 10 to 20, the amount of precipitate also goes down to zero. The region of the maximum precipitation is narrow, corresponding to the narrowness of the region where $\bar{\nu}=10$.

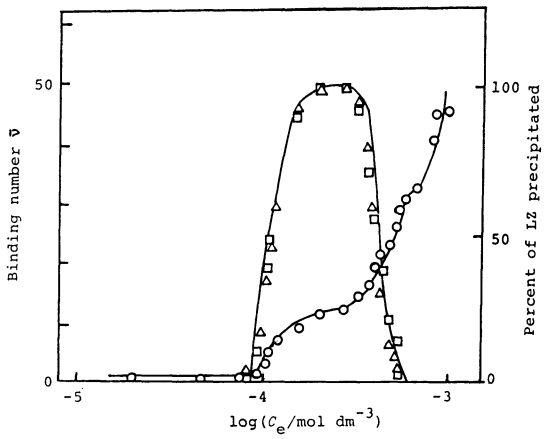

Fig. 2. The binding isotherm of SDS (○) and precipitation curve of lysozyme in NaCl solution (pH 5.8) at 25 °C. △: Lysozyme concentration 4.20×10^{-5} mol dm^{-3}, □: lysozyme concentration 2.56×10^{-5} mol dm^{-3}.

Figure 3 shows the binding isotherm and the precipitation curves in a borate buffer system, the pH and the ionic strength of which are 9.2 and 0.1 respectively. The binding isotherm shows a gradual rise in the range of 1×10^{-4}—1×10^{-3} mol dm^{-3} SDS up to the binding number of 35, and then it shows an abrupt increase up to 55, which seems to be followed by a levelling-off tendency near the CMC. Each precipitation curve has a maximum at $\bar{\nu}=9$. In this case, 100% precipitation does not occur, and the amount of the precipitate depends on the lysozyme concentration, unlike the case in Figs. 1 and 2.

The α-helix, β-structure, and random coil of proteins each have their own characteristic SDS spectra. The CD spectra of synthetic polypeptides are changed by various factors, such as the pH, the solvent, the temperature, and the addition of a surfactant, and have been used as the criteria of the α-helix, the β-structure, and the random coil.[12-15] However, the magnitudes of the CD spectra do vary among different helical, β-structured, and random-coiled polypeptides. In this work, we

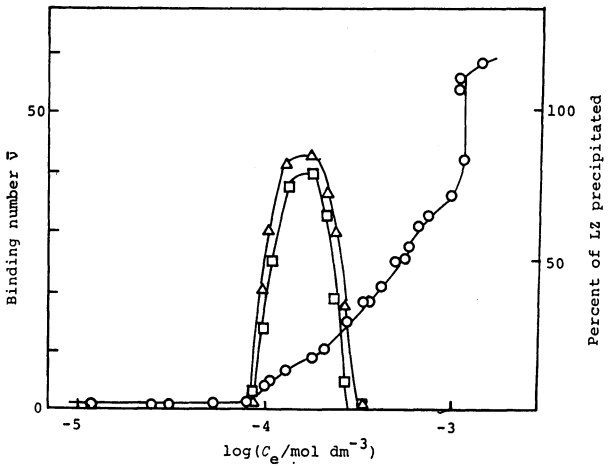

Fig. 3. The binding isotherm of SDS (○) and precipitation curves of lysozyme in borate-buffer solution (pH 9.2) at 25 °C. △: Lysozyme concentration 3.95×10^{-5} mol dm^{-3}, □: lysozyme concentration 2.70×10^{-5} mol dm^{-3}.

applied the criteria of the α-helix, the β-structure, and the random coil, which had been deduced from the CD spectra of 5 proteins (myoglobin, lysozyme, lactate dehydrogenase, papain, and ribonuclease) as analysed by means of X-rays.[16] The optical activity of the above three conformations is assumed to be additive and can be expressed as described below.

At any wavelength, λ,[16]

$$[\theta]_\lambda = f_H X_{H,\lambda} + f_B X_{B,\lambda} + f_R X_{R,\lambda}, \quad (2)$$

where $f_H+f_B+f_R=1$. $[\theta]$ is the mean residue ellipticity. The f is the fraction of each conformation in a protein molecule. The $X_{H,\lambda}$, $X_{B,\lambda}$, and $X_{R,\lambda}$ are the mean ellipticities for the helix (H), the β-structure (B), and the random coil (R) at a certain wavelength λ, respectively. The f_H, f_B, and f_R were determined by fitting the CD spectra of lysozyme to Eq. 2. The calculation by Eq. 2 was made by the least-squares method at 3 nm intervals from 240 to 200 nm. Figure 4a shows the typical CD spectra of lysozyme alone and those of lysozyme saturated with SDS in a pure-water system. The results calculated for lysozyme alone are consistent with those deduced from X-ray study.[5] In Fig. 5, we can see the correlation of the binding isotherm with the fraction of each conformation in the borate-buffer system. Below the concentration at which SDS binding begins to occur, none of the content of the conformations was affected by adding SDS. The dashed line shows the precipitation region where CD spectra can not be observed. The fractions of the α-helix and the β-structure change above the concentration where the redissolution of lysozyme takes place. With an increase in $\bar{\nu}$, the fraction of the α-helix increases, but that of the β-structure decreases. The fraction of the random coil is almost constant over the whole range of equilibrium concentrations of SDS. Similar results were also obtained in pure-water and NaCl-solution systems.

The typical near-ultraviolet CD spectra of lysozyme alone and that saturated with SDS in a pure-water system are shown in Fig. 4b. The change in the CD spectra in the near-ultraviolet region is more drastic than

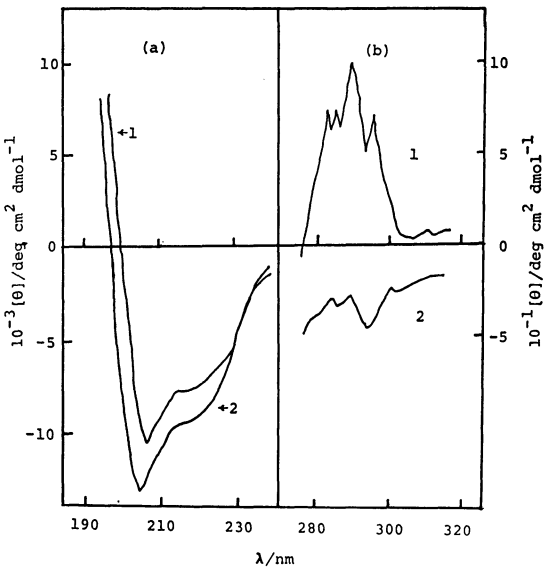

Fig. 4. The effect of the addition of SDS on CD spectrum of lysozyme (1) in pure water, (2) in SDS solution 1.03×10^{-2} mol dm^{-3}.

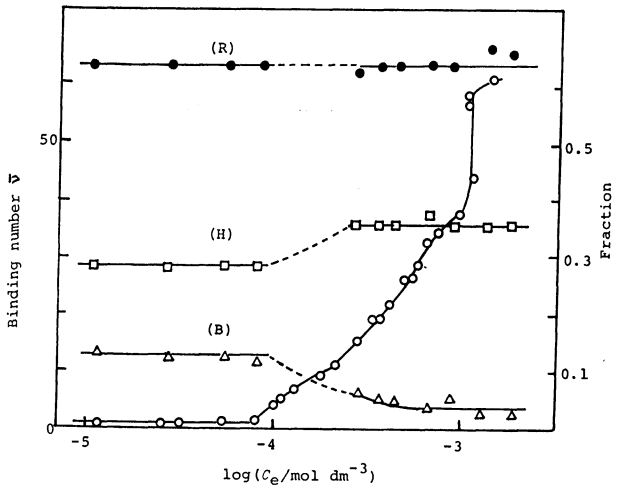

Fig. 5. The correlation of binding isotherm with the fraction of three conformations in borate-buffer solution. (H), (B), and (R) indicate the fractions of α-helix, β-structure, and random coil, respectively.

that in the far-ultraviolet region. Figure 6 shows the binding isotherm and the corresponding change in $[\theta]$ at 294 nm in a NaCl solution. The $[\theta]_{294}$ of the redissolved lysozyme solution is negative in contrast with that of a lysozyme solution without binding. In both pure-water and borate-buffer solutions, the corresponding plots showed the same tendency as that in a NaCl solution.

Discussion

The solubility of protein in an aqueous solution is on a critical balance between the net charge and the hydrophobicity. The net charge effect on the solubility of protein is typically demonstrated in the case of the precipitation at the isoelectric point. As is shown in Figs. 1—3, the binding number which gives the maximum

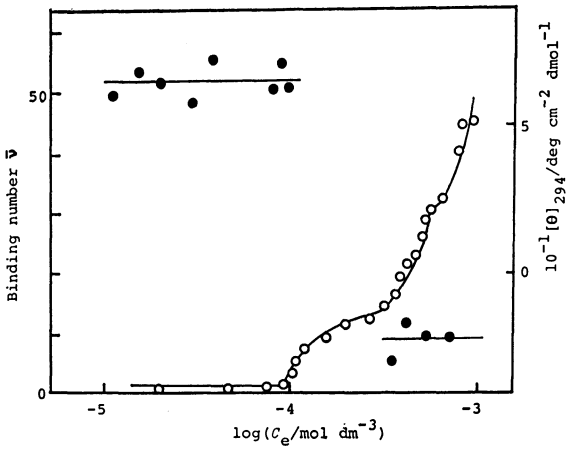

Fig. 6. The change of $[\theta]_{294}$ due to SDS binding in NaCl solution. (○): Binding isotherm, (●): $[\theta]_{294}$.

precipitation is about 10 in each case. If the change in the net charge of lysozyme caused by the binding of DS^- ion can be estimated, it must give a means of interpreting the precipitation.

The lysozyme molecule has ionizable residues (*i.e.*, 6 Lys's, 11 Arg's, and 1 His) as basic amino acid residues and 7 Asp's and 2 Glu's as acidic residues. At the pH's studied (pH 5.8 for pure water and a NaCl solution; pH 9.2 for a borate buffer solution), Lys and Arg are positively ionized, since their pK values on lysozyme are within the ranges of 10.3—10.6 and 12.7—13.3[17] respectively. This means that the lysozyme molecule has 17 positive charges. On the other hand, the negatively ionized residues are 7 Asp's and one Glu^7 in pure water and the NaCl solution system, since their pK values are within the ranges of 3.0—4.7 and 2.7—4.7[15] respectively. In the borate buffer solution of pH 9.2, Glu^{35}, whose pK is between 6 and 6.5,[17] is also ionized besides 7 Asp's and one Glu^7. Therefore, the value of the net charge of lysozyme is +9 in the solutions with and without NaCl (+17−8=+9) and +8 in the borate-buffer solution (+17−9=+8). The number of binding which gives the maximum precipitation seems to be consistent with the number of negative ions necessary to neutralize the net charge of lysozyme. Here, we have assumed that, in the competitive binding to lysozyme, the DS^- ion is much superior to its co-ion, Cl^-, because there exists a hydrophobic interaction between DS^- and lysozyme.

The binding of the DS^- ion introduces the hydrophobicity to the lysozyme (LZ) surface in addition to the neutralization of the positive charges, since DS^- consists of both ionic and hydrophobic parts. Once the DS^- ions have neutralized the net positive charge, the solubility of the LZ^+–DS^- complex will depend on the hydrophobicity of the complex itself. The number of DS^- neutralizing the net charge (+8) in a borate-buffer solution is smaller than those (+9) in pure water or a NaCl solution. Therefore, a number of DS^- ions necessary for the complete neutralization of lysozyme in the borate-buffer solution is one less than in pure water or the NaCl solution; *i.e.*, the surface of the LZ^+–DS^- complex with a zero net charge is less hydrophobic in the borate-buffer solution. This causes less precipitation than in pure water or the NaCl solution. On the other hand, Sophianopoulos and Van Holde have suggested that the lysozyme is in equilibrium with the dimer in alkaline pH.[18] If the dimerization was caused by the aggregation of the hydrophobic part of the lysozyme, the reduction of the hydrophobic area by dimerization would lead to the easy dissolution of lysozyme.

As is shown in Figs. 1—3, each isotherm has two or more steps. This means that there exist two or more kinds of mechanisms in the binding. It is well-known that the BET equation is applicable to the analysis of such a type of binding isotherm. According to Hill,[19] the statistical expression for the BET equation is given as:

$$\frac{\bar{N}}{M} = \frac{q_1\lambda}{(1-q_2\lambda+q_1\lambda)(1-q_2\lambda)}, \tag{3}$$

where $\bar{N}$ is the average number of molecules bound to protein; M, the number of sites; q_1, the partition function for the molecule in the first layer; q_2, the partition function for the molecule in the second and higher layers, and λ, the absolute activity of the free molecule in equilibrium with the bound molecule. Equation 3 is then rewritten as:

$$\frac{\bar{N}}{M} = \frac{KX}{(1-X+KX)(1-X)}, \tag{4}$$

where

$$K = q_1/q_2, \text{ and } X = q_2\lambda. \tag{5}$$

The chemical potential of SDS in solution, μ_{sol}, can be written, using the concentration, C, as:

$$\mu_{sol} = \mu^\ominus + kT\ln(C/\mathrm{CMC}), \tag{6}$$

where $\mu^\ominus$ is the chemical potential of SDS at CMC. From Eqs. 5 and 6, we obtain:

$$X = q_2\exp(\mu^\ominus/kT) \times \frac{C}{\mathrm{CMC}}. \tag{7}$$

By analogy with the adsorption of vapor on an absorbent, we can consider that, as the SDS concentration increases, the amount of the binding species increases, especially on the hydrophobic surface of the lysozyme–DS complex, where the DS^- ion is concentrated. In a bulk solution, a subsequent increase in SDS causes micelle formation, and simultaneously the amount of DS^- per site, $\bar{N}/M$, rises abruptly. We assume that $\bar{N}/M\to\infty$ when $C\to$ CMC; thus, $\bar{N}/M\to\infty$ means that $X\to 1$,as is known from Eq. 4. Therefore, from Eqs. 6 and 7 we may obtain $q_2\exp(\mu^\ominus/kT)=1$, and then;

$$X = \frac{C}{\mathrm{CMC}}. \tag{8}$$

Using Eq. 8, Eq. 4 may be rewritten as follows:

$$\frac{C}{\bar{N}(\mathrm{CMC}-C)} = \frac{1}{MK} + \frac{K-1}{MK}\times\frac{C}{\mathrm{CMC}}. \tag{9}$$

Thus, if the BET equation reproduces the data, the plot of $C/\bar{N}(\mathrm{CMC}-C)$ against C/CMC will be linear, while M and K may be obtained from the slope and the intercept of the line. The plots of $C/\bar{N}(\mathrm{CMC}-C)$ against C/CMC for the pure-water, NaCl, and borate-buffer solution systems are given in Fig. 7. The slopes and the intercepts give values of $M=18$ and $K=10$

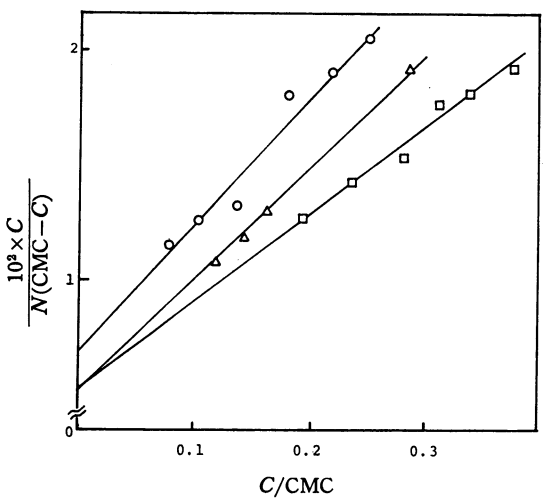

Fig. 7. BET plots for SDS binding on lysozyme in pure water (△), NaCl solution (○), and borate-buffer solution (□).

for the pure-water system, $M=16$ and $K=9$ for the NaCl-solution system, and $M=21$ and $K=11$ for the borate-buffer solution system. According to the BET theory, M is the number of sites, *i.e.*, the number of molecules forming the first layer. As has been mentioned above, the number of positively charged residues of lysozyme is 17 in the pH range studied. The values of M for both the pure-water and NaCl-solution systems are in fair agreement with the number of positively charged residues, but the value of $M=21$ for the borate-buffer system is somewhat large. This aspect of the borate-buffer system is attributable to the dimerization of lysozyme in an alkaline pH.[18]

Recalling that the precipitation is interpreted in terms of the neutralization of the net charge, and that the site number corresponds to the number of positively charged residues, the driving force for the binding of SDS to the first layer may be mainly attributed to the electrostatic interaction with the positively charged residues of lysozyme. The hydrocarbon tail of DS^- of the first layer can be expected to interact with the hydrophobic portion of the lysozyme surface, in addition to the ion-pairing of the head with the positively charged residue of lysozyme. Consideration by photo of the space-filling molecular models of lysozyme and SDS leads us to consider that the surface of lysozyme can be roughly covered with 17 DS^- ions, corresponding to the site number of the first layer, if they lay on lysozyme. This implies that mutual hydrophobic interaction between DS^-'s can be expected for the subsequent binding over the first layer. The subsequent binding is reflected in the sharp uprise of the binding isotherms. This mechanism is supported by the fact that the equilibrium SDS concentration at which the binding isotherms rise sharply is lower in NaCl and borate-buffer solution than in the pure-water solution, as in the case of the salt effect on the micelle formation.

The change in the secondary structure of lysozyme is caused by binding above an SDS concentration where the binding of SDS to the second layer begins to occur in each case. With an increase in the binding number, the helical content increases, but the β-structure decreases; later both reach constant values. An increase in the helical content of lysozyme in an aqueous solution containing more than 60% (v/v) of ethanol, methanol, and 2-propanol has been reported by Ikeda and Hamaguchi.[20] The increase in the helical content with the binding of SDS may be related to their finding that a development of the hydrophobic environment enhances the helical conformation. The tendency for the fraction of each conformation to reach a constant value indicates that the environment of lysozyme is no longer changed above a certain limit of the binding number of SDS, at which the surface of lysozyme is completely covered.

Taking into account the constant fraction of the random coil, it seems that the β-helix transition is induced partly by binding ($\bar{\nu}>10$). Hayakawa *et al.* have reported the β-helix transition of poly (L-lysine) upon the addition of sodium 1-octanesulfonate.[21] With respect to the surfactant-concentration dependence of the secondary structure, the renin substrate has been investigated by Yang and Wu.[22] At a low SDS concentration, the renin substrate shows the characteristic CD spectrum of β-structure, but at a high SDS concentration it shows the characteristic CD spectrum of the α-helix with double minima. These results surpport the idea that the β-structure of lysozyme changes to the α-helix above a certain SDS concentration.

The CD bands in the region between 275 and 300 nm originate in Trp and Tyr. The drastic change from the positive CD spectrum to the negative one upon binding, as is shown in Fig. 4b, means that the environment of these residues is largely altered.

Ikeda and Hamaguchi have demonstrated that the positive CD spectrum of lysozyme in the range from 275 to 300 nm is enhanced by the addition of alcohols (methanol, ethanol, and 2-propanol), but in a concentration of alcohols above 60% (v/v) it begins to decrease, finally becoming a negative CD spectrum. A similar decreasing tendency of the CD spectrum of lysozyme has been observed in the case of the oxidation of Trp's by *N*-bromosuccinimide.[20] These findings mean that Trp gives a clue to the CD spectrum in the range from 275 to 300 nm. The effect of the polarity of a solvent on the CD spectrum of amino-acid derivatives has been examined by Shiraki.[23] He showed that the positive CD spectrum of *N*-acetyltryptophan amide in water changed to a negative one in dioxane, and so he suggested that the sign of the CD spectrum of Trp residues in protein may be changed by the change in their surroundings. It seems reasonable that the reduction of the positive CD spectrum of lysozyme is attributable to the enhancement of the hydrophobic nature of surroundings of Trp on the binding SDS. Thus, the idea that lysozyme is covered with DS^- ions clustering like a micelle can explain the present CD spectrum obtained experimentally.

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