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Characterization of a heat modifiable protein, *Escherichia coli* outer membrane protein OmpA in binary surfactant system of sodium dodecyl sulfate and octylglucoside

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Abstract

A membrane protein, OmpA of *Escherichia coli*, in the process of refolding from its heat-modified form in the presence of sodium dodecyl sulfate (SDS) to its non-heated one by the addition of systematic amounts of octylglucoside (OG) was characterized by means of dynamic light scattering and the size exclusion chromatography combined with low angle laser light scattering photometry. Upon heating in the presence of SDS only, the amount of SDS bound to OmpA was increased from 1.8 to 2.3 g/g of protein and its hydrodynamic radius increased from 3.7 to 4.7 nm. On the addition of OG, the once denatured OmpA regained its original size above the weight fraction of OG in the total amount of surfactants, 0.8. During the process, the hydrodynamic radius was observed to decrease cooperatively at the weight fraction of 0.6, while no change took place in the molar mass of the protein. The refractive index increment of OmpA reflecting the amount of surfactant binding also regained the value before the heating in parallel with the change of size. Examination of the amount of surfactants bound to the membrane protein according to known properties of the binary surfactant micellar system of the surfactants showed that SDS was principally responsible for the denaturing phenomena of OmpA.

Keywords: Membrane protein; OmpA; Refolding; Sodium dodecyl sulfate; Octylglucoside; Surfactant binding

1. Introduction

Membrane proteins form their tertiary and higher order structure in biomembranes which are mainly constructed from lipid bilayer, or in the presence of other amphiphiles such as surfactants. For biochemical studies of a membrane protein integrated in a biomembrane, the membrane is solubilized by addition of surfactants to isolate the protein. Proteins are often modified or denatured upon solubilization, while by the use of a suitable surfactant membrane proteins can be solubilized keeping their intrinsic structure and activity. In some cases, lipid-surfactant mixtures are employed in practical experiments for studying folding properties of a membrane protein [1,2]. The effect and action of amphiphiles are, therefore, primarily important for physicochemical studies of integrated membrane proteins. Some membrane

Abbreviations: SDS, sodium dodecyl sulfate; OG, *n*-octyl-β-D-glucoside; SEC, size exclusion chromatography; LALLS, low angle laser light scattering; BSA, bovine serum albumin; OVA, ovalbumin; DLS, dynamic light scattering; h-OmpA, heat-modified OmpA

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proteins denatured in the presence of an anionic surfactant, sodium dodecyl sulfate (SDS), were shown to refold by the addition of the other kind of amphiphiles such as lipids or non-ionic surfactants. For instance, refolding experiments of bacterial opsins are concerned with mixed solvents containing sodium cholate, lipids and SDS [3], and a study of conformational change of diacylglycerol kinase involves a mixed surfactant solution of SDS and dodecylmaltoside [4]. In such circumstances of aqueous solutions, the mixed amphiphiles afford a place in which membrane proteins fold or unfold. For understanding the folding nature of a membrane protein from such experimental studies, properties of the solvent systems employed and their interactions with proteins should be clarified to derive quantitative knowledge about the behavior of proteins in mixed surfactant solutions.

In the present study, we examined properties of a major outer membrane protein of *Escherichia coli*, OmpA solubilized in the presence of SDS and *n*-octyl-β-D-glucoside (OG) and their mixtures with various compositions. This membrane protein is known to show heat modifiability, namely it changes its properties by heating in the presence of SDS [5,6]. This suggests that the protein is solubilized in the presence of SDS retaining a part of its original structure and the structure is lost by heating on this condition. The membrane protein in the heat modified form is denoted as h-OmpA in this paper. Refolding of OmpA from the denatured form, h-OmpA, can be induced by the addition of another amphiphile such as a non-ionic surfactant, OG [7]. Since the solvent in such experiments contains two kinds of surfactants, it should be considered as a binary mixed surfactant system of SDS and OG. Solution properties of aqueous solutions of the respective surfactants are well established and their binary mixed surfactant system has recently been characterized [8–10].

In order to understand the properties of this membrane protein, especially detailed aspects of the properties related with its conformational change, it is important to characterize the protein interacting with amphiphiles in relevant conditions to the unfolding and refolding phenomena. The amount of surfactant binding must reflect such interaction between the polypeptide and the amphiphilic medium. OmpA solubilized in the binary surfactant system of SDS and OG is suitable for investigating the change of interaction between the membrane protein and amphiphiles, not only because the conformational change of refolding can be observed accompanied with systematic addition of OG but also because the amount of surfactant binding can be estimated experimentally. In this context, complexes between surfactants and OmpA in the non-heated form and heat modified form, h-OmpA, in the various compositions of mixed surfactant solutions were characterized by means of dynamic light scattering and low angle laser light scattering photometry combined with size exclusion chromatography in terms of molar mass, hydrodynamic radius and amount of surfactant binding. Similar experiments were performed also for a few water-soluble proteins to compare their properties with those of OmpA in both forms.

2. Materials and methods

2.1. Materials

Sodium dodecyl sulfate (SDS) was obtained from BDH (Anala R grade), and *n*-β-octyl-β-glucoside (OG) was from Dojindo Laboratories (Kumamoto, Japan). Bovine serum albumin, BSA, was purchased from Armour Pharmaceutical, and ovalbumin, OVA, from ICN Pharmaceuticals. Other chemicals were of reagent grade.

2.2. OmpA preparation

OmpA was isolated and purified from the outer membrane of *E. coli* K-12, TNE001 strain, which lacks OmpF, OmpC, and LamB proteins. Isolation of OmpA was performed mainly according to the procedure as reported by Van Alphen et al. [11]. The preparation thus obtained was further purified by the use of hydroxyapatite chromatography according to the method of Watanabe et al. [12]. The fraction containing OmpA was applied to a hydroxyapatite column (20×2.5 cm I.D.) purchased from Mitsui-Toatsu Chemicals (Tokyo, Japan) (this column is at present distributed by Koken, Tokyo, Japan). OmpA fraction eluted at about 160 mM of the buffer concentration during the gradient from 10 mM of sodium phosphate buffer (pH 6.9) containing

1 mg/ml of SDS to 500 mM of the same buffer. This fraction gave a single band of OmpA upon SDS-PAGE.

This purified OmpA fraction was heated in boiling water for 5 min to obtain the h-OmpA fraction. OmpA (non-heated form) and h-OmpA thus prepared were equilibrated against buffers to be used for subsequent measurements by the use of a size exclusion column of Sephacryl S-300 HR (Pharmacia LKB Biotechnology, Uppsala, Sweden).

2.3. SDS-polyacrylamide gel electrophoresis (PAGE)

All experiments of polyacrylamide gel electrophoresis in the presence of SDS were carried out according to Weber-Osborn's method [13]. Low molecular weight (LMW) calibration kit proteins (Pharmacia LKB Biotechnology, Uppsala, Sweden) were employed as standards.

2.4. Size exclusion chromatography combined with low angle laser light scattering photometry

The mode of molecular assembly of a protein in surfactant solutions and surfactant binding to the protein were studied by size exclusion chromatography using a TSKgel G3000SW$_{XL}$ column (30×0.78 cm I.D.) with a TSK SW$_{XL}$ guard column (7.5×0.75 cm I.D.) combined with three detectors, a low angle laser light scattering photometer (LS-8000), a UV spectrophotometer (UV-8010) and a differential refractometer (RI-8012) connected in this series [14]. All of the detectors were products of Tosoh (Tokyo, Japan).

According to the procedure to analyze the data obtained from the detectors described elsewhere [14,15], the specific refractive index increment of a solution, dn/dc, and molar mass of an eluted substance, M, can be expressed by the outputs of the three detectors as follows.

$$R \equiv A\frac{(\mathrm{RI})}{(\mathrm{UV})} = K_{\mathrm{R}}(\mathrm{d}n/\mathrm{d}c) \quad (1)$$

$$W \equiv \frac{(\mathrm{UV})(\mathrm{LS})}{(\mathrm{RI})^2 A} = K_{\mathrm{W}}M \quad (2)$$

Here, A is the specific extinction coefficient of an eluted substance and (LS), (UV) and (RI) are peak heights, read in mV on the recorder for the light scattering photometer, the UV spectrophotometer, and the refractometer, respectively. In the above equations, K_{R} and K_{W} are instrumental constants. For the values of A, 0.68 and 0.75 l g^{-1} cm^{-1} were adopted for BSA and OVA, respectively. The values for folded OmpA and unfolded OmpA were determined to be 1.78 and 1.73 l g^{-1} cm^{-1} by means of quantitative amino acid analysis, respectively.

The specific refractive index increment determined for a protein in the presence of a surfactant is related to the amount of surfactant bound to the protein. When the protein solution is regarded to be a three-component system composed of protein, surfactant, and water, the refractive index increment for a solution at constant chemical potential for diffusible components, $(\partial n/\partial c)_{\mu}$, can be expressed as

$$\mathrm{d}n/\mathrm{d}c \equiv (\partial n/\partial c)_{\mu} = (\partial n/\partial c_{\mathrm{p}})_{c_{\mathrm{s}}} + \delta s(\partial n/\partial c_{\mathrm{s}})_{c_{\mathrm{p}}} \quad (3)$$

where δ_{s} is the gram of surfactant bound to one gram of the protein, and c_{s} and c_{p} are concentration of surfactant and protein, respectively. Using this relation, we can calculate δ_{s} according to the procedure described previously [15]. The value of $(\partial n/\partial c_{\mathrm{s}})_{c_{\mathrm{p}}}$ was mostly constant for simple proteins and was assumed to be 0.193 ml/g [14]. The value of $(\partial n/\partial c_{\mathrm{p}})_{c_{\mathrm{s}}}$ was assumed to be 0.119 ml/g in the presence of SDS [8], and 0.138 ml/g in the presence of OG [9].

All the proteins were solubilized and equilibrated against the 50 mM sodium phosphate buffer (pH 6.9) containing a defined composition of SDS and OG by the use of gel chromatography with Sephacryl S-300 HR column (30×1.5 cm I.D.). An appropriate volume of sample solution ranging 50–500 μl with concentration of 0.4–1.2 mg/ml of proteins was applied to the column at a flow-rate of 0.3 ml/min. Temperature was kept at 25°C. Mixed surfactant solutions containing SDS and OG were prepared keeping SDS concentration constant at 1 mg/ml. The composition of this mixed surfactant system will be described as X_{OG}, that is, the weight fraction of OG in total concentration of the surfactants. The solution denoted as $X_{\mathrm{OG}} = 1$ contains 8 mg/ml of OG and no SDS.

2.5. Dynamic light scattering photometry

Sample solutions (0.5–2.0 mg protein per ml) were prepared to be equilibrated against 50 mM sodium

phosphate buffer (pH 6.9) containing defined compositions of SDS and OG by the chromatography system used in the SEC-LALLS measurements. The total concentration of the surfactants was adjusted to be just above the critical micelle concentration of the SDS-OG mixed system [10], so as to prevent the presence of excess mixed surfactant micelles. All the measurements were performed by the use of a dynamic scattering photometer, DLS-700 (Otsuka Electronics, Hirakata, Japan) at 25°C to obtain hydrodynamic radii of the complexes between surfactant and protein.

3. Results

3.1. Hydrodynamic properties

OmpA and its heat modified form, h-OmpA, kept in various compositions of SDS-OG mixed surfactant system, were analyzed by SDS-PAGE using 10% (w/v) of polyacrylamide gel. Fig. 1 shows an electrophoretic diagram of them thus obtained. OmpA solubilized in the presence of SDS only migrated giving a single band at the position of 29 kDa (lane 2) and h-OmpA solubilized on the same condition migrated at the position of 35 kDa (lane 3). By the addition of OG, this heat modified protein restored its mobility. This is consistent with the previous work by Dornmair et al. [7]. In the present experiments, the restoration of the mobility was observed at X_{OG} above 0.69 (lane 4–12).

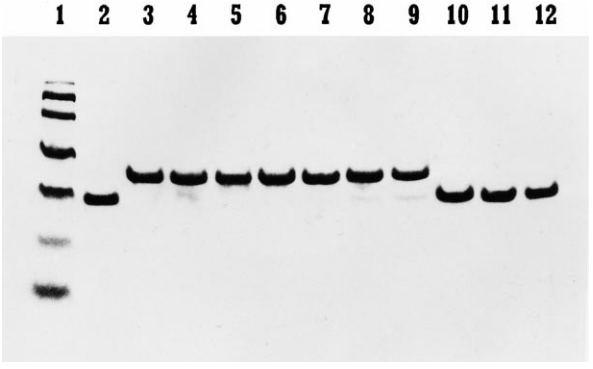

Fig. 1. SDS-PAGE diagram of h-OmpA (lanes 3–12) kept in the various compositions of the SDS-OG mixed surfactant system. The compositions of the mixed surfactant system were 0, 0.37, 0.45, 0.55, 0.61, 0.69, 0.72, 0.79, 0.84, 0.92 of X_{OG} for lanes 3–12, respectively. Standard proteins on lane 1 were phosphorylase *b* (94 kDa), bovine serum albumin (64 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa), respectively (top to bottom). The single band on lane 2 is OmpA in the non-heated form.

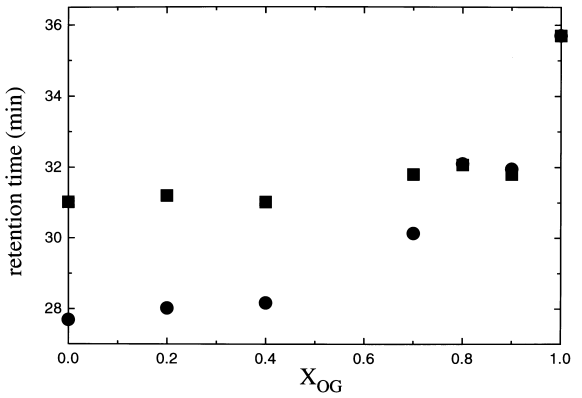

Fig. 2. Changes of retention time of OmpA (■) and that of h-OmpA (●) in the size exclusion chromatography with composition of the SDS-OG mixed surfactant system. A TSK-gel G3000 SW_{XL} column (30×0.78 cm I.D.) with a TSK SW_{XL} guard column (7.5×0.75 cm I.D.) was employed and the flow rate was 0.3 ml/min.

This change of the membrane protein was also observed as that of retention time in size exclusion chromatography which is correlated with that in its hydrodynamic volume [16]. Fig. 2 shows the retention times of OmpA and h-OmpA at various compositions of the SDS-OG mixed system, X_{OG}. Up to 0.7

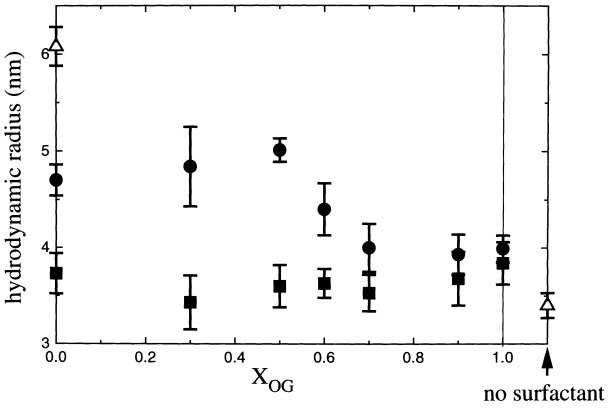

Fig. 3. The hydrodynamic radii of the proteins (OmpA (■), h-OmpA (●), and bovine serum albumin (△)) in the SDS-OG mixed system. These values were calculated from the diffusion coefficients which were obtained through the dynamic light scattering measurements. Error bars are the standard deviations of the measurements for each protein.

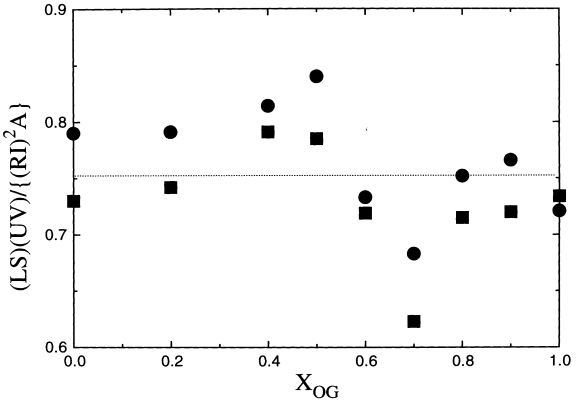

Fig. 4. The values of $W\{=(LS)(UV)/(RI)^2A\}$ in Eq. 2 for OmpA (■) and h-OmpA (●) measured for various compositions of the SDS-OG mixed system. The W is in proportion to the molar mass of the protein.

of X_{OG}, h-OmpA was eluted in advance of OmpA. Above the composition, no difference in elution behavior was observed between them. This suggests that the hydrodynamic volume of OmpA increased on heating is kept in the former range of X_{OG}, and is restored in the latter range. The retention time of OmpA in the presence of OG only was significantly larger than those at the other compositions containing corresponding populations of SDS. This can be ascribed to ionic interaction between the OmpA-surfactant complexes and the column material. Elution

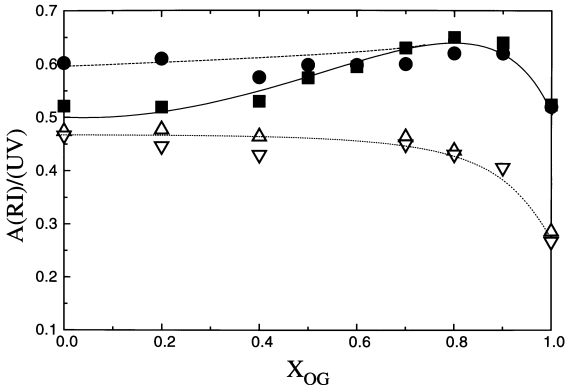

Fig. 5. Changes of refractive index increments of the proteins (BSA (△), OVA (▽), OmpA (■) and h-OmpA (●)) with the composition of the SDS-OG mixed system. The value of $R\{=A(RI)/(UV)\}$ in Eq. 1 is proportional to the refractive index increment of the protein. Lines are according to the following symbols sequentially. The solid line represents OmpA, the dashed line h-OmpA and the dotted line the water soluble proteins (BSA and OVA).

of a negatively-charged solute is accelerated due to the ionic exclusion effect in such a column as TSKgel G3000SW$_{XL}$ where the supporting material contains negative charges [17].

The hydrodynamic radii, r_H, of the protein-surfactant complexes for OmpA and h-OmpA were obtained through DLS measurement and plotted against X_{OG} in Fig. 3. In the presence of SDS only, the r_H of OmpA increased from 3.73 ± 0.21 to 4.70 ± 0.16 nm upon heating. This change in r_H is in agreement with the former study by Reithmeier and Bragg [18], although the values obtained in the present study are smaller than their values which were estimated by gel filtration with reference to water soluble proteins as standard materials. The r_H of h-OmpA decreases at around X_{OG} of 0.6–0.7 in a cooperative manner to become identical with that of OmpA, 3.88 ± 0.26 nm.

The r_H for BSA in a surfactant-free aqueous solution and in the presence of SDS were determined to be 3.40 ± 0.13 nm and 6.08 ± 0.20 nm, respectively. The former is consistent with the values reported by both Tanford et al. [19] and Tarvers and Church [17]. The r_H of the SDS-denatured BSA with intact disulfide bonds obtained in this study is consistently smaller than the value, 7.8 nm, reported by Tanford et al. [19] for the SDS-denatured BSA without the bonds. The r_H of BSA in the OG solution is identical to that of BSA in the absence of surfactant. This is consistent with the observation that BSA scarcely binds OG [15].

3.2. Molar masses of the proteins

Molar mass of a protein can be determined by application of the outputs of the three detectors to

Table 1
Amounts of SDS and OG bound to proteins in the presence of either SDS or OG

	δ^a_{SDS} (g/g protein)	δ^b_{OG} (g/g protein)
OmpA	1.8	1.6
h-OmpA	2.3	1.6
BSA	1.5	0.2
OVA	1.4	0.1

[a]Values for 50 mM of sodium phosphate buffer (pH 6.9) containing 1 mg/ml of SDS only.
[b]Values for the same buffer containing 8 mg/ml of OG only.

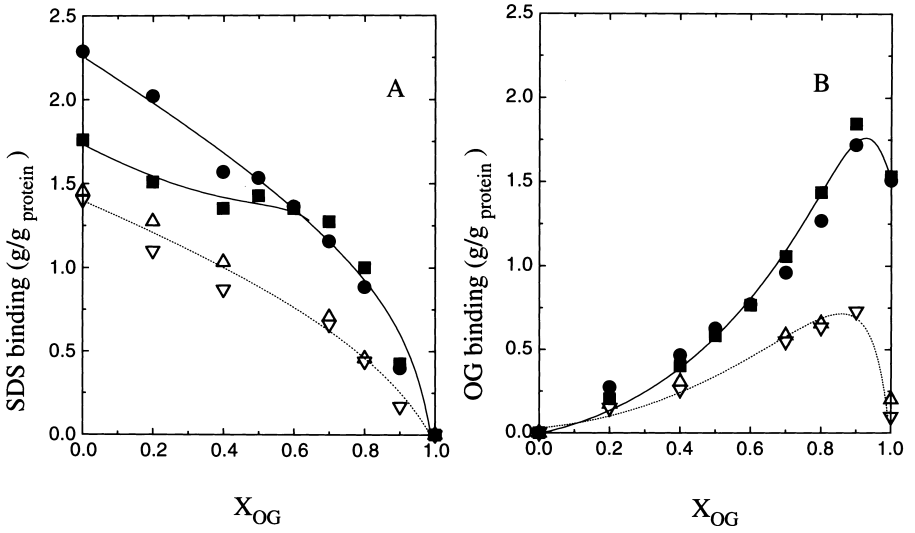

Fig. 6. The amounts of SDS (A) and OG (B) bound to the proteins (BSA (△), OVA (▽), OmpA (■) and h-OmpA (●)) in the SDS-OG mixed system calculated from Eq. 4 under the assumption that the compositions of bound surfactant to the proteins are the same as those of the mixed micelles in the solutions. The compositions of the mixed micelles of SDS and OG were estimated by the data of Kameyama et al. [10].

Eq. 2. The W in the equation is proportional to molar mass (see Section 2). The values obtained for OmpA and h-OmpA are plotted in Fig. 4 against the weight fraction of OG in the SDS-OG mixed system. This result indicated that the molar masses of OmpA and h-OmpA are the same and invariant with X_{OG}. Clearly both OmpA and h-OmpA show no association or dissociation despite the change of surfactant composition. Thus, the conformational change of the membrane protein between non-heated form and heat modified form is shown to be accompanied with no change in the molecular assembly but that in the folding compactness.

3.3. Amount of surfactant binding

The R in Eq. 1 (see Section 2) was measured for BSA, OVA, OmpA, and h-OmpA and plotted in Fig. 5 against the weight fraction of OG in the SDS-OG mixed system, respectively. For X_{OG} up to 0.6, the line for h-OmpA is above that for OmpA. The two lines approach each other, and finally assume identical values. This indicates that the specific refractive index increment of h-OmpA is significantly larger than that of OmpA in the range of X_{OG} up to 0.6. We will assume that there is no significant difference between them above that.

In the cases of X_{OG} of 0 and 1, the amounts of surfactant bound to proteins were calculated according to Eq. 3, and shown in Table 1. The amounts bound to BSA and OVA thus determined are in agreement with those reported previously [15,20]. The amount of SDS bound to OmpA was thus found to increase upon heating from 1.8 g/g protein to 2.3 g/g. These results are consistent with those reported by Reithmeier and Bragg [5]. On the other hand, the amount of OG bound to OmpA was shown to be the same as that to h-OmpA, suggesting that heat modified OmpA refolds to the non-heated form also in terms of the amount of surfactant binding by the addition of OG.

4. Discussion

In the present study, we have studied solution properties of the outer membrane protein of *E. coli*, OmpA and its heat modified form, h-OmpA in the mixed surfactant solutions of SDS and OG. The observed changes will be discussed with reference to the conformational changes of the protein therein. We have focused attention in making clear the correspondence of the conformational change to the change of amount of its surfactant binding as that

of interaction between the protein and the amphiphiles.

While the amount of surfactant binding was determined using Eq. 3 in the presence of either SDS or OG, matters are not so simple in the mixed surfactant system containing both SDS and OG. Nevertheless, one can roughly evaluate the change in amount of the surfactant binding through the ordinate R values in Fig. 5 that are proportional to the refractive index increments. Since water soluble proteins, BSA and OVA, scarcely bind OG, the ordinate value for protein moiety is thus expected to be about 0.28 for all the proteins involved. The increment above that value can be taken as a measure of surfactant binding. The values for BSA and OVA are on the same line, indicating that the amount of surfactant binding progressively decreases with the addition of OG in the same manner for these water soluble proteins. The lines for OmpA and h-OmpA are definitely above the line for BSA and OVA. This shows that OmpA, as expected for a membrane protein, has higher affinity to both of the surfactants. Notably the line for h-OmpA is roughly in parallel with that for BSA and OVA. The lines share the feature that they are flat and then descend progressively. This feature may be interpreted as follows: (i) the protein polypeptides are unfolded and maximally bind SDS; (ii) the bound SDS is progressively replaced by OG, and the cluster of bound surfactant assumes a nature of mixed micelle; (iii) with the decrease of the charge density on the cluster, the protein polypeptide refolds to the non-heated state; (iv) in the folded state, OG binds significantly to OmpA but scarcely to BSA or OVA. OmpA retains a folded structure in the presence of SDS unless heated, and binds less amount of SDS than h-OmpA. This property causes OmpA to assume a line with a unique feature in Fig. 5.

The observation shown in this figure and the interpretation described above illustrate that such a simple approach is efficient to sketch out the feature of interaction of a protein in a binary surfactant system. We have then proceeded to further detailed examination of the interaction of OmpA and h-OmpA with SDS and OG.

Each amount of SDS and OG bound to the protein can be related to the refractive index increment of the complex formed among SDS, OG, and protein in a solution containing the three components by the following equation.

$$(\partial n/\partial c_p)_\mu = (\partial n/\partial c_p)_{c_s} + \delta_{SDS}(\partial n/\partial c_{SDS})_{c_p,c_{OG}} + \delta_{OG}(\partial n/\partial c_{OG})_{c_p,c_{SDS}}. \quad (4)$$

Here, δ_{SDS} and δ_{OG} are the grams of bound SDS and OG to gram of the protein, respectively. Subscripts SDS and OG indicate that the values are those of the corresponding surfactant. Because the composition of the bound surfactants is not necessarily equal to the nominal composition of total solution, δ_{SDS} and δ_{OG} cannot be simply determined. Kronberg et al. [21] estimated the amount of bound surfactants on the surface of polystyrene latex in an anionic and non-ionic surfactant mixed system containing SDS and nonylphenol deca(oxyethylene glycol) monoether. They assumed the composition of the bound surfactants to be equal to that of the coexisting micelles. We have also assumed the same situation. Then, the composition of the micelles in the binary surfactant system, namely the ratio of δ_{SDS} to δ_{OG}, has been estimated through surface tentiometry by Kameyama et al. [10]. Knowledge of the ratio allows determination of the surfactant binding using Eq. 4.

The amounts of bound SDS and OG could be thus evaluated separately, and plotted against the weight ratio of SDS to OG used in the preparation of the sample solutions in Fig. 6. The amount of SDS bound to h-OmpA decreases monotonously with the addition of OG. The amount bound to the water soluble proteins, BSA and OVA, also changed in a similar manner. The amount of SDS bound to OmpA is significantly less than that bound to h-OmpA up to 0.6 of X_{OG} where the lines for these two merged. This again indicates that the mode of binding of SDS to OmpA differs from that to h-OmpA. On the other hand, the latter rather behaves similarly to the water-soluble proteins.

On the contrary, no difference was observed between OmpA and h-OmpA in the mode of binding of OG in the entire range of X_{OG}. As shown in Fig. 5, h-OmpA showed higher capacity for surfactant binding. The above result indicates that SDS is solely responsible for their difference. Therefore the following assumption as for the conformational change of OmpA in the presence of SDS is made: the unfolded

'heat modified' state is attributed by the influence of SDS, which is accelerated by heating. The denaturation of the membrane protein by SDS is considered to be so slow that OmpA in the non-heated form retains a folded structure unless being heated. Upon the unfolding by heat treatment, parts of OmpA polypeptide are exposed to provide additional binding sites. These sites have significantly higher affinity to SDS than to OG. The addition of OG decreases the denaturing activity of SDS through formation of mixed micelle to lead to the loss of such sites and subsequent refolding. This is supported by the result from another refolding study of a water-soluble globular protein in the binary surfactant system of SDS and OG: unfolded ribonuclease A in the presence of SDS was shown to refold by the addition of OG in a similar manner (Kameyama et al., unpublished data).

OmpA in the non-heated form in the presence of SDS is suggested to retain its apparent folded structure owing to the extremely low denaturing rate by the SDS. This denaturing reaction must be accelerated by heating. Our proceeding kinetic study as for the conformational change of OmpA in the SDS-OG mixed surfactant system is giving the results supporting this assumption.

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