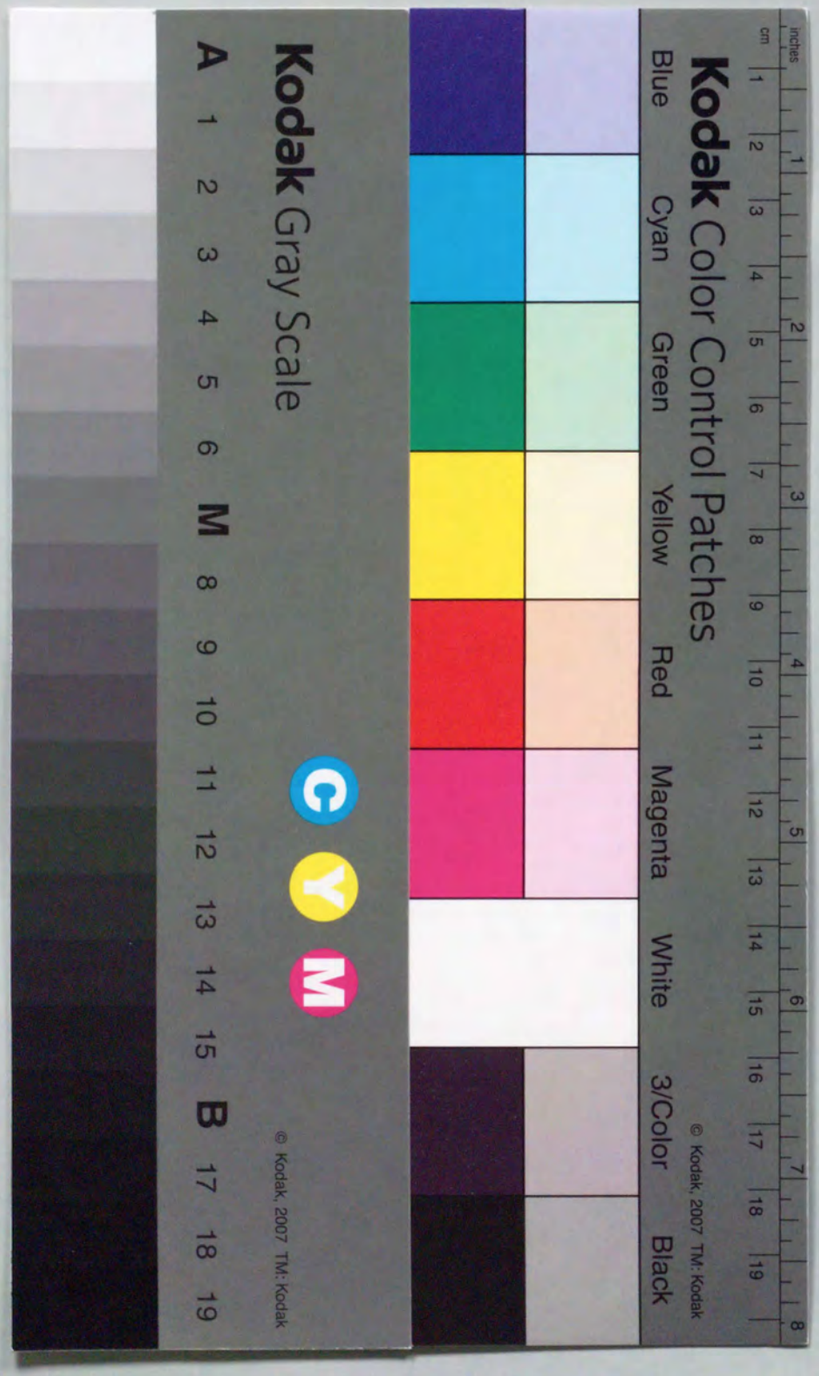

Solution Properties and Conformational Change of a Membrane Protein in Surfactant Solutions

An Approach for Solving the Protein Folding Problem for Membrane Proteins

A Doctoral Thesis

By

Satoshi Ohnishi

Submitted to

The Faculty of Science, Osaka University

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APPROVALS

February, 1999

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Satoshi Ohnishi

Satoshi Ohnishi

February 1999

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Abbreviations

(A)	aggregate form
BR	bacteriorhodopsin
BSA	bovine serum albumin
CD	circular dichroism
cmc	critical micelle concentration
δ_{SDS}	grams of SDS bound to gram of protein
δ_{OG}	grams of OG bound to gram of protein
DGK	diacylglycerol kinase
DLS	dynamic light scattering
D_{max}	maximum particle dimension
ESR	electron spin resonance
(F)	folded form
ϕ	inside diameter
h-OmpA	OmpA in the heat modified form
(I)	partially folded intermediate state
IR	infrared
LALLS	low angle laser light scattering
LHCII	light-harvesting complex from photosystem II
NaPB	sodium phosphate buffer
OG	octylglucoside
OVA	ovalbumin

P(r) function	pair-distance distribution function
[θ]	molar ellipticity
RCAM-	reduced-carboxyamidomethylated
R_G	radius of gyration
R_H	hydrodynamic radius
RNaseA	ribonuclease A
SAXS	small angle X-ray scattering
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
(U)	unfolded form
UV	ultraviolet
X_{OG}	weight fraction of OG in the mixed surfactant system of SDS and OG

Terminology

This study deals with the folding properties of a membrane protein, *Escherichia coli* OmpA. This protein is a major outer membrane protein of *Escherichia coli*, which is produced owing to the *omp* gene *in vivo*. OmpF and OmpC are also stemmed from the omp gene, and called "porin" since they have a pore in the native form *in vivo* through which substances can go inside and outside of the outer membrane.

Chapter I General Introduction

1.1 Protein Folding

Proteins are produced to play their specific roles in organisms; some catalyze physiological reactions with their own specificity, some are used as constructional materials of bodies, some are involved in electron transfer or transport of physiological materials and products, and so on. In this context, the biopolymers consisting of 20 kinds of amino acids can be regarded as ultimate functional and sophisticated macromolecules.

Twenty kinds of natural L-amino acids are polymerized to make a single polypeptide chain without branching. This chain folds into its intrinsic native conformation to exert its proper function. In other words, forming the native tertiary structure of a polypeptide is essential for exhibiting its physiological function. What gene governs is, however, only the sequence of its constituents, amino acids. A polypeptide chain of a protein which has its inherent amino acid sequence can fold spontaneously to have its inherent activity even *in vitro*. The tertiary structure of a protein should, therefore, be dominated by its amino acid sequence (Anfinsen, 1973). For these few decades, a huge number of studies on protein folding has been performed to make the mechanism clear how proteins adopt the native folding. Recent development in biotechnology or protein engineering makes it possible to synthesize a polypeptide chain with a facultative amino acid sequence. Actually, in the field of industry, such techniques are applied nowadays, and elucidation of the mechanism of protein folding is looked forward to not only from academic interest but also from industrial motivation.

Protein folding has been studied in particular from the following four points of view (Creighton, 1994).

1) Through what kinetic process or folding pathway does a protein adopt its native and biologically active folded conformation?

2) What is the physical basis of the stability of folded conformations?

3) Why does an amino acid sequence give only one native folding through the limited folding pathway?

4) Given the amino acid sequence of a protein, how can its 3-dimensional structure be predicted?

Various methods have been exploited to answer these problems. Kinetic and statistic aspects of the folding-unfolding phenomena of a protein between the unfolded and folded states have been revealed by means of procedures in physical chemistry, such as spectroscopies; ultraviolet and visible (UV) absorption, circular dichroism (CD), tryptophan fluorescence, infrared (IR), nuclear magnetic resonance (NMR) or photometries; low angle laser light scattering (LALLS), dynamic light scattering (DLS), small angle X-ray scattering (SAXS) or small angle neutron scattering (SANS). Calorimetry has been used to evaluate the thermodynamical stability of a protein directly. X-ray crystallographic analyses, which give the 3-dimensional structure of a protein molecule, afford significant insight into the protein folding. NMR may give knowledge about both of the structure and dynamics of a protein with atomic resolution. Advances in protein engineering accelerate such studies to elucidate the contribution of specific amino acids in the sequence to the stability or folding mechanism of the protein. In parallel with such experimental studies, theoretical works in molecular dynamics or calculation of stability have made progress in the simulation for

protein folding exhibiting the behavior of a real protein. Very recently, attempts have started to observe the folding behavior of a single protein molecule directly by applications of new techniques such as atomic force microscopy or fluorescence labeling spectroscopy.

These studies have made advances in approaches to solve many problems of protein folding. There is, however, a pitfall in the advances. That is, these advances are made only for very limited kinds of water-soluble globular proteins. For instance, membrane proteins, which fold and function in biomembranes, are much poorly investigated in regard to the problems of protein folding described above compared with such kinds of proteins. Studies of membrane proteins must be of great importance since they are essential and quite unique for living activity as well. What is the reason for the one-sided current of the studies? Difficulty in studying the folding of a membrane protein will be pointed out in the following section. In this thesis, we will proceed to an approach to tackle the problems.

1.2 Structure and Folding of Membrane Proteins

1.2.1 Structure of Membrane Proteins

Membrane proteins form their tertiary and higher order structures in biomembranes which are mainly constructed from lipid bilayer. Figure 1.1 shows an illustration of integral membrane proteins in a biomembrane. Since a membrane protein integrated in a biomembrane has large hydrophobic surface area which is in contact with hydrocarbons of the lipid molecules to make its native conformation stable, such a protein cannot be solubilized in a simple aqueous salt. In many cases, it has also hydrophilic regions extruded from the lipid bilayer to the aqueous environment, and thus cannot be solubilized even in a

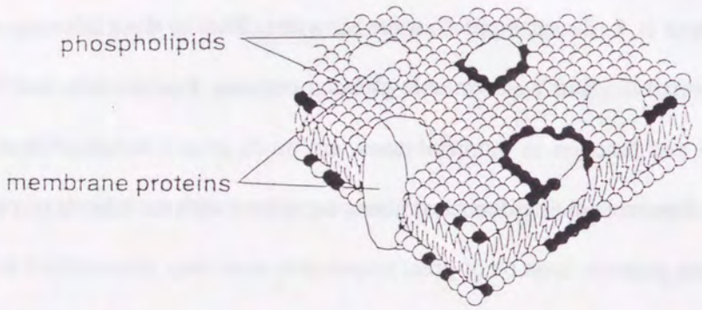


Figure 1.1 Paradigm of membrane proteins in a biomembrane, which were proposed as a fluid mosaic model by Singer and Nicolson (1972). In this model, membrane proteins can move horizontally along the planar membrane fluidly. Some membrane proteins penetrate the membrane completely, some are buried partially and some are only adsorbed on the surface of the membrane. The first type of membrane proteins, called integral membrane proteins, are referred to as "membrane proteins" and studied in this thesis.

simple organic solvent either. In this context, a membrane protein in its native folding is properly solubilized in the presence of amphiphiles such as surfactants or lipids.

For biochemical studies of a membrane protein, the membrane is solubilized commonly by the addition of surfactants to isolate the protein. While proteins are often modified or denatured upon the solubilization, a membrane protein can be solubilized by the use of a suitable surfactant keeping its intrinsic structure and activity. Upon the solubilization, surfactant molecules bind to the hydrophobic region of the protein to form the complex between the surfactant and the protein, resulting in the stable dispersion of the membrane protein in aqueous solution. In order to determine the 3-dimensional structure of a protein with atomic resolution, X-ray analysis for the crystal of the protein is one of the most powerful procedures. Crystallization of the membrane protein and the crystal growth require the ordered contacts between the proteins or the protein and the surfactant employed. The selection of an amphiphile often affects critically the crystallization of a membrane protein. Actually, surfactant molecules coordinated with a membrane protein molecule are observed in the X-ray crystallographic structure for the oxidized bovine heart cytochrome *c* oxidase (Tsukihara *et al.*, 1995, 1996). It is, hence, not easy to obtain the crystal of a membrane protein.

In spite of such difficulties, especially during the last decade, experimental knowledge concerning X-ray crystallographic analysis has been gradually accumulated for several membrane proteins. The 7 Å resolution structure of bacteriorhodopsin (BR) was determined by Henderson and Unwin (1975), and followed by the subsequent high resolution structural analysis at 3.5 Å resolution (Henderson *et al.*, 1990). The 2.3 Å resolution structures of the photosynthetic reaction center from *Rhodopseudomonas viridis*

(Deisenhofer *et al.*, 1984) and the homologous reaction center from *Rhodobacter sphaeroides* (Allen *et al.*, 1986, 1987; Chang *et al.*, 1986, 1991), the 1.8 Å resolution structures of OmpF, or porin, from *Rhodobacter capsulatus* (Weiss *et al.*, 1990) and the homologous *Escherichia coli* porin structure (Cowan *et al.*, 1992), the 3.4 Å resolution structure of the light-harvesting complex from photosystem II (LHCII) (Kühlbrandt *et al.*, 1994) and the 2.8 Å resolution structure of the oxidized bovine heart cytochrome *c* oxidase (Tsukihara *et al.*, 1995, 1996) were determined. Such successes in crystallographic studies afford not only their 3-dimensional structures with atomic resolution but also the general prospect of crystallization for membrane proteins. The general approach for solving the problems specific to crystallizing membrane proteins, which aims to determine the tertiary structure of a new membrane protein, is developing to be established on the basis of the knowledge from such studies (Kühlbrandt, 1988, Garavito *et al.*, 1996).

Although the number of the structural data is very small, such studies verified unique features of the folding nature of membrane proteins, which had been discussed formerly by speculation. That is, membrane proteins can be classified into those consisting of either α-helices or β structures in the transmembrane region in terms of the secondary structure (Stowell and Rees, 1995). Figure 1.2 shows the paradigm of membrane proteins in a biomembrane in this context. For instance, BR, the photosynthetic reaction center, LHCII and bovine heart cytochrome *c* oxidase are of the α-helical type and OmpF porin has a typical β-barrel structure with anti-parallel β–sheets. It is, however, noteworthy that electron crystallographic work on the acetylcholine receptor (Unwin, 1993) intriguingly suggests another type of membrane protein which has an α–β hybrid secondary structure in the transmembrane region (Stowell and Rees, 1995).

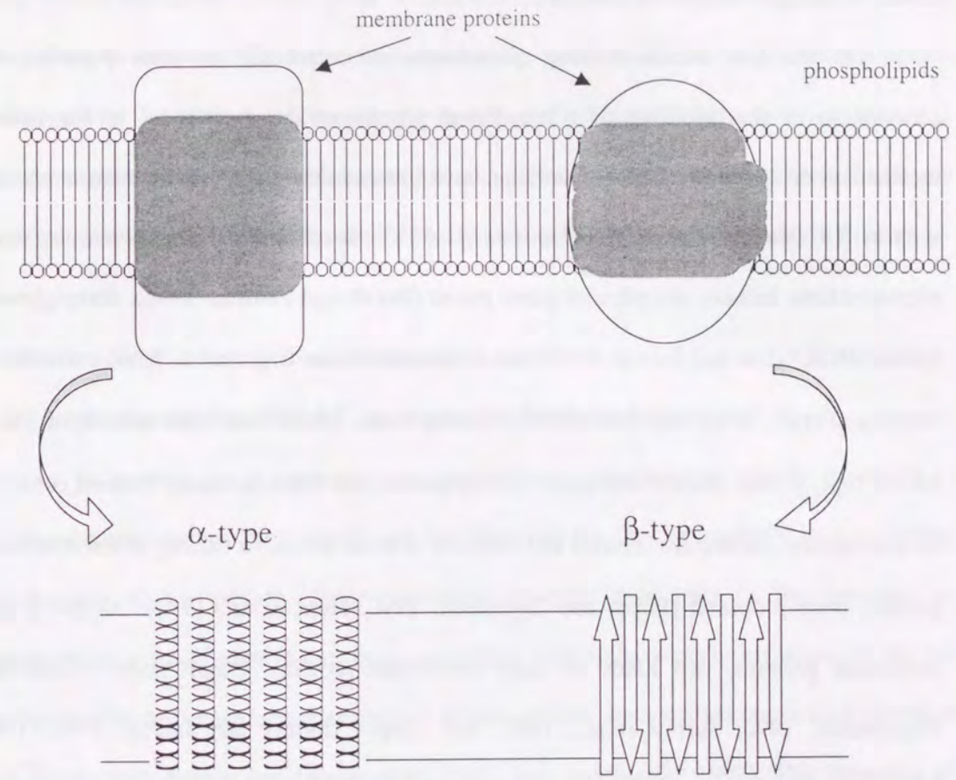


Figure 1.2 Schematic representation of integral membrane proteins in phospholipids. Membrane proteins can be categorized into two types, α-type and β-type, in terms of their secondary structures of the transmembrane segments. Crystal structures for membrane proteins reveal such a unique feature of membrane proteins.

1.2.2 Folding of Membrane Proteins

In the last decade, folding phenomena of membrane proteins regarding the observation of the refolding of a membrane protein or the assessment of the folding mechanism or the conformational stability have been studied for a few membrane proteins such as BR (Huang *et al.*, 1981, Popot *et al.*, 1987, Booth *et al.*, 1995, 1996), the major photosynthetic antenna complex of green plants (Booth and Paulsen, 1996), diacylglycerol kinase (DGK) (Lau and Bowie, 1997) and a transmembrane fragment of ferric enterobactin receptor (FepA) (Klug and Feix, 1998). Among them, BR, a membrane protein of the α-helical type, is well studied and some folding models, in which primarily formed α-helices on a membrane surface are inserted and oriented to form the native folding of the membrane protein, were proposed (Popot and Engelman, 1990, Hunt *et al.*, 1997). As for β type membrane proteins, two kinds of outer membrane proteins, OmpF porin (Eisele and Rosenbusch, 1990, Surrey *et al.*, 1996) and OmpA (Surrey and Jähnig, 1992, 1995, Kleinschmidt and Tamm, 1996), were investigated with regard to the folding mechanism as well as α type membrane proteins.

Most of such studies deal with the conformational change of a membrane protein between the folded form in a lipid vesicle or biomembrane directly and the unfolded form in the presence of a denaturant. It should be noted that the study of the folding of DGK is concerned with the conformational change between the folded form in a surfactant with mild denaturing ability and the unfolded form in the presence of another surfactant with stronger denaturing ability (Lau and Bowie, 1997). The use of two kinds of surfactants is considered to be a mixed surfactant system. Namely, the conformational change of DGK was observed by changing the composition of the mixed surfactant system in this study.

This mixed surfactant system gives a suitable experimental condition under which the membrane protein unfolds and refolds reversibly, while most of solubilized membrane proteins with their folded structures in the presence of surfactants are empirically known to denature irreversibly. Establishment of the conditions under which a protein folds and unfolds reversibly is essential for the thermodynamical assessment of the protein folding.

1.3 Experimental Conditions – Application of Surfactant System -

In general, it is essential to establish an experimental system in which a conformational change of a protein can be observed for the investigation of the folding mechanism of the protein. Since membrane proteins fold only in the presence of amphiphiles, most of experiments concerning their folding have been performed by the use of lipid-surfactant or surfactant-surfactant mixed systems as described above (*e.g.* Booth *et al.*, 1995, 1996, Surrey and Jähnig, 1992, 1995, Lau and Bowie, 1997). The formation of tertiary structures of the membrane proteins under such conditions should be dominated by interactions among the polypeptides and the amphiphilic media surrounding them. Quantitative assessment of such interactions is essential to understand the physico-chemical mechanism of the folding phenomena of the membrane proteins.

Surfactants are commonly used for the solubilization of membrane proteins. These amphiphilic molecules associate to form assemblies called micelles above a defined concentration, *i.e.* the critical micelle concentration (cmc). By the use of an appropriate surfactant, membrane proteins can be solubilized with their native folding retained. In the solution, surfactants are considered to bind to the surface of the protein with native folding

covering its hydrophobic surface with their hydrocarbon tails. Consequently, their hydrophilic heads cover the most external surface of a complex formed between the protein and the surfactants to be dispersed in aqueous solution, as is shown Figure 1.3. This ability of a surfactant in the solubilization of a membrane protein is considered to depend on the monomer concentration rather than that of the micelles or total concentration of the surfactant. To obtain a stable dispersion of a membrane protein, hence, the surfactant should be used with keeping its monomer concentration constant above a sufficient level. Since the effective concentration of the surfactant is decreased by forming the complex with the protein, using concentration above its cmc of the surfactant can keep its monomer concentration in the bulk solution constant. Therefore, the surfactant is usually used for the solubilization of a membrane protein above the cmc.

For understanding the folding mechanism of a protein, assessment of conformational stability of the protein is indispensable. The native state of a protein is known to be stabilized with a small energy difference to its denatured state, not more than 50 kJ mol^{-1}, which is called marginal stability. This is one of the characteristic properties of proteins which dominate the protein folding problems described in the section 1.1. Practically, the stability of the folded state of a protein relative to the unfolded one in terms of the Gibbs free energy difference between them, ΔG, can be determined through measuring the change in the equilibrium constant or that in the heat capacity directly accompanied with the heat denaturation. In the case of a membrane protein, one can obtain ΔG through similar experiments in the presence of amphiphiles. However, ΔG thus obtained for the membrane protein must include not only the energetic difference originated from the conformational change of the polypeptide but also from the change in the interaction of the

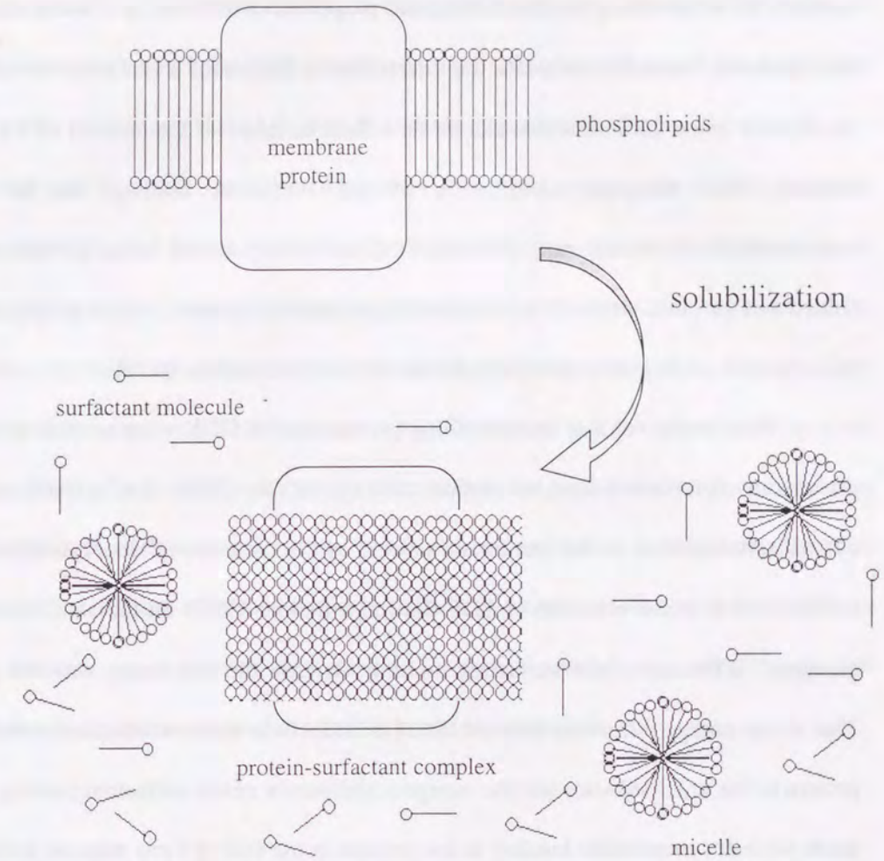


Figure 1.3 Schematic representation of a solubilized membrane protein in the presence of a surfactant. The membrane protein forms a complex with surfactant molecules upon the solubilization. The surfactant molecules are considered to bind to the hydrophobic region of the protein, which interacts with hydrocarbon chains of phospholipids in biomembrane. Surfactants in the bulk solution form aggregate called micelles.

polypeptide with the amphiphiles surrounding it. Hence, evaluating the interaction is essential for determining the thermodynamic properties of the folding of membrane proteins. One plausible method to evaluate the interaction is the use of a surfactant as a supporting amphiphile since the interaction can be described in terms of the amount of the surfactant binding. This energetic contribution of the surfactant binding can be evaluated experimentally by determining the amount of surfactants bound to the protein both in the folded and unfolded states. In this context, experimental systems involving surfactants seem suitable for studying the conformational stability of a membrane protein.

Practically, folding and unfolding phenomena of DGK were studied by the use of the mixed surfactant system of sodium dodecyl sulfate (SDS) and dodecylmaltoside to evaluate the stability of the membrane protein in the presence of dodecylmaltoside to the unfolded state in the presence of SDS (Lau and Bowie, 1997). In the study, however, the energetic difference of the surfactant binding between the two states was not accounted. That is, the energy difference thus evaluated must include the conformational stability of the protein to the unfolded state and the energetic difference of the surfactant binding. Since the mode of dodecylmaltoside binding to the protein in the folded form must be different from that of SDS binding to the protein in the unfolded form, one should evaluate such energetic difference of the surfactant binding in order to evaluate the conformational stability of the protein. In the present study, the mixed surfactant system of SDS and octylglucoside (OG) was employed to establish the approach for solving the protein folding problem in an outer membrane protein from *Escherichia coli*, OmpA. The use of the mixed surfactant system of SDS and OG seems rather suitable than that of SDS and dodecylmaltoside, since the solution properties of SDS (Hayashi and Ikeda, 1980) and OG (Kameyama and Takagi,

1990) and the mixed surfactant system itself have been well established (Kameyama *et al.*, 1997). The amount of surfactant binding to the protein could be determined by means of size exclusion chromatography (SEC) combined with refractive index and low angle laser light scattering photometry (LALLS), and this will be described in Chapter II.

It should be noted that the conformation of a membrane protein solubilized in the presence of a surfactant cannot always be identical with that in the biomembrane in detail, even though the protein thus solubilized exerts its intrinsic activity. The structure of the hydrophilic region of the protein, which is extruded from a planar lipid bilayer of the biomembrane and exposed in aqueous space, may be altered in a surfactant solution, since this region should be feasible to access to some interaction with micelles or monomers of the surfactant in the bulk solution. However, this may not be critical in considering the basic properties of the folding of a membrane protein with respect to change in the backbone structure between one state with the folded structure and another one, when the discrete thermodynamically stable states of the protein are to be concerned.

1.4 Outer Membrane Protein A (OmpA) from *Escherichia coli*

Escherichia coli OmpA is one of the well studied and characterized integral membrane proteins (Reightmeir and Bragg, 1974, 1977, Nakamura and Mizushima, 1976, Chen *et al.*, 1980, Dornmair *et al.*, 1990, Sugawara and Nikaido, 1992, 1994, Rodionova *et al.*, 1995). Its physiological function is not clear but is reported to act as a receptor for pili upon the cell conjugation (Van Alphen *et al.*, 1977), as a mechanical support of the membrane construction (Datta *et al.*, 1977, Lugtenberg and Van Alphen, 1983), or as a pore

through which substances were transported (Sugawara and Nikaido, 1992).

Genetic and biochemical analyses provided the basis for establishing a topological profile of the protein (Morona *et al.*, 1984). The model proposed for OmpA predicts eight N-terminal transmembrane segments followed by a long periplasmic C-terminal tail. No transmembrane segment was proposed in the C-terminal region of the protein, because no loop was identified to be exposed to surfactants in that region by bacteriophage mapping and also because the C-terminal domain underwent complete digestion by exogenous proteases in studies conducted with outer membranes and was completely protected in studies conducted with intact cells (Schweizer *et al.*, 1978). An alternative topological model for the protein was proposed according to the following three points (Stathopoulos, 1996): 1) Computer-aided predictions which were developed specifically to predict topology of bacterial outer membrane porins, such as OmpC and OmpF. 2) Identified sequence homologies between OmpA and other peptidoglycan-associated proteins. 3) Biochemical, immunochemical, and genetic topological data on proteins of the OmpA family provided by numerous previous studies. In this model, this protein consists of a single domain with sixteen transmembrane segments like OmpF porin structure. The latter model is, however, little supported by the spectroscopic data of the protein solution in the presence of various kinds of surfactants (Sugawara, *et al.*, 1996) and the structural properties of the protein obtained in the present study as will be described in Chapter III. Very recently, the crystallographic structure of only the N-terminal domain of the membrane protein was determined by Pautsch and Schultz (1998). The structure is shown in Figure 1.4. This structure seems to support the model by Morona *et al.* rather than the model by Stathopoulos. However, the reported structure may not deny the latter model completely

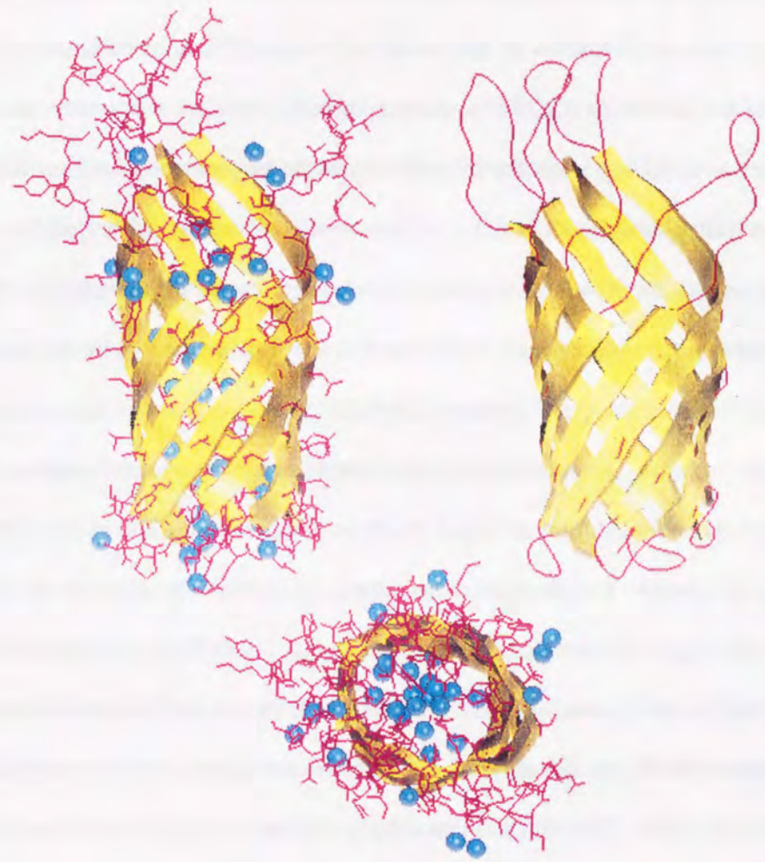


Figure 1.4 Crystal structure of the N-terminal domain of OmpA determined by Pautsch and Schultz (1998). The eight stranded β-barrel of the protein is drawn in horizontal (upper panels) and perpendicular (from cytoplasm to extracellular; lower panel) directions against a planar membrane (not included). The upper panels show the view in the same topology. Ribbon diagram of the barrel in the upper right panel shows that the long loops extrude to extracellular (top) and the short turns face to periplasmic space (down). In the upper left and lower panels, side chains of the domain and water molecules (blue globules) are included.

since that of the other half of the protein, the C-terminal domain, has been still unknown.

This protein is known to be solubilized monomerically retaining some folded structures in the presence of SDS at room temperature, while such structures are unfolded upon heating with boiling water for several minutes in the presence of the surfactant. This property is called heat modifiability, which was observed as a unique behavior on polyacrylamide gel for some bacterial outer membrane proteins such as OmpA, OmpC and OmpF (Nakamura and Mizushima, 1976). That is, the mobility of this kind of proteins on SDS-polyacrylamide gel electrophoresis (PAGE) changes upon heating, while such a change in the mobility cannot be shown in the case of most water-soluble globular proteins. In this thesis, the unfolded form of OmpA thus heat modified in the presence of SDS will be referred to as h-OmpA. This conformational change is irreversible since the unfolded form is kept by lowering temperature (Reithmeier and Bragg, 1974). It was shown, however, that the addition of excess amount of a nonionic surfactant, OG, to the h-OmpA fraction results in the restoration of the mobility of the non-heated OmpA fraction on SDS-PAGE (Dornmair *et al.*, 1990). This suggests that OmpA in the heat modified form can be refolded by the addition of excess amount of OG.

OmpA is considered to be a suitable example for studying the folding properties of membrane proteins for the following three reasons;

1) This protein is generated abundantly in the outer membrane of *Escherichia coli*, and an adequate amount of the protein can be prepared for physico-chemical experiments.

2) The protein in the heat modified form in the presence of SDS is known to refold by the addition of OG (Dornmair *et al.*, 1990), suggesting that a reversible conformational change of the protein can be observed in the mixed surfactant solution of SDS and OG.

3) OmpA is known to be monomeric both in the folded and unfolded forms in the presence of SDS (Reithmeier and Bragg, 1974, Nakamura and Mizushima, 1976), while many membrane proteins associate to form their higher order structures *in vivo* and such kind of the proteins often associate even in the presence of surfactants or lipid vesicles.

Additionally, OmpA is interesting from the point of view that the structure or solution properties of the membrane protein may be related to its unclear physiological functions. In the present study, the conformational change between its non-heated form and its heat modified one in the presence of SDS and/or OG is investigated in terms of its solution properties, hydrodynamic properties and kinetic aspects.

1.5 Scope of This Study

As described in the previous section (1.1), the study for protein folding is a significant and urgent proposition. Although a huge number of studies in this field have been carried out all over the world, most of them are concerned with water-soluble globular proteins and very limited knowledge is available as for membrane proteins. One reason for the bias originates from the difficulty in establishing experimental conditions under which membrane proteins unfold and refold reversibly.

This study was started to establish an approach for solving the folding problem about a membrane protein from a foothold of the qualitative observation by Dornmair *et al.* (1990), in which the refolding of OmpA from its heat modified form in the presence of SDS to its non-heated form was induced by the addition of an excess amount of OG. Purpose of the present study is to establish an approach for understanding the folding mechanism of the

membrane protein from a physico-chemical point of view. The present experimental system, in which the refolding reaction of OmpA from the unfolded form to the folded one can be controlled by systematically varying the composition of the mixed surfactant system of SDS and OG, is expected to afford advances in the understanding in a quantitative way.

Following general introduction of Chapter I, spectroscopic properties of OmpA in the non-heated form or the heat modified form in the presence of either SDS or OG, and solution properties of the protein during the conformational change between these forms in the mixed surfactant system of SDS and OG will be described in Chapter II. The heat modifiability of the protein will be accounted for in terms of hydrodynamic properties and the results will give an account for the refolding phenomena of the protein induced by the addition of OG. In order to obtain the structural information, OmpA in surfactant solutions was investigated through SAXS measurements to obtain the knowledge concerning the size and shape of the protein in the folded form under the solubilized condition. This will be described in Chapter III. In Chapter IV, kinetic aspects of the conformational change of OmpA in the mixed surfactant system will be described. The folding mechanism of the protein under this condition will be brought to light from this kinetic knowledge. In Chapter V, the folding mechanism of the membrane protein in the mixed surfactant system will be discussed with respect to the kinetic and thermodynamic insights. This is valuable since the present study is concerned with the folding properties of a β-type membrane protein. Additionally, we will discuss how one can investigate the folding mechanism and the conformational stability of a membrane protein, in relation to the approach of solving the folding problem about membrane proteins. Finally, conclusions and summary of the present results will be given in Chapter VI.

Chapter II Characterization of OmpA in Binary Surfactant System of SDS and OG

2.1 Introduction

In order to understand solution properties of OmpA, especially detailed aspects of those related with its conformational change, it is important to characterize the protein interacting with amphiphiles in conditions relevant to the unfolding and refolding phenomena. Such interactions between the protein and the amphiphiles can be referred to the amount of surfactant binding. OmpA solubilized in the binary surfactant system of SDS and OG is an object suitable for investigation of 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 Chapter, structural feature of OmpA in the non-heated form in the presence of either SDS or OG and that in the heat modified form in the presence of SDS examined by CD measurements are described in terms of spectroscopic properties. Subsequently, properties of both forms of the protein at various compositions of the mixed surfactant solutions examined by means of dynamic light scattering and low angle laser light scattering photometry combined with size exclusion chromatography (SEC-LALLS) are described 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. This content was mainly reported by Ohnishi *et al.* (1998).

2.2 Materials and Methods

2.2.1 Materials

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

2.2.2 OmpA Preparation

OmpA was isolated and purified from the outer membrane of *Escherichia 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.* (1977). The preparation thus obtained was further purified by the use of hydroxyapatite chromatography technique according to the method of Watanabe *et al.* (1992). The fraction containing OmpA was applied to the hydroxyapatite column (20 × 2.5 cm ϕ) purchased from Mitsui-Toatsu Chemicals Inc. (Tokyo, Japan) (this column is presently distributed from Koken Inc., Tokyo, Japan). The OmpA fraction eluted at about 160 mM of the buffer concentration during the gradient from 10 mM of sodium phosphate buffer (NaPB, pH 6.9) containing 3.47 mM 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 minutes to obtain heat modified OmpA. 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 (30 × 1.5 cm ϕ, Pharmacia LKB Biotechnology,

Uppsala, Sweden).

2.2.3 SDS-Polyacrylamide Gel Electrophoresis

All experiments for SDS-PAGE were carried out according to Weber-Osborn's method (Weber *et al.*, 1972). Low molecular weight calibration kit proteins (Pharmacia LKB Biotechnology, Upsala, Sweden) were employed as standards.

2.2.4 Size Exclusion Chromatography Combined with Low Angle Laser Light Scattering Photometry

The mode of molecular assembly of a protein in surfactant solutions and the surfactant binding to the protein were studied by the size exclusion chromatography using a TSKgel G3000SW$_{XL}$ column (30 × 0.78 cm ϕ) with a TSK SW guard column (7.5 × 0.75 cm ϕ) 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 (Hayashi *et al.*, 1989). All these detectors were products of Tosoh Co. Ltd. (Tokyo, Japan).

The output from each of the detectors can be expressed as follows.

$$(\mathrm{LS}) = k_1 (dn/dc)^2 Mc \qquad [2.1]$$

$$(\mathrm{UV}) = k_2 Ac \qquad [2.2]$$

$$(\mathrm{RI}) = k_3 c(dn/dc) \qquad [2.3]$$

Here, (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_1, k_2, and k_3 are instrumental constants for the corresponding detectors, *dn/dc* is the specific refractive index increment, *c* is the weight concentration of the protein, *M* is the molar mass of the protein, and *A* is the specific extinction coefficient. The values of *A* for BSA and OVA were adopted as 0.68 and 0.75 (l g^{-1} cm^{-1}), respectively, and those for the folded OmpA and the unfolded OmpA were determined to be 1.78 and 1.73 (l g^{-1} cm^{-1}) by means of quantitative amino acid analysis. The refractive index increment and the molar mass of a protein can be expressed according to equations [2.1], [2.2] and [2.3] as follows, respectively.

$$R \equiv A\frac{(\mathrm{RI})}{(\mathrm{UV})} = \frac{k_3}{k_2}(dn/dc) \qquad [2.4]$$

$$W \equiv \frac{(\mathrm{UV})(\mathrm{LS})}{A(\mathrm{RI})^2} = \frac{k_1 k_2}{k_3^2} M \qquad [2.5]$$

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

$$dn/dc \equiv (\partial n/\partial c_\mathrm{P})_\mu = (\partial n/\partial c_\mathrm{P})_{c_\mathrm{S}} + (\partial n/\partial c_\mathrm{S})_{c_\mathrm{P}} \qquad [2.6]$$

where δ_S is the gram of surfactant bound to gram of the protein. Using the relation, one can calculate δ_S according to the procedure described previously (Lundahl *et al.*, 1990). The value of $(\partial n/\partial c_\mathrm{P})_{c_\mathrm{S}}$ was mostly constant for simple proteins and was assumed to be 0.193 ml g^{-1} (Hayashi *et al.*, 1989). The value of $(\partial n/\partial c_\mathrm{S})_{c_\mathrm{P}}$ was assumed to be 0.119 ml g^{-1} in the presence of SDS (Hayashi and Ikeda, 1980), and 0.138 ml g^{-1} in the presence of OG (Kameyama *et al.*, 1990).

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 SEC with Sephacryl S-300 HR column (30 × 1.5 cm ϕ). An appropriate volume of sample solution ranging 50-500 µl with concentration of 11.0-35.0 µM of the proteins was applied to the column at a flow-rate of 0.3 ml min^{-1}. The temperature was kept at 25 °C. Mixed surfactant solutions containing SDS and OG were prepared keeping SDS concentration constant at 3.47 mM. The composition of this mixed surfactant system will be described as X_{OG}, that is, the weight fraction of OG in the total surfactants. Exceptionally, the solution denoted as X_{OG}=1 contains 27.4 mM of OG and no SDS.

2.2.5 Dynamic Light Scattering Photometry

Sample solutions (0.5-2.0 mg protein per ml) were prepared by equilibration against 50 mM sodium phosphate buffer (pH 6.9) containing defined compositions of SDS and OG by gel filtration using the same column system as used in the SEC-LALLS measurements. The total concentration of the surfactants was adjusted to be just above the critical micelle concentration of the mixed surfactant system of SDS and OG (Kameyama *et al.*, 1997), so as to prevent the presence of excess mixed micelles.

Mutual diffusion coefficients were measured with a dynamic scattering photometer, DLS-700 (Otsuka Electronics Co., Ltd. Hirakata, Japan) at 25 °C. Hydrodynamic radii, R_H's, of the complexes between surfactant and protein were calculated according to the Einstein-Stokes' formula (equation [2.7]).

$$D = \frac{k_B T}{6\pi\eta \quad R_H} \qquad [2.7]$$

Here, D is the self diffusion coefficient which is assumed to be equal to the mutual one for dilute solutions, and k_B, T and η are the Boltzmann's constant, the absolute temperature and the viscosity of the solvent, respectively. The solvent viscosity was assumed to be equal to the viscosity of water at the same temperature.

2.2.6 CD Measurements

CD spectra of the proteins were taken on J-720 (Jasco, Tokyo, Japan) using a cuvette with 1 mm and that with 1 cm of light path lengths for far UV region (205-250 nm) and for near UV region (250-320 nm), respectively. Protein concentrations were 7.7-12.0 μM for far UV region, and 17.2-21.7 μM for near UV region. The temperature of the cells was controlled at 25 °C by the use of a thermostat bath.

2.3 Results

2.3.1 CD spectra of OmpA

Figure 2.1 shows CD spectra in near (250-340 nm) and far UV (205-250 nm)

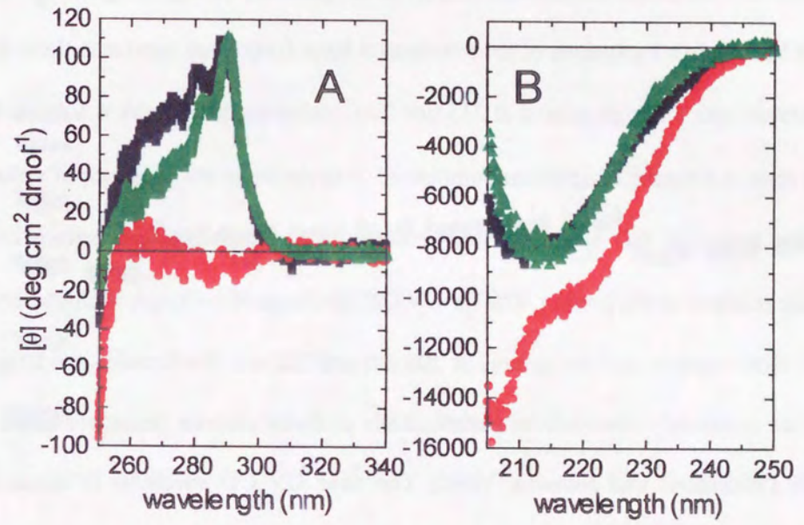


Figure 2.1 CD spectra in the near (A) and far (B) UV regions for OmpA in the non-heated form in the presence of either 3.47 mM SDS (■) or 27.4 mM OG (▲) and for OmpA in the heat modified form in the presence of 3.47 mM SDS (●) all at 25 °C. Protein concentrations were 7.7-12.0 μM for the far UV region, and 17.2-21.7 μM for the near UV region.

regions for OmpA in the non-heated form in the presence of either SDS or OG and for the protein in the heat modified form (h-OmpA) in the presence of SDS. The far UV CD spectra of both SDS and OG solutions of the non-heated form OmpA are similar to those for typical β-structures with a trough around at 215 nm. This indicates that OmpA is solubilized with a folded form containing a significant amount of β-structure in the presence of either SDS or OG. The near UV CD spectra assure that the folded form retains tertiary contacts of aromatic residues of the protein. The far UV CD spectrum of h-OmpA is characteristic of α-helices with negative maxima around at 208 nm and 220 nm (Reithmeier and Bragg, 1977), which are commonly observed for water-soluble globular proteins denatured in the presence of SDS (Waterhous and Johnson, 1994). The near UV CD spectrum is characteristic of unfolded proteins which have no tertiary contacts of the aromatic residues. These spectra suggest that OmpA denatures upon heating in the presence of SDS to an unfolded form containing some α-helical structures.

2.3.2 Hydrodynamic Properties

OmpA and h-OmpA kept in various compositions of the mixed surfactant system of SDS and OG were analyzed by SDS-PAGE using 10 % (w/v) of polyacrylamide gel. Figure 2.2 shows an electrophoretic diagram thus obtained. OmpA solubilized in the neat SDS solution migrated, giving a single band at the position of 29 K (lane 2), and h-OmpA solubilized on the same condition migrated at the position of 35 K (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.* (1990). In the present experiment, the restoration of the mobility was observed at X_{OG} above around 0.7 (lane 4-12).

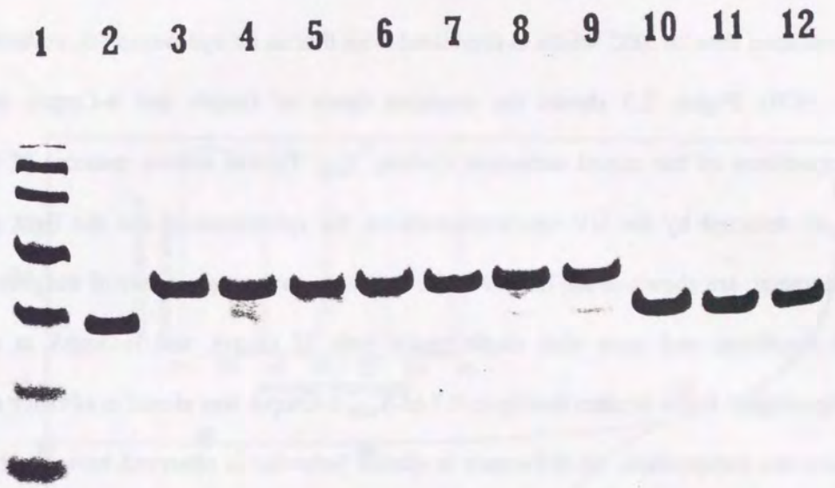


Figure 2.2 SDS-PAGE diagram of h-OmpA (lane 3-12) kept in various compositions of the mixed surfactant system of SDS and OG. The compositions of the mixed surfactant system were 0, 0.37, 0.45, 0.55, 0.61, 0.69, 0.72, 0.79, 0.84, 0.92 of X_{OG} for lanes from 3 to 12, respectively. Standard proteins on lane 1 were phosphorylase b (94 K), bovine serum albumin (64 K), ovalbumin (43 K), carbonic anhydrase (30 K), trypsin inhibitor (20.1 K), α-lactalbumin (14.4 K), respectively (top to bottom). The single band on lane 2 is OmpA in the non-heated form.

This change in the apparent size of the membrane protein was also observed as that of retention time in SEC which is correlated with that in its hydrodynamic volume (Fish *et al.*, 1970). Figure 2.3 shows the retention times of OmpA and h-OmpA at various compositions of the mixed surfactant system, X_{OG}. Typical elution patterns of OmpA at X_{OG}=0 detected by the UV spectrophotometer, the refractometer and the light scattering photometer, are shown in the inset. All the detectors gave single peaks of the protein under this condition, and gave also single peaks both of OmpA and h-OmpA at the other compositions. It can be seen that up to 0.7 of X_{OG}, h-OmpA was eluted in advance of OmpA. Above the composition, no difference in elution behavior is observed between them. This suggests that the hydrodynamic volume of OmpA increased on heating is almost kept in the former range of X_{OG}, and that the protein restores the hydrodynamic size to that of non-heated form in the latter range. The retention time of OmpA in the presence of OG only is significantly larger than those at the other compositions containing the respective fractions of SDS. This may be ascribed to ionic interactions between the OmpA-surfactant complexes and the column material. Namely, the electrostatic charge of the complex containing SDS changes with the population of the surfactant, since SDS is an anionic surfactant. Elution of such 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 (Tarvers and Church, 1985). In this context, the retention time at X_{OG}=1 may give the correct hydrodynamic volume of the protein-surfactant complex.

The hydrodynamic radii of the protein-surfactant complexes for OmpA and h-OmpA were obtained through DLS measurement and are plotted against X_{OG} in Figure 2.4. In the neat SDS solution, the R_H of OmpA increases from 37.3 ± 2.1 to 47.0 ± 1.6 Å upon

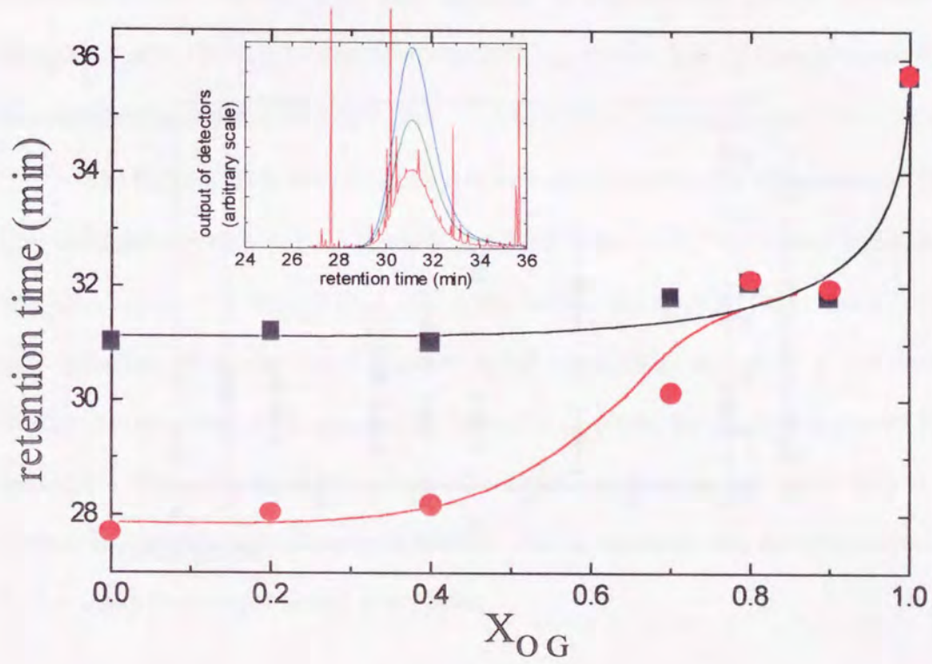


Figure 2.3 Changes in retention time for OmpA (■) and h-OmpA (●) in the size exclusion chromatography with composition of the mixed surfactant system of SDS and OG. Typical elution patterns of OmpA at X_{OG}=0 detected by UV (——), RI (——) and LALLS (——) are shown in the inset. A TSK-gel G3000 SW_{XL} column (30 × 0.78 cm ϕ) with a TSK SW guard column (7.5 × 0.75 cm ϕ) was employed and the flow rate was 0.3 ml min^{-1}.

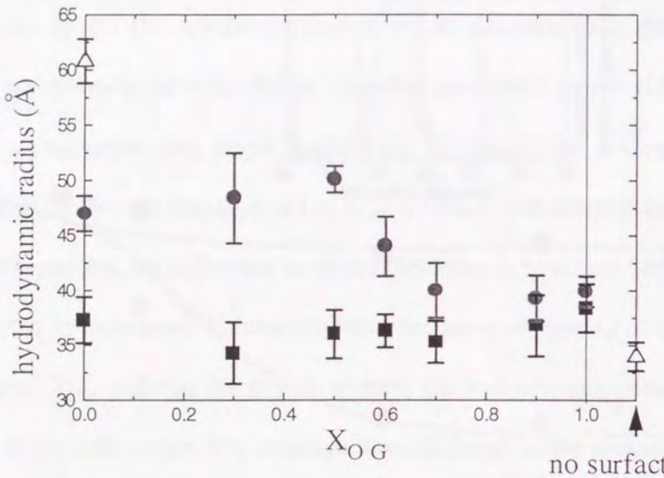


Figure 2.4 The hydrodynamic radii of the proteins (OmpA (■), h-OmpA (●), and BSA (△)) in the mixed surfactant system of SDS and OG. These values were calculated from the diffusion coefficients which were obtained through the dynamic light scattering measurements. Error bars are the standard deviations of the measurements for each protein.

heating. This change in R_H is consistent with the previous study by Reithmeier and Bragg (1977), 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, 38.8 ± 2.6 Å, within experimental error.

The R_H's for BSA in a surfactant-free aqueous solution and in the presence of SDS were determined to be 34.0 ± 1.3 Å and 60.8 ± 2.0 Å, respectively. The former is consistent with those reported by Tanford *et al.* (1974) and Tarvers and Church (1985). The R_H of the SDS-denatured BSA with intact disulfide bonds obtained in this study is consistently smaller than the value, 78 Å, reported by Tanford *et al.* (1974) for the SDS-denatured BSA without the bonds. The R_H of BSA in the OG solution was identical with that of BSA in the absence of surfactant (not shown in Figure 2.4). This is consistent with the observation that BSA scarcely binds OG (Lundahl *et al.*, 1990).

2.3.3 Molar Masses of the Proteins

The molar mass of a protein can be determined by application of the outputs of the three detectors to equation [2.5]. The value of W in the equation is proportional to the molar mass (see Materials and Methods). The values obtained for OmpA and h-OmpA are plotted in Figure 2.5 against the weight fraction of OG in the mixed surfactant system. We see that the molar masses of OmpA and h-OmpA are almost the same and substantially invariant with X_{OG}. Clearly neither OmpA nor h-OmpA shows association despite the change of surfactant composition. Thus, the conformational change of the membrane protein between the non-heated and heat modified forms is accompanied with virtually no change in the

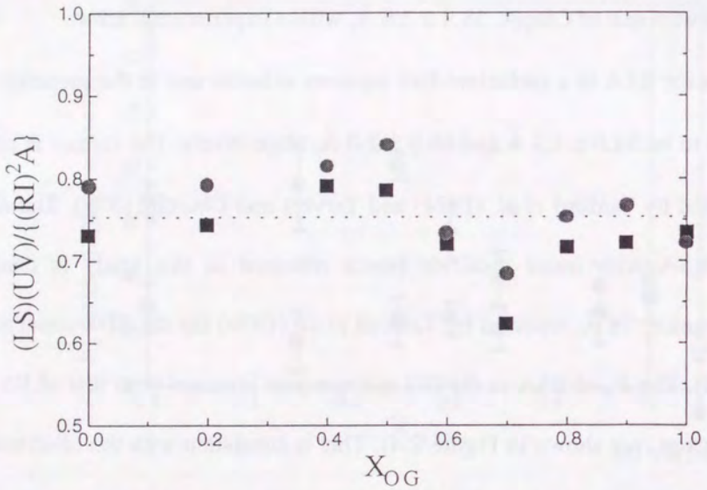


Figure 2.5 The values of W {=(LS)(UV)/(RI)^2A} in the equation [2.5] for OmpA (■) and h-OmpA (●) measured at various compositions of the mixed surfactant system of SDS and OG. The W is proportional to the molar mass of the protein.

molecular assembly.

2.3.4 Amount of Surfactant Binding

The value of R in equation [2.4] was measured for BSA, OVA, OmpA and h-OmpA and plotted in Figure 2.6 against the weight fraction of OG in the mixed surfactant system. For X_{OG} up to 0.6, the line for h-OmpA is above that for OmpA. The two lines approach with each other with increasing X_{OG}, converging to almost identical values above X_{OG}=0.7. Thus, the specific refractive index increment of h-OmpA is significantly larger than that of OmpA in the range of X_{OG} below 0.6, but there is no significant difference between them above that.

The amounts of surfactant bound to proteins at X_{OG}=0 and 1 were calculated according to equation [2.6], and shown in Table 2.1. The necessary value of $\frac{k_3}{k_2}$ in equation [2.4] was determined with (dn/dc_p)=0.376 ml g^{-1} for BSA (Kameyama et al., 1982). The amounts bound to BSA and OVA thus determined are in agreement with those reported previously (Lundahl *et al.*, 1990, Takagi *et al.*, 1975). The amount of SDS bound to OmpA is seen to increase upon heating from 1.8 gram to 2.3 gram per 1 gram of the protein. These results are consistent with those reported by Reithmeier and Bragg (1977). On the other hand, the amount of OG bound to OmpA is the same as that to h-OmpA, suggesting that heat modified OmpA refolds to the non-heated form in terms of amount of surfactant binding by the addition of OG.

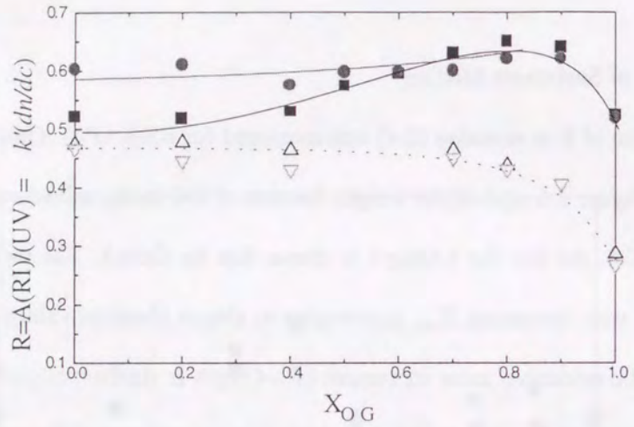


Figure 2.6 Changes in refractive index increment for the proteins (BSA (△), OVA (▽), OmpA (■) and h-OmpA (●)) with the composition of the mixed surfactant system of SDS and OG. The value of R {=A(RI)/(UV)} in equation [2.4] is proportional to the refractive index increment of the protein.

Table 2.1 Amounts of SDS and OG bound to proteins in the presence of either SDS or OG

	δ_{SDS}[1]	δ_{OG}[2]
OmpA	1.8	1.6
h-OmpA	2.3	1.6
BSA	1.5	0.2
OVA	1.4	0.1

[1] Grams of SDS per gram of protein for 50 mM of NaPB (pH 6.9) containing 3.47 mM SDS solely.

[2] Grams of OG per gram of protein for the same buffer containing 27.4 mM OG solely.

2.4 Discussion

2.4.1 Conformations of OmpA in the Presence of Surfactants

We have studied three states of OmpA in the presence of either SDS or OG and conformational changes in the mixed surfactant system. This section discusses the three states of OmpA, *i.e.*, in the non-heated form in the presence of SDS, in the presence of OG and in the heat modified form, h-OmpA, in the presence of SDS. The hydrodynamic radius of h-OmpA was larger than those of OmpA in the forded forms, while the molar masses of the protein in the non-heated and heat-modified states were essentially the same. This indicates that OmpA denatures upon heating in the presence of SDS. The far and near UV CD spectra in Figure 2.1 support this. Addition of OG resulted in the refolding of the protein from the heat modified form to the non-heated form in terms of the mobility on PAGE and the hydrodynamic radius.

The folded forms of OmpA at X_{OG}=0 and 1 were very similar in terms of the hydrodynamic volume, the molar mass and the mobility on PAGE. The near and far UV CD spectra of OmpA in the non-heated form in the presence of SDS were, however, not identical to those in the presence of OG. This is consistent with the observation by Sugawara *et al.* (1996), who found that the CD spectrum of OmpA in the presence of SDS is not identical with that in lipid vesicle. This difference in the spectra may be generated from a minor structural change in such flexible regions as loops in the protein, since hydrodynamic properties were very similar between them.

2.4.2 "Heat-Modifiability" of OmpA on SDS-PAGE

Some of *Escherichia coli* outer membrane proteins change their mobilities on SDS-

PAGE upon heating. This is called "heat modifiability" in general. This feature is commonly observed for proteins which are not denatured without heating in the presence of SDS. Porins such as OmpF and OmpC are known to increase their mobilities upon heating (Nakamura and Mizushima, 1976). This heat modifiability of porins can be ascribed to dissociation of their trimeric structures into monomeric ones.

OmpA is also one of such heat modifiable proteins. This protein solubilized in the presence of SDS decreases its mobility on SDS-PAGE upon heating. The present result shows that the molar mass of the protein does not change upon heating. This indicates that the decrease in the mobility is not due to association. The heat modifiability of OmpA is also related to frictional and electrostatic properties of the complex between the protein and SDS. The hydrodynamic radius of the complex formed between OmpA in the non-heated form and SDS was shown to increase upon heating through the DLS measurement. This increases friction to retard the electrophoretic migration of the protein. On the other hand, SEC-LALLS measurements made clear that the amount of SDS bound to OmpA was increased by heating. This increase in anionic charge can promote the migration. In practice, however, this electrostatic effect for forward migration is overcome by the above frictional effect for the retardation, and consequently this membrane protein decreases its mobility upon heating.

2.4.3 Amount of Surfactant Binding to Protein

In the present study, solution properties of OmpA in the non-heated form and the heat modified form in the mixed surfactant solutions of SDS and OG have been studied. The observed changes will be discussed with reference to the conformational changes of the

protein. We have focused attention in making clear the correspondence of the conformational change to the change in interaction between the protein and the amphiphiles, examined in terms of that in the amount of its surfactant binding.

While the amount of the surfactant binding was determined using equation [2.6] in the presence of either SDS or OG, things are not so simple in the mixed surfactant system containing both SDS and OG that estimation of the amount of mixed surfactants is difficult. For instance, composition of the mixed surfactants bound to a protein is not always equal to the prepared mixed surfactant composition owing to the difference of the ability for micelle formation between the two surfactants. Actually, the composition of mixed surfactant micelles of SDS and OG was found to be different from the prepared composition (Kameyama *et al.*, 1997). Nevertheless, one can roughly evaluate the change in amount of the surfactant binding through the ordinate R values in Figure 2.6 that are proportional to the refractive index increments. Since water-soluble proteins such as BSA and OVA scarcely bind OG, the ordinate value for protein moiety is expected to be about 0.28 for all the proteins involved. The increment above that value may 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. 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. These lines share the feature that they are flat and then descend progressively. This feature may be interpreted as follows: 1) the protein polypeptides are unfolded and maximally bind SDS; 2) the bound SDS is progressively replaced by OG, and the cluster of bound surfactant

assumes a nature of mixed micelle; 3) with a decrease in the charge density on the cluster, the protein polypeptide refolds to the non-heated state; 4) in the folded state, OG binds significantly to OmpA but scarcely to BSA or OVA. OmpA retains the folded structure in the presence of SDS unless being heated, and binds less amount of SDS than h-OmpA. This property of OmpA seems responsible for the R versus X_{OG} curve with a unique feature as shown in Figure 2.6.

The observation shown in this figure and the interpretation described above illustrate that such a simple approach for following the change in the specific refractive index increment with the composition of the mixed surfactant solution is efficient to sketch out the feature of interaction of a protein in the binary surfactant system. We further examine the interactions of OmpA and h-OmpA with SDS and OG in the following section.

2.4.4 Effect of Each of the Surfactants on the Conformational Change of OmpA in the Mixed Surfactant System of 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 the protein in a solution containing the three components by the following equation.

$$(\partial n / \partial c_P)_\mu = (\partial n / \partial c_P)_{c_S} + \delta_{SDS}(\partial n / \partial c_{SDS})_{c_P,c_{OG}} + \delta_{OG}(\partial n / \partial c_{OG})_{c_P,c_{SDS}} \quad [2.8]$$

Here, δ_{SDS} and δ_{OG} are the grams of bound SDS and OG per gram of the protein, respectively. Subscripts SDS and OG indicate that the values are associated with the corresponding surfactants. Because the composition of the bound surfactants is not

necessarily equal to the nominal composition in the solution, δ_{SDS} and δ_{OG} cannot be simply determined. Kronberg *et al.* (1986) estimated the amount of bound surfactants on the surface of polystyrene latex in an anionic and nonionic surfactant mixed system containing SDS and nonylphenol deca (oxyethylene glycol) monoether. They assumed that the composition of the bound surfactants is equal to that of the coexisting micelles. The same situation was also assumed here. Kameyama *et al.* (1997) estimated the composition of the micelles in the binary surfactant system through surface tentiometry, which corresponds to the ratio of δ_{SDS} to δ_{OG} under this assumption. Knowledge of this ratio with equation [2.8] allows determination of the surfactant binding.

The amounts of bound SDS and OG thus evaluated are plotted against the weight ratio of SDS to OG used in the preparation of the sample solutions in Figure 2.7. In panel A, the amount of SDS bound to h-OmpA decreases monotonously with the addition of OG. This is also the case for the water-soluble proteins, BSA and OVA. 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 merge. This again indicates that the mode of binding of SDS to OmpA differs from that to h-OmpA; the latter behaves rather similarly to the water-soluble proteins.

On the contrary, in panel B of Figure 2.7, no difference is observed between OmpA and h-OmpA in the mode of binding of OG over the entire range of X_{OG}. As shown in Figure 2.6, h-OmpA has higher capacity for SDS binding, so that SDS is solely responsible for the difference between OmpA and h-OmpA observed in panel A. We may therefore draw the following conclusion on the conformational change of OmpA in the mixed surfactant system of SDS and OG: The unfolded "heat modified" state is attributed to the

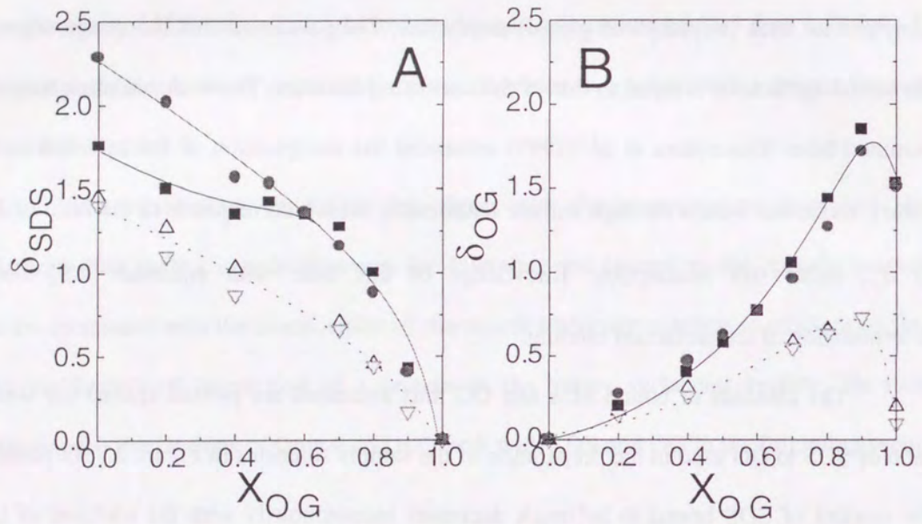


Figure 2.7 The amounts of SDS (A) and OG (B) bound to the proteins (BSA (△), OVA (▽), OmpA (■) and h-OmpA (●)) in the mixed surfactant system of SDS and OG calculated from the equation [2.8] on the assumption that the compositions of bound surfactant to the proteins are the same as those of the mixed micelles in the solutions. The compositions of the mixed micelles of SDS and OG were estimated from the data of Kameyama *et al.* (1997).

influence of SDS, which is accelerated by heating. The denaturation of the membrane protein by SDS may be so slow that OmpA in the non-heated form retains the folded structure unless being heated. Upon unfolding by heat treatment, parts of the 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, thereby leading to the loss of such sites and the 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 the 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. The kinetic study of the conformational change of OmpA in the mixed surfactant system, described in Chapter IV, will give results supporting this idea.

Chapter III Size and Shape of OmpA

3.1 Introduction

Structural information of a protein is very helpful in understanding its folding mechanism. Folding properties have been studied for water-soluble globular proteins through spectroscopic methods to propose some plausible folding schemes of the protein molecules. In this context, structural information of such a protein in its native state is usually obtained from the X-ray crystallographic analyses, and is also helpful to the understanding of biological activities *in vivo*. However, a small number of crystal structures have been determined for membrane proteins. OmpA, one of the well studied membrane proteins, is considered to be a nice example for studying the folding properties as described in Chapter I, but its 3-demensional structure has not been determined except for the transmembrane N-terminal domain of the protein (Pautsch and Schultz, 1998). Two topological models for OmpA were proposed by Morona *et al.* (1984) and by Stathopoulos (1996). In the former model, this protein has eight anti-parallel β-strands in the transmembrane N-terminal domain and the rest of the protein is considered to form an extra-membrane domain which was speculated to be exposed in periplasmic space. In the latter model, this protein has sixteen transmembrane segments consisting of fifteen anti-parallel β-strands to form a β-sheet and one helix. Some spectroscopic data (Sugawara *et al.*, 1996) and the X-ray crystallographic analysis of the N-terminal half of the protein (Pautsch and Schultz, 1998) seem to support the former model. Besides such spectroscopic and crystallographic knowledge, structural information obtained by small angle X-ray scattering

(SAXS) method is also important for characterizing the membrane protein in surfactant solutions. This chapter is concerned with the size and shape studied by SAXS on OmpA in the presence of SDS or OG.

Membrane proteins are sometimes denatured in the presence of surfactant upon their solubilization. However, OmpA in the solubilized form in the presence of SDS maintains some folded structure which can be detected by means of spectroscopic methods such as CD, intrinsic fluorescence, and so on. It should be noted that the structure under this condition is reported not to be identical with that in lipid vesicles (Sugawara *et al.*, 1996). The difference in the protein structure does not seem significant in its essential folding properties. In fact, the spectroscopic data obtained in the present study show that the CD spectrum of OmpA in a SDS solution converts reversibly to that in an OG solution, resulting in only a small difference between these spectra in the near (250-300 nm) and far UV (205-250 nm) regions (Figure 2.1). This suggests that the small difference may be generated from a minor structural change in such flexible regions as loops in the protein. The trivial difference in the structure is, hence, not critical for considering the basic folding structure of the protein.

OmpA solubilized in the presence of a surfactant, SDS or OG, forms a complex with the surfactant molecules. In this study, the properties of the complex of OmpA with SDS (OmpA/SDS) and that with OG (OmpA/OG) as well as its heat modified form in the presence of SDS (h-OmpA/SDS) were studied by means of SAXS measurement. The SAXS method is very useful for analyzing both overall structures and their conformational changes of proteins in solution in terms of structural parameters such as the radius of gyration and the distance distribution function. The SAXS measurements were also made on some water-

soluble proteins, OVA and ribonuclease A (RNaseA), in the present of SDS as reference to the complexes formed between the membrane protein and surfactants. In this chapter, the size and shape of the complexes are examined with the SAXS results together with the hydrodynamic properties determined from DLS measurements and the amount of surfactant binding from LALLS measurements described in Chapter II, to afford quantitative structural properties for the overall structure of the membrane protein solubilized in the presence of the surfactant. Our discussion refers to which of the models proposed by Morona *et al.* (1984) and by Stathopoulos (1996) is more reasonable for OmpA.

3.2 Materials and Methods

3.2.1 Chemicals

Sodium dodecyl sulfate (SDS) was obtained from BDH (Anala R grade), and *n*-octyl-β-D-glucoside (OG) was from Dojindo Laboratories Inc. (Kumamoto, Japan). Other chemicals were of reagent grade.

3.2.2 Sample Preparation

OmpA was isolated from *Escherichia coli* K-12 strain according to the procedure described in section 2.2.2. The OmpA fraction thus obtained was equilibrated through size exclusion chromatography against 50 mM NaPB (pH 6.9) containing 1.5 mM SDS or 22 mM OG to obtain OmpA solutions with the protein concentrations of about around 75-95 μM. Heat modified OmpA was also equilibrated against 50 mM NaPB (pH 6.9) containing 1.5 mM SDS to obtain h-OmpA/SDS complex.

OVA from hen egg white and RNaseA from bovine pancreas were purchased from Seikagaku Corporation (Tokyo, Japan) and Sigma Co. Ltd. (St. Louis, U.S.A.), respectively. These proteins were reduced and carboxyamidomethylated to attain their completely unfolded conformations lacking their disulfide links, according to the following procedure. Each protein was dissolved at 250 μM in 50 ml of 0.2 M Tris-acetate buffer (pH 8.0) containing 8M urea, 1 mM EDTA and dithiothreitol with molar concentration of 15 times against that of disulfide linkages in the protein solution. After stirring the mixture for 90 minutes at room temperature, the resulted sulfhydryl groups (-SH) were carboxyamide-methylated by the addition of 0.68 g of monoiodoacetatamide. The pH of the mixture was adjusted to 8.2 by the addition of 1 M aqueous sodium hydride and kept at room temperature for 60 minutes with stirring. The resulted solution was dialyzed against deionized water at room temperature for removing urea and salts to obtain white precipitation. This precipitation was resolubilized upon dialysis against 0.05 M acetic acid. Insoluble components in the resulted solution were removed by centrifugation and the supernatant was lyophilized to obtain the reduced-carboxyamidomethylated (RCAM) water-soluble proteins. RCAM-OVA and RCAM-RNaseA thus obtained were solubilized in the presence of SDS and equilibrated as in the case of OmpA/SDS to obtain RCAM-OVA/SDS and RCAM-RNaseA/SDS solutions with protein concentrations of about 250 μM.

3.2.3 Measurements of Small Angle X-ray Scattering

The X-ray source was a 0.4×8 mm spot on the copper anode of a Philips fine-focus X-ray tube, which was operated at 40 kV, 45mA with a Rigaku sealed type X-ray generator. Line focus geometry with a single mirror focusing optics was used to obtain sufficiently

intense scattered X-rays. Scattered X-ray intensities were recorded in a scattering-angle range from 3.0×10^{-3} to 7.7×10^{-2} radian with a one dimensional position sensitive proportional counter at a sample-to-detector distance of 297.5 mm, the primary X-ray beam being introduced at the center of the counter. The effective width of the counter (50 mm) was divided into 512 channels with a multi-channel analyzer. The X-ray path from a sample holder to the counter was evaluated to avoid scattering and adsorption by air. Sample solutions and their reference buffer solutions were sucked into a thin-walled quartz capillary (1.0 mm ϕ). The temperature was regulated to 25 °C by the use of a thermostat bath. Eight successive measurements were made for each solution with an exposure time for 8000 per measurement. The resulting data were collected after inspection for X-ray radiation damage to the solution and the existence of instrumental artifacts.

3.2.4 Analysis of Small Angle X-ray Scattering Data

The scattering intensities recorded on both sides of a primary beam were averaged at equivalent points after subtracting the background intensities. The subtraction was performed with a scale factor of 1.02 applied to the back ground intensities to obtain the scattering intensities from the net protein solution. The position of the primary beam (zero angle) was determined to the 296th channel so as to minimize the reliability factor, R_{sym}, of the scattering intensities at equivalent points on both sides of the zero angle. The blind region, in which accurate intensities cannot be obtained due to the obstacle by the beam stopper, was determined to be at the 296th $\pm$ 10 channels. The slit-smearing effect was corrected by the method of Glatter (Pilz *et al.*, 1979). The interparticle interference effects that appear in the smaller angle scattering region were negligible for all solutions with low

concentrations.

The radius of gyration, R_g, and the zero-angle scattering intensity, $I(0)$, were derived by a least-squares procedure from a best-fit straight line of the Guinier plot of scattering intensity data according to the following equation.

$$\ln I(q) = \ln I(0) - R_g^2 q^2 / 3 \qquad [3.1]$$

Where q is the magnitude of the scattering vector defined by $q = 4\pi\sin(\theta/\lambda)$, where 2θ and λ being the scattering angle and the wavelength of X-rays (λ = 1.5418 Å). The pair-distance distribution function, $P(r)$, was calculated by the Fourier transform of the scattering profile, $I(q)$, to facilitate understanding structural features, including internal structure, of proteins in solution.

$$P(r) = \frac{1}{2\pi^2} \int_0^\infty I(q)\, qr \sin(qr) dq \qquad [3.2]$$

This function provides the probability density finding a pair of volume elements separated in a distance of r, weighted with respective excess-electron densities, and therefore estimates the maximum particle dimension, D_{max}, defined as the distance where the $P(r)$ finally reaches zero.

3.3 Results

3.3.1 Profile of Scattering Intensity

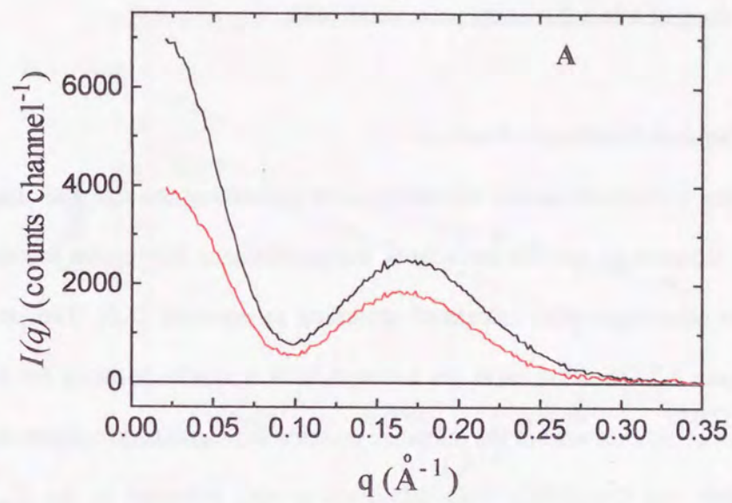
Figure 3.1A shows the SAXS profiles for the complexes of RCAM-OVA with SDS, RCAM-OVA/SDS and RCAM-RNaseA/SDS. These two water-soluble protein complexes show similar profiles with subsidiary maxima around q=0.17 Å^{-1}. These maxima were also

observed in the complex of RCAM-BSA with SDS (Shinagawa *et al.*, 1994). This suggests that these proteins in the presence of SDS have a common structural feature. In fact, as shown in Figure 3.1B, the scattering profile for h-OmpA/SDS also has a similar maximum around q=0.17 Å^{-1}. These results clearly indicate that the structure of h-OmpA/SDS is similar to those of unfolded water-soluble proteins in the presence of SDS. In contrast, as shown in Figure 3.1B, the SAXS profile of OmpA/OG is remarkably different from those of OmpA/SDS and h-OmpA/SDS. It has no subsidiary maximum around q=0.17 Å^{-1}. In other words, the structure of OmpA in OG solution is significantly different from that in SDS solution. This may be consistent with the spectroscopic data described in Chapter II (Figure 2.1) and those in the study by Sugawara *et al.*(1996).

It should be noted that $I(0)$ for OmpA/OG is stronger than that for OmpA/SDS. Since the molar mass of OmpA does not change in these surfactant solutions (Ohnishi *et al.*, 1998), the difference in $I(0)$ can be ascribed to that in average electron density among the protein-surfactant complexes.

3.3.2 Guinier Plot and Radius of Gyration

Figure 3.2 shows Guinier plots for OmpA/SDS, h-OmpA/SDS and OmpA/OG. The radii of the complexes obtained from these Guinier plots are summarized in Table 3.1. For the water-soluble proteins, the radii were not determined because the Guinier region, which gives a linear relation between $ln\ I(q)$ and q^2, could not be specified. The fact that R_g of h-OmpA/SDS is significantly larger than that of OmpA/SDS indicates that the protein is extended upon heating in the presence of SDS. On the other hand, R_g of OmpA/OG is much lager than that of OmpA/SDS, suggesting that the shape of the protein in the presence of



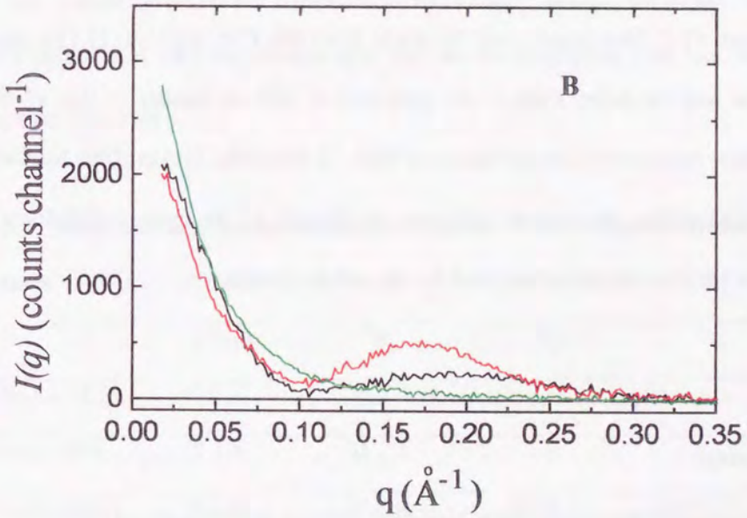


Figure 3.1 (A) SAXS profiles for the complexes formed between SDS and water soluble proteins; RCAM-OVA (——), RCAM-RNaseA (——). (B) SAXS profiles for the complexes formed between surfactants and OmpA; OmpA/SDS (——), h-OmpA/SDS (——), OmpA/OG (——). Experiments were performed at 25°C with 50 mM NaPB (pH 6.9) containing 1.7 mM SDS or 22mM OG.

OG is rather elongated than that in the presence of SDS.

3.3.3 Pair-Distance Distribution Function

In order to evaluate further detailed aspects concerning the size and shape of the complexes of the proteins with the surfactants, the pair-distance distribution functions, $P(r)$, for these five complexes were calculated according to equation [3.2]. The profiles are shown in Figure 3.3. The $P(r)$ curve for h-OmpA/SDS is similar to those for the water-soluble protein complexes used as the reference proteins and significantly differs from those for OmpA/SDS and OmpA/OG. This difference is also reflected in the D_{max} values estimated from $P(r)$. Two points may be made from the $P(r)$ profiles: 1) The structure of OmpA in the heat modified form in the presence of SDS is similar to that of the water-soluble proteins denatured in the presence of SDS. 2) Since the $P(r)$ profiles for OmpA/SDS and OmpA/OG differ, the overall structure of OmpA in the non-heated form changes depending on the kind of surfactant used for the solubilization.

3.4 Discussion

3.4.1 Structure of OmpA in the Heat Modified Form in the Presence of SDS

As described in the previous chapter, the heat modifiability of OmpA is observed due to the conformational change of the protein induced by the denaturing activity of SDS, of which very slow reaction rate at room temperature is accelerated upon heating (Ohnishi et al., 1998). The structure of OmpA in the heat modified form is, therefore, considered to be similar to those of water-soluble proteins denatured in the presence of SDS. This is

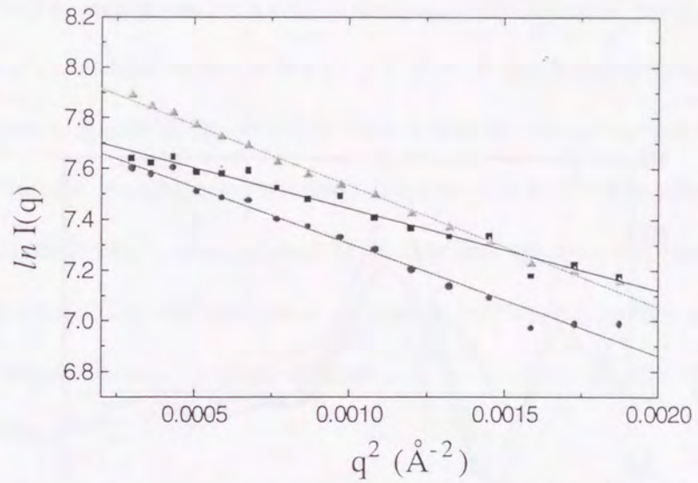


Figure 3.2 Guinier plots for the complexes between OmpA and the surfactants. The symbols are (■) for OmpA/SDS, (●) for h-OmpA/SDS and (▲) for OmpA/OG. Lines are determined by the least-squares procedure.

Table 3.1 Statistical and hydrodynamic parameters of the complexes between OmpA and surfactants

	[1] R_H	[2] R_g	[3] R_V	[4] V_0	[5] D_{max}
h-OmpA/SDS	47.0 Å	36.5 Å	---	---	55 Å
OmpA/SDS	37.3 Å	31.1 Å	---	---	69 Å
OmpA/OG	38.8 Å	37.9 Å	31.24 Å	1.22×10^5 Å^3	85 Å

[1] Obtained from DLS measurements as described in Chapter II (Ohnishi *et al.*, 1998).

[2] Obtained from Guinier plots in Figure 3.2 and equation [3.1].

[3] Obtained from SANS measurements (Kameyama *et al.*, unpublished data)

[4] Obtained using the partial specific volume for OmpA polypeptide, 0.72 ml g^{-1}, the partial specific volume of OG, 0.86 ml g^{-1} (Kameyama and Takagi, 1990) and the amount of OG bound to OmpA, 1.6 grams per gram of protein as described in Chapter II (Ohnishi et al., 1998).

[5] Obtained from $P(r)$ function profiles in Figure 3.3.

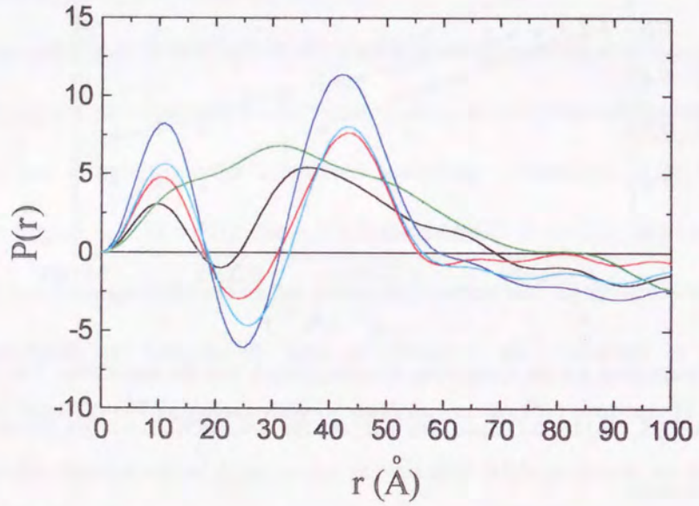


Figure 3.3 Pair distance distribution functions, $P(r)$, for the complex between the proteins and the surfactants. The symbols are (——) for RCAM-OVA/SDS, (——) for RCAM-RNaseA/SDS, (——) for OmpA/SDS, (——) for h-OmpA/SDS and (——) for OmpA/OG.

supported by the similarity in the scattering intensity profile shown in Figure 3.1A and 3.1B and in the $P(r)$ function shown in Figure 3.3. That is, the shape of OmpA in the heat modified form is similar to the shapes of water-soluble protein complexes. This is further supported from the spectroscopic study that CD spectrum in far UV region for the protein in the heat modified form is characteristic of an α-helical spectrum with negative maxima around 208 nm and 220 nm (Reithmeier and Bragg, 1977), which are commonly observed for water-soluble globular proteins denatured in the presence of SDS (Waterhous and Johnson, 1994).

The common characteristic features in $I(q)$ and $P(r)$ profiles for the water-soluble proteins and h-OmpA are the appearance of a subsidiary maximum around q=0.17 Å^{-1} and a negative maximum around r=25 Å (Figure 3.3), respectively. These features have also been observed for the complex formed between RCAM-BSA and SDS (Shinagawa *et al.*, 1994). The structure of this complex investigated by SAXS using the contrast variation method led to a proposal of its model (Shinagawa, 1994), that three globular micelle-like clusters of SDS, each having 28 Å radius, bind to the unfolded polypeptide chain to form a necklace like complex. The radius of gyration for a SDS micelle at 25 °C and at the ionic strength of 50 mM NaPB used in the present study is calculated to be about 22 Å, a value consistent with the cluster radius from the aggregation number, 75 and the partial specific volume, 0.866 g ml^{-1} (Anacker *et al.*, 1964). The common features of the present SAXS results may thus be taken to show that the structure of h-OmpA/SDS can be represented by the necklace-like cluster model proposed for the complex between RCAM-BSA and SDS (Shinagawa et al., 1994).

It should be noted that the amount of SDS bound to h-OmpA, 2.3 grams per gram of

protein, is significantly larger than that bound to water-soluble proteins, 1.5 grams per gram of protein, as described in the previous chapter (Ohnishi *et al.*, 1998). In this respect, the structure of OmpA in the heat modified form may not be identical with those of the water-soluble proteins denatured in the presence of SDS. This difference in the amount of bound SDS may originate from that in the affinity of the proteins to amphiphiles. The amount of SDS bound to h-OmpA, 2.3 grams per gram of protein, corresponds to that one molecule of the polypeptide binds 280 SDS molecules. If the dimension of each SDS cluster in h-OmpA is the same as that in RCAM-BSA, one molecule of the membrane protein is found to bind 4 SDS clusters. The proposed model shown in Figure 3.4 is drawn under these considerations.

3.4.2 Structure of OmpA in the Folded Forms Solubilized in the Presence of Surfactants

Membrane proteins are usually solubilized and isolated by the use of a surfactant, while some membrane proteins are denatured by inadequate surfactants. OmpA can be solubilized keeping a folded form by the use of either SDS or OG. The present SAXS results for the scattering intensity profile, R_g and $P(r)$ suggest that the structures of OmpA/SDS and OmpA/OG are not identical. This is consistent with the results in the spectroscopic data described in Chapter II (Figure 2.1) and the results in the study by Sugawara *et al.*(1996) that the structure of the protein in the presence of SDS is different from that in lipid vesicles.

The profile of scattering intensity $I(q)$ for OmpA/SDS (Figure 3.1B) has a subsidiary maximum around q=0.2 Å^{-1} which is not observed for OmpA/OG. This maximum maybe originates from SDS bound to the protein, because the $I(q)$ profile for SDS micelles has a subsidiary maximum around at 0.17 Å^{-1}. Amounts of the SDS and OG

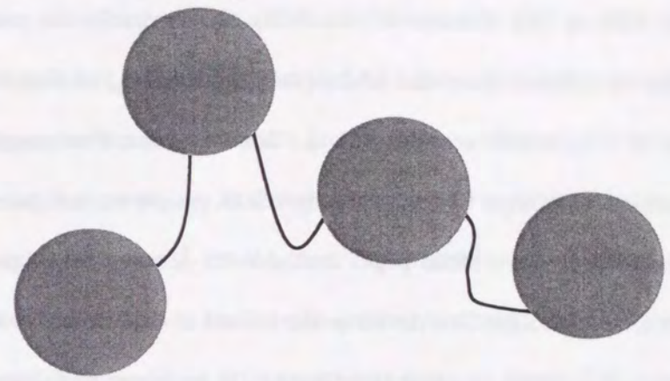


Figure 3.4 Schematic representation of the complex between SDS and OmpA in the heat-modified form constructed according to the model for the complex between SDS and RCAM-BSA (Shinagawa, 1994) with the amount of SDS bound to the protein taken into account (see Figure 5.1 for more details).

binding to OmpA in the folded form were determined as 1.8 and 1.6 grams per gram of protein, respectively, and the molar mass of the protein was confirmed to be constant in the mixed surfactant system of SDS and OG, *i.e.* the monomeric protein does not associate in the presence of SDS or OG (Ohnishi *et al.*, 1998). Consequently, the molar mass of OmpA/OG is not so different from that of OmpA/SDS. The D_{max} of OmpA/OG (85 Å) larger than that of OmpA/SDS complex (69 Å, Table 3.1) therefore suggests that the dimension of OmpA/OG is larger than that of OmpA/SDS. As shown in Figure 3.3, the $P(r)$ function of OmpA/OG has two broad peaks around r=15 Å and r=32 Å and no trough, whereas that for OmpA/SDS has two distant peaks around at r=10 Å and r=35 Å and one trough around at r=20 Å. These two peaks for OmpA/SDS are however different from those for h-OmpA/SDS, RCAM-OVA/SDS, or RCAM-RNaseA/SDS in terms of the peak positions. These findings substantiated that the overall structures of OmpA/OG and OmpA/SDS are different from those of h-OmpA/SDS, RCAM-OVA/SDS and RCAM-RNaseA/SDS, which have common structures to complexes formed between unfolded proteins and SDS as illustrated in Figure 3.4. Since OmpA/SDS and OmpA/OG are composed of two domains corresponding to the N-terminal and C-terminal ones in the topological model of Morona *et al.* (1984), the two peaks observed in the $P(r)$ functions for OmpA/SDS and OmpA/OG may result from the intra-domain and inter-domain distance distributions for the smaller and larger distances, respectively.

3.4.3 Size and Shape of OmpA in the Folded Form in the Presence of Octylglucoside

Compared with SDS, OG is known to be a surfactant with mild denaturing activity (Baron and Thompson, 1975) and often used for the crystallization of membrane proteins

(Frank *et al.*, 1987, Jalal and van der Helm, 1989, Forst *et al.*, 1993, Blaauw *et al.*, 1995). OmpA complex with OG is, therefore, considered to assume a conformation closer to the native one in the biomembrane than that in the complex with SDS. From this point of view, it is intriguing to evaluate the size and shape of the protein in the presence of OG.

The radius of gyration obtained by SAXS for a complex composed of discrete units, such as a complex formed between a protein and a surfactant, is affected by the spatial distribution of electron density in the whole structure. For evaluating the size and shape of such a complex, one should use the "true" R_g determined, for example, by means of the contrast variation method or small angle neutron scattering (SANS) measurement. SANS measurements (Kameyama *et al.*, unpublished data) on OmpA/OG gave a value, R_V of 31.24 Å, which corresponds to the net dimensional radius of gyration of the complex (see Table 3.1). The hydrodynamic radius, R_H, was obtained for this complex as described in Chapter II. The molecular volume, V_0, of this complex may be obtained as 1.22×10^5 Å^3 using the partial specific volume for OmpA polypeptide, 0.72 ml g^{-1} (calculated from the amino acid composition), the partial specific volume of OG, 0.86 ml/g (Kameyama and Takagi, 1990) and the amount of the surfactant bound to OmpA, 1.6 grams per gram of protein described in Chapter II. With these data, we estimate the geometry of OmpA/OG assuming two kinds of ellipsoids, prolate and oblate, for the complex (Van de Sande and Persoons, 1985).

1) Prolate ellipsoid

The volume and radius of gyration of a prolate ellipsoid with axes a, b and b ($a>b$) are given by

$$V=\frac{4}{3}\pi\ ab^2 \qquad [3.3]$$

$$R_g = \sqrt{\frac{(a^2+2b^2)}{5}} \qquad [3.4]$$

These equations with V=1.22×10^5 Å and R_g=31.24 Å allow us to evaluate a and b to be 62.8 Å and 21.6 Å, respectively. The hydrodynamic radius, R_H, of this ellipsoid is calculated to be 34.1 Å from the following relation.

$$R_H = \sqrt{a^2-b^2} \,/\, \ln\frac{(a+\sqrt{a^2-b^2})}{b} \qquad [3.5]$$

This value is referred to as R_H^{cal} and presented in Table 3.2.

2) Oblate ellipsoid

When an oblate ellipsoid with axes a, b and b (a>b) is assumed, V and R_g of OmpA/OG are written as

$$V = \frac{4}{3}\pi\, a^2 b \qquad [3.6]$$

$$R_g = \sqrt{\frac{(2a^2+b^2)}{5}} \qquad [3.7]$$

In this case, a and b are evaluated to be 48.5 Å and 12.4 Å, respectively, which in turn give R_H^{cal} of this ellipsoid a value of 35.7 Å when the following equation is used.

$$R_H = \sqrt{a^2-b^2} \,/\, \tan^{-1}\frac{\sqrt{a^2-b^2}}{b} \qquad [3.8]$$

The results of the above calculations summarized in Table 3.2 indicate that OmpA/OG appears to be prolate ellipsoidal rather than oblate ellipsoidal by the following reasons: 1) Since the thickness of outer membrane of *Escherichia coli* is considered to be about 35 Å (Vogel and Jähnig, 1986), it is impossible for the oblate ellipsoid to integrate the

membrane with the minor axis of 12.4 Å. 2) According to the crystallographic structure of the N-terminal domain of OmpA shown in Figure 1.4 (Pautsch and Schultz, 1998), which corresponds to the half of the protein, this domain forms a β-barrel with cylinder-like shape. Provided the crystallographic structure is a cylinder, it has about 60 Å length and 25 Å diameter. Although the structure of the other half of the protein is yet unknown, the cylinder with this dimension is plausibly included in the prolate ellipsoid.

The model proposed of Stathopoulos (1996) consisting of sixteen transmembrane segments is likely to be simulated as an oblate ellipsoid. On the other hand, the earlier topological model of Morona *et al.* (1984) consisting of the transmembrane N-terminal domain and the periplasmic C-terminal domain appears to have an elongated shape like a prolate ellipsoid. Thus, the present evaluation supports the earlier prolate-like model rather than the later oblate-like model of Stathopoulos.

Table 3.2 Geometry of the simulated prolate and oblate ellipsoids

	Major axis	minor axis	R_H^{cal}
Prolate ellipsoid	62.8 Å	21.6 Å	34.1 Å
Oblate ellipsoid	48.5 Å	12.4 Å	35.7 Å

Chapter IV Kinetic Aspects of the Conformational Change of OmpA in the Mixed Surfactant System of SDS and OG

4.1 Introduction

OmpA solubilized in the presence of SDS unfolds upon heating and can refold to the non-heated form by the addition of a sufficient amount of OG (Dornmair *et al.*, 1990). In Chapter II, this protein solubilized in the presence of SDS, OG or their mixtures was characterized in terms of the amount of surfactant bound to the protein, the molar mass, and the hydrodynamic radius (Ohnishi et al., 1998). The results revealed the following two aspects concerning the conformational change of the protein in the mixed surfactant system: 1) Refolding from h-OmpA is induced by the addition of OG in a cooperative manner. 2) The protein adopts its heat modified form owing to the denaturing activity of SDS. Consequently, the "heat modifiability" of the protein was suggested to be ascribed to its kinetic property upon the SDS denaturation. In other words, OmpA is solubilized keeping its folded structure in the presence of SDS at room temperature due to an extremely slow denaturation rate, and its denaturation is induced by the accelerated reaction rate upon heating.

Results described in Chapter III support the topological model proposed earlier for OmpA by Morona *et al.* (1984). This suggests that the membrane protein consists of the transmembrane N-terminal domain and the hydrophilic periplasmic C-terminal domain. The folding process of the protein consisting of such separate domains may not be simple, so that it seems reasonable to follow the refolding process of the protein by means of multi-

procedures. This membrane protein has five tryptophan residues only in the transmembrane N-terminal domain. Tryptophan residue is known to give an intrinsic florescence and an unique circular dichroism. The behavior of the N-terminal domain of the protein can, hence, be detected by means of tryptophan fluorescence and CD spectroscopies in a near UV region, while the whole part of the protein can be observed conventionally by means of CD spectroscopy in a far UV region. In the present chapter, refolding reactions of OmpA from the heat modified form to the non-heated form accompanied by the addition of OG were kinetically followed by multiple use of such spectroscopic properties. Unfolding reactions of the protein upon heating at various temperatures in the presence of SDS were also followed through CD measurements in the far UV region. The results may be expected to give light on the process of the conformational change of the protein in the mixed surfactant system of SDS and OG.

4.2 Materials and Methods

4.2.1 Materials and Sample Preparation

Sodium dodecyl sulfate (SDS) and *n*-octyl-β-D-glucoside (OG) were obtained from BDH (Anala R grade) and Dojindo Laboratories Inc. (Kumamoto, Japan), respectively. OmpA was purified from *Escherichia coli* K-12, TNE001 strain and isolated as described in Chapter II. The OmpA fraction thus obtained was equilibrated by SEC against 50 mM sodium phosphate buffer (NaPB, pH6.9) containing 3.47mM SDS to prepare an OmpA/SDS solution. OmpA in the heat modified form in the presence of 3.47mM SDS, h-OmpA/SDS, was prepared by heating the OmpA/SDS solution in boiling water for 5 minutes. To this h-

OmpA/SDS solution, an appropriate amount of NaPB (pH 6.9) containing 3.47mM SDS and 342.5 mM OG was added to obtain a desired h-OmpA solution with a defined OG composition in the SDS-OG mixed surfactant system. The composition was expressed in terms of weight fraction X_{OG} of OG as in Chapter II. OmpA solubilized in the presence of OG was also prepared by equilibration against NaPB (pH 6.9) containing 27.4 mM of OG by SEC.

4.2.2 CD Measurements

All CD measurements were performed at desired temperatures by the use of J-720 (Jasco, Tokyo, Japan) as described in Chapter II. Protein concentrations were 7.2-16.0 μM for the far UV region and 17.2-23.4 μM for the near UV region.

4.2.3 Fluorescence Measurements

Measurements were made at protein concentrations of 0.8-1.4 μM by the use of F-4500 (Hitachi, Ltd. Tokyo, Japan) using a cuvette with 1 cm of light path length at a defined temperature. Wavelengths for excitation and emission were 296 nm and 330 nm, respectively.

4.2.4 Refolding Experiments

The time course measurement of either CD or fluorescence was started 15 seconds after the manual addition of OG to the h-OmpA solution. The change in ellipticity was followed at 207 nm in the far UV region and at 289 nm in the near UV region and that in fluorescence intensity was followed at 330 nm of emission.

4.2.5 Temperature Jump Unfolding Experiments

In the temperature jump unfolding experiments, the OmpA/SDS solution was transferred into the cell preserved preliminarily at a defined temperature and the change in ellipticity at 207 nm in far UV region was started to follow within 10 seconds. The time-lag for the temperature raising of the solution was estimated to be a few minutes by separate experiments with a blank solution.

4.3 Results

4.3.1 Changes in Spectroscopic Properties with Composition of the Mixed Surfactant System of SDS and OG

Figure 4.1 shows near UV (A) and far UV (B) CD spectra of OmpA and its heat-modified form, h-OmpA, kept in the mixed surfactant system at various compositions for 15 hours at room temperature. In panel A, the near UV spectrum of h-OmpA at X_{OG}=0 shows no characteristic peak in the range from 280 nm to 320 nm, suggesting that the conformation of the membrane protein under the condition has no specific configuration of the aromatic side chains. By the addition of OG, two peaks appear around 284 nm and 289 nm, and their ellipticities increase with increasing X_{OG}. These peaks observed for the folded OmpA are considered to reflect the specific configuration of the aromatic residues, especially tryptophan residues, of the protein. Since the membrane protein is known to have five tryptophan residues only in the N-terminal domain, which is considered to form transmembrane β-strands (Morona *et al.*, 1984, Vogel and Jähnig, 1986), the observed spectral change in the wavelength region primarily reflects the formation of the tertiary

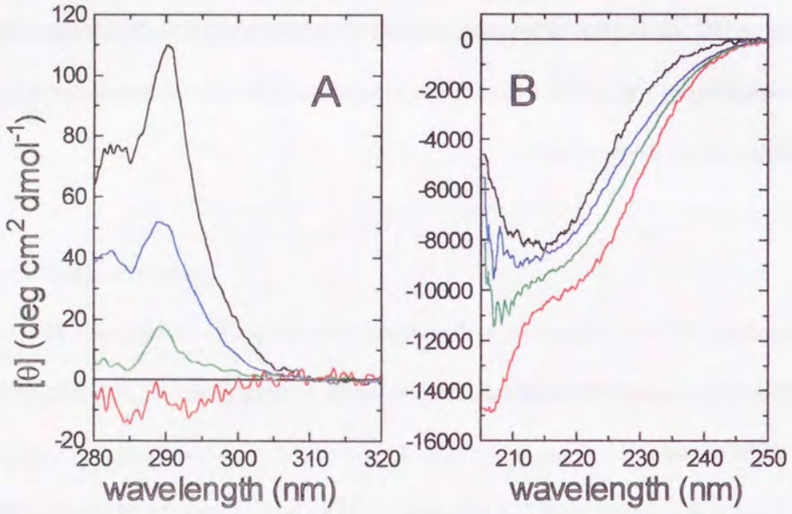


Figure 4.1 CD spectra in near (A) and far (B) UV regions for h-OmpA at various compositions of the mixed surfactant system of SDS and OG. The final compositions of the mixed surfactant system after the addition of OG are X_{OG}= 0 (——), 0.79 (——), 0.81 (——) and 0.97 (——). All the spectra were measured at 30 °C 15 hours after the addition of OG. The final protein concentrations were 5.7-17.0 μM for the far UV region and 14.2-25.7 μM for the near UV region.

structure of the N-terminal domain.

As can be seen in Figure 4.1B, the CD spectrum of h-OmpA at X_{OG}=0 in the far UV region from 205 nm to 250 nm has a large negative peak around 207 nm and a negative shoulder around 222 nm. These negative peak and shoulder disappear with increasing X_{OG} and the spectrum changes to a simple trough shape with a minimum around 215 nm. The spectrum for X_{OG}=0.9 was identical with that of OmpA in the non-heated form (see Figure 2.1). This suggests that h-OmpA refolds by the addition of OG to the non-heated form in terms of the secondary structure.

The ellipticities of h-OmpA at the indicated wavelengths in the range from 289 nm and 207 nm are plotted against the composition of the mixed surfactant system in Figure 4.2A. They change with the increase of the weight fraction of OG in a cooperative manner with one transition of which mid-point is around X_{OG}=0.78. This is consistent with the findings from SEC-LALLS measurements in Chapter II that h-OmpA refolds above X_{OG}=0.7.

When the measurement was extended to a large elapsed time of several days, the transition was found to shift to a smaller X_{OG}. Figure 4.2B shows the changes of ellipticity with X_{OG} for three elapsed times since the protein was transferred to the mixed surfactant solutions. It can be seen that the conformational change of the membrane protein proceeds very slowly, especially in the transition region, and that the rate of the refolding from the heat modified to the non-heated form depends on the final composition of the mixed surfactant system.

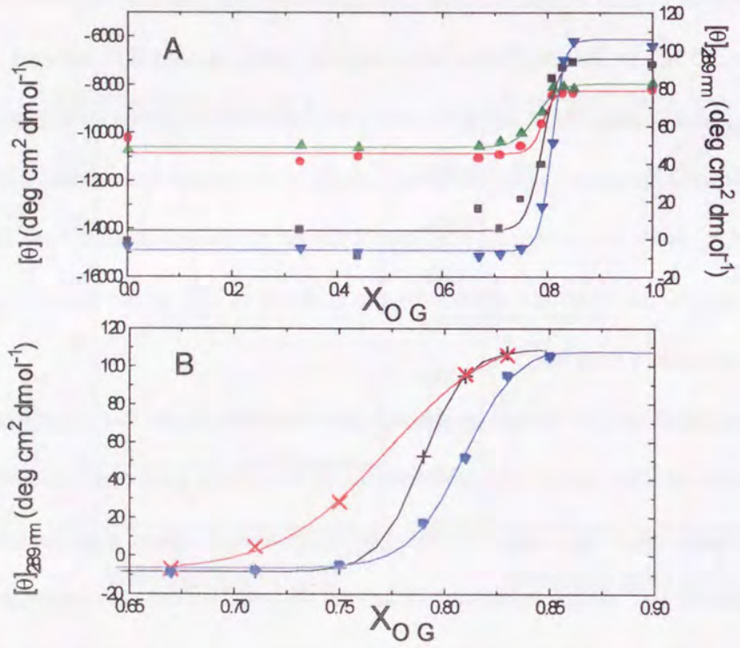


Figure 4.2 (A) Molar ellipticities of OmpA plotted against the composition of the mixed surfactant system of SDS and OG. These ellipticities at 207 nm (■), 215 nm (●), 217 nm(▲) and 289 nm (▼) were obtained from the spectra of the protein measured 15 hours after the addition of OG. The left ordinate corresponds to the ellipticities at wavelengths in far UV region, (207 nm, 215 nm and 217 nm), and the right ordinate in near UV region (298 nm). The lines are superimposed assuming the two-state transition. (B) Changes of the ellipticity of OmpA at 289 nm of wavelength with the composition of the mixed surfactant system measured after 15 hours (▼), 4 days (+) and 14 days (×) from the addition of OG. All the protein solutions were kept at 30 ℃ for the incubation and included 2mM sodium azide, an antiseptic agent.

4.3.2 Kinetics of Refolding of OmpA from its Heat Modified Form

The refolding process of h-OmpA accompanying the addition of OG was studied at various final compositions of the mixed surfactant system. The lower panels of Figure 4.3A, B and C show typical profiles of the kinetics observed for ellipticity at 289nm and 207 nm and for tryptophan fluorescence intensity, respectively. These profiles show that the conformational change proceeds so slowly that the manual mixing of the h-OmpA solution with the refolding buffer solution containing OG is adequate to studying the kinetics of the present system.

All the profiles were analyzed on the basis of three exponential functions with three rate constants k_1, k_2 and k_3:

$$Y = Y_0 + A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \exp(-k_3 t) \qquad [4.1]$$

Here, A_1, A_2 and A_3 are amplitudes and Y_0 is the final value of the profile.

The upper panels of Figure 4.3A, B and C show the differences between the fitted functions and observed values. These residuals scatter uniformly through the measuring time scales for any profiles, indicating the data are reasonably fitted by equation [4.1].

The rate constants thus obtained are plotted against X_{OG} in Figure 4.4. They increase with the fraction of OG, indicating that the refolding of OmpA is accelerated by the increase in the fraction of OG. Besides, the nine rate constants obtained from the three procedures are classified into four groups, thus indicating that the over all process of the refolding reaction consists of four elementary processes. The fastest process could be detected only by the fluorescence measurement. The second and third processes could be detected by all of the spectroscopic measurements and the slowest one, only in the far and near CD spectra.

Figure 4.5 shows the estimated amplitudes plotted against the composition of the mixed surfactant system. The symbols have been colored according to the four steps (or

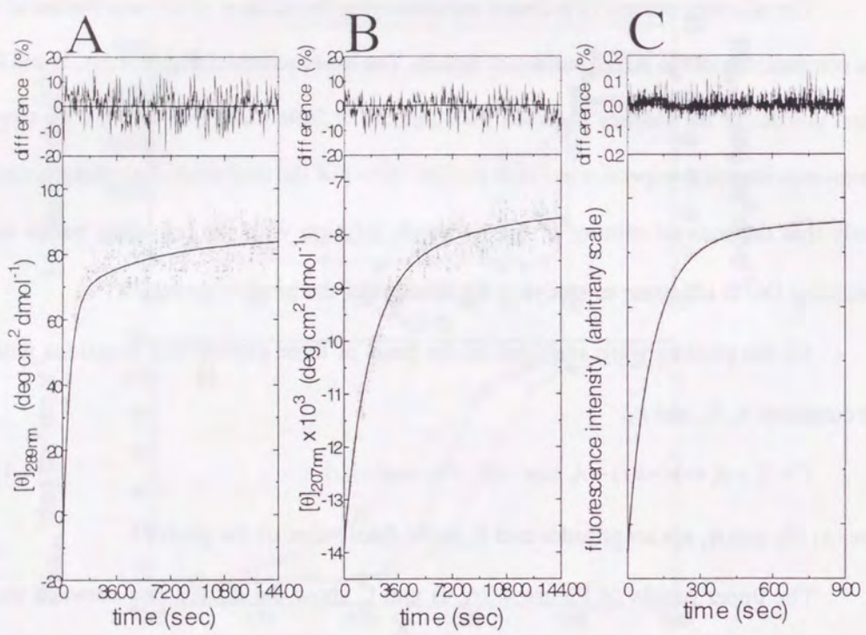


Figure 4.3 Typical profiles of the kinetics of the refolding of h-OmpA accompanying the addition of OG, observed for ellipticity at 289 nm (A), 207 nm (B) and intrinsic tryptophan fluorescence intensity (C) at 30 °C. The final compositions of the mixed surfactant system after the addition of OG are X_{OG}= 0.880 (A), 0.862 (B) and 0.882 (C). The final protein concentration were 24.5 μM (A), 10.8 μM and 0.57 μM (C). To initiate the refolding reaction, 342.5 mM OG solution containing 3.47 mM SDS was added manually. In the fluorescence experiments, the wavelengths for excitation and emission were 296 nm and 330 nm, respectively. The upper panels show the differences relative to the overall change in ellipticity or fluorescence intensity between the fitted functions (line) by the use of the equation [4.1] and the observed values.

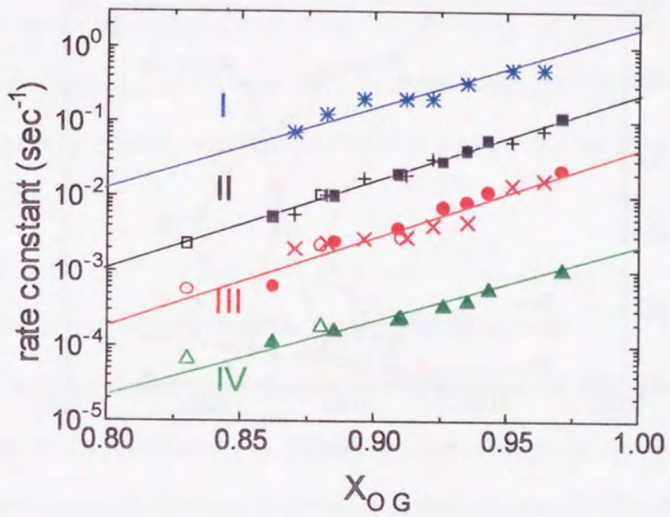


Figure 4.4 Rate constants of OmpA for refolding from the heat modified form to the non-heated form, determined by the use of equation [4.1]. The details of the analyses are described in text. The largest rate constants (k_1), the second ones (k_2) and the smallest ones (k_3) obtained from the analyses for the fluorescence kinetic profiles, are symbolized as (✳), (+) and (×), respectively. Those for the near UV CD profiles are symbolized as (□), (○) and (△), respectively, and those for the far UV CD profiles, as (■), (●) and (▲), respectively. Each of the lines is drawn assuming a logarithmic linear correlation with X_{OG} for each of the four classes of rate constants.

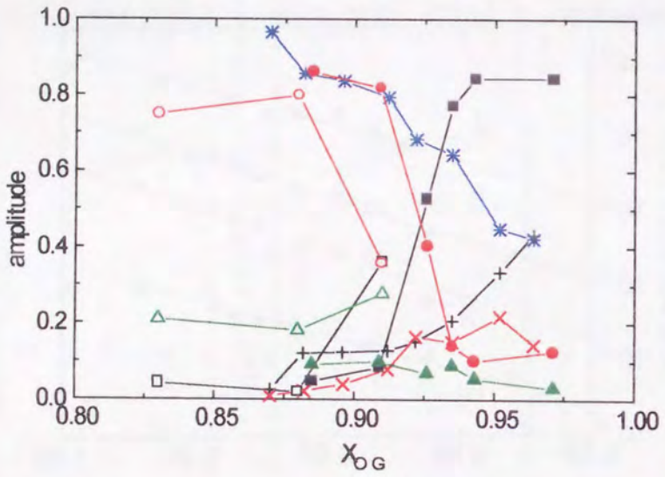


Figure 4.5 Amplitudes obtained from the analyses of the refolding kinetic profiles of OmpA by the use of the equation [4.1] at various final compositions of the mixed surfactant system of SDS and OG. In the analyses, the fluorescence profiles gave three amplitudes, A_1 (✳), A_2 (+) and A_3 (×), which correspond to the rate constants, k_1 (✳), k_2 (+) and k_3 (×) in Figure 4.5, respectively. The near UV CD profiles gave three amplitude, A_1 (□), A_2 (○) and A_3 (△), and far UV CD profiles gave A_1 (■), A_2 (●) and A_3 (▲). These amplitudes correspond to the rate constants represented by the same symbols as in Figure 4.4.

kinetic phases) I, II, III and IV classified by rate constants in Figure 4.4. The amplitudes in the step I (blue), which were detected by the fluorescence measurement, decrease with an increase in X_{OG}. Those in the step II (black), which were detected by all the techniques, increase with increasing in X_{OG}. Those in the step III (red) from the near and far UV CD profiles increase with X_{OG}, while those from the fluorescence profiles are nearly constant. Those in the step IV (green), were appeared both in the near and far UV CD profiles, are insensitive to X_{OG}.

4.3.3 Kinetics of Unfolding of OmpA to the Heat Modified Form

The unfolding reaction of OmpA in the presence of SDS was followed by monitoring the change in ellipticity at 207 nm in temperature jump experiments, in which temperature was jumped from room temperature to desired values *T*. The observed profiles were well fitted by a single exponential function, and unfolding rate constants at various temperatures were determined. The largest rate constant obtained at *T*=78.2 °C, *i.e.*, that for the fastest unfolding reaction among the experiments, was 7.2×10^{-4} sec^{-1}. The half-life for the unfolding time course under the condition corresponded to 23 minutes. The dead time, during which the temperature was raised to 78.2 °C, was measured separately with a blank buffer solution to be 2.2 minutes. The dead times similarly determined for other temperatures were within 10 % of the half-life of the unfolding reaction. The rate constants obtained from the time course profiles in all the unfolding experiments were, therefore, adopted without any correction for the dead time. Figure 4.6 shows the Arrhenius plot of the unfolding rate constants *k* for OmpA thus determined. The plots give a fairly linear correlation with the reciprocal of *T*. Assuming that the indicated linear correlation holds

down to lower temperatures, the unfolding rate constant at 30 °C is estimated to be 1.9×10^{-8} sec^{-1}. This rate constant indicates that the denaturation of the membrane protein in the presence of SDS will take 2.4 years in the presence of SDS at the temperature. Thus, the denaturation of the protein is not virtually observable under this condition.

4.4 Discussion

4.4.1 Apparent Conformational Transition of OmpA in the Mixed Surfactant System of SDS and OG

In this study, the refolding phenomena of OmpA from its unfolded form caused by SDS was examined by systematically observing its conformational change with the composition of the mixed surfactant system of SDS and OG. In this experimental system, SDS acts as a denaturant for the membrane protein. The addition of OG can be hence considered to dilute the denaturing activity of SDS. In this context, one can expect that there are certain equilibrium between the unfolded and folded forms of the protein, and that the equilibrium changes with the composition of the mixed surfactant system. Practically, the conformational change of OmpA was observed as shown in Figure 4.2A, which appears to show a cooperative transition around at X_{OG}=0.78.

In the present study, this conformational change, namely the refolding of OmpA, in the mixed surfactant system was found to proceed very slowly, especially for the final compositions below the transition region. In addition to the fact, the reverse reaction toward the unfolding form was extremely slow at room temperature, even in the neat SDS solution as will be discussed later. It is, therefore, impossible to determine the equilibrium

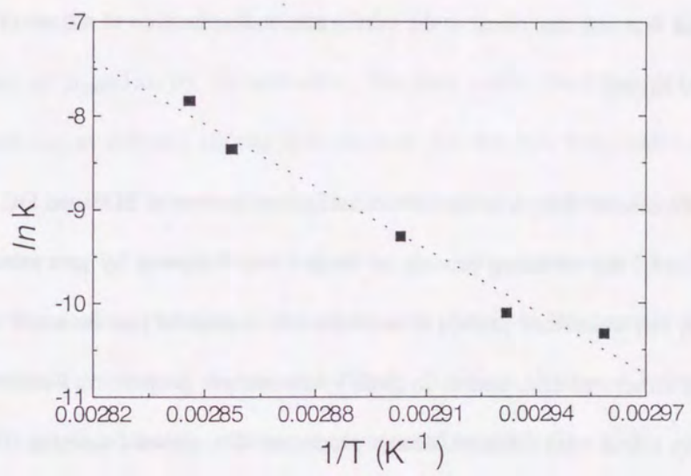


Figure 4.6 Arrhenius plot for the unfolding rate constant *k* for OmpA in the presence of SDS. The unfolding rate constants were obtained through temperature jump experiments in the presence of SDS. Unfolding kinetics of the protein was observed by ellipticity at 207 nm, and the profiles thus obtained were analyzed by the use of a single exponential function to obtain unfolding rate constants at various temperatures. The time-lags in which the temperature rises up to the determined value were within 10 % of the halflife in the profiles. The dashed straight line is the best-fit one determined by the least-square procedure.

denaturation curve for the present experimental system. The subsequent kinetic measurements reveal that the rate of the conformational change of the protein in the mixed surfactant solution depends on the composition of the mixed surfactant system exponentially and that one can observe the conformational transition at a time of the order of a week around X_{OG}=0.7.

4.4.2 Folding Process of OmpA in the Mixed Surfactant System of SDS and OG

In this study, the refolding process of OmpA was followed by spectroscopies. As described before, this membrane protein is considered to consist of two domains that can be different in their structures and, hence, in their spectroscopic properties. Besides that, the two domains may refold with different kinetic processes. The present analyses of the time-dependent spectroscopic changes clearly showed that the overall process of the refolding of OmpA can be classified into the four steps with different kinetic rate constants. The multi-step folding character of the membrane protein has also been reported in the refolding experiments on the protein from the fully unfolded state in the presence of 8 M of urea (without surfactant) to the folded state in a lipid vesicle (Surrey and Jähnig, 1995). In their study, they proposed a sequential folding scheme with four steps. In the refolding of the protein in the presence of liposomes from the urea-denatured state, the rate constants in the folding pathway were described as follows: The first step proceeds within a second and is concerned with a change from the fully unfolded form in the presence of urea to a misfolded form on the membrane surface; the second step occurs within minutes toward the partially folded form adsorbed to the membrane; the third step proceeds in the order of minutes to hour to the partially folded form inserted into the membrane; the fourth step takes hours to

be converted to the fully folded and inserted form in the membrane (Surrey and Jähnig, 1995).

From the present results one can estimate the rate constants for the refolding of OmpA from the heat modified to the folded form in the presence of OG only, under the condition of X_{OG}=1.0, by extrapolation. The time scales thus estimated for the four rate processes are as follows: shorter than seconds for the first step, order of seconds for the second one, order of minutes for the third one and order of hours for the forth one. These time scales can be compared with those in the scheme presented by Surrey and Jähnig (1995). The folding pathway of the membrane protein in the present system is shown in comparison with that of Surrey and Jähnig (1995) in Figure 4.7 (for the refolding at X_{OG}=1.0), provided that the reaction steps proceed sequentially.

The first step in the present scheme is compared to the isotropic collapse formation involving tryptophan residues. Since this step is not detected by the CD measurements but only by the tryptophan fluorescence, no specific orientation of these residues should be attained in this step. The two steps that follow may be analogues of intermediate steps in the scheme of Surrey and Jähnig. However, these intermediates shown in Figure 4.7 can be different from those in their scheme, since no aggregation was observed during the refolding of the protein in the mixed surfactant system. The slowest step may be associated with an orientation or configuration of the domains which have been formed already, since this step was not detected by the fluorescence measurements but only by the CD measurements.

According to the topological model proposed by Morona *et al.* (1984), OmpA has five tryptophan residues only in the transmembrane N-terminal domain, whereas the C-terminal domain is predicted to extrude into the periplasmic space. The folding of the

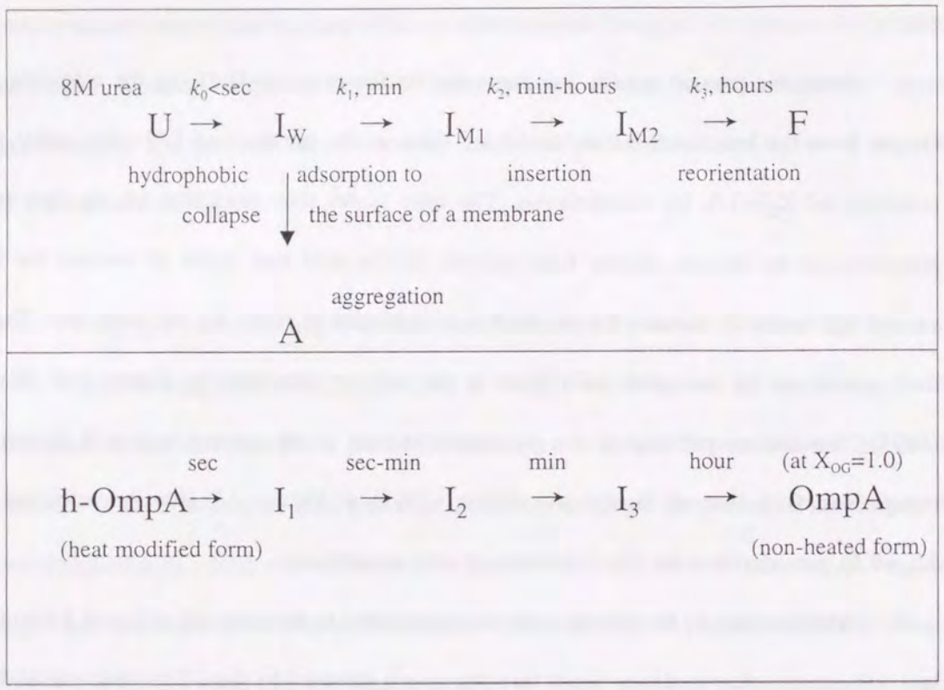


Figure 4.7 Hypothetical pathway of *in vitro* folding of OmpA in the mixed surfactant system of SDS and OG (lower scheme). The upper scheme of the folding pathway of the protein from the unfolded form in the presence of 8 M of urea (U) to the folded form in lipid vesicles (F) is presented by Surrey and Jähnig (1995), where I_W is reported to be a misfolded collapse form of the protein on the surface of the lipid membrane, which leads to an aggregate form (A), I_{M1} and I_{M2} are intermediate forms which bound to the membrane. The lower scheme is presented for the present system by the comparison between the rate constants in the upper scheme and those obtained in the present study. In the lower scheme, I_1, I_2 and I_3 are considered to be intermediate forms of the protein between the refolding in a surfactant solution. In particular, I_1 may be the one involved with hydrophobic collapse around tryptophan residues, since this state was detected only through the fluorescence measurements.

transmembrane N-terminal domain and the hydrophilic C-terminal domain may be expected to be observed separately by CD spectroscopy both in near and far UV regions. The kinetic steps obtained from the CD measurements in the near UV region in Figure 4.4 were, however, identical with those in the far UV region. It is interesting to ask whether this indicates that the N-terminal and C-terminal domains fold simultaneously. The conformational change of a polypeptide around aromatic residues often affects the ellipticity in both near and far UV regions (Manning and Woody, 1989). Besides, the N-terminal domain can dominantly contribute to the overall change of the residual ellipticity in the far UV region, since this domain has a tightly folded β-barrel structure (Pautsch and Schulz, 1998) while the C-terminal domain may have a looser folded structure with unknown secondary structures (Vogel and Jähnig, 1986). On account of these, it is unclear whether these two domains fold simultaneously. In order to make this clear, kinetic refolding data for each domain of the membrane protein in the mixed surfactant system should be compared with those for intact OmpA, namely, with the present results.

4.4.3 Stability of OmpA in the Presence of SDS

As described in Chapter I, a protein polypeptide folds to the native conformation to exert its intrinsic function. The native conformation is maintained with the energetic difference between the unfolded and folded states. It is, therefore, important to the problem of protein folding to understand the physical basis of the stability of the folded conformations. The conformational stability of a protein has been examined for a large number of water-soluble proteins mainly through two approaches: In one approach, we can estimate the conformational stability of a protein by means of calorimetry. This method

directly affords the change in the heat capacity accompanying the heat denaturation. In the other approach, we can estimate the stability of the protein from the equilibrium knowledge. In this method, difference of Gibbs free energy of the protein between the folded and unfolded states can be obtained from the equilibrium constant. Figure 4.2A shows a denaturation curve of OmpA against SDS. However, this curve allows no estimation of the stability of the protein against the denaturation, since it is not equilibrated but transient one.

Besides these two conventional approaches, kinetic data for conformational change of a protein concerning its unfolding and refolding also give information on the conformational stability. That is, one can experimentally obtain a correlation between the unfolding rate constant for a protein and the concentration of a denaturant, which provides the unfolding rate constant in water by extrapolation. This unfolding rate constant in water and the refolding rate constant in water obtained as well afford an equilibrium constant and hence ΔG for the conformational change of the protein. Although such extrapolations provide only approximate rate constants in water, the ΔG thus obtained is known not to be so different from those obtained from the conventional approaches. In this context, the conformational stability of OmpA will be examined in terms of ΔG assessed from the kinetic measurements. The ΔG thus assessed will be referred to as kinetic stability.

As for the refolding of OmpA from its heat modified form to the folded one, the refolding rate is found to depend on the composition of the mixed surfactant system in the present study. As shown in Figure 4.4, the logarithm of the rate constant in each step varies linearly with X_{OG}. If the linear relationship is extrapolated to X_{OG}=0, the refolding rate constant for OmpA in neat SDS solution is obtained. In actuality, however, the refolding of the protein contains four kinetic steps. Since the rate constants for these four steps are

logarithmically distinguishable, we may use the late-limiting refolding rate constant 2.4×10^{-10} sec^{-1} for the phase IV at 30 °C extrapolated to X_{OG}=0. The Arrhenius plot (Figure 4.6) for the protein gave an unfolding rate of 1.9×10^{-8} sec^{-1} in the presence of SDS at 30 °C. These values yield 7.9×10 for the equilibrium in the SDS solution. Therefore, this membrane protein is destabilized by 12 kJ mol^{-1} under this condition. In other words, the Gibbs free energy for the denaturation, ΔGu, of the protein is -12 kJ mol^{-1}. This value may be compared with usual values less than 40 $kJmol^{-1}$ (ΔGu= +40 kJ mol^{-1}) for the conformational stability of water-soluble globular proteins (Privalov and Gill, 1988), and seems consistent with the fact that h-OmpA in the presence of SDS solely does not refold to the non-heated form by lowering the temperature to 30 °C. This membrane protein can, therefore, be solubilized keeping its folded form in SDS solution at lower temperatures owing to the extremely slower denaturing rate under the condition. Consequently, the "heat modifiability" is observed due to the acceleration of the denaturing rate by raising the temperature. This notion supports the postulate proposed in Chapter II that SDS was principally responsible for the denaturing phenomena of OmpA in the mixed surfactant system of SDS and OG (Ohnishi et al., 1998).

Chapter V Folding of OmpA, a Membrane Protein

Owing to some experimental difficulties, studies of folding phenomena in membrane proteins are as yet very limited as mentioned in Chapter I. Dornmair *et al.* (1990) reported an intriguing phenomenon that the heat modified form of OmpA refolds to the non-heated form by the addition of a sufficient amount of OG. The present study aimed to clarify the detailed mechanism of the folding phenomena and conformational change of the membrane protein in the mixed surfactant system of SDS and OG on the basis of that knowledge. Characterization of the protein in this mixed surfactant system by SEC-LALLS made clear the role of the surfactants in the conformational change of the protein in terms of the heat modifiability and the refolding, the latter of which was observed by Dornmair *et al.* (1990) for the first time. In Chapter III, structural aspects of the protein in the presence of each of the surfactant examined through SAXS measurements were described to afford structural properties of the protein in a surfactant solution. In Chapter IV, kinetic aspects of the conformational change of the protein between its folded form and unfolded form in the presence of surfactants were investigated in a systematic way. In this chapter, we discuss a feature of the conformational change of the protein in the mixed surfactant system of SDS and OG in terms of the secondary structure, and consider folding properties of the membrane protein in the mixed surfactant system on the basis of experimental findings in the previous chapters. Finally, prospect for the application of a binary mixed surfactant system upon studying membrane protein folding is also included.

5.1 Conformational Conversion between α-Helices and β-Structures in OmpA

The conformational change of OmpA in the mixed surfactant system of SDS and OG may partly involve the secondary structure conversion between α-helices and β-structures, as observed in CD spectroscopic studies (see Figure 2.1, Figure 4.1, for example). Namely, the folded form of OmpA including β-structures converts to the unfolded form in the presence of SDS that contains significant amounts of α-helices. Upon refolding, the β-structures in the folded form are to be formed by conversion of the α-helical structures.

Such conversions between α-helical and β-structural forms are reportedly known for some water-soluble proteins: The native form of bovine β-lactoglobulin with β-structures converts to the denatured form with α-helical structures upon the denaturation induced by guanidine hydrochloride, various kinds of alcohols, trifluoroethanol or hexafluoroisopropanol (Hamada *et al.*, 1996, Hamada and Goto, 1997). The native form of a few mutant human lysozymes with α-helical structures causes amyloidosis (Booth *et al.*, 1997). Some diseases like Alzheimer's disease, maturity onset diabetes, and the prion-related transmissible spongiform encephalopathies are considered to be attributed to such a conformational conversion of the native conformations of the related proteins to their β-structural fibril formations (Sunde *et al.*, 1997). Such intriguing phenomena concerning the α-β conversion of a protein are attracting much attention from pathological points of view.

Among the above examples, the mechanism of the conversion of bovine β-lactoglobulin may be different from those of such disease related proteins, since the conversion of the protein is considered to be caused by the influence of a denaturant. This protein is known to be a predominantly β-sheet protein, although it has a markedly high intrinsic preference for α-helical structure (Hamada *et al.*, 1996). High environmental

polarity in the presence of alcohols affects the intra-chain interactions of the protein in the native form (Hirota *et al.*, 1997), resulting in the α-helix formation, which is predicted from the statistical data for intrinsic preference for the α-helical structure as for the amino acid sequence (Kuwata *et al.*, 1998).

The conformational conversion of the folded form of OmpA to the heat modified form in the presence of SDS seems to be similar to that of bovine β-lactoglobulin in terms of the denaturation induced by a denaturant. It is, however, not clear whether this membrane protein has any intrinsic preference for α-helical structure. Besides, the α-helical conformation is commonly observed for SDS denatured water-soluble proteins in spite of a variety of their secondary structures in their native states (Waterhous and Johnson, 1994). In this context, the conversion of the native conformation of a protein to an α-helical one induced by the influence of SDS may not be accounted for by the mechanism of the conversion of β-lactoglobulin induced in the presence of alcohols or guanidine hydrochloride.

5.2 Folding Mechanism of OmpA in the Mixed Surfactant System of SDS and OG

A hypothetical scheme of the conformational change of OmpA in the mixed surfactant system of SDS and OG was considered based on the four rate constants and their similarity to those obtained by Surrey and Jähnig (1995). This is shown in Figure 5.1, in which the mode of the surfactant binding to the protein is drawn with a very simple depiction, since the structure of the protein in the folded form in a surfactant solution is yet unknown. Small angle neutron scattering work under progress by the author and co-workers

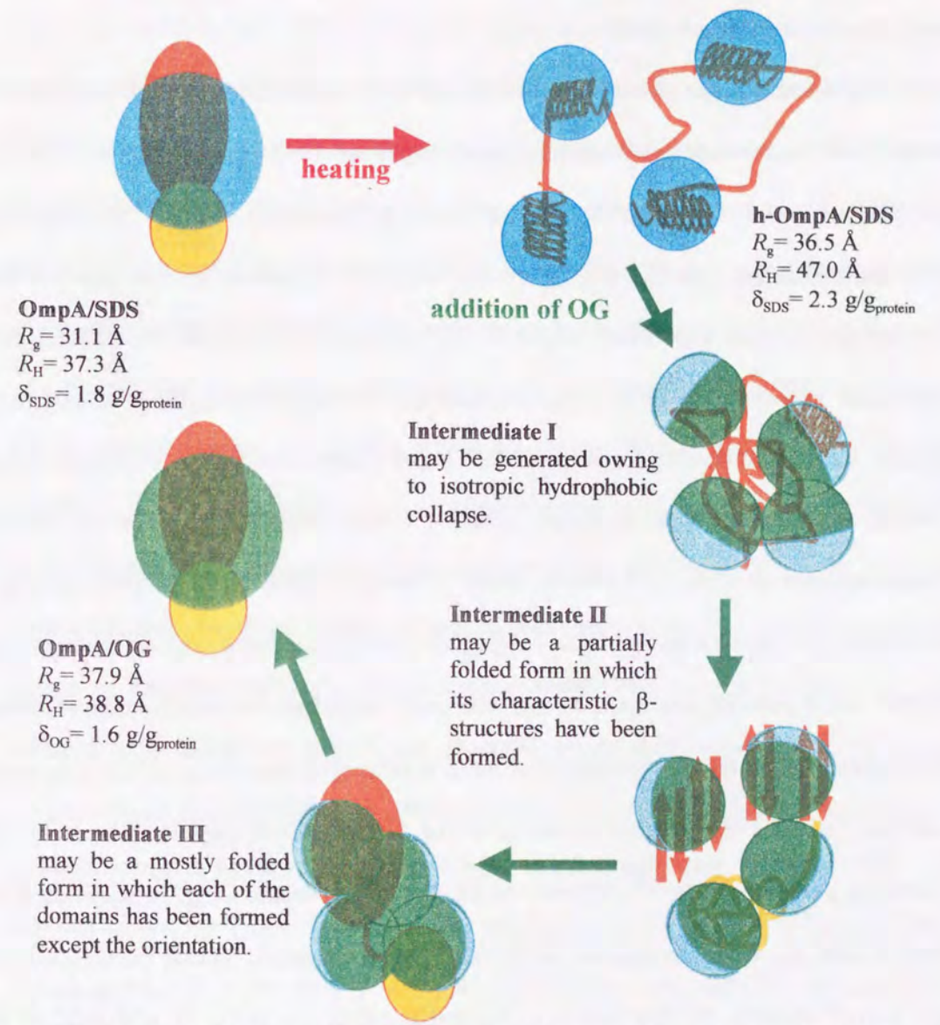


Figure 5.1 Hypothetical scheme of the conformational change of OmpA in the mixed surfactant system of SDS and OG. The structures of the protein in the non-heated form in the presence of SDS or OG and that in the heat modified form in the presence of SDS are drawn mainly based on the results from the SAXS and DLS measurements mentioned in the previous chapters. The folding scheme in this figure is shown according to the results from kinetic experiments described in Chapter IV. Three intermediates in the scheme were speculated from these results. For further details, see text.

will make clear the structural details.

For a membrane protein of α type consisting of transmembrane α-helices, a detailed folding scheme has been proposed (Popot and Engelman, 1990). In this scheme, formation of the α helices takes place on a membrane surface prior to the insertion of the polypeptide into the membrane, and the folding of the protein is completed by the insertion and subsequent orientation of these helices in the membrane. Studies on the folding of a membrane protein of β type from its unfolded form in the presence of denaturants to its folded one in lipid vesicles are reported for OmpA (Surrey and Jähnig, 1995) and OmpF (Surrey *et al.*, 1996). In these studies, a similar folding scheme is proposed for the two membrane proteins. That is, formation of the β structures takes place on the surface of a membrane prior to the insertion of the polypeptide into the membrane, and the folding of the protein is completed by the insertion and subsequent orientation of these β structures in the membrane. The preliminary formation of the β structures on the surface of the membrane can also lead to the aggregate formation of the protein, which may be induced by the formation of an intermediate β structure. In the case of the trimeric OmpF, association of its polypeptides was observed after the orientation of the β structures, namely the formation of the tertiary structure, of each monomer (Watanabe, 1994). Except for the formation of the quaternary structure, the folding process of membrane proteins of β type seems similar to that of α type. In other words, the two events in the folding process, 1) the preliminary formation of the transmembrane segments and the insertion and 2) the subsequent orientation of these segments, may be in common with membrane proteins both of α and β types. In this respect, the folding behavior of OmpA in the mixed surfactant system of SDS and OG found in the present study seems to be consistent with this perspective.

It should be noted that properties of the mixed surfactant system used in the present study are different from those of the experimental system used in previous studies through which such folding schemes are proposed (*e.g.* Popot and Engelman, 1990, Surrey and Jähnig, 1995, Surrey *et al.*, 1996) in terms of the amphiphilic environment involved. Namely, the present system preserves a micellar amphiphilic environment during the folding of a membrane protein from the unfolded form, while the other systems involve the transfer of a protein from an aqueous environment in the presence of a denaturant to amphiphilic one with lipid bilayers. It is simply presumed that the initial hydrophobic collapse of a membrane protein polypeptide under the condition with the latter environment takes place on the interface between the lipid bilayer and water, namely on the surface of the membrane. This is an analogue to the collapsed state of water-soluble proteins, which is reportedly known to have high affinity to the membrane surface (Ptitsyn, 1995). Since the initial collapse for a membrane protein was observed during their transfer from an aqueous environment to an amphiphilic one, the hydrophobic region of the protein generated by the collapse seems to be more favorable to bind to the amphiphilic membrane than to be exposed in the aqueous environment. The conformational change of the polypeptide of OmpA around the tryptophan residues, which may be responsible for the hydrophobic collapse, was also observed in the mixed surfactant system, *i.e.* under an overall amphiphilic condition. This suggests that the hydrophobic collapse of the protein is accounted for not by the preference of its hydrophobic region for binding to the membrane rather than being exposed in water, but by the a dominant force gathering its hydrophobic residues which is intrinsic for the membrane protein. In this respect, the initial hydrophobic collapse may be a common property of protein folding between water-soluble proteins and membrane ones.

5.3 Stability of OmpA in the Presence of SDS

We studied the dependence of the refolding rate constants of OmpA on the composition of OG in the mixed surfactant system and the temperature dependence of unfolding rate constant of the protein in the presence of SDS. The analysis of these rate constants indicate that this protein in the presence of SDS at 30 °C is more stable in the unfolded form by 12 kJmol^{-1} than in the folded form (ΔGu=-12 kJmol^{-1}). Namely, this protein is solubilized keeping the folded form in the presence of SDS in spite of the thermodynamical disadvantage. This form is preserved by a kinetic factor with a considerably small unfolding rate constant and denatures upon heating with an increase in the rate constant, resulting in the heat modifiability. The heat modifiability in the presence of SDS is commonly observed for the outer membrane porins, such as OmpC and OmpF (Nakamura and Mizushima, 1976), which are typical β barrel membrane proteins. Such stability, however, has not been observed for membrane proteins of α type such as BR (Booth *et al.*, 1990, 1995) and DGK (Lau and Bowie, 1997). Although it is uncertain whether any membrane proteins of β type have heat modifiability in common, at least such outer membrane proteins including OmpA maintain their folded structure in the usual time scale under similar denaturing conditions owing to extraordinary slow unfolding rates. Apparently stabilized, unfolded states with extraordinary slow unfolding rates are reported for other proteins, *e.g.* a hyperthermophilic protein, pyrrolidone carboxyl peptidase from *Pyrococcus furiosus* (Ogasahara *et al.*, 1998). Outer membrane proteins are often exposed to severe extracellular circumstances and, therefore, may be required to acquire much higher stabilities of their conformations than those of cytoplasmic proteins. This may be the same reason why proteins from hyperthermophilic microorganisms, which grow at extraordinary

high temperatures, acquire their higher stability.

The slow refolding rate of a protein is usually considered to be concerned with the height of the energy barrier on the transition from the unfolded state to the folded state (Creighton, 1994). In the case of the conformational change of OmpA in the presence of SDS at 30 °C, the energetic barrier, $\Delta G^{\ddagger SDS}$, expressed in terms of the activation free energy is calculated by the use of Eyring's equation (Frost and Pearson, 1961),

$$\Delta G^{\ddagger SDS} = RT \ln (k_B T/hk_{SDS}) \qquad [5.1]$$

to be 119 kJ mol^{-1}. Here, k_B is Boltzmann's constant, h is Planck's constant and k_{SDS} is unfolding rate constant in the presence of SDS at 30 °C. Unfortunately, this value cannot be compared with those for other proteins, for which no numerical data in the presence of SDS are available. Figure 5.2A schematically shows that OmpA in the unfolded form, destabilized by 12 kJ mol^{-1} in the presence of SDS at 30 °C, is preserved by the activation energy of 119 kJ mol^{-1}. Figure 5.2B shows a hypothetical potential profile including the unfolded (U) and folded (F) forms at various compositions of OG. We may interpret the heat modifiability and the refolding phenomena in terms of the potentials.

In the neat SDS solution at a low temperature, around room temperature, the unfolding reaction from the folded form of proceeds very slowly due to the extremely high activation potential barrier. The folded form of the protein, kept in the presence of a strong denaturant, SDS, is hence referred to as the "kinetically stable state". At a sufficiently high temperature, the protein realizes the unfolded form in the presence of the denaturant, as the unfolding reaction is enhanced. OmpA in the unfolded form once realized will not refold by simply lowering the temperature, because the unfolded form is thermodynamically stable in the neat SDS solution. By the addition of OG to the SDS-unfolded form of OmpA, the

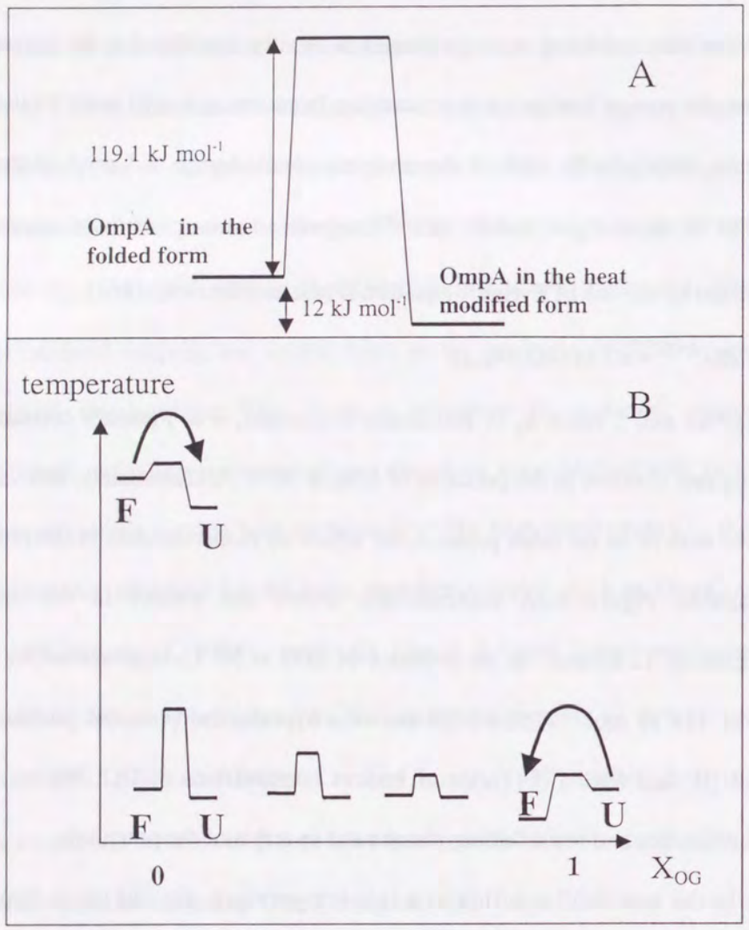


Figure 5.2 (A) An energetic scheme of OmpA in the presence of SDS at 30 °C. (B) Hypothetical energetic profiles of two conformational states of OmpA varying with temperature and the composition of the mixed surfactant system of SDS and OG. The folded form and unfolded form of OmpA are expressed with the notations of (F) and (U), respectively.

protein refolds spontaneously. This indicates that the folded form of the protein is certainly thermodynamically stabilized by the presence of OG. The thermodynamical stability may be evaluated if equilibrium constant could be obtained. However, it was difficult to determine the equilibrium denaturation curve for the isothermal conformational transition of OmpA in the SDS-OG mixed surfactants system, because the refolding and unfolding reactions were very slow, especially in the transition region. The present study has shown that the refolding rate depends on the final composition of the mixed surfactant solution of SDS and OG and is accelerated with increasing X_{OG}. This fact can be interpreted as the result of the potential barrier between the two forms reduced with X_{OG}. No unfolding reaction could be observed at any compositions of the mixed surfactant system unless heating. This suggests that the potential barrier between the two state is significantly reduced only at high X_{OG}.

5.4 Approach for Solving the Problem of Protein Folding as for Membrane Proteins –Application of Mixed Surfactant System-

The previous observation by Dornmair *et al.*(1990), in which OmpA in the unfolded state refolds to the folded state by the addition of excess amount of OG, gave an example of examining folding phenomena of the membrane protein quantitatively. In the present study, this conformational change was characterized thermodynamically and kinetically by changing the composition of the mixed surfactant system of SDS and OG systematically. The results gave significant information concerning the unique folding property of the membrane protein. It is noticeable that the folding rate of the protein could be controlled by changing composition of the mixed surfactant system. Furthermore, the present study

showed that changing composition of the mixed surfactant system of SDS and OG corresponds to changing the denaturing ability of SDS. This indicates that an experimental system with the mixture of the surfactants is an analogue of aqueous experimental systems with a denaturant such as urea or guanidine hydrochloride, which have been conventionally used for studying folding properties of water-soluble globular proteins, in terms of denaturing ability. In order to compare the knowledge about the folding properties of the membrane protein obtained from the present study with the well-known folding properties of water-soluble proteins, more examples for various kinds of proteins using the mixed surfactant system are necessary. So far, a very limited number of the studies on membrane protein folding have been reported as mentioned in Chapter I, since only a few experimental system, in which a membrane protein folds and unfolds reversibly, has been known. In this context, utilization of the mixed surfactant system is expected to be a foothold for advancing research concerning the folding mechanism of membrane proteins.

It is prerequisite, however, to have detailed knowledge in advance about the solution properties of the respective surfactants concerned and their mixture. For example, these surfactant molecules form micelles above the cmc, or solubility of them decreases critically below a temperature, called the Kraft point, or their solutions show phase separation above a temperature, called the clouding point. Such properties should be examined preliminarily. The mixed surfactant system of SDS and OG is suitable in this context, because each of the surfactants and the mixed surfactant system have been well examined (Hayashi and Ikeda, 1980, Kameyama and Takagi, 1990, Kameyama *et al.*, 1997). Further advances in this field are expected by the use of a mixed surfactant system combined with modern methods, which give the detailed insight into protein folding with

atomic resolution, such as NMR or ESR techniques.

Chapter VI Summary of the Thesis

6.1 Characterization of OmpA in the Mixed Surfactant System of SDS and OG

A membrane protein, OmpA of *Escherichia coli*, in the process of refolding from its heat-modified form in the presence of SDS to its non-heated one by the systematic addition of OG was characterized by means of dynamic light scattering and size exclusion chromatography combined with low angle laser light scattering photometry. Upon heating in the presence of SDS, the amount of SDS bound to OmpA increased from 1.8 to 2.3 grams per gram of protein and its hydrodynamic radius increased from 37.3 to 47.0 Å. On 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 denaturation phenomena of OmpA.

6.2 Size and Shape of OmpA in a Surfactant Solution

OmpA solubilized in the presence of a surfactant forms a complex between the protein and the surfactant. The complexes formed between OmpA in the folded form and

SDS (OmpA/SDS), between OmpA in the unfolded form and SDS (h-OmpA/SDS) and between OmpA in the folded form and OG (OmpA/OG) were examined by means of small angle X-ray scattering, SAXS. Complexes between water-soluble proteins and SDS were also examined for RCAM-OVA, RCAM-OVA/SDS, and RCAM-RNaseA, RCAM-RNaseA/SDS as references to those of the membrane protein. The SAXS profiles of scattering intensities and of pair distance distribution function, $P(r)$ function, for h-OmpA/SDS were very similar to those for RCAM-OVA/SDS and RCAM-RNaseA/SDS, indicating that the structure of OmpA in the unfolded form is similar to those of water-soluble proteins in the presence of SDS. The structure of OmpA in the folded form in OG solution was shown to be different from that in SDS solution according to their scattering intensities and $P(r)$ function profiles. Radii of gyration for OmpA/OG, OmpA/SDS and h-OmpA/SDS were obtained according to their Guinier plots to be 37.9 Å, 31.1 Å and 36.5 Å, respectively. The structure of OmpA/OG was well simulated as a prolate ellipsoid with a major axis of 62.8 Å and a minor axis of 21.6 Å. While the simulation did not give the details, the dimension of the simulated ellipsoid appeared reasonable for the membrane protein.

6.3 Kinetic Aspects of the Conformational Change of OmpA in the Mixed Surfactant System of SDS and OG

Refolding reactions of OmpA from the heat modified form to the folded form accompanying the addition of various amounts of OG were kinetically investigated by measurements of CD in far and near UV region and intrinsic fluorescence. The results gave four refolding rate constants, which were relatively small compared to those reported for

water-soluble proteins. These refolding rate constants were shown to depend on the composition of the mixed surfactant system of SDS and OG. This refolding process of the protein was considered to consist of four steps, which is consistent with the results by Surrey and Jähnig (*Biochemistry*, **270**, 1995, 28199-28203). Unfolding of OmpA upon heating in the presence of SDS was also kinetically investigated through temperature jump experiments by means of CD spectroscopy in the far UV region. The comparison of the unfolding rate at 30 °C estimated by extrapolation of the Arrhenius plot with the refolding limiting rate at the temperature in the SDS solution estimated by extrapolation to zero OG indicated that the former rate was significantly greater than the latter one, while both constants were very small. The structure of OmpA in the non-heated form is thus presumed to be thermodynamically unstable in the presence of SDS, though the unfolding is not observed in the usual time scale at the temperature.

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List of Publications

1. Characterization of a heat modifiable protein, *Escherichia coli* outer membrane protein OmpA in binary mixed surfactant system of sodium dodecyl sulfate and octylglucoside., Ohnishi, S., Kameyama, K. and Takagi, T. *Biochim. Biophys. Acta* **1375** (1998) pp. 101-109

2. Kinetic aspects of the conformational change of a membrane protein, *Escherichia coli* OmpA in the mixed surfactant system of sodium dodecyl sulfate and octylglucoside., Ohnishi, S. and Kameyama, K., to be submitted.

3. Common structures in SDS-denatured proteins observed by small angle X-ray scattering., Ohnishi, S., Ozawa, T., Sato, M. and Kameyama, K., to be submitted.

Related Publications

1. Denaturation behavior and conformational stability of *Escherichia coli* outer membrane protein A in the presence of urea and octylglucoside. Kameyama, K., Noguchi, S. and Ohnishi, S., to be submitted.

2. Structure of *Escherichia coli* OmpA solubilized in the presence of octylglucoside studied by small angle X-ray and neutron scattering., Kameyama, K., Ohnishi, S., Sato, M. and Nagao, M., in preparation to be published.