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Characterization of the refolding and reassembly of an integral membrane protein OmpF porin by low-angle laser light scattering photometry coupled with high-performance gel chromatography

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Abstract

The refolding and reassembly of an integral membrane protein OmpF porin denatured in sodium dodecylsulfate (SDS) into its stable species by the addition of n-octyl-β-D-glucopyranoside (OG) have been studied by means of circular dichroism (CD) spectroscopy and low-angle laser light scattering photometry coupled with high-performance gel chromatography. The minimal concentration where change in the secondary structure was induced by the addition of OG was found to be 6.0 mg/ml in CD experiments. A species unfolded further than the SDS-denatured form of this protein was observed at an early stage (5–15 min) of refolding just above the minimal OG concentration. In addition, the CD spectrum of protein species obtained above the minimal OG concentration showed that the protein is composed of a β-structure which is different from the native structure of this protein. In light scattering experiments, no changes in molecular assemblies were observed when the OG concentration was below its minimal refolding concentration determined by CD measurements. Above the minimal concentration, a compact monomeric species was observed when denatured OmpF porin was incubated for 5 min at 25 °C in a refolding medium containing 1 mg/ml SDS and 7 mg/ml OG, and then injected into columns equilibrated with the refolding medium. After an incubation of 24 h before injection into the columns, predominant dimerization of this protein was observed in addition to incorrect aggregation.

Keywords: Refolding; Molar mass determination; Light scattering; Membrane protein; OmpF porin; Proteins

1. Introduction

Folding and assembly problems of oligomeric integral membrane proteins are still a subject of interest. OmpF porin is a trimeric integral membrane protein that functions as a general diffusion pore in the outer membrane of *Escherichia coli* [1]. The subunit with a molar mass of 37 kg/mol [2,3] has a 16-stranded β-barrel structure [4]. As summarized by Buchanan [5], in vitro reassembly of the denatured monomer of OmpF porin into its stable trimer has been investigated. Eisele and Rosenbusch [6] have demonstrated that a dialysis method using soybean lecithin and polydisperse octyl-oligoethyleneoxide (not commercially available) was useful for the renaturation of OmpF porin denatured in 6 *M* guanidine hydrochloride. Yields of refolded OmpF porin trimer improve when the unfolded material is added to mixed lipid-surfactant micelles of dodecylmaltoside and dimyristoylphosphatidylcholine for insertion into the lipid bilayer [7]. These reports show that the usage of amphiphiles, such as non-ionic surfactants, is necessary for the reassembly of OmpF porin from its denatured monomer. However, the dialysis method and the

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membrane insertion method are not suitable for characterization of protein association under refolding conditions. Therefore, in this study, information was obtained about the refolding and reassembly process of this protein using a renaturation method that required only the addition of non-ionic surfactants, but no subsequent dialysis. The species might include those formed as the result of incorrect folding often observed among the products of the process. Such species have been characterized through determination of their molar mass by low-angle laser light scattering photometry coupled with high-performance gel chromatography. In particular, this technique is suitable for the molar mass determination of the protein moieties of a membrane protein solubilized with a surfactant [8,9].

As a non-ionic surfactant for renaturation, n-octyl-β-D-glucopyranoside (OG) was selected in the present study. The surfactant has been used for the solubilization of a membrane protein, keeping its biological function. The reassembly of denatured monomeric OmpF porin with such non-ionic surfactants has been already observed in gel electrophoresis experiments that were reported in detail by Watanabe [10]. In this study, in order to understand the refolding and reassembly of denatured monomeric OmpF porin, the protein structures in the presence of OG were followed by circular dichroism (CD) spectroscopy and low-angle laser light scattering photometry to monitor the secondary structure and the molecular assemblies of the protein, respectively.

In CD experiments, the minimal concentration of OG where change in secondary structure was induced by the addition of the surfactant was found to be 6 mg/ml. A species unfolded further than the SDS-denatured form of this protein was transiently observed at an early stage (5–15 min after the addition of OG) during refolding of OmpF porin in a medium containing OG in a concentration just above the minimal concentration. In addition, the CD spectrum of protein species obtained by the addition of OG in a concentration above the minimal concentration showed that the protein is composed of a β-structure that is different from that of native OmpF porin. Light scattering technique revealed the following points. No changes in molecular assemblies of the protein were observed upon the addition of OG in a concentration below the minimal refolding concentration determined by CD measurements. Above the minimal concentration of OG, a compact monomeric species, presumed to be a folded monomer, was observed when OmpF porin denatured by SDS on heating was incubated for 5 min at 25 °C in a refolding medium that contained 7 mg/ml OG and 1 mg/ml SDS, and then injected into columns equilibrated with the refolding medium. After an incubation of 24 h before injection into the columns, the dimerization of this protein was observed in addition to incorrect aggregation. The incorrect aggregates were dissociated to a denatured monomer by the addition of excess SDS. This report describes the effective dimerization of denatured monomeric OmpF porin during its refolding, as observed by low-angle laser light scattering photometry combined with high-performance gel chromatography.

2. Experimental

2.1. Materials

Sodium dodecylsulfate (SDS) was purchased from BDH Chemicals (Poole, UK) and n-octyl-β-D-glucopyranoside (OG) from Dojin Chemicals (Kumamoto, Japan) and Nacalai Tesque (Kyoto, Japan). Other chemicals were of analytical grade.

2.2. Preparation of sample solutions

OmpF porin was purified from *Escherichia coli* B as described previously [9]. A sample solution (5 mg/ml protein, 10 mg/ml SDS, 50 m*M* sodium phosphate buffer, pH 7) obtained in a sealed glass tube was placed in a water bath of 95 °C for 5 min to obtain the denatured protein. After cooling to room temperature, the solution was passed through a 0.45-μm pore size HV membrane filter (Nihon Millipore, Yonezawa, Japan), and concentrated ~10-fold using a Centricon-30 centrifugal ultrafilter (Nihon Millipore). The concentrate was then injected into gel permeation chromatography (GPC) columns, a tandem array of two TSK-G3000SWXL columns (300×7.8 mm I.D., Tosoh, Tokyo) equipped with a

guard column TSK-GCSWXL (40×6.0 mm I.D., Tosoh), equilibrated with 2 mg/ml SDS, 0.1 *M* sodium chloride and 10 m*M* 3-[*N*-morpholino]-propanesulfonic acid (MOPS), pH 6.9, at a flow-rate of 0.4 ml/min at 25 °C. OmpF porin denatured by SDS on heating was eluted as a single peak. The fractions were collected and diluted with the elution buffer to a final protein concentration of 0.4 mg/ml. The protein concentration was determined spectrophotometrically assuming that the absorbance value of a 1% solution at 278 nm was 14.1 in a 1-cm light-path cuvette [11].

2.3. Refolding procedure

The refolding (reassembly) of denatured monomeric OmpF porin (0.4 mg/ml) solubilized in 2 mg/ml SDS was attempted by adding a buffer solution that contained OG in a concentration twice as high as needed in the final solution to an equal volume of the solution of the denatured protein. The final concentrations of the protein and SDS were always kept constant at 0.2 and 1.0 mg/ml, respectively. The solution also contained 0.1 *M* sodium chloride and 10 m*M* MOPS, pH 6.9.

2.4. Circular dichroism spectrum measurements

CD spectra were recorded with a 1-mm light-path quartz cell using a J-720 (or J-820) spectropolarimeter (Jasco, Tokyo) at 25 °C. Spectra were collected in 0.01-nm increments at a scan rate of 20 nm/min, 1-nm bandwidth, and 20-mdeg sensitivity using a 0.25-s time constant. Background corrections were made on all measurements. Molar ellipticity [θ] (deg cm^2/dmol) was calculated using a mean residue molar mass of 109 g/mol [2,3].

2.5. SDS–polyacrylamide gel electrophoresis

For gel electrophoresis of the fractions obtained by gel chromatography, reassembly (refolding) of the protein was quenched by the addition of a large excess of SDS. The final solution contained 20 mg/ml SDS, one-fourth of the original concentration of non-ionic surfactants, 0.08 *M* sodium chloride, 5 m*M* EDTA, 10% glycerol and 8 m*M* MOPS, pH 7. A 10-μl volume of the solution was applied to each sample well on the top of the slab gel. SDS–polyacrylamide gel electrophoresis was performed with a mini-sized apparatus (12.5% gel; 60 mm in length and 1 mm in thickness) at room temperature (25 °C) according to the method of Laemmli [12]. Proteins in the gel were visualized using the Wako silver staining kit (Wako, Osaka, Japan).

2.6. Low-angle laser light scattering photometry combined with high-performance gel chromatography

This system consists of an HPLC pump system, GPC columns and three sequential detectors, a low-angle laser light scattering photometer (TSK LS-8000, Tosoh), an ultraviolet spectrophotometer (TSK UV-8000, Tosoh) and a differential refractometer (TSK RI-8000, Tosoh). The GPC columns were a tandem-array of two TSK-GEL G3000SWXL equipped with a TSK-GEL GCSWXL guard column (Tosoh). The temperature of the columns and the flow-through cell in the low-angle laser light scattering photometer was kept at 25 °C using a column jacket and a metallic cell holder through which water of constant temperature was circulated. The eluent was a MOPS buffer solution (0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9) containing several concentrations of OG and 1 mg/ml SDS.

Data handling has been described in connection with the molar mass determination of proteins [8]. In the present experiment, samples are eluted as the complex of a protein and a surfactant (or surfactants). The molar mass of the protein moiety, M_p, was obtained according to the following equations:

$$M_p = k_1(dn/dc_p)^{-1}(LS)(RI)^{-1} \quad (1)$$

$$(dn/dc_p) = k_2A(RI)(UV)^{-1} \quad (2)$$

where dn/dc_p is the specific refractive index increment expressed in terms of weight concentration of the protein (c_p); k_1 and k_2, constants, which depend on the experimental conditions; *A*, the extinction coefficient at 280 nm (ml/mg protein for 1-cm light-path); (*LS*), (*UV*) and (*RI*), the outputs of the low-angle laser light scattering photometer, the ultraviolet

spectrophotometer and the differential refractometer, respectively. From the above two equations, the molar mass can be expressed as:

$$M_p = kA^{-1}(LS)(UV)(RI)^{-2} \quad (3)$$

where k is a constant. The constant, k, was determined by using the following proteins of known molar mass (kg/mol) and extinction coefficients at 280 nm (ml/mg, for a 1-cm light-path): bovine carbonic anhydrase ($M_p = 29$, $A = 1.9$ [13]), ovalbumin ($M_p = 42.7$, $A = 0.735$ [13]), bovine serum albumin ($M_p = 66.3$, $A = 0.678$ [13]) and OmpF porin trimer ($M_p = 111$ [2,3], $A = 1.38$ [11]). Solutions of water-soluble proteins used as molar mass standards were prepared by dissolution of ~1 mg of protein in 5 ml of a MOPS buffer containing 1 mg/ml SDS and respective concentrations of a non-ionic surfactant. Trimeric OmpF porin was prepared in the above MOPS buffer to give a final concentration of 0.2 mg/ml. The sample solutions were filtered through a 0.45-μm pore size HV membrane filter (Nihon Millipore, Yonezawa, Japan).

3. Results and discussion

3.1. Changes in secondary structure by the addition of a renaturing (non-ionic) surfactant

The overall conformational change of denatured OmpF porin upon the addition of a renaturing surfactant, OG, was first examined by circular dichroism (CD) measurements with respect to the secondary structure of the protein. This experiment was needed to determine the conditions for the next light scattering experiments. Fig. 1 shows typical CD spectra of OmpF porin under a few sets of conditions. The far-UV CD spectrum of OmpF porin in the presence of SDS indicates that the native trimer is rich in β-structure, and the monomer denatured by SDS on heating forms an α-helix-like structure [11,14–16]. The CD spectrum of the native trimer is characterized by a minimum near 217 nm. That of the denatured monomer is characterized by a minimum at 207 nm and a negative shoulder at 222 nm.

Fig. 2 shows the change in the ellipticity at 217 nm of denatured OmpF porin upon the addition of OG while the concentration of SDS was kept constant at 1 mg/ml. The addition of OG up to 5 mg/ml progressively decreased the negative ellipticity of OmpF porin. No time-dependent change in the ellipticity was observed as monitored from 5 min up to 24 h. The CD spectra showed a feature suggesting that the species is rich in an α-helix-like structure similar to that of the denatured OmpF porin. On the other hand, the ellipticity became time-dependent above an OG concentration of 5 mg/ml as shown in Fig. 2. The negative ellipticity progressively increased during the period observed. The curve obtained after 24 h (circles) shows a steep increase in the ellipticity at an OG concentration of 6 mg/ml, leveling off to a larger negative ellipticity than that

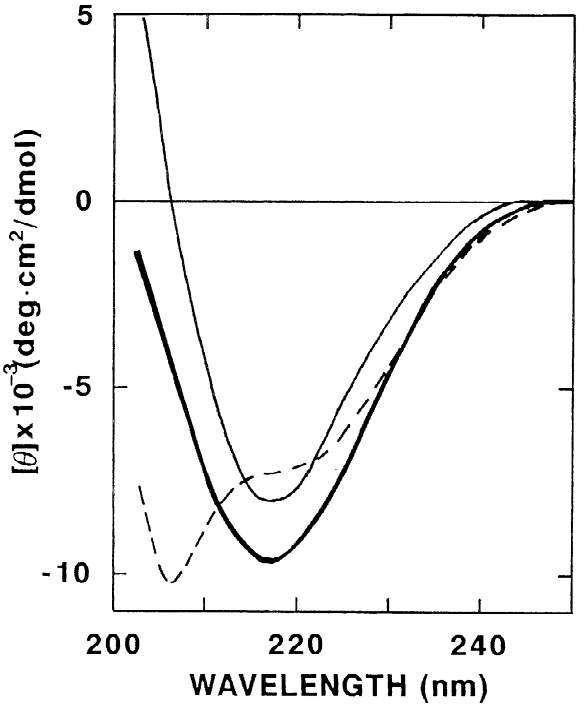


Fig. 1. Typical CD spectra of OmpF porin solubilized with surfactants. Thin solid and dashed lines correspond to the spectra of native trimer and denatured monomer in a MOPS buffer solution containing 1 mg/ml SDS, respectively. The spectrum shown in thick solid line was obtained for a solution prepared as follows: a 200-μl volume of a MOPS buffer solution containing denatured OmpF porin (0.4 mg/ml) and SDS (2 mg/ml) was mixed with an equal volume of a MOPS buffer solution containing 14 mg/ml OG. The CD spectrum of the protein in the mixture obtained was measured after incubation for 24 h at 25 °C. CD spectra were scanned 20 times (for ~30 min) at 25 °C. Protein concentration was kept constant at 0.2 mg/ml.

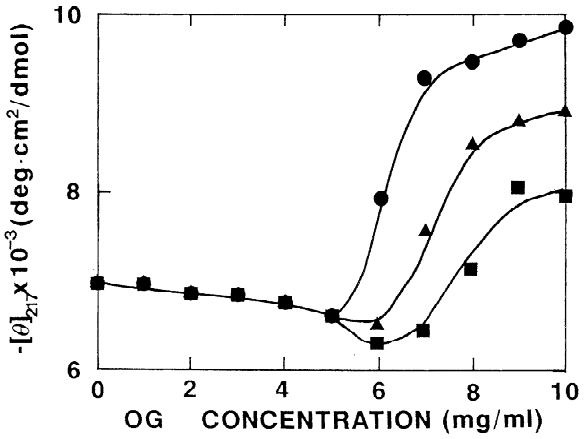


Fig. 2. Changes in the ellipticity at 217 nm of denatured OmpF porin upon the addition of OG. SDS concentration was kept constant at 1.0 mg/ml. Squares, triangles and circles correspond to ellipticities obtained when a spectrum was scanned eight times (for ~10 min) after 5, 60 min and 24 h. Other conditions were the same as those in Fig. 1. The three symbols overlap below an OG concentration of 5 mg/ml. Errors are within the order of a few percent.

of the native OmpF porin (about -8000 deg cm^2/dmol). Hereafter, this concentration will be called the minimal refolding concentration. Spectra obtained after standing for 24 h in a refolding medium that contained OG above the minimal refolding concentration show that the species have a β-structure (thick solid line in Fig. 1) not identical to that of the native OmpF porin as shown in Fig. 1.

3.2. Molecular assembly below the minimal refolding concentration of a renaturing surfactant

The molar mass of the species of OmpF porin denatured by SDS on heating, the starting material of the refolding and reassembly experiments, was determined by a light scattering technique at 25 °C. A sample had been equilibrated with the solution containing 1 mg/ml SDS, 0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9 before the light scattering measurements by gel chromatography. The molar mass of the OmpF porin species in the solvent was found to be 37 ± 2 kg/mol ($n = 3$), which corresponds to the molar mass of the subunit polypeptide of OmpF porin [2,3].

Similarly, we tried to determine the molar mass of species present in a reaction mixture that contained OG and 1 mg/ml SDS. OG was added to bring about refolding and reassembly of a protein, while SDS was previously added to cause denaturation. When the concentrations of OG were below the minimal refolding concentration determined by CD measurements (6 mg/ml; Fig. 2), no change in molecular assembly was observed as the OmpF porin denatured by SDS on heating was incubated for 24 h, and injected into gel columns equilibrated with the same medium.

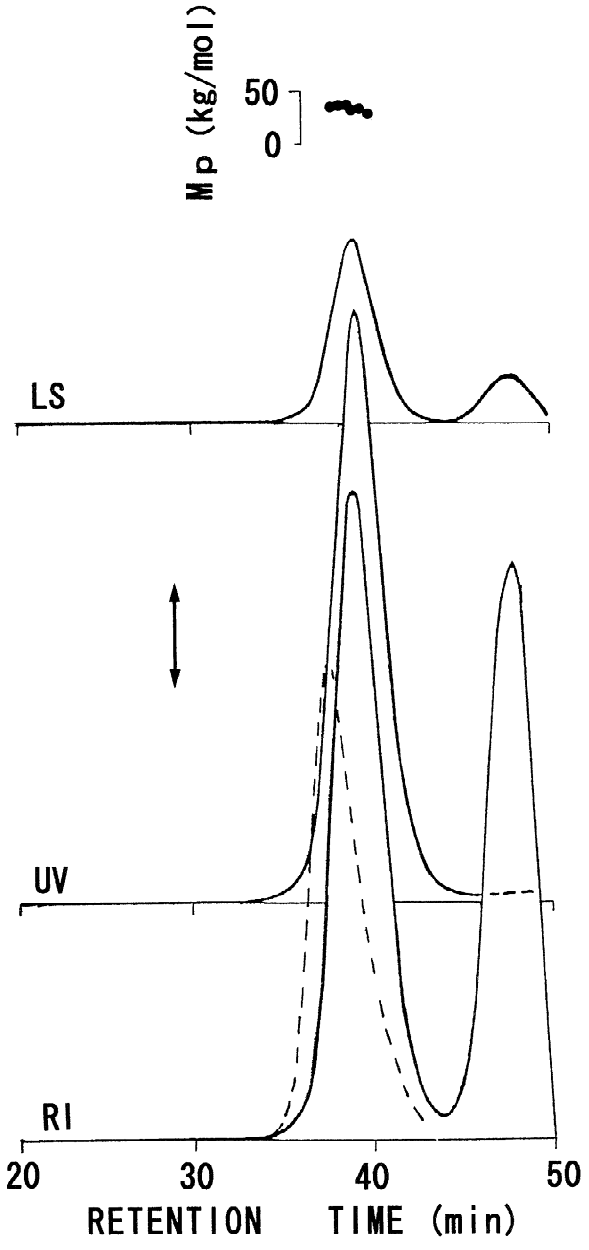
Fig. 3 shows typical elution curves obtained by using three detectors, the low-angle laser light scattering photometer (LS), the UV-spectrophotometer (UV) and the differential refractometer (RI). The elution buffer contained 5 mg/ml OG and 1 mg/ml SDS. Comparison of the three curves indicates that OmpF porin was eluted as a single peak at a retention time of 40 min. The peak at 48 min has been assigned to non-protein materials, probably micelles consisting of SDS and OG, because it lacks UV absorption. Hereafter, this non-protein peak will not be discussed.

Fig. 4 shows a typical, fairly linear calibration plot of the molar mass of proteins including native trimeric OmpF porin versus the values of $A^{-1}(LS)(UV)(RI)^{-2}$, confirming the reliability of this technique for molar mass determination of membrane proteins. As shown with an open circle in Fig. 4, the molar mass of protein species at apex of the 40-min peak was 36.8 ± 0.9 kg/mol. The values of molar mass determined for a region covered by the protein peak are shown in the top of Fig. 3 and coincide well with that of the single polypeptide, ~37 kg/mol, calculated from its amino acid sequence [2,3] indicating that OmpF porin is eluted as a monomer. The dashed line at the bottom of Fig. 3 shows an elution curve of a standard trimeric OmpF porin. The trimer peak overlaps with the monomeric form, since both are comparable in size [17,18] necessitating the use of the light scattering technique for the determination of the molar mass of OmpF porin species.

3.3. Molecular assembly above the minimal refolding concentration of a renaturing surfactant

Fig. 5 shows a set of elution curves obtained when

OmpF porin denatured by SDS on heating was incubated for 5 min in a medium containing 7 mg/ml OG and 1 mg/ml SDS before injection into the columns equilibrated with the same medium. The molar mass and *RI*/*UV* signal ratio profiles are also included on the top of Fig. 5. The *RI*/*UV* ratio reflects the amount of surfactants bound to the protein. A small peak was observed at 28 min only by the light scattering photometer (LS in Fig. 5). Judging from the retention time and the lack of response by the other two detectors, the peak can be



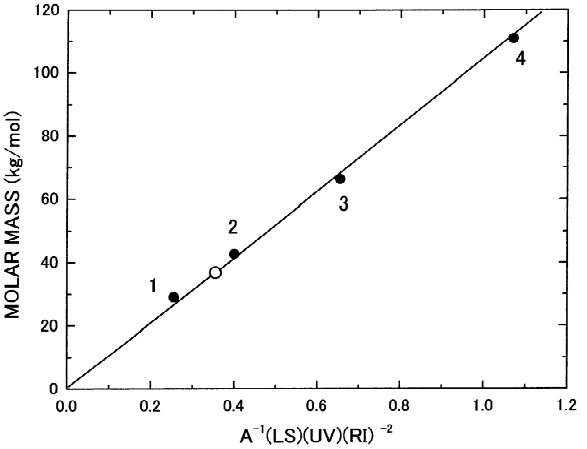


Fig. 4. Relationship between the value of $A^{-1}(LS)(UV)\ (RI)^{-2}$ and the molar mass of proteins. Experimental conditions are the same as Fig. 1. Closed circles show data points for bovine carbonic anhydrase (1), ovalbumin (2), bovine serum albumin (3), and OmpF porin trimer (4) standards. The open circle shows the data point at the peak apex of 40 min determined for three independent experiments. Error bars are within the symbols.

Fig. 3. Elution curves when the OG concentration is just below the minimal refolding concentration. Elution curves (solid lines) were obtained by a low-angle laser light scattering photometer (LS), a UV-spectrophotometer (280 nm, UV) and a differential refractometer (RI). The gain-settings for these detectors were 32, 0.16 and 16, respectively. The retention time refers to that observed for the light scattering photometer. The actual retention times observed for the other detectors were corrected for the time lag caused by their sequential arrangement. Molar mass (circles) shown on the top of a figure was calculated from the signal intensities of the three detectors according to Eq. (3). Sample solution was prepared as follows: 300 μl of a MOPS buffer solution containing 0.4 mg/ml denatured monomeric OmpF porin and 2 mg/ml SDS was mixed with 300 μl of a MOPS buffer solution containing 10 mg/ml OG. After standing for 24 h at 25 °C, 500 μl of the mixture obtained were injected into a tandem-array of two TSK-G3000SWXL columns equipped with a TSK-GCSWXL guard column equilibrated with a MOPS buffer solution containing 1 mg/ml SDS, 5 mg/ml OG. Flow-rate was 0.4 ml/min. The length of a vertical bar with arrowheads corresponds to one-tenth of the full-scale for the three detector signals. The dashed line in the RI trace indicates the elution curve of native trimeric OmpF porin.

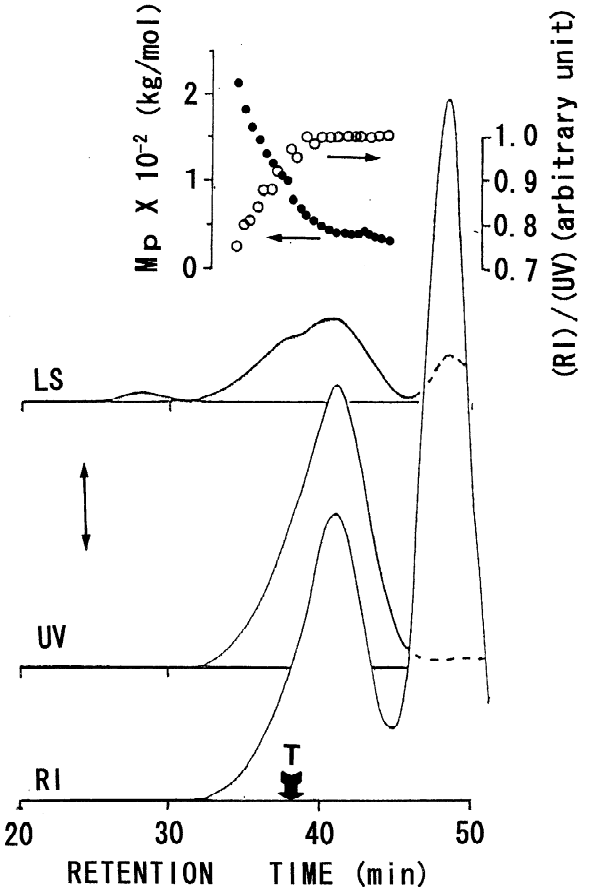


Fig. 5. Elution curves obtained when denatured OmpF porin was incubated for 5 min before injection into columns in a medium containing more than 6 mg/ml OG. Molar mass (closed circles) and the *RI/UV* signal ratio (open circles) estimated for the protein peak are also included on the top of a figure. The sample was prepared as follows: a 300-μl volume of a denatured monomer solution was mixed with an equal volume of a buffer solution containing 14 mg/ml OG, and 500 μl of the mixture after incubation for 5 min at 25 °C were injected into GPC columns equilibrated with a MOPS buffer solution containing 1 mg/ml SDS, 7 mg/ml OG at 25 °C. The arrow with T corresponds to the retention time (38 min) of purified native trimer. Other conditions were the same as in Fig. 3.

assigned to a negligibly small amount of large particles. Protein species were eluted between 32 and 45 min. The peak was much broader than that observed below minimal refolding concentrations (compare Fig. 5 and Fig. 3), especially on the less retained side where the light scattering photometer indicated a shoulder.

The apex of the UV absorbance signal at 280 nm was near 42 min, later than the elution time of native trimeric OmpF porin (indicated by the arrow marked T in Fig. 5). The molar mass of protein species at 42 min in three independent experiments was found to be 40±2 kg/mol indicating that the major components are still monomers. Furthermore, the RI/UV ratio increased with retention time, reaching 1.0 while the value for native OmpF porin trimer was 0.84±0.03 ($n=4$). These results suggest that the denatured polypeptide of OmpF porin refolds to a product with a molecular size smaller than that obtained below the threshold concentration while binding larger amounts of surfactants than native OmpF porin.

Fig. 6 shows the elution curves for the sample incubated for 24 h in the medium used for Fig. 5. The profiles of molar mass and *RI/UV* values are also shown on the top of Fig. 6. Two protein peaks were observed at 31 and 40 min. The molar mass started from above 500 kg/mol and decreased to a plateau of ~70 kg/mol corresponding to the molar mass of the dimer of this protein (74 kg/mol) [2,3]. The molar mass at the first and second peak apexes, determined in three independent experiments, was found to be 622±55 and 77±5 kg/mol, respectively, clearly indicating the presence of higher aggregates and the dimer of OmpF porin. On the basis of the RI/UV profile, it is conceivable that the dimer binds still larger amounts of surfactants than the native trimer, but slightly smaller amounts than the monomeric species in Fig. 5, while the aggregates bind smaller amounts of surfactants than native trimeric OmpF porin.

In addition, fractions of the first and second peak (a and b in Fig. 6) were collected and analyzed by SDS–polyacrylamide gel electrophoresis. The inset in Fig. 6 shows that the aggregates of OmpF porin dissociated into its denatured monomer after the addition of excess amounts of SDS at room temperature, while most of fraction b was detected as an intermediate band in the SDS–polyacrylamide gel electrophoresis experiments. The latter result indicates that the band between the bands of trimeric and monomeric OmpF porin is the SDS-resistant stable dimer of this protein, although very small amounts of monomer and SDS-resistant stable trimer were also detected due to the high sensitivity of the silver

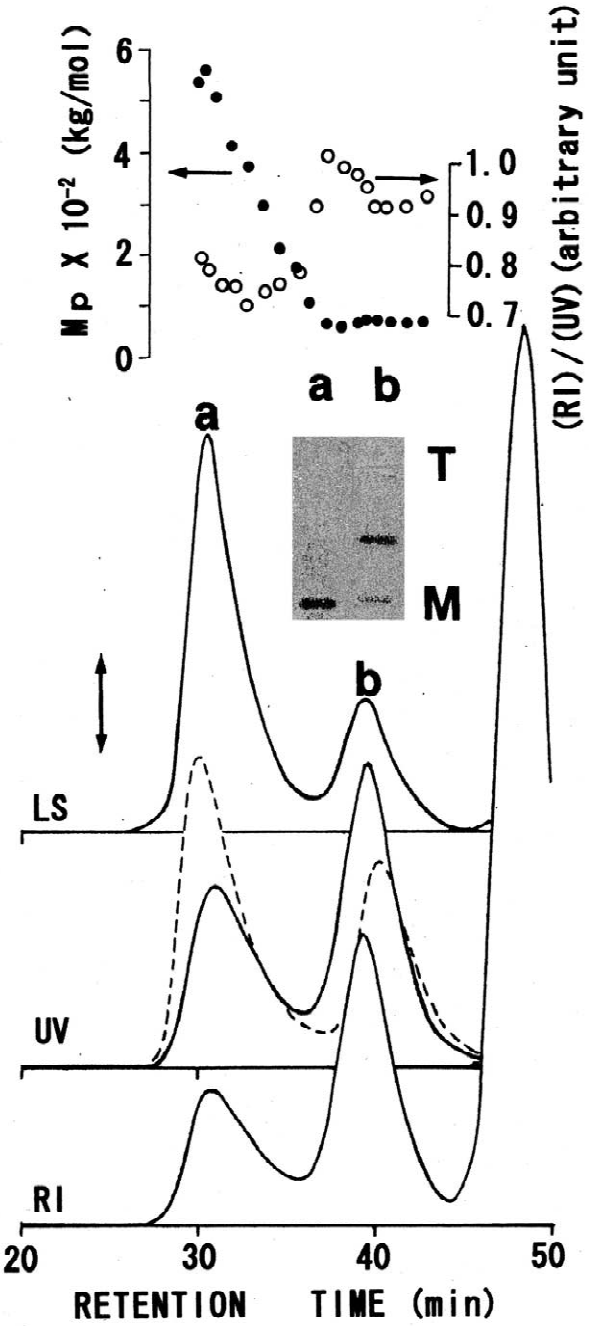


Fig. 6. Elution curves obtained after 24-h incubation of the same sample as in Fig. 5 above 6 mg/ml OG. Molar mass (closed circles) and the *RI*/*UV* values (open circles) are also included on the top of a figure. The dashed line indicates the elution curve obtained in the presence of 10 mg/ml OG and 1mg/ml SDS. Other conditions were the same as those in Fig. 5. Fractions designated a and b on the light scattering tracing were analyzed by SDS–polyacrylamide gel electrophoresis by mixing 60 μl of each fraction with 20 μl of 8% SDS, 20 m*M* EDTA and 40% glycerol and applying 10 μl to the sample well of the gel. The protein was visualized with a silver staining kit. T and M are the positions of the bands expected for the trimer and denatured monomer of this protein.

staining method. The existence of the dimer in the in vivo folding pathway of OmpF porin has been reported by pulse-chase experiments using immunoprecipitation and SDS–polyacrylamide gel electrophoresis [19]. Fig. 6 also shows the UV elution curve of the sample at 280 nm in the presence of 10 mg/ml OG and 1 mg/ml SDS (dashed curve) clearly indicating that the yield of reassembled dimer in 7 mg/ml OG and 1 mg/m SDS is higher (~60%) than that in 10 mg/ml OG and 1 mg/ml SDS (less than ~40%).

3.4. In vitro folding and assembly pathway of OmpF porin

The results obtained in this study allow us to postulate the hypothetical in vitro folding and assembly pathway of denatured monomeric OmpF porin (Fig. 7). First of all, in CD experiments described in Section 3.1, it was found that denatured (unfolded) monomeric OmpF porin forms an α-helix-like structure (U in Fig. 7). The structure was converted to a β-structure under conditions where refolding was expected to occur (Fig. 1). In the early stage (within 15 min) during conversion under conditions just above the threshold of refolding shown in Fig. 2, a further unfolded species (I1 in Fig. 7) other than the SDS-denatured form of this protein was transiently observed (Fig. 2). Moreover, light scattering (Fig. 6) indicates that the CD spectra obtained after conversion (Fig. 1) reflect the secondary structure of the incorrectly folded species that forms irregular aggregates (A in Fig. 7). Therefore, the irregular aggregates are composed of a β-structure which is different from that of native OmpF porin. They bind a smaller amount of surfactants than the native trimer (Fig. 6), and are easily dissociated into denatured monomer (U in Fig. 7) by the addition of SDS at room temperature (Fig. 6). These observations suggest that an incorrect intermolecular β-structure is dominant in the irregular aggregates.

In the light scattering experiments, folded monomeric OmpF porin (I2 or M in Fig. 7) was observed during the early stage (within 40 min) of refolding above the threshold conditions of refolding shown in Fig. 2 (Fig. 5). The species is believed to be more

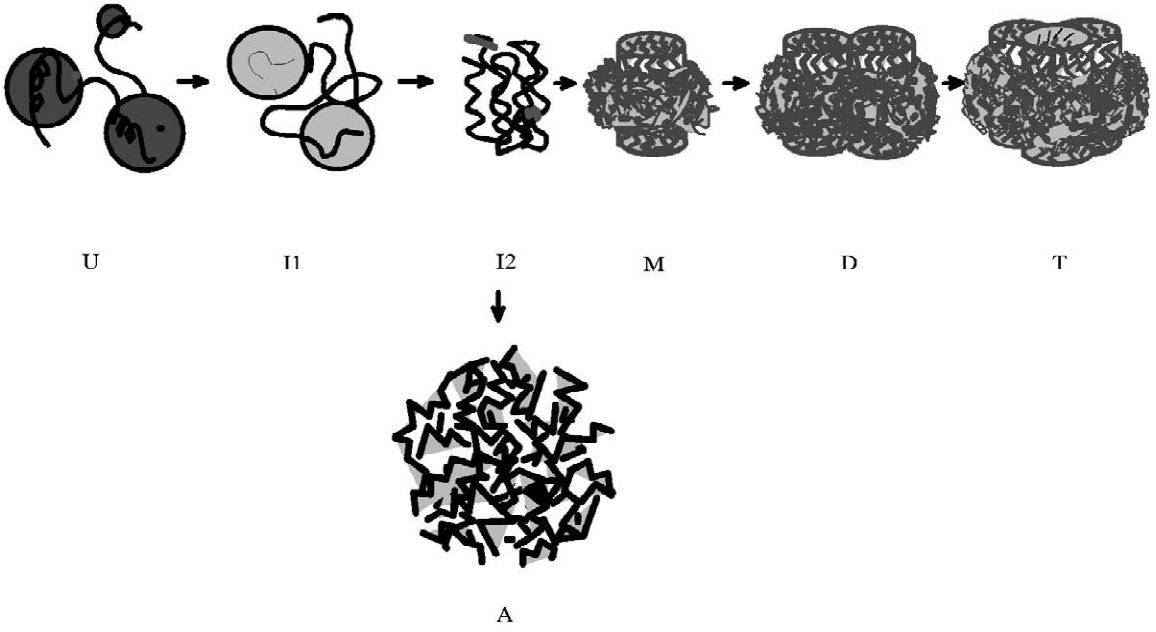


Fig. 7. Hypothetical in vitro folding and assembly pathway of OmpF porin. A denotes incorrect aggregates; D, folded dimer; I1, species unfolded further; I2, partially folded monomer; M, folded monomer assumed to have a β-barrel structure; T, folded trimer; U, denatured (unfolded) OmpF porin monomer. A circular cylinder represents the β-barrel structure of OmpF porin. The size of U and I1 is comparable to that of T.

compact than the denatured monomer, and binds larger amounts of surfactants than native trimeric OmpF porin (Fig. 5). A protein species in the I2 state may have a character of the molten-globule state of soluble proteins [20]. We assume that if a folded and compact monomer (M in Fig. 7) with the β-barrel structure characteristic of OmpF porin is produced, it spontaneously assembles into the SDS-resistant dimer (D in Fig. 7) and then into the SDS-resistant trimer (T in Fig. 7). The dimeric OmpF porin (D in Fig. 7) was suggested to be an intermediate during assembly in intact cells [19], and it was also observed during in vitro assembly from the monomer secreted by spheroplasts of *Escherichia coli* [21].

Fig. 7 also shows the state of surfactants bound to the protein. The model of U state was proposed from neutron scattering data of SDS–protein complexes [22,23]. The complex consists of spherical micelles (dark gray areas) and a polypeptide chain. The chain forms a helical structure in part and is localized in the interior or interface of the SDS micelles. In the I1 state, the complex consists of mixed micelles of SDS and OG (light gray areas) and a polypeptide chain that has a structure unfolded further than the chain structure in the U state. Surfactants bound to the protein species in stage I2 are not shown. In states M, D and T, surfactants interact with the outside areas because the outside of the β-barrel is hydrophobic. Therefore, the forms are not spherical assemblies as shown.

4. Conclusion

Low-angle laser light scattering photometry combined with high-performance gel chromatography is a powerful tool for the study of the folding and assembly of membrane proteins in the presence of surfactants. The present study is the first report on the effective dimerization of denatured monomeric OmpF porin in its refolding in the presence of OG. The method will be useful for further characterization of the intermediate dimer during assembly of this protein. The folding and assembly pathway proposed in this study may reproduce a part of the physiological step of this protein. However, since the signal peptide, as well as other components such as chaperone protein, is expected to affect the targeting of the protein and the kinetics of their folding process, further studies should clarify this point.

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