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Physicochemical Characterization of the Reassembled Dimer of an Integral Membrane Protein OmpF Porin

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The *in vitro* reassembled species of OmpF porin, which was renatured from its denatured monomer using *n*-octyl-β-D-glucopyranoside, was characterized by low-angle laser light scattering photometry, circular dichroism spectroscopy and synchrotron radiation small-angle X-ray scattering measurements. The light scattering measurement reconfirmed that the reassembled species was the dimer of the protein. Circular dichroism spectra of the reassembled dimer showed a native-like β-structure. A small-angle X-ray scattering measurement indicated that the size of the reassembled dimer was nearly equal to that of the native trimer under the present experimental conditions. In a thermal denaturation experiment followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the reassembled dimer was less stable than the native trimer.

KEY WORDS: CD; light scattering; membrane protein; reassembly; SAXS.

1. INTRODUCTION

Although investigating the *in vitro* refolding of hydrophobic membrane proteins is a notoriously difficult task, the folding and assembly problem of oligomeric integral membrane proteins is an important subject in the life and biomaterial sciences. OmpF porin is a trimeric integral membrane protein as a general diffusion pore of the outer membrane of *Escherichia coli*. X-ray analysis revealed that the porin subunit with a molecular weight of 37,000 has a sixteen-stranded β-barrel structure (Cowan *et al.*, 1992). As previously summarized (Buchanan, 1999), the *in vitro* reassembly of the denatured monomer of the OmpF porin into its stable trimer has been investigated. A dialysis method using soybean lecithin and polydisperse octyl-oligoethyleneoxide (not commercially available) was useful for the trimerization of the OmpF porin monomer denatured in 6 *M* guanidine hydrochloride (Eisele and Rosenbusch, 1990). The yields of the refold OmpF porin trimer are improved when the unfolded material is added to mixed lipid-surfactant micelles of dodecylmaltoside and dimyristoylphosphatidylcholine (Surrey *et al.*, 1996).

Recently, we found that mild surfactants such as *n*-octyl-β-D-glucopyranoside (OG) induced a reassembled species that migrated between the denatured monomer and native trimer of the OmpF porin during sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Watanabe, 2002a). Furthermore, the predominant dimerization of this protein was observed in addition to an incorrect aggregation when the denatured OmpF porin was incubated for 24 h in a refolding medium containing 1 mg/ml SDS and 7 mg/ml OG, and then injected into columns equilibrated with the refolding medium (Watanabe, 2002b). The existence of a dimer species

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Abbreviation: CD, circular dichroism; GPC, gel permeation chromatography; MOPS, 3-[N-morpholino]propanesulfonic acid; OG, *n*-octyl-β-sc d-glucopyranoside; SAXS, small-angle X-ray scattering; SDS, sodium dodecyl sulfate; UV, ultraviolet.

in the *in vivo* folding pathway of the OmpF porin has been reported by pulse-chase experiments using immunoprecipitation and SDS-polyacrylamide gel electrophoresis (Reid *et al*., 1988). The dimer band in the gel electrophoresis was also found during the trimerization of an *in vitro* synthesized OmpF porin monomer (Sen and Nikaido, 1990). The dimer species has been considered to be an important assembly intermediate based on the previous studies. However, little is known about the physicochemical properties of the OmpF porin dimer. In this study, therefore, to clarify the characteristics of the dimer, the reassembled species, which was renatured from the denatured monomer using OG, was characterized by low-angle laser light scattering, circular dichroism (CD) spectroscopy and small-angle X-ray scattering (SAXS). The reassembled species was reconfirmed to be the dimer of the protein based on the light scattering measurement. The CD spectra of the dimer show a native-like β-structure. Furthermore, the compactness of the dimer is suggested by the SAXS experiment.

2. MATERIALS AND METHODS

2.1. Chemicals

Sodium dodecyl sulfate (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). All other chemicals were of analytical grade.

2.2. Sample Preparation

OmpF porin was purified from *Escherichia coli* B as previously described (Watanabe and Takagi, 1993). In order to denature the protein, 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 95°C water for 5 min. The sample was then prepared in order to exchange the solvent for a MOPS buffer solution (0.1 *M* sodium chloride, 10 m*M* 3-[N-morpholino] propanesulfonic acid (MOPS), pH 6.9) containing 1 or 2 mg/ml SDS using a gel permeation chromatography (GPC) column (TSK-G3000SWXL column, 300 × 7.8 mm I.D., Tosoh, Tokyo, Japan) equipped with a guard column (TSK-GCSWXL, 40 × 7.8 mm I.D., Tosoh).

The refolding and reassembly of the denatured monomeric porin (0.4 mg/ml) solubilized in 2 mg/ml SDS were performed by adding an equal volume of the MOPS buffer solution (0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9) containing 14 mg/ml OG (Watanabe, 2002a, b). The reaction mixture was incubated for 24 h and then concentrated about 10-fold using a Centricon-YM30 centrifugal filter (Millipore, Bedford, MA, USA). An aqueous solution of the concentrate was applied to a TSK-G3000SWXL column equipped with a guard column of TSK-GCSWXL that have been equilibrated with the MOPS buffer (0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9) containing 8 mg/ml OG. A single protein peak was obtained and the resultant solution was used as a refolded (reassembled) sample for subsequent characterization. The protein concentration was spectrophotometrically determined assuming the absorbance value of a 1% solution at 278 nm for a 1-cm light-path to be 14.1 (Rosenbusch, 1974)

2.3. CD Measurements

The CD spectra were recorded using a J-820 spectropolarimeter (Jasco, Tokyo) at 25°C. Far-ultraviolet (UV) (200–250 nm) and near-UV (250–320 nm) CD spectra were collected with 1 and 5-mm light path quartz cells, respectively. A correction for the base line was made for the buffer solutions. Molar ellipticity, expressed in deg m^2/dmol, was calculated using the mean residues molar mass of 109 g/mol (Chen *et al*., 1979; Inokuchi *et al*., 1982).

2.4. Low-Angle Laser Light Scattering Photometry

This system consists of an HPLC pump system equipped with a sample loop having an internal volume of 200 μl, GPC columns and three sequential detectors, a low-angle laser light scattering photometer (TSK LS-8000, Tosoh), a UV spectrophotometer (TSK UV-8000, Tosoh) and a differential refractometer (TSK RI-8000, Tosoh). The GPC columns were a 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 24°C using a column jacket and a metallic cell holder through which constant temperature water was circulated. The eluent was a

MOPS buffer (0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9) containing 8 mg/ml OG.

Data handling has been described in connection with the molecular weight determination of proteins (Takagi, 1990). In the present experiment, samples are eluted as the complex of a protein and a surfactant. The molecular weight of the protein moiety, M_p, was obtained on the basis of the following equations.

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

$$(dn/dc_p) = k_2 A(RI)(UV)^{-1} \quad (2)$$

where dn/dc_p is the specific refractive index increment expressed in terms of the weight concentration of the protein (c_p); k_1 and k_2 are constants, which depend on the experimental conditions; A is the extinction coefficient at 280 nm (ml/mg protein for 1 cm light-path); and (LS), (UV) and (RI) are the outputs of the low-angle laser light scattering photometer, the UV spectrophotometer and the differential refractometer, respectively. From the above two equations, the molecular weight can be expressed as:

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

where k is a constant. The constant, k, was determined using the following proteins of known molecular weight and extinction coefficients at 280 nm (ml/mg, for a 1 cm light-path): bovine serum albumin (M_p = 66.300 (monomer), 132.600 (dimer), A = 0.678 (Fasman, 1976) and the OmpF porin trimer (M_p = 111,000 (Chen *et al.*, 1979; Inokuchi *et al.*, 1982), A = 1.41 (Rosenbusch, 1974). Bovine serum albumin (1mg) was dissolved in 5 ml of a MOPS buffer (0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9) containing 8 mg/ml OG. The trimeric OmpF porin was prepared in the same MOPS buffer at 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).

2.5. Synchrotron Radiation Small-Angle X-ray Scattering

SAXS measurements were performed with an optics system at the beamline BL-10C station in the Photon Factory of the High Energy Accelerator Research Organization as previously described (Watanabe *et al.*, 1999). A wavelength (λ) of 0.1488 nm was used. The temperature of the cell with a 1-mm light path and a pair of 15 μm-quartz windows was kept constant at 24°C using the metallic cell holder through which constant temperature water was circulated. Data were collected for 20 min with a position sensitive proportional counter at a sample-to-detector distance of 900 mm. The obtained signals were corrected for solvent scattering, sample concentration and normalized to the beam intensity to yield the net scattering intensity $I(q)$, where q ($=4\pi\sin\theta/\lambda$, 2θ is the scattering angle) is the scattering vector. The q-value was calibrated using a diffraction pattern of dried chicken collagen. The solution X-ray scattering measurements of the samples in the presence of SDS were performed using an X-ray scattering spectrometer equipped with RIGAKU R-AXIS IV (imaging plate, 30 cm × 30 cm) at beamline BL-40B2 of SPring8 at the Japan Synchrotron Radiation Research Institute, Harima, Japan. The camera length, X-ray wavelength and sampling time were 1 m, 0.15 nm and 300 s, respectively. Two-dimensional scattering data were circle-averaged about the beam center. The other data-treatment was the same as already described.

2.6. Gel Electrophoresis

A final sample solution contained 0.15 mg/ml protein, 20 mg/ml SDS, 4 mg/ml OG, 0.08 *M* sodium chloride, 5 m*M* EDTA, 10% glycerol and 8 m*M* MOPS, pH 7. The sample solution contained in a sealed glass tube was placed in water of several different temperature (25–95°C) for 5 min. 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 using a mini-sized apparatus (12.5% gel; 60 mm length and 1 mm thickness) at room temperature (24°C) according to the method of Laemmli (Laemmli, 1970). Proteins in the gel were stained with coomassie brilliant blue R-250.

3. RESULTS

3.1. Light Scattering Measurements

Figure 1 shows typical elution curves obtained by using three detectors (the low-angle laser light scattering photometer, UV-spectrophotometer and differential refractometer) when a sample solution containing the reassembled species was injected into the columns. A few peaks were observed at 11–13 min only in the light scattering trace. These peaks

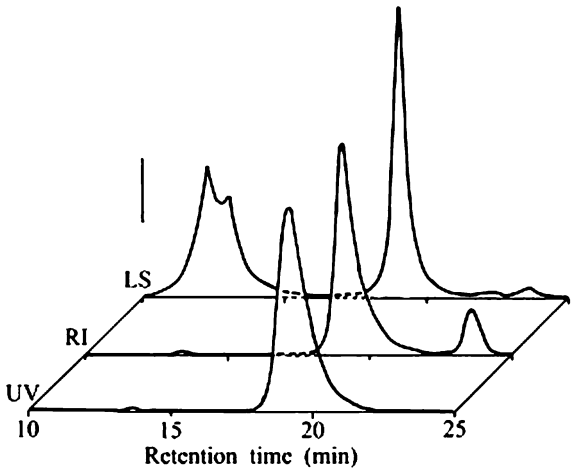

Fig. 1. Typical elution curves obtained by using three detectors, the low-angle laser light scattering photometer (LS), UV-spectrophotometer (UV) and differential refractometer (RI). The gain-settings for these detectors were 32, 0.5 and 64, respectively. The retention time refers to that observed for the light scattering photometer. The retention times for other detectors were corrected for the time lag caused by their sequential arrangement. Flow rate was 0.5 ml/min. A 200 μl of the reassembled sample solution (0.4 mg/ml) was applied to the columns. The length of a vertical bar corresponds to one-tenth of the full-scale for the three detector signals.

are assignable to large particles of which the weight content is negligibly small because the signals from the UV-spectrophotometer and refractometer are very small at the same time. The peak of the reassembled species was observed at 19 min in all three elution curves. The peak at 24 min was assigned to non-protein materials, probably surfactant micelles, because it lacks UV absorption. Figure 2 shows a typical calibration plot of standard proteins and the data of the reassembled species. The molecular weight of the reassembled species was 74,500 ± 500 (n = 5). Moreover, Fig. 3 shows the protein concentration dependence of the apparent molecular weight. The molecular weight extrapolated to zero protein concentration was calculated to be 74,700 ± 600 corresponding to the molecular weight of the dimer of this protein (74,000), which was calculated from its amino acid sequence (Chen *et al.*, 1979; Inokuchi *et al.*, 1982). Figure 3 also shows the plots of the outputs of the refractometer versus protein concentrations for the native trimer and reassembled species.

3.2. CD Spectra

The secondary structure of the reassembled dimer was examined on the basis of the far-UV CD measurements. Figure 4 shows the typical CD patterns of the native trimer and reassembled dimer in the presence of OG and the denatured monomer in SDS. The CD spectrum of the native trimer is rich in the β-structure that is characterized by a minimum (about −8,000 deg cm^2/dmol) near 217 nm and a crossover point near 207 nm. The CD spectrum of the reassembled dimer was similar to that of the native trimer, while the minimal ellipticity at 217 nm is slightly decreased to about −7000 deg cm^2/dmol. The CD spectrum of the denatured monomer in SDS is characterized by a minimum at

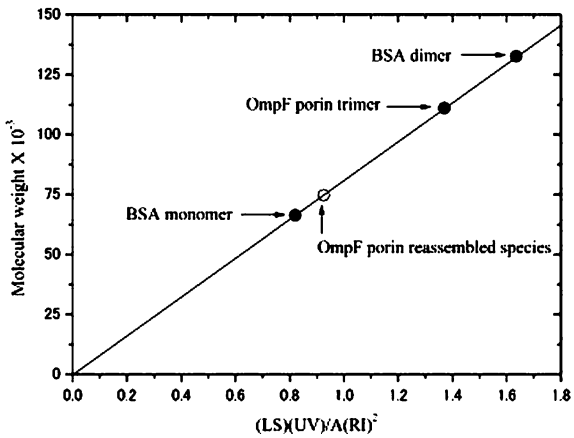

Fig. 2. Relationship between the value of A^{-1}(LS)(UV)(RI)$^{-2}$ and molecular weight of the proteins. The open circle shows the data point at the peak of 19 min for five independent experiments. Error bars are within the symbols.

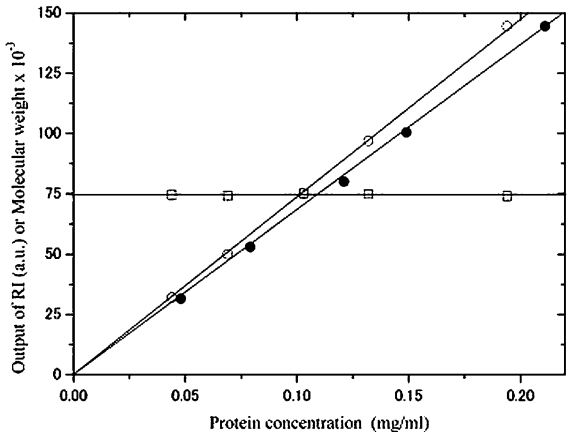

Fig. 3. Protein concentration dependence of the apparent molecular weight of the reassembled species (□) and the outputs of the differential refractometer for the native trimer (●) and the reassembled species (○).

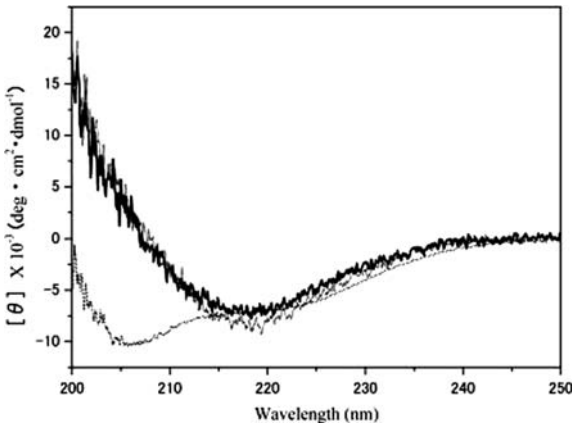

Fig. 4. Far-UV-CD spectra of the native trimer (—), reassembled dimer (—) and denatured monomer (----) The protein concentration was 0.77 mg/ml. The native and reassembled proteins were in 8 mg/ml OG, 0.1 *M* sodium chloride, 10 m*M* MOPS, pH 6.9. The denatured protein was in the same buffer containing 1 mg/ml SDS instead of 8 mg/ml OG.

207 nm and a shoulder at 222 nm, as previously described (Watanabe, 2002b).

Figure 5 shows the near-UV CD spectra under the same conditions as that of Fig. 4. The near-UV CD spectrum of the native trimer shows a form with positive signals below 270 nm and twin peaks near 280 and 290 nm. The near-UV CD spectrum of the reassembled dimer was similar to that of the native trimer while the signal of the peaks is smaller than that of the native trimer. On the other hand, the near-UV CD spectrum of the denatured monomer in SDS represents negative signals as shown by the dashed line in Fig. 5.

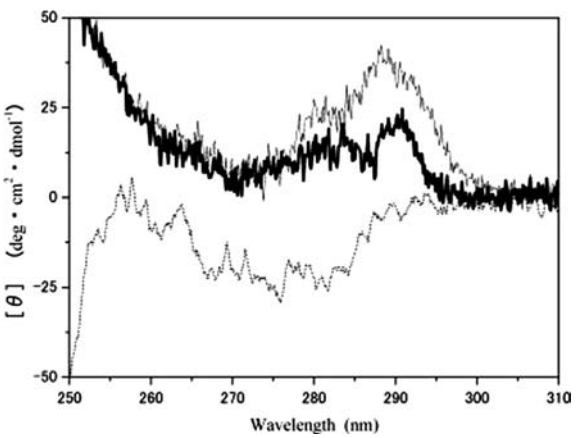

Fig. 5 Near-UV-CD spectra. The other conditions were the same as those listed in Fig. 4.

3.3. SAXS Measurements

Figure 6 shows the solution X-ray scattering profiles (q versus ln$I$, $q = 4\pi\sin\theta/\lambda$ (λ is X-ray wave length, 2θ is the scattering angle), I is scattering intensity) of the native trimer and reassembled dimer in the presence of OG. The scattering curves were analyzed using the Guinier approach (Guinier and Fournet, 1955). The low q-region of the scattering curves was approximated by $I(q) = I(0)\exp(-q^2R_g^2/3)$, where $I(0)$ is the scattering intensity at zero scattering angle and R_g is the radius of gyration. Thus, the plot of ln$I(q)$ versus q^2 (a Guinier plot) yields the straight line, the slope of which provides an estimation of the R_g value. Figure 7 shows an example of the Guinier plots of the SAXS data of the native trimer and reassembled dimer in the presence of OG. The curves in the region of $q^2 < 0.12$ nm^{-2} were well approximated by a straight line while the low-q limit of the Guinier rule ($qR_g < 1$) was $q^2 < 0.06$ nm^{-2}. No clear sign of significant aggregation, as would be indicated by an upward deviation from the straight-line fit in the small-angle region, is observed. The R_g values were estimated from the data in the region of q from 0.13 to 0.23 nm^{-1}. The R_g values of the native trimer and reassembled dimer were calculated to be 4.1 ± 0.1 and 4.2 ± 0.1 nm, respectively. The zero-angle scattering intensity was estimated to be 105,000 for the native trimer and 68,000 for the reassembled dimer under this condition. The molecular weight of the reassembled dimer was calculated to be 72,000 from

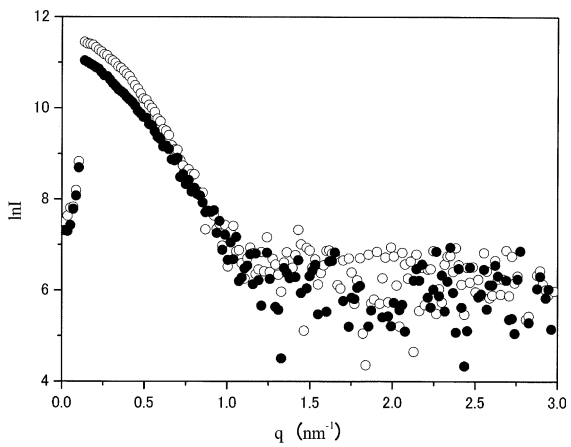

Fig. 6. An example of the scattering curves for the native trimer (○) and reassembled dimer (●) of the OmpF porin. The protein concentration was 0.8 mg/ml. The abrupt decrease in the scattering intensity below $q = 0.12$ nm^{-1} results from the beam stopper.

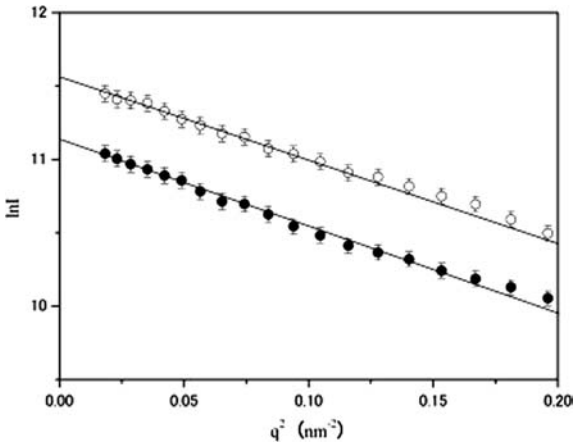

Fig. 7. Guinier plots (q^2 versus ln$I$) of SAXS data. The other conditions were the same as those listed in Fig. 6.

the zero-angle scattering intensities. Figure 8 shows the plots of q^2I versus q (Kratky plots; Kratky, 1982) in a few states of the protein.

3.4. Stability of the Reassembled Dimer

The reassembled dimer of the OmpF porin is resistant to the denaturing action of sodium dodecyl sulfate (SDS) at room temperature (Watanabe, 2002a and b), as has been shown for the native trimer (Rosenbusch, 1974). The oligomers are dissociated into the denatured monomer by heat-treatment in the presence of SDS. The denatured monomer migrates faster than the dimer and trimer in a gel

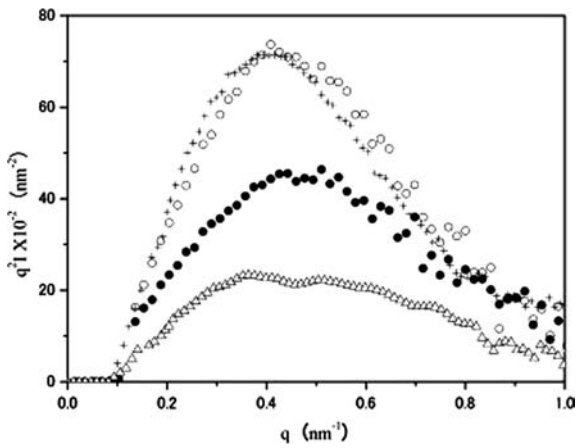

Fig. 8. Kratky plots (q versus q^2I) of SAXS data. Conditions in the presence of OG were the same as those listed in Fig. 6. The data of the native trimer and denatured monomer in the presence of SDS are also shown as crosses (+) and triangles (△), respectively. In these cases, the protein concentration was 0.3 mg/ml. The y axis of the data in SDS was normalized to that of the data in OG.

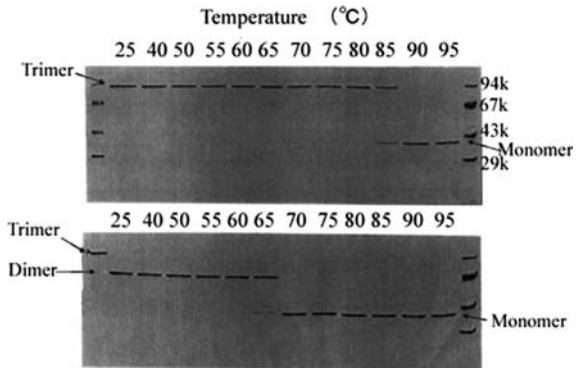

Fig. 9. Thermal denaturation followed by SDS-polyacrylamide gel electrophoresis.

during SDS-polyacrylamide gel electrophoresis. Therefore, the heat-stabilities of the native trimer and reassembled dimer were monitored by SDS-polyacrylamide gel electrophoresis. Figure 9 shows the effect of temperature on the dissociation of the native trimer and reassembled dimer in excess SDS. The native trimer migrated at the position of 94,000 and the reassembled dimer band was observed in the middle between two bands assignable to the trimer and denatured monomer of the OmpF porin under these experimental conditions. The native trimer and reassembled dimer were dissociated in the denatured monomer (Mw:37,000) above 85 and 65 °C, respectively.

4. DISCUSSION

In this study, we investigated the physicochemical properties of the reassembled species of the OmpF porin that was renatured from its denatured monomer using OG. Low-angle laser light scattering photometry combined with gel chromatography is a reliable method for the determination of the molecular weight of a membrane protein as previously described (Watanabe, 1993). Therefore, the reassembly of the OmpF porin in the presence of SDS and OG has been characterized by this method (Watanabe, 2002b). In a previous study, the aggregation of this protein was observed in a refolding medium containing 1 mg/ml SDS and 7 mg/ml OG. In the present study, however, we found that the incorrect protein peak almost disappeared in a chromatogram when denatured OmpF porin was incubated for 24 h in a refolding medium containing 1 mg/ml SDS and 7 mg/ml OG, and then injected into columns equilibrated with a buffer

solution containing 8 mg/ml OG. The incorrect aggregates will be adsorbed on the column matrix. An example of the chromatograms of the isolated sample containing only the reassembled species is shown in Fig. 1. The light scattering measurements reveal that the reassembled species is the dimer of the OmpF porin (Figs. 2 and 3). On the other hand, the output ratio of the refractometer versus the protein concentrations corresponds to the specific refractive index increment, as described in Eq. (2) (see section 2.2). The output ratio of the refractometer for the dimer was greater than that of the native trimer (Fig. 3). This result indicates that the specific refractive index increment of the reassembled dimer is greater than that of the native trimer, therefore, the dimer binds larger amounts of surfactants than the native trimer. In the presence of SDS and OG, a similar observation was also obtained in a previous study (Watanabe, 2002b).

The far-UV CD spectrum clearly indicates the native-like β-structure of the reassembled dimer of the OmpF porin (Fig. 4). The intact dimer of the OmpC porin has produced a similar observation for the far-UV CD spectrum while the signal at 217 nm of the dimer was about 70% of that of the native trimer (Rocque and McGroarty, 1989). They have also shown that the dimer of the OmpC porin might represent the smallest functional, channel-forming aggregate. On the other hand, the near-UV CD spectrum of the native OmpF porin shows a form with a positive signal below 270 nm and twin peaks near 280 and 290 nm (Fig. 5). The OmpF porin contains 2 tryptophan, 29 tyrosine and 19 phenylalanine residues in the polypeptide chain (Chen *et al*., 1979; Inokuchi *et al*., 1982). Furthermore, the crystal structure of the OmpF porin showed that the two tryptophan residues exist on the external side of the third and tenth β-strands of the barrel, and the tyrosine and phenylalanine residues are distributed all over the barrel and the loops (Cowan et al., 1992). Since the near-UV region originates from these aromatic amino acid residues, the CD spectrum represents a probe of the protein conformation. Although the ellipticity of the reassembled dimer at 280–290 nm is smaller than that of the native trimer, the near-UV CD spectrum of the reassembled dimer is very similar to that of the native trimer, and clearly different from that of the denatured monomer, which shows only negative signals (Fig. 5). Thus, the above observations suggest that the reassembled dimer consists of a similar β-barrel structure to that of the native trimer.

A SAXS measurement reveals that the size of the reassembled dimer is nearly equal to that of the native trimer (Fig. 7). This result is consistent with the previous observation that the reassembled dimer was eluted at a similar time as that of the native trimer during gel chromatography in the presence of SDS and OG (Watanabe, 2002b). The slightly large size of the reassembled dimer versus the native trimer will correspond to the surfactant binding to the protein because the reassembled dimer binds larger amounts of surfactants than the native trimer, as described about Fig. 3. These observations suggest the compactness of the reassembled dimer. Since the zero-angle scattering intensity is related to the molecular weight of solutes in solution, the present result is consistent with the results of the light scattering measurements described above. The Kratky plot (Fig. 8) is related to the protein configuration (Kratky, 1982). The peak around $q = 0.4\ \mathrm{nm}^{-1}$ is typical for globular molecules. The peak-height of the reassembled dimer is two-thirds that of native trimer in the presence of OG. As a reference, the peak-height of the denatured monomer is one-third that of the native trimer in the presence of SDS. In these cases, the Kratky plot may be more sensitive than the Guinier plots (Fig. 7) to the changes in the protein molecular assembly of this protein. Therefore, the Kratky plots will be useful for a study of the reassembly process of the OmpF porin in the future. A heat-stability experiment showed that the reassembled dimer was less stable and denatured at 65 °C compared to 85 °C for the native trimer (Fig. 9). The present result reveals that oligomerization contributes to the thermostability of the OmpF porin. Pulse-chase experiments also showed that a metastable 50-kDa dimer was an intermediate in the assembly pathway of the OmpF porin in growing cells (Reid *et al*., 1988).

In conclusion, the *in vitro* reassembled species of the OmpF porin, which was renatured from the SDS-denatured monomer using OG (Watanabe, 2002a and b), was characterized in detail by physicochemical methods such as light scattering, CD and SAXS. The present results show that the reassembled species is a metastable compact dimer that consists of a native-like β-structure.

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