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Characterization of further association of the trimeric membrane protein porin by low-angle laser-light scattering photometry coupled with high-performance gel chromatography

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ABSTRACT

Porin (OmpF), a trimeric membrane protein, in an extract of the outer membrane of *Escherichia coli* gave a twin-peaked elution pattern on Sephacryl S-300HR gel chromatography in the presence of sodium dodecyl sulphate. The species eluting earlier and later were found to be the hexamer and trimer, respectively, from molecular mass determination by low-angle laser-light scattering photometry coupled with TSK-G3000SWXL gel chromatography. As the hexamer was dissociated into the trimer under the conditions of sodium dodecyl sulphate polyacrylamide gel electrophoresis, its presence had been overlooked. The addition of lipopolysaccharide, another component of the outer membrane, and subsequent dialysis induced association of the trimer, the product containing an appreciable amount of the hexamer.

INTRODUCTION

Pores formed by the outer membrane protein of *Escherichia coli*, porin (OmpF), allow the restricted passage of hydrophilic substances with molecular masses of less than about 600 [1]. Porin is a trimeric integral membrane protein and is highly resistant to proteases and surfactants such as sodium dodecyl sulphate (SDS). Recent X-ray analysis revealed that the porin subunit with a molecular mass of 37 000 [2,3] has a sixteen-stranded β-barrel structure [4]. Lipopolysaccharide (LPS) is another predominant component of the outer membrane, and binds tightly to porin [1]. The formation of a channel requires the presence of both LPS and further association of the trimer [5]. The product of such an association has not been well characterized with respect to the molecular mass.

In the course of the isolation of porin, we extracted it from the outer membrane of *Escherichia coli* and found that the preparation gave a twin-peaked elution curve on Sephacryl S-300HR gel chromatography. Yamada and Mizushima [6] observed a similar mode of elution from a Sephadex G-200 column. These observations suggested that the extract contains two species of porin differing in the degree of association. The latter group, however, could only detect trimeric porin for both the peaks on SDS polyacrylamide gel electrophoresis (PAGE) [6]. In this study, the molecular masses of the protein species giving the peaks were determined in the same medium as that for the Sephacryl gel chromatography by low-angle laser-light scattering photometry coupled with high-performance gel chromatography. This technique is suitable for the molecular mass determination of the

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protein moiety of a membrane protein solubilized by a surfactant [7].

The first and second peaks could be assigned to the hexamer and trimer of porin, respectively, by the light scattering technique. The hexamer was found to dissociate into the trimer under the conditions of SDS-PAGE. The addition of LPS to the purified trimer was found to give a further associated species comprising the hexamer.

EXPERIMENTAL

SDS was purchased from BDH (Poole, UK) and LPS (type B055) from Sigma (St. Louis, MO, USA). Other chemicals were of analytical-reagent grade.

Porin was isolated from *Escherichia coli* B specifically producing porin of the OmpF type by the following modification of the method reported previously [8]. Wet bacterial cells (80 g) were suspended in 240 ml of 50 m*M* Tris–HCl buffer (pH 7.6) containing 0.1% mercaptoethanol. The suspension was then sonicated twice for 10 min with an interval of 5 min at below −10°C. The cell wall debris was collected by centrifugation at 27 000 *g* for 110 min, dispersed in 0.01 *M* Tris–HCl buffer (pH 7.5) and re-collected by centrifugation at 80 000 *g* for 60 min. The cell envelope obtained as a pellet was stored at −80°C until used.

The cell envelope (15 g) was washed with 320 ml of 2% SDS at 30°C for 30 min to remove peripheral proteins and then collected by centrifugation at 80 000 *g* for 30 min at 20°C. The pellet was washed three times by suspension in water and subsequently collected by centrifugation at 80 000 *g* for 30 min at room temperature. The pellet was suspended in 320 ml of 50 m*M* Tris–HCl buffer (pH 7.6) containing 1% SDS, 5 m*M* EDTA and 0.03% sodium azide and then left overnight at 37°C. The porin thus obtained is in the form of a complex with the peptidoglycan matrix. The complex was collected by centrifugation at 80 000 *g* for 60 min at 20°C and then washed twice by centrifugation as a suspension in water at 80 000 *g* for 60 min at 20°C.

Porin was removed from the matrix by suspending the complex in 100 ml of 15 m*M* Tris–HCl buffer (pH 7.6) containing 0.5 *M* NaCl, 5 m*M* EDTA and 1% SDS. The insoluble material was removed by centrifugation at 80 000 *g* for 90 min after allowing the extract to stand overnight with stirring, and the supernatant was concentrated about tenfold using a PM-30 Amicon ultrafilter at room temperature. The concentrate was applied to a Sephacryl S-300HR (Pharmacia, Uppsala, Sweden) column (details are given in the legend to Fig. 1).

The fraction rich in trimeric porin obtained by the above-mentioned gel chromatography was further purified as follows, and used as the starting material in experiments carried out to examine the potential of the trimer for further association. The trimer-rich fraction was further purified by hydroxyapatite chromatography in the presence of SDS according to the procedure described previously [9]. A tandem array of a guard column (type A-4001) and a main column (HCA column type A-7610; 100 mm × 4.0 mm I.D.), both from Mitsui-Toatsu Chemicals (Tokyo, Japan) (now distributed by Koken, Tokyo, Japan), was used. The columns were equilibrated with 50 m*M* sodium phosphate buffer (pH 6.9) containing 0.1% SDS and 0.01% sodium azide. The sample solution (15 ml) was applied to the columns after dialysis against the above buffer. After passage of the starting buffer for 15 min, the concentration of phosphate in the buffer was increased linearly to 500 m*M* over 25 min at a flow-rate of 1.0 ml/min, the concentrations of other components being kept constant. Fractions containing porin were collected and concentrated about fivefold with a PM-30 Amicon ultrafilter and then applied to a TSK-G3000SW column (600 mm × 21.6 mm I.D.) (Tosoh, Tokyo, Japan) equipped with a guard column of TSK-GCSW (50 mm × 21.6 mm; I.D.) (Tosoh) equilibrated with 50 m*M* sodium phosphate buffer (pH 6.9) containing 0.1% SDS and 0.01% sodium azide. The temperature and flow-rate were 25°C and 2.0 ml/min, respectively. Porin was eluted as a single peak and the peak fraction was used as the stock solution of trimeric porin. The concentration of porin was determined spectrophotometrically assuming the absorbance value of a 1% solution at 278 nm for a 1-cm light path to be 14.1 [10].

The molecular mass was determined by moni-

toring elution from high-performance gel chromatographic columns using a measuring system for molecular mass determination. The solvent delivery system was the same as that described previously [7], and equipped with a sample loop with an internal volume of 500 μl. The columns and detectors were products of Tosoh and were arranged along the flow-line in the following sequence: guard column of TSK-GEL GCSWXL (40 mm × 6.0 mm I.D.), two tandem-linked TSK-GEL G3000SWXL columns (300 mm × 7.8 mm I.D.), low-angle laser-light scattering photometer (LS-8000), UV spectrophotometer (UV-8000) and differential refractometer (RI-8000). The temperature of the columns and the flow-through cell in the low-angle laser-light scattering photometer was kept constant at 25°C using a column jacket and a metallic cell holder through which water of constant temperature was circulated.

The molecular mass of the protein moiety of a surfactant–protein complex, M_r, is related to the signal intensities from the three detectors mentioned above according to the following equation [7]:

$$M_r = kE^{-1}(\text{Output})_{LS}(\text{Output})_{UV}(\text{Output})_{RI}^{-2} \quad (1)$$

where k is an instrumental constant, E the specific absorptivity at 280 nm (ml/mg, for a 1-cm light path) and (Output) the output of the respective detectors specified by the subscripts LS (low-angle laser-light scattering photometer), UV (spectrophotometer) and RI (differential refractometer). The retention times refer to those observed for the light scattering photometer. The actual retention times observed for the other detectors were corrected for the time lag due to their sequential arrangement.

The constant k was determined by using the following proteins of known molecular masses and absorptivities: bovine carbonic anhydrase ($M_r = 29\,000$, $E = 1.9$ [11]), bovine serum albumin ($M_r = 66\,300$, $E = 0.678$ [11]) and heat-treated porin monomer and purified trimer {$M_r = 37\,000$ (monomer), 111 000 (trimer) [2,3] $E = 1.38$ [10]}.

SDS-PAGE was performed with a mini-sized apparatus (12.5% gel; 60 mm long and 1 mm thick) at 6°C according to the method of Laemmli [12].

LPS was determined through the determination of 2-keto-3-deoxyoctonic acid by the thiobarbituric acid method described by Osborn *et al.* [13].

RESULTS

For the purification of porin, it was extracted from the complex between the protein and the peptidoglycan matrix with a buffer solution containing 0.5 *M* sodium chloride, 5 m*M* EDTA and 1% SDS. As shown in Fig. 1, when the crude extract was applied to a Sephacryl S-300HR

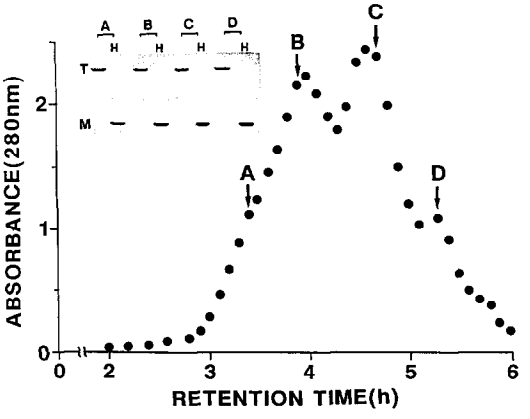

Fig. 1. Typical elution pattern of the crude extract of porin in the presence of SDS. A 10-ml aliquot of the extract from 15 g of wet cell envelope fraction was applied to a Sephacryl S-300HR column (85 cm × 2.6 cm I.D.) equilibrated with 50 m*M* sodium phosphate buffer (pH 6.9) containing 0.1% SDS, 5 m*M* EDTA and 0.03% sodium azide at a flow-rate of 1.1 ml/min. The eluate was collected in 7-ml fractions and monitored for absorbance at 280 nm with a cell of 1-cm light path. The fractions designated A, B, C and D were analysed by SDS-PAGE as described below, and the electrophoretic patterns obtained are shown in the inset. Each of the fractions was diluted with the elution buffer to give an absorbance of 0.3 at 280 nm for a 1-cm light path. A 60-μl volume of each of the diluted solutions was mixed with 20 μl of an aqueous solution with the composition 8% SDS, 20 m*M* EDTA and 40% glycerol. A 10-μl volume of each of the solutions was applied to the unmarked lanes. Each of the lanes marked H was loaded with 10 μl of the respective sample heated at 95°C for 5 min. The protein was stained with Coomassie Brilliant Blue R-250. T and M are the positions of the bands expected for the intact trimer and denatured monomer of porin, respectively.

column after concentration, a twin-peaked elution profile was observed in the frontal region.

Four fractions were chosen from those from the Sephacryl column, and designated A, B, C and D, as shown in Fig. 1. They were analysed by SDS-PAGE for the detection of porin and possible contaminating proteins. The electrophoretic patterns obtained are included as an inset in Fig. 1. For fractions A, B and C, only a single protein band was observed. Only for fraction D, another protein band with a faster electrophoretic mobility was detected in addition to the band described above (not shown). The mobilities of the commonly observed bands were significantly enhanced on heating. This heat sensitivity is the so-called heat modifiability characteristic of porin. The results obtained by electrophoresis indicate that fractions A, B and C contain only the porin trimer.

To examine further the molecular assembly in the fractions A, B and C, they were analysed by the light scattering technique coupled with a tandem array of two TSK-G3000SWXL columns in the same medium. Elution from the columns was monitored with a low-angle laser-light scattering photometer, a UV spectrophotometer and a differential refractometer. The three sets of elution curves are shown in Fig. 2, together with the change in the molecular mass of porin with retention time in the peak region. In contrast to the conclusion obtained from SDS-PAGE, none of the fractions was dominated by the porin trimer. Moreover, they differed significantly with regard to the profile of the change in molecular mass. The molecular mass observed for fraction A was found to start from more than 400 000 and to decrease rapidly.

Fraction A is clearly rich in a highly associated species. The molecular mass profiles for both fractions B and C show plateaux at about 200 000–210 000 and about 110 000, respectively. These values correspond roughly to molecular masses of six and three times that for the subunit molecular mass ($M_r = 37\,000$) [2,3] of porin, respectively. Clearly, porin in the extract from the outer membrane matrix is highly heterogeneous as to the degree of association. To summarize, a highly associated species is eluted in the frontal region and the porin hexamer and trimer

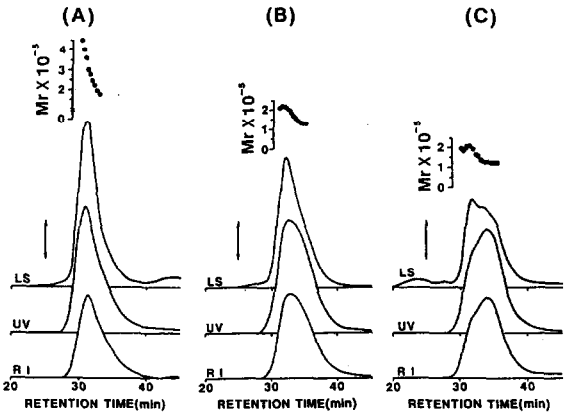

Fig. 2. Characterization of the fractions obtained on Sephacryl gel chromatography (Fig. 1) with respect to molecular mass. (A), (B) and (C) show the sets of elution patterns obtained with the measuring system for molecular mass determination described under Experimental and the molecular masses (●) determined according to eqn. 1. LS, UV and RI are as in eqn. 1. The gain settings of the detectors were 32, 0.16 and 32, in the order given above. A 500-μl volume of each of the solutions with an absorbance of 0.3 prepared as described in Fig. 1 was applied to TSK-G3000SWXL columns in the system. The same buffer solution as described in Fig. 1 was used for equilibration and elution (0.4 ml/min). The length of the vertical bar with arrowheads corresponds to one tenth of the full scale for the respective detector.

are dominant in the first and second peaks, respectively. Rechromatography of the trimer fraction gave only the trimer peak, but that of the hexamer fraction gave a trimer peak corresponding to a few per cent of the total area.

In addition, the amounts of LPS bound to porin were estimated to be 0.6, 8 and 18 mg/g protein for fractions A, B and C, respectively. Assuming the molecular mass of LPS to be 4300 [6] and that all proteins in the fractions are porin, the molar ratios of LPS to the porin trimer were calculated to be 0.02, 0.2 and 0.5 mol/mol of the trimer, respectively.

Fraction B was subjected to harsh conditions and then analysed with the measuring system for molecular mass to examine the stability of the hexamer. Incubation with 8 *M* urea for 24 h at room temperature failed to dissociate the hexamer. Neither 10 m*M* EDTA nor 0.5 *M* sodium chloride was effective. Conversion to the trimer was only observed when the fraction was heated at 55°C for 30 min in the presence of 1% SDS.

These results indicate that the hexamer is fairly stable.

Starting from the porin trimer purified by hydroxyapatite chromatography, we tried to convert the trimer into the hexamer. As LPS has been suggested to play a crucial role in the oligomerization of the porin trimer [1,5], it was incubated with LPS in the presence of SDS. As shown in Fig. 3A, incubation with LPS for 120 h induced association of the trimer only slightly, as is suggested by the small shoulder peak in the light scattering trace and the upward shift of the molecular mass profile in the frontal region. It should be noted, however, that the retention time of the sample prepared with LPS was significantly shorter than that of the purified trimer used as the starting material (Fig. 3A). This result suggests that the porin trimer binds with LPS, and consequently its molecular size increases.

The presence of SDS was presumed to be responsible for the inefficient conversion to the hexamer. SDS was, therefore, removed from the sample solution by dialysis (for details, see the legend to Fig. 3). As shown in Fig. 3B, extensive aggregation of the trimer was observed. A shoulder was observed in the molecular mass profile, indicating that the trimer could be partially converted into the hexamer.

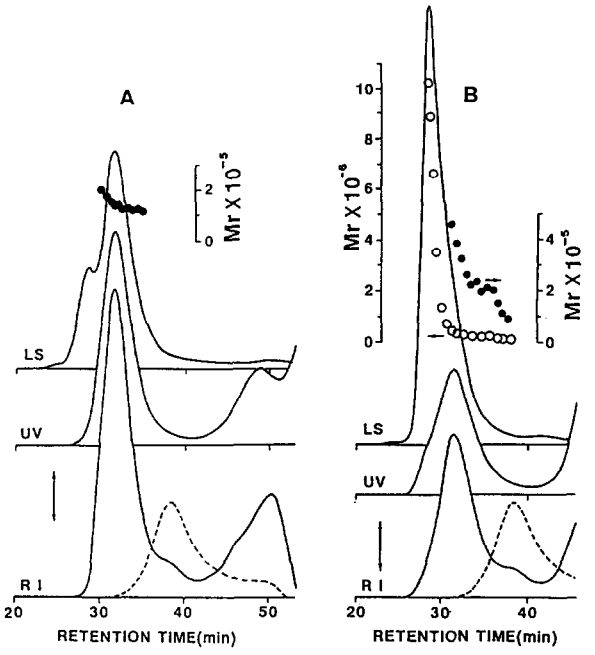

Fig. 3. Elution patterns obtained on TSK-G3000SWXL gel chromatography carried out for examination of the effectiveness of attempts at the preparation of the hexamer from the purified trimer. Two sets of elution patterns and molecular masses (circles), like those in Fig. 2, were obtained for samples prepared as follows. (A) A 600-μl volume of the purified trimer solution (0.4 mg/ml) in 50 m*M* sodium phosphate buffer (pH 6.9) containing 0.1% SDS and 0.01% sodium azide was mixed with an equal volume of an LPS solution (1.2 mg/ml) in the same buffer as above. After standing for 120 h at 25°C, 500 μl of the mixture were applied to the same measuring system as in Fig. 2, except for the absence of EDTA. The elution conditions were the same as those in Fig. 2. The gain settings of the detectors were 16 for LS, 0.16 for UV and 32 for RI. (B) A mixture was prepared in the same manner as described above and then dialysed against 10 m*M* sodium phosphate buffer (pH 7.0) for 24 h. As the solution became turbid, 50 μl of 2% SDS in the 50 m*M* buffer mentioned above were added to 450 μl of the suspension to clarify it. After standing overnight at room temperature, the solution obtained was applied to the same system. The gain settings of the detectors were 8 for LS, 0.16 for UV and 32 for RI. Note that the scale for the molecular masses shown by the open circles is different from that for the closed circles. Other conditions were the same as in (A). In both (A) and (B), the broken line in the refractive index (RI) trace indicates the elution pattern obtained for the purified porin trimer used as the starting material.

DISCUSSION

Yamada and Mizushima [6] obtained a twin-peaked elution curve similar to that in Fig. 1 on Sephadex G-200 chromatography of a crude preparation of porin. They collected the eluate in the frontal region and used it in reconstruction of the membrane with a lattice structure in which LPS is involved. The porin used in their study was assumed to exist as a trimer. They presumed that a difference in the amount of LPS produced the twin-peaked elution curve. In this study, however, the species in the frontal region on Sephacryl chromatography was found by light scattering photometry coupled with high-performance gel chromatography to be an associated form of the porin trimer, as shown in Fig. 2. Our results for the amounts of LPS bound are in agreement with those obtained by Yamada and Mizushima. As the amounts of LPS bound are very small (see Results), the observation of the associated species suggests that the trimers are

directly assembled with each other in such an associated species.

The results obtained with the light scattering technique contradict the observation that the hexamer is not detected on SDS-PAGE. SDS-PAGE according to the method of Weber and Osborn [14], in which a sodium phosphate buffer is used, also reportedly failed to detect the hexamer [6], and we found this to be reproducible (not shown). There are significant differences in the conditions between the two separation techniques with regard to both the medium conditions and the mechanism of separation. We have no immediate explanation for the discrepancy, however. In any case, the present results indicate that the porin trimer can associate to form the hexamer, and the stability of the product is comparable to but slightly lower than that of the trimer. The trimer could be converted into the hexamer in a low yield, as shown in Fig. 3.

Schindler and Rosenbusch [5] reported that the presence of LPS and the association of the porin trimer are necessary for the formation of stable channels in a lipid bilayer. Our results together with theirs strongly suggest that porin exists not as a trimer but as a further associated form in the outer membrane. In this study, a major product of such an associated species was found to be the porin hexamer even in the presence of SDS. This finding was only possible using the measuring system for molecular mass determination in which a light scattering photometer plays a pivotal role. The technique allows the unique determination of the molecular mass of a protein moiety without reference to the retention time of a sample, as shown in Fig. 3A. Further studies on the relationship between the channel activity and the well characterized associated state of porin trimers will lead to a better understanding of the molecular organization and functions of the bacterial outer membrane.

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