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Separation of gate- and channel-forming domains in the pore-forming protein of the outer membrane of *Pseudomonas aeruginosa*

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The domains of the pore-forming protein responsible for the gate and channel formations were separated and identified in the outer membrane of *Pseudomonas aeruginosa*. The proteolytic cleavage of the 46K channel protein, protein D2, yielded two major domains with apparent M_r of 27K and 19K. We identified the 27K polypeptide to be the channel-forming domain by an in vitro permeability assay. The channel size of purified 27K domain was indistinguishable from that of native protein D2. Degradation of the 19K domain into small subfragments increases the channel activity about ten times suggesting that the 19K polypeptide forms the gate or cap.

Channel; Gate; Outer membrane; Porin; *Pseudomonas aeruginosa*

1. INTRODUCTION

The outer membrane of *Pseudomonas aeruginosa* contains 3 species of porins, proteins C, D2 and E1 (OprC, OprD and OprE), forming small pores when compared to the *Escherichia coli* porin pore [1]. Protein F was reportedly a porin forming very large, yet dreadfully inefficient pores [2]. Probably because the outer membrane of *P. aeruginosa* contains small pores or less likely, large yet inefficient pores, the organism is highly resistant to many structurally diverse antibiotics. Imipenem, a small carbapenem antibiotic (M_r 299) is a powerful drug against *P. aeruginosa*. The imipenem-resistant *P. aeruginosa* produces reduced amounts of OprD [3] and the introduction of cloned *opr*D into *opr*D-negative hosts recovered imipenem susceptibility [4]. It was thus demonstrated that imipenem penetrates the *P. aeruginosa* outer membrane mainly through the OprD pore.

OprD showed two functions: (i) the protein allowed less specific diffusion of saccharides with M_r less than about 300 to 400 [1]; (ii) the protein has specificity towards the diffusion of carbapenem antibiotics and basic amino acids [5]. OprD forms channels by trimeric aggregates of identical subunits with M_r of 46,000 [6]. In this study, OprD was enzymatically dissected into two major domains. One of the domains showed channel forming activity and another domain was responsible for gate or cap formation.

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2. MATERIALS AND METHODS

2.1. *Purification of OprD*

OprD was purified from *P. aeruginosa* PA01 or KG1079 as described previously [1].

2.2. *Trypsin treatment*

In a typical experiment, purified OprD (33 μg) was mixed with 3.3 or 33 μg of trypsin in 100 μl of octaethyleneglycol dodecyl ether ($C_{12}E_8$) buffer at 23°C for desired times.

2.3. *Reconstitution of proteoliposome and the permeability assay*

These were performed by the procedures described earlier [1].

2.4. *SDS-acrylamide gel electrophoresis*

Acrylamide gel electrophoresis in the presence of SDS was carried out by the procedure described by Laemmli [7].

2.5. *Materials*

L-Tosylamino-2-phenylethylchromomethylketone-treated trypsin, egg yolk phosphatidyl choline and diacetylphosphate were purchased from Sigma. $C_{12}E_8$ and β-octylglucoside (β-OG) were obtained from Nikko Chemicals and Dojin Chemicals, respectively. Imipenem was obtained from Banyu Pharmaceuticals. All other chemicals used were of the highest purity commercially available.

3. RESULTS AND DISCUSSION

To characterize the OprD structure, the trimer was subjected to limited proteolysis in the non-ionic surfactant $C_{12}E_8$ solution and in the reconstituted membrane. The results showed that trypsin quantitatively cleaved OprD into two major fragments with an apparent M_r of about 27K and 19K (Fig. 1a). Large amounts of trypsin and prolonged incubation caused further fragmentation of the 19K domain into two subfragments with apparent M_r of roughly 12K and 8K (Fig. 1a). The trypsin susceptibility of the OprD reconstituted into li-

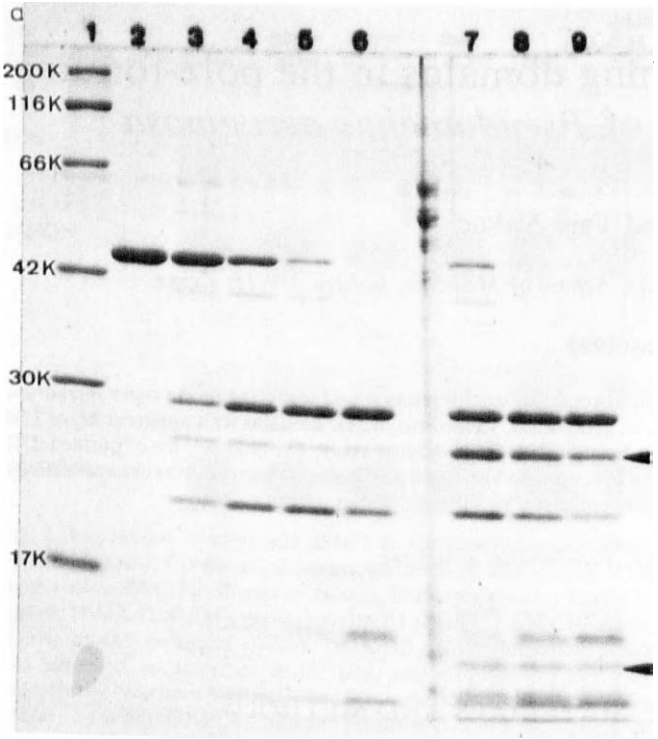

Fig. 1. (a) Trypsin susceptibility of OprD. Purified OprD (33 μg) was mixed with 3.3 or 33 μg of trypsin in 100 μl of $C_{12}E_8$ buffer and incubated at 23°C for the desired times. Aliquots of the sample (10 μl) were mixed with 10 μl of the electrophoresis sample buffer, heated at 95°C for 5 min and subjected to SDS-PAGE (13.5% gel). Lane 1, M_r marker (Daiichi Pure Chemicals); lane 2, untreated OprD; lanes 3 to 6, OprD treated with 3.3 μg of trypsin for 0.5 (lane 3), 2 (lane 4), 5 (lane 5) and 24 h (lande 6); lane 7 to 9, OprD treated with 33 μg of trypsin for 0.5 (lane 7), 2 (lane 8) and 5 h (lane 9). Arrow heads indicate trypsin.

posome membrane was indistiguishable from that in $C_{12}E_{18}$ (Fig. 1b). Trypsin completely digested heat-denatured OprD into small fragments (data not shown). The results suggest that OprD consists of two major domains (M_r, 27 and 19K).

To test the function of these domains, we examined the effect of trypsin treatment on channel activity by determining the permeability of saccharides. The permeability rate of glucose increased markedly and it is proportional to the amounts of trypsin used (Fig. 2a). When 5 μg of OprD was treated with 10.1 μg of trypsin for 2 h, the diffusion rate of glucose increased about 10 times. Trypsin alone at this concentration (113 μg/ml, 4.7 μM) showed no detectable effect on the liposome swelling assay (data not shown).

Two interpretations may be possible to explain the channel activation: (i) trypsin treatment caused conformational change of OprD dilating the channel size, and (ii) trypsin cleaved off the gate-forming domain without affecting the channel size. We tested these possibilities by determining the diffusion rate of the uncharged saccharides with different M_r. Fig. 2b shows that the diffusion rates of saccharides through trypsin-treated OprD

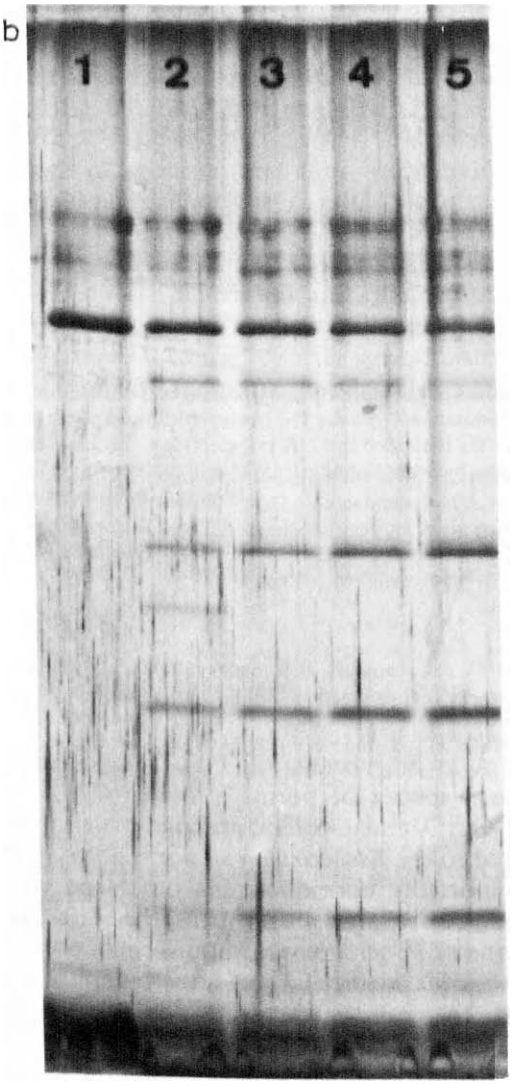

Fig. 1. (b) Trypsin susceptibility of OprD reconstituted in the membrane. The proteoliposome containing 12 μg of OprD was treated with 12 μg of trypsin in the Tris-HCl, pH 8.0 buffer at 23°C for 0.5 (lane 2), 2 (lane 3), 6 (lane 4), and 24 h (lane 5). Lane 1, untreated OprD. The protein bands were visualized by silver staining [8].

channels appeared to be fully comparable with those of untreated porin, indicating that the trypsin treatment did not affect the size of the channel. Determination of imipenem permeability showed consistent results (data not shown). Thus, we favour interpretation (ii) as the more likely mechanism for channel activation.

In order to find the gate-forming domain, we examined the polypeptide composition of the proteoliposomes used for the permeability experiments (Fig. 2c). In comparison of the polypeptide composition of the proteolytic products with the increment in channel activity, we found that cleavage of the 19K domain into two subfragments paralleled the channel activity. Therefore, it is most likely that the 19K domain forms the gate (or cap).

The question to be answered is whether only the 27K

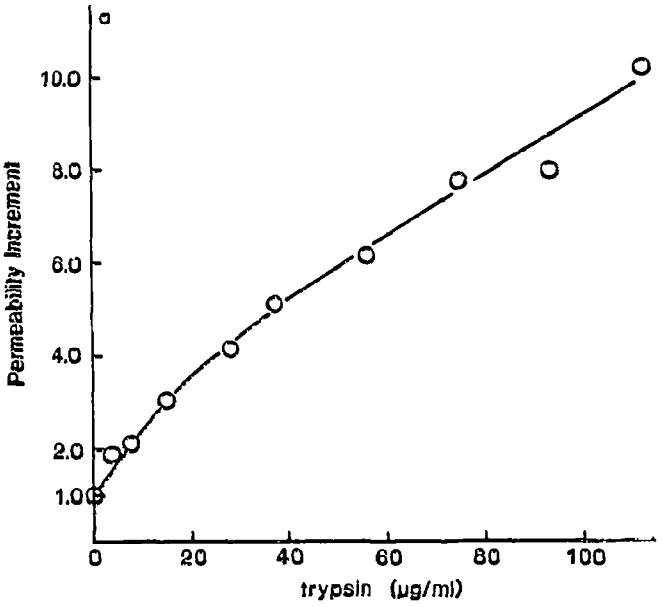

Fig. 2. (a) Permeability property of the trypsin-treated OprD. Liposome membranes containing 3.38 μg of OprD per 0.675 μmol lipids per tube were dried, suspended in 90 μl of solution containing 40 mosM stachyose, 1 mM MOPS, pH 7.2 and various amounts of trypsin and then blended on a Vortex mixer. The proteoliposomes were incubated at 37°C for 2 h. (a) The diffusion rate was determined by the liposome swelling method as described previously [1]. The figure shows the relative permeability of glucose normalized against that through untreated OprD. One point is an average of 4 independent experiments.

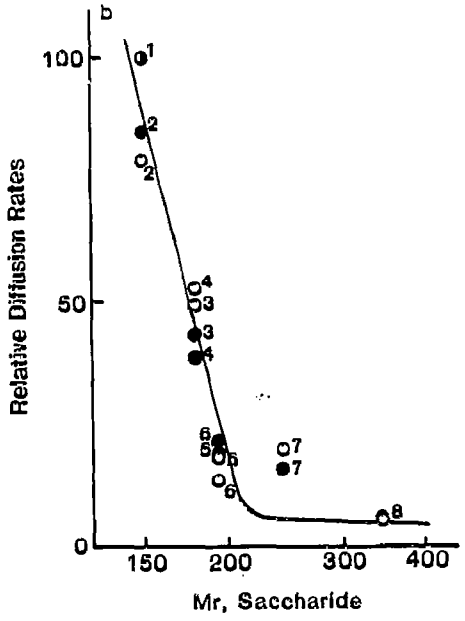

Fig. 2. (b) Permeability of various saccharides. Proteoliposome identical to that of lane 5 of Fig. 2c was used for the liposome swelling assay. The saccharides used were: 1, arabinose; 2, ribose; 3, glucose; 4, mannose; 5, α-methylglucoside; 6, α-methylmannoside; 7, *N*-acetyl glucosamine; and 8, sucrose. The figure shows the relative diffusion rates normalized to that of arabinose of the respective liposome. Symbols; (○), untreated; (●), trypsin treated OprD.

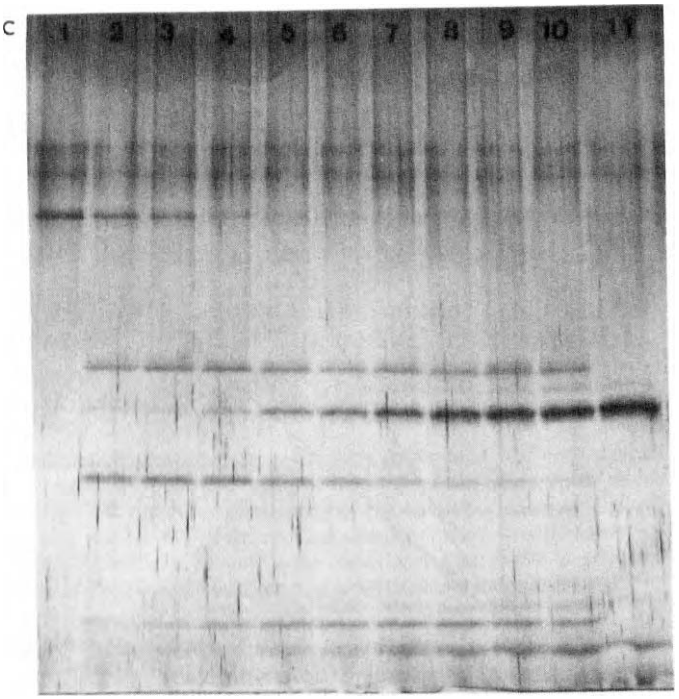

Fig. 2. (c) Polyacrylamide gel electrophoretogram of the OprD in the liposome membranes used for the permeability assay above (a). A portion of the proteoliposomes was subjected to SDS-PAGE. Lane 1, untreated OprD; lanes 2 to 10, proteoliposome treated with 3.75 (lane 2), 7.5 (lane 3), 15 (lane 4), 28 (lane 5), 38 (lane 6), 56 (lane 7), 75 (lane 8), 94 (lane 9), and 113 μg (lane 10) of trypsin/ml; and lane 11, trypsin alone, 113 μg/ml. The protein bands were visualized by silver staining.

domain has the pore-forming activity. We purified the 27K domain to near homogeneity from trypsin-treated OprD (Fig. 3, inset) and tested for saccharide permeability in the reconstituted liposomes. Results depicted in Fig. 3 clearly show that purified 27K domain formed the channel. The relative diffusion rates of arabinose and glucose were about 2 to 1 as was the case in native OprD. The channel forming activity appeared to be 8 times higher than that of equimolar native OprD, confirming the results shown in Fig. 2a. Thus, it was firmly established that 27K and 19K domains of 46K OprD form the channel and gate (or cap), respectively.

Then, another question to be answered is whether the channel-forming domain is indeed embedded in the hydrophobic region of the lipid bilayer. To identify the domain(s) anchored in the membrane, OprD in the liposome membrane was treated with trypsin and subjected to gel chromatography in the presence of 1 M NaCl. The fragments corresponding to 27K, 19K and 8K were co-eluted with the liposome, suggesting that channel-forming and also gate-forming domains were associated with the liposome membrane (data not shown).

Based on the results presented in this paper, we propose the current model for OprD. OprD forms a functional unit by trimeric aggregates of 46 K polypeptides. Each 46K polypeptide consists of 2 major domains, 27K and 19K forming the channel and the gate, respec-

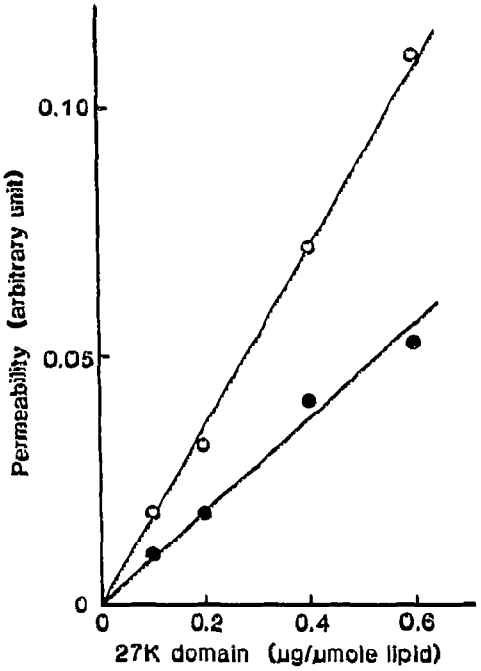

Fig. 3. The channel-forming activity of purified 27K domain. Purified OprD (2 mg) was treated with 2 mg of trypsin in $C_{12}E_8$ buffer at 37°C for 60 min. The mixture was subjected to repeated DEAE-HPLC chromatography in a solution of 1% β-OG/1 mM EDTA/10 mM Tris-HCl, pH 8. Permeability of glucose (●) and arabinose (○) was determined as described in the legend to Fig. 2a. Inset, polyacrylamide gel electrophoretogram of purified 27K domain. Lane 1, trypsin-treated OprD; lane 2, purified 27K domain.

tively. This is the first substantial demonstration of gate- and channel-forming domains in isolated channel protein to our knowledge.

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