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Lipopolysaccharide regions involved in the activation of *Escherichia coli* outer membrane protease OmpT

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OmpT is an integral outer membrane protease of *Escherichia coli*. Overexpression of OmpT in *E. coli* and subsequent *in vitro* folding of the produced inclusion bodies yielded protein with a native-like structure. However, enzymatically active protease was only obtained after addition of the outer membrane lipid lipopolysaccharide (LPS). OmpT is the first example of an enzyme that requires LPS for activity. In this study, we investigated the nature of this activation. Circular dichroism analysis showed that binding of LPS did not lead to large structural changes. Titration of OmpT with LPS and determining the resulting OmpT activity with a fluorimetric assay yielded a dissociation constant of 10^{-4} M for *E. coli* K-12 LPS. Determining the dissociation constants for different LPS chemotypes revealed that a fully acylated lipid A part is minimally required for activation of OmpT. The heptose-bound phosphates in the inner core region were also important for activation. The affinity for LPS was not dependent on the concentration of substrate, neither was affinity for the substrate influenced by the concentration of LPS. This indicated that LPS most likely does not act at the level of substrate binding. We hypothesize that LPS induces a subtle conformational change in the protein that is required for obtaining a native active site geometry.

Keywords: OmpT; membrane protease; lipopolysaccharide; activation.

The outer membrane of Gram-negative bacteria has an asymmetrical architecture with phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. Various proteins are found in the outer membrane, and LPS has been shown to play an important role in their folding and assembly *in vivo* and *in vitro* [1–4]. Furthermore, enzymatic activity of *in vitro* folded *E. coli* outer membrane protease OmpT was found to depend on the presence of LPS [5]. OmpT is a 33.5-kDa protease that cleaves preferentially between two basic amino acids [6–8]. The protein has been classified as a serine protease, which was supported by the identification of putative active site residues Ser99 and His212 [9]. However, the recently solved X-ray structure of OmpT [10] and mutagenesis studies of conserved acidic residues [11] indicate that OmpT might be a novel protease with an Asp/His catalytic dyad. The enzyme has been suggested to be involved in urinary tract disease [12], in DNA excision repair [13] and in the breakdown of antimicrobial peptides [14], but the exact biological function remains unclear. We previously developed an *in vitro* folding procedure to facilitate large-scale purification of OmpT from inclusion bodies [5]. No enzymatic activity was observed for the purified protein in the absence of LPS, although it was folded into a native-like structure according to its heat-modifiable migration behavior on an SDS/PAGE gel. After varying several conditions, it was concluded that active OmpT could only be obtained when LPS was added to the protein [5]. The X-ray structure of OmpT in the absence of LPS did not explain this absolute requirement for LPS to yield active enzyme [10].

The single known three-dimensional structure of a protein in complex with LPS is that of the *E. coli* outer membrane protein FhuA [15]. In the X-ray structure, a single LPS molecule was found to be noncovalently associated with FhuA. A more detailed study on the interaction between FhuA and LPS revealed that FhuA contains an LPS binding motif that is conserved in several other proteins known to bind LPS [16]. This putative binding site consists of four residues that form specific interactions with the LPS molecule [16]. Three of these amino acids were found to be present in a similar constellation in the X-ray structure of OmpT (Arg138, Arg175 and Lys226), indicating that OmpT may have a specific LPS binding site as well [10].

In *E. coli* K-12 and other so-called 'rough' strains, the amphipathic LPS molecules consist of two distinct regions; a largely hydrophobic lipid A part and a hydrophilic oligosaccharide region. Several of the sugar residues in LPS contain carboxylate and/or phosphate moieties, which render the molecule a large negative charge. The anionic

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Abbreviations: Abz, *o*-aminobenzoyl; Dap(DNP): *N*-β-dinitrophenyl-L-diaminopropionic acid; $DodMe_2NPrSO_3$: *N*-dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate; LPS, lipopolysaccharide; OPOE, *n*-octyl-oligo-oxyethylene; Tween 20, polyoxyethylene sorbitanmonolaurate.

Enzyme: OmpT (EC 3.4.21.87; SWISS-PROT P09169).

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LPS molecules in the outer membrane are linked to each other through salt bridges via divalent cations (Ca^{2+}, Mg^{2+}), forming a highly ordered structure that acts as an effective permeability barrier. As LPS contains various sugars, acyl chains and negatively charged substituents, several parts of this complex macromolecule could potentially be involved in the mechanism of activation. In the present study, a set of structurally distinct LPS chemotypes was used to investigate the interaction of LPS with OmpT.

MATERIALS AND METHODS

Materials

Fast Flow S-Sepharose was from Amersham Pharmacia Biotech. Polyoxyethylene sorbitanmonolaurate (Tween 20) was from Bio-Rad and *n*-octyl-oligo-oxyethylene (OPOE) was obtained from Alexis. *N*-Dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate ($DodMe_2NPrSO_3$) from Fluka was purified by passing a solution of the detergent in methanol/chloroform (1 : 1) over an Al_2O_3 column, which removed all acidic impurities present in the commercial preparation. The resulting solution was evaporated under vacuum and the resulting white powder was stored at room temperature. The fluorogenic substrate Abz-Ala-Arg-Arg-Ala-Dap(DNP)-Gly (Abz, *o*-aminobenzoyl; Dap (DNP), N-β-dinitrophenyl-L-diaminopropionic acid) was a generous gift of R. C. Cox (Utrecht University).

Purification of OmpT

OmpT was purified from inclusion bodies as described previously with some modifications [5]. To prevent autoproteolysis at the most susceptible cleavage site Lys217–Arg218, a variant with mutations G216K and K217G was used. This variant was previously shown to fold and behave like wild-type OmpT, although the catalytic efficiency of the protease for cleavage of peptide substrates was decreased [5]. In summary, mature G216K/K217G OmpT was produced as inclusion bodies in *E. coli* BL21(DE3) cells [17] harboring the T7 expression plasmid pRAK11 [5]. After dissolving 200 mg of isolated inclusion bodies in 8 M urea, OmpT was folded by dilution in detergent $DodMe_2NPrSO_3$ (final concentration 25 mM). The solution was adjusted to pH 4.0 using 10% acetic acid and loaded onto a 40-mL Fast Flow S-Sepharose column equilibrated with buffer A (10 mM $DodMe_2NPrSO_3$, 20 mM NaAc, pH 4.0). The column was washed with buffer A and OmpT was eluted with a gradient of 0–1 M NaCl in buffer A. Peak fractions were pooled and dialyzed against buffer A. To change the buffer composition and increase the protein concentration, a fraction of the dialyzed pool containing 54 mg OmpT was loaded onto a 5-mL Fast Flow S-Sepharose column equilibrated with buffer A. The column was washed with buffer B (1% OPOE, 5 mM NaAc, pH 4.0) and OmpT was eluted using 1 M NaCl in buffer B. A sample of the pooled peak fractions containing 28 mg OmpT was dialyzed against buffer C (1% OPOE, 10 mM Tris, pH 8.3), resulting in a final fraction of 4.2 $mg \cdot mL^{-1}$ pure OmpT, which was stored at −20 °C. The concentration of OmpT was determined by measuring the absorbance at 280 nm. A molar absorption coefficient of 74 960 $M^{-1} \cdot cm^{-1}$ was used, which was calculated using the method of Gill & von Hippel [18].

Purification of LPS

E. coli K-12 DH5α cells [19] were grown in Luria–Bertani medium [20], isolated by centrifugation, washed with distilled water and freeze-dried. LPS was extracted according to a modified PCP (PCP1, phenol/chloroform/petroleum ether, 2 : 5 : 8, v/v/v) procedure [21]. A model of the chemical structure of *E. coli* K-12 DH5α LPS (schematically given in Fig. 1) was constructed based on unpublished compositional analysis data kindly provided by U. Zähringer

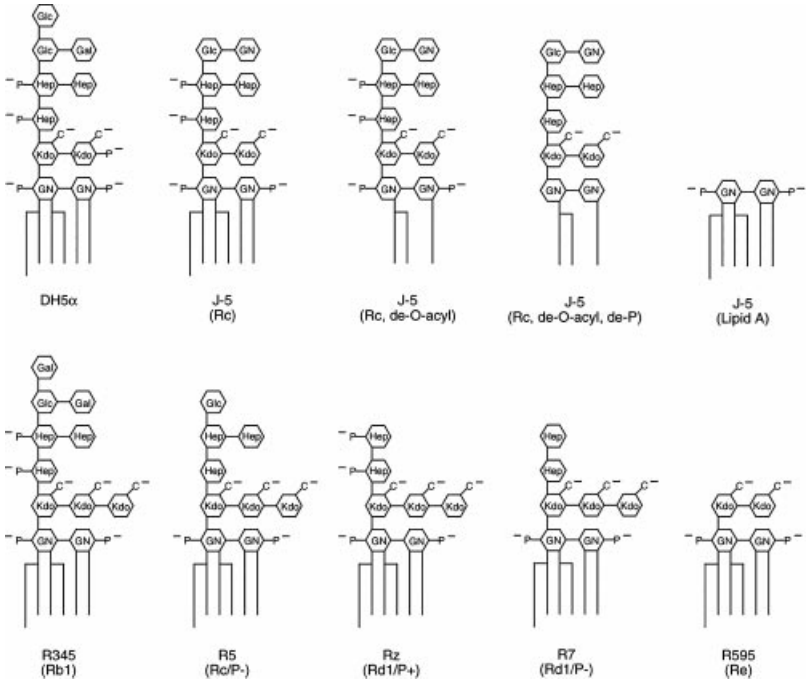

Fig. 1. Schematic chemical structures of various LPS chemotypes from *E. coli* (upper five) and *S. minnesota* (lower five). Additional structural information can be found in references [4,22–24]. Unusual abbreviations used in this Figure are: C, carboxylate; GN, GlcNAc; Hep, L-*glycero*-D-*manno*-heptose; P, phosphate or aminoethyl phosphate.

(Research Center Borstel). The purification and modification of various LPS from *E. coli* J-5 and *Salmonella minnesota* were as described previously [4]. The chemical structures of these LPS chemotypes have been reported [4,22–24] and are schematically given in Fig. 1. Freeze-dried samples of all LPS types were dissolved in 0.05% triethylamine to a concentration of 5 mg·mL^{-1} and stored at −20 °C. Molar concentrations of all LPS chemotypes except lipid A were determined by quantifying the amount of Kdo sugars, using the method described by Dröge *et al.* [25]. Lipid A does not contain any Kdo residues, therefore its molar concentration was determined using the calculated molecular mass of 1.8 kDa [22]. Due to the presence of impurities (e.g. salt) in the freeze-dried samples, the lipid A concentration determined in this way was not highly accurate.

Circular dichroism spectroscopy

CD spectra were recorded at room temperature on a Jasco J-810 spectropolarimeter. Samples containing 15 μM OmpT in buffer C with or without 200 μM of *E. coli* K-12 DH5α LPS were analyzed using quartz cells with a path length of 0.2 mm.

Enzymatic activity assay

The internally quenched fluorogenic peptide substrate Abz-Ala-Arg-Arg-Ala-Dap(DNP)-Gly was used in a fluorimetric assay as described previously [5]. Standard assay conditions were 5 μM substrate, 1 mM Tween 20, 5 mM EDTA, 10 mM Tris, pH 8.3. Activity was measured in a fluorimeter using excitation and emission wavelengths of 325 and 430 nm, respectively. Prior to measurement, OmpT was preincubated on ice for at least 3 h at a concentration of 0.2 mg·mL^{-1} in buffer C with or without LPS. Typically, 10 μL of the preincubation solution (2 μg OmpT) was added to 2 mL of assay mixture. Although OmpT and LPS were diluted 200-fold, the initial increase in fluorescence was linear for at least one minute, indicating that the complex of OmpT and LPS, formed during the preincubation, apparently did not dissociate during the measurement. The increase in fluorescence was linear for a longer time period when LPS was already present in the assay mixture before addition of preincubated OmpT, but the initial activity was equal to that in the absence of additional LPS in the assay mixture. It was therefore concluded that the relevant conditions were those during preincubation.

The activation of OmpT by LPS was studied by preincubating OmpT with 0–180 μM LPS and fitting of the resulting titration curves using the equation $A = A_{max}/(1 + K_d/[L])$ describing a hyperbolic saturation curve, in which A is the activity of OmpT in U·mg^{-1}, A_{max} is the activity at saturating LPS concentration, K_d is the dissociation constant of the OmpT–LPS complex, and [L] is the concentration of LPS during preincubation. The turnover number k_{cat} (s^{-1}) was calculated from the value of A_{max} (U·mg^{-1}). For several LPS chemotypes the values of A_{max} and K_d could not be determined separately under the conditions used due to low affinities. In these cases, the value of A_{max}/K_d, which is equal to the slope of the curve at low concentrations of LPS, was used to compare the efficiencies of the corresponding LPS chemotypes. The effect of substrate concentration on OmpT activation by LPS was studied by varying the concentration of substrate from 1 to 8.5 μM. The influence of ionic strength was studied by addition of 300 mM NaCl during preincubation. We previously found that binding of the positively charged substrate by OmpT was impaired at high ionic strength [8]. Therefore, it was necessary to carry out activity assays at low salt concentration in the assay mixture. This was accomplished by 200-fold dilution of the preincubated sample into the assay, resulting in a final concentration of 1.5 mM NaCl.

RESULTS

We have previously shown that protease OmpT is only enzymatically active in the presence of LPS [5]. OmpT is found in *E. coli* K-12, therefore LPS from the K-12 strain DH5α was isolated and used to characterize the activation of OmpT. To determine which parts of LPS are involved in the activation of OmpT, a set of previously described LPS chemotypes from *E. coli* and *S. minnesota* [4] was used as well. These LPS chemotypes are similar to DH5α LPS, but vary in size and charge as shown in Fig. 1.

Structural analysis of OmpT activation

CD spectra of OmpT with or without *E. coli* K-12 DH5α LPS were recorded. Both spectra are indicative of a β barrel fold and show that addition of LPS does not lead to large structural changes in the protein (Fig. 2).

Kinetic analysis of OmpT activation

OmpT was preincubated with 0–180 μM LPS of varying chemotype and subsequently activities were measured in a fluorimetric activity assay. For DH5α, J-5 (Rc), R345 (Rb1) and Rz (Rd1/P +) LPS, hyperbolic saturation curves were obtained (see Fig. 3 for DH5α and Rz LPS) from which the turnover number k_{cat} and the dissociation constant K_d could be determined (Table 1). As might have been expected from their similar chemical structures, these four LPS chemotypes yielded comparable values for k_{cat} (average

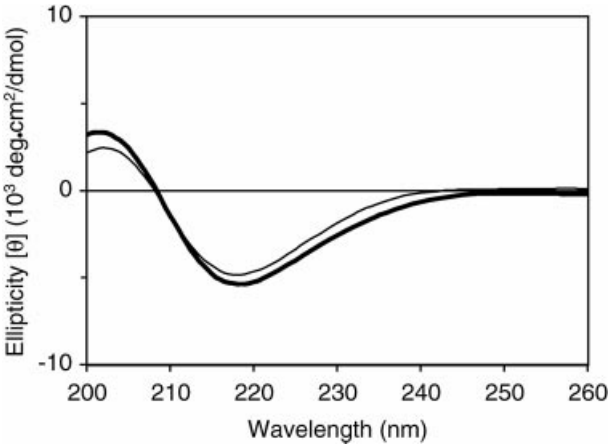

Fig. 2. CD spectra of OmpT. Spectra were recorded for 15 μM OmpT in the absence (light trace) or presence (bold trace) of 200 μM DH5α LPS.

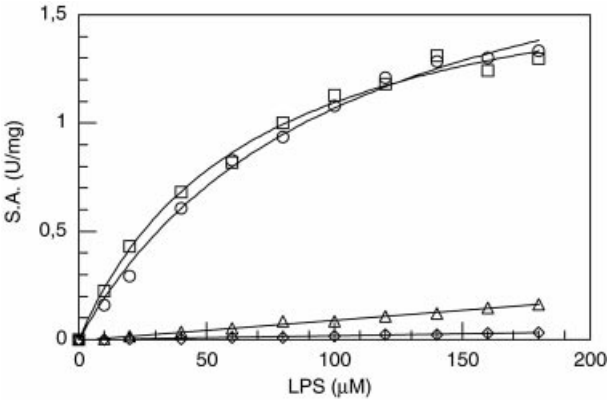

Fig. 3. Titration of LPS to OmpT. After preincubation of OmpT with 0–180 μM of DH5α LPS (○), Rz LPS (□), R7 LPS (△) or J-5 lipid A (◇), specific activities were measured in a fluorimetric assay at standard conditions.

1.15 ± 0.13 s^{-1}) and K_d (99 ± 32 μM). For J-5 (lipid A), R5 (Rc/P-), R7 (Rd1/P-) and R595 (Re) LPS, the titration curves were linear up to 180 μM LPS due to relatively low affinities (K_d > 1 mM; Fig. 3). As a consequence, only the ratio of k_{cat} over K_d could be determined from the slopes of the linear regions. The values of k_{cat}/K_d for these four chemotypes were between 50 and 1300 $M^{-1}·s^{-1}$ (Table 1), which is ninefold to 250-fold lower than the average value for DH5α, J-5, R345 and Rz LPS (12 300 ± 2400 $M^{-1}·s^{-1}$). This indicates that lipid A, R5, R7 and R595 LPS lack important parts for the interaction with OmpT. Detailed comparison of the structures in Fig. 1 shows that particularly the heptose-bound phosphates are important for efficient activation (compare R345 with R5 and Rz with R7). For J-5 LPS that had been dephosphorylated and/or de-O-acylated, no OmpT activity could be detected at all up to 180 μM of LPS. This clearly indicates that full acylation of LPS is essential for interaction with OmpT. To further study the contribution of hydrophilic and hydrophobic interactions, 300 mM NaCl was added during preincubation of OmpT with DH5α or R7 LPS. This increase in ionic strength weakens polar interactions and strengthens hydrophobic interactions. The affinity of OmpT for DH5α LPS (with phosphates and intact lipid A) was somewhat lower in the presence of salt (K_d = 147 vs. 104 μM). In contrast, the values of k_{cat} and k_{cat}/K_d were increased at higher ionic strength (2.2 vs. 1.2 s^{-1} and 15 000 vs. 12 000 $M^{-1}·s^{-1}$, respectively). Also for R7 LPS (without phosphates, but with native lipid A), the efficiency of activation was increased when salt was added (k_{cat}/K_d = 1100 vs. 500 $M^{-1}·s^{-1}$). Unfortunately, the value of K_d for R7 LPS could not be determined separately for reasons mentioned above.

In order to estimate the on-rate (k_{on}) and off-rate (k_{off}) of the complex formation, OmpT was preincubated with 20 μM DH5α LPS and subsequently activity was followed for 30 min. The gradual decrease in specific activity caused by dissociation of the OmpT–LPS complex was analyzed using first order kinetics and corrected for substrate conversion. The resulting fit (not shown) yielded a k_{off} of 5×10^{-4} s^{-1}, indicating a rather slow dissociation rate. The dissociation constant K_d was 10^{-4} M for DH5α LPS (see above) and equals k_{off}/k_{on}, leading to a value of 5 $M^{-1}·s^{-1}$ for k_{on}. A similar experiment with R5 LPS yielded a k_{off} of 7×10^{-3} s^{-1}, which indicates that deletion of the heptose-bound phosphates destabilizes the complex between OmpT and LPS considerably. As already pointed out above, a value for K_d, and therefore k_{on}, could not be obtained for R5 LPS. Interestingly, the respective values of the parameter k_{off} and of the ratio k_{cat}/K_d both differ about 10-fold between DH5α and R5 LPS.

To investigate whether the heptose-bound phosphates and a fully acylated lipid A displayed an additive effect, OmpT was preincubated with R7 LPS (with native lipid A, but without phosphates) in the presence or absence of de-O-acylated J-5 LPS (with phosphates). It was found that the activity of OmpT in the presence of 50 or 100 μM R7 LPS could not be increased by addition of 50 or 100 μM

Table 1. Influence of LPS chemotypes from various bacterial strains on OmpT activation. After preincubation of OmpT with 0–180 μM LPS (structures shown in Fig. 1), activity was measured in a fluorimetric assay and the parameters k_{cat} and K_d were determined as described in Materials and Methods.

Strain (LPS chemotype)	Sugar residues	Acyl chains	Heptose-bound phosphates	k_{cat} (s^{-1})	K_d (μM)	k_{cat}/K_d ($M^{-1}·s^{-1}$)
E. coli						
DH5α	10	6	+	1.2	104	12000
J-5 (Rc)	9	6	+	1.3	141	9200
J-5 (Rc, de-O-acyl)	9	3	+	[a]	[a]	0[a]
J-5 (Rc, de-O-acyl, de-P)	9	3	–	[a]	[a]	0[a]
J-5 (Lipid A)	2	6	–	[b]	[b]	50–250[c]
S. minnesota						
R345 (Rb1)	11	6	+	1.1	84	13000
R5 (Rc/P–)	9	6	–	[b]	[b]	1300
Rz (Rd1/P +)	7	6	+	1.0	67	15000
R7 (Rd1/P–)	7	6	–	[b]	[b]	500
R595 (Re)	4	6	–	[b]	[b]	400

[a] No OmpT activity could be detected up to 180 μM LPS. [b] The parameters k_{cat} and K_d could not be determined individually due to a linear relationship between LPS concentration and OmpT activity up to 180 μM LPS. [c] Lipid A concentration could not be determined accurately due to its lack of Kdo (see Materials and methods).

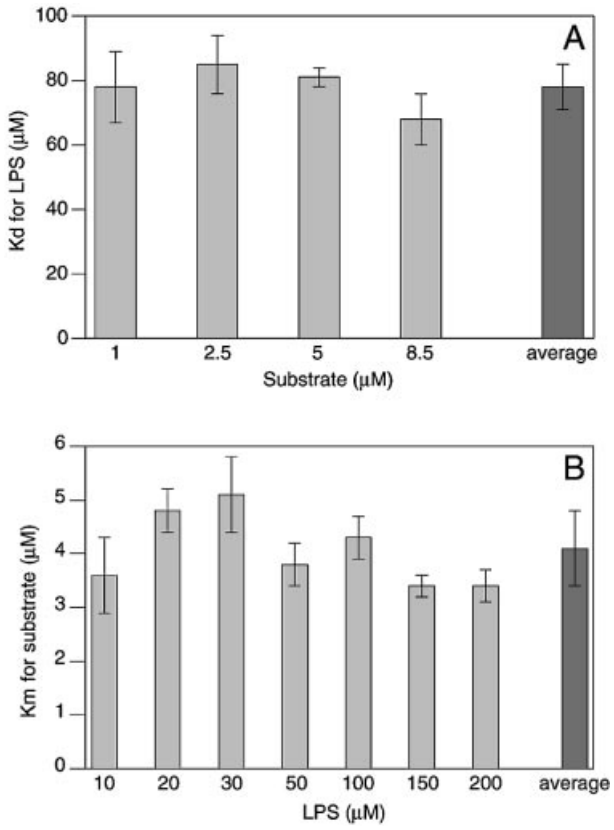

Fig. 4. Absence of an allosteric effect between the binding of LPS and substrate to OmpT. After preincubation of OmpT at 0–180 μM DH5α LPS, activities were measured at 1, 2.5, 5 or 8.5 μM of Abz-Ala-Arg-Arg-Ala-Dap(DNP)-Gly. The affinities for LPS at each substrate concentration (A) and the affinities for the substrate at each LPS concentration (B) were determined from the resulting saturation curves.

de-O-acylated J-5 LPS (activity was even slightly decreased, data not shown). These results indicated that the heptose-bound phosphates and native lipid A should be present within the same LPS molecule in order to give full enzymatic activity.

The relationship between substrate binding and LPS binding was studied by varying the concentration of DH5α LPS between 0 and 200 μM at 1, 2.5, 5 and 8.5 μM of substrate, respectively. The resulting affinities for LPS were not significantly dependent on the substrate concentration, with an average K_d of 78 ± 7 μM (Fig. 4A). The same data were used to construct Michaelis–Menten curves at each LPS concentration. No significant relationship between LPS concentration and affinity of OmpT for the substrate could be observed, with an average K_m of 4.1 ± 0.7 μM (Fig. 4B).

DISCUSSION

We previously demonstrated by SDS/PAGE [5] and X-ray crystallography [10] that inclusion bodies of *E. coli* outer membrane protease OmpT can be folded *in vitro* into an apparently native β barrel structure. Surprisingly, OmpT activity was obtained only after the addition of LPS [5]. We studied the details of this activation using various techniques. We used CD spectroscopy to investigate the effect of LPS on the structure of the enzyme (Fig. 2). The CD spectrum of OmpT without LPS indicated a large content of β strand conformation, supporting the existence of a β barrel fold in the absence of LPS. A similar spectrum was obtained for OmpT in the presence of LPS, indicating that LPS binding does not induce large conformational changes in the protein. This suggests that the β barrel structure of OmpT in the absence of LPS as determined by X-ray crystallography [10] closely represents the protein in its active state in the presence of LPS. The only outer membrane enzyme besides OmpT that has been extensively characterized to date is *E. coli* outer membrane phospholipase A [26]. This enzyme does not depend on LPS, but its activity was shown to be regulated by reversible dimerization [27]. We have used two different methods to investigate whether dimerization of OmpT could be involved in the activation mechanism by LPS. Chemical cross-linking of OmpT with glutaraldehyde at pH 6.5 or 8.3 in the absence or presence of LPS and subsequent analysis by SDS/PAGE did not reveal evidence for dimer formation (L.Vandeputte-Rutten & R. A. Kramer, unpublished data). Dimers were observed, however, when the experiment was carried out at pH 4.0 in the presence of LPS. Similar results were found using analytical ultracentrifugation, where indications for dimerization were obtained only at pH 4.0 (L.Vandeputte-Rutten & R. A. Kramer, unpublished data). As the calculated isoelectric point of OmpT is 5.3, dimer formation at acidic pH may be due to aspecific aggregation of the positively charged OmpT with the negatively charged LPS. Moreover, as *E. coli* only grows in a limited pH range of ≈ 6.0–8.0 [28], the relevance of OmpT behavior at pH 4.0 is questionable. Therefore, dimerization of OmpT is not likely to play a role in activation of the protease. A technique that has been applied successfully to study LPS binding by the peptide antibiotic polymyxin B is surface plasmon resonance, where polymyxin B was covalently immobilized on sensor chips [29]. This technique might in principle be useful for the characterization of LPS binding by OmpT, but immobilization of an integral membrane protease is complicated (e.g. in terms of protein stability and enzymatic activity).

A logical strategy to obtain more insight into the effect of LPS on the enzymatic activity of OmpT is to use an enzymatic activity assay, and this has indeed been the most successful approach so far. To determine the role of specific regions in the LPS molecule, we preincubated OmpT with a set of well-characterized LPS chemotypes and subsequently measured proteolytic activity in a fluorimetric assay. During preincubation, OmpT was present in micelles formed by detergent OPOE (concentration OPOE 1%, ≈ 30 mM). The physical state of the amphipathic LPS molecule in an LPS–water system has not been fully established yet, but the concentration of free monomers is probably below 1 μM [30]. It is therefore reasonable to assume that the majority of LPS molecules (10–180 μM during preincubation) formed mixed micelles with OPOE and OmpT. It is worth noting that the addition of phosphatidylcholine, phosphatidylglycol and *n*-dodecylmaltoside to micelles containing OmpT did not result in enzymatic activity (R. A. Kramer & N. Dekker, unpublished data), indicating the requirement for a specific complex between LPS and OmpT rather than a physicochemical effect.

A fully acylated lipid A part was found to be essential for OmpT activation as judged from the inability of de-O-acylated J-5 LPS to activate OmpT. This probably indicates that hydrophobic interactions between OmpT and the LPS molecule play an important role in the activation process. In addition to this hydrophobic interaction, at least one of the heptose-bound phosphates in the inner core region of LPS was found to be important for activation of OmpT as well. In this respect, it is interesting to note that one of these phosphates has previously been suggested to facilitate trimerization of outer membrane porin OmpF *in vitro* [2], and one or both of the phosphates were required for efficient *in vitro* folding of porin PhoE [4]. However, neither the phosphates alone nor any other part of the core region are absolutely essential for activation of OmpT, as lipid A (without core sugars) was also capable of activating the enzyme to some extent. The combined importance of the acyl chains and the phosphates was further studied by addition of 300 mM of NaCl during preincubation, which abolishes most ionic interactions and increases the contribution of hydrophobic interactions. The observed higher activation efficiencies in the presence of salt suggest that hydrophobic interactions are indeed important for activation. Furthermore, the involvement of negatively charged phosphates in binding was supported by the observation that the affinity of OmpT for DH5α LPS (with phosphates) was decreased at higher ionic strength. Interestingly, the low activity of OmpT in the presence of dephosphorylated LPS could not be increased by addition of de-O-acylated LPS containing all phosphates. Apparently, the heptose-bound phosphates and a native lipid A part should be present within the same LPS molecule for optimal activation. Activity was even slightly decreased upon addition of de-O-acylated LPS, therefore it is conceivable that the two types of LPS competed for the single LPS binding site proposed previously [10]. Binding of LPS chemotypes DH5α, J-5 (Rc), R345 (Rb1) or Rz (Rd1/*P* +) to OmpT was characterized by K_d values of 10^{-4} M. Although the experimental conditions and LPS chemotypes differed for previously published affinities of other LPS binding peptides and proteins, it appears that most of these were found to display higher affinities. Examples of reported dissociation constants include 10^{-8} M for polymixin B and 10^{-10} M for Sushi-1 [31].

To investigate whether LPS allosterically influences the affinity of OmpT for peptide substrates, the concentration of DH5α LPS was varied at different substrate concentrations. It was found that substrate binding and LPS binding did not influence each other significantly (Fig. 4), suggesting that OmpT may be able to bind peptide substrates in the absence of LPS. This hypothesis is supported by the X-ray structure of LPS-free OmpT, which contains a large anionic cleft where the substrate is thought to bind [10]. We propose that changes in the putative preformed substrate binding site are not required to facilitate substrate binding and are therefore not involved in the activation mechanism.

Figure 5 shows the LPS molecule from the FhuA structure [15] modeled on the proposed LPS binding site in the structure of OmpT [10]. In this model, the LPS molecule is located at the side of OmpT where the fourth extracellular loop (L4, residues 200–230) is present. Interestingly, mutations D208A, D210A, H212A, H212N, H212Q, G216K/K217G, K217T and R218L in loop L4 all resulted in partial or virtually complete loss of enzymatic activity [5,9,11]. Asp210 and His212 are currently considered to be catalytic residues, with their side chains pointing into the anionic cleft [10,11]. The function of the other mutated residues remains to be determined, although Arg218 may be of structural importance since it forms a salt bridge with Glu211. We propose that LPS induces a subtle conformational change in OmpT, possibly in loop L4, thereby changing the properties of the active site in such a way that it becomes capable of hydrolyzing substrates. An alternative mechanism where LPS participates directly in catalysis was also considered. However, the native *E. coli* K-12 LPS molecule is clearly not large enough to bend over the rim of the OmpT molecule and reach into the active site. Moreover, even relatively short LPS chemotypes were still able to activate the protease. Additional studies are obviously required to establish the precise mechanism of activation by LPS. To this end, efforts are currently made to solve the X-ray structure of OmpT in complex with LPS.

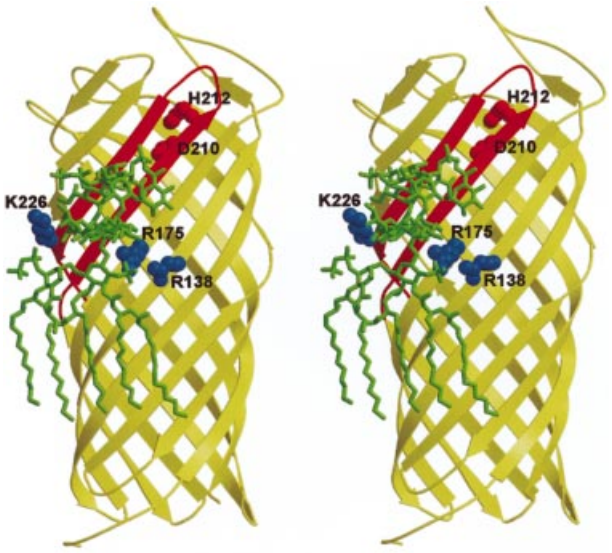

Fig. 5. Model in stereo of OmpT with a bound LPS molecule. The *E. coli* K-12 LPS molecule found in the X-ray structure of FhuA [15] was modeled at the putative LPS binding site in the X-ray structure of OmpT [10]. The view is perpendicular to the barrel axis, with the membrane-embedded part of the protein at the bottom and the extracellular loops at the top. The LPS molecule (stick model) is shown in green. OmpT (ribbon model) is presented in yellow, except for loop L4 which is highlighted in red. The side chains of five residues (space-filled model) are indicated by their one letter code abbreviation and residue number; the putative active site residues Asp210 and His212 in loop L4 are colored red, the putative LPS binding site residues Arg138, Arg175 and Lys226 are in blue.

To the best of our knowledge, OmpT is the first example of an enzyme that specifically requires LPS for its activity. As OmpT is fully surrounded by LPS *in vivo*, the activation is not a regulatory mechanism for OmpT activity in the outer membrane. An obvious question that remains is why OmpT activity is dependent on the presence of LPS. During biogenesis, outer membrane proteins are transported from the cytosol over the inner membrane and subsequently via the periplasm to the outer membrane. If OmpT would be proteolytically active during this route to its final destination,

it could be harmful to other cellular proteins that it encounters. In this respect, it is worth noting that the outer membrane protein PhoE was found to fold at least partially in the periplasm before membrane insertion [32]. Therefore, we propose that the dependence of OmpT activity on the presence of LPS is required to keep the protease inactive until it is assembled at the site where it should perform its function.

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