Substrate Specificity of the Integral Membrane Protease OmpT Determined by Spatially Addressed Peptide Libraries†

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ABSTRACT: *Escherichia coli* outer membrane protease T (OmpT) is an endopeptidase that specifically cleaves between two consecutive basic residues. In this study we have investigated the substrate specificity of OmpT using spatially addressed SPOT peptide libraries. The peptide acetyl-Dap(dnp)-Ala-Arg↓Arg-Ala-Lys(Abz)-Gly was synthesized directly onto cellulose membrane. The peptide contained the aminobenzoyl (Abz) fluorophore, which was internally quenched by the dinitrophenyl (dnp) moiety. Treatment of the SPOT membrane with the small, water-soluble protease trypsin resulted in highly fluorescent peptide SPOTs. However, no peptide cleavage was observed after incubation with detergent-solubilized OmpT, a macromolecular complex with an estimated molecular mass of 180 kDa. This problem could be solved by the introduction of a long, polar polyoxyethylene glycol linker between the membrane support and the peptide. Peptide libraries for the P_2, P_1, P_1', and P_2' positions in the substrate were screened with OmpT, and peptides of positive SPOTs were resynthesized and subjected to kinetic measurements in solution. The best substrate Abz-Ala-Lys-Lys-Ala-Dap(dnp)-Gly had a turnover number k_{cat} of 40 s^{-1}, which is 12-fold higher than the starting substrate. Peptides containing an acidic residue at P_2 or P_2' were not substrates for OmpT, suggesting that long-range electrostatic interactions are important for the formation of the enzyme−substrate complex. OmpT was highly selective toward L-amino acids at P_1 but was less so at P_1' where a peptide with D-Arg at P_1' was a competitive inhibitor (K_i of 19 μM). An affinity chromatography resin based on these findings was developed, which allowed for the one-step purification of OmpT from a bacterial lysate. The implications of the determined consensus substrate sequence (Arg/Lys)↓(Arg/Lys)-Ala for the proposed biological function of OmpT in defense against antimicrobial peptides are discussed.

Knowledge of the substrate specificity of proteolytic enzymes is indispensable for the rational design of inhibitors and will help to select target proteins in biological databases. The specificity can be determined by a wide variety of techniques using synthetic peptides, phage display, and combinatorial peptide libraries. Synthetic peptides can be kinetically characterized with a given protease to determine the sequence preference of the enzyme in an iterative procedure. To accelerate this tedious process, mixtures of synthetic peptides each with a different sequence can be used. Analysis of the reaction products by N-terminal sequencing (*1*) or sequence determination of both N- and C-termini (*2*) allows for the determination of the preferred cleavage site in peptides. Phage display (*3*) combines the advantages of very large library sizes, the selection of preferred substrate sequences by repetitive panning, and the usage of proteinaceous substrates and sequence analysis by DNA sequencing. Combinatorial peptide libraries have also been applied for the characterization of the substrate specificity of proteases (*4*, *5*). The hydrolytic action of a protease toward peptides attached to resin beads can be monitored using fluorescence with an appropriate couple of fluorophore and quencher, e.g., aminobenzoyl (Abz)[1] and Tyr(NO_2), respectively (*6*). The identity of the peptide can be determined after cleavage of the peptides from the support by protein microsequencing or mass spectrometry. More recently, large random libraries synthesized on solid support have been described that are defined only at a single position of the amino acid sequence (*7*, *8*). The individual peptides with known composition were cleaved from the resin, and the activity of the protease toward each library member was determined. Such approaches have the advantage that sequence identification of the cleavage products afterward is not necessary and that catalysis takes place in solution.

SPOT libraries (*9*) have proven to provide a flexible and economic procedure for the analysis of the specific recognition of peptides by biomolecules. In a SPOT library peptides

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[1] Abbreviations: Abz, aminobenzoyl; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; Dap(dnp), 3-(dinitrophenyl)diaminopropionic acid; DMF, dimethylformamide; Fl, Abz fluorophore; Fmoc, 9-fluorenylmethyloxycarbonyl; LPS, lipopolysaccharide; NHS, *N*-hydroxysuccinimide; OG, octyl β-glucopyranoside; OmpT, outer membrane protease T; Qn, Dap(dnp) quencher; PEG, polyoxyethylene glycol; Pfp, pentafluorophenyl ester; TFA, trifluoroacetic acid.

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are synthesized at defined positions on a cellulose membrane support yielding a positionally addressed library. The analysis of protein binding (e.g., monoclonal antibodies) is done on the solid support that allows for the direct interpretation of the sequence specificity of the interaction. The SPOT technique has also been used to determine the substrate specificity of small, water-soluble enzymes. Phosphorylation of immobilized peptides by kinases using [γ-^{32}P]ATP allowed for the determination of the substrate specificity of these enzymes (*10*).

The outer membrane protease T (OmpT) is a 33.5 kDa endoprotease present in *Escherichia coli*. Recently, the large-scale purification of OmpT has been described (*11*). OmpT is proposed to adopt a β-barrel structure within the outer membrane with the active site of the enzyme exposed at the cell surface (*11*). Site-directed mutagenesis experiments have shown that residues Ser99 and His212, both located in large extracellular loops in the topology model, are active site residues (*12*), which suggests that OmpT is a serine hydrolase although the classical consensus sequences are absent. OmpT is proposed to fulfill a protective function in the bacterium by the degradation of antibacterial peptides such as protamine (*13*). In general, these peptides have a high content of basic residues for binding the negatively charged cell surface of Gram-negative bacteria, and OmpT is specific for cleavage between two consecutive basic amino acids (*14*). Interestingly, cleavage of proteins by OmpT at sites lacking the dibasic motif has been reported several times (e.g., refs *15*−*17*), and the consensus sequence for cleavage has not been determined yet.

Our aim was to use SPOT libraries for determining the substrate specificity of OmpT. A potential drawback of this technique is the inaccessibility of large enzymes to peptides on a solid support, which has limited its use to small, water-soluble enzymes, whereas OmpT is an integral membrane enzyme. OmpT can be solubilized in detergent micelles, resulting in an enzyme−detergent complex with a molecular mass of 180 kDa (*14*). In this study we have improved and widened the scope of the SPOT technique by the introduction of a polyoxyethylene glycol (PEG) linker between the solid support and the peptide. This approach allowed for the successful application of such peptide libraries to determine the substrate specificity of a membrane enzyme present in a large macromolecular complex. The results of the library screen were verified by kinetic analysis of the OmpT-catalyzed hydrolysis of peptides with the newly determined optimal cleavage sequences. On the basis of the outcome of the library screen, D-amino acid containing inhibitors against OmpT were developed. An affinity chromatography Sepharose resin was prepared with such an inhibitor as the immobilized ligand, and this resin allowed a one-step purification of OmpT from a bacterial lysate.

EXPERIMENTAL PROCEDURES

Chemicals. Side-chain-protected 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids and the corresponding pentafluorophenyl ester (Pfp), Fmoc-Dap(dnp)-OH [Fmoc-3-(dinitrophenyl)diaminopropionic acid], and Fmoc-Lys(Abz-Boc)-OH (*tert*-butyloxycarbonyl-Lys with a Boc-protected Abz group attached to the N$^{\epsilon}$ group) were purchased at Bachem AG (Bubendorf, Switzerland). Fmoc-NH-PEG-CO_2-NHS (molecular mass of 3400 Da) was from Shearwater Polymers (Huntsville, NY). K12 lipopolysaccharide (LPS) was a generous gift from Dr. Klaus Brandenburg (Research Center Borstel, Center for Medicine and Biosciences, Borstel, Germany). OmpT refers to a recombinant, autoproteolytically stable variant of wild-type *E. coli* enzyme with the substitutions G216K and K217G. The protein was expressed and purified as described by Kramer et al. (*11*). The concentration of OmpT was determined by measuring the absorbance at 280 nm, using a molar absorption coefficient of 74 960 M^{-1} cm^{-1} (corresponding to an OD_{280nm} of 2.24 at 1 mg/mL). A kit with all reagents for the manual synthesis of the SPOT libraries was obtained from Genosys Biotechnologies Inc. (Cambridge, U.K.). Cellulose membrane Whatman 50 was from Whatman (Maidstone, U.K.). Wang resin was from NovaBiochem (Läufelfingen, Switzerland). NHS-activated HiTrap Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of the highest purity commercially available.

Synthesis of Building Blocks. Fmoc-Dap(dnp)-OH and Fmoc-Lys(Abz-Boc)-OH were converted into the corresponding Pfp ester as described by Christensen et al. (*18*).

Peptide Synthesis. Peptides were synthesized using standard Fmoc chemistry on Wang resin with a Labortec SP640 peptide synthesizer (Bachem AG, Bubendorf, Switzerland). Fmoc-protected amino acids with acid-labile side-chain protection groups were activated with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate in dimethylformamide (DMF) in the presence of diisopropylethylamine prior to addition to the resin. Abz was coupled as a Boc-protected derivative. After synthesis the peptide was deprotected in the presence of scavengers and cleaved off from the resin by trifluoroacetic acid (TFA). Purification was achieved using C18 reversed-phase HPLC. The peptides were analyzed by mass spectrometry and analytical HPLC.

Preparation of SPOT Membrane. Cellulose paper was derivatized with β-alanine, and SPOTs were defined in a second synthesis reaction with β-alanine as described by Kramer and Schneider-Mergener (*19*). The SPOTs (8 mm diameter each on 96-well microtiter plate format) corresponded to 70 nmol of free amine on average as determined by bromophenol blue staining. The SPOTs were modified by the addition of 4 μL of 75 mM Fmoc-NH-PEG-CO_2NHS in DMF. After 15 min at ambient temperature a second 4 μL aliquot was added, and the reaction was allowed to proceed for another 15 min. The membrane was washed with DMF, unreacted free amines were blocked with acetic anhydride, and the Fmoc protecting group was cleaved by 20% piperidine, all following standard procedures from the manufacturer [Genosys manual; see also Kramer and Schneider-Mergener (*19*)]. The efficiency of the PEG coupling was determined by bromophenol blue staining to be 20%. Subsequent double amino acid couplings (described below) resulted in over 97.5% efficiency each.

Library Synthesis. SPOT libraries were synthesized on the PEG-derivatized SPOT cellulose membranes following the procedures in the manufacturer's manual (Genosys). The P_1 library had the following amino acid sequence, acetyl-Dap-(dnp)-Ala-Xxx-Arg-Ala-Lys(Abz)-Gly, with the C-terminal glycine covalently attached to the ω-amino function of the PEG linker. Xxx is one of the 20 natural amino acids and corresponds to the positionally addressed SPOT in the library.

The other libraries were prepared in a similar fashion.

Library Screen with OmpT. The SPOT library (typically on 4 × 5 cm of cellulose paper) was soaked in distilled water twice for 5 min each, followed by (two times) a buffer of 10 mM Tris, pH 8.3, with 1 mM EDTA. The library was incubated with 4 mL of 10 μM OmpT and 24 μM K12 LPS in 2% octyl β-glucopyranoside (OG), 10 mM Tris, pH 8.3, and 1 mM EDTA for 24 h on a shaking platform at room temperature. Hereafter, a fluorescent image was recorded. The membrane was illuminated with a 310 nm lamp, and fluorescence was recorded on a Bio-Rad Model GS-700 imaging densitometer system equipped with a CCD camera. The blackening of the spots in the inverted images corresponds with the increase in fluorescence due to removal of the N-terminal peptide fragment carrying the dinitrophenyl quencher.

Fluorometric Assay. Kinetic parameters were determined in a fluorometric assay using various internally quenched peptide substrates [e.g., Abz-Ala-Arg-Arg-Ala-Dap(dnp)-Gly]. The peptide was dissolved in water (stock at 1 mg/mL), and the concentration was determined by absorption measurements at 353 nm using an ϵ of 13 780 M^{-1} cm^{-1}. The stock solution was diluted to a final concentration of 0.1−10 μM in 1 mM Tween-20, 10 mM Tris, pH 8.3, and 5 mM EDTA. Excitation of Abz at 325 nm results in a fluorescence signal with an emission maximum at 430 nm. In the intact substrate this fluorescence is quenched by the dinitrophenyl moiety in Dap(dnp). Cleavage between the two arginines by OmpT (typically 10 μL of a solution of 418 nM OmpT with 200 mM K12 LPS in 10 mM Tris, pH 8.3, and 1% OG added to 2 mL of the substrate solution) separates the fluorophore from the quencher, resulting in an increase in fluorescence (up to 40-fold). For the kinetic measurements rates were determined at substrate conversions less than 10%. The kinetic parameters K_M and k_{cat} were determined from the resulting hyperbolic saturation curves.

Preparation of the Affinity Chromatography Resin. NHS-activated HiTrap Sepharose (2.5 mL of swollen matrix corresponding to 185 mg of dried resin) was modified with 1,3-diaminopropane, yielding amino-functionalized resin with an amine content of 166 μmol/g as described by Tegge and Frank (*20*). The peptide acetyl-Ala-Lys-(D)Arg-Val-Gly-(β)-Ala was synthesized onto this resin following the procedure for the peptide synthesis described by Gast et al. (*21*) with the following modifications. Synthesis was carried out using a Labortech SP640 peptide synthesizer. All coupling steps were extended to 2 h, and the coupling reactions were monitored using the Kaiser test (*22*). After completion of the synthesis the protecting groups were removed by treatment of the resin with 2 mL of a solution of 2% (v/v) water and 3% (v/v) triisobutylsilane in TFA overnight. The resin was washed extensively with DMF and ethanol and stored in ethanol at −20 °C.

Affinity Purification of OmpT. OmpT was expressed in *E. coli* DH5α transformed with plasmid pND9 as described by Kramer et al. (*11*). Cells of a 100 mL culture grown overnight were collected by centrifugation and resuspended in 10 mL of an ice-cold buffer of 20 mM Tris, pH 8.3. The cells were lysed by sonication. The detergent OG was added to a final concentration of 2% (w/v). Resin [1 mL of a 50% suspension (v/v) in ethanol] was washed once with 10 mL of 1 M Tris, pH 8.3, and three times with 10 mL of 10 mM Tris, pH 8.3, each. The lysate was incubated with resin on a rotating wheel for 30 min at 4 °C. The supernatant was carefully removed, and the resin was transferred to a column. The column was washed with 10 mL of buffer A (2% OG in 10 mM Tris, pH 8.3). OmpT was eluted by a linear gradient of 0−100 mM N^{α}-benzoyl-L-arginine ethyl ester (BAEE) in buffer A (2 × 10 mL), and 1 mL fractions were collected. The enzymatic activitiy in each fraction was determined in the fluorometric assay [at 10 μM Abz-Ala-Arg-Arg-Ala-Dap-(dnp)-Gly]. Fractions containing OmpT were pooled and dialyzed twice against 20 volumes of buffer A at 4 °C. Samples collected during the purification were analyzed on SDS−PAGE.

Table 1: Kinetic Parameters of Substrates of OmpT

substrate[a]	mass calcd (Da)	mass measd[b] (Da)	K_M[c] (μM)	k_{cat}[c] (s^{-1})	k_{cat}/K_M[c] ($\times 10^6$ $M^{-1}\cdot s^{-1}$)
(1) Fl-A-R-R-A-Qn-G	900.87		3.1	3.2	1.0
(2) Fl-R-R-Qn-G	758.73	758.30			0.001
(3) Fl-A-R-R-Qn-G	829.80	829.51			0.002
(4) Fl-R-R-A-Qn-G	829.80	829.51	1.3	1.3	1.0
(5) Fl-A-K-R-A-Qn-G	872.86		8.3	12.3	1.5
(6) Fl-A-R-K-A-Qn-G	872.86		10.4	10.2	1.0
(7) Fl-A-K-K-A-Qn-G	844.85		52	39.6	0.8
(8) Fl-A-R-I-A-Qn-G	857.97				<0.001
(9) Fl-A-R-R-I-Qn-G	942.96				<0.001
(10) Fl-A-R-R-V-Qn-G	928.93				0.06
(11) Fl-I-R-R-A-Qn-G	942.96		3.6	1.4	0.4

[a] The single letter code for the amino acid residues is used. Fl and Qn are abbreviations for the Abz fluorophore and the Dap(dnp) quencher, respectively. [b] Molecular masses determined with MS were within 0.5 Da accuracy. [c] Errors in the kinetic parameters are 10%.

RESULTS

Substrate Specificity of OmpT. Recently, we described two new assays to measure the enzyme activity of OmpT (*11*), and we found that OmpT displays only very low activity toward an Arg-Arg dipeptide. With the introduction of flanking Ala residues OmpT displays a large increase in both affinity and activity. In this study the tetrapeptide Ala-Arg-Arg-Ala was the starting point for further analyses of the substrate specificity. To determine the kinetic parameters, a fluorescent Abz group and a quenching dnp group attached to the side-chain amino function of Dap were introduced in the substrate at the N- and C-termini, respectively (substrate 1 in Table 1). The substrate binds to OmpT with an affinity of 3.1 μM, and the enzyme has a turnover number of 3.2 s^{-1} (Table 1). In substrate 2 lacking the flanking Ala residues there is a large decrease in the specificity constant k_{cat}/K_M. The large loss in affinity prohibited the individual determination of K_M and k_{cat}. The introduction of an Ala residue at P_2 [nomenclature according to Schechter and Berger (*23*)] leading to substrate 3 did not improve the specificity constant. In contrast, solely the introduction of Ala at P_2' in substrate 4 resulted in kinetic parameters comparable to those of the starting substrate 1. Replacement of the flanking alanines by glycines resulted in a large decrease in activity of OmpT toward this substrate, suggesting that OmpT has certain preferences for the type of flanking residues (data not shown). The results obtained with substrates 1−4 indicate that the sequence Arg-Arg-Ala is the minimal substrate requirement.

Design of the SPOT Peptide Library. To determine the

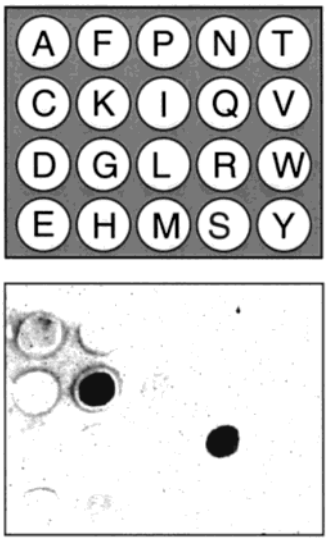

FIGURE 1: Fluorescent image of the SPOT library screened with trypsin. The peptides had the following composition, acetyl-Dap-(dnp)-Ala-Gly-Xxx-Ala-Lys(Abz)-Gly, and were synthesized directly onto the cellulose membrane (see text). The identity of the amino acid at position Xxx was different for each SPOT and is given in the top panel. Incubation of the SPOT membrane with bovine pancreatic trypsin (2 mg/mL in 20 mM Tris, pH 8.3, 10 mM $CaCl_2$) resulted in highly fluorescent SPOTs for Arg and Lys (bottom panel).

preference of OmpT for the amino acid residues present in the substrate, we used SPOT libraries. In initial trials, peptides with the sequence acetyl-Dap(dnp)-Ala-Arg-Arg-Ala-Lys(Abz)-Gly were synthesized directly onto the cellulose membrane. Under UV light the membrane SPOTS were not fluorescent. If OmpT would cleave these peptides at the recognition site, the SPOTS should become fluorescent; however, no fluorescence was observed. To validate the method, trypsin was used as a control. Incubation of the membrane with trypsin at 2 mg/mL resulted in a rapid development of highly fluorescent SPOTS due to cleavage after one of the basic residues, resulting in removal of the dnp quencher (data not shown). In a library with peptides of the general sequence Dap(dnp)-Ala-Gly-Xxx-Ala-lys-(Abz)-Gly, trypsin was only active toward peptides on the SPOTS with Xxx corresponding to Arg and Lys (Figure 1), in agreement with the known substrate specificity for cleavage after a basic amino acid. The absence of OmpT activity toward the peptides could result from shielding of the substrate by crowding on the solid support. To test this hypothesis, the amount of peptide per SPOT was varied using mixtures of Boc-Gly-Pfp and Fmoc-Gly-Pfp in the first coupling step. The reaction of the Boc derivative results in chain termination. The ratio of the two compounds was varied between 100:1 and 1:100. Lowering the amount of peptide per SPOT did not lead to detectable activity of OmpT. For trypsin we could see little effect on activity although below 10% peptide present the detection of fluorescence became difficult.

Whereas trypsin is a relatively small and water-soluble enzyme, OmpT is larger and, more importantly, is solubilized in detergent micelles. We reasoned that this large enzyme−micelle complex with an estimated mass of 180 kDa may limit the accessibility of the enzyme to the peptide on the solid support. To improve presentation of the substrate to the enzyme, we incorporated the linker 6-aminocaproic acid, which has previously been successfully used for enzymatic cleavage of peptides on a cellulose support (*24*). The introduction of up to five of these linker units did not result in detectable activity of OmpT toward the peptides present at the tip of the linker. A potential drawback of the caproic acid linker is the hydrophobic character of the pentane chain. Therefore, we tested whether polyoxyethylene glycol (PEG) was a suitable linker. Cellulose membrane SPOTS were derivatized with Fmoc-NH-PEG-CO_2NHS, a compound with 70 oxyethylene glycol moieties. The synthesis step with this large building block had a strongly reduced coupling efficiency of only 20% compared to over 97.5% for single amino acid building blocks. Peptides displayed on the PEG linker were efficiently cleaved by both trypsin and OmpT, indicating that the long polar PEG linker strongly improves the accessibility of the peptides for the large macromolecular enzyme complex. The conditions, such as pH, ionic strength, temperature, enzyme concentration, and type and concentration of detergent and LPS, were optimized for the cleavage of the peptides on the solid support. During this process it appeared that the cleavage results were reproducible and consistent with the outcome of the library screen discussed below.

Library Screen. Libraries were synthesized on the basis of the starting sequence, acetyl-Dap(dnp)-Ala-Arg-Arg-Ala-Lys(Abz)-Gly. In the P_1 library the first residue N-terminal of the scissile bond was varied according to the scheme presented in Figure 2. The P_2, P_1', and P_2' libraries had variations at the corresponding positions in the starting sequence. After incubation of the libraries with OmpT, several SPOTS became fluorescent, indicative of preferential cleaveage by OmpT (Figure 2). The peptide libraries were incubated with a relatively high concentration of OmpT (10 μM). Therefore, the experiments represented a selection toward k_{cat}, whereas the affinity for the substrate was not used as a selection criterion. Moreover, K_M is ill-defined for immobilized peptides in the SPOT technique. A good correlation was found between k_{cat} values obtained for the free peptides and their effect in the SPOT analysis. For the P_1 library the cleavage was highly selective for peptides with a positively charged Lys or Arg present at this position, with the SPOT of the Lys-containing peptide being most fluorescent. For the P_1' position the preference of OmpT was less exclusive, with Lys being preferred and a number of other amino acids also being allowed. For P_2' there is a clear preference for a branched and hydrophobic Ile or Val and Ala. For P_2 there is a preference for hydrophobic residues (Ala, Ile, Phe), with other neutral residues being accepted as well. Interestingly, the SPOT for Lys in the P_2 library is clearly positive. Given the preference for neutral residues at P_2, a lysine here is probably an artifact where cleavage occurs directly after this Lys, since substrate 4 is a good substrate for OmpT. This implies that the Lys in the P_2 library represents a false positive, whereby this residue actually is a P_1 residue. For all libraries there was no or hardly any cleavage observed for peptides containing a negatively charged Asp or Glu or for a bulky, aromatic residue such as Trp. The results of the screen of the SPOT libraries are summarized in Table 2.

Resynthesis of Library Substrates and Kinetic Evaluation. The library screen was carried out with the peptides still attached to the solid support to facilitate the identification.

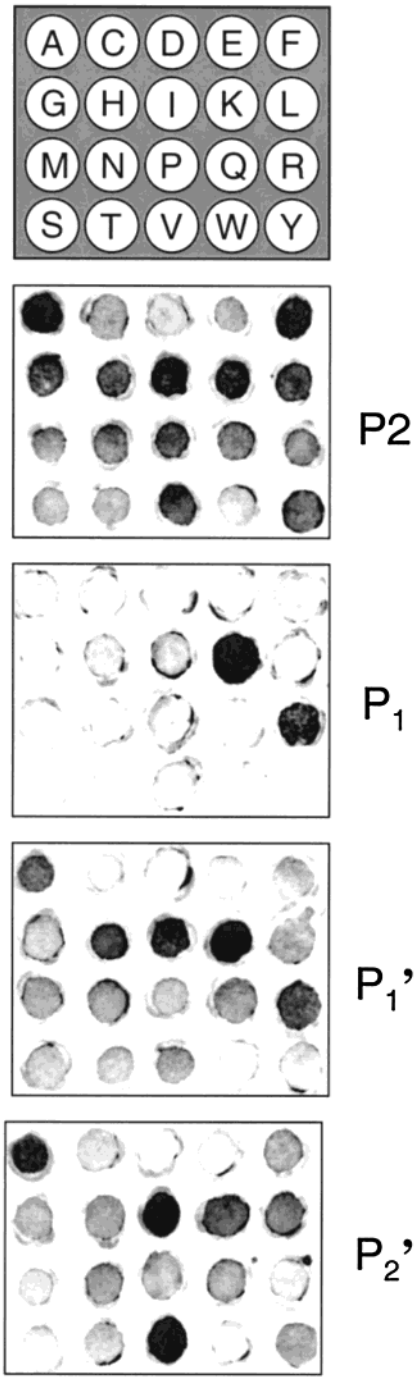

FIGURE 2: Fluorescent images of the SPOT libraries after incubation with OmpT. The peptides had the following composition: acetyl-Dap(dnp)-Ala-Arg-Arg-Ala-Lys(Abz)-GlyPEG-support. In each library one position in the recognition sequence Ala-Arg-Arg-Ala (P_2-P_1-P_1'-P_2') was varied according to the scheme in the top panel. The varied position in each library is indicated at the right side of each panel.

To validate this approach, several peptides were resynthesized and were subjected to kinetic measurements in solution. The activity of OmpT toward the individual peptides was determined at various substrate concentrations (typically 1−10 μM), and the kinetic parameters k_{cat} and K_M were determined by fitting of the resulting hyperbolic saturation curves (Figure 3). In the SPOT library peptides with Lys or Arg at P_1 were degraded by OmpT, with the peptide with Lys producing the brightest fluorescent SPOT (Figure 2). In solution OmpT displayed higher activity toward substrate 5 with a Lys at P_1 when compared to the starting substrate 1, but the enzyme displayed a decreased affinity toward substrate 5, resulting in only a small change in the specificity constant (Table 1). A similar observation was made for Lys at P_1' (compare substrate 6 with substrate 1). The introduction of lysines at both P_1 and P_1' resulted in accumulation of the effects for each single substitution, yielding substrate 7 that displayed a very high k_{cat} of 12 times that of the starting substrate. The increase in k_{cat} coincided with a strong reduction in apparent binding affinity. At the P_1' position

Table 2: Substrate Specificity of OmpT As Determined by the SPOT Library[a]

library	preferred (95−100%)	favored (50−95%)	allowed (5−50%)	not allowed (0−5%)
P_2	Ala (100) Phe (98) Ile (96)	Lys[b] (90) Leu (68) Val (64) Pro (61) His (60) Gly (55)	Tyr (41) Asn, Gln (31) Arg (20) Met (16) Cys (9)	Glu (4) Asp, Ser, Thr, Trp (<1)
P_1	Lys (100)	Arg (59)		Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val (<1)
P_1'	Lys (100)	Ile (68) His (56) Arg (52) Ala (43)	Gln (28) Asn, Val (22) Met (20) Leu (18) Phe, Pro (10) Ser, Thr (7)	Gly (5) Asp, Cys, Glu, Trp, Tyr (<1)
P_2'	Ile, Val (100)	Ala (88)	Lys (49) Leu (30) Asn (14) Tyr (8) His (6)	Gln (4) Phe (2) Pro (1) Arg, Asp, Cys, Glu, Gly, Met, Ser, Thr, Trp (<1)

[a] Numbers in parentheses represent the intensities of the SPOTs relative to the intensity of the darkest SPOT for the corresponding library. Intensities were measured using a Bio-Rad GelDoc 2000 system with Quantity One software. [b] Lys at position P_2 represents a false positive (see text for explanation).

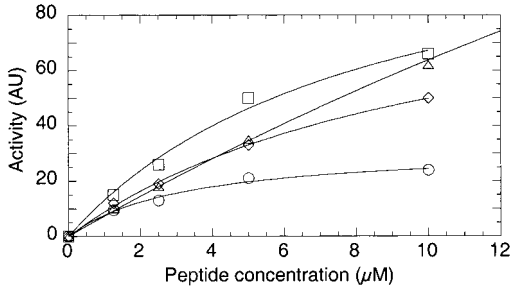

FIGURE 3: Kinetic analysis of OmpT toward representative peptides from the SPOT library. The substrate had the following general composition: Abz-Ala-Xxx-Yyy-Ala-Dap(dnp)-Gly. The activity of OmpT was measured by fluorescence at various concentrations of substrate as described in Experimental Procedures, and activities are given in arbitrary fluorescent units. The data shown are for substrate 1 (Xxx-Yyy = Arg-Arg; circles), substrate 5 (Lys-Arg, squares), substrate 6 (Arg-Lys, diamonds), and substrate 7 (Lys-Lys, triangles). The data were fitted with a hyperbolic saturation curve using Kaleidagraph. For substrate 7, data were collected at higher substrate concentration as well to obtain an accurate value for K_M.

Table 3: Kinetic Parameters of Inhibitory Peptides of OmpT

peptide[a]	mass calcd (Da)	mass measd[b] (Da)	K_i[c] (μM)
(12) Fl-A-(D)R-(L)R-A-Qn-G	900.87	900.68	>100
(13) Fl-A-(L)R-(D)R-A-Qn-G	900.87	900.55	19
(14) Fl-A-(D)R-(D)R-A-Qn-G	900.87	900.61	>100

[a] The single letter code for the amino acid residues is used. Fl and Qn are abbreviations for the Abz fluorophore and the Dap(dnp) quencher, respectively. [b] Molecular masses determined with MS were within 0.5 Da accuracy. [c] Errors in the kinetic parameters are 20%.

Ile was positive in the SPOT library screen (Table 2). However, for the corresponding substrate 8 OmpT displayed very low activity in solution; it appears that this fluorescent SPOT represents a false positive for unknown reasons. For P_2' OmpT has a clear preference for small hydrophobic amino acids (e.g., Ala, Ile, and Val; see Table 2). The three substrates (1, 9, and 10) were compared, and it turned out that amino acids bigger than Ala were poor substrates with only 6% and 0.1% activity compared to the starting substrate 1 for Val and Ile, respectively. The sensitivity in the SPOT library screen is apparently sufficiently high to detect these low activities. A clear preference for Ile and Val at P_2' was indicated by the SPOT technique, which could not be confirmed, however, for the free peptides. Possibly kinetics on immobilized peptides are at variance with free peptide kinetics due to solubility effects, which will require more detailed investigation. For P_2 an Ile instead of Ala was tested as substrate (11). This replacement had little effect on the kinetic parameters, in agreement with the observation that an amino acid at P_2 can completely be omitted (substrate 4).

Stereospecificity of OmpT. The stereospecificity of OmpT for recognition of the residues around the scissile bond was studied using substrates in which the residues at P_1 or P_1' were replaced by D-amino acids (peptides 12−14, Table 3). OmpT did not display any hydrolytic activity toward these peptides. Even when the peptides were incubated with a large excess of enzymes for several days, there was no sign of degradation using thin-layer chromatographic analysis (data not shown). For these peptides the inhibition constant K_i was determined by measuring kinetics with substrate 1 at various concentrations of the inhibitory peptides. Interestingly, the peptides 12 and 14, having a D-Arg at P_1 did not bind competitively to the substrate in the active site of the enzyme, suggesting that OmpT is highly specific for this position in the substrate. For peptide 13 with the D-Arg at P_1' a K_i of 19 μM could be determined (Table 3), which indicates that this peptide binds with 6-fold lower affinity than the substrate in the active site of OmpT. This result suggests that OmpT is much less descriminative for the P_1' position.

Affinity Chromatograpy. The observation that peptides with D-Arg at P_1' are good competitive inhibitors was used to develop an affinity chromatography resin for the purification of OmpT. The sequence acetyl-Ala-Lys-(D)Arg-Val-Gly was selected as a noncleavable, substrate-like ligand for OmpT. This ligand was directly synthesized on Sepharose resin. The resin was used to purify OmpT from a bacterial lysate. *E. coli* DH5α cells transformed with plasmid pND9 produced large amounts of OmpT. On SDS−PAGE the overexpressed OmpT migrated at 29 kDa (Figure 4, lane 2).

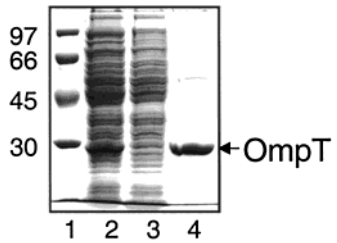

FIGURE 4: SDS−PAGE analysis of the affinity purification of OmpT. Proteins were analyzed on 11% SDS−PAGE and Coomassie Brilliant Blue stained (*29*). Lanes: 1, marker proteins with molecular mass indicated on the left; 2, bacterial lysate of DH5α cells overexpressing OmpT encoded on plasmid pND9; 3, supernatant obtained after incubation of the lysate with affinity chromatography resin; 4, OmpT eluted from the resin with BAEE.

The bacterial cells were lysed by sonication, and the membrane proteins were solubilized by the addition of the detergent OG. Incubation of the lysate with resin resulted in the quantitative binding of all OmpT present, based on activity measurement (data not shown) and SDS−PAGE (Figure 4, lane 3). The resin was washed with buffer, and bound OmpT was eluted by a linear gradient of BAEE. BAEE is a derivative of arginine and binds competitatively to OmpT with a K_i of 1 mM. Fractions from 45 to 65 mM BAEE, containing 86% of the original activity, were pooled and dialyzed to remove BAEE. From 100 mL of bacterial culture 1.7 mg of OmpT was isolated, and the protein was over 90% pure as determined by SDS−PAGE (Figure 4, lane 4). For the affinity chromatography resin a loading capacity of at least 3.4 mg of OmpT/mL of swollen beads was estimated for crude protein mixtures.

DISCUSSION

In this work a combinatorial peptide library on a solid support was used to investigate catalytic properties of OmpT. Solid supports have the advantage that synthesis and screening are carried out on the same support, either resin beads or cellulose membranes (SPOT). The latter approach has the additional advantage that the library is positionally addressed; that is, for each position on the solid support the chemical identity is known a priori. Solid supports have been used for the determination of the substrate specificity of enzymes such as the 38 kDa PKA kinase (SPOT membrane; *10*) and the 27 kDa protease subtilisin (resin beads; *25*). A drawback of the existing methods is that even with the highly permeable polar SPOCC resin, specifically designed for enzymatic solid-phase methods (*25*), there still are limitations concerning accessibility to substrates for large enzymes. Peptides on SPOCC resin could be completely cleaved by subtilisin but not by the 72 kDa matrix metalloprotease MMP-9 (*25*). It has been suggested that the usage of longer PEG chains for the preparation of the SPOCC resin could overcome these permeability problems (*25*). For SPOT membranes we observed similar results. Peptides, synthesized directly onto the cellulose support, were sequence specifically cleaved by the 26 kDa protease trypsin but not by OmpT. This fully integral membrane enzyme has a molecular mass of 33.5 kDa. However, the presence of detergents, needed to solubilize the protein, gives rise to a much larger macromolecular complex with an estimated molecular mass of 180 kDa (*14*). The detergent is likely present as a flexible continuous phase surrounding the proposed, membrane-

spanning β-barrel as is the case for the structurally related porins (*26*). To overcome the accessibility problem, a linker was introduced to present the peptide toward the enzyme. The hydrophobic ϵ-aminocaproic acid linker did not improve accessibility, even not when up to five of such units were used (corresponding to roughly 40 carbon−carbon bonds). This linker has been successfully used for peptide cleavage by chymotrypsin and papain (*24*), but these enzymes are relatively small (26 and 23 kDa, respectively). One could envisage two reasons why the linker was not successful in the case of OmpT: (1) the hydrophobic nature of the pentane chain, leading to clustering and thereby reducing accessibility, and (2) the limited length of the linker. To circumvent both problems, we introduced a long, polar PEG linker (corresponding to roughly 200 carbon−carbon bonds). Peptides displayed on the tip of this linker were readily cleaved by OmpT. Interestingly, the accessibility problems for large enzymes were not reported for antibodies, despite the large molecular mass (150 kDa) of these glycosylated proteins. Actually, the first application of the SPOT technique was the determination of the sequence of the epitope recognized by a monoclonal antibody (*9*). For antibodies the binding to the peptides on the solid support is detected by highly sensitive immunological methods, whereas for enzymes it is likely that a considerable amount of peptide needs to be processed before a fluorescent signal develops.

With the SPOT libraries clear rules for the specificity of OmpT were revealed. OmpT is highly specific for cleavage between two basic residues and has preference for an alanine following this sequence, leading to the consensus sequence (Arg/Lys)↓(Arg/Lys)-Ala. OmpT is a true endoprotease, since a dibasic sequence at either the N- or C-terminus of a peptide is not cleaved (*14*). There is no clear preference for any amino acid residue at P_2, and even the Abz fluorophore is accepted at this position. Apparently, a peptide linkage N-terminal of the dibasic motive is minimally required.

The kinetic analysis of substrates with either Lys or Arg at P_1 or P_1' revealed a clear inverse correlation between activity and affinity. An Arg-Arg substrate was bound with high affinity and was cleaved at moderate rates, whereas a Lys-Lys substrate displayed reduced binding affinity (17-fold) concurrent with a 12-fold increase in activity. A reduced affinity correlated with a high turnover rate may point to a dominant influence of the turnover rate constant in the Michaelis−Menten constant. Consequently, the dissociation rate of the enzyme−substrate complex is relatively slow. When also release of reaction products is slow, the turnover rate will be negatively affected. This will lead to high-affinity constants and low turnover rates, as were found for the Arg-Arg substrates.

Most remarkably, the introduction of a negatively charged amino acid at either P_2 or P_2' diminishes all activity of OmpT toward such substrates. This effect has also been observed for the substrate Abz-A-R-R-A-Tyr(NO_2)-G (*11*). OmpT displays optimal activity at pH 6.5 toward this substrate. Above pH 8, where the side-chain hydroxyl group of the nitrotyrosine present at P_3' is deprotonated, OmpT loses all activity toward the peptide. These results suggest that long-range electrostatic interactions play an important role in the formation of the enzyme−substrate complex. Recently, it has been shown that such long-range electrostatics are an important determinant for the association rates of protein−protein complexes, without much effect on dissociation rates (*27*). We examined the importance of such electrostatic interactions between the substrate and OmpT. An increase of the ionic strength by the addition of 50 mM NaCl already had a significant effect on affinity (5-fold reduction) with only a minor change in activity (unpublished results). Furthermore, OmpT activity heavily relies on the presence of the highly acidic lipopolysaccharide (LPS, net charge of −6 at neutral pH) (*11*). Presumably, LPS associates with OmpT and favorably contributes to the electrostatic potential around the enzyme leading to high turnover of substrate.

OmpT is not or is poorly inhibited by commercially available inhibitors such as phenylmethanesulfonyl fluorides (*14*). We observed that D-amino acid containing peptides based on the optimal substrate sequence were not degraded by OmpT. The enzyme absolutely requires L-amino acids at the P_1 position for turnover. In competition experiments in the presence of substrate, it turned out that peptides with a D-Arg at P_1 did not bind to the active site of OmpT. Surprisingly, the D-stereoisomer at P_1' was accepted by the enzyme, although OmpT likely has a specific S_1' binding site for the recognition of the basic amino acid at P_1'. An affinity chromatography resin based on such inhibitory peptide was developed, which allowed for the one-step purification of OmpT from a complex mixture. The introduction of sequence alterations in the immobilized ligand might be useful for the selection of OmpT variants displayed at the bacterial cell surface with new specificities engineered by directed evolution techniques.

OmpT has been proposed to fulfill a protective role against the action of antimicrobial peptides such as protamine (*13*). It has been shown that the presence of OmpT is essential for the virulence of *E. coli* strains in the human urinary tract (*28*). The antimicrobial peptides are, in general, highly basic molecules that supposedly interact with the negatively charged cell surface of the bacterium made up of LPS molecules. Human protamine P_1, for example, is a 50 amino acid peptide that contains 24 arginine residues and no lysines. In general, protamines have a very high content of arginines, and this property might be reflected in the specificity of OmpT with high affinity toward an Arg-Arg sequence.

Structural analysis of the protease would provide a basis for understanding the high specificity of the interaction of OmpT with the substrate. For such exercise an overexpression system for the production of OmpT has been set up (*11*). This approach has provided us with large amounts of pure OmpT, and the protein has recently been crystallized (L. Vandeputte-Rutten and Dr. P. Gros, unpublished results). The forthcoming structure will hopefully provide insight in specific substrate recognition and catalytic mechanism.

CONCLUSION

To the best of our knowledge this is the first time that peptide libraries on solid support have been used to determine the substrate specificity of a large membrane enzyme. Modification of the solid supports by a long, polar linker proved to be essential for this application.

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