

The Binding Specificity of OppA Determines the Selectivity of the Oligopeptide ATP-binding Cassette Transporter*

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The purification and functional reconstitution of a five-component oligopeptide ATP-binding cassette transporter with a remarkably wide substrate specificity are described. High-affinity peptide uptake are dependent on liganded substrate-binding protein OppA, which interacts with the translocator OppBCDF with higher affinity than unliganded OppA. Transport screening with combinatorial peptide libraries revealed that (i) the Opp transporter is not selective with respect to amino acid side chains of the transported peptides; (ii) any peptide that can bind to OppA is transported via Opp, including very long peptides up to 35 residues long; and (iii) the binding specificity of OppA largely determines the overall transport selectivity.

ATP-binding cassette (ABC)¹ transporters belong to a large superfamily of membrane proteins that are abundantly present among all kingdoms of life. Members of this family share a common four-domain architecture composed of two transmembrane domains that span the membrane several times in α -helical conformation and two membrane-associated nucleotide-binding domains that fuel the translocation process at the expense of ATP. The existence and arrangement of this four-domain structure has been confirmed by the high-resolution crystal structure of the vitamin B₁₂ ABC transporter BtuCD from *Escherichia coli* (1). In addition, bacterial ABC importers use substrate-binding proteins (SBPs), located at the extracellular surface or in the periplasmic space, to scavenge for substrate and subsequent delivery to the translocator. The individual subunits of ABC transporters can be present as separate proteins or fused together in almost any possible combination (2, 3).

The oligopeptide transport system (Opp) from *Lactococcus lactis* is a five-component ABC transporter composed of a membrane-anchored SBP, OppA, two transmembrane proteins, OppB and OppC, and two nucleotide-binding domains, OppD and OppF (4). OppA binds a peptide and delivers it to the translocator complex, where upon ATP hydrolysis the peptide

is transported into the cell. In addition to nutrition of the cell, in microorganisms the Opp system is also involved in various signaling processes including regulation of gene expression, chemotaxis, competence development, sporulation, DNA transfer by conjugation, and virulence development (reviewed in Ref. 5). In *L. lactis* (and other lactic acid bacteria), the Opp system is an essential part of the proteolytic system because it transports into the cell β -casein-derived peptides formed by the cell wall-associated protease PrtP, enabling the organism to grow in milk. On the basis of intracellular amino acid accumulation in peptide-containing media, the lactococcal Opp system has been proposed to transport peptides in the range of 4 to at least 18 residues (6).

The mechanism of peptide binding to OppA has been examined in detail using combinatorial peptide libraries and fluorescence techniques (7–9). OppA from *L. lactis* binds oligopeptides as long as 35 residues and has the highest affinity for nonameric peptides. OppA is not able to completely enclose peptides longer than 6 amino acid residues. The remaining residues stick out and interact with the protein surface actively, thereby contributing to the peptide binding affinity. This mechanism of substrate binding by OppA is different from that proposed for other SBPs that, on the basis of crystal structures, are thought to enclose the substrate completely (10). Recently, Charbonnel *et al.* (11) concluded, on the basis of OppA sequence comparisons and peptide utilization patterns of six *L. lactis* strains, that OppA is not the main determinant of peptide transport specificity of Opp. However, *in vivo* peptide uptake experiments are often complicated by rapid breakdown of internalized peptides by peptidases, subsequent efflux of labeled amino acids, and binding of peptides to the cell wall (6, 12). These complications prohibit any detailed analysis of transport kinetics, translocation mechanism, and substrate specificity of Opp.

Here, we describe an *in vitro* system for the study of peptide uptake via Opp. The purification and functional reconstitution in artificial membranes of this five-component ABC transport system enabled the analysis of the interaction of liganded and unliganded OppA with OppBCDF and determination of the overall transport specificity of Opp. Using radiolabeled combinatorial peptide libraries, we now demonstrate that the transport selectivity of Opp matches the binding specificity of OppA.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Growth Conditions—Plasmid pAMP42 is a pNZ8048-derivative with a 3804-bp NcoI-BamHI insert containing the *oppDFBC* genes under control of the nisin A promoter, yielding OppD, OppF, OppB, and C-terminal His₆-tagged OppC.² *L. lactis* NZ9000 was used as a host for pAMP42. Cells were grown in 5% (w/v) whey-permeate, 0.5% (w/v) yeast extract, 0.5% (w/v) glucose, and 65 mM potassium

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¹ The abbreviations used are: ABC, ATP-binding cassette; SBP, substrate-binding protein; DDM, dodecyl- β -D-maltoside; Ni²⁺-NTA, nickel-nitrilotriacetic acid.

² A. Picon, unpublished data.

P_i , pH 7.0, supplemented with 5 $\mu\text{g}/\text{ml}$ chloramphenicol in a volume of 10 liters in a fermentor with pH control (ADI 1065 fermentor; Applikon Dependable Instruments B.V., Schiedam, The Netherlands). The pH was kept constant at pH 6.5 by titrating with 2 M KOH. Transcription was induced at an OD_{660} of 2 by adding 1:1000 (v/v) of the culture supernatant of *L. lactis* NZ9700. Cells were harvested by centrifugation, washed with 100 mM potassium P_i , pH 7.0, resuspended in 100 mM potassium P_i , pH 7.0, plus 20% (v/v) glycerol and stored at -80°C . OppA was produced by *L. lactis* AMP2/pAMP31 as described (8).

Purification of Opp—Membrane vesicles were prepared by rupturing the cells with a high-pressure homogenizer (Kindler Maschinen AG, Zürich, Switzerland) in the presence of 20% (v/v) glycerol. OppBCDF-containing membranes were resuspended to 5 mg/ml of total membrane protein in 50 mM potassium P_i , pH 8.0, 200 mM KCl, and 20% (v/v) glycerol (buffer A) and solubilized with 0.5% DDM. After 12 min of centrifugation at $280,000 \times g$, the supernatant was diluted five times with buffer A and incubated with nickel-nitriloacetic acid (Ni^{2+} -NTA) resin (0.05 ml/mg of total membrane protein) for 1.5 h at 4°C while shaking. Subsequently, the resin was poured into a column and washed with 20-column volumes of buffer A, supplemented with 0.05% DDM and 10 mM imidazole. The proteins were eluted with 100 mM histidine, 0.05% DDM in buffer A, pH 7.0. OppA was purified as described (8), except that DDM was not exchanged for Triton X-100 during the washing and elution steps.

Immunoblotting and Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry—Proteins were separated by SDS-PAGE (12.5% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semidry electrophoretic blotting. Immunodetection was done using monoclonal antibodies raised against the six-histidine tag (Sigma) or polyclonal anti-OppD antibodies at titers of 1:3,333 and 1:10,000, respectively. Detection was performed by using the Western-Light chemiluminescence detection kit with CSPD as a substrate according to the manufacturer's instructions (Tropix Inc., Bedford, MA). Protein identification by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was done by performing in-gel tryptic digestion in combination with CNBr cleavage as described previously (13).

Membrane Reconstitution of the Opp System—Reconstitution of Opp was done in three steps. In step 1, liposomes (20 mg/ml in 50 mM potassium P_i , pH 7.0) of acetone/ether-washed *E. coli* lipid and L- α -phosphatidylcholine from egg yolk in a ratio of 1:3 (w/w) were extruded through 400-nm pore size polycarbonate filters, diluted to 4 mg/ml, and titrated using Triton X-100 (14). Purified OppBCDF in elution buffer was added at a 1:100 (w/w) protein:lipid ratio, and the mixture was incubated for 30 min at room temperature while shaking gently. To remove the detergent, 40 mg/ml wet weight polystyrene beads (BioBeads SM2) was added, followed by a 15-min incubation at room temperature. Biobeads SM2 were added four more times, and the incubation times were 15 min, 30 min, overnight and 1 h at 4°C . After dilution five times with 50 mM potassium P_i , pH 7.0, proteoliposomes containing OppBCDF were collected by centrifugation for 1.5 h at $150,000 \times g$ and 4°C and stored in liquid nitrogen. In step 2, OppA was anchored to the liposomes via its lipid moiety, and the final concentration of OppA after membrane reconstitution was estimated from the binding of peptide, as described by Detmers *et al.* (8). Unless stated otherwise (see the legend to Fig. 2), an OppA concentration of 0.35 nmol/ m^2 of membrane surface was used. In step 3, an ATP-regenerating system, composed of 9 mM ATP, 9 mM MgSO_4 , 24 mM creatine phosphate, and 2.4 mg/ml of creatine kinase, was included into Opp-containing proteoliposomes by two freeze-thaw cycles. After extrusion through 400-nm pore size polycarbonate filters, the proteoliposomes were washed twice and resuspended in 50 mM potassium P_i , pH 7.0, to a concentration of 20 mg/ml of lipid.

Uptake of Bradykinin—Transport assays were started by diluting proteoliposomes into 200 μl of 50 mM potassium P_i , pH 7.0 (final lipid concentration, 4 mg/ml) containing [^3H]bradykinin at various concentrations. Uptake experiments were performed at 30°C . At given time points, 40 μl of samples were taken and mixed 1:1 with 200 μM unlabeled bradykinin in 50 mM potassium P_i , pH 7.0. The sample was then diluted with 2 ml of ice-cold 8% (w/v) PEG-6000, filtered over 0.15- μm pore size cellulose acetate (OE65) filters (Schleicher & Schuell, Dassel, Germany), and washed twice with 2 ml of ice-cold 8% (w/v) PEG-6000. The amount of radioactivity retained on the filter was determined by liquid scintillation counting. The concentrations of bound and free bradykinin and of liganded and unliganded OppA were calculated from the total concentration of OppA, the total concentration of bradykinin, and the K_D for bradykinin binding of 0.1 μM (7, 8). For the calculation of

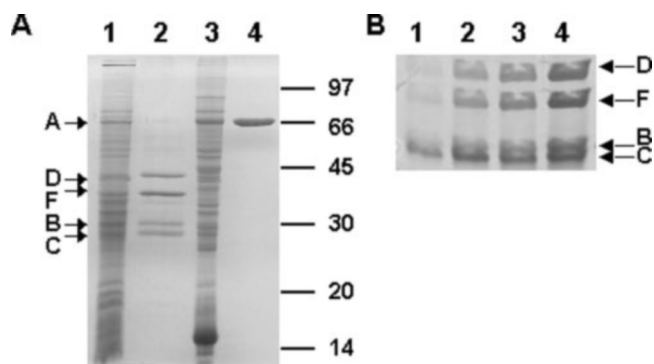


FIG. 1. Amplification and purification of the Opp system. A, overexpressed (membrane vesicles, lanes 1 and 3) and purified (lanes 2 and 4) OppBCDF and OppA are shown, respectively. The positions of molecular weight marker proteins are indicated (kDa). B, purification of OppBCDF with 10, 5, 2.5, and 0 mM imidazole present (lanes 1, 2, 3, and 4, respectively) during binding to the Ni^{2+} -NTA column.

peptide accumulation levels, an internal volume of 0.5 $\mu\text{l}/\text{mg}$ of lipid was used, which corresponds to 50 $\mu\text{l}/\text{mg}$ of OppBCDF.

Screening of Combinatorial Peptide Libraries—Uptakes were done in a custom-built microwell filtration unit (8×6 wells, 300- μl volume each) which allowed a 4-fold scaling down of the assay volumes as compared with the setup described above. After filtration over 0.15- μm pore size cellulose acetate (OE65) filters (Schleicher & Schuell), samples were washed twice with 200 μl of 50 mM potassium P_i , pH 7.0. The amount of radioactivity retained on the filter was determined with gamma counting. Randomized peptide libraries used in this study have been described previously (8, 15). Peptides were iodinated as described (16) using chloramine T, and free iodine was removed by DOWEX 1X8 (Serva).

Concentration of OppA at the Membrane Surface—The two-dimensional concentration of lipid-anchored OppA (in nmol/ m^2) at the membrane surface was calculated from the lipid and OppA concentration and a lipid surface area of 0.6 nm^2 . For comparison of affinities with other SBP-dependent ABC transport systems, this two-dimensional concentration was converted into a local concentration of OppA (in μM). For this, a shell of ~ 3.4 nm around the liposomal surface was used (estimated from the crystal structure of OppA from *Salmonella typhimurium*; 10).

Miscellaneous—Protein concentrations were determined according to the method of Lowry *et al.* (17), using bovine serum albumin as a standard. Peptide concentrations were determined using the bicinchoninic acid assay (Pierce). The concentrations of purified OppBCDF or OppA were determined spectrophotometrically by measuring the absorption at 280 nm and using extinction coefficients of 0.990 and 1.605 ($\text{mg}/\text{ml})^{-1} \text{cm}^{-1}$, respectively.

RESULTS

Purification of OppBCDF—The components of the oligopeptide translocator complex were amplified in *L. lactis* using the nisin expression system (18, 19). OppBCDF was overproduced to an estimated level of $\sim 5\%$ of total membrane protein content after 2 h of induction (Fig. 1A). Membrane solubilization and purification were done with DDM as a detergent. Initial conditions for maintaining an intact OppBCDF complex during purification were screened for ionic strength (0–500 mM KCl) and pH (6.0–8.0) using 20 mM imidazole, 0.05% DDM, and 20% (v/v) glycerol as a basal medium. This did not lead to purification of the complete translocator but resulted in purification of the His₆-tagged component OppC only (data not shown). However, when imidazole was omitted from the buffer during binding of the complex to the Ni^{2+} -NTA resin, the OppBCDF proteins could be obtained in an approximate 1:1:1:1 ratio (Fig. 1B). Varying the glycerol concentration from 0 to 40% (v/v) did not have any effect on the domain stoichiometry obtained after purification (data not shown). The individual translocator subunits were identified by Western blotting, using monoclonal anti-His-tag or polyclonal anti-OppD antibodies for detection of OppC or OppD, respectively (data not shown). OppF was iden-

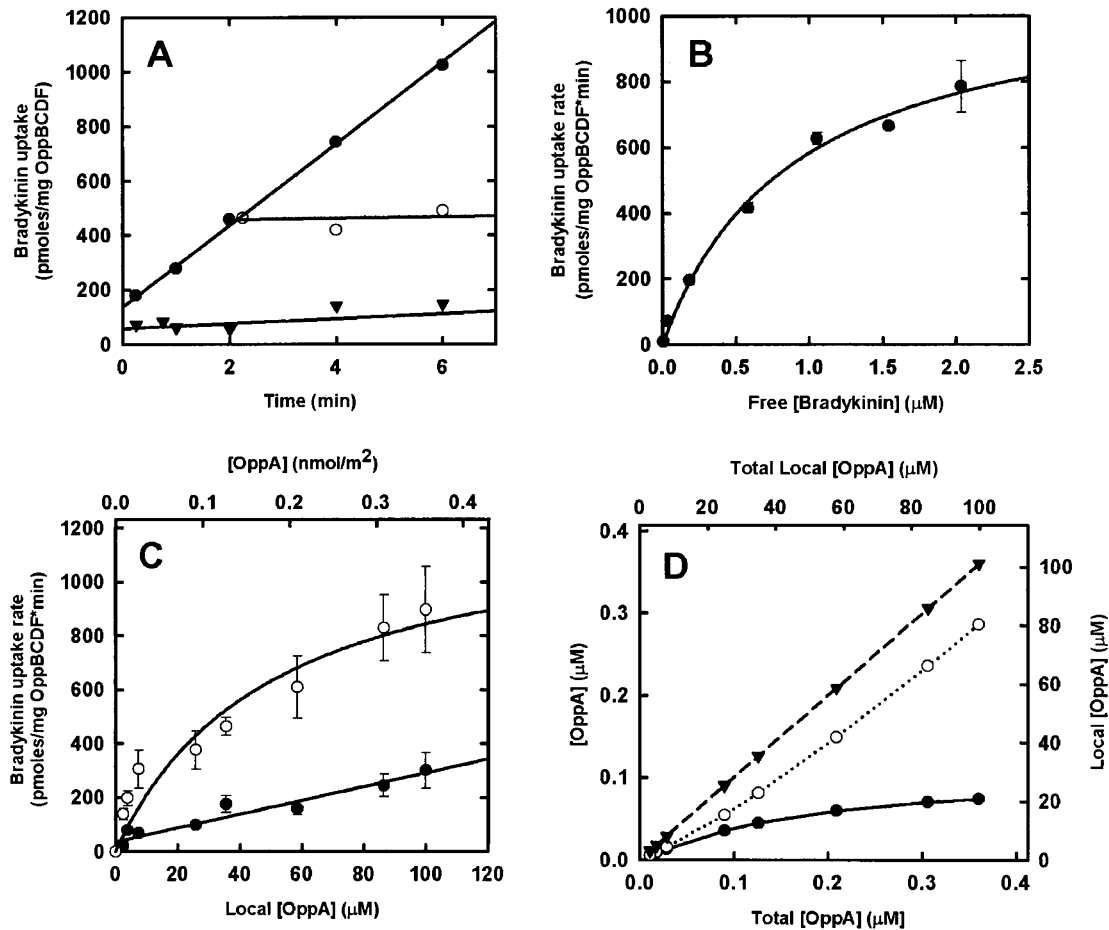


FIG. 2. **Functional reconstitution and characterization of the Opp system.** A, [^3H]bradykinin ($0.7 \mu\text{M}$) uptake was assayed in the presence (●) and absence (▼) of ATP plus the ATP-regenerating system enclosed in the proteoliposomes. The open circles denote samples to which $100 \mu\text{M}$ unlabeled bradykinin was added after 2 min of [^3H]bradykinin uptake. B, kinetics of [^3H]bradykinin uptake. Initial rates of uptake were calculated from the linear part of the curves as depicted in A. Data were fitted with the Michaelis-Menten equation. C, uptake rates at increasing OppA levels were determined in the presence of saturating (○, $1.5 \mu\text{M}$) and low (●, $0.1 \mu\text{M}$) [^3H]bradykinin concentrations. The concentration of membrane-anchored OppA was varied from 0 to $0.36 \mu\text{M}$, which corresponds to two-dimensional concentrations of 0 to 0.35 nmol/m^2 of membrane surface (top axis) and local concentrations at the membrane of 0 to $100 \mu\text{M}$ OppA (bottom axis, see "Experimental Procedures" for details of the calculations). The error bars indicate the standard deviation of experiments performed in duplicate. D, calculation of the concentrations of liganded (●) and unliganded (○) OppA at a bradykinin concentration of $0.1 \mu\text{M}$. The total OppA concentration (▼) was varied from 0 to $0.36 \mu\text{M}$ (as in C). For easy comparison, the local concentration of OppA at the membrane is shown on the top axis.

tified by peptide mapping with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an in-gel digestion procedure developed for integral membrane proteins (13). The mass spectrum of the putative OppB band did not yield enough peaks to unambiguously confirm the identity of this protein.

Functional Reconstitution of the Opp System—Membrane reconstitution of the oligopeptide transporter was done via a three-step procedure. First, the purified translocator complex OppBCDF was incorporated into Triton X-100 destabilized liposomes. Thereafter, purified OppA was anchored to the outside of OppBCDF containing liposomes via its N-terminal lipid modification by absorbing the purified protein in 0.05% DDM to the (proteo)liposomes and removal of residual detergent with BioBeads. This resulted in proteoliposomes that contained all five proteins of which the Opp system is composed. Next, after inclusion of ATP and the ATP-regenerating system into the vesicle lumen by two freezing-thawing cycles, bradykinin transport could be measured (Fig. 2A). Control experiments indicated that bradykinin does not interact specifically with the liposomes, and uptake was not observed when AMP-PNP, a nonhydrolyzable ATP analog, was used instead of ATP, ruling out specific effects on the membrane of the components of the ATP-regenerating system (8, 20). To increase the signal-to-

noise ratio, background binding of radiolabeled substrate to OppA was diminished by chasing with a large excess (~ 100 -fold) of unlabeled bradykinin. Chasing background binding did not lead to an increase of the amount of label inside or efflux of accumulated [^3H]bradykinin from the proteoliposomes (Fig. 2A). The apparent K_m of transport for bradykinin was $0.9 \pm 0.2 \mu\text{M}$ (Fig. 2B). To verify that bradykinin uptake had taken place, the accumulation level ($[\text{bradykinin}]_{\text{in}}/[\text{bradykinin}]_{\text{out}}$) was calculated (see "Experimental Procedures"). At a bradykinin concentration of $1.5 \mu\text{M}$, the accumulation level was ~ 50 after 4 min of uptake, indicating that the peptide was transported against the concentration gradient.

Peptide Uptake as a Function of OppA Concentration—Because liganded OppA rather than the free peptide is the actual substrate of OppBCDF, the effect of increasing the amount of OppA on the transport rate was determined. For easy comparison with other ABC transporters that use a soluble SBP, the local concentrations of OppA at the membrane surface (in μM) were calculated as described in "Experimental Procedures." A concentration of OppA in the assay of $0.36 \mu\text{M}$ equals 0.35 nmol/m^2 of membrane surface, which corresponds to a local concentration at the membrane of $\sim 100 \mu\text{M}$. At a bradykinin concentration of $1.5 \mu\text{M}$ (a concentration >10 -fold above the K_D for bradykinin binding; $K_D = 0.1 \mu\text{M}$), the K_m of the translocat-

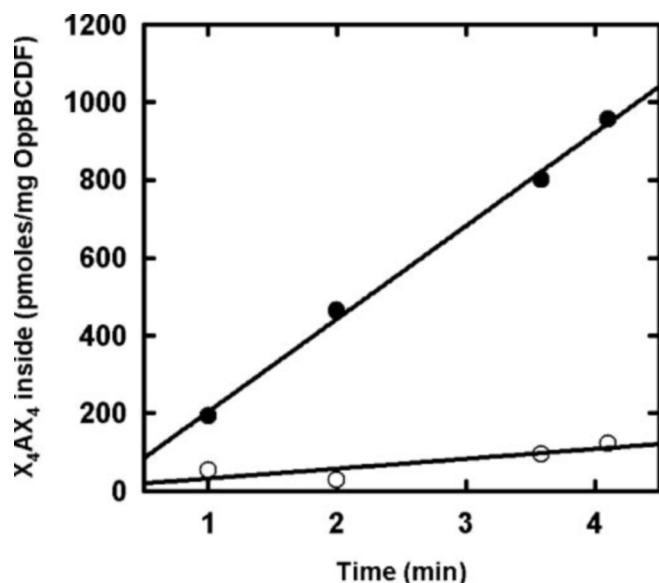


FIG. 3. Uptake of the randomized nonapeptide X_4AX_4 ($3.2 \mu\text{M}$) into Opp-containing proteoliposomes in the absence (●) or presence (○) of $100 \mu\text{M}$ bradykinin.

tor OppBCDF for liganded OppA was $\sim 50 \mu\text{M}$ (Fig. 2C). At a low substrate concentration, *i.e.* with $0.1 \mu\text{M}$ bradykinin, the uptake rate increased linearly with the concentration of OppA, and no inhibition attributable to the large excess (up to 4-fold) of unliganded OppA was observed (Fig. 2C). Fig. 2D shows how the ratio of liganded *versus* unliganded OppA varied in the titration and confirms that at the highest level of OppA, a 4-fold excess of unliganded over liganded OppA was present. These results indicate that the translocator has a much higher affinity for liganded than for unliganded OppA.

Uptake of Randomized Peptides via Opp—Because unliganded OppA does not inhibit the uptake of peptides, at least under our experimental conditions, the specificity of Opp for peptides can be estimated directly from the rate of uptake. Because the peptide libraries were available in relatively small quantities, a microwell plate setup was used for these transport assays. The peptide libraries were used at concentrations ranging from 3.2 to $5.0 \mu\text{M}$. Randomized peptides were labeled at tyrosine residues with tracer amounts of ^{125}I to monitor the transport reaction. Control experiments showed that uptake and (a) specific binding of randomized peptides could be specifically blocked by the addition of $100 \mu\text{M}$ bradykinin (Fig. 3). The chase with a large excess of bradykinin allowed us to separate transport from possible nonspecific interaction of the peptides with the liposomes.

Length Specificity—To determine the length dependence of transport, randomized peptide libraries ranging from 5 to 35 amino acid residues were used. All peptides, even the extremely long ones of up to 35 residues, were transported by the Opp system (Fig. 4A). The inhibition of bradykinin binding to OppA by the combinatorial peptide libraries, determined previously by Detmers *et al.* (8), is shown for comparison. Peptides with nine amino acid residues showed the highest uptake rates and the best binding to OppA. Longer and shorter peptides were transported with slower rates compared with the X_9 library, but overall, the longest and shortest peptides were transported better than expected on the basis of the binding data. Because partly translocated peptides might be trapped in the transporter (and not be chased by the excess of bradykinin), we calculated the uptake of the largest peptides in terms of accumulation levels and number of peptides translocated per transporter. The accumulation level of X_{35} ($[X_{35}]_{\text{in}}/[X_{35}]_{\text{out}}$)

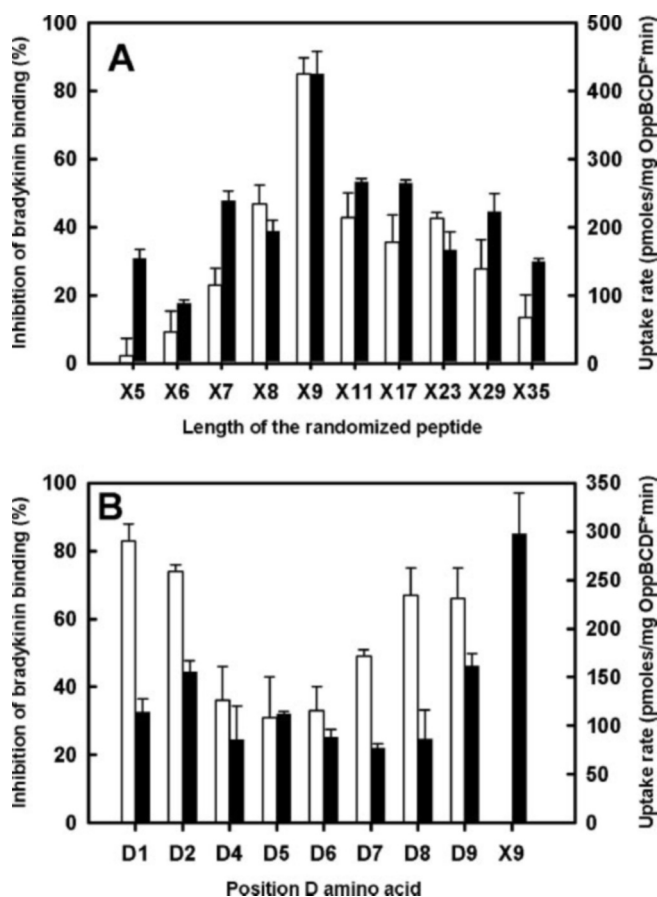


FIG. 4. Transport of randomized peptide libraries of different length (A) and nonamers with D-amino acids at a defined position (B). The peptide concentration was $3.2 \mu\text{M}$ (A) or $5 \mu\text{M}$ (B). Each rate determination was based on four data points; samples were taken after 1, 2, 3.5, and 5 min and were carried out at least twice. Error bars indicate the standard deviation. The transport data are shown as black columns; the binding data are shown for comparison (white columns; from Ref. 8)

was 2.5, and at least 30 peptides were transported per translocator after 5 min of uptake (see “Experimental Procedures”). These numbers, and the fact that X_{35} uptake still increased linearly with time after 5 min (not shown), demonstrate that even the largest peptides could be fully translocated.

Influence of D-Amino Acids—The effect of D-amino acids on transport was determined with randomized nonameric peptide libraries containing D-amino acids at a single position. The D-amino acid-containing peptides showed lower uptake rates compared with the all L-amino acid-containing X_9 library (Fig. 4B). Whereas in previous binding assays the negative effect of the D-amino acids was significant for the positions 4, 5, and 6, the differences in transport were less pronounced.

Transport of Peptides with Modified Termini—Randomized peptides with modified termini were used to test whether a free N or C terminus was required for transport via Opp. Uptake rates of octameric peptides (X_8) with an acetylated N or amidated C terminus or both were compared with the uptake rate of the unmodified randomized octameric peptide. The results indicate that free N or C termini are not essential for transport (Fig. 5). Acetylation of the N terminus had no effect, whereas amidation of the C terminus reduced the uptake rate by a factor of two. In addition, a nonameric randomized peptide library with a formylated N-terminal methionine, fMX₉, was transported equally well as MX₉.

Nonameric Randomized Peptides—Nonameric randomized peptide libraries were used in which amino acids at all but one

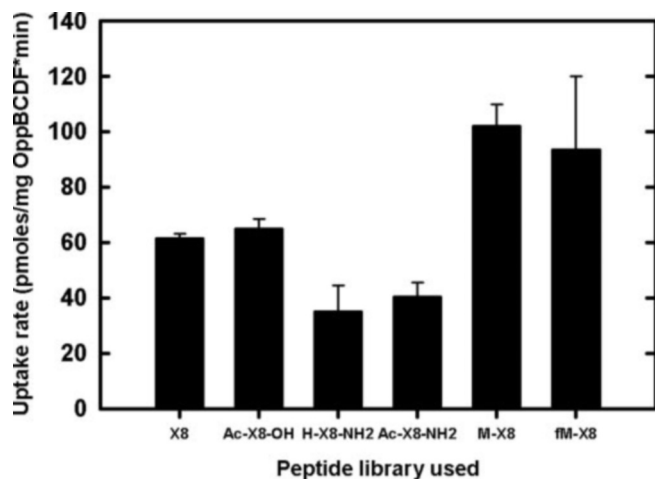


FIG. 5. Transport of randomized peptide libraries with modified termini. The peptide concentration was $3.2 \mu\text{M}$. For further details, see the legend to Fig. 4.

position were random. This position contains one of 19 different amino acids (cysteine was excluded from the libraries). In this way, the effect of a single amino acid at a given position can be determined independently of the sequence context of the rest of the peptide. For comparison, the transport via Opp and binding to OppA were screened for two positions. Generally, the transport data differed little from the binding results (Fig. 6). All peptides tested were transported, and peptides that bound best to OppA showed the highest transport rates. At position one, bulky and hydrophobic amino acids were somewhat disfavored (His, Leu, Val, Trp, and Tyr). At position five, no clear differences between the binding specificity of OppA and the transport selectivity of the Opp system as a whole could be discerned.

To investigate further whether the overall transport specificity differs from the binding selectivity of OppA, two amino acids, methionine and valine, were chosen for screening using randomized nonameric peptide libraries. These two amino acids showed almost no differences in binding specificity, irrespective of the position in the peptide (8). Transport specificity of the methionine-containing libraries matched perfectly with the binding selectivity (Fig. 7A). In the case of valine, some differences were observed (Fig. 7B), of which the negative effects of valine at positions 1, 4, and 9 on the transport rates were most pronounced. However, again all peptides could bind to OppA and were well transported by the Opp system.

DISCUSSION

We developed an *in vitro* assay system for the analysis of the kinetics, mechanism, and specificity of peptide transport, and we present the unique selectivity properties of the oligopeptide (Opp) ABC transporter from *L. lactis*.

Opp transporters in microorganisms are more complex than most other ABC systems because they are composed of five separate polypeptides, and this has hampered their purification and membrane reconstitution for many years. The translocator complex OppBCDF is embedded in the membrane, whereas the oligopeptide-binding protein is tethered to the external surface of the membrane. The Opp translocator was produced in *L. lactis* and purified as a complex using a single His₆-tag on OppC. Although Ni²⁺-NTA-based purification protocols generally use low concentrations (5–30 mM) of imidazole in the protein binding to the resin and washing steps, imidazole severely compromised the stability of the translocator. All four proteins could be co-purified only when imidazole was absent in the binding step and histidine was used for the elution of the complex.

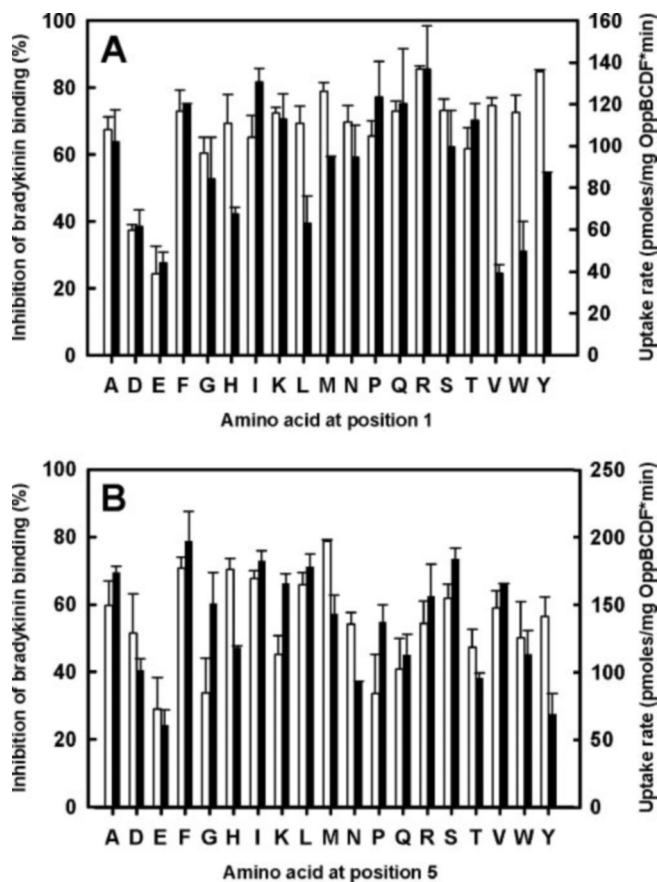


FIG. 6. Transport of randomized nonameric peptide libraries with a defined amino acid at position 1 (A) or 5 (B). Peptides were tested at $3.2 \mu\text{M}$. For further details, see the legend to Fig. 4.

Membrane reconstitution of Opp was done by inserting the OppBCDF complex into detergent-destabilized liposomes, after which OppA was anchored to the outside of the membranes via its N-terminal lipid modification. Because of the high affinity of OppA for the reporter substrate bradykinin ($K_D = 0.1 \mu\text{M}$) and the use of relatively high concentrations of OppA, a ~ 100 -fold excess of unlabeled bradykinin was used to stop the transport reaction and chase all OppA-bound [³H]bradykinin. The bradykinin that accumulated inside of the proteoliposomes was not affected by the chase with unlabeled bradykinin, because the transporter functions unidirectionally.

The affinity of the translocator for its substrate, liganded OppA, was determined by measuring uptake rates at increasing OppA concentrations at the membrane surface and of saturating amounts of bradykinin. The K_m of the translocator for liganded OppA was $\sim 50 \mu\text{M}$, which is in the same range as the values of $\sim 65 \mu\text{M}$ determined for the histidine system HisJQMP₂ (21) and 25 – $50 \mu\text{M}$ for the maltose system MalEFGK₂ (22). The translocator could already be saturated at relatively low absolute amounts of OppA because OppA is attached to the membrane. Even at an OppA concentration in the assay of only $0.36 \mu\text{M}$, which corresponds to 0.35 nmol/m^2 of membrane surface, the local concentration of OppA at the membrane could be as high as $100 \mu\text{M}$. Inhibition of transport activity by unliganded SBP has been observed for the periplasmic binding protein-dependent histidine and maltose ABC transport systems (21, 23), and this has been ascribed to competition between unliganded and liganded SBP for the translocator. In the case of the histidine permease, it even was suggested that liganded and unliganded HisJ interact with equal affinity with the translocator complex (24). We observed

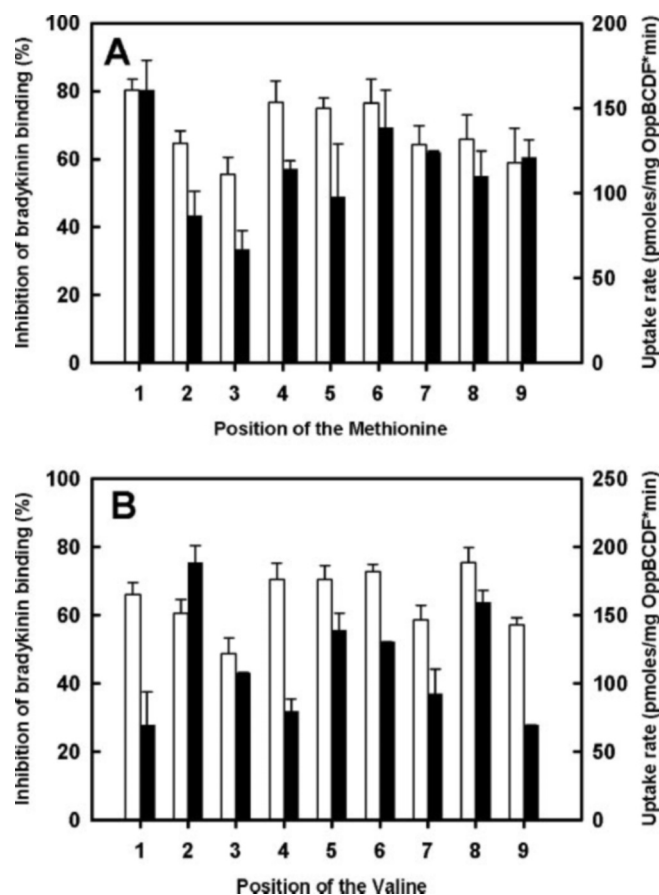


FIG. 7. Transport of randomized nonameric peptide libraries with methionine (A) or valine (B) at a defined position. Peptides were tested at $3.2 \mu\text{M}$. For further details, see the legend to Fig. 4.

no inhibition at low peptide concentrations, corresponding to local concentrations of $20 \mu\text{M}$ liganded and $80 \mu\text{M}$ unliganded OppA, which indicates that the affinity of the translocator for liganded OppA is much higher than for unliganded OppA.

It is often assumed that the SBP of bacterial ABC transporters determines the overall transport selectivity of the system, but unlike Opp, the specificity of most systems is narrow, and binding and transport of only a few substrates have been compared. Recently, Charbonnel *et al.* (11) proposed, by comparing the peptide utilization patterns of six different *L. lactis* strains, that OppA is not the main determinant of peptide transport specificity. It was claimed that the translocator OppBCDF plays an important role in determining the overall transport selectivity. It must be noted, however, that several of the strains used were not well characterized, and other factors (*e.g.* other peptide transporters or lack of certain peptidases) might have interfered with the observed peptide utilization patterns. The sequences of the *opp* genes of two of the strains used, *L. lactis* MG1363 and IL1403, differ for the transmembrane proteins OppB and OppC at only two positions (the system studied by us is from the MG1363 strain) (4, 25). This makes it unlikely that the observed differences in peptide transport can be ascribed solely to different properties of the transmembrane moieties of the Opp systems. In a follow-up paper, Juillard and colleagues (26) proposed that some peptides bind to OppA without subsequently being transported, but with the exception of one tetrapeptide, there is no real evidence for this notion. By using combinatorial peptide libraries, we now clearly show that the binding specificity of OppA compares well with the overall transport selectivity of the Opp system. For the specificity studies, we used saturating OppA concentrations,

implying that substrate binding or delivery to the translocator is not rate-determining. This mimics the *in vivo* situation where overexpression of OppA in wild-type *L. lactis* cells increased the amount of peptide binding but did not affect the rate of uptake (27). Furthermore, the K_m for bradykinin transport *in vivo* ($0.27 \pm 0.03 \mu\text{M}$) (27) and *in vitro* ($0.9 \pm 0.2 \mu\text{M}$) are in the same range. Thus, both *in vivo* and *in vitro* there is a large excess of OppA over OppBCDF, and the rate-determining step for bradykinin uptake is in translocation and not in peptide binding.

A striking feature of the *L. lactis* Opp system is that it binds and transports exceptionally long peptides. Here, we show that the Opp ABC transporter is capable of transporting peptides as long as 35 residues. Peptides shorter or longer than 9 residues are transported relatively better than expected, solely on the basis of the binding data. This fits well with the observation made previously that small peptides are bound relatively poorly but transported rather well (6). On the basis of these experiments and pre-steady state peptide-binding kinetics, it was suggested that the dissociation rate constant (k_{-1}) is an important determinant of the rate of transport. Tight binding to OppA was associated with low k_{-1} values, which might limit the transfer of peptide from OppA to the translocator and yield a low rate of transport. On the other hand, poorly binding peptides had a high k_{-1} and were transported relatively well. A similar explanation may hold for the difference in binding and transport of the penta- and hexameric peptides.

In a previous report, we showed that there is no strict requirement for a free amino and carboxyl group for a peptide to bind to OppA (9). Acetylation of the N-terminal amino group or amidation of the C-terminal carboxyl group of the peptide SLSQSLSQS decreased the binding affinity only 4- and 7-fold, respectively. Here, we show that a free amino or carboxyl group is also not essential for peptide transport by Opp.

To further analyze the transport specificity of the Opp system, randomized nonameric peptides containing D-amino acids or a particular residue at one single position were used. The uptake rate of D-amino acid-containing libraries was 2–3-fold lower than the X₉ library. This is in agreement with the binding studies in which a 5-fold higher peptide concentration had to be used to obtain similar levels of inhibition of bradykinin binding, as with all L-amino acid-containing libraries. Overall, there are only small differences between the binding and transport profiles. Positions 7 and 8 seem a little more selective than expected on the basis of the binding to OppA. The screening of positions 1 and 5 of the randomized nonameric libraries revealed little differences between binding and transport specificity. In general, it appears that any peptide that can bind to OppA can also be transported by Opp. There are no indications for any amino acid causing a peptide to be completely excluded from transport.

The fact that some peptides with bulky and hydrophobic amino acids (His, Leu, Trp, and Tyr at position 1 and Val at positions 1, 4, and 9) show somewhat reduced uptake rates can be explained in two ways. Either these peptides bind relatively strongly to OppA, resulting in low k_{-1} values and consequently lower transport rates, or a peptide-binding site within OppBCDF disfavors these amino acids at the positions 1, 4, and 9.

In conclusion, the affinity of OppBCDF for liganded OppA is in the same range as the corresponding interaction in other SBP-dependent ABC transporters, but because OppA is membrane-anchored, much lower amounts are needed to saturate the translocator than with soluble SBPs. The affinity of the transmembrane complex for liganded OppA is much higher than for unliganded OppA. The Opp system has the remarkable capacity to transport peptides ranging from 4 to 35 resi-

dues. Overall, the transport selectivity seems to be determined by the binding specificity of OppA.

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