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Specificity Mutants of the Binding Protein of the Oligopeptide Transport System of *Lactococcus lactis*

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The kinetic properties of wild-type and mutant oligopeptide binding proteins of *Lactococcus lactis* were determined. To observe the properties of the mutant proteins in vivo, the *oppA* gene was deleted from the chromosome of *L. lactis* to produce a strain that was totally defective in oligopeptide transport. Amplified expression of the *oppA* gene resulted in an 8- to 12-fold increase in OppA protein relative to the wild-type level. The amplified expression was paralleled by increased bradykinin binding activity, but had relatively little effect on the overall transport of bradykinin via Opp. Several site-directed mutants were constructed on the basis of a comparison of the primary sequences of OppA from *Salmonella enterica* serovar Typhimurium and *L. lactis*, taking into account the known structure of the serovar Typhimurium protein. Putative peptide binding-site residues were mutated. All the mutant OppA proteins exhibited a decreased binding affinity for the high-affinity peptide bradykinin. Except for OppA(D471R), the mutant OppA proteins displayed highly defective bradykinin uptake, whereas the transport of the low-affinity substrate KYKG was barely affected. Cells expressing OppA(D471R) had a similar *Kd* for transport, whereas the *Vmax* was increased more than twofold as compared to the wild-type protein. The data are discussed in the light of a kinetic model and imply that the rate of transport is determined to a large extent by the donation of the peptide from the OppA protein to the translocator complex.

In bacteria, the binding protein-dependent permeases constitute an important group of transport systems for the uptake of nutrients such as sugars, amino acids, anions, and peptides (1, 8). In gram-negative bacteria, the systems consist of a periplasmic substrate binding protein, a membrane-bound complex formed by two hydrophobic integral membrane proteins (or a single protein with two domains), and two membrane-associated proteins that carry the ATP-binding cassette motif (8). The periplasmic substrate binding protein is usually present in large excess, serving to capture the substrate with high affinity and to deliver it to the membrane-bound complex. The substrate binding proteins determine the specificity of the transport systems and therefore the range of molecules that may enter the cell (31).

The oligopeptide transport system (Opp) possesses one of the most versatile binding proteins, since it is able to handle a large variety of peptides present in the medium. Experiments with amino acid auxotrophic strains of *E. coli* indicate that the protein has a higher affinity for tri- and tetrapeptides than for di- and pentapeptides (7). The Opp system of *Lactococcus lactis* is homologous to the Opp systems of enteric bacteria. As for many other binding proteins in gram-positive bacteria, the OppA protein is anchored to the cytoplasmic membrane by a lipid-modified cysteine (6). The Opp system of *L. lactis* has the capacity to transport peptides from 4 to at least 18 residues (4). Kinetic analysis of binding of the peptides SLSQS, SLSQSKVLPVPQ, SLSQSKVLPVPQ, RDMPIOQA, and RDMPIQAF to OppA of *L. lactis* showed a relationship between the peptide dissociation constants (*Kd*) and the length of the ligand (14), varying from millimolar values for SLSQS to submicromolar values for SLSQSKVLPVPQ.

The crystal structures of the oligopeptide binding protein (OppA) from *Salmonella enterica* serovar Typhimurium in complex with tripeptides (34), tetrapeptides (33), or dipeptides as well as unliganded binding proteins (31) have been solved, and the residues involved in interactions with the peptides have been identified. The main chain of the peptide is in an extended conformation and forms parallel and antiparallel β-sheet interactions with some residues of OppA. The N terminus of the peptides forms a salt bridge with the side chain of Arg-413 and His-371 each form a salt bridge with the carboxylate groups of the tri- and tetrapeptide ligands, respectively, and Lys-307 has been postulated to form a salt bridge with the C terminus of pentapeptides. In the case of the dipeptide, the C-terminal interaction with OppA is indirect and occurs via a water molecule that interacts with the side chain of Arg-404 and Arg-413. The side chains of the peptides are accommodated in spacious and hydrated pockets, where few direct contacts are made with the protein. Water molecules act as flexible adapters that match the hydrogen-bonding requirements of OppA and the ligand and/or shield charges on the buried ligand (35). The peptides are buried within OppA, according to the Venus flytrap mechanism (19).
In line with the similar three-dimensional structures of the OppA protein of serovar Typhimurium (OppASt) and the dipeptide binding protein DppA of E. coli and the relatively low degree of identity in primary sequence between these proteins, it seems likely that OppASt and L. lactis (OppALl) also have a similar structural fold (27); the amino acid identity between these proteins is 21 to 22%. Comparison of OppALl and OppASt shows that of the important residues that interact with the peptides in OppASt, only Lys-307 is conserved in OppALl (Fig. 1). On the basis of the structure of the OppASt protein, we made amino acid substitutions in OppALl that should be near or at the peptide binding site. The effects of these substitutions on the growth of L. lactis as well as in vivo peptide transport and peptide binding to purified OppA are reported in this paper.

MATERIALS AND METHODS

Strains, growth conditions, media, and chemicals. All strains and plasmids are listed in Table 1. L. lactis NZ234 was grown at 37°C with vigorous aeration in Luria broth (29), supplemented with 500 μg of erythromycin per ml when carrying plasmids pAMP21 or pAMP31. L. lactis strains were grown in M17 broth (Difco Laboratories, East Molesey, United Kingdom) at 30°C as stand cultures or on M17 broth solidified with 1.5% agar (36) supplemented with 0.5% (wt/vol) glucose and 5 μg of erythromycin per ml, if required. For purification purposes, the L. lactis strains were grown in fed batch in 10-liter fermentors with pH control (ADI 1065 fermentor; Applikon Dependable Instruments, B.V., Schiedam, The Netherlands). The pH value was kept constant at 6.5 by the addition of 1 M KOH. Complementation studies were performed on plates or liquid control (AD1 1065 fermentor; Applikon Dependable Instruments, B. V., Schiedam, The Netherlands). The pH value was kept constant at 6.5 by the addition of 1 M KOH. Complementation studies were performed on plates or liquid controls.

General DNA techniques. Plasmid and chromosomal DNA were isolated by the alkaline lysis method as previously described (29). PCR was performed with VENT DNA polymerase (New England Biolabs). After 30 cycles of amplification, the PCR products were purified using the QiAquick spin PCR purification kit (Qiagen). DNA modification enzymes were obtained from Boehringer GmbH (Mannheim, Germany). Digestions were carried out according to the manufacturer’s recommendations. Ligation of DNA fragments was performed as previously described (29). L. lactis was transformed by electroporation as described (9). DNA was sequenced by the dideoxy-chain termination method (30) using T7 DNA polymerase.

Construction of oppA deletion mutants. The oppA gene was deleted from the chromosome of L. lactis MG1363 and IM15 via homologous recombination (16). An integration plasmid, pAP2, which contains the 5’ and 3’ flanking sequences of oppA, was constructed for this purpose. Both flanking regions were amplified by PCR using pVS8 (37) as template and the primers FBB plus RCP (5) as primers. Primers are listed in Table 2. The PCR products correspond to the 5’ (1,086 bp) and 3’ (1,096 bp) flanking regions were restricted with BamHI plus PstI and PvuI plus SphI, respectively, and ligated into the multiple cloning site of pORI280 (15). L. lactis MG1363 and IM15 were transformed with pAP2, and transformants were selected on CDM plates supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 200 μg of erythromycin per ml, if required. For purification purposes, the L. lactis strains were grown in fed batch in 10-liter fermentors with pH control (ADI 1065 fermentor; Applikon Dependable Instruments, B.V., Schiedam, The Netherlands). The pH value was kept constant at 6.5 by the addition of 1 M KOH. Complementation studies were performed on plates or liquid controls.

FIG. 1. Alignment of parts of the OppA proteins from L. lactis and S. enterica serovar Typhimurium. Sequences of the homologous peptide binding region were aligned using CLUSTAL X program. Conserved residues are marked with an asterisk, while similar residues are marked with a single or double dot. N, C3, C4, an dC5 correspond to interactions of OppASt with the N terminus of peptides and the C terminus of tri-, tetra-, and pentapeptides, respectively. Characters in boldface represent the identified peptide binding residues in serovar Typhimurium and their putative counterparts in L. lactis. Substitutions made in OppALl are also indicated by arrows.
Construction of mutants of OppA. Oligonucleotide-directed site-specific mutagenesis was used to generate single mutations in OppA. The mutants were generated using the CLUSTAL X program. A gap penalty of 30 and an extension gap penalty of 0.05 were used. The alignment was then manually modified to prevent gaps in the sequences that aligned with the known secondary structure and R, reverse primer) and XnYF (F, forward primer) plus RAB were used as primers in the first PCR step with plasmid pAMP31 as template. Subsequently, both PCR products were purified together and used as template for the second PCR step with the oligonucleotides XbaF plus RAB. The resulting 1,483-bp fragments were digested with XbaI plus BamHI and exchanged for the equivalent fragment of pAMP31. All 1,465-bp XbaI-BamHI fragments were checked by nucleotide sequencing.

Western analyses. L. lactis cells were harvested at the end of the exponential phase of growth, washed once with water, and resuspended in water to a density of 10^8 cells/ml. The cells were sonicated for nine cycles of 5 s at an amplitude of 4 μm with 15 s cooling, on ice, using an MSE Soniprep 150-probe sonicator (Crawley, United Kingdom). Subsequently, sample buffer was added and the lysates were boiled for 5 min. Cell debris was removed by centrifugation (12,000 g, 10 min, 4°C).

Sequence alignment. A multiple alignment of the OppA protein from serovar Typhimurium, DppA protein from E. coli (22), and OppA from L. lactis was generated using the CLUSTAL X program. A gap penalty of 30 and an extension gap penalty of 0.05 were used. The alignment was then manually modified to prevent gaps in the sequences that aligned with the known secondary structure elements of OppA and DppA.

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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</thead>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BZ234</td>
<td>C600 derivative; Em*</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pVS8</td>
<td>Cm*: pSH71 replicon; opp operon of <em>L. lactis</em></td>
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<tr>
<td>pORI280</td>
<td>Em*: lacZ*; deletion derivative of pPW01 lacking rep4</td>
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<td>pAP2</td>
<td>pORI280 containing the 5' and 3' flanking regions of oppA</td>
<td>This work</td>
</tr>
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<td>pSKII(+)</td>
<td>Carb*: high-copy-number expression vector</td>
<td>Stratagene</td>
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<tr>
<td>pSKE8HiS</td>
<td>pSKII(+) derivative carrying lacS with NcoI site on the initiation codon and a C-terminal His tag</td>
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<tr>
<td>pGK13</td>
<td>Em*: pPW01 replicon; <em>E. coli</em> oppL shuttle vector</td>
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<tr>
<td>pGKGS8</td>
<td>pGK13 derivative carrying lacS as a 3,784-bp EcoRI-DraI fragment from pSKE8HiS ligated into the EcoRI-EcoRV sites</td>
<td>10</td>
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<td><strong>Strains</strong></td>
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<tr>
<td>N422H</td>
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<td></td>
</tr>
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<td>AMP2</td>
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<tr>
<td>L. lactis</td>
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<td>MG1363</td>
<td>Plasmid-free derivative of NCD0712; Lac^- Pro^-</td>
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<td>IM15 ΔoppA</td>
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<tr>
<td>A477DR</td>
<td>5'-GGTTCAGAATCAATGACC-3'</td>
<td>A477D</td>
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* Nucleotides in boldface type correspond to restriction site; nucleotide changes that give rise to an amino acid substitution are underlined.
Transport assays. Cells grown to an optical density at 600 nm of 1.0 were harvested by centrifugation, washed twice, and resuspended in buffer A (100 mM potassium phosphate [pH 6.5], 5 mM magnesium sulphate). A total of 50 μl of the cell suspension (∼1.2 mg of protein/ml for KYGK uptake; 0.17 mg of protein/ml for bradykinin uptake) was added to 200 μl of buffer A supplemented with glucose (25 mM final concentration). Cells were incubated for 3 min at 30°C (in assays with KYGK as substrate) or 10°C (bradykinin as substrate), after which the transport reaction was initiated by the addition of 5.85 μM 125I-KYGK or 0.7 μM bradykinin (‘H-RPPGFSPFR diluted with RPPGFSPFR), unless specified otherwise. At given time points, 50-μl samples were withdrawn and diluted with 2 ml of ice-cold 0.1 M LiCl. The samples were rapidly filtered through 0.45-μm-pore-size cellulose-aceate filters (Schleicher & Schuell GmbH, Dassel, Germany) and washed with 2 ml of ice-cold 0.1 M LiCl. The radioactivity of the filter was determined by liquid scintillation. To estimate the binding, the same procedure was followed except that the cells were incubated in buffer A without glucose for 6 min, and the amount obtained for strain AMP2 was subtracted in all cases to determine the kinetic constants for bradykinin uptake, the amounts of bradykinin were varied from 0 to 5 μM. The uptake rate for each concentration was calculated by linear regression from the intracellular peptide concentration at different time points up to 90 s. The uptake rate as a function of the substrate concentration was fitted to the Michaelis-Menten equation.

Purification of OppA-His₆. Membrane-bound OppA-His₆ was isolated from inside-out membrane vesicles of L. lactis. The membrane vesicles were isolated as previously described (26) and solubilized at 5 mg of protein/ml in buffer B (50 mM potassium phosphate; 100 mM KC1; 10% glycerol [pH 7.6]) plus 0.2% (wt/vol) DDM. The mixture was incubated on ice for 30 min, and the insoluble material was removed by centrifugation (280,000 × g; 15 min). The solubilized membrane proteins were mixed with Ni-nitriloacetic acid resin previously equilibrated with buffer B. The mixture was incubated for 1 h at 4°C under continuous shaking and subsequently poured into a Bio-spin column (Bio-Rad). The column was washed with 20 column volumes of buffer B, pH 6.5, plus 0.05% DDM supplemented with 15 mM imidazole. The protein was eluted with buffer B plus 0.05% DDM containing 500 mM imidazole. A desalting step on a PD10 column (Bio-Rad) was performed in order to remove the imidazole. All handlings were done in an Aminco 4800 spectrofluorimeter. The effect of peptide addition on changes in intrinsic protein fluorescence, as previously described (14), except that 0.05% DDM was present in the buffer solution. All measurements were done in an Amino 4800 spectrofluorometer. The effect of peptide addition on fluorescence was measured at 15°C by exciting OppA (0.6 μM) at 280 nm with a silt width of 2 nm and measuring the emission at 315 nm with a silt width of 8 nm. Data analyses were performed as previously described (14).

Miscellaneous. Protein content was determined according to Lowry et al. (17) with bovine serum albumin as standard. The concentration and stability of purified OppA proteins were evaluated by measuring the absorption spectrum between 240 and 340 nm. The extinction coefficient of OppA was calculated as previously described (24), obtaining a value of 1.605 (mg/ml)⁻¹·cm⁻¹.

RESULTS

Analysis of oppA deletion mutants of L. lactis. To study the properties of wild-type and mutant alleles of OppA in vivo, the oppA gene was deleted from the chromosome of strains MG1363 and IM15 by a crossover in each of the flanking regions with the integration plasmid pAP2. This procedure allows the complete deletion of oppA, leaving intact the other genes of the opp operon. Some putative mutants were analyzed by PCR, and the absence of the OppA protein was confirmed by immunoblotting. One mutant of each parent strain was chosen for further studies and named L. lactis AMP15 (MG1363 ΔoppA) and L. lactis AMP2 (IM15 ΔoppA) (Fig. 2).

Lactic acid bacteria are multiple amino acid auxotrophs (3), and their nitrogen requirements are met by taking up free amino acids or peptides from the medium. Since it has been proved that the Opp system is essential for the uptake of peptides longer than three residues (12), the deletion of the oppA gene should result in a strain unable to grow on peptides as the source of one of these essential amino acids. Indeed, L. lactis AMP15 was unable to grow on CDL medium with one of the tetra- or pentapeptides GI, GL, LWL, LSQ, and YGGFL as the sole source of leucine, whereas the strain grew normally on CDL plates containing 1-leucine. L. lactis IM15 is impaired in the degradation of peptides due to the deletion of four peptidases, but it is still able to use leu- enkephalin (YGGFL) as a source of leucine. As anticipated, L. lactis AMP2 was unable to grow on CDL plates containing 100 μM of leu-enkephalin as a sole source of leucine, whereas the strain grew normally on CDL plates containing 1-leucine. To show directly that L. lactis AMP2 is defective in oligopeptide uptake, we monitored the uptake of 3H-bradykinin. Transport of bradykinin was completely abolished in L. lactis AMP2, whereas the uptake rate in the parent strain (IM15) was about 400 pmol · min⁻¹ · mg of protein⁻¹ (Fig. 3).
L. lactis AMP2 was tested as host for the expression of OppA with the plasmid pAMP21 or pAMP31. Both plasmids contain the oppA gene under the control of the P32 promoter, and the genes are fused to a sequence that specifies a 6-His tag. Unlike in pAMP31, the oppA gene in pAMP21 lacks the signal sequence. The protein specified by oppAΔss is referred to as OppA*. As anticipated, pAMP21(oppAΔss) was unable to restore the ability of L. lactis AMP2 to utilize leu-enkephalin as a source of leucine (data not shown), and the transport of bradykinin was negligible (Fig. 3). L. lactis AMP2/pAMP31(oppA) was able to use leu-enkephalin as a source of leucine and transported bradykinin with an uptake rate of about 550 pmol min⁻¹ mg⁻¹ of protein⁻¹ (Fig. 3).

Overall, the results demonstrate that the oppA gene has been deleted from the chromosome of L. lactis MG1363 and IM15 and that OppA is the only binding protein that allows the organism to transport the tested oligopeptides. Complementation occurs with the oppA gene in trans.

Overexpression and localization of OppA. The amount of OppA present in AMP2/pAMP31 was approximately eight times higher than the level present in the parent strain (data not shown). Electron microscopy studies showed that all OppA produced by AMP2/pAMP31 was localized at the surface of the cell (Fig. 4). As was anticipated, OppA* was found in the cytoplasm of strain AMP2/pAMP21, due to the lack of a signal sequence. The observation that the rate of bradykinin uptake by AMP2/pAMP31 is at best 40% higher than that of IM15, whereas the expression level of OppA increased eightfold, indicates that transport of this peptide is not to a large extent rate determined by OppA activity.

Expression of site-specific mutant OppA proteins. The tertiary structure of OppA of serovar Typhimurium has been elucidated, and the specific residues that may interact with the termini of different peptides have been identified (33). OppA of serovar Typhimurium (OppA₃₃) and OppA of L. lactis (OppA₄₄) are homologous, but the identity between the two proteins is only 21 to 22%. A comparison of the primary sequence of both proteins (Fig. 1) shows that only Lys-307 in OppA₃₃, which interacts with the carboxy terminus of the pentapeptides, is conserved in OppA₄₄ (Lys-349). The identification of the other residues that, on the basis of the OppA₃₃ structure, could interact with the termini of the peptides is more ambiguous. The residues equivalent to Asp-419 (N terminus of peptides), Arg-413 (C terminus of tripeptides), and His-371 (C terminus of tetrapeptides) in OppA₃₃ could be Ala-477, Asp-471, and Asn-422 or Ala-423. To establish the possible role of these residues in peptide binding and transport, substitutions were made on the basis of the structure of OppA₃₃, yielding the following OppA₄₄ mutants: K349Q, A477D, D471R, N422H, and A423H.

The plasmids bearing the mutant genes were transformed to strain AMP2. Expression of these mutant OppA proteins was tested by Western analysis in whole cells and in membrane vesicles. In all cases, OppA was present in the membrane fraction and the mutant proteins were produced in amounts comparable to that of the wild-type protein expressed from plasmid pAMP31 (Fig. 2). The expression level of OppA present in AMP2/pAMP31 was approximately eight times higher than the level present in the parent strain (data not shown).

In vivo function of mutant OppA proteins. To determine if these mutant proteins were able to complement the deletion mutants, L. lactis AMP15 and AMP2 were transformed with one of the following plasmids: pAM31(K349Q), pAM31(N422H), pAMP31(A423H), pAMP31(D471R), or pAMP31(A477D). Transformants were tested for their ability to use oligopeptides (GLGL, LWMR, SLSQS, and YGGFL) as the sole source of leucine. All mutant OppA proteins sustained growth on these tetra- or pentapeptides as the sole source of leucine and had growth rates similar to that of the wild-type protein (data not shown).

Binding of bradykinin to cells expressing wild-type or mutant OppA proteins. The data presented in Fig. 3 show that the increased amount of OppA present in strain AMP2/pAMP31 resulted in a slightly increased uptake rate but a highly increased binding (340 pmol min⁻¹ mg⁻¹ of protein⁻¹ when the uptake curve is extrapolated to time zero). To evaluate the binding of bradykinin quantitatively, the cells were incubated in buffer A without glucose for 6 min, and the amount of bound bradykinin was determined. Under these conditions, the cells
did not accumulate the substrate and since the binding of bradykinin to OppA appeared to be tight, it could be quantified by the filtration assay. L. lactis AMP2/pAMP31 bound approximately seven times more bradykinin than IM15, whereas binding to the OppA mutants K349Q, A423H, D471R, and A477D was similar to that of IM15, at a bradykinin concentration of 0.7 μM (Table 3). The N422H mutant displayed intermediate binding. Since the expression levels of these mutant proteins were similar to that of wild-type OppA and functional complementation was observed in growth experiments, the data are consistent with a reduced affinity for bradykinin (see below) but, at this point, it cannot be ruled out that part of the mutant proteins is inactive.

Transport of peptides by cells expressing wild-type or mutant OppA proteins. KYGK and bradykinin are low- and high-affinity substrates, respectively, of the Opp system of L. lactis (4, 14). Moreover, KYGK was used as substrate because it is not degraded by strains IM15 and AMP2 due to their multiple peptidase deficiencies. The rates of uptake of 125I-KYGK and 3H-bradykinin are shown in Table 3. Each of the mutant OppA proteins restored the uptake of KYGK and bradykinin in the AMP2 background, albeit to different levels. With the exception of OppA(D471R), the rates of KYGK uptake were comparable to that of the system with the wild-type OppA protein (Table 3). The rates of bradykinin uptake by strains expressing OppA proteins K349Q, N422H, and A477D were significantly lower than that of the wild type. The rate of uptake by strain expressing OppA(D471R) was much higher for both peptides (fivefold for KYGK and threefold for bradykinin).

Strain AMP2/pAMP31(D471R) was studied further because of its exciting properties, that is an apparent decreased binding affinity for bradykinin and an increased transport activity. To establish whether these properties are also manifested in the kinetic parameters of transport (Km and Vmax), we determined the uptake rate as a function of the bradykinin concentration and compared the obtained Km and Vmax values to the data obtained for strain AMP2/pAMP31 (Table 4). Strain AMP2/pAMP31(A477D) was also characterized as an example of a system that displayed reduced binding and transport activity. The differences in transport rate between the wild type and mutants were mainly at the level of Vmax but a small but significant change in Km was also observed for OppA(A477D). Thus, the apparent decrease in binding affinity for bradykinin by OppA(D471R) is accompanied by a higher Vmax for uptake (see Discussion for interpretation).

Table 3. Binding of bradykinin (RPPGFSPFR) and uptake of peptides by L. lactis OppA mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>1H-RPPGFSPFR bounda</th>
<th>125I-KYGK uptake rateb</th>
<th>3H-RPPGFSPFR Uptake rateb</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP2</td>
<td>0°</td>
<td>&lt;0.2</td>
<td>15 ± 10</td>
</tr>
<tr>
<td>IM15</td>
<td>39 ± 5</td>
<td>ND</td>
<td>400 ± 40</td>
</tr>
<tr>
<td>AMP2/pAMP31(WT)</td>
<td>286 ± 20</td>
<td>5 ± 1</td>
<td>550 ± 40</td>
</tr>
<tr>
<td>AMP2/pAMP31(K349Q)</td>
<td>56 ± 6</td>
<td>4 ± 1</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>AMP2/pAMP31(N422H)</td>
<td>106 ± 9</td>
<td>ND</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>AMP2/pAMP31(A423H)</td>
<td>32 ± 4</td>
<td>3 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>AMP2/pAMP31(D471R)</td>
<td>51 ± 4</td>
<td>28 ± 3</td>
<td>1,600 ± 50</td>
</tr>
<tr>
<td>AMP2/pAMP31(A477D)</td>
<td>30 ± 3</td>
<td>6 ± 2</td>
<td>53 ± 10</td>
</tr>
</tbody>
</table>

a The amount of bradykinin bound to AMP2 (40 ± 4) was subtracted in all cases. Bradykinin (1H-RPPGFSPFR), (0.7 μM) final concentration.
b Uptake rates are in picomoles of peptide per milligram of protein per minute. Rates are shown as means ± standard errors. ND, not determined.

Table 4. Kinetic parameters for bradykinin uptake in L. lactis cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vmax (pmol/mg/min)</th>
<th>Km (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP2/pAMP31(WT)</td>
<td>1,095 ± 106</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>AMP2/pAMP31(D471R)</td>
<td>2,407 ± 228</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>AMP2/pAMP31(A477D)</td>
<td>1,118 ± 48</td>
<td>0.54 ± 0.06</td>
</tr>
</tbody>
</table>

a Vmax values are in picomoles per milligram of protein per minute. Rates are shown as means ± standard errors.
latter step was performed because the lipid anchor prevented entry of the protein into the polycrylamide gel. NCE of tryp- 
sin-treated OppA(WT) and OppA(A477D) yielded two spe-
cies that correspond to the open unliganded and the closed 
ligated forms of OppA (14). Upon addition of bradykinin or 
SLSQSKVPQ, the fast-migrating form became predomi-
nant. In the case of OppA(D471R), only one form was ob-
served before and after incubation with peptides (data not 
shown). Due to the altered electrophoretic mobility of 
OppA(D471R) even in the presence of SDS, it was not possi-
bile to conclude if this unique form corresponds to the open 
or closed conformation.

**Peptide binding studied by intrinsic protein fluorescence.** 
To study the binding of peptides in a more quantitative man-
ner, purified and guanidinium-HCl-treated OppA(WT), 
OppA(D471R), and OppA(A477D) were used in intrinsic pro-
tein fluorescence assays. The emission spectrum of OppA 
showed a maximum at 332 nm. Upon binding of peptides, a 
blue shift of approximately 2 nm was observed. Binding of 
bradykinin or SLSQKSVLP resulted in an increase in fluores-
cence below 340 nm and in a decrease above 340 nm (data not 
shown). The increase in fluorescence at 315 nm was concen-
tration dependent and could be used to determine the kinetic parameters for peptide binding. Binding of bradykinin and SLSQKSVLP to 
OppA(WT), OppA(D471R), and OppA(A477D) yielded a de-
pendence on the peptide concentration that could be predomi-
nant for the observed fluorescence change. The dissociation 
constants determined for bradykinin and SLSQKSVLP were 
0.29 μM for OppA(WT), 2.83 μM for OppA(D471R), and 
20.9 μM for OppA(A477D). The dissociation constants, and [S] is the peptide concentra-
tion (Fig. 5). The dissociation constants determined for bradykinin 
were of the same order of magnitude when the nonapeptide 
SLSQKSVLP was used as test substrate. These results indicate 
that the mutations affect the affinity of OppA for bradykinin but 
ot the affinity for peptides in general.

**DISCUSSION**

In this paper, we show that deletion of the oppA gene from 
the chromosome rendered L. lactis MG1363 and IM15 inactive 
in the uptake of oligopeptides. These strains could be comple-
mented with the oppA gene in trans. The presence of a His tag 
at the C terminus did not affect the functionality of OppA; all 
of the overexpressed protein was directed to the cell surface as 
shown by the electron microscopy studies. The increase in 
the expression of OppA resulted in a highly increased peptide 
binding capacity, whereas the uptake rate was only marginally 
affected. These initial studies have set the stage for the in vivo 
and in vitro analyses of mutations in the peptide binding pro-
tein of the Opp system of L. lactis.

Several site-directed mutants of OppA were constructed on 
the basis of a comparison between the primary sequence of 
OppA from serovar. Typhimurium and L. lactis, taking advan-
tage of the three-dimensional structure of OppA (14). The expres-
sion of all of these mutant OppA proteins restored the trans-
port of peptides as well as the growth of ΔoppA mutants of 
MG1363 and IM15 on peptides as a source of essential amino 
acids. Mutant D471R displayed a five- and threefold-higher 
uptake rate for KYGK and bradykinin (RPPGFSPFR), respecti-
vely. The rate of transport of KYGK was not significantly 
affected in the other mutants, whereas that of bradykinin was 
approximately 10-fold lower. The apparent increase in uptake 
rate in OppA(D471R) and the decrease in OppA(A477D) 
correspond to a change in Vmax rather than to large alterations 
in the affinity constants for uptake. Studies of peptide binding to 
wild-type and mutant OppA proteins showed that the Kf 
values for bradykinin binding to OppA(D471R) and OppA(A477R) 
increased by 1 and 2 orders of magnitude, respectively, as compared 
with OppA(WT). The same proteins exhibited wild-type binding 
kinetcs for the other nonapeptide tested (SLSQKSVLP). The 
consequences of these differences in Kf and Kd values and their 
dependence on the peptide used are discussed below.

Since the Kf for bradykinin binding to OppA(D471R) and 
OppA(A477R) was greatly increased, it was not possible to 
determine the binding stoichiometry for these mutants. To 
determine the actual number of binding sites, one needs a 
high-affinity ligand such that Kf << [OppA] under the exper-
imental conditions (see our previous analysis in reference 14). 
In our opinion, the diminished amount of bradykinin binding is 
consistent with the increase in Kd and does not involve a 
decrease in the binding stoichiometry as a result of a fraction 
of inactive protein. This notion is supported by the observation 
that the kinetics of SLSQKSVLP binding to OppA(D471R) is 
very similar to that of the wild-type protein.
Comparison of the specificities of OppA\textsubscript{Ll} and OppA\textsubscript{St} in relation to the structure. All the mutations introduced into OppA\textsubscript{Ll} seem to affect the specificity of the protein for peptides. The positions were selected for mutagenesis studies on the basis of their proposed interactions with the tri-, tetra-, or pentapeptides in OppA\textsubscript{St}. The selected residues in OppA\textsubscript{Ll} clearly have a more global effect on the interactions with the peptides, as pronounced differences in transport and binding activities were observed when the nonameric peptide bradykinin was used as test substrate. Since the transport of peptides by Opp is rate determined by the kinetics of bradykinin binding to only a small extent, changes in this parameter may not be observed in the overall transport reaction. The same may apply for other peptides, and it would require a full analysis of both peptide binding and transport. Unfortunately, the availability of radiolabelled oligopeptides for transport studies is limited, whereas the dissociation constants of small peptides (five or fewer residues) are too high (in the millimolar range) to be analyzed by NCE or intrinsic fluorescence. As a consequence, we cannot rule out the possibility that some of the mutants have an altered $K_d$ for tripeptides (D471R) or tetrapeptides (N422H or A423H) specifically.

In our opinion, however, the fact that these residues are not conserved may reflect the differences in function of both OppA proteins; that is, OppA\textsubscript{Ll} serves to accumulate rather long peptides (>5 residues) (4), whereas the optimal activity of OppA\textsubscript{St} is for tri- and tetrapeptides (7). Part of the binding affinity of OppA\textsubscript{St} for tri- and tetrapeptides will be obtained from the interactions of the carboxyl-terminal ends of these peptides with the corresponding residues in the protein. The dissociation constants of OppA\textsubscript{Ll} for tri- and tetrapeptides are much higher than those of OppA\textsubscript{St}, most likely because the interactions with the termini of the peptides are absent. In this regard, it is worth emphasizing that, despite the high dissociation constants of OppA\textsubscript{Ll} for tri- and tetrapeptides, all the peptides tested thus far are taken up by Opp of L. lactis (4); the capacity of Opp\textsubscript{Ll} to transport tripeptides is more ambiguous.

A moderate decrease in binding affinity results in an increased uptake rate. The $K_d$ obtained for bradykinin for OppA(D471R) is about 10-fold higher than that of OppA(WT). This difference is in agreement with the observed lower-binding activity in cells expressing OppA(D471R). Due to its very fast association, it was not possible to determine the association ($k_1$) and dissociation ($k_{-1}$) rates for bradykinin by stopped-flow fluorescence measurements. Nevertheless, we speculate that the increased $K_d$ of OppA(D471R) for bradykinin is caused by an increased dissociation rate constant ($k_{-1}$). This suggestion follows from the observation that the large variation in $K_d$ of OppA* for a range of peptides relates to differences in $k_{-1}$ (14). Site-directed mutagenesis studies of the arabinose-binding protein of E. coli (40, 41) also showed that variations in $K_d$ relate to an altered $k_{-1}$ rather than to a change in the association constant ($k_1$). This implies that bradykinin gains access to the active site of OppA(WT) and OppA(D471R) equally well but that the dissociation rates from these binding proteins are different.

The consequences of this suggestion on the overall transport by Opp can be analyzed from a previously published scheme (14). According to this model (Fig. 6) transport takes place in four steps: I, binding of the ligand to the binding protein; II, docking of the liganded binding protein to the membrane complex; III, donation of the ligand to the membrane complex; and IV, translocation of the substrate across the membrane. It has been proposed that the donation of the ligand from the binding protein to the membrane-bound complex determines the rate of the whole transport process (14, 21), which corre-

sponds to step III of the scheme. In this case, the rate of transport can be described by the following equation:

$$v = \frac{k_1[M_0][L]}{K_{0K} + [L]}$$

in which $K'_{0K}$ is the rate constant of this donation step, $[M_0]$ is the total concentration of membrane-bound complex, $[L]$ is the concentration of the ligand, $K_{0K}$ corresponds to the equilibrium constant for binding of the ligand to the binding protein ($k_{-1}/k_1$), $K_{0K}$ is the equilibrium constant for binding of the unliganded binding protein to the membrane complex. If we assume that $k_{-1}$ and $k_{-2}$ are related, that is, that the rate of dissociation of the peptide from OppA is the same for free (EL) and membrane-docked (EL/M) binding protein, then the rate of transport will increase in proportion to $k_{-2}$. In other words, the increase in $V_{max}$ for bradykinin uptake in OppA(D471R) reflects an enhanced donation of the peptide from the binding protein to the membrane complex.

A large decrease in binding affinity results in a lower uptake rate. In cells expressing OppA(A477D), the lower $V_{max}$ value for bradykinin uptake parallels a dramatic decrease in the binding affinity of the OppA(A477D) protein for bradykinin. If we assume that the increased $K_d$ is a consequence of a higher value for $k_{-1}$, and thus $k_{-2}$, then following the same line of reasoning as in OppA(D471R), one would also expect an increased rate of uptake for OppA(A477D). However, if the $K_d$ becomes too low, the equilibrium between liganded and unliganded OppA will be towards unliganded binding protein and step I in the scheme may become rate determining. In this regard, it is worth noting that unliganded and liganded binding proteins are believed to have a similar affinity for the membrane complex in the case of the histidine system (2).

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