Kinetics and Specificity of Peptide Uptake by the Oligopeptide Transport System of *Lactococcus lactis*†

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Received July 16, 1998; Revised Manuscript Received August 27, 1998

ABSTRACT: To obtain amino acids for growth, *Lactococcus lactis* uses a proteolytic system to degrade exogenous proteins such as caseins. The extracellular cell wall-attached proteinase PrtP and the oligopeptide transport system Opp mediate the first two steps in the utilization of caseins. β-Casein is degraded by PrtP to fragments of 5−30 amino acid residues, and only a limited number of peptides are selected from this pool for uptake via Opp. To study the specificity of Opp and the kinetics of peptide uptake in *L. lactis* in detail, we used the following strategy: (i) the Opp system was overexpressed; (ii) a 4-fold peptidase mutant was used that is unable to degrade KYGK; (iii) iodinated KYGK was used as the reporter peptide; (iv) libraries of peptides, in which one amino acid position is systematically varied, were used as competitive peptides; and (v) peptides were synthesized on the basis of the β-casein degradation products, their inhibition of KYGK uptake was determined, and the uptake of these peptides was followed by high-performance liquid chromatography (HPLC). These studies indicate that (i) the Opp system can transport a broad range of peptides from 4 up to at least 18 residues with very little preference for particular side chains and (ii) the kinetics of peptide uptake differ for different substrates tested. Whereas class I peptides such as KYGK exhibit normal Michaelis−Menten kinetics, the level of uptake of the majority of peptides (class II) increases sigmoidally with concentration. Different models for explaining the apparent cooperative effects that are observed for peptide uptake are discussed.

Lactic acid bacteria possess a proteolytic system that is involved in the degradation of exogenous proteins such as the caseins in milk. The proteolytic system of *Lactococcus lactis* consists of a proteinase PrtP, several peptidases, and amino acid and peptide transport systems. The extracellularly located proteinase PrtP and the oligopeptide transport system Opp are central components for which no alternative activity is present (*1*). In vivo studies of the breakdown of β-casein by PrtP have shown that the carboxyl-terminal end is degraded preferentially. The casein hydrolysate contains peptides varying in length from 5 to more than 30 amino acid residues (*2*). Mass spectrometry has been used to identify substrates of Opp present in the peptide mixture by following the accumulation of peptides and amino acids inside the cell. These studies indicated that peptides up to a length of at least 10 residues are taken up via Opp (*2*); peptides that appeared in the cytoplasm include SLSQS, KAVPYPQ, KVLPVPQ, VLPVPQ, SKVLPVPQ, QSKVLPVPQ, and SQSKVLPVPQ. However, it has not been possible to derive rules for the specificity of Opp because of competition between the peptides, which is influenced by putative differences in affinity constants and maximal rates of uptake as well as temporal differences in substrate concentration.

The Opp system of *L. lactis* consists of five proteins: the integral membrane proteins OppB and OppC, the ATP-binding proteins OppD and OppF, and the substrate-binding protein OppA; the system belongs to the ABC superfamily (*3*). In addition to the ATP-dependent oligopeptide transport system, *L. lactis* possesses two other peptide transporters: a proton motive force-driven di- and tripeptide carrier (DtpT) (*4*) and an ATP-driven di- and tripeptide transporter (DtpP) with a preference for hydrophobic di- and tripeptides (*5*). These systems are not directly relevant to the utilization of β-casein because di- and tripeptides are not formed in detectable amounts by the proteinase.

The OppA protein is homologous to the oligopeptide binding protein of *Salmonella typhimurium* (*6*), but the amino acid similarity (∼20% identity in the primary sequence) is so low that the X-ray structure of OppA of *S. typhimurium* is of limited help in assigning residues critical for substrate binding in OppA of *L. lactis*. In fact, OppA of *L. lactis* is an outsider within the family of peptide binding proteins; i.e., the identity is low (20−25%) even with peptide binding proteins from Gram-positive bacteria such as Spo0K from *Bacillus subtilis*. The structure of OppA of *S. typhimurium* predicts a broad substrate specificity, but this aspect has not been studied in great detail; the upper size limits of peptide binding and transport are unknown. The upper size exclusion

† This work was financially supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, and the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrially relevant research program of The Netherlands Association of Biotechnology Centres in The Netherlands (ABON).

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10.1021/bi981712t CCC: $15.00
Published on Web 11/05/1998

limits of a peptide transport system may not be of immediate physiological relevance in Gram-negative bacteria, as the outer membrane may form a barrier for large peptides. In Gram-positive bacteria, on the other hand, there is no such barrier, and larger peptides may, in principle, be available for the transporter.

In this paper, the kinetics and specificity of the Opp system of *L. lactis* were studied through competitive inhibition of the uptake of a radiolabeled reporter peptide that is not degraded by the cell. The kinetics of uptake of distinct peptides were also inferred from HPLC analysis of extra- and intracellular fractions.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture Conditions, and Growth Media. The following *L. lactis* ssp. *lactis* strains were used: MG1363 (Lac^-, $Proteinase^-$), the plasmid-free derivative of NCDO712 which is Lac^+, $Proteinase^+$ (*7*); the 5-fold peptidase deletion mutant IM16 (*8*), which is a derivative of MG1363 that lacks pepX, pepT, pepO, pepC, and pepN; and the Opp^- deletion strain IM17, which is otherwise isogenic to MG1363 (*8*). When appropriate, the strains were transformed with plasmid pVS8, which carries the genes of the *opp* operon together with *pepO* (*3*). Lactococcal strains were grown at 30 °C in a chemically defined medium (CDM) at pH 6.5 (*9*), which was supplemented with 0.5% (w/v) glucose, or in M17 broth (Difco) and 0.5% glucose (GM17); when appropriate, chloramphenicol was added to a concentration of 5 μg/mL. The strains were stored at −80 °C in CDM and glucose supplemented with 10% (v/v) glycerol.

Iodination of the Tetrapeptide KYGK. The tetrapeptide KYGK was iodinated at the tyrosine residue using the iodinating reagent 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Pierce Chemical Co., Rockford, IL) and 200 μCi of $Na^{125}I$ (2145 mCi/μmol, Amersham). The iodination was performed according to the instructions of the manufacturer. Briefly, 1 mg of iodinating reagent was dissolved in 2 mL of chloroform, and 100 μL of this solution was pipetted into an Eppendorf tube. The solvent was slowly evaporated using nitrogen gas, leading to the formation of a coating of the Eppendorf tube with the iodinating reagent. KYGK (100 μL, 1 mg/mL) was incubated for 15 min at room temperature in the coated tube together with the radiolabeled iodine, and the reaction was stopped by transferring the solution into a noncoated Eppendorf tube containing 1 μL of 1 M DTT. Free iodine was removed by anion exchange chromatography using a DOWEX (OH^-) column, and iodinated KYGK was eluted with 100 mM potassium phosphate (pH 6.5) (*10*). Peak fractions were pooled and stored in small aliquots at −80 °C.

Transport Assays. Prior to transport, the cells were washed twice with 100 mM potassium phosphate and 5 mM $MgSO_4$ (pH 6.5) (buffer A) and resuspended to a final A_{660} of approximately 25. To de-energize the cells and deplete them of amino acids, this washed cell suspension was incubated with 20 mM 2-deoxyglucose for 30 min at 30 °C (*11*). Subsequently, the cells were washed twice with buffer A. The transport assay mixture consisted of buffer A, supplemented with 0.5% glucose; the concentration of total cell protein in the mixture was in the range of 1.5−2.0 mg/mL. The cells were pre-energized for 3 min at 30 °C. Uptake was initiated by adding the peptide(s) in the appropriate concentration. Samples of 1 mL were taken, and the cells were separated from the medium by filtration using 0.45 μm pore size cellulose acetate (OE 67) filters (Schleicher & Schuell GmbH, Dassel, Germany). The cells were subsequently washed twice with 2 mL of ice-cold buffer A. Peptide transport was monitored by determining the extracellular and intracellular concentrations of peptides and their component amino acids by reversed-phase high-performance liquid chromatography (*12*). Amino acids and peptides were analyzed after derivatization with dansyl chloride and detected with a Jasco fluorescence detector FP-920 spectrophotometer (excitation and emission wavelengths of 315 and 500 nm, respectively).

For transport assays with ^{125}I- or ^3H-labeled peptides, the cells were used directly after washing with buffer A. The assay mixture consisted of 100 μL of buffer A supplemented with 0.5% glucose, and the protein concentration was in the range of 1.5−2.0 mg/mL. Uptake was initiated by adding ^{125}I- or ^3H-labeled peptide at the appropriate concentration. The cells were separated from the medium by filtration using 0.45 μm pore size cellulose acetate (OE 67) filters (Schleicher & Schuell GmbH). The cellulose acetate filters exhibited a low background binding level of KYGK as compared to the more generally used cellulose nitrate filters. Transport reactions were stopped by adding a 20-fold excess of ice-cold 100 mM LiCl, and the filters were washed once more with 2 mL of this solution. All transport assays were carried out at 30 °C.

Data Analysis. For the determination of IC_{50} values, the data were fitted to the logistic function

$$V = \frac{V_{100} - V_0}{\left[1 + \left(\frac{[I]^m}{IC_{50}{}^m}\right)\right]} + V_0 \qquad (1)$$

in which V_{100} and V_0 correspond to the rate of uptake in the absence of inhibitor and the rate of uptake at an infinite inhibitor concentration, respectively, [I] is the concentration of inhibitor peptide, m is related to the slope of the curve at the inflection point, and IC_{50} is the concentration of inhibitor peptide at which the uptake is inhibited by 50%.

The data of the kinetic experiments were fitted according to the equation

$$V = \frac{V_{max}[S]^n}{[S]^n + K_m} \qquad (2)$$

in which V and V_{max} correspond to the actual and maximal rate of uptake, respectively, [S] is the substrate concentration, K_m is the apparent affinity constant, and n is the Hill coefficient. If n is 1, the equation takes the form of a normal Michaelis−Menten equation.

Competitive inhibition was analyzed according to the equation

$$V = \frac{V_{max}[S]^{n1}}{[S]^{n1} + K_m\left[1 + \left(\frac{[I]^{n2}}{K_I{}^{n2}}\right)\right]} \qquad (3)$$

in which V, V_{max}, and K_m have the same meaning as above and [I] and K_I correspond to the concentration of inhibitor peptide and apparent inhibition constant, respectively. Since the Hill coefficient (n) is not necessarily the same for the substrate and inhibitor peptides (see Results), we denote the corresponding coefficients *n1* and *n2*.

The data used for the different kinetic experiments represent the arithmetic average of three independent measurements; the standard deviation is given. The data were routinely analyzed using the nonlinear least-squares regression program of SigmaPlot (Jandel Scientific Software).

Miscellaneous. Protein concentrations were determined by the method of Lowry et al. (*13*), with bovine serum albumin (BSA) as the standard. For immunoblotting, the proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, 10% polyacrylamide) and transferred to poly(vinylidene difluoride) membranes (PVDF) by semi-dry electrophoretic blotting (*14*). The antibodies directed against OppA were used at a serum dilution of 1:20000 (*8*).

RESULTS

Increased Level of Expression of the Opp System. To study accurately the transport of peptides, it was necessary to amplify the levels of the Opp proteins. By introduction of extra copies of the *opp* genes (pVS8) into *L. lactis* MG1363, the rate of leu-enkephaline uptake was increased approximately 8-fold (data not shown). The increased activity correlated with an amplified expression of OppA (Figure 1A,B), OppD (not shown), and most probably OppB, OppC, and OppF as well. The SDS−PAGE showed an increase in the intensity of a ±65 kDa band for the strain containing the plasmid pVS8, whereas this band was absent in the Δ*opp* strain IM17. Immunoblot analysis with antibodies directed against OppA of *L. lactis* confirmed the enhanced expression of OppA in MG1363/pVS8 (Figure 1A,B).

Subsequently, various growth media were tested for their inducing and/or repressing effects on the expression of Opp. Complex broth (GM17, most likely containing peptides of various sizes) and chemically defined medium (CDM), to which the essential amino acid leucine was added in the form of L-leucine, L-leucyl-L-leucine, L-leucyl-L-leucyl-L-leucine, or L-leu-enkephaline, were compared. Figure 1C shows that the highest uptake rates were obtained in CDM and leucine. Peptides seem to inhibit the expression of Opp and, consequently, to lower the peptide transport activity. This effect was most pronounced for L-leucyl-leucyl-leucine, which abolished nearly all activity irrespective of whether free leucine was present or absent in the medium.

Selection of a Reporter Peptide. Because of endogenous peptidase activity, intracellular leu-enkephaline was degraded to amino acids, and efflux of radiolabeled tyrosine took place (data not shown; see also the transient uptake depicted in Figure 1C). To lower the extent of breakdown of the peptide, the 5-fold peptidase mutant IM16 was used; however, degradation of leu-enkephaline to the tripeptide tyrosyl-glycyl-glycine and the amino acids phenylalanine and leucine still occurred (*15*). A good reporter peptide should meet all of the following criteria: (i) to be taken up exclusively and with a high rate by the Opp system, (ii) to avoid being hydrolyzed intracellularly, (iii) to contain a tyrosine residue that can be used for iodination of the peptide, and (iv) to have the transport of the iodinated peptide be indistinguishable from the uptake of unmodified reporter peptide. The tetrapeptide L-lysyl-L-tyrosyl-L-glycyl-L-lysine (KYGK) met all four criteria. It was anticipated that KYGK would not be broken down by the peptidase mutant IM16/pVS8 on the basis of previous studies (*15*). Figure 2A shows that KYGK accumulates intracellularly; the initial rate of uptake at a substrate concentration of 8 μM was 0.5 nmol min^{-1} mg^{-1} of protein. As anticipated, the uptake of KYGK was competitively inhibited by tetraalanine, whereas dialanine and tyrosine had no significant effect. The apparent affinity constant (K_m^{app}) for the uptake of KYGK by the Opp system was 0.7 ± 0.1 mM, and the V_{max} was 63 ± 7 nmol min^{-1} (mg of protein)$^{-1}$. The inhibition constant (K_I) for tetraalanine using KYGK as reporter peptide was 0.04 mM (Figure 2B).

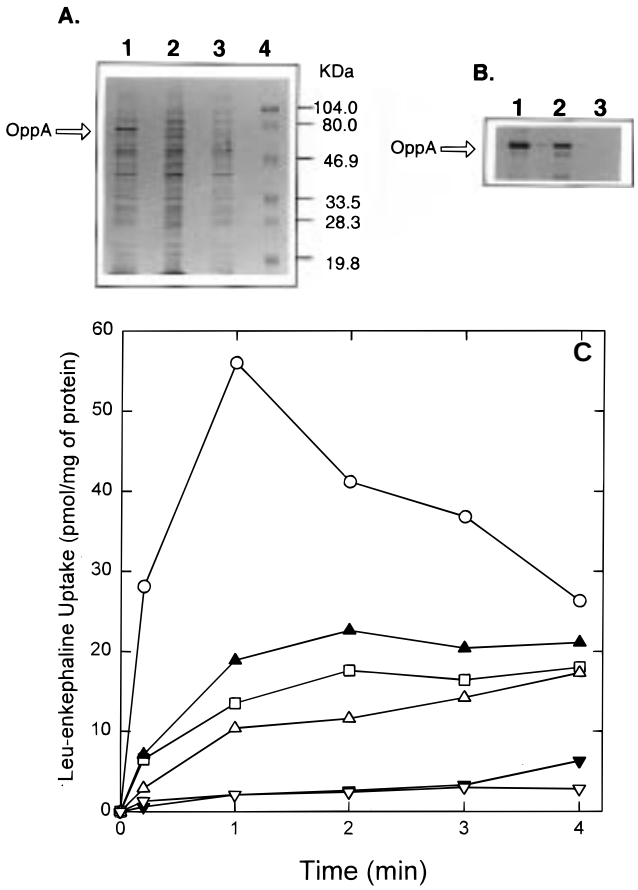

FIGURE 1: Expression levels of OppA and Opp transport activity in *L. lactis*. SDS−polyacrylamide gel (10% v/v) stained with Coomassie Brilliant Blue (A) and immunoblotted using antibodies directed against OppA of *L. lactis* (B): lane 1, cell extract of *L. lactis* IM16/pVS8 cells grown on CDM; lane 2, cell extract of IM16 cells grown on CDM; lane 3, cell extract of IM17 (Δ*opp*) cells grown on CDM; and lane 4, molecular mass marker; 15 μg of total protein was loaded per lane. (C) The uptake of [^{3}H]-leu-enkephaline (final concentration of 3.6 nM) by MG1363/pVS8 cells grown in different media. After growth to an A_{660} of about 1.0, the cells were washed and resuspended, reaching a final protein concentration of 0.5 mg/mL in buffer A. Culture conditions were as follows: (○) CDM containing 3.6 mM leucine, (□) M17, (△) CDM and 3.6 mM cold leu-enkephaline, (▲) CDM without leucine with 3.6 mM cold leu-enkephaline, (▽) CDM and 1.2 mM leucyl-leucyl-leucine, and (▼) CDM without leucine with 1.2 mM leucyl-leucyl-leucine.

Kinetics of Uptake of β-Casein-Derived Peptides. A number of peptides formed from β-casein by PrtP and used

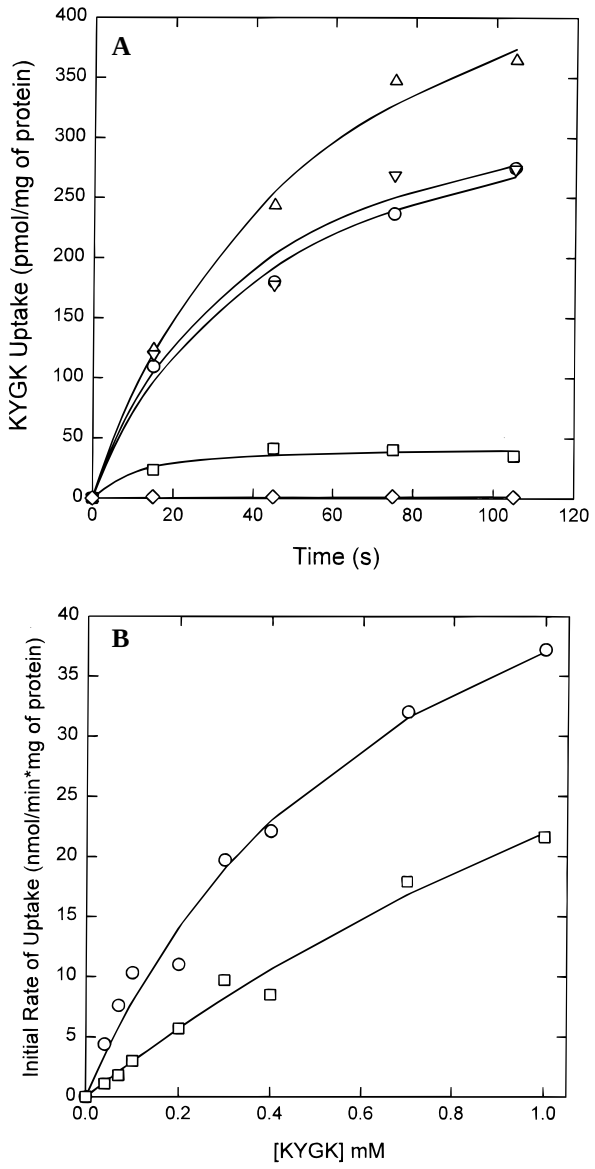

FIGURE 2: Transport of L-lysyl-L-tyrosyl-L-glycyl-L-lysine (KYGK) by the Opp system of *L. lactis*. (A) Uptake of the iodinated KYGK by IM16/pVS8 grown on CDM with 0.5% (w/v) glucose. The final protein and peptide label concentrations were 1.8 mg/mL and 8 μM, respectively: (○) no addition or with (□) 1 mM tetraalanine, (△) 1 mM dialanine, (▽) 1 mM tyrosine, and (◇) Δ*opp* strain without further additions. (B) Concentration dependence of the initial rate of KYGK uptake in IM16/pVS8 cells: (○) no addition or with (□) 0.2 mM tetraalanine. The data were fitted to eqs 2 (no addition) and 3 (with tetraalanine); the *n* values approached 1.

by *L. lactis* were synthesized (*2*). Figure 3A shows the carboxyl-terminal region of β-casein and the peptide fragments formed by the proteinase PrtP; the figure also depicts which of these peptides were taken up (thick arrows) from a casein hydrolysate and which were not (thin arrows). Of these peptides, six were tested as possible inhibitors of the uptake of KYGK by the Opp system. Figure 3B shows the inhibition of K[^{125}I]YGK uptake by the undecameric peptide RDMPIQAFLLY and by nonlabeled KYGK. It is important to note that inhibition may reflect competition for binding of KYGK to OppA and does not necessarily reflect transport of the inhibitor peptide (see later). Surprisingly, the inhibition by RDMPIQAFLLY showed kinetics different than those by KYGK. Figure 3B shows fits of the data using eq 1 (Experimental Procedures). Although it is difficult to differentiate between *m* values of 2 or 3, it is clear that *m* exceeds 1 for RDMPIQAFLLY. An *m* value of >1 suggests that peptide binding and/or transport is a cooperative process. The inhibition of K[^{125}I]YGK uptake by nonlabeled KYGK fits best with an *m* value of 1, and this result is consistent with the Michaelis−Menten kinetics observed in Figure 2B. Cooperative effects were also observed for SLSQS, SLSQSKVLP, SLSQSKVLPVPQ, RDMPIQA, and RDMPIQAF; the *m* values varied between 2 and 3 (Table 1).

Since these casein-derived peptides were still partially degraded in the peptidase mutant used, whereas KYGK was not, we needed to establish whether the transport kinetics were affected by the intracellular fate of the transported peptide. Previous work (*9*) showed that the tetrapeptide GLGL is not degraded in a Δpep*XTCN* strain. The inhibition of KYGK uptake by GLGL clearly exhibited cooperative effects ($m = 2.3 \pm 0.4$), similar to those observed for RDMPIQAFLLY and other peptides, indicating that the difference in kinetic behavior was not due to peptide or amino acid accumulation. Furthermore, it was not exclusively a feature of large peptides.

Cooperativity should be observed not only in assays in which RDMPIQAFLLY is used as an (competitive) inhibitor of KYGK uptake but also when uptake of RDMPIQAFLLY is measured directly. Since this peptide is hydrolyzed intracellularly, and amino acids such as tyrosine are partially released from the cell via carrier-mediated efflux, iodinated RDMPIQAFLLY could not be used in the uptake assays. However, some amino acids, including proline and methionine, are almost quantitatively retained by the cell under our experimental conditions (initial rate measurements; data not shown), and this property was used to follow the kinetics of RDMPIQAFLLY uptake. Figure 3C shows that the rate of RDMPIQAFLLY uptake depends sigmoidally on the substrate concentration, as determined from the accumulation of proline. Similar results were obtained when the accumulation of other amino acids was followed (data not shown).

Transport of Peptide Libraries. The X-ray structure of OppA of *S. typhimurium* indicates that the protein binds the polypeptide backbone and accommodates most, if not all, side chains of natural L-amino acids at the 1−3 positions of the peptide. Whether, and how, peptides longer than five residues are bound is unclear. To investigate whether OppA of *L. lactis* has a clear preference for particular amino acids at the amino terminus or the carboxyl terminus of relatively large peptides, we used nona- or decameric peptides as competitive inhibitors of KYGK uptake. In these peptide libraries, which were kindly supplied by J. J. Neefjes, either the amino- or the carboxyl-terminal residue was varied while the other positions were kept constant. The effect of a 12-fold excess of the nonameric peptide XVDNKTRAY on the uptake of iodinated KYGK was analyzed first; this peptide library consists of 19 peptides that have a different amino acid at position X. All of the peptides strongly inhibited transport (Figure 4A). The apparent K_Is for the nonameric peptides varied from 45 μM when X = N to 78 μM when X = Q (see Table 1 for details of the calculations) when calculated for the kinetic parameters of KYGK uptake, the extent of inhibition of uptake of KYGK at a substrate concentration of 4 μM, and inhibitor peptide concentrations

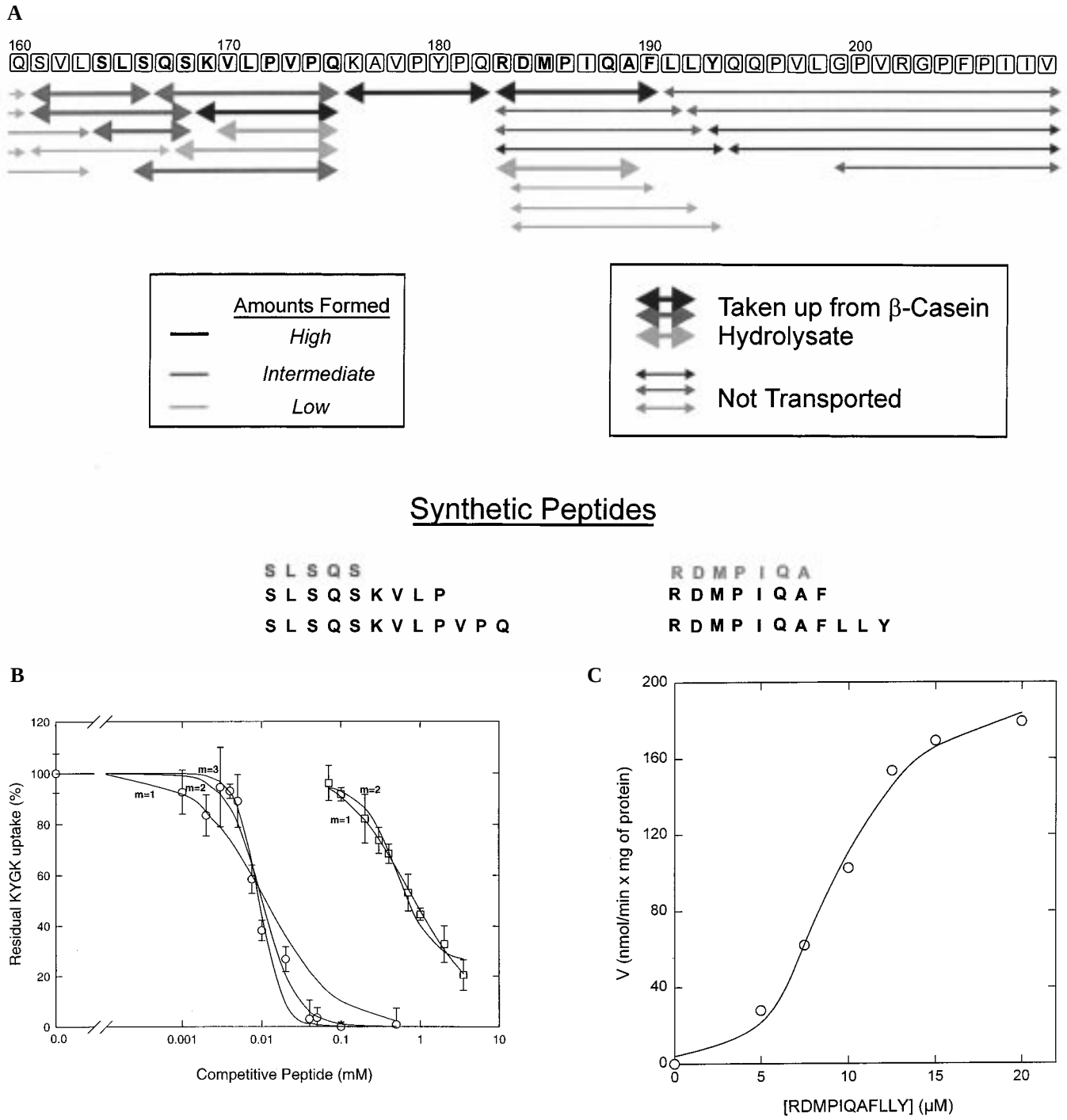

FIGURE 3: Inhibition of KYGK uptake by β-casein-derived peptides. (A) Peptides of the carboxyl-terminal part of β-casein that are formed by PrtP in vivo (for details, see ref *2*). The synthetic peptides based on the β-casein sequence are indicated. (B) Inhibition of iodinated KYGK uptake by the β-casein-derived peptide RDMPIQAFLLY (○) and unlabeled KYGK (□) using IM16/pVS8 cells. The data were fitted to eq 1 using different *m* values. The final concentration of iodinated KYGK was 4 μM, and the protein concentration was 1.8 mg/mL. The IC_{50} values for RDMPIQAFLLY and KYGK were 10 ± 1 and 700 ± 18 μM, respectively. The 100% transport rate was 470 pmol min^{-1} (mg of protein)$^{-1}$. (C) Uptake of RDMPIQAFLLY by MG1363/pVS8 cells. The uptake rates were calculated from the intracellular proline accumulation rate as determined by HPLC. The apparent K_m for RDMPIQAFLLY was 8.8 ± 0.4 μM.

of 10 or 49 μM and using Hill coefficients for KYGK (*n1*, eq 3) of 1 and inhibitor peptide (*n2*) of 2. The peptide with a cysteine residue at the N-terminal position inhibited the least, most likely because the cysteine was partially oxidized. Reducing the peptide with 1 mM DTT gave a much higher extent of inhibition, and 1 mM DTT alone had no effect on transport. The effect of a large excess (1250-fold) of unlabeled KYGK on the uptake of iodinated KYGK is shown as a positive control.

For variations of the carboxyl-terminal position, the decameric peptides RYWANATRSX were used. In general, the apparent K_I values of the decameric peptides were lower than those for the nonameric peptides, which may not (only) reflect the differences in size but (also) be related to the differences in the composition of the two libraries. The extents of inhibition of KYGK uptake by a 12- or 2.5-fold excess of decameric peptide are shown in Figure 4B. The estimated K_I values for the peptides were 10 μM when X =

Table 1

peptide	K_m (μM)	IC_{50} (μM)[a]	K_I (μM)[b]	K_I^{est} (μM)[c]		
				n2 = 1	*n2* = 2	*n2* = 3
KYGK	700 ± 100	700 ± 18 (*m* = 1.1 ± 0.3)	−	−	−	−
AAAA	−	37 ± 9 (*m* = 1.4 ± 0.5)	40 (*n2* = 1)	−	−	−
GLGL	−	26 ± 2 (*m* = 2.3 ± 0.4)	30 (*n2* = 2)	−	−	−
XVDNKTRAY	−	−	−	9−60	45−78	46−81
RYWANATRSX	−	−	−	2−30	10−38	17−41
SLSQS	−	75 ± 6 (*m* = 2.1 ± 0.5)	82 (*n2* = 2)	−	−	−
SLSQSKVLP	−	27 ± 5 (*m* = 1.9 ± 0.3)	23 (*n2* = 2)	−	−	−
SLSQSKVLPVPQ	−	20 ± 3 (*m* = 2.0 ± 0.2)	17 (*n2* = 2)	−	−	−
RDMPIQA	−	22 ± 2 (*m* = 2.7 ± 0.7)	30 (*n2* = 3)	−	−	−
RDMPIQAF	−	25 ± 2 (*m* = 1.8 ± 0.2)	33 (*n2* = 2)	−	−	−
RDMPIQAFLLY	8.8 ± 0.4	10 ± 1 (*m* = 2.1 ± 0.3)	10 (*n2* = 2)	−	−	−

[a] The *m* values in the IC_{50} column were obtained by fitting the experimental data to eq 1. [b] The *n2* values in the K_I column reflect the rounded-off *m* values (IC_{50} column) and were used to convert the IC_{50} values into K_I values. [c] K_I values were obtained for different values of *n2* (*n2* as defined in eq 3).

V to 38 μM when X = E (for *n1* = 1 and *n2* = 2).

Transport of Nonameric and Octadecameric Peptides. The experiments with the β-casein-derived peptides show that peptides up to a length of 12 residues effectively compete for transport via Opp. To establish unequivocally that these peptides not only inhibit KYGK uptake by competing for binding to OppA but also are transported via Opp, the extracellular fractions of the transport assay mixtures of *L. lactis* strains MG1363/pVS8 and IM17 were analyzed by HPLC after incubation of the cells with 200 μM SLSQSKVLP, RDMPIQAFLLY, or RPPGFSPFR (bradykinin) in the presence of 0.5% (w/v) glucose.

The data for SLSQSKVLP are shown in Figure 5, but similar results were obtained with RDMPIQAFLLY and RPPGFSPFR (data not shown). The SLSQSKVLP peptide disappeared rapidly from the external medium when it was incubated with MG1363/pVS8 (Opp^+) cells, whereas the extracellular concentration of SLSQSKVLP did not change significantly in the presence of IM17 (Opp^-) cells (Figure 5). In fact, the overall HPLC pattern did not change significantly when the Opp^- strain was incubated with SLSQSKVLP (Figure 5B), which rules out the possibility that extracellular breakdown of the peptide occurs under our experimental conditions. The appearance of some new peaks and the increase in the extent of others when the Opp^+ strain was incubated with SLSQSKVLP must therefore be due to the excretion of amino acids and/or smaller peptides following uptake of SLSQSKVLP and intracellular degradation. Further evidence for this notion comes from the analysis of intracellular fractions, which did not change over time when strain IM17 was used, whereas the size of the pools of amino acids present in SLSQSKVLP increased in MG1363/pVS8. The uptake of SLSQSKVLP was also followed in the 5-fold peptidase mutant IM16 as this peptide was found to accumulate in strain IM16 when taken up from a complex casein hydrolysate (*2*). The intracellular concentration of SLSQSKVLP was small compared to that of cells that take the peptide from a complex casein hydrolysate (data not shown), most likely because all the residual peptidase activity in strain IM16 is directed against a single peptide.

To establish further the upper size exclusion limits of Opp of *L. lactis*, an octadecameric peptide (KILFLNNAINHGIFSPLG) was used in the transport assay (Figure 6). In this experiment, we show the changes in intracellular amino acid pools following incubation of the Opp^+ and Opp^- strains with KILFLNNAINHGIFSPLG. The sizes of the pools of intracellular peptides and amino acids in MG1363/pVS8 (Opp^+) were already significantly increased after incubation for 15 s with the peptide, whereas no such increases were observed in strain IM17 (Opp^-). Extracellular breakdown of the octadecamer to amino acids or small peptides, e.g., due to cell lysis, can also be ruled out in this experiment, since this would have led to an increase in the size of the intracellular amino acid pools in strain IM17 as a result of uptake of amino acids and/or di- and tripeptides via the corresponding amino acid and di- or tripeptide transport systems. Therefore, we conclude that *L. lactis* is able to transport peptides as long as 18 amino acid residues, and possibly even longer ones.

DISCUSSION

In this paper, we describe the kinetics and specificity of the oligopeptide transport system of *L. lactis.* The following findings are the most important. (i) Opp is able to bind and transport peptides as long as 18 residues. (ii) The specificity of the system is broad, and the affinity differs at most 2−4-fold when the amino- or carboxyl-terminal residues of nona- and decameric peptides are varied. (iii) Non-Michaelis−Menten kinetics are observed for the transport of many, but not all, of the peptides tested.

For these studies, the expression levels of the Opp proteins were increased by using a multicopy plasmid carrying the *opp* genes and selection of appropriate growth conditions. Maximum levels of expression were observed for strains carrying plasmid pVS8 and grown on a chemically defined medium containing amino acids. Strains grown on a chemically defined medium containing di- and tripeptides show very low levels of uptake of [^{3}H]leu-enkephaline, whereas the presence of this pentapeptide in the growth medium has a moderately repressing effect. The mechanism of regulation of expression of Opp is not yet fully understood, but our data (to be published elsewhere) suggest that di- and tripeptides with hydrophobic residues act as effector molecules in the transcriptional regulation of *opp*. The inhibition by leu-enkephaline is therefore most likely caused by the formation of di- and/or tripeptides intracellularly (e.g., YGG and FL; *15*).

An unexpected finding of our work is related to the non-Michaelis−Menten kinetics observed with RDMPIQAFLLY,

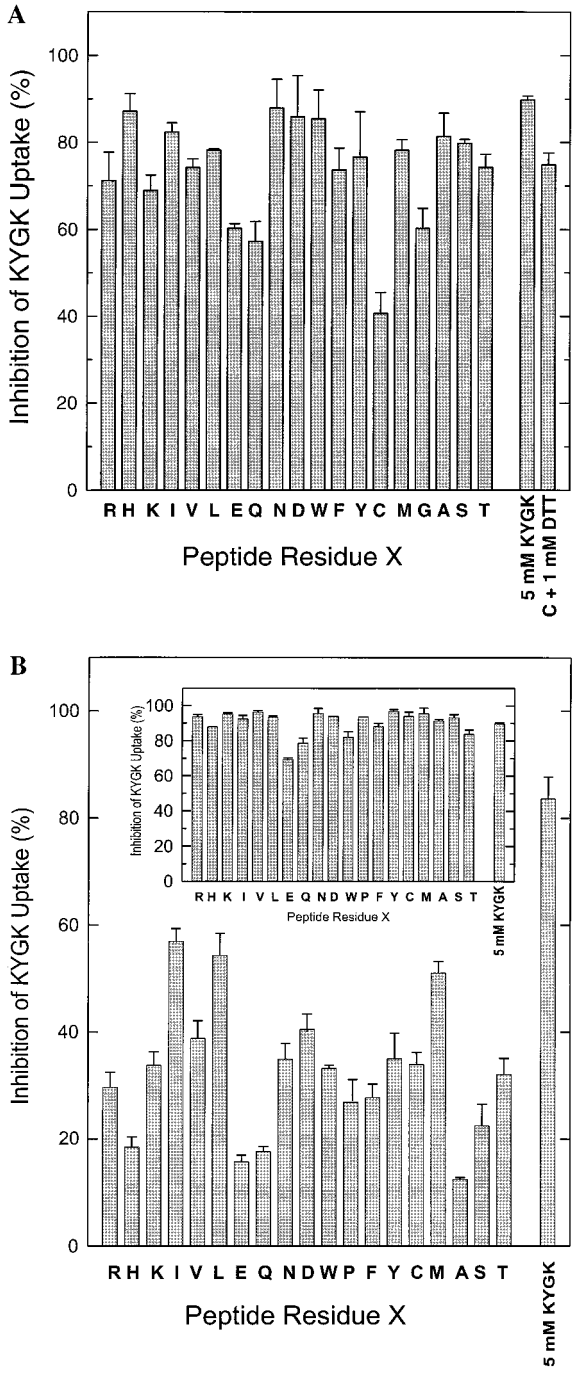

FIGURE 4: Inhibition of KYGK uptake by nonameric and decameric peptides using IM16/pVS8 cells. (A) Inhibition of KYGK uptake by XVDNKTRAY, in which X corresponds to the amino-terminal residue as indicated in the figure. The concentrations of labeled KYGK and inhibitor peptide were 4 and 49 μM, respectively. The uninhibited transport rate corresponds to 430 pmol min^{-1} (mg of protein)$^{-1}$. (B) The inhibition of KYGK uptake by RYWANATRSX, in which X corresponds to the carboxyl-terminal residue. The concentration of KYGK was 4 μM, whereas the concentration of RYWANATRSX was kept at 10 (main figure) and 49 μM (inset). The protein concentration in the transport assay was 2.0 mg/mL. The error bars indicate the standard deviation from the mean of two independent experiments.

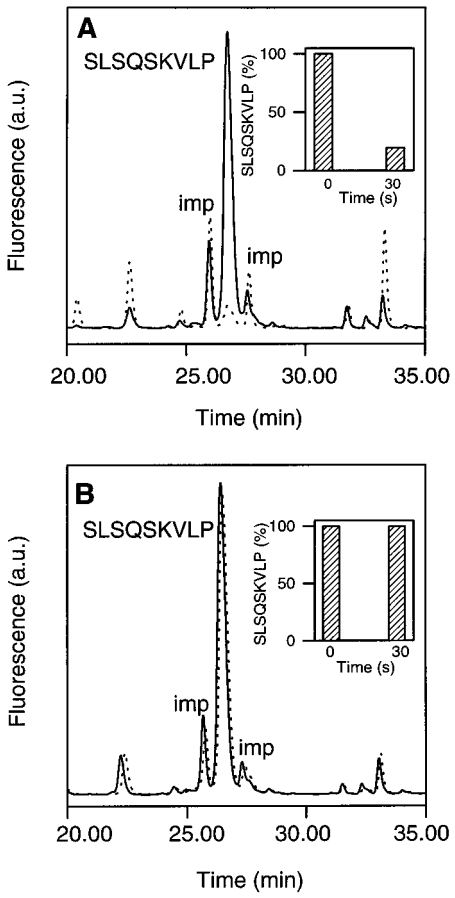

FIGURE 5: Intact nonameric peptide SLSQSKVLP is taken up via Opp. Panel A shows the HPLC chromatograms of the extracellular medium after incubation of 200 μM SLSQSKVLP with MG1363/pVS8 (Opp$^+$) cells in the presence of 0.5% (w/v) glucose after 0 (solid line) and 30 s (dotted line). Panel B shows the same chromatograms using IM17 (Opp$^-$) cells. The peak indicated as SLSQSKVLP was identified by mass spectrometry (*2*); imp refers to impurities present in the SLSQSKVLP sample. Importantly, these peaks did not change significantly during the course of the experiment (either in MG1363/pVS8 or in IM17). The inset shows the relative amount of peptide present after incubation for 0 and 30 s. The protein concentration in the assay mixture was 2.0 mg/mL.

GLGL, and other peptides (class II), whereas the system obeys Michaelis−Menten kinetics with KYGK and AAAA as substrates (class I peptides). How do we explain these observations? The sigmoidal rate−substrate relationship points to an allosteric mechanism in which either binding of the peptide to OppA or the association of liganded OppA with the translocator complex occurs in a cooperative manner. Since the receptors of binding protein-dependent transport systems are thought to have only a single substrate-binding site, the binding of peptides to OppA is unlikely to be cooperative. In fact, in recent experiments with purified OppA, we have shown that binding of class II peptides SLSQSKVLP and SLSQSKVLPVPQ to OppA exhibits simple saturation kinetics, from which K_d values in the micromolar range could be estimated (*16*). Thus, the kinetics of peptide binding do not reflect the kinetics of transport.

By analogy with the histidine permease of *S. typhimurium* (*17*) and the maltose permease of *Escherichia coli* (*18*), and founded mathematically (*18*, *19*), both ligand-loaded and unloaded OppA may interact with the membrane components and compete with one another for docking on OppBCDF. This implies that, in the transport assays, peptides may associate with either "free" or "docked" OppA. This interaction now resembles a random-order mechanism in which the ternary complex, i.e., liganded OppA bound to OppBCDF, is formed via either binding of the peptide to

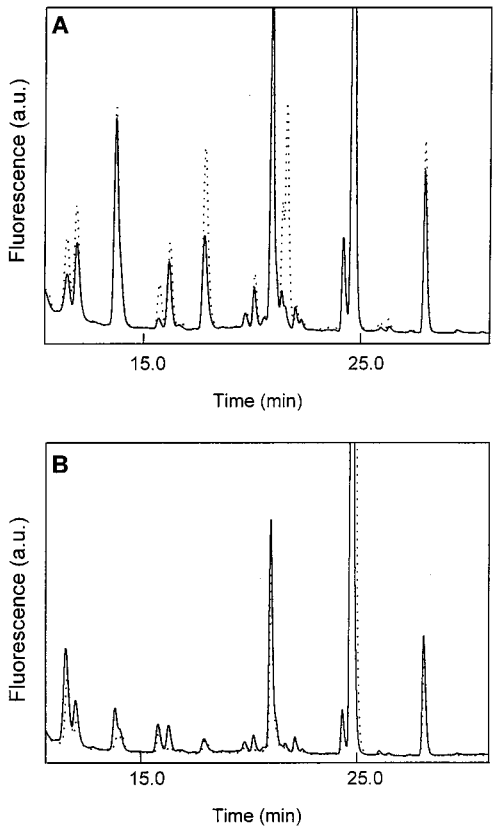

FIGURE 6: Uptake of the octadecameric peptide KILFLNNAINHGIFSPLG. Panel A shows the HPLC chromatograms of the internal amino acid pool of MG1363/pVS8 cells after incubation with 100 μM octadecameric peptide KILFLNNAINHGIFSPLG after 0 (solid line) and 15 s (dotted line). Panel B shows the corresponding chromatograms of the internal amino acid pool of the IM17 (Δ*opp*) strain after incubation after 0 (solid line) and 5 min (dotted line).

"docked" OppA or binding of the peptide to "free" OppA, followed by docking of liganded OppA to OppBCDF. A sigmoidal dependence of the rate of uptake on the peptide concentration is observed when the slower of the two pathways (e.g., docking of free OppA to OppBCDF, followed by peptide binding) contributes most to the overall flux at low peptide concentrations, whereas the more rapid one does at high peptide concentrations (*20*). Since class I peptides exhibit Michaelis−Menten kinetics in the uptake assays, the forward rates of the two alternative pathways to the ternary complex should be similar for these substrates.

As shown by computer simulation studies (*20*, *21*), sigmoidal rate−substrate relationships can also be observed if a protein (OppA) exists in two or more forms that interact differently with the substrate, a situation that may be relevant for binding protein-dependent transport. In the simplest model (Figure 7; *22*), the substrate associates with either one of the two conformational isomers of the protein (E_1 and E_2), of which E_1 is thermodynamically more stable; L_O refers to external ligand, whereas L_T corresponds to ligand that is transferred to the membrane components of Opp. If interconversion of E_1 to E_2 is slow compared to the other reactions, and the rate-determining step in going from E_1 and L_O to E_2 and L_T is $E_1L \rightharpoonup E_2L$ ($k_{+2} \ll k_{+3}$; to make the reverse isomerization $E_2L \rightharpoonup E_1L$ kinetically unimportant we assume that $k_{+2} \gg k_{-2}$), then E_2 is formed in appreciable amounts after the first turnover of the system. The slow

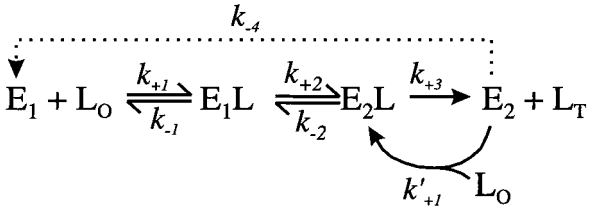

FIGURE 7: Kinetic scheme for explaining the observed non-Michaelis−Menten kinetics.

step in the reaction sequence is bypassed when, subsequently, L_O associates with E_2 rather then E_1. Since the rate of conversion of E_1 to E_2 is a function of the ligand concentration, the overall reaction rate will accelerate with the concentration, which will give rise to a sigmoidal curve. For the Opp system, E_1 and E_2 would correspond to two different conformations of unliganded OppA. Since class I and II peptides exhibit different kinetics, one would have to assume that $K_d(E_1) > K_d(E_2)$ or $k'_{+1} > k_{+1}$ for class II, whereas these parameters are similar for class I.

At present, it is difficult to discriminate between these models or alternative, more complicated, ones (*20*) to explain the apparent cooperative effects. Experimentally, it would require a kinetic analysis of transport in which peptide and OppA concentrations could be varied independently. It would also require pre-steady-state analysis for following the initial steps of the transport process, which is not feasible with the present experimental setup.

The observation that different ligands (peptides) impose distinct kinetic behavior on the uptake processes is unusual, but it may have its equivalent in other binding protein-dependent transport systems. For the maltose transport system of *E. coli*, there is ample evidence that different sugars lead to distinct conformational isomers (R and B mode binding) of the binding protein MalE (*23*). Sugars that are bound via the B mode are not transported, whereas R mode substrates are. The R and B modes of MalE are related to the closed-liganded and open-liganded state of the binding protein, respectively. Within the scheme depicted in Figure 7, the R mode of binding would correspond to E_2L. Since sigmoidal rate−substrate relationships are not observed for the maltose system, the R mode substrates would be analogous to class I in case of Opp. Within the same scheme, B mode substrates would trap the system in E_1L, i.e., a situation that requires k_{+2} to approximate zero.

The uptake and inhibition experiments using the peptides from the libraries as competitive substrates show that the Opp system of *L. lactis* bind peptides without much preference for the side chain of the amino-terminal residue; i.e., bulky, hydrophobic, polar, and charged residues are tolerated, albeit with some variation in affinity. The side chain of the carboxyl-terminal residue of a decameric peptide affects the affinity over a 4-fold range, suggesting that this portion of the molecule is interacting with OppA; the possibilities for such interaction are discussed below. Taken together, the uptake and inhibition experiments show that peptides in the range of 4−12 residues compete with the reporter peptide KYGK with K_I values in the range of 10−700 μM (Table 1).

To facilitate the comparison of the various inhibitor studies, we have converted the IC_{50} values into K_I values on the basis of the assumption that inhibition of KYGK uptake

by the reporter is competitive. In the case of the peptide libraries, the K_I values were estimated from a limited number of data points. Consequently, we do not have estimates of the Hill coefficients and could only calculate the K_I values for given values of *n* (Table 1). The variations in the K_I values of the nona- and decameric peptides are 7- and 15-fold for a particular position when one assumes that *n2* (eq 3) is 1. These values decrease to 2−4-fold when a more plausible *n2* value of 2 is used.

Although the upper size limits for binding to OppA and transport by Opp of *S. typhimurium* are not known, the system is able to bind and transport dipeptides. This suggests a functional and structural difference with OppA from *L. lactis* as we have never observed any transport, or (competitive) inhibition of oligopeptide uptake, by dipeptides. In the closed-liganded state of OppA of *S. typhimurium*, the peptides of two to five amino acid residues are completely enclosed in the protein interior. The main chain of the peptide is in an extended conformation and forms parallel and antiparallel β-sheet interactions with OppA, thereby satisfying the hydrogen bonding capacity of the peptide backbone (*6*). The crystal structure of OppA provides no information about the interactions of the carboxyl-terminal residues of peptides longer than five amino acids. To our knowledge, there is no experimental evidence against or in favor of binding to OppA of anything larger than a pentapeptide. The observation that the K_I values for inhibition of Opp-mediated transport by RYWANATRSX in *L. lactis* differ approximately 4-fold (*n2* = 2), when the carboxyl-terminal residue of RYWANATRSX is varied, suggests that the carboxyl-terminal residues are interacting with OppA. Most probably, the carboxyl-terminal residues of peptides longer than five residues hang out of the binding site of OppA but make contact with the surface of the binding protein. Similar to what is known for sugar transport proteins such LacY of *E. coli* and LacS in *Streptococcus thermophilus* (cited in ref *24*), these contacts could contribute to the affinity, but do not necessarily reside in a well-defined binding site that recognizes the peptide. In other words, the specificity of OppA for peptides is determined by the interactions between the main chain of the first four to five amino acids, whereas additional residues added onto a peptide may increase the binding affinity but do not participate in the recognition mechanism.

Our studies indicate that every residue is, in principle, tolerated at the 1 and 10 positions of a peptide, albeit with moderate variations in affinity. The affinities for given peptides may vary by several orders of magnitude and determine the extent of utilization of peptides from complex protein hydrolysates if residues other than the amino- and carboxyl-terminal amino acids also contribute to the interaction with OppA. In fact, our in vivo studies on the utilization of β-casein-derived peptides indicate that some peptides that are formed in relatively large amounts are not taken up by *L. lactis*, whereas others that are formed in small amounts are efficiently removed from the medium (*2*; see also Figure 3A).

At present, we cannot explain the behavior of the undecameric β-casein-derived peptide RDMPIQAFLLY, which is formed in large amounts during breakdown of β-casein by the proteinase PrtP. It is not transported by Opp from the β-casein-derived peptide pool (*2*), whereas it is taken up with a high rate and high affinity when present as a single peptide in the external medium. Although our data set is limited, it seems that the larger the peptide the higher the affinity, whereas estimates of their maximal rates of uptake (HPLC experiments) do not reveal large differences (unpublished).

ACKNOWLEDGMENT

We acknowledge Dr. Jacques J. Neefjes of the Dutch Cancer Institute for kindly providing the different peptide libraries. We also acknowledge Dr. Anja Hagting for valuable suggestions throughout the work.

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BI981712T