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# Kinetics and Structural Requirements for the Binding Protein of the Di-tripeptide Transport System of *Lactococcus lactis*

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ABSTRACT: The gene (*dppA*) encoding the binding protein of the di-tripeptide ABC transporter of *Lactococcus lactis* (DppA) was cloned under the control of the nisin promoter. Amplified expression ($\approx$200-fold increase) of the protein fused to a carboxyl-terminal six-histidine tag allowed the purification of DppA-(His)$_6$ by nickel-chelate affinity and anion-exchange chromatography. Ligand binding to DppA-(His)$_6$ elicited an electrophoretic mobility shift, a decrease in the intrinsic fluorescence, and a blue shift of the emission maximum. Each of these parameters detected conformational changes in the protein that reflect ligand binding, and these were used to determine the structural requirements of DppA-(His)$_6$ for binding peptides. The major features of peptide binding include (i) high affinity for di- and tripeptides, (ii) requirement of a free N-terminal $\alpha$-amino group and an $\alpha$-peptide bound contiguous with the N-terminal amino group, (iii) stereospecificity for L-isomers, and (iv) preference for dipeptides containing methionine or arginine, followed by hydrophobic tripeptides consisting of leucine or valine residues. Maximal binding affinity was detected at pH 6.0, and the $K_d$ for binding increased 1 order of magnitude for every unit increase in pH. This suggests that the ionization of protein residues (p$K > 6.0$) in or in close proximity to the binding site is critical in the binding mechanism.

Most if not all (micro)organisms have evolved specific transport systems that mediate the uptake of peptides from their environment. Nutrient accumulation is the most obvious function of these transporters, but they also play a role in biological processes such as sporulation, heme synthesis, gene expression, and chemotaxis *(1−4)*. Structurally diverse peptide transporters have been characterized, but the majority of the high-affinity systems belong to the ABC transporter superfamily *(1, 5−7)*. These are multicomponent/domain transport systems composed of two integral membrane units and two peripheral ATP-binding units, which function together with an extracytoplasmic solute-binding protein *(8)*. The substrate binding proteins provide the primary interaction site for the ligand and largely define the specificity of the transport systems *(9)*.

Current understanding of the mechanism of peptide recognition and binding mainly comes from studies carried out in the Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* and the Gram-positive bacterium *Lactococcus lactis (10−13)*. In these organisms at least three partly complementary peptide transporters coexist *(11, 14)*. The crystal structures of the peptide binding proteins OppA and DppA, from *S. typhimurium* and *E. coli*, respectively, in complex with their corresponding ligands have provided

structural basis for the notion that the proteins accept a wide range of peptides *(9, 15−19)*. The peptide ligands bind in a deep cleft between the two globular domains, and these close around the substrate in a manner reminiscent of a Venus fly trap *(9)*. High-affinity peptide binding to OppA and DppA arises from hydrogen-bonding and electrostatic interactions between the protein and the main chain and termini of the ligand. The amino acid side chains are projected into spacious and hydrated pockets in which few direct contacts are made with the protein *(18)*. Thus, the relative sequence-independent ligand binding is based on the avoidance of potentially unfavorable interactions with the ligand side chains *(16, 19)*. Despite these structural studies, relatively little is known about the relative affinities for peptides of OppA of *S. typhimurium* and DppA of *E. coli*. The specificity has largely been inferred from growth and/or transport experiments *(11)*; systematic studies of the binding kinetics have not been made.

From mutant analysis it was known that *Lactococcus lactis* MG1363 possessed at least three peptide transport systems: an ABC transporter for oligopeptides (Opp), a secondary transporter for di- and tripeptides (DtpT), and a third di-tripeptide transport system *(14)*. Inhibitor studies indicated that the third system is driven by ATP, and recent genome analysis revealed an operon coding for a putative ABC peptide transporter. The binding protein (DppA) of the system was amplified and purified; DppA of *L. lactis* shares 30%, 25%, and 30% identity with OppA of *S. typhimurium*, OppA of *L. lactis*, and DppA of *E. coli*, respectively. The functionality of DppA, as a high-affinity di- and tripeptide

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binding protein, is demonstrated by electrophoretic and spectroscopic techniques. The binding properties and affinities for a large number of peptides differing in length, side-chain composition, stereochemistry, and/or modifications at the amino or carboxyl terminus have been determined.

## EXPERIMENTAL PROCEDURES

*Strains and Growth Conditions.* Lactococcus lactis NZ9000 (MG1363 derivative, *pepN::nisRK*) was kindly provided by O. Kuipers and used to overexpress the *dppA* gene of *L. lactis* MG1363 (20) after transformation with the vector pNZDppA. The supernatant of cultures of *L. lactis* NZ9700 (21) was used as a source of nisin A to trigger the transcription from the *nisA* promoter. The expression of wild-type DppA was analyzed in *L. lactis* AG500 (IM17 derivative, Δ*dtpT*; 22, 23). The strains were grown, at 30 °C, in M17 broth or agar (Difco, East Molesey, U.K.) supplemented with 0.5% (w/v) glucose and 5 μg/mL chloramphenicol, when appropriate.

*Plasmid Construction and General DNA Manipulations.* For the construction of pNZDppA, the coding region of *dppA* was amplified by the polymerase chain reaction with the vector pNZAE (CmR; pHLP5 derivative carrying the *dppA* gene as a 1919-bp chromosomal DNA fragment from *L. lactis* MG1363; Sanz et al., manuscript in preparation) as template. In the forward primer (5′-CGC GCC ATG GGT TCA AAA ACA AGT GAG C-3′), a *Nco*I restriction site (underlined) was introduced 3′ of the sequence encoding the signal peptide. In the reverse primer (5′-GCG GAT CCT TTA ATA TAA GCC GAT TTT AAG TCG-3′), a *Bam*HI restriction site (underlined) was created that overlaps with the stop codon of the *dppA* gene. The PCR product was digested with *Nco*I and *Bam*HI and ligated with the 3.7 kb fragment obtained by digestion of pHLP5 (24) with the corresponding enzymes. This procedure resulted in the directional cloning of *dppA* under the control of the *nisA* promoter and in frame with the sequence specifying a carboxyl-terminal factor Xa cleavage site and a six-histidine tag (24).

Molecular cloning techniques were performed essentially as described by Sambrook et al. (25). Plasmid DNA from *L. lactis* was isolated by either the method of Birnboim and Doly (26), with minor modifications (27) or the Qiagen column purification kit (Qiagen, GmbH, Hilden, Germany). PCR was performed with Vent DNA polymerase according to the instructions of the supplier (New England Biolabs, Beverly, MA). *L. lactis* was transformed by electroporation as described by Holo and Nes (28).

*Expression and Purification of DppA-(His)₆.* *L. lactis* NZ9000, carrying the vector pNZDppA, was grown to an $A_{660}$ of approximately 1.0. At that stage, the expression of DppA-(His)₆ was triggered by the addition of 1:1000 dilution of the filtered supernatant of a *L. lactis* NZ9700 culture (containing about 10 ng of nisin A/mL). The induction time was 2 h, and at the end of this period the cells had reached a final $A_{660}$ of around 3.0. Cells were harvested by centrifugation at 6000g for 10 min at 4 °C, washed twice in 50 mM sodium phosphate, pH 7.0, and resuspended in the same buffer, supplemented with 20 mM imidazole, 100 mM NaCl, and 1 mM EDTA plus 1 mM phenylmethanesulfonyl fluoride. Cells were disrupted by four passages through a French pressure cell at 20 000 psi. Unbroken cells and cell debris were removed by low-speed centrifugation (13000g for 20 min, at 4 °C) and, subsequently, membranes were removed by high-speed centrifugation (290000g for 1 h at 4 °C). DppA-(His)₆ was purified from the cytosolic fraction by two consecutive chromatographic steps. First, the cytosol was applied onto a Bio-Spin column (Bio-Rad, Hercules, CA) containing Ni²⁺-NTA resin (Quiagen GmbH, Hilden, Germany), preequilibrated with sodium phosphate, pH 7.0, supplemented with 20 mM imidazole. The washing step was performed with 50 mM imidazole (12 column volumes) and DppA-(His)₆ was eluted with 300 mM imidazole (2 column volumes) in sodium phosphate, pH 7.0. Fractions containing DppA-(His)₆ were pooled and desalted with a PD-10 column (Bio-Rad, Hercules, CA). The desalted sample was then applied onto an anion-exchange column (Q Sepharose, Pharmacia Biotech, Uppsala, Sweden), preequilibrated with 50 mM sodium phosphate, pH 7.0, containing 20 mM NaCl. DppA-(His)₆ eluted at 20 mM NaCl, in 50 mM sodium phosphate, pH 7.0 (four column volumes). Purified DppA-(His)₆ contained endogenous ligand, which was removed upon partial denaturation/renaturation of the protein as described by Lanfermeijer et al. (13) except that the initial guanidine hydrochloride concentration was 3 M.

*Protein Concentration.* Protein concentration was determined by the method of Lowry et al. (29), with bovine serum albumin as the standard. The concentration of the purified protein was also estimated from the absorption at 280 nm, with a calculated extinction coefficient of 1.164 $(mg/mL)^{-1}$ $cm^{-1}$.

*Electrophoresis.* SDS−polyacrylamide gel electrophoresis was performed according to Laemmli (30), using 6% acrylamide stacking gels and either 10% or 12% acrylamide resolving gels. Native cationic gel electrophoresis was basically carried out by the method of Reisfield et al. (31) and modified by Lanfermeijer et al. (13), using 12% acrylamide resolving gels. Protein samples of 1 μg were loaded in a final volume of 10 μL. When appropriate, ligands at 1 mM final concentration were preincubated with the protein in 50 mM sodium phosphate, pH 7.0. Proteins were visualized by Coomassie Brilliant Blue staining.

The isoelectric point of the free and ligand-bound forms of DppA-(His)₆ was experimentally determined by use of the Pharmacia Phast gel system and a broad pI range (from 3 to 10) gel, according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). In this assay the dipeptide Leu-Leu was used as a ligand at 1 mM concentration. The p*I* was also calculated from the primary sequence of the protein with Proteomics tools of ExPASy molecular biology server.

*Western Analysis.* For quantitative analysis of the expression levels of DppA in *L. lactis*, cells from cultures grown up to a final $A_{660}$ of about 3 were washed and resuspended in 50 mM sodium phosphate, pH 7.0, to $A_{660}$ of about 20. The cell suspension was then sonicated for 10 cycles (15 s on/15 s off) at an amplitude of 6 μm, on ice. Proteins were separated by electrophoresis in SDS−10% polyacrylamide gels and subsequently transferred to poly(vinylidene difluoride) membranes (Boehringer, Almere, The Netherlands) by semidry electroblotting (32). Polyclonal antibodies were raised against the purified DppA-(His)₆ protein and used at a serum dilution of 8000-fold. Monoclonal antibodies (Dianova GmbH, Apeldoorn, The Netherlands) raised against

the six-histidine tag were used to monitor the protein throughout the purification procedure. Primary antibodies were detected with the Western-Light chemiluminescence kit using CSPD as substrate (Tropix Inc., Bedford, MA).

*Fluorometric Assays.* Fluorescence spectra were obtained on an Aminco 4800 spectrofluorometer at 15 °C. Excitation was at 280 nm and emission was scanned from 290 to 390 nm with 2 nm bandwidths. Titration of intrinsic fluorescence of DppA-(His)$_6$ with different ligand concentrations was performed by excitation at 280 nm, with a 2 nm bandwidth, and by monitoring the emission at 340 nm, using an 8 nm bandwidth. Ligand binding studies were generally performed in 50 mM sodium phosphate, pH 6.0. When ligand binding was analyzed as a function of pH, the following buffers were used: 50 mM sodium acetate/acetic acid (pH 5.0−5.5), 50 mM sodium phosphate (pH 6.0−8.0), and 50 mM Tris-HCl (pH 8.0−8.5). Binding curves were analyzed by saturation kinetics (eq 1) and the general equilibrium binding equation (eq 2, *33*). The saturation kinetic equation was used when dissociation constants were at least 3-fold higher than the protein concentration, whereas the general equilibrium binding equation was favored when dissociation constants were at least 3-fold lower than the protein concentration. When the values of the protein concentration and dissociation constants were in the same range, both procedures were applied, with this exception: that in the case of analyzing the data by the Michaelis−Menten equation (eq 1), the free peptide concentration was used, and a reiterative fit procedure was applied. Both procedures resulted in similar values.

$$\Delta F = \frac{\Delta F_{\max} L}{K_d + L} \quad (1)$$

$$\Delta F = \Delta F_{\max} \frac{\left(1 + \frac{K_d}{P} + \frac{L}{P}\right) - \left[\left(1 + \frac{K_d}{P} + \frac{L}{P}\right)^2 - 4\frac{L}{P}\right]}{2} \quad (2)$$

In these equations, $\Delta F$ is the observed fluorescence change upon addition of ligand L, $\Delta F_{\max}$ is the maximum fluorescence change at infinite ligand concentration, $K_d$ is the equilibrium dissociation constant, and $P$ is the protein concentration. Nonlinear least-squares regression was performed with the Sigma Plot program (Jandel Scientific Software). The estimated kinetic parameters are the average of three independent determinations. The standard deviations are given.

## RESULTS

*Overexpression and Purification of DppA-(His)$_6$.* To facilitate the purification and characterization of the dipeptide binding protein, a gene construct was made that specifies DppA (without the signal sequence) fused to a carboxyl-terminal six-histidine tag [DppA-(His)$_6$]. In this construct, the codon for the amino-terminal cysteine, required for lipid modification of the native protein, was replaced by a methionine. The use of a vector carrying the gene *dppA-6H* translationally fused to the *nisA* promoter *(34)* resulted in about a 200-fold increase in expression level over that of the strain *L. lactis* AG500 (Figure 1). No signal above the background level was observed in the absence of nisin, indicating that the *dppA-6H* gene was under tight control of the *nisA* promoter.
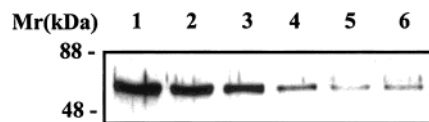


FIGURE 1: Expression level of wild-type DppA and DppA-(His)$_6$. Proteins from total cell lysates were separated by SDS−(10%) PAGE and analyzed by immunoblotting, using antiserum raised against purified DppA-(His)$_6$. Lanes 1−5, samples from *L. lactis* NZ9000/pNZDppA induced with nisin, containing 2, 0.8, 0.4, 0.2, and 0.1 $\mu$g of total protein, respectively; lane 6, sample of *L. lactis* AG500 (wild-type derivative) containing 40 $\mu$g of total protein.
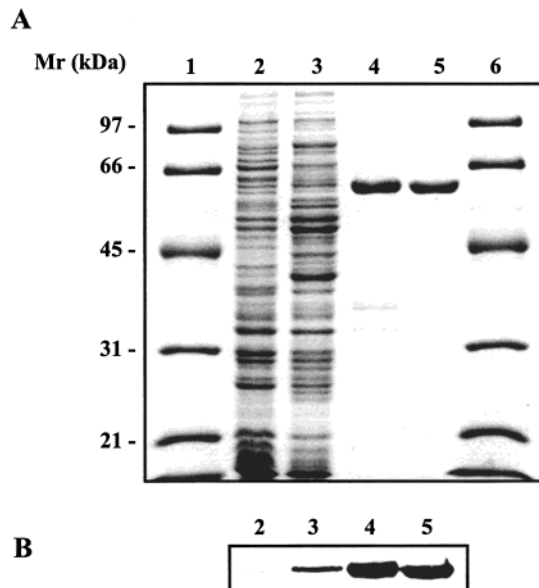


FIGURE 2: SDS−PAGE and Western analysis of the purification of DppA-(His)$_6$. Protein samples were separated by SDS−(10%) PAGE and visualized by Coomassie Brilliant Blue staining (A) or analyzed by immunoblotting, using monoclonal anti-6-histidine tag antibodies (B). Lanes 1 and 6, molecular weight markers; lane 2, membranes after disruption of cells in a French press cell (4 $\mu$g); lane 3, cytosolic fraction (4 $\mu$g); lane 4, eluate from Ni−NTA column (2 $\mu$g); lane 5, flowthrough from anion-exchange column corresponding to the purified DppA-(His)$_6$ protein (1.7 $\mu$g).

The protein profiles of SDS−PAGE and Western analysis during cell fractionation and purification are shown in Figure 2. DppA-(His)$_6$ was isolated from the cytosolic fraction and constituted about 2% of the total cell soluble protein (Figure 2, lane 3). Less than 10% of the protein was associated with the membrane fraction, and this fraction was even smaller when 100 mM NaCl was added to the buffer used during cell fractionation (Figure 2B, lane 2). The cytosolic fraction was first applied to a Ni$^{2+}$-NTA column, resulting in a high degree of purification (≥90% purity; Figure 2, lane 4). The remaining contaminants were removed in the second chromatographic step on Q-Sepharose anion-exchange resin; DppA-(His)$_6$ eluted at 20 mM NaCl (flowthrough), whereas the contaminants remained bound to the anion-exchange resin up to a salt concentration of 300 mM NaCl (Figure 2, lane 5). The overall procedure yielded about 3.5 mg of protein (purity ≥95%)/L of cell culture of approximately $A_{660}$ of 3.

*Isoelectric Point of DppA-(His)$_6$.* The p*I* of DppA-(His)$_6$, including the six-histidine tag, is 6.3 when calculated from the primary sequence. However, DppA-(His)$_6$ behaved as a basic protein in anion-exchange chromatographic experiments. Therefore, the p*I* of both the free and ligand-bound forms of the protein were determined experimentally to
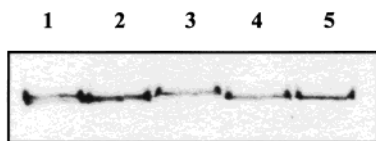
FIGURE 3: Native cationic gel electrophoresis of free and ligand-bound forms of purified DppA-(His)$_6$. Protein samples (1 $\mu$g) were separated in a native 12% acrylamide gel and visualized by Coomassie Brilliant Blue staining. Lane 1, purified protein; lane 2, purified protein preincubated with 1 mM Leu-Leu; lane 3, protein free of ligand after guanidinium chloride treatment; lanes 4 and 5, protein treated with guanidinium chloride and preincubated in the presence of 1 mM Leu-Leu and Val-Val, respectively.

Table 1: Peptide Specificity of DppA-(His)$_6$ As Analyzed by Native Cationic Gel Electrophoresis[a]

| series | peptide | mobility shift | peptide | mobility shift |
|---|---|---|---|---|
| **A** | Ala-Glu | + | Glu-Ala | − |
| | Ala-Phe | + | Phe-Ala | + |
| | Ala-Leu | + | Leu-Ala | + |
| | Ala-Arg | + | Arg-Ala | + |
| | Ala-Pro | + | Pro-Ala | + |
| **B** | Ala-Ala | + | Ala-Ala-Ala | + |
| | Val-Val | + | Val-Val-Val | + |
| | Leu-Leu | + | Leu-Leu-Leu | + |
| | Phe-Phe | + | Phe-Phe-Phe | nt[a] |
| | Met-Met | + | Met-Met-Met | nt |
| | Ile-Ile | + | Ile-Ile-Ile | nt |
| | Glu-Glu | − | Glu-Glu-Glu | − |
| | Arg-Arg | + | Arg-Arg-Arg | − |
| | Gly-Gly | − | Gly-Gly-Gly | − |
| **C** | Leu-Gly-Gly | − | Gly-Gly-Leu | − |
| | Phe-Gly-Gly | − | Gly-Gly-Phe | − |
| | Met-Gly-Gly | − | Met-Gly-Gly | nt[b] |
| | Arg-Gly-Gly | + | Gly-Gly-Arg | nt |
| **D** | Ala-Ala-OMe | + | NAc-Ala-Ala | − |
| | L-Ala-D-Ala | − | D-Ala-L-Ala | − |
| | Ala-His | + | $\beta$-Ala-His | − |

[a] Each peptide was tested at a final concentration of 1 mM. [b] nt, not tested.

explore in somewhat more detail this finding. In a broad pH gradient gel, from 3 to 10, a p$I$ value of 8.5 was observed irrespective of whether the ligand was present (data not shown), confirming the basic nature of the protein.

*Specificity of DppA-(His)$_6$ Analyzed by Native Cationic Gel Electrophoresis.* The analysis of the purified DppA-(His)$_6$ by native cationic gel electrophoresis revealed the presence of two species, presumably the free and ligand-bound forms (Figure 3, lane 1). Upon addition of ligand, e.g., Leu-Leu, only the band with the highest mobility was observed (Figure 3, lane 2). The copurified endogenous ligand was successfully removed by reversible partial denaturation of DppA-(His)$_6$ with guanidine hydrochloride, with a recovery of about 70% of the total amount of protein. DppA-(His)$_6$ devoid of endogenous ligand exhibited a slow mobility in the native gel (Figure 3, lane 3), and all of the protein migrated with a higher mobility when the sample was preincubated with ligand (Figure 3, lanes 4 and 5).

The difference in electrophoretic mobility was exploited to screen the substrate specificity of DppA-(His)$_6$ (Table 1). Of the various dipeptides tested, only those composed of two glutamic acid or glycine residues or just a glutamic acid residue at the amino-terminal position failed to elicit a shift in the migration of DppA-(His)$_6$ (Table 1, series A and B). The esterification of the $\alpha$-carboxyl group at the C-terminus
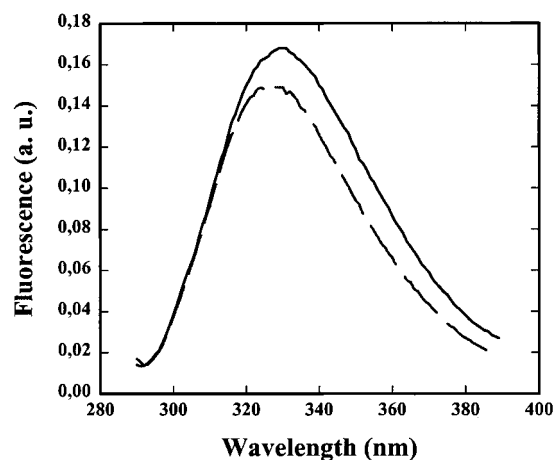


FIGURE 4: Effect of ligand binding on the intrinsic fluorescence of DppA-(His)$_6$. Fluorescence emission spectra of 0.4 $\mu$M DppA-(His)$_6$ in the presence (- - -) or absence (—) of saturating concentrations of ligand (0.4 mM Val-Val). The spectrum was recorded in 50 mM sodium phosphate, pH 6.0, at 15 °C. The excitation wavelength was at 280 nm.

of the dipeptide Ala-Ala was tolerated but not the acetylation of the $\alpha$-amino group at the N-terminus (Table 1, series D). Dialanine containing a D-residue at the first or second position did not provoke a mobility shift (Table 1, series D). The dipeptide Ala-His caused a change in mobility, whereas the equivalent peptide with a $\beta$-amino acid at the N-terminal position (carnosine) did not (Table 1, series D). Tripeptides composed of either basic, acid, or glycine residues did not provoke a mobility shift (Table 1, series B). This was also the case for tripeptides containing two contiguous glycine residues, regardless of their position (Leu-Gly-Gly, Phe-Gly-Gly, Gly-Gly-Leu, and Gly-Gly-Phe; Table 1, series C); the only exception was Arg-Gly-Gly. Peptides longer than three residues up to a concentration of 1 mM did not induce a mobility shift, indicating that DppA-(His)$_6$ is specific for di- and tripeptides.

*Binding Affinities of DppA-(His)$_6$ Estimated from Changes in Intrinsic Protein Fluorescence.* The intrinsic fluorescence of DppA-(His)$_6$, in the pH range from 6.0 to 8.5, is characterized by excitation and emission maxima at 279 and 330 nm, respectively (Figure 4). The emission spectra of DppA-(His)$_6$ at more acid pH values (5.0−5.5) were essentially the same as those at pH 6.0 to 8.5 although the amplitude of the signal was slightly reduced (data not shown). The addition of saturating concentrations of ligand resulted in a blue shift in the emission peak of 1−3 nm and an overall decrease in the fluorescence (Figure 4). The decrease in fluorescence at 340 nm upon addition of ligand was used to determine the kinetic parameters of peptide binding. In every case, peptide binding obeyed classical saturation kinetics, but the maximal changes in fluorescence varied from 7% to 28% (Figure 5, Tables 2−5). The stoichiometry of peptide binding to DppA-(His)$_6$ could be determined by titrating the protein with tightly bound substrates, such as Ala-Ala at pH 6.0 (data not shown) or Val-Val-Val at pH 7.5 (Figure 6). Extrapolation of the limiting asymptotes of the data indicated a stoichiometry of 1.1 ± 0.1 mol of peptide bound/mol of protein.

*pH Dependence of Peptide Binding.* The pH dependence of peptide binding was tested in the pH range from 5.0 to 8.0, with dialanine as ligand (Figure 7). The highest affinity
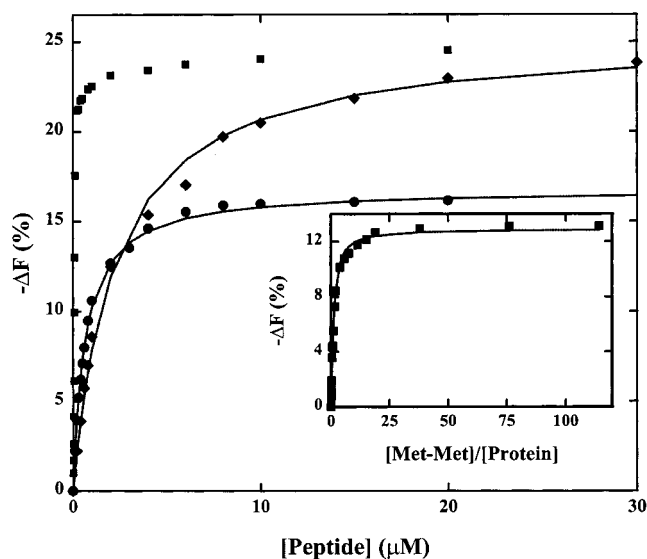
FIGURE 5: Fluorescence titration of DppA-(His)$_6$ with various peptides. Intrinsic protein fluorescence of 0.2 $\mu$M DppA-(His)$_6$ upon addition of increasing concentrations of Ala-Ala (●), Arg-Arg-Arg (◆), and Met-Met (■) in 50 mM sodium phosphate, pH 6.0, at 15 °C. The inset shows the intrinsic protein fluorescence of 0.5 $\mu$M DppA-(His)$_6$ upon addition of increasing concentrations of Met-Met (■), in 50 mM Tris-HCl, pH 8.5, at 15 °C. The solid line represents the best fits of the data to eq 1 (main figure) or the generalized binding equation (eq 2; inset of the figure), respectively. The $K_d$ values at pH 6.0 for Ala-Ala, Arg-Arg-Arg, and Met-Met are 0.66, 3.0 and less than 0.02 $\mu$M, respectively. The $K_d$ value at pH 8.5 for Met-Met is 0.46 $\mu$M.
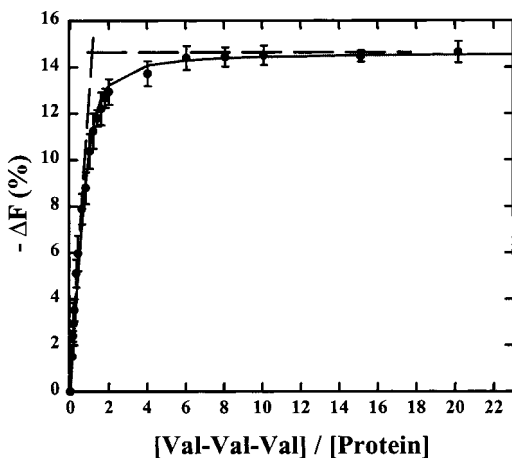


FIGURE 6: Fluorescence titration of DppA-(His)$_6$ with Val-Val-Val. Intrinsic fluorescence of 0.5 $\mu$M DppA-(His)$_6$ upon the addition of increasing concentrations of Val-Val-Val (0−0.1 mM) in 50 mM sodium phosphate, pH 7.5, at 15 °C. Data are the mean of three independent determinations, and the standard deviations are represented by error bars. The solid line represents the best fit of the data to the generalized binding equation (eq 2), yielding a $K_d$ of 0.06 $\mu$M. The intersection point of the dashed lines corresponds to the binding stoichiometry.

of peptide binding was observed at pH 6.0 and the $K_d$ increased approximately 1 order of magnitude per unit increase in pH (Figure 7B). This suggests that deprotonation of a protein residue interferes directly with peptide binding. $\Delta F_{max}$ remained almost constant in the pH range 6.0−8.0 but decreased below pH 6.0 (Figure 7A). On the basis of these results, it was decided to further analyze peptide binding at pH 6.0, except when the binding affinity became too high for an accurate estimation of the $K_d$ (Table 3).
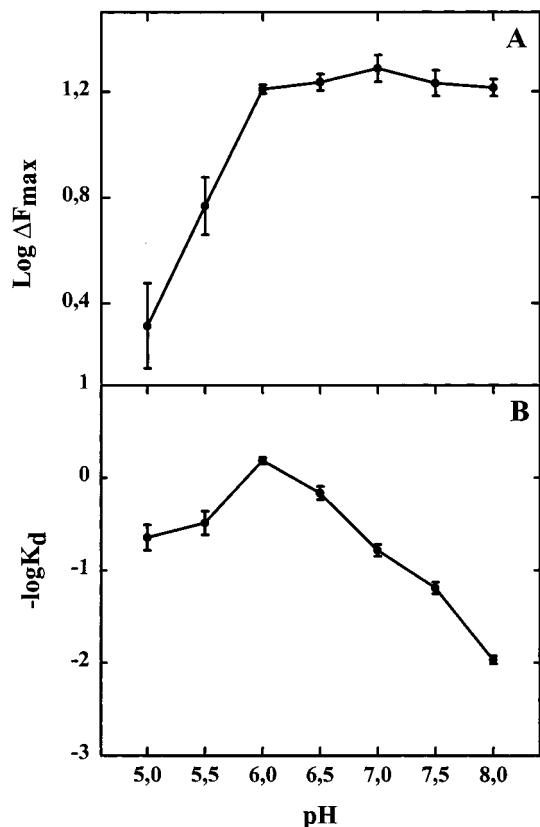


FIGURE 7: pH dependence of kinetic parameters for peptide binding. Binding saturation curves were determined for Ala-Ala at different pH values (5.0−8.0), with 0.2 $\mu$M DppA-(His)$_6$, and kinetic parameters ($K_d$ and $\Delta F_{max}$) were calculated from the corresponding fits (see Experimental Procedures section for details). (A) pH dependence of $\Delta F_{max}$; (B) pH dependence of $K_d$.

Table 2: Effect of Aminoacyl Side Chains and Peptide Length on Kinetics of Binding[a]

| peptide | $\Delta F_{max}$ (%) | $K_d$ ($\mu$M) |
|---|---|---|
| Val-Val | ~15 | <0.02 |
| Leu-Leu | ~15 | <0.02 |
| Met-Met | ~20 | <0.02 |
| Ala-Ala | 16.4 ± 0.6 | 0.66 ± 0.05 |
| Gly-Gly | 11.6 ± 0.8 | 57 ± 7 |
| Arg-Arg | ~25 | <0.02 |
| Glu-Glu | 21.6 ± 2.0 | 58 ± 3 |
| Val-Val-Val | ~20 | <0.02 |
| Leu-Leu-Leu | ~20 | <0.02 |
| Ala-Ala-Ala | ~15 | <0.02 |
| Gly-Gly-Gly | nd[b] | nd |
| Arg-Arg-Arg | 24.4 ± 1.4 | 3.0 ± 0.7 |
| Glu-Glu-Glu | 15.2 ± 1.2 | 182 ± 53 |

[a] Kinetic parameters were estimated from the quenching of the intrinsic protein fluorescence upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.0, at 15 °C (see Experimental Procedures section for details); the final protein concentration varied from 0.2 to 0.5 $\mu$M. [b] nd, binding not detected by fluorescence spectroscopy.

*Aminoacyl Side-Chain Composition and Peptide Length.* The effect of the composition of the aminoacyl side chain on the kinetic parameters of peptide binding is shown in Tables 2 and 3. DppA-(His)$_6$ displayed the highest affinities ($K_d$ values in the nanomolar range) for peptides composed of hydrophobic or basic amino acid residues, while the affinities for peptides composed of acidic or glycine residues were in the micromolar range. The nonpolar peptide dialanine

Table 3: Dissociation Constants ($K_d$)[a] for Binding of High-Affinity Peptides to DppA-(His)$_6$ at Different pH

| peptide | pH | | | |
|---|---|---|---|---|
| | 5.0 | 7.5 | 8.0 | 8.5 |
| Ala-Ala | | 15.64 ± 2.19 | 367 ± 126 | |
| Val-Val | | 0.22 ± 0.07 | | |
| Leu-Leu | | 0.35 ± 0.11 | | |
| Arg-Arg | 0.04 ± 0.01 | <0.02 | <0.02 | <0.020 |
| Phe-Ala | | 4.31 ± 0.15 | | |
| Leu-Ala | | 2.27 ± 0.89 | | |
| Arg-Ala | | <0.02 | 0.06 ± 0.02 | |
| Ala-Arg | | 0.16 ± 0.03 | | |
| Met-Met | | <0.02 | <0.02 | 0.46 ± 0.03 |
| Ala-Ala-Ala | | 1.37 ± 0.18 | | |
| Val-Val-Val | | 0.06 ± 0.02 | | |
| Leu-Leu-Leu | | 0.06 ± 0.02 | | |

[a] Dissociation constants ($K_d$, expressed as micromolar) were estimated from the quenching of the intrinsic protein fluorescence upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.0–7.5, 50 mM Tris-HCl, pH 8.0–8.5, or 50 mM sodium acetate/acetic acid, pH 5.0, at 15 °C (see Experimental Procedures section for details); the final protein concentration varied from 0.2 to 1 $\mu$M.

Table 4: Effect of Position of Amino Acid Residues on Kinetics of Binding[a]

| peptide | $\Delta F_{max}$ (%) | $K_d$ ($\mu$M) |
|---|---|---|
| Ala-Phe | 24.2 ± 3.9 | 0.09 ± 0.04 |
| Phe-Ala | ~25 | <0.02 |
| Ala-Leu | 14.3 ± 1.6 | 0.12 ± 0.04 |
| Leu-Ala | 15.1 ± 0.4 | 0.07 ± 0.02 |
| Ala-Pro | 27.0 ± 1.5 | 12.55 ± 2.14 |
| Pro-Ala | 26.4 ± 0.3 | 5.26 ± 0.26 |
| Ala-Arg | 7.2 ± 0.2 | 0.05 ± 0.01 |
| Arg-Ala | ~25 | <0.02 |
| Ala-Glu | 22.3 ± 0.3 | 4.24 ± 0.38 |
| Glu-Ala | 21.1 ± 2.5 | 66.90 ± 10.82 |
| Leu-Pro | 27.5 ± 1.5 | 1.28 ± 0.29 |
| Pro-Leu | 25.4 ± 1.6 | 1.01 ± 0.21 |

[a] Kinetic parameters were estimated from the quenching of the intrinsic protein fluorescence upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.0, at 15 °C (see Experimental Procedures section for details); the final protein concentration varied from 0.2 to 0.5 $\mu$M.

showed an intermediate affinity with a $K_d$ of 0.66 $\mu$M. Homodipeptides and tripeptides composed of amino acid residues of similar hydrophobicity, such as valine and leucine, had similar affinities (Table 3). Comparable trends were observed for homotripeptides, but the $K_d$ values for these were lower than those of the corresponding dipeptides (Table 3). The effect of the peptide chain length on the affinity varied for peptides with different composition. Thus, tripeptides composed of alanine, leucine, or valine showed higher affinities (4–12-fold differences) than the corresponding dipeptides, whereas those composed of arginine, glutamic acid, and glycine displayed lower affinities (>3-fold differences) than the respective dipeptides (Tables 2 and 3).

*Position of Amino Acid Residues.* The relative contribution of the position of a particular residue to the peptide binding was systematically studied with series of alanine-, leucine-, or proline-containing dipeptides (Tables 3 and 4). Overall, the data indicate that the more hydrophobic the N-terminal residue, the higher the affinity. In fact, dissociation constants for Leu-Ala and Phe-Ala are so low (below 0.02 $\mu$M) that they could only be determined accurately at pH 7.5 (Tables

Table 5: Effect of Stereochemistry, Peptide Modifications at Carboxyl and Amino Termini, and $\beta$-Linked Amino Acid Residues on Kinetics of Binding[a]

| peptide | $\Delta F_{max}$ (%) | $K_d$ ($\mu$M) |
|---|---|---|
| L-Ala-L-Ala | 16.0 ± 0.6 | 0.66 ± 0.05 |
| L-Ala-D-Ala | 16.8 ± 1.6 | 311 ± 85 |
| D-Ala-L-Ala | nd[b] | nd |
| D-Ala-D-Ala | nd | nd |
| Ala-Ala-OMe | 14.9 ± 1.2 | 13 ± 2 |
| NAc−Ala-Ala | nd | nd |
| Ala-His | 14.7 ± 1.9 | 0.42 ± 0.05 |
| $\beta$-Ala-His | 21.5 ± 1.5 | 340 ± 107 |

[a] Kinetic parameters were estimated from the quenching of the intrinsic protein fluorescence upon the addition of increasing concentrations of ligand in 50 mM sodium phosphate, pH 6.0, at 15 °C (see Experimental Procedures section for details); the final protein concentration was 0.2 $\mu$M. [b] nd, binding not detected by fluorescence spectroscopy.

3 and 4). The N-terminal position of basic (arginine) or acidic (glutamic acid) residues had drastic and opposite effects on the binding affinities, revealing the importance of positive charges at the N-terminus. An acidic residue at the N-terminus (e.g., Glu-Ala) caused a reduction in affinity of more than 15-fold (Table 4), while a basic residue at that position (e.g., Arg-Ala) increased the affinity (Tables 3 and 4). Proline-containing peptides at the N- or C-terminus displayed moderate to low affinities; this may be related to the structural restrictions imposed by the rigid pyrrolidinic ring (Table 4).

*Stereochemistry, N- and/or C-Terminus Modification, and $\beta$-Linked Amino Acid Residues.* The stereochemical specificity was studied by analyzing the affinities of dialanine containing one or two D-residues. Binding of D-Ala-L-Ala and D-Ala-D-Ala was not observed while the affinity for L-Ala-D-Ala was markedly reduced (Table 5). Acetylation of the $\alpha$-amino group at the N-terminus of dialanine impaired binding, while methylation of the $\alpha$-carboxyl group at the C-terminus did not prevent binding but caused a decrease in affinity of about 20-fold (Table 5). The presence of a $\beta$-alanine residue at the N-terminal position of the dipeptide Ala-His reduced the affinity about 800-fold, which emphasizes the importance of an $\alpha$-peptide bound contiguous with the N-terminal amino group (Table 5).

*Nonbound Peptides and Restriction Size.* Triglycine was the only tested tripeptide that did not seem to be a suitable ligand for DppA-(His)$_6$. Neither free amino acids nor tetra- nor pentaalanine modified the emission spectra of the protein. Some oligopeptides at concentrations in the millimolar range caused fluorescence changes, but these are most likely due to breakdown products (that is, di- or tripeptides) present in the sample. Notice that the affinity of DppA-(His)$_6$ for di- and tripeptides is in the submicromolar to micromolar range, implying that minute breakdown (<1%) may already cause artifactual signals when peptides are tested at micromolar to submillimolar concentrations.

Overall, proline-containing peptides (Pro-Ala, Ala-Pro, Leu-Pro, and Pro-Leu) exerted the largest effects on protein fluorescence, with saturating concentrations of peptide quenching the protein fluorescence almost 30% at pH 6.0. Dipeptides containing methionine or arginine at the N-terminus bound tightest, with dissociation constants in the nanomolar range (Table 3). These high affinities were

followed by those of hydrophobic tripeptides consisting of leucine and valine residues (Table 3).

## DISCUSSION

In this paper, the overexpression, purification, and characterization of the substrate binding protein of the third peptide transport system (Dpp) of *L. lactis* is reported. The functionality of DppA (substrate binding protein without lipid anchor), initially identified on the basis of its sequence similarity with known peptide binding proteins, has been explored biochemically with special emphasis on kinetics and structural requirements for peptide binding. This work, thus, constitutes the first thorough study of the binding properties of a di-tripeptide binding protein based on direct measurements of ligand binding.

The differences in electrophoretic mobility were initially exploited to screen the substrate specificity of DppA-(His)$_6$. Despite the advantages of this technique in terms of the low amount of protein (1 $\mu$g) required for binding detection, it is not suitable for low-affinity binding assays. For instance, binding of L-Ala-D-Ala, $\beta$-Ala-His, Glu-Ala, Glu-Glu, Gly-Gly, and Glu-Glu-Glu ($K_d$ values > 50 $\mu$M) and Arg-Arg-Arg ($K_d$ value $\approx$ 3 $\mu$M) could not be detected by the electrophoretic assay, not even with peptide concentrations of 1 mM, while binding was clearly observed in the fluorescence assay. On the other hand, Ala-Glu, Ala-Pro, and Pro-Ala are also low-affinity substrates, with dissociation constants in the micromolar range ($K_d$ values of 4−12 $\mu$M), but these peptides did cause a mobility shift in the native gels. The reason for this different behavior of protein−ligand interactions with similar $K_d$ values has not been investigated further, but it can be related to differences in the dissociation ($k_{off}$) and association ($k_{on}$) rate constants. Notice that $K_d$ equals $k_{off}/k_{on}$ and a high $k_{off}$ may result in an underestimation of the binding by native cationic electrophoretic assay as the equilibrium is changing continuously when the protein and peptide are migrating in the gel.

The specificity and kinetics of peptide binding to DppA-(His)$_6$ were investigated in detail by fluorescence spectroscopy. Systematic studies were carried out in order to draw conclusions about the structural requirements of the protein for ligand binding. The major features of peptide binding include (i) high affinity for di- and tripeptides, (ii) requirement of a free N-terminal $\alpha$-amino group and an $\alpha$-peptide bound contiguous with the N-terminal amino group, (iii) stereospecificity for L-isomers, and (iv) preference for dipeptides containing methionine or arginine and hydrophobic tripeptides consisting of leucine or valine residues.

Information about the specificity of peptide transport systems (Opp, Dpp, and Tpp) of *E. coli* and *S. typhimurium* is largely based on the capacity of amino acid auxotrophs to grow in the presence of defined peptide-containing medium (*11*). These studies indicated that a positively charged primary or secondary N-terminal $\alpha$-amino group is critical for peptide binding, while a free $\alpha$-carboxyl group at C-terminus is desirable but not essential for Opp and Tpp. Dpp appears to have a stricter requirement for a free $\alpha$-carboxyl group at C-terminus as its loss or derivatization decreased dramatically the uptake capacity. Binding studies with OppA of *E. coli* by equilibrium dialysis confirmed the main specificity characteristics determined from the growth

experiments, but only a limited number of peptides were tested (*10*). The three-dimensional structures of OppA of *S. typhimurium* and DppA of *E.coli* reveal that the N-terminus of the ligand forms a salt bridge with the side chain of a conserved aspartic acid residue, while salt bridges or water-mediated interactions are formed between the $\alpha$-carboxyl group of the peptide and basic residues of the protein (*16, 19*). DppA-(His)$_6$ imposes stricter structural requirements for the N-terminal residue than for the C-terminus of the peptide, as manifested by the absence or drastic reduction of binding affinity upon modification of the amino-terminus. Alignment of the primary sequences of OppA and DppA of *S. typhimurium* and *E. coli*, respectively, and that of DppA of *L. lactis* indicates that the aspartate interacting with the ligands' $\alpha$-amino group is conserved and corresponds to the Asp-422 in DppA of *L. lactis* (data not shown). This aspartate residue is not conserved in OppA of *L. lactis*, which is consistent with the observation that modification of the $\alpha$-amino group of oligopeptides does not affect binding as markedly as observed in the other binding proteins (F. C. Lanfermeijer, W. N. Konings, and B. Poolman, manuscript in preparation).

The basic residue (Arg-355) of DppA from *E. coli* that interacts with the C-terminus of the bound dipeptide is not conserved in DppA of *L. lactis*. DppA of *L. lactis* has a neutral residue (Ala-378) at the equivalent position, which is consistent with the observation that it does not show such a marked preference for dipeptides nor such a strict requirement for a free $\alpha$-carboxylate group at the C-terminus as is the case for DppA of *E. coli*.

DppA-(His)$_6$ has a strong selectivity for L-residues. Some binding activity (500-fold higher $K_d$) was observed when the D-isomer was present at the C-terminus of the dipeptide. The Opp system and the dipeptide permease of *E. coli* also show stereochemical specificity with a strong preference for L-residues at each position, but D-isomers are tolerated at the C-terminus (*11*).

Although peptide binding proteins are often thought to have little selectivity for different peptides, this study reveals that the $K_d$ values for peptides vary more than 3 orders of magnitude, depending on the side-chain composition. In *E. coli*, peptide uptake via the Dpp system is influenced more by side-chain modifications (largest variation of about 100-fold) than via Opp (*35*). Binding studies with purified OppA of *E. coli* revealed that the nature of the side-chain residues can influence the individual kinetic parameters by a factor of about 10 (*10*).

The absence of binding of triglycine to DppA-(His)$_6$ reveals that this peptide is a poor substrate. Also, tripeptides composed of two contiguous glycine residues were unable to induce an electrophoretic mobility shift. Low binding affinities for peptides consisting of either glycine or proline residues have also been observed for OppA of *E. coli*, and these observations have been explained in terms of peptide conformation (*10*). Peptides exclusively composed of proline were not tested in our study but all single-proline-containing dipeptides displayed low affinities.

In *E. coli* and *S. typhimurium*, the transport and binding properties of the three peptide transport systems are partially overlapping with regard to the length of the transported ligand (*11*). In *L. lactis,* di- and tripeptides are taken up via DtpT and Dpp (*14;* (G. Fang, W. N. Konings, and B.

Poolman, manuscript in preparation). DtpT shows higher affinity for dipeptides than for tripeptides and, the highest affinity is observed for peptides with at least one hydrophobic residue (G. Fang, W. N. Konings, and B. Poolman, manuscript in preparation). DppA-(His)$_6$ does not show a clear preference for either di- or tripeptides. The dissociation constants indicate that the binding of tripeptides of hydrophobic nature and diarginine and dimethionine is favored. It should be stressed, however, that tight binding to the binding protein may result in poor transport rates due to restricted delivery of the ligand from the binding protein to the membrane complex *(13)*.

DppA-(His)$_6$ is active over a broad range of pH (at least from 5.0 to 8.5). The reduction in affinity with increasing pH was observed for peptides of different composition and charge, indicating that the effect can be attributed to the ionization of some protein residue(s) critical for binding. The primary sequence of DppA of *L. lactis* contains two nonconserved histidines, that is, at positions 478 and 514, one of which could be responsible for the pH dependence of binding. However, on the basis of sequence alignments and the structural analysis of DppA of *E. coli*, the residues corresponding to the quoted histidines are located in surface-exposed loops and, thus, are unlikely to be involved in ligand binding.

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