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Binding Specificity of the Periplasmic Oligopeptide-Binding Protein from *Escherichia coli*

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The structural properties required for the binding of peptide substrates to the *Escherichia coli* periplasmic protein involved in oligopeptide transport were surveyed by measuring the ability of different peptides to compete for binding in an equilibrium dialysis assay with the tripeptide Ala-Phe-[^{3}H]Gly. The protein specifically bound oligopeptides and failed to bind amino acids or dipeptides. Acetylation of the peptide amino terminus of $(Ala)_3$ severely impaired binding, whereas esterification of the carboxyl terminus significantly reduced but did not completely eliminate binding. Peptides composed of L-amino acids competed more effectively than did peptides containing D-residues or glycine. Experiments with a series of alanyl peptide homologs demonstrated a decrease in competitive ability with increasing chain length beyond tripeptide. Competition studies with tripeptide homologs indicated that a wide variety of amino acyl side chains were tolerated by the periplasmic protein, but side-chain composition did affect binding. Fluorescence emission data suggested that this periplasmic protein possesses more than one substrate-binding site capable of distinguishing peptides on the basis of amino acyl side chains.

Peptides play a major nutritional role by acting as a source of carbon and nitrogen (13) in enteric bacteria such as *Salmonella typhimurium* and *Escherichia coli*. Of the transport systems responsible for uptake of peptides in these bacteria, the oligopeptide permease is the most completely characterized. This permease is capable of transporting peptides composed of two to six amino acids (11, 24, 37). The upper limit for transport depends on the hydrodynamic volume of the peptide rather than on the number of amino acyl residues (32). It has been suggested that this property results from the sieving properties of the outer membrane, rather than from inherent characteristics of the permease (32). The oligopeptide permease requires its peptide substrates to be α-linked (26, 29) and to possess an ionizable primary or secondary N-terminal group (11, 25, 28). Although a free carboxyl terminus is not required for transport, the oligopeptide permease shows a reduced affinity for peptides in which the terminal carboxyl group has been modified (30, 31). In terms of stereospecificity, the permease prefers L-isomers as the first two residues but can tolerate D-isomers in subsequent positions (3, 21). Although the amino acyl side chains affect both the affinity (33) and rate (30) of peptide uptake, the oligopeptide system is capable of transporting a wide variety of peptide substrates (2, 7, 8, 12, 24, 30). This is understandable in light of the vast number of possible peptide permutations that might be available for transport.

The oligopeptide permease has been genetically characterized. The *opp* locus maps at 34 min on the *S. typhimurium* chromosome and 27 min on the *E. coli* chromosome (17). The locus consists of four genes, *oppA*, *oppB*, *oppC*, and *oppD*, organized as a single operon and cotranscribed in order from *oppA* to *oppD* (18). Oligopeptide transport is sensitive to osmotic shock and requires a source of phosphate-bond energy (6; D. G. Morgan, Ph.D. dissertation, Vanderbilt University, Nashville, Tennessee 1985), indicating that this permease can be classified as a shock-sensitive transport system (15). The *oppA* locus encodes a 52,000-dalton periplasmic protein in *S. typhimurium* (16). The equivalent periplasmic protein from *E. coli* W has been purified, physically characterized, and shown to bind peptides (14). The *oppA*-encoded protein from *S. typhimurium* has also recently been purified, and the amino acid sequence has been deduced from the nucleotide sequence of the gene (17a).

To further analyze the process of oligopeptide transport in bacteria, the substrate specificities conferred upon the system by each transport component must be identified. Since the oligopeptide-binding protein appears to be the initial receptor in peptide transport, its specificities for binding may reflect those of the entire system. In this study, central structural features of peptides were surveyed to determine their effect upon binding. Among the properties investigated were the amino and carboxyl termini, the stereochemistry of the residues, the length of the peptide chain, and the amino acyl side chains.

MATERIALS AND METHODS

Peptides. All peptides were composed of L-amino acids unless otherwise stated. The following peptides were purchased from Chemical Dynamics Corp.: Ala-Phe-Gly, $(Ala)_3$, $(Ala)_4$, L-Ala-D-Ala-L-Ala, Ala-Gly-Gly, $(Gln)_3$, $(Glu)_3$, $(Gly)_3$, $(Leu)_3$, $(Met)_3$, $(Phe)_3$, and $(Pro)_3$. Peptides obtained from Bachem were $(Ser)_3$, $(Thr)_3$, $(Tyr)_3$, and $(Val)_3$. Peptides obtained from Sigma Chemical Co. (St. Louis, Mo.) were as follows: $(Ala)_2$, $(Ala)_5$, $(D\text{-}Ala)_3$, *N*-acetyl-Ala-Ala-Ala, and Ala-Ala-Ala-*O*-methyl ester. Ala-Phe was obtained from Research Organics. $(Lys)_3$ and $(Orn)_3$ were from Serva (Heidelberg, Federal Republic of Germany). $(Orn)_3$ was also obtained from Miles Scientific (Div. Miles Laboratories, Inc., Naperville, Ill.). Ala-Phe-[^{3}H]Gly (90 mCi/mmol) was synthesized as previously described (14). All peptides and amino acids were analyzed by thin-layer chromatography to ensure purity.

Chemicals. ACS scintillation fluid was from Amersham Corp. (Arlington Heights, Ill.). Imidazole, obtained from

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TABLE 1. Characterization of binding

Competing peptide or amino acids	% Ala-Phe-[^{3}H]Gly remaining bound with the following concn of competitor[a]:		
	50 μM	100 μM	1 mM
None	(100)	(100)	(100)
Ala, Phe, Gly	106 ± 4	105 ± 5	108 ± 5
Ala-Phe-Gly	2 ± 1	0[b]	ND[c]
Ala-Phe	100 ± 0	87 ± 15	23 ± 17
Ala-Phe, Gly	107 ± 2	ND	ND

[a] The ability to compete for binding was determined by equilibrium dialysis with 1.7 μM oligopeptide-binding protein and 1.5 μM Ala-Phe-[^{3}H]Gly. Under conditions in which no competing peptide was present, the percentage of Ala-Phe-[^{3}H]Gly bound was considered to be 100%. All results are the average of two to three independent assays ± range.

[b] Binding of Ala-Phe-[^{3}H]Gly was below the level of detection.

[c] ND, Not determined.

Sigma, was recrystallized from benzene at 40°C and then again from benzene at room temperature. All other chemicals were reagent grade or better. Water was deionized and then glass distilled.

Oligopeptide-binding protein. The oligopeptide-binding protein was purified from *E. coli* W (ATCC 9637) as previously described (14). Preparations of the binding protein were greater than 97% homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focusing, and immunoblotting.

Competitive binding assays. Competition with Ala-Phe-[^{3}H]Gly for binding to the purified oligopeptide-binding protein was monitored by equilibrium dialysis. Assays were carried out in a Hoefer equilibrium microvolume dialyzer with Spectra/Por 1 (flat sheet, M_r cutoff = 6,000 to 8,000) dialysis membranes (Spectrum Medical Industries). One side of each cell contained a 70-μl sample of 1.7 μM (0.1 mg/ml) purified oligopeptide-binding protein. The other side contained an equal volume solution of 1.5 μM Ala-Phe-[^{3}H]Gly plus the competing peptide or amino acid at a specified concentration. Competition studies were carried out in 10 mM imidazole acetate (pH 7.3) containing 100 mM NaCl. The dialysis chambers were rotated for 18 h at 4°C to allow equilibration, and multiple samples were removed from each side and counted in a Beckman LS-7500 liquid scintillation counter.

Fluorescence emission spectroscopy. Fluorescence emission spectra were determined in an SLM-Aminco SPF-500C ratio recording spectrofluorometer. Excitation was at 290 nm, and the bandpass was 2 nm. Spectra were obtained of the oligopeptide-binding protein at a concentration of 1.7 μM (0.1 mg/ml) in 10 mM imidazole acetate (pH 7.3)–100 mM NaCl in the presence or absence of each specified peptide at a concentration of 100 μM. Fluorescence emission was monitored from 300 nm to 450 nm at room temperature.

RESULTS

The tripeptide Ala-Phe-[^{3}H]Gly, which is transported via the oligopeptide permease, binds to the purified oligopeptide-binding protein with a dissociation constant of 0.1 μM and a binding stoichiometry of 1.1 mol of tripeptide bound per mol of binding protein (14). In the present studies, the ability of a number of amino acids and peptides to compete with Ala-Phe-[^{3}H]Gly for binding to the purified oligopeptide-binding protein was measured to characterize the structural properties of peptides required for binding.

Specificity for oligopeptides. The amino acids Ala, Phe, and Gly, either individually or in combination, were unable to reduce binding of Ala-Phe-[^{3}H]Gly, even when present in greater than 600-fold excess (Table 1). In contrast, unlabeled Ala-Phe-Gly was an excellent competitor, completely eliminating Ala-Phe-[^{3}H]Gly binding. The dipeptide Ala-Phe demonstrated some degree of competition, but only at elevated concentrations. These results indicate that the purified protein specifically binds peptides and displays a strong preference for tripeptides over dipeptides.

Effect of termini. Acetylation of the amino terminus of $(Ala)_3$ drastically impaired the ability of this peptide to compete with Ala-Phe-[^{3}H]Gly for binding (Table 2). Even at 1 mM, *N*-acetyl-$(Ala)_3$ displayed virtually no competition. In comparison, unmodified $(Ala)_3$ reduced the binding of labeled peptide by 96% when present at a concentration of 10 μM. Esterification of $(Ala)_3$ significantly reduced but did not completely eliminate the ability of this peptide to compete for binding. These results imply that both the amino and carboxyl termini are structurally important for the recognition and binding of peptide to the protein. However, the amino terminus appears to play a more critical role in this function than does the carboxyl terminus.

Effect of stereochemistry. As indicated in Table 2, (D-Ala)$_3$ did not compete with Ala-Phe-[^{3}H]Gly for binding. Moreover, introduction of a D-residue in the second position, as in L-Ala-D-Ala-L-Ala, was sufficient to abolish competition. Peptides containing amino acids with L-stereochemistry, as opposed to glycine which lacks a chiral center, were preferred. Replacement of the third residue in $(Ala)_3$ with glycine resulted in significantly reduced competition. Additional substitution of glycine for alanine in the second position further decreased the ability of the peptide to compete for binding compared with $(Ala)_3$. Thus, stereochemistry of the peptide contributes significantly to its binding properties with the protein showing a strong preference for peptides composed of L-amino acids.

Effect of peptide chain length. Inhibition of Ala-Phe-[^{3}H]Gly by a series of alanyl peptide homologs was investigated as a function of concentration to determine the effect of chain length on peptide binding (Fig. 1). $(Ala)_3$ and $(Ala)_4$ display similar competition curves, both effectively inhibiting Ala-Phe-[^{3}H]Gly binding at concentrations below 10 μM. However, a concentration of $(Ala)_5$ approximately 2 orders of magnitude greater was required to achieve the same degree of competition observed for $(Ala)_3$. $(Ala)_2$ exhibited almost no ability to compete for binding, even at concentrations up to 1 mM. These results support the conclusion that the binding protein prefers oligopeptides to dipeptides and

TABLE 2. Effect of termini and stereochemistry on binding

Competing peptide[a]	% Ala-Phe-[^{3}H]Gly remaining bound with the following concn of competitor[b]:		
	10 μM	100 μM	1 mM
None	(100)	(100)	(100)
$(Ala)_3$	4 ± 0	ND[c]	ND
N-Acetyl-$(Ala)_3$	100 ± 0	98 ± 3	96 ± 2
$(Ala)_3$-*O*-methyl ester	96 ± 2	40 ± 15	2 ± 1
(D-Ala)$_3$	99 ± 3	96 ± 13	91 ± 10
L-Ala-D-Ala-L-Ala	96 ± 3	96 ± 3	98 ± 6
Ala-Ala-Gly	32 ± 2	4 ± 1	0[d]
Ala-Gly-Gly	90 ± 5	72 ± 6	31 ± 0

[a] All peptides are composed of L-amino acids unless otherwise noted.

[b] The ability of a peptide to compete for binding was determined as described in Table 1, footnote *a*.

[c] ND, Not determined.

[d] Binding of Ala-Phe-[^{3}H]Gly was below the level of detection.

suggests that a decrease in affinity for binding occurs with increasing chain length beyond tripeptide.

Effect of amino acyl side chains. A number of tripeptide homologs were used to study the effect of amino acyl side chains on peptide binding (Table 3). While certain peptides composed of hydrophobic or nonpolar amino acids were excellent inhibitors, other peptides in this same classification were very poor competitors of Ala-Phe-[^{3}H]Gly binding. In general, peptides consisting of polar amino acids such as $(Ser)_3$ or $(Gln)_3$ competed as well or better than hydrophobic peptides. Peptides composed of amino acids with ionic side chains were as a class consistently poor inhibitors of Ala-Phe-[^{3}H]Gly binding. Similarly, results from assays in which peptides competed with Ala-Lys-[^{14}C]Gly for binding (data not shown) indicated that peptides with hydrophobic or polar side chains effectively inhibited binding of labeled peptide but that peptides possessing ionic amino acyl groups showed very little competition.

The effect of amino acyl side chains was also investigated by fluorescence emission spectroscopy. The oligopeptide-binding protein exhibited native tryptophan fluorescence when excited at 290 nm (Fig. 2) with an emission maximum at 349 nm. Upon addition of 100 μM $(Ala)_3$, an excellent competitor for the Ala-Phe-Gly binding site, no change in the fluorescence spectrum was observed. Similar results were also observed in the presence of 100 μM Ala-Phe-Gly (data not shown). However, the addition of 100 μM $(Lys)_3$, a peptide which displayed no competition for binding to the Ala-Phe-Gly site, resulted in a substantial increase in tryptophan fluorescence with a shift to longer wavelengths. Thus, $(Lys)_3$ affects the fluorescence spectrum of the periplasmic protein differently from $(Ala)_3$ or Ala-Phe-Gly.

TABLE 3. Effect of amino acyl side chains on peptide binding

Competing peptide[a]	% Ala-Phe-[^{3}H]Gly remaining bound with the following concn of competitor[b]:		
	50 μM	100 μM	1 mM
Nonpolar, hydrophobic			
$(Ala)_3$	0[c]	ND[d]	ND
$(Gly)_3$	99 ± 0	96 ± 4	68 ± 8
$(Leu)_3$	93 ± 3	88 ± 2	ND
$(Met)_3$	0	0	ND
$(Pro)_3$	104 ± 3	104 ± 1	ND
$(Phe)_3$	31 ± 1	19 ± 1	ND
$(Tyr)_3$	56 ± 2	44 ± 0	ND
$(Val)_3$	15 ± 1	8 ± 1	ND
Polar			
$(Gln)_3$	3 ± 1	0	ND
$(Ser)_3$	7 ± 2	5 ± 1	ND
$(Thr)_3$	76 ± 2	64 ± 1	ND
Ionic			
$(Glu)_3$	102 ± 5	92 ± 18	44 ± 14
$(Lys)_3$	100 ± 6	100 ± 8	74 ± 10
$(Orn)_3$	105 ± 0	106 ± 10	99 ± 3

[a] All peptides are composed of L-amino acids.
[b] The ability of a peptide to compete for binding was determined as described in Table 1, footnote *a*.
[c] Binding of Ala-Phe-[^{3}H]Gly was below the level of detection.
[d] ND, Not determined.

DISCUSSION

Equilibrium dialysis has been used extensively for the purification and characterization of the periplasmic binding component from a variety of transport systems, including those for leucine (9, 10, 41), glutamine (39), cystine (4), galactose (5), ribose (40), and oligopeptides (14). In the present study, a microvolume equilibrium dialysis technique was used to minimize the amount of purified oligopeptide-binding protein and labeled peptide substrate needed for each assay. In cases in which periplasmic binding proteins have been purified containing a stoichiometric amount of tightly bound endogenous ligand (1, 34), microvolume assays have yielded nonlinear binding curves owing to the isotopic dilution of the radioactive substrate by the bound ligand. However, linear double-reciprocal binding plots were obtained for the oligopeptide-binding protein (14), suggesting that no endogenously bound ligand is associated with this protein and that microvolume equilibrium dialysis should be an accurate method for measuring binding. Competition with Ala-Phe-[^{3}H]Gly for binding to the purified oligopeptide-binding protein was used to investigate the structural properties of peptide substrates that are required for peptide-protein interactions. Although dissociation constants were not determined, the ability of a given peptide to compete for binding was taken as a relative measure of the affinity of the binding protein for the peptide at that site.

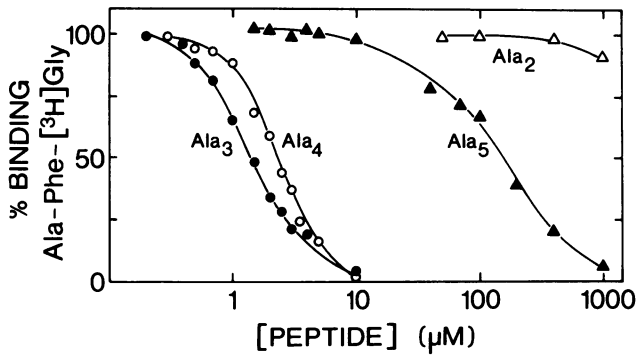

FIG. 1. Effect of peptide chain length on binding. Competition with Ala-Phe-[^{3}H]Gly (1.5 μM) for binding to purified oligopeptide-binding protein (1.7 μM) was determined by equilibrium dialysis for a series of alanyl peptide homologs. The percentage of Ala-Phe-[^{3}H]Gly remaining bound was plotted versus the log of the initial concentration of competing peptide. Under conditions in which no competitor was present, binding of Ala-Phe-[^{3}H]Gly was considered to be 100%. Each curve represents the combined results from two to three independent assays.

The results demonstrate that the binding protein possesses little or no affinity for free amino acids and dipeptides. The protein recognized features characteristic of two-peptide amide bonds since combinations of individual amino acids or of an amino acid and a dipeptide did not produce competition comparable to that exhibited by an intact tripeptide. These results are consistent with the transport and genetic evidence which indicates that the uptake of amino acids (19, 20), dipeptides (7, 24, 38), and oligopeptides (2, 7, 17, 24) is mediated by separate systems. The binding protein thus appears to be responsible for conferring on the permease the specificities that distinguish the oligopeptide system from the dipeptide system.

The oligopeptide permease absolutely requires its peptide substrates to possess an ionizable primary or secondary N-terminal amino group (3, 11, 25, 28). However, peptides lacking a C-terminal α-carboxyl group (27, 31) or having the functionality variously replaced or derivatized (8) are still transported, albeit with reduced affinities compared with the parent peptides. In the present study, acetylation of the

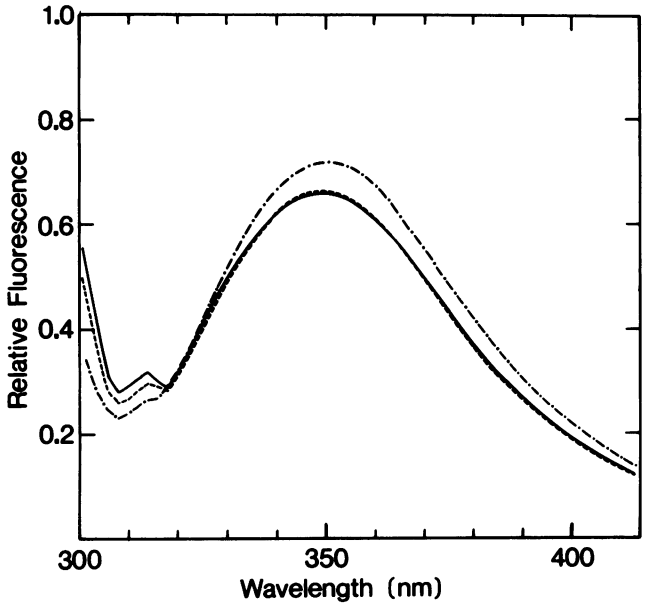

FIG. 2. Effect of peptide substrates on the fluorescence emission spectrum of the oligopeptide-binding protein. The relative fluorescence of the oligopeptide-binding protein at a concentration of 1.7 μM (0.1 mg/ml) in 10 mM imidazole acetate (pH 7.3)–100 mM NaCl was determined as a function of wavelength with excitation at 290 nm. Symbols: ——, fluorescence of the protein in buffer alone; - - - -, fluorescence of the protein in the presence of 100 μM $(Ala)_3$; - · - · - · , fluorescence of the protein in the presence of 100 μM $(Lys)_3$. All spectra were obtained 10 min after mixing the peptide with the binding protein.

amino terminus of $(Ala)_3$ severely diminished the inhibition by this peptide of Ala-Phe-[^{3}H]Gly binding, while esterification of the carboxyl terminus of $(Ala)_3$ resulted in a pronounced, but less serious decrease in inhibition. These binding results parallel the characteristics observed for transport (21, 25, 28); the preferences of the transport system probably reflect the preferences of the binding protein for peptides with unmodified termini.

Another factor influencing substrate specificity is the length of the peptide chain. Analysis of the binding data obtained for a series of alanyl peptide homologs indicates that the affinity of the periplasmic protein for these peptides decreased with increasing chain length from $n = 3$ to 5. It should be noted that the molecular weight of the longest homolog, $(Ala)_5$, is well below the cutoff allowed by the porins in the outer membrane (32).

The oligopeptide permease displays a wide tolerance toward the amino acyl side chains of peptide substrates (2, 7, 8, 12, 24, 30), with the side chains primarily exerting an effect on the affinities (33) and rates of uptake (30). Therefore, the influence of amino acyl side chains on binding specificity was measured with a variety of tripeptide homologs. The ability to compete for binding with Ala-Phe-[^{3}H]Gly varied widely within the classes of peptides composed of nonpolar, polar, and hydrophobic amino acids, but generally paralleled the competitive abilities reported for transport (30) with the ranking as follows: $(Ala)_3 > (Ser)_3 >> (Gly)_3$.

Peptides composed predominantly of glycine are poorer substrates for transport than similar peptides consisting of alanine (30, 33). These results may be accounted for on the basis of peptide binding. Each subsequent replacement of glycine for alanine in $(Ala)_3$ (Table 2) resulted in a significant reduction in the ability of the peptide to compete for binding. The apparent low affinities for $(Gly)_3$ and for $(Pro)_3$ may be explained on the basis of peptide conformation. In $(Pro)_3$, the series of rigid pyrrolidine rings places severe constraints on the peptide backbone and may prevent adoption of the conformation necessary for binding. In $(Gly)_3$, it has been suggested that a fairly unrestricted range of rotational angles can be accommodated by the peptide bonds and may reduce the effective concentration of the particular conformation recognized by the binding protein (33). Moreover, adoption of a particular backbone conformation upon binding may result in a significant loss of entropy. This negative contribution to the overall free energy of association could be manifested as a low apparent binding affinity.

Peptides composed exclusively of amino acids with ionic side chains failed to compete for binding to the purified protein. $(Lys)_3$ and $(Orn)_3$ competed for uptake with each other, as well as with a variety of other oligopeptides containing hydrophobic or nonpolar side chains, implying that these peptides share a common transport system (24, 37). More importantly, mutations in the genetic locus coding for the oligopeptide permease prevent uptake of a wide range of oligopeptides, including $(Lys)_3$ and $(Orn)_3$ (18). These results suggest that the permease is capable of transporting peptides composed of ionic amino acyl side chains.

Two hypotheses rationalizing the discrepancy between the transport and binding data can be suggested. First, one (or more) separate and distinct binding site(s) for peptides consisting primarily of amino acids with charged side chains may exist on the periplasmic protein. Second, the uptake of peptides such as $(Lys)_3$ and $(Orn)_3$ may bypass the periplasmic binding protein and may rely directly on the membrane-associated proteins. A mutation in the transport system for maltose has been characterized which allows transport directly through the membrane components, circumventing the involvement of the binding protein (36).

The first of these hypotheses was investigated in a preliminary experiment by fluorescence emission spectroscopy. The periplasmic proteins involved in histidine (35), arabinose (23), galactose (22), and glutamine (39) transport undergo a conformational change upon binding substrate that results in an alteration in their fluorescence emission spectrum. A significant increase in the fluorescence emitted by the oligopeptide-binding protein was observed upon the addition of $(Lys)_3$, suggesting that peptides composed of ionic amino acids do interact with the protein and induce a change in conformation. The fact that $(Ala)_3$ and Ala-Phe-Gly fail to produce similar changes in the fluorescence spectrum but do compete for binding in the radioactive assays suggests that the binding protein possesses more than one binding site capable of distinguishing peptides on the basis of their amino acyl side chains.

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