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THE COOPERATIVE CHARACTER OF PHENYLALANINE BINDING BY A PROTEIN FRACTION ISOLATED FROM BAKER'S YEAST MEMBRANES

JOSEF VOŘÍŠEK*

Laboratory for Cell Membrane Transport, Institute of Microbiology, Czechoslovak Academy of Sciences, Praha (Czechoslovakia)

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SUMMARY

The non-ionic detergent Tween 80 (1%) and $MgCl_2$ (0.1 M) were employed for the extraction of isolated yeast membrane fraction. Solubilized proteins obtained from this fraction were separated from lipoprotein particles by gel chromatography (Sephadex G-100) and partially purified (DEAE-cellulose). Significant and specific binding affinity for phenylalanine was reproducibly demonstrated by equilibrium dialysis. The binding of phenylalanine was thermolabile. A cooperative character of the observed binding was deduced from Lineweaver–Burk plot of the measured biphasic saturation curve. The results are in keeping with the role of binding protein in the membrane transport

INTRODUCTION

Only indirect evidence implicated the participation of pericytoplasmic binding proteins in the membrane transport[1]. These proteins were often considered as possibly identical with a membrane carrier, which was postulated as an integral part of the active transport model[2,3]. Proteins with specific binding properties have already been isolated from bacterial periplasma and characterized[1].

Similarly, we presumed that the binding proteins would act in the transport systems of the constitutive amino acid uptake by the yeast *Saccharomyces cerevisiae*. While the first *in vivo* experiments indicated that the enzymic translocation of amino acids across yeast membranes is a non-specific process[4,5], specific permease systems were later found to play an important role. Permeases for arginine[6], lysine[7], methionine[8] and dicarboxylic amino acids[9] were then associated with the unequivocally demonstrated general amino acid permease[10].

We found that the extraction of yeast membranes was a crucial step in the isolation of binding proteins. All the treatments formerly employed, *e.g.* extraction with 8 M urea, sonication[11] and extraction with 1 M NaI[12] are known to involve a denaturation, the releasing agents used acting as denaturing agents[13] rather than solvents for the lipoproteins present in the yeast cell membrane. The use of the non-

* Present address: Laboratory for Electron Microscopy, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

ionic detergent Tween 80 was found to be essential for extraction of material absorbing at 280 nm and for preserving the phenylalanine binding activity, the only reproducibly detectable binding activity in our experiments with commercial yeast preparations.

MATERIALS AND METHODS

Yeast strain and reagents

The prototrophic industrial strain of *Saccharomyces cerevisiae* (Distillery Kolín) was employed. At least 3-day-old commercial preparations of yeast, with no dead cells detectable after staining with methylene blue, were used. The non-ionic detergent Tween 80 was obtained from Spofa, Praha; Trizma base and L-phenylalanine were products of Sigma Co., 2-mercaptoethanol of Koch–Light Laboratories; Sephadex G-100 and G-200 of Pharmacia, Uppsala; DEAE-cellulose of Fluka A.G., Buchs. L-Tyrosine was obtained from Reanal, Budapest; the Visking cellophane tubing was purchased from Union Carbide Corporation; and the Diaflo PM-10 membranes were from Amicon Corporation. Labelled amino acids were from the Ú.V.V.V.R., Praha. Specific radioactivities: L-[^{14}C]glycine, 50 Ci/mole; L-[^{14}C]valine, 105 Ci/mole, L-[^{14}C]-isoleucine, 25 Ci/mole; L-[^{14}C]phenylalanine, 117 Ci/mole; L-[^{14}C]histidine, 62 Ci/mole, L-[^{14}C]aspartic acid, 100 Ci/mole; L-[^{14}C]lysine, 120 Ci/mole; L-[^{14}C]arginine, 54 Ci/mole; L-[^{14}C]methionine, 29 Ci/mole; L-[^{14}C]-proline, 80 Ci/mole. All other reagents were of reagent grade and were purchased from Lachema, Brno.

Buffer solutions

The standard Tris buffer contained 20 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The standard phosphate buffer was 20 mM sodium phosphate buffer (pH 5.5), 10 mM 2-mercaptoethanol and 10 mM $MgCl_2$. Redistilled water was used for all the buffer solutions.

Preparation of membrane extract

800 g of yeast, washed five times with physiologic saline, were suspended in 250 ml of ice-cold standard Tris buffer and disintegrated with glass beads (Pb free) in a rotating glass disc apparatus[14] for 6 min. The washed membrane fraction pellet[11] was extracted for 60 min with 250 ml standard Tris buffer (this and all the following procedures were carried out at 4 °C) containing 1.0% Tween 80 and then once more with the same solution *plus* 0.1 M $MgCl_2$. The resulting combined extracts (500 ml) were centrifuged at 104 000 × *g* for 30 min and the volume was reduced to 60 ml by ultrafiltration (Visking tubing). This solution was applied to a Sephadex G-100 column (4.5 cm × 100 cm).

Sephadex G-100 chromatography

The Sephadex G-100 in standard phosphate buffer was packed in a 2.5 cm × 40 cm column, onto which at least 5 ml of protein solution were loaded. Fractions of 6 ml were collected.

DEAE-cellulose chromatography

10 ml (70 mg of protein) were adjusted to pH 7.5 by adding Trizma-base crystals

and applied to a DEAE-cellulose column (2.5 cm × 40 cm) which had been equilibrated with standard Tris buffer. The proteins were fractionated by means of NaCl gradients produced by gradual mixing of 300 ml of standard Tris buffer with 300 ml of the same buffer *plus* 1.0 M NaCl. Fractions of 10 ml were collected and the absorbance measured at 280 nm.

Binding of phenylalanine to isolated membranes

Washed membrane fragments (8 mg protein) were suspended in 0.5 ml 2 μM L-[^{14}C]phenylalanine in standard phosphate buffer, incubated at 25 °C for 30 min and centrifuged (15000 × *g*, 20 min). Suspensions of membranes boiled for 5 min were used as controls. Radioactivity of supernatants was measured in 50-μl aliquots. Values were corrected for dilution of substrate in the membrane pellet, as calculated from dilution of xylose solution added in a parallel experiment[15].

Equilibrium dialysis method

An equilibrium dialysis method was used for demonstration of binding activity of the prepared proteins. A protein solution (0.3 ml, not more than 1 mg of protein) was pipetted into a Visking tubing bag and immersed in 2.5 ml of standard phosphate buffer. Equal concentrations of radioactive amino acid were added to both compartments and the dialysis was carried out for 20 h on a reciprocal shaker at 4 °C. Non-labelled L-phenylalanine was used for isotope dilutions beginning with 30 μM phenylalanine. The solutions and glass employed for equilibrium dialysis were suitably sterilised before use The 50-μl aliquots of the resulting solutions were put onto aluminium planchets, diluted with 2 ml of distilled water and dried in an air stream.

Radioactivity measurement

Radioactivity was measured using a 2π proportional gas flow counter (Frieseke–Hoepfner) using infinitely thin samples. The radioactivity of chromatograms was checked using a Frieseke–Hoepfner chromatogram scanner with GM tubes FH 215a, 1.13 mg/cm^2.

Chromatography of amino acids

After 20 h of dialysis with radioactive phenylalanine, the binding protein preparation was heated to 90 °C and the supernatant was applied to chromatographic paper Whatman No. 1. Amino acids were separated by ascending paper chromatography in *n*-butanol–acetic acid–water (60:20:20, by vol.) and identified by comparison with standard samples and by means of tabulated R_F values after ninhydrin detection.

Protein concentration

The modified[16] Lowry method or the spectrophotometric method of Warburg and Christian[17] were used. For the calculation of protein content in isolated yeast membranes, an aliquot of membrane suspension was hydrolysed overnight in 0.1 M NaOH[16] and the protein concentration in the supernatant was determined by the Lowry method.

Calculations

From the Langmuir–Michaelis dissociation equation for protein–ligand complex

formed during the equilibrium dialysis[18], the final form was derived:

$$[CS] = C_t \frac{[S]}{K_m + [S]}$$

and this was employed in the equilibrium dialysis scheme expressed as:

$$\frac{[S] + [CS]}{[S]} > 1$$

(S, ligand; C_t, total carrier, CS, ligand–carrier complex) which served for calculating the theoretical proportion of binding protein in the preparation.

RESULTS

Purification procedure

A fraction principally representing cell membranes was prepared from disintegrated yeast as described in Materials and Methods. No binding of 2 μM L-[^{14}C]-phenylalanine was detectable in this fraction. The amount of hydrolysable protein in the isolated membrane fragments prepared from 800 g of yeast was 6.15 g. The values characterizing the yield of the purification procedure are listed in Table I.

TABLE I

BALANCE OF THE PURIFICATION OF PHENYLALANINE BINDING PROTEINS

The membrane fragments were prepared from 800 g of washed commercial yeast by disintegration with glass beads and centrifugation The standard Tris–HCl buffer (pH 7 4) containing 1% Tween 80 was used for extraction of the membranes and the extraction was repeated with the same buffer solution *plus* 0 1 M $MgCl_2$ The details are given in Materials and Methods The subsequent column chromatography of proteins on Sephadex G-100 and DEAE-cellulose is described in Results Only half of the AB fraction was used for the next purification step

Step (fraction)	*Protein (mg)*	*Yield (%)*	*Specific binding at 2 μM phenylalanine (pmoles per mg protein)*
(Membrane fragments)	6150	100	—
Sephadex G-100 chromatography (AB)	146	2 3	35
DEAE-cellulose chromatography (BC)	11 4	0 36	115

For the further treatment of isolated membranes the buffered Tween 80 (1%) with 0.1 M $MgCl_2$ was used. Besides soluble proteins, a mass of particles floating even at 104000 $\times$ g was extracted. This solution was applied to the Sephadex G-100 column and eluted with standard phosphate buffer.

The $A_{280\ nm}$ values measured in the effluent fractions are presented in Fig. 1. The A_1 fraction contained all non-sedimenting particles (probably a lipoprotein). The fractions A_2 to A_4 were found to exhibit a binding affinity to phenylalanine after reducing their volume to 5% by ultrafiltration through Diaflo PM-10 membrane. The volume of the combined active fraction (AB in the sequel) was reduced to 20 ml and the protein concentration was determined. About one-half of the material was used to assess saturation characteristics and specificity of binding. Simultaneously,

the second half of the AB fraction was purified on a DEAE-cellulose column. The profile of the absorbance values measured as $A_{280\ nm}$ in the collected fractions is presented in Fig. 2. The fractions were combined as indicated by arrows (BA, BB and BC fractions). The volume of each fraction was reduced to 3 ml and the protein solutions were dialysed overnight against 100 vol. of standard phosphate buffer The binding activity was found only with the BC fraction

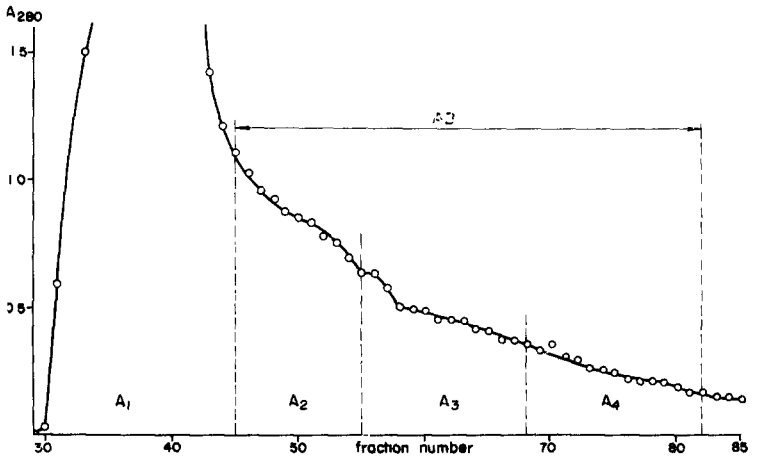

Fig 1 Fractionation of proteins extracted from yeast membrane fragments The washed membranes prepared from 800 g of commercial yeast were extracted by 500 ml of 20 mM Tris–HCl buffer (pH 7 4), 0 1 mM EDTA, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1% Tween 80 in the ice bath for 30 min and then once more with the same solution *plus* 0 1 M $MgCl_2$ The volume of combined extracts was reduced to 60 ml by ultrafiltration (Visking tubing) This material was loaded on a Sephadex G-100 column (4 4 cm × 100 cm) and eluted with 2 mM sodium phosphate buffer (pH 5 5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol The A_1 fraction contained all the non-sedimenting particles (lipoprotein) The concentrated A_2 to A_4 fractions were equivalent as concerns the binding of 2 μM phenylalanine and were combined as the AB fraction

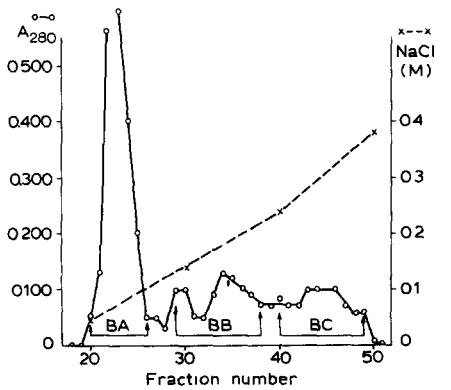

Fig. 2. Purification of phenylalanine binding proteins. The AB fraction of binding proteins (10 ml, 70 mg protein) was adjusted to pH 7 4 by adding Trizma-base crystals and loaded on a column of DEAE-cellulose (2 5 cm × 40 cm) which had been equilibrated with standard Tris–HCl buffer (pH 7 4) The proteins were eluted using a linear gradient of NaCl from 0 to 0 1 M (2 × 300 ml). Titration with $AgNO_3$ in the presence of potassium chromate was used for determination of the profile of the NaCl gradient The BA, BB and BC protein fractions were combined, as indicated by arrows The binding activity for phenylalanine was present in the BC fraction

The absence of Tween 80 from the AB fraction was demonstrated by ultraviolet spectroscopy. The protein preparations were centrifuged (40000 × *g* for 30 min) to remove any contaminations which might disturb the binding activity determination. The sterility of the preparations was tested on blood agar.

Specificity of binding

The following labelled amino acids (at a concentration of 2 μM each) were used for testing the binding specificity of the AB fractions: Gly, Val, Ile, Phe, His, Asp, Lys, Arg, Met and Pro. Significant binding was reproducibly found only with phenylalanine. After 20 h of equilibrium dialysis and deproteinisation of samples by heating, the ^{14}C activity was found solely in the spot corresponding to phenylalanine.

Saturation curves

The saturation of the AB and BC fractions with L-[^{14}C]phenylalanine was measured in the concentration range of 0.5 μM-10 mM Fig. 3 shows an example of the biphasic curve obtained with the AB fraction. The wide range of employed substrate concentrations made it necessary to plot them on a logarithmic scale against the values of binding expressed in percent of the equilibrium value. The point of breakage of the curve was found between 4 and 8 μM phenylalanine with different preparations. The enhancement of binding at concentrations of ligand higher than 8 μM (4 μM) was surprising, and in the following paragraphs we demonstrate that it was due to the change of the binding affinity. Our preparations (AB) were not purified to homogeneity and therefore the presence of two systems for phenylalanine binding was the most obvious explanation of the observed saturation kinetics.

Properties of the binding system with higher affinity to the ligand are better illustrated by the plot of absolute binding values against phenylalanine concentration (Fig. 4). The sigmoid curve obtained and the concave character of the Lineweaver–Burk plot (inserted graph) made accurate specification of the dissociation constant impossible. However, for control calculations of C_t it was necessary to estimate the

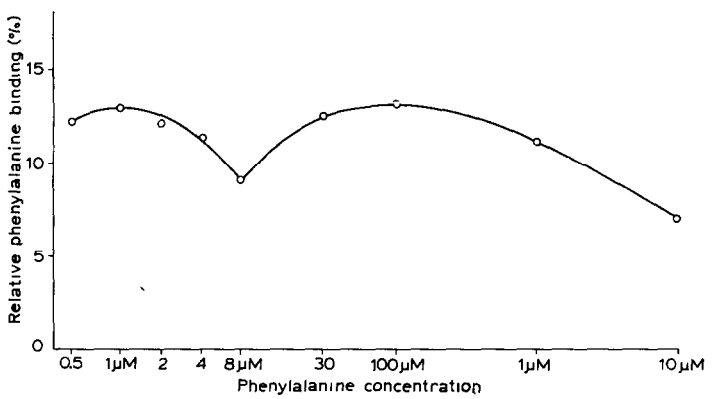

Fig 3 Binding of phenylalanine as a function of substrate concentration The AB fraction, containing 2.6 mg protein per ml, was used for equilibrium dialysis The wide range of employed substrate concentrations made it necessary to plot them on a logarithmic scale against the values of binding as the percent enrichment in radioactivity in protein compartment The values were obtained as an average of a series of four parallel samplings

apparent K_{diss} ($K_{1/2}$) which was found to be approximately 2.5 μM. From the values of binding of 2 μM L-phenylalanine and assuming a molecular weight similar to the average in bacterial periplasmic binding proteins (35000, ref. 1), it was then calculated that the AB fraction contained about 10 μg of binding protein per mg total protein. This value was well reproducible with different preparations. Also the fluctuation of specific binding activity values was found reasonable, the values fitting between 35 and 50 pmoles per mg total protein

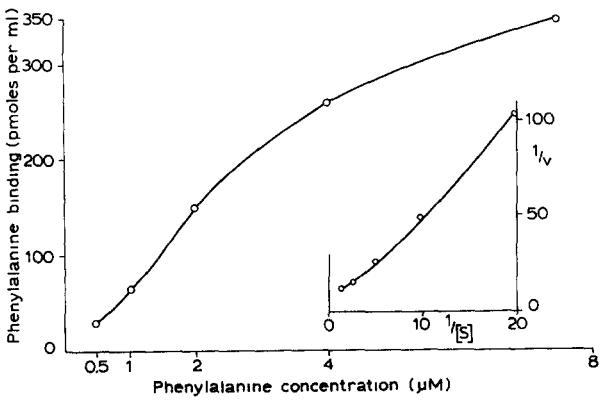

Fig 4 The absolute values of phenylalanine binding as a function of substrate concentration. The AB fraction, containing 2 6 mg protein per ml, was used for equilibrium dialysis The values of binding in pmoles were plotted against the substrate concentrations, both on a linear scale The inserted graph shows the Lineweaver–Burk replot of the values

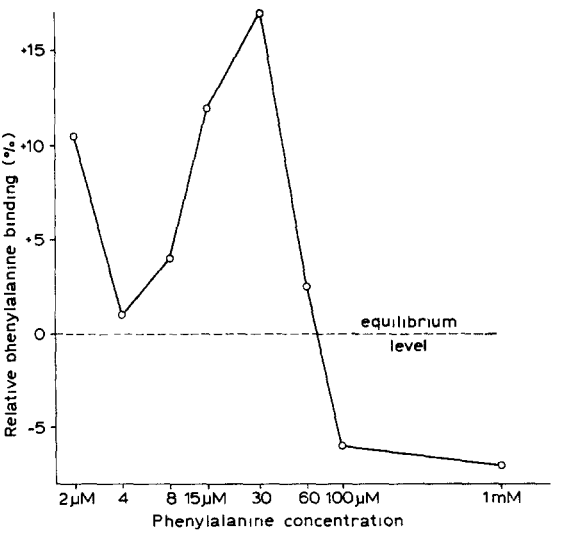

Fig 5 Binding of phenylalanine as a function of substrate concentration. The BC fraction, containing 1.9 mg protein per ml, was used for equilibrium dialysis The substrate concentrations were plotted on a logarithmic scale against the values of binding plotted as the percent enrichment in radioactivity in protein compartment The equilibrium level indicates the value of radioactivity in the external buffer compartment after 20 h of equilibrium dialysis

The system possessing the lower affinity to ligand behaved in a rather surprising manner when the AB preparation was investigated (Fig. 3). Very little "chase out" effect was observable and even at 10 mM phenylalanine the binding was very high. This was not in accord with the estimations quoted in the preceding paragraph. The situation changed after the DEAE-cellulose purification step. The phenylalanine saturation function for the BC protein fraction is shown in Fig. 5. The binding is plotted again as percent enrichment in the protein compartment after equilibrium dialysis and the ligand concentrations are plotted on a logarithmic scale. Now the complete "chase out" of the bound ligand is discernible at phenylalanine concentrations of about 0.1 mM. The negative values of binding are sustained by the fact that on dialysis the nonactive fractions never reached the exact equilibrium value, the radioactivity in the protein compartment was significantly lower than in the external buffer compartment. This was probably due to the self-absorption of radioactivity in samples prepared from protein solutions.

After the DEAE-cellulose purification step the shape of both parts of the ligand saturation curve was distinctly sigmoid, the Lineweaver–Burk plot being concave. For the system with lower affinity to phenylalanine, the half-saturation constant could not even be estimated. However, the system was saturated with 30 μM ligand and the $K_{1/2}$ may be approximately equal to 15 μM.

The purification on DEAE-cellulose enhanced the percent of binding at phenylalanine concentrations above 8 μM when compared with the binding at 2 μM. Nevertheless, the specific binding at 2 μM phenylalanine was enhanced to 115 pmoles per mg total protein in the BC fraction. Specific activity of binding at 30 μM phenylalanine varied between 0.29 and 0.43 nmole per mg total protein with the AB fraction, with the BC fraction the range was 1.35–1.55 nmoles per mg total protein.

Five different preparations were used for the characterization of the AB fraction and three of them were further purified on DEAE-cellulose. Sephadex G-200 column chromatography did not change the kinetic properties when compared with the AB fraction, nor separate the two binding systems. The binding properties of the first and last effluent fractions containing protein were identical, the fractions formed a broad single peak

The values of binding measured by equilibrium dialysis were not in linear proportion with the protein concentration above 3 mg/ml. The maximum binding observed with the preparation containing 7.7 mg protein per ml was 35% greater than the equilibrium value. It has to be mentioned here that the preparations were kept at 0 °C, as freezing caused precipitation of proteins and inactivation It was found that during the 10 days, which were necessary for accomplishing the described purification, the binding of phenylalanine to the AB fraction was not significantly reduced. Nevertheless, the yield of the purification procedure is low and any further purification will be difficult because of obvious technical limits in the first steps of membrane preparation and extraction. All our purification experiments were successful as concerns the phenylalanine binding activity in the protein preparations.

Competitors of binding

The nonlabelled L-phenylalanine, L-tyrosine, L-arginine or L-valine (1 mM) were tested as competitors of L-[^{14}C]phenylalanine binding (2 μM) by the AB fraction. All the amino acids had little effect (see the point corresponding to 1 mM phenylalanine

on Fig. 3). When the BC fraction was employed, the binding of 2 μM L-[^{14}C]phenylalanine was completely abolished by 1 mM L-phenylalanine and L-tyrosine.

pH dependence

The binding was found to be independent of pH in the range of 4.0–7.5 (sodium phosphate buffer).

Thermostability

The binding affinity was diminished by heating the preparation for 30 s at 65 °C.

Ultraviolet spectrum of preparations

The continuous ultraviolet spectrum of the dialysed BC fraction was measured between 190 and 310 nm. The only peak was found at 280 nm.

Homogeneity of preparations

The binding specificity of the isolated membrane proteins for phenylalanine was not paralelled by polyacrylamide gel electrophoretic homogeneity[19]. Both the AB and BC fractions were composed of six principal bands, the purification changed only the proportion of their relative density.

DISCUSSION

Isolation of binding material from the membranes of microorganisms is one of the methods used for demonstration of a "carrier" acting in the active transport systems[18]. The main problem which will necessarily influence any discussion at this moment, is the uncertainty of the precise function of binding proteins in the transport. They are certainly specific with respect to the transported substrate and are therefore assumed to constitute a certain component of the membrane translocation system. However, a direct demonstration of their function, as represented by the reconstitution of the membrane transport system *in vitro*, has not yet been possible.

The soluble protein material from the yeast membrane with binding affinity for phenylalanine has been characterized in the present paper. The only amino acid which could displace phenylalanine from binding was its analogue tyrosine. Such a narrow specificity is surprising, as no special system for the phenylalanine uptake in yeast has been reported so far. Instead, the competition of different, structurally unrelated amino acids with the phenylalanine was observed[4]. This was explained by demonstrating an existence of a general amino acid permease[10]. When this enzyme was inactivated by the presence of NH_4^+, the initial rate of uptake of aromatic amino acids was reduced as a consequence of a strong limitation of the accumulative power of the permeases[20]. Therefore, the presence of single binding activity in our preparations made from viable commercial yeast cells results most probably from the technical procedure used.

The purification of yeast membranes before the extraction and the chromatographic verification enabled us to exclude the possibility of phenylalanine binding by an intracellular enzyme of phenylalanine metabolism. Such a clear localization of binding proteins serves as an important indication of their role in the membrane

translocation process, which was not obvious with pericytoplasmic proteins isolated by osmotic shock from bacteria[1]. This concept is supported also by the observed kinetic properties. The biphasic saturation can be compared with amino acid uptake by the yeast *in vivo* which exhibited two half-saturation constants for the majority of the amino acids studied[21] Unfortunately, phenylalanine was not among them, so that in this case the parallel is uncertain.

In experiments with purified galactose binding protein of *Escherichia coli* it was found that the binding of a certain concentration of ligand changed the conformation of the binding protein molecule and reduced the value of binding affinity by two orders of magnitude[22,23]. Consequently, a better interpretation of our kinetic observations would be to attribute the second wave of ligand binding to a modified form of the binding protein with lower affinity. Generally, the two affinities of a binding protein correspond to properties postulated for a carrier molecule and their maintenance on respective sides of the membrane can explain the active transport itself[22]. Because of specific limitation of the exit process in the active transport of amino acids in yeast[5,21] we assume that it is possible to compare the observed difference in half-saturation constants of our binding systems with the concentration gradients formed after the accumulation of amino acids in yeast cell pool. The order of magnitude of binding affinities observed for phenylalanine *in vitro* corresponds well to values of half-saturation constants of amino acid uptake *in vivo*[21], and this may also indicate the carrier role of the isolated binding protein.

With our present knowledge the explanation discussed in the above paragraph seems more probable than the assumption of two binding proteins for a single amino acid; especially since the Sephadex G-200 purification step showed very similar, if not identical, molecular size of the two binding systems in question. Nevertheless, both concepts include the participation of binding proteins in the membrane translocation.

The molecular transition of the binding protein molecule at a certain concentration of ligand, yielding an additional binding site with a lower affinity was discussed. On the other hand, the sigmoid saturation functions indicate unequivocally the homotropic allosteric character of phenylalanine binding over the whole concentration range. Hence, the Lineweaver–Burk plot was concave so that all the given control calculations based on the dissociation constants are only approximate. This phenomenon was observed with all our preparations and it was not changed during the purification procedure.

The experiments with amino acid transport in intestine[24] indicated cooperativity of two or more binding sites of a similar type existing on the molecule of a carrier. Recently, the cooperative kinetics of galactose binding by "shock proteins" from *E. coli* were described and compared with similar properties of methylgalactose permease *in vivo*[25]. Our results, which also indicate the presence of more active sites on the molecule of binding protein, might mean another interesting contribution to the pool of data necessary for the evaluation of different models proposed for membrane carrier action.

A significant binding of amino acids other than phenylalanine was nonreproducibly present in our preparations. A study of the phenomenon is in progress.

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