A role for aminopeptidase N in Na⁺-dependent amino acid transport in bovine renal brush-border membranes

Stella PLAKIDOU-DYMOCK, Michael J. TANNER and John D. McGIVAN

Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

A monoclonal antibody FD19 which removes reconstitutable Na⁺-dependent amino acid transport activity from solubilized bovine renal brush-border membrane vesicles was found to react specifically with the enzyme aminopeptidase N. Cleavage of aminopeptidase N from the membranes with papain inhibited Na⁺-dependent amino acid transport activity without affecting that of α -methyl D-glucoside. Removal of aminopeptidase substantially increased the K_m values for the Na⁺-dependent transport of alanine, glutamine, leucine and phenylalanine without

affecting the V_{max} . Both Na⁺-dependent amino acid transport and aminopeptidase activity in intact vesicles were competitively inhibited by amino acids with very similar specificity. These results suggest that the amino acid-binding sites of aminopeptidase N and the transporter interact in some way to increase the K_m of the transport process for its substrates. However, independent direct inactivation of the transport system by papain cannot be ruled out.

INTRODUCTION

The Na⁺-dependent transport of neutral amino acids across the brush-border membrane of bovine renal proximal tubule cells is mediated by a single transport system of broad specificity [1] which has been termed System B^o [2]. A transport system of similar specificity has been described in rabbit intestine [3] and in the bovine renal epithelial cell line NBL-1 [2]. The broad-specificity transport system found in epithelial cells is distinct from the classical amino acid-transport systems A, ASC and L which are widely distributed in non-epithelial cell types (see [4] for a review).

The protein that mediates Na⁺-dependent amino acid transport in renal brush-border membranes has not yet been identified. Procedures for the solubilization and reconstitution into proteoliposomes of Na⁺-dependent alanine-transport activity from rabbit [5] and bovine [6] kidney have been reported. Radiation inactivation studies have indicated that the functional amino acid transporter *in situ* may have a molecular mass of 274 kDa [7]. Na⁺-dependent amino acid transport activity has been expressed in *Xenopus* oocytes after injection of rabbit renal cortex mRNA [8], but there are as yet no reports of the isolation of a cDNA clone encoding the transport protein.

In a recent study in this laboratory [9], it was shown by fractionation and reconstitution studies that the Na⁺-dependent amino acid-transport protein is a glycoprotein. A monoclonal antibody prepared against a concanavalin A-binding fraction of solubilized brush-border membranes reacted with a protein of molecular mass 132 kDa. Removal of this protein from solubilized membranes by immunoaffinity chromatography resulted in substantial removal of reconstitutable Na⁺-dependent alanine-transport activity when measured at low substrate concentrations. It was proposed that this protein constituted at least a component of the transport system.

In this paper it is shown that the protein identified in our previous work is the enzyme aminopeptidase N (EC 3.4.11.2). This enzyme splits off the N-terminal amino acid from peptides

and has a broad specificity, preferring peptides with neutral N-terminal amino acids. It is one of the best characterized of the membrane hydrolases (see [10,11] for reviews). The tissue distribution and physiological importance of these hydrolases has been described (see [12,13]). In intestinal microvilli, aminopeptidase N is important for the hydrolysis of dietary peptides before amino acid absorption. In the central nervous system, it is thought to be involved in the breakdown of neurotransmitters. The enzyme is abundant in renal brush-border membranes, but has no clearly defined physiological function in these membranes. Aminopeptidase N has also been found to be one of the cluster of differentiation (CD) glycoproteins in human myeloid cell plasma membranes [14], and more recently it has been shown to be the major receptor for the enteropathogenic coronavirus TGEV [15] and for human coronavirus 229E [16].

The molecular sizes of aminopeptidase N and its postulated multimeric forms have been extensively characterized [see 11,12]. cDNA clones encoding the enzyme from human intestine [17] and rat kidney [18] have been isolated and sequenced and part of the gene for porcine aminopeptidase N has also been isolated [19]. Aminopeptidase N has been expressed in MDCK cells after transfection with the human cDNA clone [20].

Aminopeptidase N is an ectoenzyme anchored to the membrane via a transmembrane helical region near the N-terminus [21] with a small region of the N-terminus protruding into the cytoplasm. The main body of the protein is joined to the anchor by a short 'stalk'. Papain cleaves the enzyme at a site near the outer membrane surface [22]. The transmembrane helix remains in place, while the rest of the molecule is detached from the membrane with no loss of enzyme activity.

The idea that membrane peptidases may be directly or indirectly involved in amino acid transport in kidney and gut is not new (see e.g. [23]). In this paper we present evidence that aminopeptidase N is functionally linked to the Na⁺-dependent neutral amino acid-transport system in bovine kidney brush-border membranes.

MATERIALS AND METHODS

Preparation and fractionation of bovine renal brush-border membrane vesicles (BBMV)

Vesicles were isolated from fresh bovine kidney by a modification of the MgCl_2 precipitation method [24] as described in detail elsewhere [1] except that mannitol was replaced by an equal concentration of sucrose in the homogenization medium. The vesicles were washed and suspended in a medium containing 0.25 M sucrose, 10 mM K^+ Hepes and 0.2 mM CaCl_2 at pH 7.4 and were rapidly frozen in liquid nitrogen and kept at -20°C until used.

Immunoaffinity chromatography and protein deglycosylation

The monoclonal antibody FD19 was prepared as described in a previous paper [9] and was covalently attached to CNBr-activated Sepharose. Brush-border membranes were solubilized in the detergent MEGA-10 [25] in 20 mM Tris/HCl, pH 7.5 (3 mg of detergent/mg of protein) and the solution was clarified by centrifugation at 100000 g for 30 min at 4°C . Portions of the suspension were passed down a column of FD19 attached to Sepharose. After being washed with the same medium, the column was eluted with 50 mM diethylamine, pH 11, and samples were collected in 0.5 M Tris/HCl, pH 7.4 (to give 50 mM Tris/HCl final concentration). Fractions containing the protein were pooled and concentrated using Amicon microconcentrators. Deglycosylated proteins were prepared using peptide N -glycosidase F (PNGase F) (supplied by Dr. W. Mawby, Department of Biochemistry, University of Bristol). Protein (20 μg) was unfolded by heating at 95°C for 2 min in 0.1 vol. of buffer containing 10 mM EDTA, 10% 2-mercaptoethanol, 1% SDS, 500 mM sodium phosphate, pH 7.4. To this mixture was added octyl glucoside (5 $\mu\text{g}/\mu\text{l}$ final concentration) plus PNGase F (1 μg). The solution was incubated at 37°C and samples were removed at 6 h and 24 h.

Preparation of samples for N-terminal sequence analysis

Purified protein samples (typically 10 μg) prepared as described above were separated by SDS/PAGE and electroblotted (LKB Semi-Dry Blotter) on to Pro-Blott membrane (Applied Biosystems). The desired bands were cut out and subjected to automated amino acid sequence analysis on a 477A/120A protein sequencer (Applied Biosystems) with the Blot cartridge using standard cycles. For N-terminal analysis of papain-cleavage products, BBMV (10 mg in 1 ml) were digested with 4 μl of 25 mg/ml papain in 50 mM Tris/HCl, pH 7.5, for 5 h at 37°C . The membranes were then solubilized as above and purified on an FD19-Sepharose column. The eluted protein fractions were concentrated and used for N-terminal sequence analysis as above.

Assay of aminopeptidase activity

Aminopeptidase activity was assayed at 37°C in a medium containing 50 mM Tris/HCl at pH 7.4 together with the appropriate concentration of amino acid- p -nitroanilide as indicated. The initial rate of increase in absorbance at 410 nm was monitored continuously. The absorption coefficient of nitroaniline at 410 nm was found to be $5280 \text{ M}^{-1}\cdot\text{cm}^{-1}$.

Measurement of transport in BBMV

This was performed at 20°C as described previously [1]. Briefly, 15 μl of vesicle suspension (40–80 μg of protein) was rapidly mixed with an equal volume of buffer containing 0.25 mM sucrose, 10 mM K^+ Hepes and 0.2 mM CaCl_2 at pH 7.4 and also

either 0.2 M NaCNS or 0.2 M KCNS together with an appropriate concentration of the substrate to be transported and ^3H -labelled substrate. The transport reaction was stopped after 10 s by the addition of 1 ml of ice-cold stop solution (0.25 M sucrose, 10 mM K^+ Hepes, 0.2 mM CaCl_2 plus 0.2 M NaCl, pH 7.4). The suspension was rapidly filtered through 0.45 μm nitrocellulose filters, which were washed with $3 \times 1 \text{ ml}$ of stop solution. The filters were dissolved in scintillant and stored for at least 30 min to allow for decay of chemiluminescence before counting.

In experiments in which the transport of 0.1 mM alanine was measured, the specific radioactivity was 200 d.p.m./pmol. In experiments in which transport kinetics were measured, the transport medium contained a constant amount of radioactive substrate together with various concentrations of unlabelled substrate. The initial rate of transport at both the highest and lowest concentrations used was linear over the first 10 s (results not shown), and rates were routinely measured after this time. In all the kinetic experiments, the rate in the presence of KCNS was subtracted from that in the presence of NaCNS at the same substrate concentrations to give the Na^+ -dependent rate.

Gels and Western blotting

SDS/PAGE was performed as described by Laemmli [26]. Western blotting was performed by the method of Towbin et al. [27] except that the nitrocellulose was blocked with phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4) containing 5% skimmed milk powder plus 0.2% Tween 20, and washed with phosphate-buffered saline containing 0.2% Tween-20.

Protein was assayed as described by Bradford [28].

RESULTS

Identification of the protein reacting with monoclonal antibody FD19

We have shown previously [9] that a specific monoclonal antibody (FD19) removes a major proportion of reconstitutable Na^+ -dependent alanine-transport activity from solubilized BBMV. The protein with which this antibody reacts is characterized in Figure 1. Figure 1(a) shows that passage of solubilized BBMV through a column of FD19-Sepharose removes a major protein of molecular mass 132 kDa. The single protein binding to FD19 is the same as that which may be purified from a column of peanut lectin (Figure 1b), and the FD19-binding protein is a glycoprotein containing about 20 kDa of sugar residues as shown by deglycosylation experiments (Figure 1c). The antibody reacts with both native and deglycosylated forms of the protein.

Table 1 presents evidence that the protein recognized by FD19 is the enzyme aminopeptidase N. The rate of hydrolysis of amino acid- p -nitroanilide derivatives (2 mM) by BBMV was greater for the alanine and leucine compounds than for those of glycine and lysine, in accordance with the known specificity of this enzyme in other tissues. Passage of 1 mg of solubilized BBMV through a FD19-Sepharose column removed approximately 95% of the aminopeptidase activity, and this was the same for each substrate. Aminopeptidase activity could be subsequently eluted from the column with 50 mM diethylamine at pH 11. Only 30–40% recovery of enzyme activity was achieved owing to the extreme conditions necessary for dissociation of the enzyme-antibody complex and to losses during subsequent concentration. The pattern of specificity of the eluted enzyme was the same as that of the enzyme in the original membranes.

In similar experiments on a different batch of brush-border

Figure 1 Purification and characterization of the protein reacting with the monoclonal antibody FD19

(a) Removal of 132 kDa protein from BBMVs on an FD19-Sepharose immunoaffinity column. (i) SDS/PAGE of BBMVs before (lane A) and after (lane B) passage down the immunoaffinity column. (ii) Western blot using FD19 of BBMVs before (lane C) and after (lane D) passage down the FD19 column. Loadings were 5 µg of protein on each lane. Lane E shows the molecular-mass values for prestained protein standards. (b) Purification of 132 kDa protein by peanut lectin chromatography and by a FD19 immunoaffinity column. (i) SDS/PAGE of protein purified on peanut lectin (lane A) and FD19 column (lane B). (ii) Western blot using FD19 of protein purified on peanut lectin column (lane D) and FD19 column (lane E). Loadings were 1 µg of protein per lane. Lanes C and F are prestained protein standards as in (a). (c) Deglycosylation of protein purified on FD19. (i) Silver-stained SDS/polyacrylamide gel of FD19 column-purified protein (lane C) after deglycosylation for 6 h (lane B) and for 12 h (lane A). Loadings were 1 µg of protein per lane. (ii) Western blot using FD19 on FD19 column-purified protein (lane F), the protein deglycosylated for 6 h (lane E) and for 24 h (lane D). Lane G shows prestained molecular-mass markers.

Table 1 Removal of aminopeptidase N activity from solubilized BBMVs by the monoclonal antibody FD19

BBMVs were solubilized in MEGA-10 and 1 ml (1.1 mg of protein) was applied to a column (2.5 cm × 1 cm) of Sepharose-FD19. Elution of the column was as described in the Materials and methods section. The Table shows total aminopeptidase activity found in the original fraction, the fraction which passed straight through the column and the fraction eluted with diethylamine.

Substrate (2 mM)	Aminopeptidase activity (µmol/min)			
	Applied to column	Column effluent	Eluted with diethyl- amine	Activity removed by antibody (%)
Leucine- <i>p</i> -nitroanilide	6.02	0.32	1.70	94.7
Alanine- <i>p</i> -nitroanilide	6.10	0.42	1.75	93.2
Glycine- <i>p</i> -nitroanilide	1.83	0.07	0.77	96.2
Lysine- <i>p</i> -nitroanilide	0.51	0.038	0.175	93.6

Membrane form

Bovine	XXKGFYISKALGILAILRGVAAV
Human	MAKGFYISKSLGILGILLGVAHV
Porcine	MAKGFYISKALGILGILLGVAHV

Papain form

Bovine	DQSKPWNRYRLPTLLPDSY
Human	DQSKAWNRYRLPNTLKPDSY
Porcine	DQSKPWNRYRLPTLLPDSY

Figure 2 Alignment of the N-terminal amino acid sequence of bovine renal aminopeptidase N with human and porcine aminopeptidase N

The sequence obtained from N-terminal amino acid sequence analysis of the membrane form and the papain digest form of bovine aminopeptidase N is shown aligned to the amino acid sequence deduced from the cDNA of human intestinal aminopeptidase N [17]. In addition, the sequence deduced from the porcine partial gene [19] is presented. The 20 amino acids obtained from the bovine papain digest form align to amino acids 66 (D) to 84 (S) of the human aminopeptidase N. This is just within the Ser/Thr-rich junctional domain (amino acids 43–68) predicted from the human sequence [17]. The differences in amino acids that occur between the three species are underlined.

membranes it was found that the specific activity of hydrolysis of leucine-*p*-nitroanilide (2 mM) was 2.22 µmol/min per mg, that of the column effluent was 0.34 µmol/min per mg and that of the protein eluted from the antibody column was 24.6 µmol/min per mg, a purification factor of 11.1. The column bound 4.2% of the total protein and 86% of the total activity. If no loss of activity occurred on the column and aminopeptidase N were the only protein bound, a purification of 20.5-fold would be expected. In fact, the recovery of activity from the column was 53% of that bound whereas the recovery of protein was 100% so that the expected purification factor is 10.9-fold, in close agreement with that obtained.

The FD19-binding protein was purified by SDS/PAGE and then electroblotted on to Pro-Blott membrane. The peptide band was cut out and the N-terminal sequence determined. Figure 2 shows that the N-terminal sequence was very similar to the N-terminal sequence of aminopeptidase N from pig and human. It is known that papain cleaves aminopeptidase N at a specific site near the N-terminus. BBMVs were digested with papain and the FD19 protein was purified by immunoaffinity chromatography. The products were separated by SDS/PAGE and the new N-terminal sequence was determined. This internal sequence was again similar to internal sequences in the enzymes from human and pig. This work precisely locates the papain-cleavage site of the bovine kidney enzyme. The isolated protein reacted weakly on Western blots with a polyclonal antibody to pig kidney aminopeptidase N (kindly supplied by Dr. A. J. Kenny, University of Leeds).

All these data, taken together, show that the antibody FD19 reacts specifically with aminopeptidase N. Such stringent tests were necessary to establish the specificity of the antibody since aminopeptidase N is a major component of brush-border membranes and the presence of this enzyme in any particular fraction could in principle be due to contamination.

Effect of removal of aminopeptidase N on Na⁺-dependent amino acid transport activity in BBMVs

Since removal of the FD19-reacting protein from solubilized BBMVs greatly reduced reconstitutable Na⁺-dependent transport activity [9] and this protein has now been identified as

Figure 3 Release of aminopeptidase from brush-border membranes using papain

Brush-border membranes suspended in buffer containing 0.25 mM sucrose, 10 mM K^+ HEPES, and 0.2 mM $CaCl_2$ at pH 7.4 at a concentration of 4.3 mg of protein/ml were incubated with 50 μ g of papain/2.3 mg of protein at 20 °C. At intervals, 0.15 ml of suspension was centrifuged in a Beckmann Airfuge at 100 000 g for 30 s. A sample of the supernatant was retained and the pellet was washed and resuspended in 0.2 ml of the same medium for measurement of activity in the pellet. Aminopeptidase activity was measured at 37 °C using 1.6 mM leucine- p -nitranilide as substrate. ●, Pellet; ■, supernatant.

aminopeptidase N, the relationship between aminopeptidase N and amino acid transport in intact BBMVs was explored.

Aminopeptidase N may be cleaved from brush-border membranes by treatment with papain, a process that leaves the membrane relatively intact [21]. Figure 3 shows the time course of the disappearance of aminopeptidase activity from the membrane and its appearance in the supernatant when BBMVs were treated with limiting concentrations of papain. Under these particular conditions, the release of enzyme activity was complete in 15–20 min; 10–15% of aminopeptidase activity was not released even after prolonged incubation. The activity released from the membranes was fully recovered in the supernatant.

Figure 4(a) shows the initial rate of Na^+ -dependent alanine

transport (measured at 0.1 mM alanine) at various times after papain treatment of membranes. A large decrease in the rate of alanine transport occurred which followed approximately the same time course as the dissociation of aminopeptidase (Figure 3). Alanine-transport activity decreased to a new steady value which was still higher in the presence of Na^+ than in the presence of K^+ . Transport activity in the absence of Na^+ was not affected by papain treatment. In order to determine whether the decrease in Na^+ -dependent alanine transport could be attributed to a non-specific increase in membrane permeability leading to the collapse of the Na^+ gradient, the uptake of the glucose analogue α -methyl D-glucoside was measured in the presence and absence of Na^+ in a parallel incubation (Figure 4b). α -methyl D-glucoside uptake was Na^+ -dependent in these membranes and papain treatment did not affect the rate in the presence or absence of Na^+ .

These results were confirmed in a series of experiments where Na^+ -dependent alanine and α -methyl D-glucoside transport were measured on the same membrane samples treated or not with papain for between 30 and 40 min. The initial rate of transport of alanine (0.1 mM) was 1.11 ± 0.084 nmol/mg in 10 s (mean $\pm$ S.E.M. of 14 observations); after papain treatment this rate was reduced to 0.383 ± 0.035 nmol/mg ($n = 14$) ($P < 0.001$ by Student's t test). The initial rate of Na^+ -dependent α -methyl D-glucoside uptake was 0.396 ± 0.035 nmol/mg in 10 s (mean $\pm$ S.E.M. for 18 observations) and after papain treatment the corresponding value was 0.358 ± 0.022 nmol/mg ($n = 18$) (not significant). The effect of papain on alanine transport is specific since collapse of the Na^+ gradient should also affect the transport of α -methyl D-glucoside.

Figure 5 shows the concentration-dependence of Na^+ -dependent alanine transport in BBMVs preincubated for 30 min in the presence or absence of papain. Non-linear regression to the Michaelis–Menten equation indicated a K_m value for alanine of 2.5 ± 0.48 mM (mean $\pm$ S.E.M.) in good agreement with the value of 2.5–3.0 mM determined in previous work [1]. In papain-treated membranes, the K_m for alanine transport increased to 8.3 ± 1.2 mM but the V_{max} was essentially unchanged.

The Na^+ -dependent amino acid-transport system in BBMVs (System B^o) has a broad specificity and has been shown to transport alanine, glutamine, leucine and phenylalanine among other amino acids. The effect of papain treatment of BBMVs on

Figure 4 Effect of papain digestion of membrane vesicles on the uptake of alanine and α -methyl D-glucoside in the presence of NaCNS and KCNS

BBMVs were incubated with papain as in the legend to Figure 3. At various times, the uptake of alanine or α -methyl D-glucoside was measured over a 10 s time interval, in the presence of either NaCNS or KCNS. (a) 0.1 mM alanine; (b) 0.1 mM α -methyl D-glucoside. ●, NaCNS; ▲, KCNS.

Figure 5 Effect of papain digestion of BBMVs on the kinetics of Na⁺-dependent alanine transport

After incubation of the membranes for 30–40 min with or without papain as in Figure 4, alanine transport was measured in the presence of NaCNS or KCNS over a 10 s time period for each alanine concentration. The rate in the presence of KCNS was subtracted from that in the presence of NaCNS to give the Na⁺-dependent rate. The rate in the presence of KCNS was proportional to the alanine concentration over the concentration range used and was 0.84 nmol/10 s per mg at 1 mM alanine; this rate was unaffected by treatment of the membranes with papain. The mean $\pm$ S.E.M. of values from three separate experiments is shown for each concentration. ●, Control; ▲, after papain digestion.

Table 2 Effect of papain treatment on the kinetics of Na⁺-dependent alanine, glutamine, phenylalanine and leucine transport in BBMVs

The Na⁺-dependent uptake of amino acids at substrate concentrations in the range 0.1–10 mM was measured as described in the Materials and methods section in experiments similar to that in Figure 5. Initial rates were measured over a 10 s time interval and the experiment was performed between 30 and 40 min after addition of papain to the membranes. The mean of three separate determinations of the rate was taken for each concentration. The kinetic parameters (mean $\pm$ S.E.M.) quoted represent the best fit to the Michaelis–Menten equation using a non-linear regression program.

Substrate	No papain		+ Papain	
	K_m (mM)	V_{max} (nmol/10 s per mg)	K_m (mM)	V_{max} (nmol/10 s per mg)
Alanine	2.6 $\pm$ 0.5	24.4 $\pm$ 1.8	8.3 $\pm$ 1.2	25.4 $\pm$ 2.2
Glutamine	0.9 $\pm$ 0.2	11.5 $\pm$ 0.7	5.0 $\pm$ 1.0	14.5 $\pm$ 1.4
Phenylalanine	2.0 $\pm$ 0.2	12.3 $\pm$ 0.7	4.6 $\pm$ 0.8	15.6 $\pm$ 1.7
Leucine	0.7 $\pm$ 0.1	6.0 $\pm$ 0.5	1.4 $\pm$ 0.1	6.4 $\pm$ 0.3

the kinetic parameters of transport of these substrates was determined (Table 2). In each case the K_m was increased and the V_{max} for transport was unaffected. The increase in K_m for the hydrophobic amino acids phenylalanine and leucine was less than for the hydrophilic amino acids glutamine and alanine, and hence the inhibition by papain treatment at low concentrations of these hydrophobic amino acids was less marked.

Comparison of some properties of aminopeptidase N and Na⁺-dependent amino acid transport in BBMVs

A comparison of some kinetic properties of these two systems was conducted. Both aminopeptidase activity and Na⁺-dependent amino acid transport were found to be inhibited competitively by

Table 3 Inhibition of aminopeptidase N activity and of Na⁺-dependent amino acid transport in renal bovine brush-border membranes

The hydrolysis of alanine-*p*-nitroanilide was measured at two different concentrations in the presence of a range of concentrations of inhibitor. K_i values were obtained from Dixon plots. The Na⁺-dependent uptake of alanine by intact BBMVs was measured and the kinetics of inhibition were analysed as in [1]. Data indicated with an asterisk are taken from reference [1]. For both transport and hydrolysis measurements, 'no inhibition' is taken to mean less than 5% inhibition of the initial rate when the inhibitor amino acid is present at 100-fold concentration excess over the substrate.

Inhibitor	Characteristics of inhibition	
	Alanine- <i>p</i> -nitroanilide hydrolysis	Na ⁺ -dependent alanine transport
L-Norvaline	Competitive, K_i 1.92 mM	Competitive, K_i 0.22 mM
L-Leucine	Competitive, K_i 2.22 mM	Competitive, K_i 0.2 mM*
L-Phenylalanine	Competitive, K_i 0.77 mM	Competitive, K_i 1.3 mM*
L-Glutamine	Competitive, K_i 1.8 mM	Competitive, K_i 0.9 mM*
L-Alanine	Competitive, K_i 6 mM	—
D-Alanine	No inhibition	No inhibition*
D-Glutamine	No inhibition	No inhibition
Glycine	Competitive $K_i > 30$ mM	$K_i > 10$ mM
L-Lysine	Competitive $K_i > 20$ mM	No inhibition*
L-Aspartate	No inhibition	No inhibition*
2-(Methylamino)isobutyrate	No inhibition	No inhibition*
N-Phenyl maleimide	No inhibition at 5 mM	Half-maximal effect at 1 mM*
Bestatin	Half-maximal inhibition at 1 μ g/ml	No inhibition at 10 μ g/ml
Na ⁺ -free medium	No inhibition	Complete inhibition

free amino acids as determined by Dixon plot analysis (detailed results not shown).

The specificity of inhibition of alanine-*p*-nitroanilide hydrolysis and Na⁺-dependent alanine transport by a range of amino acids is shown in Table 3. There was a close similarity in the spectrum of amino acid inhibition of the two systems. As indicated by the K_i values, leucine, norvaline, phenylalanine and glutamine were the best inhibitors in each case. D-amino acids, glycine and the charged amino acids lysine and aspartate were ineffective inhibitors of both aminopeptidase activity and transport. 2-(Methylamino)isobutyrate, the specific substrate of amino acid-transport System A which is absent from brush-border membranes, inhibited neither process. Bestatin, a very effective inhibitor of aminopeptidase activity at micromolar concentrations, did not affect transport, whereas *N*-phenylmaleimide, which inhibits transport [1], did not affect aminopeptidase activity. In agreement with previous work of others, aminopeptidase activity in intact membranes was not affected by the presence or absence of Na⁺ ions. These observations indicate that the processes of peptide hydrolysis and amino acid transport are separate, but the specificities of recognition of free amino acids by the aminopeptidase and the transporter are similar.

DISCUSSION

We have shown previously [9] that removal of a specific protein from solubilized BBMVs using the monoclonal antibody FD19 results in greatly decreased Na⁺-dependent alanine-transport activity at low alanine concentrations in subsequently reconstituted proteoliposomes. The results presented in this paper

describing both enzyme activity and partial amino acid sequence establish the identity of this protein as aminopeptidase N. The high degree of similarity between the partial amino acid sequences obtained for the bovine kidney enzyme and those aminopeptidases characterized from other sources [14,17–19] indicate that the sequence of the enzyme is highly conserved.

The above finding indicates that aminopeptidase is functionally associated in some way with amino acid transport. Further evidence for this proposition is provided by the finding that removal of aminopeptidase N from intact membranes by papain treatment considerably increased the K_m of Na^+ -dependent amino acid transport without changing the V_{max} . Papain has been shown to remove relatively few proteins from brush-border membranes, with no gross effect on membrane permeability [22]. It is unlikely that the limiting concentrations of papain used cause a non-specific increase in Na^+ permeability since Na^+ -dependent α -methyl D-glucoside uptake was unaffected. Further, a change in the Na^+ electrochemical gradient might be expected to affect the V_{max} , rather than the K_m of transport; this has been shown to be the case for Na^+ -dependent amino acid transport in hepatocytes [29].

An alternative explanation of the effect of papain digestion on Na^+ -dependent amino acid transport is that papain acts directly on the carrier protein in such a way that the K_m for amino acids is increased whereas the V_{max} is unchanged. The available data do not rule out this possibility.

It appears unlikely that aminopeptidase N is the actual transport protein. There is no Na^+ requirement for enzyme activity or for the inhibition of enzyme activity by free amino acids. Reconstitution of aminopeptidase N produces proteoliposomes which associate with amino acids with the expected specificity, but with no Na^+ -dependence [9]. Removal of 90% of the aminopeptidase from the membranes with papain did not affect the V_{max} of transport. However, aminopeptidase N appears to regulate amino acid transport in that removal of this enzyme has a large effect on the rate of amino acid transport at low substrate concentrations.

Sufficient data are not at present available to allow the proposal of a detailed mechanism for the interaction between aminopeptidase and the transport protein. Aminopeptidase N and the transporter appear to recognize free amino acids with similar specificity. The broad-specificity amino acid-transport system has been identified only in kidney and intestine, tissues that also contain high aminopeptidase N activity. It is possible that the amino acid-binding sites of the aminopeptidase N and the transport system are in close proximity and that the transport system accepts amino acids from the aminopeptidase binding site more readily than from free solution. This 'channelling' effect could be driven by the energy-requiring removal of amino acids from the external transporter site across the membrane. The probable importance of such channelling of metabolites to the transport of proteins in the highly organized membrane structure of the kidney has been considered [30].

Recently a number of reports have appeared describing stimulation of Na^+ -dependent amino acid transport in *Xenopus* oocytes after injection of mRNA derived from single cDNA clones. The rBAT clone from rabbit kidney [31] encodes a 78 kDa protein which has a cytoplasmic N-terminus, single membrane-spanning domain and large putative extracellular domain which is highly glycosylated. When the cRNA was expressed in oocytes, a stimulation of the endogenous Na^+ -dependent transport System $\text{b}^{0,+}$ occurred. It was concluded that this protein was either a constitutive element or specific activator of System $\text{b}^{0,+}$. The isolation of a clone (D2) from rat kidney which had a high degree of sequence similarity to rBAT was

independently reported at the same time [32]. D2 was also suggested to encode a transport activator or regulatory subunit of System $\text{b}^{0,+}$. These two clones show sequence similarity to the NAA-Tr cDNA previously isolated by Tate et al. [33].

The rBAT protein (and also by analogy D2) shows amino acid sequence similarity to another membrane glycoprotein, the human and mouse 4F2 cell surface antigen heavy chain 4F2hc. Injection of *Xenopus* oocytes with cRNA from a clone of 4F2hc stimulated the endogenous Na^+ -dependent cation-preferring amino acid-transport activity (System y^+ -like) [34].

The isolation of clones encoding two similar proteins which affect the activities of two different amino acid-transport systems has suggested the existence of a class of glycoproteins that act as regulators or structural elements of amino acid-transport systems in eukaryotic cells [34]. These proteins are characterized by a single transmembrane region near the N-terminus with a cytoplasmic N-terminal tail and a large glycosylated extracellular domain. Aminopeptidase N also has these properties, and now has been shown to influence Na^+ -dependent amino acid transport in BBMV.

Final elucidation of the role of aminopeptidase N in amino acid transport should come from expression of the cDNA for the enzyme in a suitable system. It is of interest to speculate that one reason why a single cDNA clone for the renal amino acid transporter has not yet been isolated is that cDNA for both the transporter and aminopeptidase N may have to be expressed simultaneously for optimal rates of transport of amino acids to be observed. Whether similar considerations apply to the different Na^+ -dependent amino acid-transport systems in other tissues is at present an open question.

This work was funded by a grant from the Wellcome Trust. We thank Dr. A. J. Kenny for supplying a polyclonal antibody to pig aminopeptidase N.

REFERENCES

- Lynch, A. M. and McGivan, J. D. (1987) *Biochim. Biophys. Acta* **899**, 176–184
- Doyle, F. A. and McGivan, J. D. (1992) *Biochim. Biophys. Acta* **1104**, 55–62
- Stevens, B. R., Kaunitz, J. D. and Wright, E. M. (1984) *Annu. Rev. Biochem.* **46**, 417–433
- Christensen, H. N. (1990) *Physiol. Rev.* **70**, 43–77
- Koepsell, H., Korn, K., Ferguson, D., Menhur, H., Ollig, D. and Haase, W. (1984) *J. Biol. Chem.* **259**, 6548–6558
- Lynch, A. M. and McGivan, J. D. (1987) *Biochem. J.* **244**, 503–508
- Beliveau, R., Demeule, M., Jette, M. and Potier, M. (1990) *Biochem. J.* **268**, 195–200
- Coady, M. J., Pajor, A. M., Toloza, E. M. and Wright, E. M. (1990) *Arch. Biochem. Biophys.* **283**, 130–134
- Doyle, F. A. and McGivan, J. D. (1992) *Biochem. J.* **281**, 95–102
- Kenny, A. J. and Maroux, S. (1982) *Physiol. Rev.* **62**, 91–128
- Semenza, G. (1986) *Annu. Rev. Cell. Biol.* **2**, 255–313
- Turner, A. J., Hooper, N. M. and Kenny, A. J. (1987) in *Mammalian Ecto-enzymes* (Kenny, A. J. and Turner, A. J., eds.), pp. 211–248, Elsevier Scientific Publishing Co., Amsterdam
- Luzio, J. P., Baron, M. D. and Bailyes, E. M. (1987) in *Mammalian Ecto-enzymes* (Kenny, A. J. and Turner, A. J., eds.), pp. 111–137, Elsevier Scientific Publishing Co., Amsterdam
- Look, A. T., Ashmun, R. A., Shapiro, L. H. and Peiper, S. C. (1989) *J. Clin. Invest.* **83**, 1299–1307
- Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjöström, H., Noren, O. and Laude, H. (1992) *Nature (London)* **357**, 417–419
- Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellicchio, C. R., Shapiro, L. H., Look, T. A. and Holmes, K. V. (1992) *Nature (London)* **357**, 420–422
- Olsen, J., Cowell, G. M., Koenigshoefer, E., Danielsen, E. M., Møller, J., Laustsen, L., Hansen, O. C., Welinder, K. G., Engberg, J., Hunziker, W., Spiess, M., Sjöström, H. and Noren, O. (1988) *FEBS Lett.* **238**, 307–314
- Watt, V. M. and Yip, C. C. (1989) *J. Biol. Chem.* **264**, 5480–5487
- Olsen, J., Sjöström, H. and Noren, O. (1989) *FEBS Lett.* **251**, 275–281
- Wessels, H. P., Hansen, G. H., Fuhrer, C., Look, A. T., Sjöström, H., Noren, O. and Spiess, M. (1990) *J. Cell Biol.* **111**, 2923–2930

-
- 21 Ferraci, H., Maroux, S., Bonicel, J. and Desnuelle, P. (1982) *Biochim. Biophys. Acta* **684**, 133–136
- 22 Louvard, D., Maroux, S., Vannier, C. and Desnuelle, P. (1975) *Biochim. Biophys. Acta* **375**, 236–248
- 23 Kenny, A. J. and Booth, A. G. (1976) *Biochem. Soc. Trans.* **4**, 1011–1017
- 24 Biber, J., Steiger, B., Haase, W. and Murer, H. (1981) *Biochim. Biophys. Acta* **647**, 169–176
- 25 Hildreth, J. E. K. (1982) *Biochem. J.* **207**, 363–366
- 26 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–681
- 27 Towbin, H., Staehelin, T. and Gordon, J. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 28 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 29 Moule, S. K. and McGivan, J. D. (1990) *Biochim. Biophys. Acta* **1031**, 383–397
- 30 Lumsden, C. J. and Silverman, M. (1990) *Methods Enzymol.* **191**, 34–72
- 31 Bertran, J., Werner, A., Moore, M. L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M. and Murer, H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5601–5605
- 32 Wells, R. G. and Hediger, M. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5596–5600
- 33 Tate, S. S., Yan, N. and Udenfriend, S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1–5
- 34 Bertran, J., Maganin, S., Werner, A., Markovich, D., Biber, J., Testar, X., Zorzano, A., Kuhn, L. C., Palacin, M. and Murer, H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5606–5610
-