Glycyl-L-proline transport in rabbit enterocyte basolateral-membrane vesicles

Jane DYER,* R. Brian BEECHEY,* Jean-Pierre GORVEL,† Roy T. SMITH,* Raymond WOOTTON‡ and Soraya P. SHIRAZI-BEECHEY*§

*Department of Biochemistry, University College of Wales, Aberystwyth, Dyfed SY23 2DD, U.K.,

†Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, Marseille Cedex 9, France, and

‡Wellcome Research Laboratories, Beckenham, Kent BR3 3BS U.K.

The properties of a peptide-transport system in rabbit enterocyte basolateral membrane were examined with glycyl-L-proline as the substrate. Basolateral-membrane vesicles prepared from rabbit proximal intestine were characterized in terms of both purity and orientation. Marker-enzyme assays show that the basolateral-membrane marker, ouabain-sensitive K^+ -activated phosphatase, is enriched 17-fold with respect to the initial homogenate. The activities of enzymes used as markers for other membranes and organelles are low, and contamination of the final membrane fraction with these is minimal. The use of immunoblotting techniques further confirms the absence of brush-border-membrane contamination. Proteins in the basolateral-membrane vesicle preparation gave no cross-reaction with antibodies against the 140 kDa antigen and the Na^+ /glucose-symport protein, markers specific to the brush-border membrane of the enterocyte. Conversely, antibodies raised against the classical basolateral-membrane marker, the RLA class I histocompatibility complex, reacted strongly with a 43 kDa basolateral-membrane protein. The orientation of the basolateral-membrane vesicles was shown to be predominantly inside-out on determination by two independent criteria. The uptake of [^{14}C]glycyl-L-proline by these vesicles is stimulated by the presence of an inwardly directed pH gradient, and this stimulation can be abolished by the proton ionophores carbonyl cyanide *p*-trichloromethoxyphenylhydrazone (CCCP) and tetrachlorotrifluoromethylbenzimidazole (TTFB). Transport is also inhibited by $HgCl_2$, thimerosal, Na^+ and other glycyl dipeptides.

INTRODUCTION

The transcellular route for the uptake of solutes in intestinal epithelia involves transport across the brush-border membrane into the cell, movement through the cell, and extrusion across the basolateral membrane into the bloodstream. The study of the mechanisms involved in these processes has been greatly facilitated by the development of methods for the isolation of both the brush-border and basolateral membranes of the cell [1,2].

It is now widely accepted, on the basis of work using vesicles, that the transport of di- and tri-peptides across the brush-border membrane of the intestinal epithelial cells is facilitated by a specific carrier system, distinct from those for amino acids, and is driven by an inward H^+ gradient ($pH_o < pH_i$) [3–6].

The transcellular flux of peptides has only been studied with whole-tissue preparations [7–9]. By using these techniques it is not possible to establish whether these peptides are hydrolysed within the cells, such that only the constituent amino acids pass through the basolateral membrane and enter the bloodstream. There have been no direct studies on the transport of peptides across the basolateral membrane.

In the present paper, we demonstrate that a carrier for glycyl-L-proline (Gly-Pro) on the basolateral membrane of the enterocyte does exist.

EXPERIMENTAL

Materials

D-[U- ^{14}C]Glucose (sp. radioactivity 10.0 GBq/mmol), L-[U- ^{14}C]glucose (sp. radioactivity 2.07 GBq/mmol), [^{14}C]glycyl-L-

proline ([^{14}C]Gly-Pro; sp. radioactivity 236.8 MBq/mmol), [U- ^{14}C]glycine (sp. radioactivity 4.0 GBq/mmol) and L-[U- ^{14}C]proline (sp. radioactivity 10.5 GBq/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. The dipeptides, amino acids and cephradine were supplied by Sigma. The membrane filters (Millipore GSWP02500) were obtained from Millipore Products Division, U.K. All other chemicals were of the highest analytical grade commercially available.

Preparation of basolateral-membrane vesicles

The method of Shirazi-Beechey *et al.* [10], was modified by the incorporation of a cation precipitation step [11] to remove brush-border-membrane contamination. New Zealand White rabbits (8–12 weeks old) were used. Sections of intestine were removed, everted, and placed in ice-cold 0.9% (w/v) NaCl. All steps in the procedure were carried out at 0–4 °C. The sections were blotted with absorbent paper to remove excess mucus. The mucosa was then scraped off and weighed before being suspended in a solution which contained 12.5 mM-NaCl, 1 mM-Tris/HCl, pH 8.0, 0.2 mM-phenylmethanesulphonyl fluoride, 0.5 mM-dithiothreitol and 0.2 mM-benzamidine. The suspension (1 g of tissue/10 ml of buffer) was then homogenized (Ystral Polytron homogenizer at setting 5) for 1 min. The homogenate was filtered through cotton gauze to remove foam and mucus before being diluted 10-fold with the suspension buffer. The subcellular-fractionation scheme is as follows. The homogenate was centrifuged at 500 *g* for 10 min to remove nuclei and other large particles of cellular material. The supernatant, S_1 , was centrifuged at 20000 *g* for 15 min. The crude membrane fraction, pellet P_2 , was then resuspended in 20 ml of buffer A (100 mM-mannitol/2 mM-Hepes/Tris, pH 7.1) and homogenized with the

Abbreviations used: CCCP, carbonyl cyanide *p*-trichloromethoxyphenylhydrazone; Gly-Pro, glycyl-L-proline; TTFB, tetrachlorotrifluoromethylbenzimidazole.

§ To whom all correspondence should be addressed.

Polytron, with two bursts of 30 s at setting 4. The volume was adjusted to 40 ml with buffer A. Then 2.5 M-MgCl₂ was added to the stirred diluted homogenate to a final concentration of 8 mM. The suspension was stirred on ice for 20 min. The precipitated membranes were sedimented by centrifugation (2000 *g* for 10 min), yielding a pellet. This pellet was washed by resuspension in 40 ml of buffer A and centrifuged at 20000 *g* for 20 min. The pellet was then resuspended in 9 ml of 250 mM-sorbitol/20 mM-Hepes/Tris, pH 7.5, and homogenized with 100 strokes in a Dounce tissue grinder with a loose-fitting pestle. Samples (1.5 ml) from the membrane suspension were layered on a 15 ml linear sorbitol gradient (35–55% w/v, in 20 mM-Hepes/Tris buffer, pH 7.5) and centrifuged in a swing-out rotor at 100000 *g*_{av} for 1 h.

A discrete white band near the top of the gradient that was enriched in the basolateral-membrane marker, ouabain-sensitive K⁺-activated phosphatase, was collected with a syringe and resuspended in 14 ml of buffer B, which contained 300 mM-mannitol, 20 mM-Hepes/Tris, pH 7.5, 0.1 mM-MgSO₄ and 0.02% (w/v) NaN₃, and recovered by centrifugation at 100000 *g* for 30 min. The membranes were then resuspended in approx. 0.3 ml of buffer B, by passing through a 27-gauge needle several times. The suspension was divided into 50 µl batches, and stored at liquid-N₂ temperatures until use. The D-glucose uptake results obtained with freshly prepared basolateral-membrane vesicles were the same as those for vesicles kept in liquid N₂ for 8 weeks. This contrasts with vesicles prepared from rat intestine, which tissue is susceptible to anoxia, and the vesicles cannot be stored in liquid N₂ [12].

Basolateral-membrane vesicles could also be prepared from mucosal scrapings which had been frozen in liquid N₂ directly after preparation and stored subsequently at -80 °C. The results of D-glucose and peptide transport obtained from vesicles prepared from both fresh and frozen mucosal scrapings were similar.

Enzyme assays

All enzymes were assayed at 37 °C, except sucrase, which was assayed at 25 °C.

Ouabain-sensitive K⁺-activated phosphatase was assayed by the method of Colas & Maroux [13], the reaction time being 10 min; aminopeptidase N activity was determined by the method of Feracci & Maroux [14], and sucrase was measured by the method of Dahlqvist [15]; α-mannosidase activity was assessed by the method of Tulsiani *et al.* [16]; Tris-resistant α-glucosidase was assayed by the method of Peters [17], and the activity of succinate dehydrogenase was measured by the method of Pennington [18].

Protein was estimated by its ability to bind Coomassie Blue, by using a commercial kit (Bio-Rad).

Polyacrylamide-gel electrophoresis and immunoblotting

Separation of protein was performed in slab gels (0.75 mm thick, 5 cm long) made of 7.5% (w/v) polyacrylamide gel, containing 0.1% SDS, with a 5% (w/v) stacking gel [19].

The immunoblotting technique was performed as described by Moktari *et al.* [19]. Mouse monoclonal anti-(140 kDa antigen) antibody (23B921) [20] was used to detect brush-border membranes. Rat anti-RLA class I histocompatibility antigen was used to detect basolateral membranes [19]. In all experiments a second antibody coupled to peroxidase was used for visual detection of the antigens.

Orientation of vesicles

The orientation of the vesicles was investigated by measuring (a) the latency of ouabain-sensitive K⁺-activated phosphatase

and (b) the inactivation of DNAase 1 by actin. To permeabilize sealed vesicles, the technique of Forbush [21] was used.

Latency of K⁺-activated phosphatase. Vesicles were preincubated for 10 min at 22 °C in a solution containing 1% (w/v) BSA, 20 mM-Hepes/Tris, pH 7.0, and 0–1.0 mg of SDS/ml before being assayed for ouabain-sensitive K⁺-activated phosphatase. The enzyme activity was assayed over a range of detergent concentrations in order to determine the maximum potential activity. The latency was measured as the ratio of the activity with SDS to that without SDS.

Distribution of actin on plasma membranes. Actin is a ubiquitous protein found on the inner surface of cell membranes [22]. DNAase 1 activity is inhibited in the presence of actin. The enzyme was therefore used as a non-penetrating probe [23], in the presence and absence of detergent to permeabilize the vesicles. The assay was performed as described in ref. [23], with 1 µg of DNAase 1 per assay. Fish milt DNA was the substrate. The sidedness of the membrane vesicle preparation was measured as the ratio of the activity in the absence of detergent to that in the presence of detergent.

Transport studies

Uptake of D- and L-glucose. The transport of D-glucose and L-glucose into basolateral-membrane vesicles prepared from rabbit intestine was studied at 25 °C and pH 7.4 by the filtration-stop technique [24]. The assay was begun by addition of 100 µl of a reaction medium containing 300 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM-MgSO₄, 0.02% NaN₃ and 1.0 mM-glucose (either D or L), to 5 µl of vesicles (100 µg of protein). At the appropriate time 1 ml of a stop buffer (300 mM-mannitol, 0.1 mM-HgCl₂, 20 mM-Hepes/Tris, pH 7.4, 0.02% NaN₃) was added. A 0.9 ml sample was taken from each tube and filtered rapidly through a 0.22 µm-pore nitrocellulose filter under vacuum and washed with 5 × 1 ml of stop solution. The residual radioactivity on the filter was then measured.

Uptake of [1-¹⁴C]Gly-Pro into basolateral-membrane vesicles. This was determined at 25 °C in the presence of an inwardly directed H⁺ gradient (pH_i = 7.5; pH_o = 5.5) and in the absence of Na⁺. The assay was begun by the addition of 100 µl of an incubation medium containing 300 mM-mannitol, 20 mM-Mes/Tris, pH 5.5, 0.1 mM-MgSO₄, 0.02% NaN₃ and 10 mM-[1-¹⁴C]Gly-Pro to 5 µl of vesicles (100 µg of protein). In some experiments NaN₃ was omitted from the intravesicular and incubation media. At the appropriate time 1 ml of an ice-cold stop buffer (300 mM-mannitol, 0.1 mM-HgCl₂, 20 mM-Hepes/Tris, pH 7.5, 0.1 mM-MgSO₄, 0.02% NaN₃) was added. A 0.9 ml sample was taken from each tube and filtered through a prewetted 0.22 µm-pore nitrocellulose filter (Millipore GSWP02500) under vacuum and washed with 5 × 1 ml of stop solution. The filters were then dissolved in 4 ml of scintillation fluid (Ecoscint) and the residual radioactivity was then measured.

Radiochemical analysis of Gly-Pro by h.p.l.c.

This was done with a Pye-Unicam PU 4003 controller and pump linked to a Ramona D-Raytest radioactivity detector. Separation was carried out on a Techsil-5-sil analytical column by isocratic elution with butan-1-ol/acetic acid/water (13:3:5, by vol.) at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Characteristics of the basolateral-membrane preparations

The homogeneity of the basolateral membranes isolated from

Table 1. Activities of marker enzymes in the basolateral-membrane fraction

Specific activities are means $\pm$ S.D. (numbers of preparations assayed are shown in parentheses). Enrichment = ratio of the specific activity of the basolateral membrane fraction to that of the homogenate. Recovery = total activity of the basolateral-membrane fraction as a percentage of the total activity in the homogenate. Abbreviations: BLM, basolateral membrane; BBM, brush-border membrane; ER, endoplasmic reticulum.

Enzyme	Marker for	Specific activity (nmol/min per mg of protein)	Enrichment	Recovery (%)
Sucrase	BBM	8.8 ± 5.0 (5)	0.4	0.15
Aminopeptidase N	BBM	20.0 ± 17.5 (6)	0.6	0.05
α -Mannosidase	Golgi	11.3 ± 3.8 (6)	2.7	0.34
α -Glucosidase (Tris-resistant)	ER	7.2 ± 7.0 (6)	2.7	0.81
Succinate dehydrogenase	Mitochondria	Not detected	—	—
K ⁺ -activated phosphatase	BLM	22.0 ± 7.5 (7)	17	15.0

rabbit intestine was assessed by two independent methods: (1) the activities of enzymes specific to the basolateral membrane and to other potential contaminating membranes and organelles were assessed; (2) the presence of proteins characteristic of the basolateral and brush-border membranes was detected with specific antibodies.

Activities of marker enzymes in the basolateral-membrane preparation. The data in Table 1 show that the recoveries of the enzymes characteristic of the brush-border, mitochondrial, Golgi and endoplasmic-reticulum membranes were low. The activities of both sucrase and aminopeptidase N, markers of the brush-border membrane, were decreased in the final basolateral-membrane vesicle preparation to values below that of the original homogenate. There was a low, but significant, enrichment of 2.7-fold for both the marker enzyme of the Golgi, α -mannosidase, and that of the endoplasmic reticulum, Tris-resistant α -glucosidase. Both of these subcellular organelles have membranes of a rather similar density to those of the basolateral membrane [25]. The recovery of all these enzymes is low (0.05–0.81%). Succinate dehydrogenase activity was undetectable in these vesicles. In contrast, ouabain-sensitive K⁺-activated phosphatase activity, a marker of the basolateral membrane, was enriched 17-fold in the final fraction with respect to the homogenate. Values previously reported for the enrichment of ouabain-sensitive K⁺-activated phosphatase in basolateral membrane vesicles prepared from rat intestine are in the range 10–17-fold [13,25–27] and 10-fold for vesicles from rabbit small intestine [10]. On the basis of these results, we conclude that a high proportion of the basolateral membranes present in the original homogenate are concentrated in the final preparation.

Presence of immunoreactive proteins in the basolateral-membrane preparations. The 140 kDa antigen is specifically located on the brush-order membrane. It is absent from basolateral membranes [19,25,26]. The 75 kDa Na⁺/glucose symporter is also specific to the brush-border membrane [28,29]. The major histocompatibility antigen (anti-RLA Class I) is uniquely located on the basolateral membrane [19,25]. The availability of antibodies to these proteins enabled (1) an independent assessment of the presence of brush-border membranes in the final preparation of basolateral membranes and (2) an unequivocal recognition of the final membrane preparation being basolateral in origin.

The protein components of the brush-border and basolateral-membrane preparations were separated on a 7.5% polyacrylamide gel containing 0.1% SDS. After electrotransfer on to cellulose nitrate sheets, the potential of the proteins to interact with the monoclonal antibody 23B921 raised against the 140 kDa antigen [19], the polyclonal antibodies raised against the major

Fig. 1. Immunodetection of the basolateral-membrane marker (RLA class I histocompatibility antigen) and of the brush-border-membrane marker (140 kDa antigen) in brush-border and basolateral-membrane fractions

(a) Coomassie Blue staining; (b) immunoblotting using monoclonal antibody 23B921 directed against the 140 kDa antigen (0.03 mg/ml); (c) immunoblotting using antiserum anti-RLA class I antigen (1/100 dilution). Total amounts of proteins in tracks (1), brush-border membranes, and (2), basolateral membranes, were 0.02 mg and 0.02 mg in (a) 0.01 mg and 0.05 mg in (b), and 0.05 mg and 0.05 mg in (c) respectively.

histocompatibility antigen (anti-RLA class I) [30,31] and polyclonal antibodies raised against the Na⁺/glucose symporter [28,29] was assessed. As shown in Fig. 1, by using the 23B921 antibody it was not possible to detect the 140 kDa antigen in the basolateral-membrane fraction, even when the sample loading was increased 4-fold. Control experiments showed that the anti-(140 kDa antigen) antibody reacted strongly with a 140 kDa protein in a brush-border membrane fraction.

Blotting with the anti-RLA class I antibody revealed a band migrating with a molecular mass of 43 kDa in the basolateral-membrane fraction. This antibody did not react with any protein in the brush-border membrane vesicles. Samples of the basolateral-membrane vesicle preparation blotted with antibodies against the brush-border Na⁺/glucose transporter

Table 2. Determination of sidedness of basolateral-membrane vesicles (BLMV) by quantification of actin exposure by DNAase 1 inactivation

Vesicles (120 µg of protein) were incubated with 1 µg of DNAase 1 in the absence or the presence of the indicated detergent, for 15 min on ice. The reaction was started by addition of 1 ml of DNA solution at a concentration giving an A_{260} of 0.8. The hydrolysis was monitored by measuring the increase in A_{260} ; 100% DNAase 1 activity represents an A_{260} change of 0.055/min. Results are means $\pm$ S.D. ($n = 5$).

Additions	DNAase activity (%)
None	100
0.1% Triton X-100	96 $\pm$ 2.4
SDS (0.2 mg/ml) + 1% BSA	102 $\pm$ 4.5
BLMV	62 $\pm$ 0.9
BLMV + 0.1% Triton X-100	58 $\pm$ 1.3
BLMV + SDS (0.2 mg/ml) + 1% BSA	59 $\pm$ 2.7

[28,29] did not show any cross-reaction. These results indicate that the basolateral-membrane fraction is devoid of detectable brush-border membrane contamination and bears a classical basolateral-membrane marker, the RLA class I histocompatibility antigen.

Orientation of basolateral-membrane vesicles

The sidedness of the basolateral-membrane vesicles was explored by determining the latency of the ouabain-sensitive K^+ -activated phosphatase and the asymmetric distribution of actin in the membranes [22].

Latency of the ouabain-sensitive K^+ -activated phosphatase. The activity of this enzyme was measured in the presence of increasing concentrations of SDS. As the SDS concentration was increased from 0 to 0.2 mg/ml there was a slight increase in the average activity, but the variation within the assay was such that it was not statistically significant. At concentrations greater than 0.2 mg/ml there was a progressive decrease in the activity.

DNAase 1 inactivation. In this experiment the vesicles were incubated, with and without either 0.1% Triton X-100 or 0.2 mg of SDS/ml, for 15 min before addition of substrate and determination of the inhibition of DNAase. The results are presented in Table 2; the vesicles cause a marked inhibition of the DNAase activity. This inhibition is not increased by preincubation of the vesicles with either detergent, a procedure which allows the DNA and the enzyme access to the interior of the vesicle [23]. From the results of these experiments, the basolateral-membrane vesicles appear to be at least 90% inside-out.

Functional characteristics of the vesicles

Transport of D- and L-glucose. The data in Table 3 show that the stereoisomers, D- and L-glucose, are transported in a Na^+ -independent stereospecific manner, in accordance with previously reported results [10,32]. In the presence of Na^+ , no overshoot was observed. This further confirms the conclusion that the basolateral-membrane vesicle preparation is free of contamination from brush-border membranes.

Transport of Gly-Pro. Uptake of Gly-Pro into basolateral-membrane vesicles isolated from rabbit proximal intestine was studied in the presence of an inward-directed H^+ gradient ($pH_i = 7.5$; $pH_o = 5.5$). The results are presented in Fig. 2. The uptake of Gly-Pro is stimulated by the presence of an inwardly

Table 3. Glucose transport by basolateral-membrane vesicles

Transport of D- and L-glucose into basolateral-membrane vesicles was studied at 25 °C and pH 7.5. To 5 µl of vesicles (100 µg of protein) was added 100 µl of reaction medium containing 300 mM-mannitol, 0.1 mM- $MgSO_4$, 0.02% NaN_3 , 20 mM-Hepes/Tris, pH 7.5, and 1 mM-glucose (D-[U- ^{14}C]glucose or L-[1- ^{14}C]glucose, 147 GBq/mol). At the appropriate time 1.0 ml of ice-cold stop solution, containing 300 mM-mannitol, 0.1 mM- $MgSO_4$, 0.02% NaN_3 , 20 mM-Hepes/Tris, pH 7.5, and 0.1 mM- $HgCl_2$, was added. A 0.9 ml sample from each tube was filtered through a 0.22 µm-pore Millipore cellulose acetate/nitrate filter, under vacuum, and the filter washed with 5 × 1 ml of stop solution. The residual radioactivity on the filter was measured. The equilibrium uptake was measured after 30 min. Results are means $\pm$ S.D. ($n = 4$).

Glucose	Initial rate of glucose uptake (pmol/s per mg of protein)	Equilibrium uptake of glucose (pmol/mg of protein)
D	32 $\pm$ 1.6	619 $\pm$ 2.7
L	17 $\pm$ 0.3	531 $\pm$ 7.3

Fig. 2. Time course of the uptake of Gly-Pro by basolateral membrane vesicles in the presence and absence of an inward H^+ gradient

Uptake of Gly-Pro was studied at 25 °C as described in the Experimental section. Vesicles were incubated in a medium containing 300 mM-mannitol, 10 mM-Gly-Pro, 0.1 mM- $MgSO_4$, 0.02% NaN_3 and either 20 mM-Hepes/Tris, pH 7.5 (●), or 20 mM-Mes/Tris, pH 5.5 (○). At the appropriate time, uptake was stopped by addition of 1 ml of ice-cold stop solution, containing 300 mM-mannitol, 20 mM-Hepes/Tris, pH 7.5, 0.1 mM- $MgSO_4$ and 0.02% NaN_3 . A 0.9 ml sample from each tube was taken immediately, filtered through a 0.22 µm-pore nitrocellulose filter, washed with 5 × 1 ml of stop solution, and then the filter was dissolved in 4 ml of scintillation fluid. The residual radioactivity on the filter was measured. Each point represents the mean $\pm$ S.D. for four assays.

directed H^+ gradient, and the time course of this shows no overshoot. As shown in Table 4, the proton ionophores CCCP and TTFB inhibited the H^+ -stimulated uptake of Gly-Pro into the basolateral-membrane vesicles. Uptake was also inhibited by $HgCl_2$, thimerosal and Na^+ . The inhibition by high concentrations of Na^+ has also been observed in the uptake of glucose by basolateral-membrane vesicles [10]. The presence or absence of NaN_3 in the assay did not affect the measured rates.

Table 4. Effects of composition of the external incubation medium on transport of Gly-Pro, glycine and proline

The vesicles were incubated at 25 °C for 10 s in an incubation medium containing 10 mM-Gly-Pro, 1 mM-glycine or 1 mM-proline, 0.1 mM-MgSO₄, 0.02 % NaN₃ and either 20 mM-Hepes/Tris, pH 7.5, or 20 mM-Mes/Tris, pH 5.5, the indicated additions and mannitol to bring the osmolality to 320 mosm. Transport assays were carried out as described in the Experimental section. Each assay was done in duplicate. Values represent means $\pm$ S.D. for three separate experiments.

Substrate	Conditions		Additions	Initial rate of uptake (pmol/s per mg of protein)
	pH _o	pH _i		
10 mM-Gly-Pro	7.5	7.5	None	13.2 $\pm$ 0.2
	7.5	5.5	None	38.2 $\pm$ 0.8
	7.5	5.5	1 mM-HgCl ₂	4.2 $\pm$ 0.9
	7.5	5.5	1 mM-CCCP	6.3 $\pm$ 1.2
	7.5	5.5	1 mM-TTFB	11.5 $\pm$ 4.0
	7.5	5.5	100 mM-NaSCN	8.1 $\pm$ 3.9
	7.5	5.5	100 mM-sodium gluconate	7.5 $\pm$ 1.4
	7.5	5.5	2 % (v/v) ethanol	40.1 $\pm$ 2.4
1 mM-Glycine	7.5	7.5	None	24.6 $\pm$ 5.2
	7.5	5.5	None	15.7 $\pm$ 5.3
1 mM-L-Proline	7.5	7.5	None	7.8 $\pm$ 2.3
	7.5	5.5	None	7.6 $\pm$ 1.0

Fig. 3. Kinetics of Gly-Pro uptake

The dependence of initial rates of Gly-Pro uptake on Gly-Pro concentration was studied over the range 0.025–10 mM in basolateral-membrane vesicles prepared from rabbit proximal intestine. Initial rates were measured with 10 s incubations under standard conditions, and were calculated by subtracting the uptake in the absence of a H⁺ gradient from those with an inward-directed H⁺ gradient (pH_i = 7.5; pH_o = 5.5). The data are presented as a Michaelis-Menten curve and (inset) as a Hanes-Woolf plot (s/v against s). Each datum point represents the mean $\pm$ S.D. for three determinations.

Uptake of the constituent amino acids, glycine and L-proline, by the basolateral-membrane vesicles was by the Na⁺-independent facilitated-diffusion mechanisms as reported by other workers [33,34]. The uptake did not respond to the presence of a H⁺ gradient, and is at rates lower than those recorded for the movement of Gly-Pro, suggesting that the dipeptide was being transported by a distinct mechanism.

The mechanism of transport for Gly-Pro across the basolateral membrane is similar to that reported for brush-border membrane vesicles [3–6]. The possibility of the peptide uptake being due to

the presence of contaminating brush-border membrane was dismissed by the results of the marker-enzyme assays and immunoblotting experiments.

Kinetics of Gly-Pro uptake

The dependence of the initial rates of Gly-Pro uptake on Gly-Pro concentration was studied over the concentration range 0.025–20 mM in basolateral-membrane vesicles prepared from jejunum. The initial rates of uptake were measured with 10 s incubations in both the presence and the absence of an inwardly directed H⁺ gradient. Values were calculated from the H⁺-gradient-driven uptake. The uptake of Gly-Pro was saturable and conformed to Michaelis-Menten kinetics. The results are presented in Fig. 3. The K_m and V_{max} were calculated by linear-regression analysis from the Hanes-Woolf plot. In the concentration range covered, the Hanes-Woolf plot was linear, suggesting the presence of a single carrier-mediated transport system with a calculated K_m value of 2.0 ± 0.2 (4) mM and a V_{max} of 0.1 ± 0.03 (4) nmol/s per mg of protein.

Gly-Pro uptake as a function of osmolality

It is possible that the Gly-Pro associated with the membrane vesicles is due to binding rather than movement into the intravesicular space. If the Gly-Pro is moving into the vesicles, the amount taken in will depend on the volume of the vesicle. The volume of the vesicles was changed by varying the osmolality of the external medium. The uptake of Gly-Pro at 60 min as a function of external-medium osmolality is shown in Fig. 4. The uptake of the dipeptide decreased with an increase in medium osmolality, and extrapolation to infinite osmolality, i.e. zero volume, indicated that no Gly-Pro was bound to the vesicles. The results show the uptake of the Gly-Pro into an osmotically reactive intravesicular space with no binding to the surface of the vesicles.

Effect of dipeptides and free amino acids on Gly-Pro uptake

The effect of 10 mM dipeptides and free amino acids on the uptake of 1 mM-Gly-Pro was investigated. A range of potentially competitive inhibitory glycyl-dipeptides was chosen to contain C-terminal amino acids from each of the groups. The effect of the constituent amino acids, glycine and proline, and the cephalosporin antibiotic, cephradine, on Gly-Pro uptake was

Fig. 4. Effect of osmolarity of the external medium on the uptake of Gly-Pro by rabbit jejunal basolateral-membrane vesicles

Basolateral-membrane vesicles were suspended in 300 mM-mannitol/20 mM-Hepes/Tris (pH 7.5)/0.1 mM-MgSO₄/0.02% NaN₃. Vesicles were incubated for 60 min at 25 °C in media containing 10 mM-Gly-Pro, 20 mM-Mes/Tris, pH 5.5, 0.1 mM-MgSO₄, 0.02% NaN₃ and mannitol in concentrations to give the indicated osmolarity. Correlation coefficient = 0.984.

Table 5. Effect of dipeptides and free amino acids on Gly-Pro uptake

Basolateral membrane vesicles were incubated at 25 °C for 10 s in an incubation medium containing 1 mM-Gly-Pro, 300 mM-mannitol, 20 mM-Mes/Tris, pH 5.5, 0.1 mM-MgSO₄, 0.02% NaN₃ and 10 mM of the indicated dipeptide, amino acid or cephalosporin antibiotic. Transport assays were carried out as described in the Experimental section. Values represent means for three determinations. The uptake of 1 mM-Gly-Pro in the absence of an inhibitor was taken to represent 100%.

Competitor	Inhibition (%)
Glycyl-L-histidine	95
Cephadrine	62
Carnosine	57
Glycylglycine	55
Glycyl-L-phenylalanine	49
Glycine	39
L-Proline	26
Glycyl-L-glutamate	0

also investigated. The results are presented in Table 5. Of the compounds tested, glycyl-L-histidine gave the greatest degree of inhibition, and glycyl-L-glutamate the least. This suggests that

the presence of a positive charge enhances the inhibitory ability of the compound.

Chemical structure of the transported radiolabel

It is possible that the measured uptake of radiolabel was due to the hydrolysis of Gly-Pro before transport of the constituent amino acids. H.p.l.c. analysis of the Gly-Pro showed that there was no free glycine present. The potential enzymic hydrolysis of the dipeptide before transport of the radiolabel was assessed. Vesicles (100 µg of protein) were incubated for periods up to 60 min with Gly-Pro under the standard conditions used for the assay of transport. The vesicles were then separated from the suspension medium by filtration, and the filtrate was collected. The vesicles on the filter were disrupted by placing the filter in 500 µl of 0.1% Triton X-100. The radiolabelled materials in the filtrate and the detergent solution were then analysed by h.p.l.c., and the recorded retention times of the radiolabelled material were identical with that of the authentic Gly-Pro. This indicates that the Gly-Pro was not hydrolysed and was transported intact by the basolateral membrane vesicles.

Our results demonstrate that a molecule capable of transporting Gly-Pro does exist on the basolateral membrane of the rabbit enterocyte. It is therefore feasible that small peptides can be absorbed in the intestine by a transcellular route.

We are grateful to Professor E. M. Wright for the use of antibodies to the enterocyte Na⁺/glucose symporter.

REFERENCES

- Hopfer, U., Nelson, K., Perotto, J. & Isselbach, K. T. (1973) *J. Biol. Chem.* **248**, 25–32
- Mircheff, A. D., van Os, C. H. & Wright, E. M. (1980) *J. Membr. Biol.* **52**, 83–92
- Ganapathy, V., Mendicino, J. F. & Leibach, F. H. (1981) *Life Sci.* **29**, 2451–2457
- Ganapathy, V. & Leibach, F. H. (1983) *J. Biol. Chem.* **258**, 14189–14192
- Himukai, M., Kameyama, A. & Hoshi, T. (1983) *Biochim. Biophys. Acta* **723**, 659–667
- Ganapathy, V., Burckhardt, G. & Leibach, F. H. (1984) *J. Biol. Chem.* **259**, 8954–8959
- Rubino, A., Field, M. & Schwachman, H. (1971) *J. Biol. Chem.* **246**, 3542–3548
- Matthews, D. M. & Payne, J. W. (1980) *Curr. Top. Membr. Transp.* **14**, 331–425
- Cheeseman, C. I. (1980) *Can. J. Physiol. Pharmacol.* **58**, 1326–1333
- Shirazi-Beechey, S. P., De Jong, M. D., van Os, C. H. & Beechey, R. B. (1987) *Biochem. Soc. Trans.* **15**, 539–540
- Biber, J., Steiger, B., Haase, W. & Murer, H. (1981) *Biochim. Biophys. Acta* **647**, 169–176
- Fell, B. F. (1961) *J. Pathol. Bacteriol.* **81**, 251–254
- Colas, B. & Maroux, S. (1980) *Biochim. Biophys. Acta* **600**, 406–420
- Feracci, H. & Maroux, S. (1980) *Biochim. Biophys. Acta* **599**, 448–463
- Dahlqvist, A. (1964) *Anal. Biochem.* **7**, 18–25
- Tulsiani, D. R. P., Opheim, D. J. & Touster, O. (1977) *J. Biol. Chem.* **252**, 3227–3233
- Peters, T. J. (1976) *Clin. Sci. Mol. Med.* **51**, 557–574
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
- Moktari, S., Feracci, H., Gorvel, J.-P., Mishal, Z. & Maroux, S. (1986) *J. Membr. Biol.* **89**, 53–64
- Gorvel, J.-P., Rigal, A., Olive, D., Mawas, C. & Maroux, S. (1986) *Biol. Cell* **56**, 121–126
- Forbush, B., III (1983) *Anal. Biochem.* **128**, 159–163
- Cohen, C. M., Jackson, P. L. & Branton, D. (1978) *J. Supramol. Struct.* **9**, 113–124
- Grinstein, S. & Cohen, S. (1983) *Anal. Biochem.* **130**, 151–157
- Shirazi-Beechey, S. P., Gorvel, J.-P. & Beechey, R. B. (1988) *J. Bioenerg. Biomembr.* **20**, 273–288

25. Ahnen, D. J., Mircheff, A. K., Santiago, N. A., Yoshioka, C. & Gray, G. A. (1982) *J. Biol. Chem.* **257**, 12129–12135
26. Nguyen, T. D., Broyart, J.-P., Ngu, K. T., Illecas, A., Mircheff, A. K. & Gray, G. M. (1987) *J. Membr. Biol.* **98**, 197–205
27. Mircheff, A. K. & Wright, E. M. (1976) *J. Membr. Biol.* **28**, 309–333
28. Hediger, M. A., Coady, M. J., Ikeda, T. S. & Wright, E. M. (1987) *Nature (London)* **330**, 379–381
29. Hediger, M. A., Ikeda, T. S., Coady, M. A., Gunderson, C. B. & Wright, E. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2634–2637
30. Feracci, H., Rigal, A. & Maroux, S. (1986) *J. Membr. Biol.* **89**, 53–63
31. Massey, D., Feracci, H., Gorvel, J.-P., Rigal, A., Soulie, J. M. & Maroux, S. (1987) *J. Membr. Biol.* **96**, 19–25
32. Wright, E. M., van Os, C. H. & Mircheff, A. K. (1980) *Biochim. Biophys. Acta* **595**, 112–124
33. Stevens, B. R., Kaunitz, J. D. & Wright, E. M. (1984) *Annu. Rev. Physiol.* **46**, 417–433
34. Davies, S., Maenz, D. D. & Cheeseman, C. I. (1987) *Biochim. Biophys. Acta* **896**, 247–255

Received 25 January 1990/9 April 1990; accepted 18 April 1990