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TRANSPORT OF 5-OXOPROLINE INTO RABBIT RENAL BRUSH BORDER MEMBRANE VESICLES

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5-Oxoproline has been shown to be transported intact into rabbit renal brush border membrane vesicles by a Na^+-gradient dependent mechanism. The transport occurs against a concentration gradient. The equilibrium transport of 5-oxoproline decreases with increasing medium osmolarity. The predominant portion of transport occurs into an osmotically responsive intravesicular space, the non-specific surface binding being negligible. Competition experiments show that transport of 5-oxoproline is inhibited by most of the neutral amino acids whereas acidic, basic and imino acids do not have any effect.

Introduction:

5-Oxoproline, also known as pyrrolidone carboxylic acid or pyroglutamic acid is a cyclic amino acid present in free form in all animal tissues and also at the N-terminus of a number of naturally occurring, biologically active peptides and proteins. The metabolic formation and utilization of 5-oxoproline in the γ-glutamyl cycle have been reviewed (1). This amino acid is produced in free form independently by the action of at least three enzymes - γ-glutamyl cyclotransferase (1), pyrrolidonyl carboxypeptidase (2,3) and γ-glutamylamine cyclotransferase (4). 5-oxoproline as the N-terminal residue of proteins is most likely formed late in protein synthesis by cyclization of N-terminal glutamine (5). Utilization of 5-oxoproline is initiated by the action of 5-oxoprolinase which converts 5-oxoproline to glutamic acid (6).

5-Oxoproline is present in all animal tissues and plasma. The level of 5-oxoproline in the mouse tissues is 20-50 μM (7). It is also a major water soluble nitrogen compound in the epidermis where its concentration is at least ten times higher (8). The urinary excretion of 5-oxoproline is 1 μmole per mg

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creatinine in the mouse (7) and 820 μmole per mg creatinine in man (9). The tissue and plasma levels of 5-oxoproline and its excretion in urine increases many fold in patients with 5-oxoprolinuria associated with either glutathione synthetase deficiency (10) or 5-oxoprolinase deficiency (11,12).

An interesting feature of 5-oxoprolinuria is the accompanying elevated level of proline and other amino acids in the plasma (13,14). It was suggested that there may be competitive interactions between proline and 5-oxoproline in metabolism or transport (7). However, no data are available on the transport of 5-oxoproline and its interaction with other amino acids during transport across any cellular membrane. We report here for the first time the characteristics of 5-oxoproline transport in kidney, using purified renal brush border membrane vesicles from rabbit.

Methods and Materials

Rabbit renal brush border membrane vesicles were prepared as described previously (15,16). The purity of the membranes was routinely evaluated by specific marker enzymes (16) and occasionally by electron microscopy. Only the membranes completely free from basal-lateral membrane contamination as assessed by the absence of (Na^+-K^+) ATPase activity were used. Transport was assayed by the Millipore filtration technique described in detail elsewhere (15). When the effect of various amino acids on transport was studied, the pH of the solutions in each case was adjusted to 7.5 with either HCl or Tris base.

Plasma 5-oxoproline was determined as glutamic acid after hydrolysis as described by Hagenfeldt et al. (17). 200 μl of deproteinized plasma was passed through a column (6 x 0.6 cm) of Dowex 50 W x 4 (50-100 mesh) and the 5-oxoproline eluted with 3 ml of water. The eluate was mixed with 3 ml of 5 N HCl and hydrolyzed for 1 hr at 100°C. The solution was evaporated to dryness and 1 ml of sodium citrate buffer (200 mM, pH 2.1) added and analyzed on the amino acid analyzer (Beckman Model 120-C).

Urine 5-oxoproline was measured as previously described (11). The organic acids in the urine were isolated on a DEAE-Sephadex column and eluted with 1.5 M pyridinium acetate buffer, pH 5.0. The eluate was evaporated to dryness and the organic acids esterified with 14% boron trifluoride in n-butanol. The butyl esters were then separated and quantified by gas chromatography (Varian, model 3700) using a 6% diethyleneglycol succinate column.

5-Oxoprolinase activity was determined by the method of Stromme and Eldjarn (18). The intravesicular contents were extracted as described in (19) and analyzed for 5-oxoproline and glutamic acid (17).

[^{14}C(U)]-5-oxoproline (286 mCi/mmol) was purchased from New England Nuclear. Unlabeled 5-oxoproline was from Sigma. Other amino acids were from California Corporation for Biochemicals. All other reagents were of analytical grade.

Results and Discussion

Plasma and urinary levels of 5-oxoproline. There is no data available on the plasma and urinary levels of 5-oxoproline in the rabbit. We, therefore, analyzed the rabbit plasma and urine for their 5-oxoproline content. The concentration of 5-oxoproline in the plasma was 30 μM and the urinary excretion was 0.36 μmole/mg creatinine.

5-Oxoprolinase activity. The conversion of 5-oxoproline to glutamic acid is catalyzed by 5-oxoprolinase in an ATP-dependent reaction. Mammalian kidneys contain very high activities of this enzyme. The reported value is about 30 nmol/hr/mg protein in the rat kidney homogenate (20,21) and 11 nmol/hr/mg protein in the guinea pig kidney homogenate (8). In the present study, we determined the 5-oxoprolinase activity in the rabbit renal cortex homogenate as well as in the purified brush border membrane vesicles. The specific activity of 5-oxoprolinase was 13 nmol/hr/mg protein in the homogenate and 0.03 nmol/hr/mg protein in the brush border membrane vesicles. Since 5-oxoprolinase is a soluble enzyme, the trace activity associated with the purified membranes represents the cytosolic contamination.

Since the transport assays were carried out in the absence of ATP, conversion of 5-oxoproline to glutamic acid during the regular transport assay using the purified membranes would be negligible. We followed the formation of glutamic acid in the incubation medium as a function of time. The amount of 5-oxoproline added to the incubation medium was 3 nmol. Glutamic acid in the medium was below detectable levels after 30 sec incubation. However, with prolonged incubation (30 minute), traces of glutamic acid (0.0006 nmol) were detected in the medium. It is, therefore, clear that there is no appreciable conversion of 5-oxoproline to glutamic acid under the normal transport assay conditions.

Transport of 5-oxoproline. Fig. 1 represents the typical pattern of transport of 5-oxoproline (20 μM) into brush border membrane vesicles in NaCl and KCl media. 5-Oxoproline exhibited the 'overshoot' phenomenon in the presence of a Na^+-gradient. The accumulation of 5-oxoproline inside the vesicles was maximal at 30 sec and then decreased due to the efflux of the

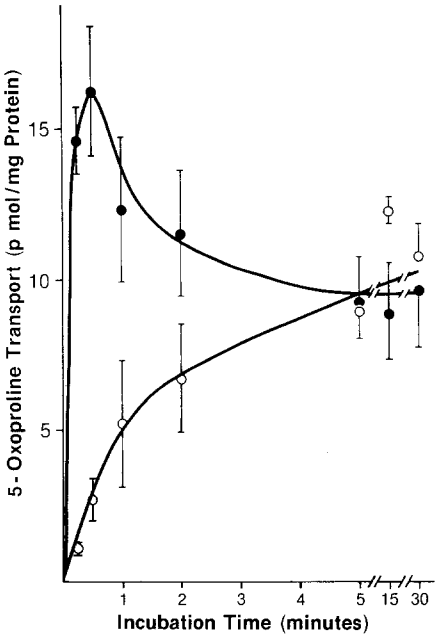

Fig. 1 Time course of 5-oxoproline transport in NaCl and KCl media.

The vesicles were incubated with labeled 5-oxoproline (20 μM) for varying periods of time in NaCl and KCl media.

●——● NaCl medium

○——○ KCl medium

amino acid. No overshoot phenomenon was observed in KCl medium. The transport of 5-oxoproline in KCl medium was lower than the transport in NaCl medium at all the initial periods of incubation. The Na^+-dependent stimulation of transport was 13 fold at 15 sec and 6 fold at 30 sec. The amount of 5-oxoproline inside the vesicles reached an equilibrium value at prolonged incubation (30 min) and this final level of transport was the same in both NaCl and KCl media. At the peak of the overshoot, the intravesicular concentration of 5-oxoproline was almost twice that of the equilibrium value.

To distinguish between non-specific binding to the membranes and transport into the intravesicular space, the equilibrium transport of 5-oxoproline (30 min incubation) was measured as a function of the medium osmolarity. The transport was found to be inversely proportional to the osmolarity of the incubation medium (Fig. 2). The non-specific binding calculated by extrapolation of the results to infinite osmolarity was 15% of the transport observed

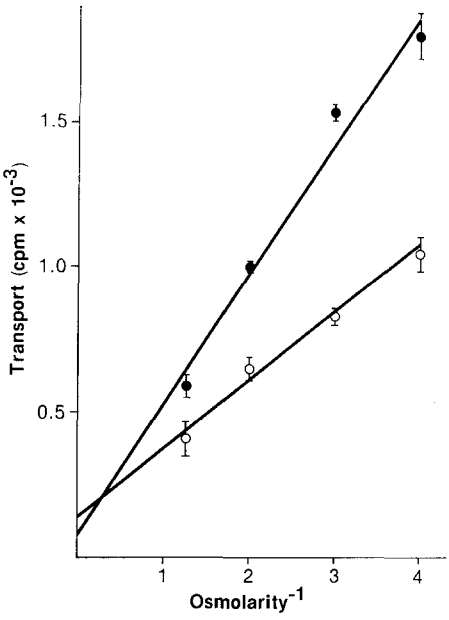

Fig. 2 Effects of medium osmolarity on the initial and equilibrium transport of 5-oxoproline in NaCl medium.

To calculate the initial rates of transport, the vesicles were first preequilibrated in Na^+-free medium of varying osmolarities for 30 min. The vesicles were then incubated with labeled 5-oxoproline (20 μM) for 30 sec. in respective osmolar solutions containing NaCl. To calculate the equilibrium transport, the vesicles were directly incubated with labeled 5-oxoproline (20 μM) for 30 min in NaCl medium of varying osmolarities.

●—● Initial rates of transport
○—○ Equilibrium transport

at 300 mosm. The effect of osmolarity on the initial rates of transport was studied using a 30 sec incubation. The vesicles were pre-equilibrated in Na^+-free media of varying osmolarities before they were used in the transport assay. The initial transport rates varied inversely with osmolarity (Fig. 2), but the binding was less than 5% of the total transport at 300 mosm. The predominant portion of 5-oxoproline transport, therefore, occurs into an osmotically responsive intravesicular space.

Intravesicular contents. Analysis of the intravesicular contents after 30 sec incubation of the vesicles with 20 μM [^{14}C]-5-oxoproline in the presence of a Na^+-gradient showed that more than 99% of the radioactivity was in the form of 5-oxoproline. Only trace amounts of radioactivity in the form of glutamic acid was detected.

Table 1

Effects of other amino acids on the initial rates of Na^+-dependent 5-oxoproline transport

Addition	Relative Transport
None	100
Isoleucine	37 ± 7
Alanine	40 ± 8
Valine	46 ± 7
Tryptophan	49 ± 4
Serine	51 ± 13
Glutamine	57 ± 2
5-oxoproline	57 ± 9
Phenylalanine	58 ± 2
Threonine	61 ± 5
Methionine	67 ± 2
Leucine	80 ± 5
Proline	75 ± 8
Hydroxyproline	102 ± 5
Glycine	104 ± 5
Lysine	113 ± 7
Arginine	116 ± 9
Glutamic acid	102 ± 9
Aspartic acid	104 ± 4
Pipecolic acid	104 ± 3
Glycyl-proline	112 ± 11

Renal brush border membrane vesicles were incubated with labeled 5-oxoproline (20 μM) at 25°C for 30 sec in NaCl and KCl media. The concentration of unlabeled amino acids in the incubation medium was 2 mM. The Na^+-dependent 5-oxoproline transport was calculated by substracting the transport observed in KCl medium from the transport in NaCl medium. The control value for the Na^+-dependent transport was 18.4 ± 1.8 pmol/mg protein and this was taken as 100.

Effects of other amino acids on 5-oxoproline transport. Table 1 shows the effects of neutral, acidic and basic amino acids and imino acids on the transport of 5-oxoproline. The Na^+-dependent transport of 5-oxoproline was markedly inhibited by all the neutral amino acids except glycine. Proline caused a small but highly reproducible inhibition while hydroxyproline had no effect. The basic amino acids lysine and arginine and the acidic amino acids aspartic acid and glutamic acid also did not have any effect on 5-oxoproline transport. Pipecolic acid and glycyl-proline, a dipeptide highly resistant to hydrolysis by renal brush border membrane vesicles (19) did not cause any inhibition. These results clearly show that 5-oxoproline and the neutral amino acids share a common transport mechanism in the rabbit renal brush border membrane.

The inhibition, though weak, by proline is an interesting observation. Because of the structural similarities between proline and 5-oxoproline, one would expect a greater inhibition of 5-oxoproline transport by proline. Glycine, proline and hydroxyproline are known to share a common transport system in the kidney. However, a number of recent investigations show that proline also interacts with many neutral amino acids during transport. Proline has been shown to inhibit alanine transport in the rabbit renal brush border membranes (22). Similarly, a number of neutral amino acids, especially phenylalanine, cause significant inhibition of proline transport in the rabbit kidney (23,24). The inhibition caused by proline is, therefore, not altogether surprising. This may be one of the possible reasons for the elevated plasma concentration of proline in 5-oxoprolinuria.

Pipecolic acid has long been known as a product of lysine metabolism in animals. There are significant structural similarities between pipecolic acid, proline and 5-oxoproline. Our recent findings with a patient with 5-oxoprolinuria associated with 5-oxoprolinase deficiency (11) suggested competition between proline and pipecolic acid during transport. But, the present study shows that pipecolic acid does not compete with 5-oxoproline during transport.

The data presented in this paper show that the rabbit kidney can efficiently reabsorb 5-oxoproline from the glomerular filtrate against a concentration gradient, thus effectively avoiding its elimination in the urine. Once inside the tubular cells, it can be utilized because of the high levels of 5-oxoprolinase activity in the renal tissue.

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