DISTRIBUTION OF GAMMA-GLUTAMYL TRANSPEPTIDASE AND GLUTAMINASE ISOENZYMES IN THE RABBIT SINGLE NEPHRON

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Abstract—"Phosphate-independent maleate-stimulated glutaminase" was investigated as a function of gamma-glutamyl transpeptidase (gamma-GTP). The activity of gamma-GTP in brush border membranes was found to be four times higher than that in the microsomal fraction of the renal cortex. This gamma-GTP activity was exclusively located in the proximal tubule of isolated single nephrons. Specific activity of gamma-GTP was 105 U/g protein (19.8 μU/mm length) in the first 2 mm portion of the proximal tubule and 1352 U/g protein (209 μU/mm) in the last 2 mm portion of the proximal straight tubule. Activity of phosphate independent glutaminase (PIG) was distributed in the same patterns as those of gamma-GTP, not only in the subcellular fractions, but also in the isolated nephron segments. On the other hand, phosphate dependent glutaminase (PDG) was distributed highly in the papillary mitochondrial fraction and in the distal tubule. Observations on the effect of pH on the enzyme activities of gamma-GTP and PDG showed that these enzyme activities were decreased significantly when the pH of the assay mixture was lowered. In the case of PIG, however, the effect of pH was just reversed. From these findings, it may be possible to interpret that gamma-GTP may play an important role in ammonia production in the brush border membrane of the proximal tubule as a function of glutaminase.

It has been recognized that the activity of gamma-glutamyl transpeptidase (gamma-GTP), clinically evaluated in obstructive liver disease, is much higher in the renal cortex than in other organs, such as the liver, spleen and brain (1). Histochemically, gamma-GTP has been shown to be located in the brush border membrane of the proximal tubule (2, 3). Heinle et al. reported that gamma-GTP activity in the lyophilized rat nephron was primarily found in the straight portion of the proximal tubule (4). Gamma-GTP catalyzes the transfer of the gamma-glutamyl group of glutathione to free amino acid to form the dipeptide, cysteinyl glycine and gamma-glutamyl acceptor (5). Glutaminase I has at least two isoenzymes, phosphate dependent glutaminase (PDG), requiring inorganic phosphate as an activator; and phosphate independent glutaminase (PIG), activated by maleate (6). Tate and Meister (7) have found that gamma-GTP purified from rat kidney hydrolyzed glutamine to glutamate.

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Curthoys and Kuhlenschmidt (8) have investigated both glutaminase and gamma-GTP reactions using PIG purified from rat kidney, reporting that the PIG reaction was catalyzed by the same enzyme catalyzing the gamma-GTP reaction. Therefore, these two independent studies (7, 8) have indicated that “maleate-stimulated phosphate-independent glutaminase” is a catalytic function of gamma-GTP.

Glutaminase and glutamate dehydrogenase are considered to function in the main pathway of ammonia production in the kidney. Curthoys et al. (9) emphasized the importance of PDG in the proximal tubules of the chronic acidotic rat. They showed a 20-fold increase of PDG activity only in the early proximal convoluted tubules of these rats.

Additionally, in the acute acidotic state, ammonia excretion was reported to be increased prior to enzymatic induction, while the activity of PDG was somewhat decreased (10). Several possible explanations have been proposed for these findings: increase of an activator such as maleate (11), increase of plasma glutamine (12), and change of the pathway producing ammonia (13, 14).

In order to clarify possible functional relationships in renal ammonia production from glutamine, the present study was designed to investigate the distribution of gamma-GTP and glutaminase isoenzymes in isolated single nephrons of rabbit.

MATERIALS AND METHODS

Materials

L-glutamine, L-glutamate, alpha-ketoglutarate, L-gamma-glutamyl-p-nitroanilide, glycylglycine and 2-amino-2-methyl-propan-1,3-diol (Amediol) were obtained from Sigma. Triethanolamine hydrochloride, nicotinamide adenine dinucleotide (NAD), iodonitrotetrazolium chloride (INT), diaphorase, and glutamate dehydrogenase (GLDH) were obtained from Boehringer. All other chemicals used were of analytical grade.

Methods

(1) Preparation of subcellular fractions of kidney slices: Male New-Zealand white rabbits (2.5–3.0 kg, B.W.), maintained on normal food intake and water ad libitum, were used. The rabbit was anesthetized intravenously with sodium pentobarbital 40 mg/kg. The left kidney was perfused with physiological saline via the renal artery, then removed. The following steps were carried out on ice. The kidney was cut into three parts (cortex, medulla, and papilla), and each section was homogenized in 9 volumes of 0.25 M sucrose. The homogenates were centrifuged 900×g for 10 min and the pellets were discarded. The supernatants were recentrifuged at 8,000×g for 10 min and the resultant pellets were resuspended (mitochondrial fractions). Centrifugation of the remaining supernatant at 105,000×g for 60 min yielded microsomal fractions (pellets) and soluble fractions.

(2) Preparation of brush border membranes: Brush border membranes were obtained from the renal cortex of New-Zealand white rabbits using the Liang and Sacktor method (15) with slight modifications.

(3) Preparation of isolated nephron segments: Male New-Zealand white rabbits (1.5–2.0 kg, B.W.) were used. Microdissection of collagenase treated kidney slices was done by the procedure of Morel et al. (16). The isolated nephron segments were dissected in ice-cold Hanks’ solution under a stereoscopic microscope. The identification of each nephron segment was according to Maunsbach (17) and Morel et al. (16). The microdissected nephron segments were collected into microcapillary pipettes under phase contrast microscopy (×100).

(4) Enzyme assays:

Gamma-glutamyl transpeptidase: Gamma-GTP activity was measured spectrophoto-
metrically at 25°C using 4.0 mM L-gamma-glutamyl-p-nitroanilide as a substrate (15, 18) in 48 mM ammonium buffer, pH 8.2, containing 50 mM glycylglycine and 10 mM MgCl₂, except when the pH optimum was studied. Gamma-GTP activity was measured on samples of kidney slices and brush border membranes in 2.1 ml final assay mixture. Each nephron segment, collected into a glass capillary pipette, was solubilized with 1% deoxycholate. This sample solution was divided into two parts: for enzyme assay and for protein determination. Usual protein amounts used were 100 to 300 ng. The final assay volume was 105 μl. Enzyme activity showed linearity with respect to time, 0 to 10 min, and with protein content, 50 to 1,000 ng. There was no detectable loss of gamma-GTP activity during collagenase treatment at 37°C for 50 min and microdissection at 0°C within at least 6 hours.

Glutaminase isoenzymes: Glutaminase isoenzymes were determined by measuring glutamate formation as described by Curthoys and Lowry (9), with modifications in microscale preparations. Glutaminase activity showed linearity with respect to time, 0 to 60 min incubation, and with protein content, 100 to 2,000 ng.

Glutamate dehydrogenase: Glutamate dehydrogenase activity was photometrically measured at 25°C according to the usual method (19). The final assay volume was 205 μl.

(5) Protein determination: Protein content was determined by the Lowry procedure (20) in a final assay volume of 22 or 65 μl using an ultra-microcuvette having 0.5 mm diameter × 7 mm light path. Protein content on the order of 10 ng was detectable. One unit (U) was defined as the enzyme activity that transforms 1 μmole of substrate in 1 min at 25°C.

RESULTS

Figure 1 shows the specific activity of gamma-GTP in the subcellular fractions of the rabbit kidney slices (cortex medulla and papilla). Gamma-GTP activity was highly concentrated in the microsomal fraction of the cortex with a specific activity of 2,375 U/g protein. This value was about 4 times as high as the activity of the total homogenate of the cortex. Soluble fractions (supernatants) revealed almost no activity.

Brush border membranes possessed high gamma-GTP activity; 10,420 U/g protein (average of two independent experiments).
In the isolated nephron segments, gamma-GTP activity was high in the proximal tubules and very low in the other segments. Figure 2 shows the distribution of gamma-GTP activity in the 7 different nephron segments. Specific activity of gamma-GTP was highly concentrated in the proximal tubules, especially in the last portion of the proximal straight tubules. Gamma-GTP activity was 105 U/g protein in the first 2 mm portion of the proximal convoluted tubule (PS₁) and 1,352 U/g protein in the last part of the proximal straight tubule (PS₃).

The enzyme activities can be calculated as a function of tubule length (mm) or a glomerulus. Table 1 shows protein contents of each nephron segment. Within the proximal tubule, the order of the enzyme activities per mm is identical to that of the specific activities in Fig. 2.

The pH dependent changes of enzyme activities in gamma-GTP and glutaminase isoenzymes (PDG, PIG) are shown in Fig. 3. The pH optimums of gamma-GTP, PIG, and PDG were 8.2, 6.6 and 7.4, respectively. Both gamma-GTP and PDG activities

**Fig. 2.** Localization of gamma-GTP activity in the 7 different nephron segments. Each nephron segment was homogenized in 1% deoxycholate and was separated into two parts for the enzyme assay and for protein determination. Usual protein amounts used for the assay were 100 to 300 ng. Assay conditions were the same as those stated in Fig. 1 except for the final volume (105 ml). Abbreviations of the nephron segments are: Glm, glomerulus; PS₁, the first 2 mm portion of the proximal convoluted tubule; PS₂, the last portion of proximal convoluted tubule; PS₃, the terminal portion of the proximal straight tubule; TA, the cortical thick ascending limb of Henle’s loop; Dist, distal convoluted tubule including macula densa; and CCT, cortical collecting tubule with branched portion. Each column shows the mean value of the activities and vertical line indicates ±1 S.D. Numbers of independent determinations are in parenthesis. Enzyme activity is expressed as unit/gram protein at 25°C.

**Fig. 3.** The pH dependent changes of activities in gamma-GTP and glutaminase isoenzymes (PDG, PIG). The PDG incubating mixture contained 20 mM glutamine, 0.2 mM EDTA, 0.02% BSA and 150 mM phosphate buffer, pH 6.0–8.6. For PIG, the assay mixture contained 10 mM glutamine, 0.2 mM EDTA, 0.02% BSA, and 60 mM Tris-Cl buffer, pH 6.0–8.6. Glutaminase activity was determined by measuring glutamate as formazan formation spectro-photometrically at 492 nm. Gamma-GTP activity was measured by the same procedure as stated in Fig. 1 with the exception of pH. The data are presented as the percent of maximum activity.
Table 1. Protein contents of the isolated nephron segments

| Nephron segment | Protein content (mean±S.D.) |
|-----------------|-----------------------------|
| Glm             | 82.8±14.0 ng/g glomerulus   |
| PTs₁            | 188.5±45.4 ng/mm            |
| PTs₂            | 246.8±31.6 ng/mm            |
| PTs₃            | 154.8±39.1 ng/mm            |
| TA              | 49.6±11.1 ng/mm             |
| Dist            | 87.4±13.8 ng/mm             |
| CCT             | 75.6±11.1 ng/mm             |

Each nephron segment from the rabbit kidney was microdissected under a stereomicroscope and photographed. Protein contents of each nephron segment was measured by the method of Lowry et al. (20) in a final assay volume of 65 µl (see Materials and methods). Abbreviations of the nephron segments are: the glomerulus (Glm), segment 1 to 3 of the proximal tubule (PTs₁–PTs₃), the thick ascending limb of Henle's loop (TA), the distal tubule (Dist) and the cortical collecting tubule (CCT).

Increased markedly with increasing pH of the assay mixture. Conversely, PIG activity decreased with increasing pH.

Glutaminase isoenzyme (PDG, PIG) activities in the subcellular fractions are represented in Fig. 4. The highest activity of PDG was found in the mitochondrial fractions of the papilla (17.6 U/g protein). Activity was very low in the soluble fractions (supernatants) and moderate (6.9–7.0 U/g protein) in the mitochondria of the cortex and medulla.

Unlike PDG, the highest activity of PIG was located in the microsomal fraction of the cortex (12.2 U/g protein). The PDG/PIG ratio in the whole homogenate for the papilla was approximately 20, but in both the cortex and medulla, this ratio in the homogenate was one or less (data are not stated).

Figure 5 shows the distribution of glutaminase isoenzymes (PDG, PIG) in the isolated nephron segments. The highest PDG activity was localized in the distal convoluted tubule (17.0 U/g protein), while activity was lower (1.5 to 6.8 U/g protein) in the proximal tubules. Conversely, PIG activity was located in the proximal tubules, especially in the straight portions (16.8 U/g protein). It could be seen that the PDG and PIG activities were distributed differently in different nephron segments.
Fig. 5. Distribution of glutaminase isoenzyme activities in the isolated nephron segments. Each nephron segment was homogenized with a 100 μl teflon-glass capillary homogenizer and divided into three parts for detecting glutaminase activity, tissue blank and protein determination. The incubating medium for PDG contained 20 mM glutamine, 0.2 mM EDTA, 0.02% BSA and 150 mM phosphate buffer, pH 7.0. For PIG, the assay mixture contained 10 mM glutamine, 0.2 mM EDTA, 0.02% BSA and 60 mM Tris-Maleate buffer, pH 7.0. Crude homogenate (500-1,000 ng protein content) was added to the first incubating medium. After 60 min incubation at 37°C, the reaction mixture was deproteinized. The supernatant (10 μl) was used to detect glutamate. The final assay mixture for detecting glutamate was 160 μl. Glutaminase activity is expressed as units per gram protein at 37°C. Numbers of independent determinations are given in parentheses. Abbreviations of the nephron segments are the same as stated in Fig. 2. PIG is shown as the white column and PDG as the grey column. The vertical line indicates ±S.E.M.

PIG activities along the nephron were approximately complementary.

In order to demonstrate a distributional relation between glutamate dehydrogenase and glutaminases in the ammoniagenesis, glutamate dehydrogenase activity was also assayed in the single nephron. As depicted in Fig. 6, the activities of this enzyme were distributed homogeneously.

**DISCUSSION**

The distribution of gamma-GTP activity along a single nephron has been reported in rats using lyophilized rat kidney slices by Heinle et al. (4). They reported that a high activity of gamma-GTP was localized in the proximal straight tubule and moderate activity was observed in the distal tubule. In our study, using rabbit nephron segments micro-dissected from collagenase treated kidney slices, the highest activity of gamma-GTP was observed in the proximal straight tubule. Negligible activity of gamma-GTP, however, was found in the
lower nephron segments (distal convoluted tubule, thick ascending limb of Henle’s loop, and cortical collecting tubule). Previous histochemical studies have shown that gamma-GTP activity was distributed almost exclusively in the brush border membranes of the proximal tubules (2, 5), which is consistent with our finding. Concerning the two contradictory findings of gamma-GTP activity in the distal tubules, differences in the preparation of the isolated nephron might be involved. We could find a similar distribution pattern for gamma-GTP in the rat single nephron treated with collagenase (21). After digestion with collagenase, microdissection of a single nephron was quite simple; we could often collect an entire single nephron from the glomerulus to the collecting tubule, except the thin and thick limb of Henle’s loop. Therefore, using the collagenase treatment, the accurate identification of dissected nephron segments is possible. Using the collagenase treatment method, not only membrane-bound proteins like adenylate cyclase (16, 22), but also soluble proteins e.g., LDH isoenzymes (23) and gluconeogenesis in isolated tubule suspension (24, 25) could have been determined.

Glutaminase and glutamate dehydrogenase are considered to function in the main pathway of ammonia production, since glutamine and glutamate, the substrates of these enzymes, are abundant in the plasma under physiological conditions (26, 27). As shown in Fig. 6, glutamate dehydrogenase activity was distributed nearly homogenously along the nephron. Additionally, as shown in Fig. 5, complementary distribution of PDG and PIG along the single nephron was observed. Therefore, it might be suggested that ammoniagenesis from glutamine is attributable to the entire nephron segment.

Concerning the intrarenal relationship between gluconeogenesis and ammoniagenesis in acidic dogs and rats, increases of ammonia production (10, 13, 25) and gluconeogenesis (24, 28) have been reported. We could find that glucose production from pyruvate was high in the microdissected rabbit proximal tubule, especially in the straight portion (29). Therefore, ammoniagenesis in the proximal tubules should be taken into consideration. In the acute acidic rat, ammonia excretion was reported to be increased prior to enzymatic induction, and the activity of PDG was rather decreased (10). For an explanation of these findings in the acute acidic state, the following possibilities have been proposed: increase of an activator such as maleate (11), increase of glutamine in the plasma (12), and change of the pathway for ammonia production (13, 14). Considering the findings of the slice examination, a permeability problem would be an important factor for PDG (30), because PDG has been reported to be located in the inner membranes of the mitochondria. The activity of PIG was distributed similarly to that of gamma-GTP, not only in the subcellular fractions, but also in the isolated nephron segments. PIG may be considered to be the catalytic function of ‘gamma-GTP’ (7, 8, 31). Therefore, a permeability change should not be important in increasing PIG activity, since PIG would be located in the brush border membranes and would thus face the glomerular filtrate.

The reverse responses of gamma-GTP and PIG to the change of pH of the assay mixture as shown in Fig. 3 might be explained by such an acidosis. From studying the effects of pH on product specificity of purified phosphate-independent glutaminase using gamma-glutamyl-p-nitroanilide as substrate, Curthoys and Kuhlenschmidt (8) suggested that decreased pH promoted glutaminase activity and that below pH 6.0, this enzyme functioned strictly as a glutaminase. Therefore, it could be speculated that in under conditions of lowered pH, ‘gamma-GTP’ may convert to glutaminase in the gamma-
GTP enriched proximal tubules, and the pH drop in the glomerular filtrate accompanied by acute acidosis (32, 33) might affect the increase of PIG activity.

It should be concluded that with respect to increasing ammonia excretion in acute acidosis, "gamma-GTP" may play an essential role; accordingly with a drop in the pH of glomerular filtrates, "gamma-GTP" would be converted to PIG, which could increase ammonia production from glutamine. Further studies to make direct measurements of ammoniagenesis in a single nephron under various conditions are necessary.

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