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THE LOCATION OF GLUTAMINE SYNTHETASE WITHIN THE RAT AND RABBIT NEPHRON

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Summary: Glutamine synthetase (EC 6.3.1.2) was measured in seven different parts of the nephron in the rat and five in the rabbit, dissected from freeze-dried microtome slices. In the rat the enzyme is essentially confined to the proximal straight tubule. Acidosis did not change the activity; methionine sulfoximine abolished it. In the rabbit the enzyme is high in both proximal convoluted and straight tubules. Assays were made with a new method which measures glutamine formation per se. One of the products, NADH, is amplified by enzymatic cycling to provide sufficient sensitivity (2-10 pmol of glutamine).

Rat kidney not only degrades glutamine when needed to provide NH_4^+ to balance excess acid, but can also synthesize glutamine from glutamate and NH_4^+ (1-3). Previous quantitative histochemical studies with large glutamine and glutamate loads, suggested that the synthetic and degradative functions might be assigned to different parts of the nephron (4). To test this possibility, the distribution of glutamine synthetase along the nephron, was determined.

MATERIALS AND METHODS

Animals: Purina chow fed male Sprague-Dawley rats (350-400 g) were used. Some were made acidotic by providing 0.3 M NH_4Cl in place of drinking water for 7 days. Others were given L-methionine-D-L-sulfoximine, 2.5 mmol/kg i.p., 100 min before kidney removal. Trypan blue was given ahead of time (to the rats only) to identify proximal convoluted tubules (5). Rat kidneys were removed after brief etherization and frozen within 1 sec in Freon-12 ($C Cl_2F_2$) at -160°. Kidneys from white New Zealand rabbits (4 to 5 kg) were removed under nembutal anesthesia and similarly frozen. Parts of single nephrons were dissected from freeze-dried microtome slices and weighed (10-40 ng) on a quartz fiber balance (6,7). Slices from four gross regions: outer cortex, outer stripe of outer medulla, inner stripe of outer medulla and medulla (papilla) were prepared at -20° (8). The gross outer stripe of the rabbit kidney was narrow and less distinct than in the rat and represented the cortico-medullary junction.

Auxilliary enzymes were from Sigma Chemical Co. or Boehringer Mannheim Corp., except beef heart lactic dehydrogenase which was from Worthington. Other biochemicals were from Sigma.

The two glutamine synthetase procedures have 5 basic steps: 1) glutamine synthesis from glutamate, NH_4^+ and ATP, 2) destruction of

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excess glutamate with glutamic dehydrogenase and NAD^+, 3) destruction with HCl of the NADH produced, 4) conversion of glutamine to glutamate and 5) measurement of glutamate by the NADH formed upon repetition of step 2. Because of the necessarily large excess of glutamate in step 1 (the K_m is 4.4 mM), destruction of glutamate in step 2 is quite demanding. In addition, NH_4^+ carried over from step 1 makes the glutamic dehydrogenase equilibrium even less favorable. (Additional NH_4^+ from the auxilliary enzymes must be avoided). In the macro procedure (for homogenate assays) pyruvate and lactic dehydrogenase are added to pull the reaction by converting NADH as it is formed back to NAD^+. In addition, before proceeding, H_2O_2 is added, which destroys α-ketoglutarate and further helps to pull the reaction. (H_2O_2 cannot be added from the beginning because it would also destroy pyruvate.) In the micro procedure, glutamate concentration is lower by a factor of 4, and H_2O_2 alone pulls the reaction sufficiently close to completion. (EDTA is added before the last step, because some of the NADH may otherwise be destroyed by H_2O_2 carried over from step 2.) Buffers are chosen to produce in succession the following approximate pH's: 7.2 at step 1, 8.0 at step 2, less than 2 at step 3, 4.6 at step 4, 8.0 at step 5 and, in the case of the micro procedure, 12 at step 6. The pH at each step and the dilution of glutamate to 200 μM or less at step 2 are critical.

In the micro procedure, all steps but the last are carried out in "oil wells" (6). This permits the use of very small volumes and thereby avoids excessive dilution of the ng sized tissue samples, which would prevent successful assays.

Because glutamine synthetase activities in some segments are almost zero, it was necessary to evaluate "tissue blanks" (ATP omitted). These averaged 48 mmol/kg (dry wt) for both species.

Analysis of homogenates (5-20 μg tissue). Homogenates were prepared at 0° in 20 mM Tris-HCl, pH 8, containing 0.02% bovine plasma albumin, and 1 mM EDTA at 1:50 to 1:200 dilution.

To 50 μl of reagent (50 mM imidazole-HCl buffer, pH 7.2, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM ammonium acetate, 2 mM glutamate, 2 mM ATP and 0.02% bovine plasma albumin) in a fluorometer tube were added 1 or 2 μl of the homogenate (total tissue dilutions of 1:2500 to 1:10,000). After 1 h at 37° the reaction was stopped in a water bath at 100° (2 min). After cooling, 400 μl were added of glutamate destroying reagent (50 mM Tris-HCl buffer, pH 8, 1 mM NAD^+, 0.3 mM ADP, 3 mM pyruvate, and 100 μg/ml of beef liver glutamate dehydrogenase (EC 1.4.1.3) in glycerol and 5 μg/ml of beef heart lactate dehydrogenase (EC 1.1.1.27) from which $(NH_4)_2SO_4$ had been removed by centrifugation). After 40 min at 30°, 25 μl of a solution containing 200 mM H_2O_2 and 15 mM EDTA were added, followed 20 min later (at room temperature) by 50 μl of 0.5 N HCl to destroy NADH. After 15 min, 200 μl were added of glutaminase reagent (210 mM sodium acetate, 30 mM acetic acid, 0.3% bovine plasma albumin, 1 mM EDTA and 10 μg/ml of E. coli glutaminase (EC 3.5.1.2)). After another 30 min, 500 μl of glutamate measuring reagent were added (80 mM Tris-HCl buffer, pH 9.4, 0.4 mM ADP, and 1.5 mM NAD^+). An initial reading was made, 50 μg of glutamate dehydrogenase were added and the final increase in fluorescence measured (about 30 min).

Analysis of parts of freeze-dried nephrons (10-40 ng)

Weighed samples were loaded at timed intervals into 0.1 μl droplets of the reagent in the oil well rack. The reagent was that described for homogenates except that glutamate was reduced to 1 mM. Standards were 0.1 μl of 25 to 100 μM glutamine in the same reagent. One hour after adding the first sample, the rack was heated 6 min in an oven at 95°. After cooling, 2 μl were added of glutamate destroying reagent as described above except for omission of pyruvate and lactic dehydrogenase and addition of 10 mM H_2O_2 and 1 mM EDTA. After 40 min at 25° the

reaction was stopped with 0.25 µl of 0.5 N HCl. Glutaminase reagent (2.5 µl of the reagent described for homogenates) was added, incubated 30 min, then followed by 2.5 µl of glutamate measuring reagent (240 mM Tris-HCl buffer, pH 9.4, 0.6 mM ADP, 2 mM NAD and 300 µg/ml of glutamate dehydrogenase).

The reaction was stopped with 1 µl of 1 N NaOH and heated at 60° for 20 min to destroy NAD^+. A 5 µl aliquot was transferred into 100 µl of enzymatic cycling reagent (9) to amplify the NADH (1000 to 3000-fold). Cycling was stopped by heating 2 min at 100° and the analysis completed as described previously (9).

In calculating the results, corrections were made for the fact that tissue samples were added at different times to the reagent, whereas all incubations were stopped at the same time.

Comment on the method. An improvement which would favor glutamate destruction at step 2 would be to increase the NAD^+ in the glutamine destroying reagent to 2.5 mM for both procedures and omit it from the glutamate measuring reagent.

Because of the difficulty of destroying glutamate in step 2, glutamate is kept low, in fact below its K_m. For glutamate, with 2 mM ATP, the apparent K_m was 4.4 mM, and for ATP, with 2 mM glutamate, 0.3-0.5 mM. The glutamate K_m agrees fairly well with that reported by Deuel et al. (10) for purified rat kidney glutamine synthetase (6 mM). They also found a K_m of 0.25 mM for NH_4^+. The K_m for ATP is similar to that for rat kidney extracts found by Iqbal and Ottaway (11).

In the homogenate assay, activity was proportional to amount of tissue up to 10 µg (15% fall off with 20 µg), and to time of incubation up to an hour. Activity increased 3-fold between 19° and 37°.

RESULTS

Among four gross regions of rat kidney (Fig. 1) the outer stripe of outer medulla has 6 times more glutamine synthetase than any other zone. The peak value of 300 mmol/kg(wet)/h at 37° may be compared with values of 42 obtained by Richterich and Goldstein for rat cortex plus outer medulla (12), and of 138 obtained by Lund (13) for cortex. The outer stripe in rat kidney is rich in proximal straight tubules. In rabbit kidney (Fig. 1) the highest activity was in the outer cortex rather than at the cortico-medullary junction. Rabbit cortex contains more proximal straight tubules than rat cortex; the outer stripe, fewer (14). Substantial glutamine synthetase activity was found in whole kidney cortex of mouse and hamster (legend to Fig. 1). No activity was found in any region of dog kidney.

Acidosis produced by 7 days with NH_4Cl in the drinking water did not alter glutamine synthetase in gross regions of rat kidney (Fig. 1). This agrees with results of others for whole kidney (3,15).

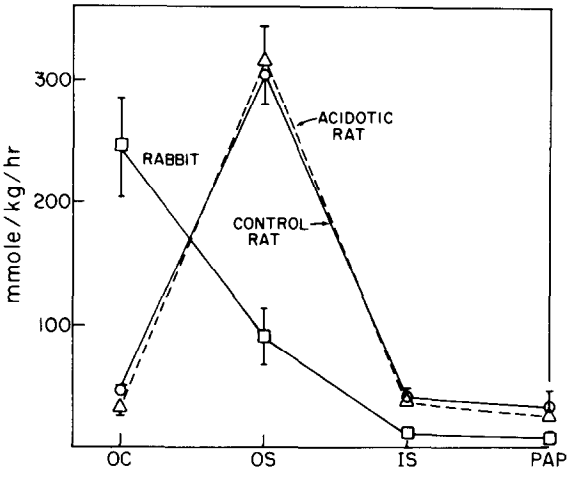

Fig. 1 Glutamine synthetase in gross regions of normal and acidotic rat and normal rabbit kidney. Averages (±SEM) are for 8 normal and 4 acidotic rats in the case of outer cortex (OC) and outer stripe (OS), 4 normal and 2 acidotic rats in the case of inner stripe (IS) and papilla (PAP), and 3 normal rabbits for all regions. Activities were measured with 2 mM glutamate at 37° and are based on wet weight. Whole cortex samples from 4 mice and 4 hamsters averaged 63 ± 16 and 46 ± 9 mmol/kg/h respectively.

Within limits of analysis, glutamine synthetase of rat nephron was found exclusively in the proximal straight tubule (Fig. 2). The early portion from the medullary ray, PST_E, and the later part from the outer stripe, PST_L, contain about equal amounts, whereas other parts of the nephron show less than 5% as much activity. The synthetase was not decreased in acidosis. Therefore, the sharp decrease in in vivo synthetase activity observed by Damian and Pitts (16) must be due to other factors. Glutamine synthetase activity was completely blocked by the administration of methionine sulfoximine, which is known to inhibit the enzyme (17).

Synthetase distribution in rabbit nephron (Table 1) differs from that in rat. Activity is high in both proximal convoluted and straight tubules. Also some activity is found in glomeruli and distal straight tubules.

DISCUSSION

Measurement of glutamine synthetase. Assay of glutamine synthetase

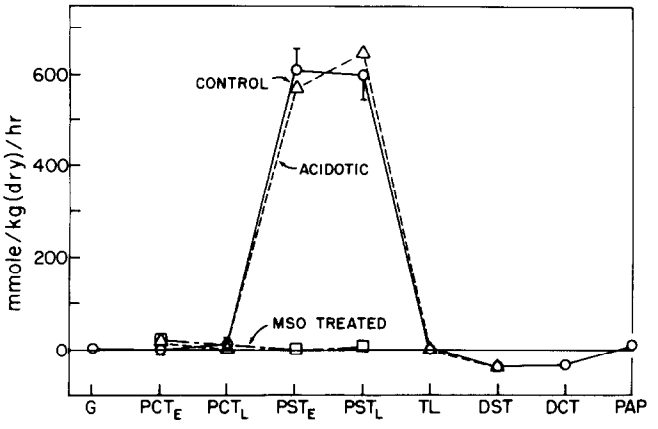

Fig. 2 Glutamine synthetase distribution in the rat nephron. For controls, each point represents the average (±SEM) of 4 animals; 4 to 12 samples of each structure were assayed for each rat. Other points represent the average of multiple samples from 2 animals. Because of the correction for average tissue blanks, some of the low activities appear negative. Abbreviations are MSO, methionine sulfoximine; G, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; TL, small patch from thin limb area; DST, distal straight tubule; DCT, distal convoluted tubule; and PAP, papilla. Subscripts E and L signify early and late. Early (near glomerulus) and late PCT represent tubules stained, respectively, darkly and lightly with trypan blue; early and late PST represent respectively, tubules from medullary ray and from outer stripe of outer medulla.

in tissues has been difficult. The method used here has several advantages over previous ones (10-11): it permits high tissue dilution which minimizes interference by enzymes such as ATPase and glutaminase, and inhibition by glutamine, ADP and P_i (10,11,13,18,19), and it provides the sensitivity needed to analyze minute tissue samples. It also ensures specificity because it is glutamine itself which is finally measured. Its disadvantage is the great care that must be taken to remove the glutamate substrate before measuring the enzyme product.

Other (successful) methods have been based on substitution of hydroxylamine for ammonia and subsequent measurement with Fe^{3+} (10,11,18, 19) or the use of radioactive glutamate with subsequent isolation and counting of the glutamine (13). Methods available for the purified enzyme (ADP formation (19), P_i formation (20), disappearance of NH_4^+

Table 1. Glutamine synthetase in parts of rabbit nephron. Each entry is the average (±SEM) of 9 to 13 samples dissected from freeze-dried microtome sections of the individual kidney. Abbreviations are as in Fig. 2.

Rabbit	G	PCT_E	PST_E	PST_L	DST
			mmol/kg(dry)/h		
A	107 ± 27	447 ± 58	581 ± 104	427 ± 110	144 ± 16
B		466 ± 104	452 ± 87	339 ± 82	111 ± 30

(12)) are clearly not suitable for tissue analysis. None of the methods applied to tissues has proven to be very sensitive. Lund, for example, recommended the use of not less than 10 mg of liver assayed at 1:50 dilution (13). Many authors have complained of analytical difficulties arising from insensitivity of the ferric-ion-hydroxylamine color reaction or from side reactions with other substances (11,13,20).

Localization of glutamine synthetase. The finding in the rat of a high level of glutamine synthetase in the proximal straight tubule was predicted from the effects of giving large loads of glutamate (4,21). Glutamine increased dramatically in the proximal straight tubules with little change elsewhere. In contrast, large loads of glutamine itself caused only modest increases in glutamine, and these were spread throughout the nephron.

It seems significant that the segment of the rat nephron which is the exclusive locus of the enzyme which makes glutamine, has the lowest activity of the enzyme which degrades it, i.e., P_i dependent glutaminase (22). (P_i independent glutaminase activity, which is very high in the proximal straight tubule, has been shown to be a side reaction of γ-glutamyl transpeptidase (23,24). It would seem to be a useful arrangement to have the synthetic and degradative capacities segregated. However, the purpose of glutamine formation is not clear. Glutamine levels are

very high in the plasma, therefore there should be no need for any segment of the nephron to make its own. The enzyme may serve to regulate ammonia excretion or simply to detoxify ammonia. Possibly ammonia which escapes from glutamate degradation in the proximal convoluted tubule is captured as glutamine in the next nephron segment.

Interpretation of the difference in distribution of the enzyme in rat and rabbit will have to wait for more quantitative histochemical information about the rabbit nephron.

Although glutamine synthetase activity averaged over the whole rat kidney is not high, activity in the proximal straight segment is rather impressive. The values in Fig. 2 were obtained at 20° with glutamate below the K_m. Calculated for saturating glutamate concentration at 38°, the activity in this segment would be 10 mol/kg(dry)/h or 37 mmol/kg(wet)/min. This is almost twice the activity of glutamic dehydrogenase in this segment (25).

The absence of demonstrable glutamine synthetase in dog (2,3) and human (26) kidney, makes it seem unlikely that the enzyme serves a crucial function. The difference between animals with and without renal glutamine synthetase may be related to a difference in urinary acidity. In man and dog the urine is habitually acid and therefore more urinary ammonia is required than in rats, rabbits and hamsters, with usually neutral or alkaline urine. The latter group may use glutamine synthetase to retrieve NH_4^+ not needed in the urine.

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