THE ACTIVITIES OF THE KEY ENZYMES OF THE γ-GLUTAMYL CYCLE IN MICRODISSECTED SEGMENTS OF THE RAT NEPHRON

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1. Introduction

In 1970, Orlowski and Meister introduced the concept of the γ-glutamyl cycle [1], a sequence of six enzyme-catalysed reactions which degrade and resynthesize the tripeptide glutathione (GSH). The authors postulated that this cycle, which is presented schematically in fig.1, represents one of the systems responsible for reabsorption of amino acids in the kidney ([2], for reviews see [3,4]).

Although the existence of this cycle is generally agreed upon, considerable doubts have been raised

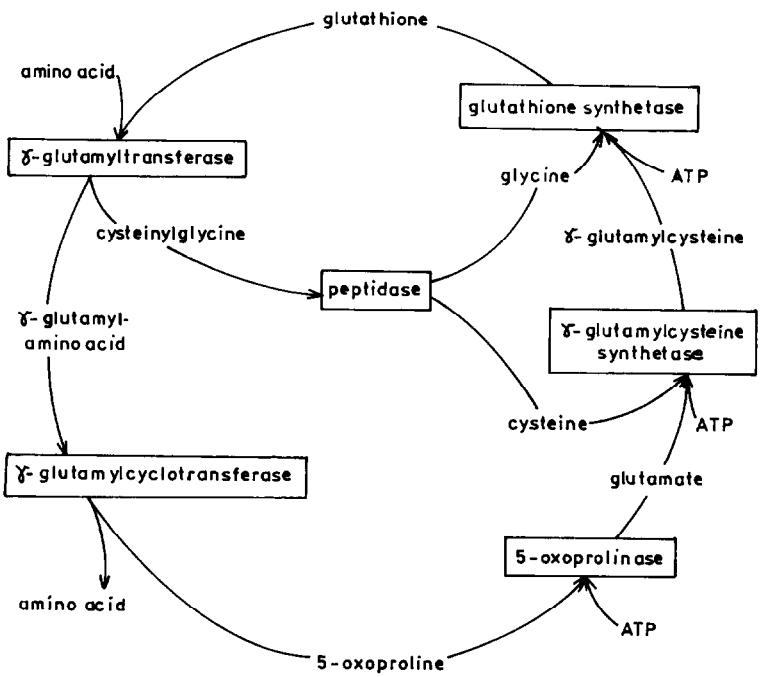

Fig.1. The γ-glutamyl cycle (according to refs. [1–4]).

regarding its alleged function. Among these are in vivo studies in mice on the rate of oxoproline formation [5], enzymatic data on the mode of action [6] and localization [7] of renal γ-glutamyltransferase, and observations on a patient with an inborn γ-glutamyltransferase deficiency [8].

In order to obtain further information on the physiological role of the cycle, this study reports on the measurements of the activities of three of the cycle's enzymes in the functional segments of the kidney responsible for the reabsorption of low molecular weight compounds.

The glomerulus, the proximal convoluted tubule, the proximal straight tubule and the distal tubule were prepared by microdissection of the rat nephron [9]. The results show a discrete distribution of the γ-glutamyl cycle enzymes along the nephron and a coincidence of the maximum specific activities in the proximal straight tubule. The absolute specific activities of the enzymes differ by decades in the order γ-glutamyltransferase >> glutamylcysteine synthetase >> 5-oxoprolinase.

2. Materials and methods

The radioactively labelled substrates L-[U^{14}C]-glutamate, spec. act. 285 mCi/mmol and 5-oxo-L-[U-^{14}C]proline, spec. act. 253 mCi/mmol, were purchased from Amersham Buchler, Ffm. and New England Nuclear GmbH, Dreieichenhain, FRG, respectively. Phosphoenolpyruvate, ATP, pyruvate-kinase, and dithioerythritol were obtained from Boehringer GmbH, Mannheim, FRG, Triton X-100 and *N*-morpholino-3-propionic acid (MOPS) from Serva GmbH, Heidelberg, FRG and L-methionine-DL-sulfoximine from Calbiochem Los Angeles, Calif., USA. Imidazolone carboxylate was synthesized according to ref. [10]. All other substances were 'pro Analysi' grade chemicals from Merck AG, Darmstadt, FRG.

2.1. *Microdissection and preparations of the samples*

The functional segments of the rat nephron were microdissected from lyophilized kidney slices as described in [9]. Up to 200–300 ng dry weight of single tubular portions were pooled for one analysis; each enzyme activity was determined three times. The samples were dissolved in 0.1 M MOPS, pH 7.8, 0.1% Triton X-100 and 5 mM dithioerythritol and placed in an ice bath for 30 min. For reference measurements, homogenate was prepared from 124 μg of whole lyophilized kidney slices by dissolving the tissue in 0.2 ml of the same buffer.

2.2. *Enzyme assays*

γ-Glutamyltransferase was measured with γ-glutamyl-*p*-nitroanilide as the substrate, according to ref. [11] with the following modifications: the buffer system was 0.1 M MOPS, pH 8.2, 0.1% Triton X-100; the reaction vols were 120 μl and 210 μl for the isolated structures and homogenate, respectively; the incubation temperature was 37°C.

γ-Glutamylcysteine synthetase activity was measured by determining the formation of glutamylcysteine from 5 mM cysteine and 44 μM L-[U-^{14}C]glutamate (0.45 μCi). The product was precipitated as the cadmium mercaptide and radiochemically determined as described in ref. [12]. The incubation conditions were modified in the following way: the reaction volume of 40 μl contained 0.1 M MOPS, pH 7.8, as buffer and additions of a mixture of 20 mM serine + borate, pH 7.8 and 10 mM imidazolone carboxylate as inhibitors of γ-glutamyltransferase [13] and 5-oxoprolinase [14] respectively. The incubation time varied between 1 and 3 h. From the actual initial rates the corresponding IU's defined for saturating substrate concentrations, were calculated by comparison of known enzyme activities (made with purified glutamylcysteine synthetase from bovine red cells [15]) determined under standard conditions with the conditions used here.

5-Oxoprolinase was tested by measuring the production of L-[U-^{14}C]glutamate from 5-oxo-L-[U-^{14}C]-proline under the following conditions: In the test volume of 20 μl 20 mM $MgSO_4$, 5 mM ATP, 5 mM phosphoenolpyruvate, 1 unit pyruvate kinase, 100 mM KCl, 5 mM L-methionine-DL-sulfoximine, 80 μM 5-oxo-L-[U-^{14}C]proline (free of glutamate purified by chromatography on cellulose, see below) 2 mM dithioerythritol and the sample in 100 mM MOPS, pH 7.8, were incubated at 37°C up to 3 h. The radioactive product was separated by thin-layer chromatography on cellulose in butanol/acetic acid/water (4 : 1 : 1). Radioactivity was compared with the pure substances and showed R_F-values of 0.24 (L-glutamate) and 0.55 (5-oxoproline) [16].

The time course of the reactions of glutamylcysteine synthetase and of 5-oxoprolinase were linear in time up to 3 h.

3. Results

Figure 2 a–c shows the specific activities of the enzymes γ-glutamyltransferase, glutamylcysteine synthetase and 5-oxoprolinase, in the pooled microdissected structures and the homogenate of rat kidney. It should be noted that the scales in the diagrams are very different and that they are interrupted in a and b. The histograms show that in the glomeruli the activities of all three enzymes are low or even absent. The structure which combines the enzymes of the γ-glutamyl cycle in high activities compared with the activities of the neighbouring segments is the proximal straight tubule, yet in extremely different absolute values. Taking the smallest value as 1, the ratios of the specific activities of 5-oxoprolinase : glutamyl-cysteine synthetase : glutamyltransferase equals 1 : 200 : 13 500. Although it is difficult to compare enzymatic rates measured in vitro with the actual in vivo conditions it can be stated that the activities of the three enzymes of the γ-glutamyl cycle are entirely different from each other.

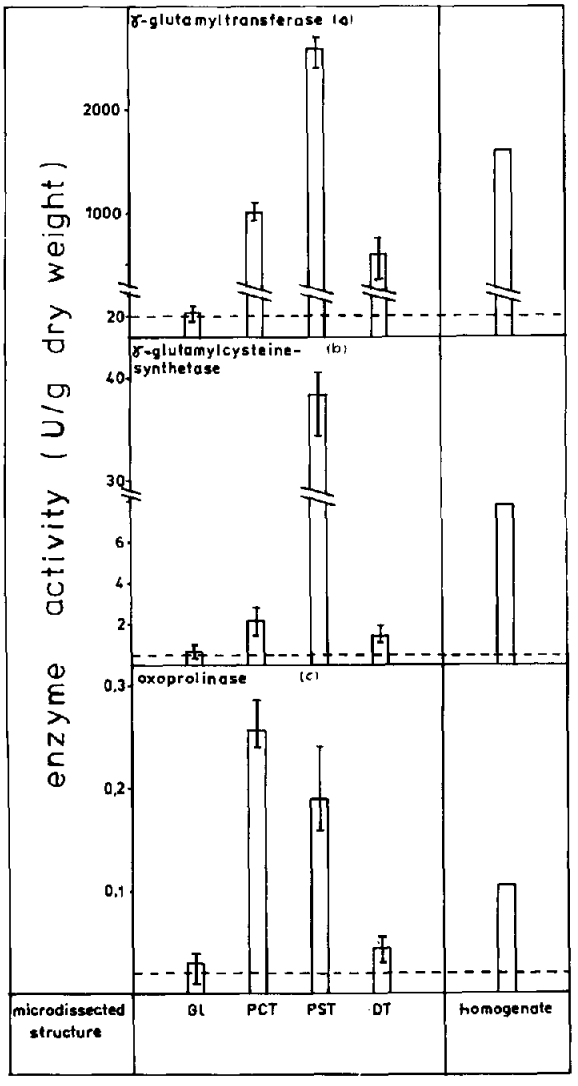

Fig. 2. Distribution of three enzymes of the γ-glutamyl cycle in various microdissected structures of rat nephron. The histograms show the specific activities of glutamyltransferase (a), glutamylcysteine synthetase (b), and 5-oxoprolinase (c) in pooled microdissected glomeruli (Gl), proximal convoluted tubules (PCT), proximal straight tubules (PST), distal tubules (DT) of rat kidney and homogenate from the organ. The dashed lines mark twice the blank of the determinations, the bars represent the minimum and the maximum single out of three independent determinations, the height of the column the arithmetic mean of the three determinations. The enzyme activities were measured according to the following principles:
(a) Formation of nitroaniline from γ-glutamyl-*p*-nitroanilide [11].
(b) Formation of [^{14}C]glutamyl – (^{12}C)cysteine [12].
(c) Formation of [^{14}C]glutamate from 5-oxo-[^{14}C]proline.
They are given as units (μmol product formed/min). For further details see Materials and methods.

The data show that the enzymes of the γ-glutamyl cycle are concentrated in the straight tubule, with the exception that the acitivity of 5-oxoprolinase is

of the same size in the convoluted tubule. The amounts of the lowest (5-oxoprolinase) and the highest (glutamyltransferase) enzyme activities, however, differ by at least four orders of magnitude in the straight tubule.

4. Discussion

The three enzymes investigated in this study were taken as representative of the γ-glutamyl cycle for the following reasons:

(a) γ-Glutamylcysteine synthetase starts the biosynthesis of glutathione from the constituent amino acids. In many organs this has been shown to be the rate-limiting step of the synthesis in vivo [17].

(b) γ-Glutamyltransferase initiates the degradation of glutathione as well as the hypothetical translocation of the amino acid to be transported.

(c) 5-Oxoprolinase links the degradation and the biosynthesis of the carrier molecule. In vivo findings [5,18] and enzymatic data [19] suggest that this step is the limiting reaction in the cycle.

Indeed the data reported here support the view that the conversion of 5-oxoproline to glutamate represents the rate-limiting step of the γ-glutamyl cycle. What seems to be very strange in an enzyme system operating in a cyclic sequence is the fact that the activities of the key enzymes are extremely unbalanced. Furthermore, the enzymes of the cycle are simultaneously concentrated on the straight portion of the tubule and not in the convoluted tubule, where the main amino acid reabsorption takes place [20,21]. Fortunately, the transport rate of an amino acid which is a potential glutamyl cycle candidate has been quantitatively determined by microperfusion of rat kidney proximal tubules: Silbernagl et al. found a rate of 2.5×10^{-11} mol/cm/s for L-methionine [22] which is an excellent substrate for γ-glutamyltransferase [23]. Based on a value of 260–300 ng dry weight/mm length of tubule [24] we calculated from our experiments maximal activities of 1.2×10^{-10} mol/cm/s for γ-glutamyltransferase and 1×10^{-14} mol/cm/s for 5-oxoprolinase in the straight part of the tubule. This suggests that although the γ-glutamyltransferase activity is sufficiently high, the turnover of the cycle which is dictated by the slowest step, the oxoprolinase reaction, is by far too slow to account for the observed transport rate for methionine.

In summary, the results and the implicated considerations force the conclusion that the physiological function of the γ-glutamyl cycle has to be re-evaluated.

Acknowledgements

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