Analysis of Kinetic Properties of γ-Glutamyl Transpeptidase from Rat Kidney

L. DVOŘÁKOVÁ, J. KRŮŠEK, F. ŠŤASTNÝ and V. LISÝ

Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic

Abstract. The initial rate kinetics of rat kidney γ-glutamyl transpeptidase were measured using L-γ-glutamyl-*p*-nitroanilide and glycyl-glycine as the donor and the acceptor substrate, respectively. Experimental data were fitted with the initial rate equation, and the obtained results indicated that: (1) Michaelis constants for transpeptidation (K_b), autotranspeptidation (K_a) and hydrolysis (K_h) are 8.56 mmol/l, 2.02 mmol/l and 0.005 mmol/l, respectively. (2) The maximum rate of transpeptidation (V_b) exceeds that of hydrolysis (V_h) and autotranspeptidation (V_a) 160 times and 5 times, respectively. (3) A comparison of the ratios of maximal rate: Michaelis constant of individual reactions shows that hydrolysis is approximately 10 times more efficient than the remaining two reactions. (4) Under routine conditions used for γ-glutamyl transpeptidase estimation, transpeptidation is the prevalent reaction.

Key words: γ-Glutamyl transpeptidase — Kinetics — Rat — Kidney

Abbreviations: GGT, γ-glutamyl transpeptidase; GpNA, γ-glutamyl-*p*-nitroanilide; Gly-Gly, glycyl-glycine.

Introduction

γ-Glutamyl transpeptidase (GGT; E.C.: 2.3.2.2) is a widely distributed membrane bound glycoenzyme with the highest activity found in the kidney (Tate and Meister 1981). As it is the only known enzyme able to cleave the γ-glutamyl bond (Spiesky et al. 1990), it has been suggested to participate in the metabolism of various γ-glutamyl compounds, such as glutathione or leukotrienes, and in the transport of amino acids and small peptides across the plasma membrane (Tate and Meister 1981; Anderson et al. 1982; Jankásková et al. 1992). GGT is of interest not only

Correspondence to: Václav Lisý, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic, E-mail: lisy@biomed.cas.cz

because of its physiological functions but also because of its clinical significance For example changes in its activity as well as in the structure of its saccharide moiety can serve as an indicator of hepatobiliary disease (Kottgen et al 1978 Boelsterli 1979 Blotman and Prince 1988)

GGT catalyzes both the hydrolysis of γ-glutamyl compounds and the transfer of γ-glutamyl residue from these donor substrates (A) to acceptor amino acids or small peptides (B) In addition some donors may also act as acceptor substrates (Allison 1985) Thus GGT catalyzes three types of reactions

1) transpeptidation γ-Glu-A + B → γ-Glu-B + A
2) autotranspeptidation γ-Glu-A + γ-Glu-A → γ-Glu-γ-Glu-A + A
3) hydrolysis γ-Glu-A + H_2O → Glu + A

Furthermore, inhibition by the acceptor substrate has to be considered (Allison 1985) Analysis of the enzyme kinetics in such a complicated system which includes all the mentioned reactions is quite difficult Consequently one of the reactions is often omitted in reports concerning this problem (Thompson and Meister 1976 Shaw et al 1977, Allison 1985) However, the routine determination of GGT activity (Tate and Meister 1974) as well as the estimation of the apparent Michaelis constant from Lineweaver Burk plot (Reyes and Barela 1980) are usually performed in a reaction mixture containing millimolar concentrations of L-γ-glutamyl-*p*-nitroanilide (L-GpNA) and glycyl-glycine (Gly-Gly) as the donor and the acceptor of the γ-glutamyl moiety, respectively In this case, any of the reactions mentioned above can not be excluded The kinetic model involving the complex of all GGT catalyzed reactions has not yet been described Thus, the initial rate equation which corresponds to this model and is valid for conditions of routine GGT activity estimation is being provided in this report and is applied to the determination of kinetic constants for rat kidney GGT The reaction scheme used for our model of GGT kinetics is based on the kinetic mechanism described by Allison (1985) to which reaction steps belonging to autotranspeptidation are added

Materials and Methods

All chemicals were purchased from Sigma Chemical Co

The crude membrane fraction (P_2 fraction) was prepared from the kidneys of 50-day-old female Wistar rats by the method of Cotman and Mathews (1971) The P_2 fraction lysed in 5 mmol/l Tris-HCl buffer, pH 7 5 was solubilized with 0 5% Triton X-100 in 50 mmol/l Tris-HCl, pH 7 5 The supernatant after centrifugation (13,000 × *g*, 20 min) was used for analysis (Kottgen et al 1976 Dvořáková et al 1992) All steps were performed at 4 °C

Initial rate measurements were performed in 80 mmol/l Tris-HCl buffer, pH 9 0 (Tate and Meister 1974), containing 75 mmol/l NaCl The concentration of

the donor substrate, L-GpNA, ranged from 0.05 mmol/l to 2.5 mmol/l. The acceptor substrate, Gly-Gly, was maintained at the concentration of 20 mmol/l in one set, and was omitted in the other set of experiments. In GGT catalyzed reaction, GpNA is split into a *p*-nitroaniline and a γ-glutamyl residue. Consequently, in the first set of experiments, γ-glutamyl-glycyl-glycine, γ-glutamyl-γ-glutamyl-*p*-nitroaniline, and glutamate are formed by transpeptidation, autotranspeptidation and hydrolytic reaction, respectively. In the absence of Gly-Gly only hydrolysis and autotranspeptidation can occur. The initial rate was determined on the basis of the formation of *p*-nitroaniline which can be monitored by the increase of absorbance at 410 nm. The absorbance was recorded continuously for 3 min at 37 °C using a Spectrophotometer PU 8700 (Philips, Netherlands). One unit (U) of GGT activity was defined as the amount of the enzyme which catalyzes the formation of 1 μmol of *p*-nitroaniline per minute (Tate and Meister 1974).

The kinetic constants were estimated by non-linear fit (Marquardt – Levenberg algorithm, Sigmaplot 4.1, Jandel Scientific) of the initial rate equation to the experimental data. The mathematical model, based on the scheme shown in Fig. 1, was derived using the method of King and Altman (1956).

Results and Discussion

The reciprocal form of the initial rate equation involving all types of GGT catalyzed reactions mentioned above (Fig. 1) was derived and drawn up using Cleland symbols (Cleland 1963).

$$v = \frac{V_h[A]\left(1 + \frac{[B]}{K_{iab}}\right) + [A]^2\frac{V_a}{K_a}}{[A]\left(1 + \frac{[B]}{K_b}\right) + \frac{[A]^2}{K_a} + K_h\left(1 + \frac{[B]}{K_{iab}}\right)\left(1 + \frac{[B]}{K_i}\right)} \tag{1}$$

[A] and [B] are concentrations of the donor and the acceptor substrates, respectively. All kinetic constants, defined in Table 1, are comprised in equation (1), when V_b is incorporated into the partitioning constant K_{iab}.

In the absence of the acceptor substrate ([B] = 0) equation (1) is reduced to equation (2).

$$\frac{1}{v} = \frac{\frac{K_h}{[A]^2} + \frac{1}{[A]} + \frac{1}{K_a}}{\frac{V_h}{A} + \frac{V_a}{K_a}} \tag{2}$$

In the course of the first set of measurements the concentration of the acceptor substrate [B] is held constant and can thus be combined with the kinetic constants from equation (1). The constant members of equations (1) and (2) can then be

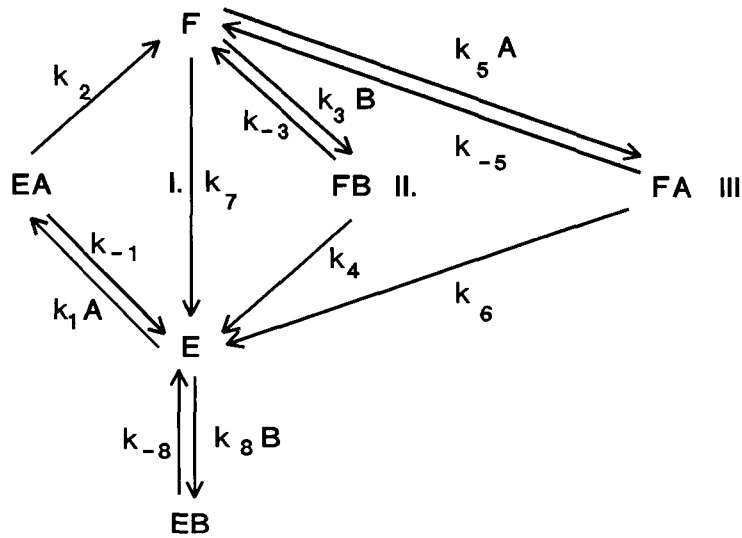

Figure 1. Reaction scheme for simultaneous hydrolysis (I) transpeptidation (II) and autotranspeptidation (III) catalyzed by GGT. Free enzyme and γ-glutamyl enzyme are denoted F and F' respectively. A and B correspond to the donor and acceptor substrates respectively. Competitive inhibition by the acceptor substrate is included.

transformed to new parameters which are included in equations (3) and (4)

$$\frac{1}{v} = \frac{\dfrac{a}{[A]^2} + \dfrac{b}{[A]} + c}{\dfrac{d}{[A]} + e} \tag{3}$$

$$\frac{1}{v} = \frac{\dfrac{f}{[A]^2} + \dfrac{1}{[A]} + c}{\dfrac{g}{[A]} + e} \tag{4}$$

The parameters are defined in Table 2.

To obtain the sets of experimental data the initial rate was measured both in the presence and in the absence of the acceptor substrate under varying enzyme concentrations (Fig. 2). The resulting data were fitted with equations (3) and (4) respectively, using the non-linear least-squares-regression analysis. By this computer fitting, the constant parameters a, b, c and f were obtained (Table 3). Comparing equations (3) and (4) it can be seen that the parameters c and e are present in both of these equations. Under the condition of identical enzyme activity

Table 1. Definition of kinetic constants

K_h	Michaelis constant for donor	$\frac{k_7(k_{-5}+k_6)(k_4+k_{-3})(k_{-1}+k_2)}{(k_{-3}+k_4)[k_5k_6(k_{-1}+k_2)+k_1(k_{-5}+k_6)(k_7+k_2)]}$
K_i	Inhibition constant for acceptor	$\frac{k_{-8}}{k_8}$
K_b	Michaelis constant for acceptor	$\frac{(k_{-3}+k_4)[k_5k_6(k_{-1}+k_2)+k_1(k_{-5}+k_6)(k_7+k_2)]}{k_1k_3k_{-5}+k_6)(k_2+k_4)+\frac{1}{K_a k_5k_6(k_{-3}+k_4)}}$
K_a	Michaelis constant for donor acting as acceptor	$\frac{(k_{-3}+k_4)[k_5k_6(k_{-1}+k_2)+k_1(k_{-5}+k_6)(k_2+k_7)]}{k_1k_5(k_{-3}+k_4)(k_2+k_6)}$
V_1	Maximum relative rate for hydrolysis	$\frac{k_1k_2k_7(k_{-3}+k_4)(k_{-5}+k_6)}{(k_{-3}+k_4)[k_5k_6(k_{-1}+k_2)+k_1(k_{-5}+k_6)(k_2+k_7)]}$
V_b	Maximum relative rate for transpeptidation	$\frac{k_1k_2k_3k_4(k_{-5}+k_6)}{k_1k_3(k_{-5}+k_6)(k_{-4}+k_4)+\frac{1}{K_a k_5k_6(k_{-3}+k_4)}}$
V	Maximum relative rate for autotranspeptidation	$\frac{k_1k_2k_5k_6(k_{-3}+k_4)}{k_1k_5(k_{-3}+k_4)(k_2+k_6)}=\frac{k_2k_6}{k_2+k_6}$
K_{eb}	Partitioning constant	$\frac{K_bV_1}{V_1}=\frac{k_{-1}(k_{-3}+k_4)}{k_3k_4}$

used for the two sets of experiments (i.e. those performed in the presence and in the absence of the acceptor substrate) these two parameters can be used to inspect the correctness of the computer fitting. The parameters containing the maximum relative rate (i.e. parameters d, e and g) as well as the maximum relative rates themselves could be estimated just for each individual experiment. Their values are not constant as a solubilized membrane fraction containing only partially purified enzyme was used for the initial rate measurements. However their ratio is constant and can be determined independently of the degree of the enzyme purity (Table 3).

The curves constructed on the basis of equations (3) and (4) are shown in Fig. 2. The nonlinearity of the curves which in the case of Fig. 2A simulates substrate inhibition arises from the nature of equations (3) and (4). If these equations

Table 2. Definition of parameters from equations (3) and (4)

a	$K_I\left(1+\frac{[B]}{K_{IA}}\right)\left(1+\frac{[B]}{K_B}\right)$	e	$\frac{1}{K_B}$
b	$1+\frac{[B]}{K_I}$	f	K_I
c	$\frac{1}{K_B}$	g	V_I
d	$V_h\left(1+\frac{[B]}{K_{IAB}}\right)$		

are handled as a ratio of two polynomials they can be rewritten in the following way

$$\frac{1}{v}=\frac{a}{d}\frac{1}{[A]}+\frac{b-a\frac{e}{d}}{d}+\frac{c-\frac{e}{d}(b-a\frac{c}{d})}{d\frac{1}{[A]}+c} \tag{5}$$

$$\frac{1}{v}=\frac{f}{g}\frac{1}{[A]}+\frac{1-f\frac{e}{g}}{g}+\frac{c-\frac{c}{g}(1-f\frac{e}{g})}{g\frac{1}{[A]}+c} \tag{6}$$

The right side of equations (5) and (6) can be divided into two parts in which the first two members correspond to a linear relationship while the third hyperbolic member is responsible for the curvature in the range of high donor substrate concentrations Because of the limited solubility of GpNA all experimental data shown in Fig 2 4 are situated in the linear part of the curve Thus under the conditions described in this paper the upward curvature only corresponds to the theoretically calculated result However the shape of the curve was experimentally demonstrated using another donor substrate, L γ-glutamyl 3-carboxy-4-nitroanilide the solubility of which is substantially higher (PetitCleir et al 1980 Solberg et al 1981) Fig 2*B* corresponds to the situation when no acceptor substrate is present (equation (4) or (6)) It is obvious that the hyperbolic member appears at substantially lower concentrations of the donor substrate when compared with Fig 2 4 Therefore the third member in equation (6) has to have a higher value than that in equation (5) Evidence for this fact can be obtained by introducing numerical values of the parameters (Table 3)

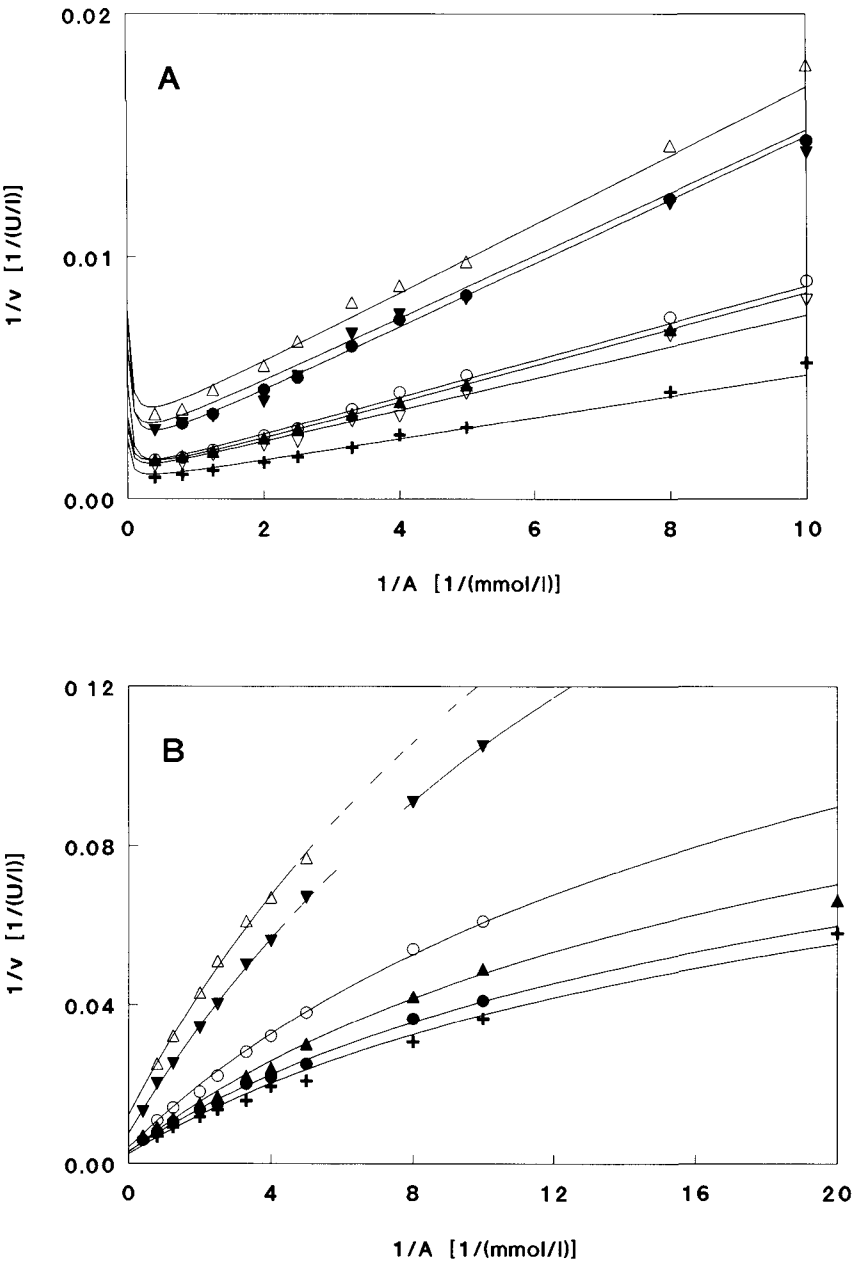

Figure 2. Double-reciprocal plots of initial rate vs donor substrate (L-GpNA) *A* In the presence of acceptor substrate (20 mmol/l Gly-Gly) *B* In the absence of acceptor substrate Lines were drawn using the parameter values of Table 3 with equation (3) (*A*) and equation (4) (*B*) Total activity of the samples (*A*) ▼ 361 U/l, △ 289 U/l ○ 631 U/l + 1148 U/l, ▲ 639 U/l, ● 348 U/l, ▽ 748 U/l (B) ▼ 361 U/l, △ 289 U/l, ○ 631 U/l + 1148 U/l, ▲ 781 U/l, ● 925 U/l

Table 3 Parameters and their ratios computed from equations (3) and (4) using the non linear least squares regression analysis

Parameter		Parameter ratio	
a	2.2 ± 0.2	$\frac{e}{g}$	15.6 ± 0.5
b	3.1 ± 0.3	$\frac{d}{e}$	23.6 ± 1.1
e	0.19 ± 0.09	$\frac{d}{g}$	397 ± 35
f	0.0051 ± 0.0003		

Note Each value represents the mean of parameter values obtained from fitting of equations (3) and (4) to 4–7 sets of experimental data ± SD

Table 4. Kinetic constants of rat kidney GGT

K_I	0.0051 ± 0.0003	$\frac{V}{V_t}$	35.0 ± 5.7	$\frac{V_t K}{V K_t}$	12.1 ± 0.7
K	2.0 ± 0.4	$\frac{V_t}{V}$	4.7 ± 0.7	$\frac{V_t K}{V K_t}$	1.2 ± 0.1
K_t	8.6 ± 1.1	$\frac{V_t}{V}$	16.1 ± 2.1	$\frac{V_t K_t}{V_t K_t}$	9.9 ± 0.7
K	> 100				

Note Definition of symbols used for kinetics constants is given in Table 1 Constant values K_I K K_t and K are given in mmol/l The data represent the mean of 5–7 experiments ± SD

The actual kinetic constants were calculated (Table 4) on the basis of parameter definitions shown in Table 2 The value of K_I is almost the same as that published by Thompson and Meister (1976) who estimated $K_I = 5.6$ μmol/l At the organ level the value of K_t shown in Table 4 is similar to K of hog kidney GGT ($K = 2.08$ mmol/l $K_t = 24.9$ mmol/l) (London et al 1976) while K_I rather corresponds to K_I of human GGT (serum $K = 7.98$ mmol/l $K_I = 12.5$ mmol/l liver $K_t = 7.2$ mmol/l $K_I = 10.6$ mmol/l kidney $K_t = 9.7$ mmol/l $K_I = 10.7$ mmol/l) (Shaw et al 1978)

The ratio of the maximum relative rate of the two reactions was estimated If GGT is saturated with its substrates the maximum relative rate of transpeptidation V_t exceeds that of hydrolysis V_I and autotranspeptidation V 160 times and 5 times respectively The maximum relative rate of autotranspeptidation V_a

Table 5. Contributions of individual reactions to the total value of initial rate when the donor substrate concentration equals K_t (i.e. 2.0 mmol/l)

Acceptor substrate concentration (mmol/l)	v_h	v_a	v_t
0	2.4%	96.98%	
20	0.5–1%	19–22%	77–80%

Note. v_h, v_a and v_t represent the initial rates of hydrolysis, autotranspeptidation and transpeptidation respectively.

is 35 times higher than that of hydrolysis V_h (Table 4). Using the value of V_i/K_s (i is h, h or a) as a measure of catalytic efficiency and enzyme specificity, it has been found that hydrolysis is the most efficient reaction, while the efficiency of both transpeptidation and autotranspeptidation is about 10 times lower (Table 4). However, a suitable substrate concentration can suppress a more efficient reaction, while another reaction becomes dominant. When the donor substrate concentration reaches the value of K_t (i.e. 2 mmol/l), the contribution of autotranspeptidation to the final value of initial rate is 1/2 V_a. Using equations (3) and (4) with parameter values listed in Table 3, the contributions of the other two reactions may be calculated (Table 5). This Table gives the approximate relative contributions of individual reactions occurring under standard experimental conditions, as the K_t value is close to the donor substrate concentration routinely used for GGT activity estimation (i.e. 2.5 mmol/l). Because the acceptor substrate is usually present in the reaction mixture, transpeptidation is the prevalent reaction, while autotranspeptidation and hydrolysis represent approximately 20% and 1% of the total value, respectively.

For the analysis of the kinetic properties presented in this paper, a relatively high specific activity of GGT was required. However, certain simplification is essential when kinetic properties of GGT are to be analyzed from sources with low activity. The simplification is based on further analysis of equation (5), which results in a definition of the constant of half saturation (K_{hs}, formal analog of Michaelis constant of one-substrate reaction) (Dvorakova et al. 1996). Although the estimation of K_h is usually sufficient for characterization of GGT kinetic properties (Reyes and Barela 1980), the complex reaction mechanism should be kept in mind. We suppose that the model proposed here can contribute to the understanding and interpretation of the kinetics of GGT-mediated reactions.

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