Biochimica et Biophysica Acta, 658 (1981) 220—231

BBA 69231

SOME KINETIC PROPERTIES OF γ-GLUTAMYLTRANSFERASE FROM RABBIT LIVER

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(Received April 8th, 1980)
(Revised manuscript received October 24th, 1980)

Key words γ-*Glutamyltransferase, Glycylglycine, (Rat liver)*

Summary

γ-Glutamyltransferase ((5-glutamyl)-peptide. amino-acid 5-glutamyltransferase, EC 2.3.2.2) of rabbit liver (detergent form) was purified 1100-fold in order to study its kinetic properties. Kinetic studies were conducted from pH 6.0 to 12.0 in the absence and presence of the acceptor substrate glycylglycine using γ-glutamyl-3-carboxy-4-nitroanilide as the donor.

The existence of more than one binding site for both donor and acceptor is postulated on kinetic evidence such as donor substrate activation, donor substrate inhibition and acceptor substrate activation.

Homotropic interaction is also observed, in the form of negative cooperativity, in donor substrate binding, in the absence of acceptor at pH less than 9.0 and positive cooperativity ($n = 2$), in the absence or presence of acceptor at pH greater than 9.0.

Hydrolase reaction reaches a maximum of activity at pH 10 (pK 8.6). Transferase activity under conditions of maximal velocity is maximal at pH 9.0 (pK 7.1). The ratio of transferase activity/hydrolase activity is maximal at pH 7.0—7.5. At low donor substrate concentrations, maximal activity is attained at pH 7.5.

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Autotransfer is not thought to play a significant role in the conditions used for the study.

Studies done with rat kidney brush border membrane at pH 6 and 7.5 corroborate the findings with the purified enzyme.

Introduction

γ-Glutamyltransferase ((5-glutamyl)-peptide amino-acid 5-glutamyltransferase, EC 2.3.2.2) which catalyzes the transfer of the γ-glutamyl moiety from γ-glutamyl peptides or synthetic substituted amides on to different peptides or aminoacids acceptors is also capable of autotransfer and has some glutaminase activity. Kinetic studies done at pH 8—8.5 suggested a ping-pong mechanism [1—8]. It is very likely that a covalent γ-glutamyl enzyme intermediate is formed [1,3,4,6,8—11] and that deacylation is the rate-limiting step since all glutamyl donors have the same efficiency regardless of the leaving group.

Many authors have reported donor substrate inhibition when the enzyme was assayed with γ-glutamyl-carboxynitroanilide [2,12] or nitroanilide [4,13] and glycylglycine [2,12—14], which is not readily explained by a ping-pong mechanism.

Interested in rabbit liver γ-glutamyltransferase as a marker of drug-metabolizing enzyme induction [15], we purified and studied the kinetic properties of this enzyme. Results obtained under conditions of maximal velocity at different pH value with and without acceptor, help us to understand the mechanism of action of γ-glutamyltransferase. Some kinetic experiments were also done on rat kidney brush border membranes to see the influence of binding of γ-glutamyltransferase to natural membrane on the mechanism.

Materials and Methods

Materials

L-γ-Glutamyl-3-carboxy-4-nitroanilide was purchased from Boehringer Mannheim and glycylglycine from Merck Darmstadt. DEAE-Sephacel and Con A-Sepharose were bought from Pharmacia Fine Chemicals, Uppsala, and Ultrogel from Industrie Biologique Française. Bovine serum albumin and α-methyl-D-mannoside were obtained from Sigma Chemical Co., U.S.A. and sodium deoxycholate and Lubrol WX from Fluka Buchs. Other chemicals used were of analytical reagent grade.

Purification of γ-glutamyltransferase

Purification procedure was carried out according to a modification of the method of Huseby [12] for the rabbit liver. Fauve de Bourgogne male rabbits (1.8—2 kg) received phenobarbital (50 mg/kg per day) for 4 days subcutaneously in the flank and were killed on the 5th day. The livers were excised immediately after death and homogenized.

Homogenization, solubilization of the enzyme from the membranes and acetone precipitation, butanol treatment and $(NH_4)_2SO_4$ precipitation were carried out according to the method of Huseby [12]. However, when a mixture

of Lubrol and sodium deoxycholate (final concentration of 5 $g \cdot l^{-1}$ each) was used to remove the enzyme from plasma membranes, all subsequent steps were carried out in the presence of Lubrol unless otherwise stated.

The preparation was then passed through DEAE-Sephacel and Ultrogel AcA-22. The column (60 × 3 cm) was equilibrated with 0.01 M Tris-HCl buffer (pH 8 0)/0.15 M NaCl/0 2 mM $CaCl_2$/0.2 mM $MnCl_2$/2 $g \cdot l^{-1}$ Lubrol. The active fractions collected from DEAE-Sephacel were applied and eluted with the same buffer. The active fractions were then dialyzed against the same buffer without Lubrol.

The sample was passed on Con A-Sepharose. The column (10 × 2 cm) was equilibrated with Tris-HCl buffer/$MnCl_2$/$CaCl_2$/NaCl, as above.

The enzyme solution was applied and the column washed with buffer until the effluent showed no absorbance at 280 nm. Elution was carried out with 1 M α-methyl-D-mannoside dissolved in buffer. 1 ml fractions were collected

Analytical methods

Protein concentration was estimated by the method of Lowry et al [16] with bovine serum albumin as a standard.

Kinetic experiments were carried out on a Rotochem IIa Analyzer in 0.1 M Tris-HCl buffer/0.1 M sodium acetate. Increasing concentrations of L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine were studied at various pH values

Assays were carried out in the following manner: 0.3 ml Tris-acetate/glycylglycine buffer was mixed with 0.05 ml L-γ-glutamyl-3-carboxy-4-nitroanilide, 0.05 ml enzyme and 0.2 ml distilled water. The rate of *p*-nitroanilide liberation at 30°C was determined from the increase in absorbance at 405 nm.

Kinetic parameters were obtained graphically according to Lineweaver-Burk plots V and $K_{0.5}$ values have been calculated by extrapolating linear fractions of the curves or taking two V values in cases of nonlinearity.

Results

The rabbit γ-glutamyltransferase was purified 1100-fold in the presence of Lubrol to a specific activity of 13.5 $\mu g \cdot min^{-1} \cdot mg^{-1}$ (Table I).

The enzyme was much less active than that reported by Huseby [12] for the human γ-glutamyltransferase. The apparent molecular weight was determined at about 200 000 by chromatography on Ultrogel AcA-34, which is consistent with the heavy form of γ-glutamyltransferase.

Kinetic studies

Hydrolase reaction in the absence of acceptor. When the concentration of L-γ-glutamyl-3-carboxy-nitroanilide (GluCNA) is increased at pH <9.0, a very important substrate activation is observed (Fig. 1A). Donor substrate activation disappears as pH increases. At pH 9.0—10.5, kinetics look Michaelian but become non-Michaelian again at pH >10.5 (Fig. 1B and inset)

The pH dependency of the hydrolase reaction is shown on Fig. 2B. The maximal rate (V) reaches a maximum at pH 9.5 and remains constant up to pH 12.0 and is dependent on an ionizing group of pK 8.6—9.0 This is true for

TABLE I

PARTIAL PURIFICATION OF γ-GLUTAMYLTRANSFERASE FROM RABBIT LIVER

Step	Volume (ml)	Total protein (mg)	γ-Glutamyltransferase activity		Purification (-fold)
			Total/U	Specific (U mg^{-1})	
Homogenate	4392	178 667	2144	0 012	—
Detergent extract	3107	59 600	2086	0 035	2 9
Acetone precipitation	2000	33 363	1949	0 058	4 8
Butanol treatment	936	3018	637	0 21	17 5
$(NH_4)_2SO_4$ precipitation	170	1240	431	0 35	29 2
DEAE-Sephacel	22	181	145	0 80	67
Ultrogel	10 5	8	31 2	3 9	325
Affinity chromatography	3	0 68	9 2	13 5 *	1125

* The molecular weight was estimated by Ultrogel AcA-34 chromatography as approx 200 000

both high and low affinity sites. Fig. 2A describes the variation of $K_{0.5}$ as a function of pH for both high and low affinity sites, indicating a large difference in the sites at pH 7.0 which vanishes at pH >9.5.

Transferase reaction in the presence of acceptor. At pH 7 0, where two sites seem to be kinetically active with the donor substrate, the increase in the acceptor glycylglycine concentration, at constant 1 mM GluCNA, points to the existence of two binding sites for glycylglycine. Fig. 3A clearly shows the anti-cooperative binding at pH 7.0. It should be noted that the rate in the absence of acceptor was substracted because it was not negligible at low concentrations of acceptor. Anticooperative binding disappears at alkaline pH where only one high affinity binding site can be seen (Fig. 3B). Binding of glycylglycine follows the same pattern as that of GluCNA as a function of pH.

Glycylglycine activates the transformation of GluCNA in taking part into the transferase reaction. At pH <9.0, activation is observed up to 200 mM glycyl-glycine (Fig. 4) possibly due to the action of a second site and at pH >9.0, the activation is maximal at 50 mM. It should be emphasized that the $K_{0.5}$ value of the high affinity site for glycylglycine barely varies from pH 7.0 to 11.0 (14—20 mM).

While in the absence of acceptor, activation by donor substrate is observed, the presence of glycylglycine on the acceptor site induces a change in kinetics. Anticooperativity is still seen but inhibition by substrate is the result (Fig. 4A and 4B). Interestingly enough, this change occurs already at 5 mM glycylgly-cine (data not shown) indicating that the binding of the acceptor on the high affinity site is determinant.

Indeed, increasing the concentration acceptor does not seem to affect donor substrate inhibition but stimulates the transferase activity as indicated by the effect of glycylglycine on Fig. 5

Donor substrate inhibition disappears at alkaline pH, following the same pattern as that of the activation in the absence of acceptor.

The pH variation of V transferase is very different from that of hydrolase (Fig. 6A). The pK of 7.1, is almost 2 pH units lower than that measured in the

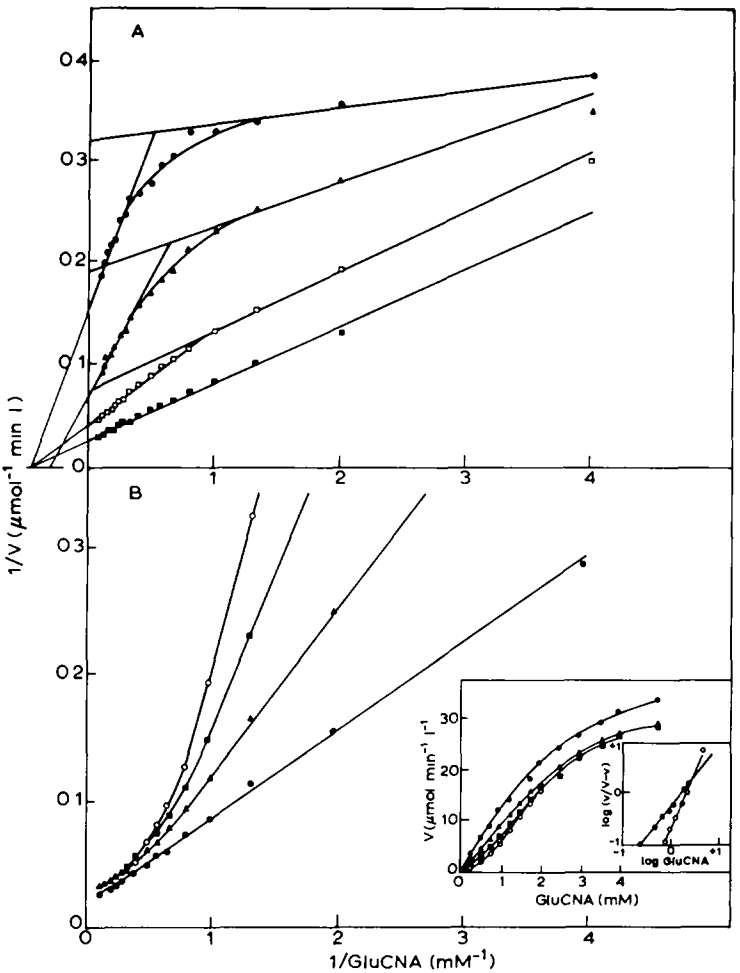


Fig 1A Double-reciprocal plot of the activity vs γ-glutamyl-3-carboxy-4-nitroanilide (GluCNA) at different pH values (●——●, 7 5, ▲——▲, 8 0, □——□, 8 5 and ■——■, 9 0) and in absence of glycylglycine Inset table summarizes the kinetic parameters calculated from the plot

pH	K_1 (mM)	V_1 (μmol min^{-1} l^{-1})	K_2 (mM)	V_2 (μmol min^{-1} l^{-1})	V_1/V_2
7 5	2 4	6 6	<0 1	3 1	2 1
8 0	3 3	14 4	0 3	5 5	2 6
8 5	2 5	25 0	0 9	14 1	1 8
9 0	2 1	36 9			

Fig. 1B Double-reciprocal plot of the activity vs γ-glutamyl-3-carboxy-4-nitroanilide (GluCNA) at different pH values (●——●, 10 0, ▲——▲, 10 5, ■——■, 11 0 and ○——○, 11 5) and in absence of glycylglycine Inset figures are shown to illustrate the apparence of positive cooperativity as pH increases

absence of glycylglycine (Fig. 2) Such a shift in pK is compatible with a change in catalysis from hydrolase to transferase, or with an important interaction of the acceptor molecule with the active site residue involved in the acylation by donor substrate. We must also consider that acceptor binding may

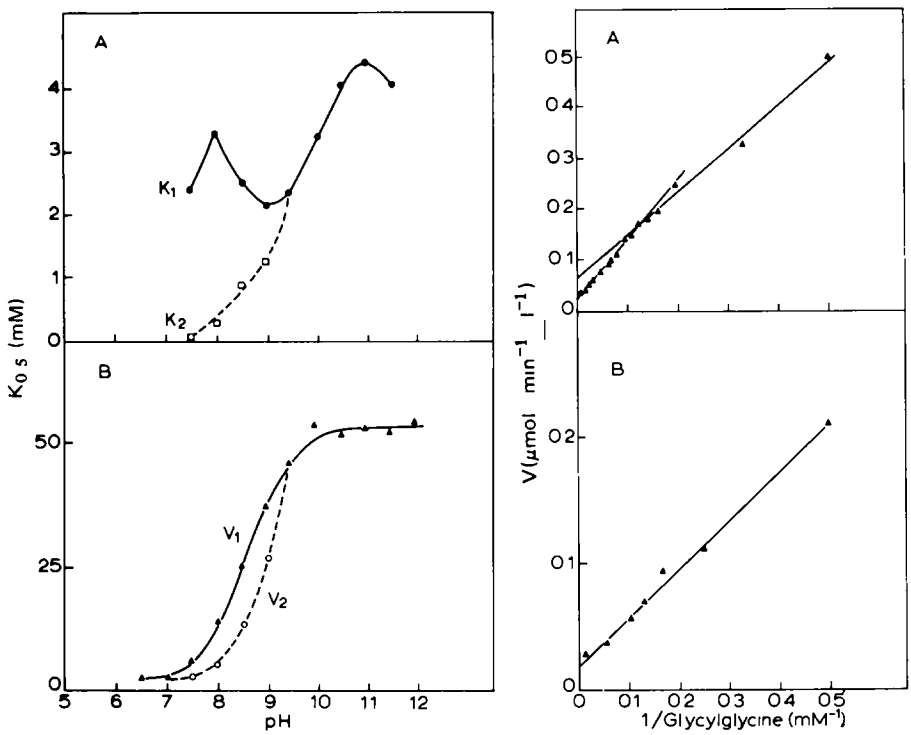


Fig 2 Kinetic parameters, V and $K_{0\,5}$ as a function of pH, in absence of acceptor (A) K_1 (●———●) is the $K_{0.5}$ of the low affinity site which barely varies with pH K_2 (□———□) is the $K_{0.5}$ of the high affinity site which increases to the level of K_1 at pH 9 5 At higher pH, only one binding site is operating or both have the same affinity (B) V_1 (▲———▲) is V of the low affinity site and V_2 (○———○) V of the high affinity site The pK of V_1 is 8 6

Fig 3A Double-reciprocal plot of the activity vs glycylglycine in presence of 1 mM GluCNA at pH 7 0 Note that v-v_0 is plotted here v_0 is determined in the absence of glycylglycine Low affinity site has a $K_{0\,5}$ of 50 mM and the high affinity site a $K_{0\,5}$ of 13 3 mM The ratio of V_1/V_2 = 3 B Double-reciprocal plot of v v_0 vs glycylglycine at 10 mM GluCNA at pH 10 5 v_0 is the activity determined in absence of glycylglycine Only one site or two sites of equal affinity are seen with a $K_{0\,5}$ of 20 mM

expose a new active site residue which contributes to transferase catalysis.

As shown in Fig. 6B, the pH dependency of $K_{0\,5}$ was studied in the presence of 100 mM glycylglycine. The $K_{0\,5}$ value increases at alkaline pH with a pK value of 8.6. It is very unlikely that increase in $K_{0\,5}$ is due to the presence of glycylglycine acting as a competitive inhibitor, since $K_{0\,5}$ also increases (pK = 9.0) in the absence of glycylglycine (Fig. 2) GluCNA has a pK of 9.3 and might be responsible in part for the variation of $K_{0\,5}$.

Fig. 6C represents $V/K_{0\,5}$ vs. pH. It is noteworthy that γ-glutamyltransferase has its peak of activity at pH 7.5 at low donor substrate concentration The two pK values, 6.3 and 9.3, are likely to be those of the carbonyl group of the donor in the acyl enzyme intermediate (histidine), and for the proper orientation of the acceptor (α-$\overset{+}{N}H_3$). One cannot exclude the pK of the donor GluCNA.

Depending on the concentration of the donor and the acceptor both hydrolase and transferase are likely to operate at the same time. Fig. 7 represents the

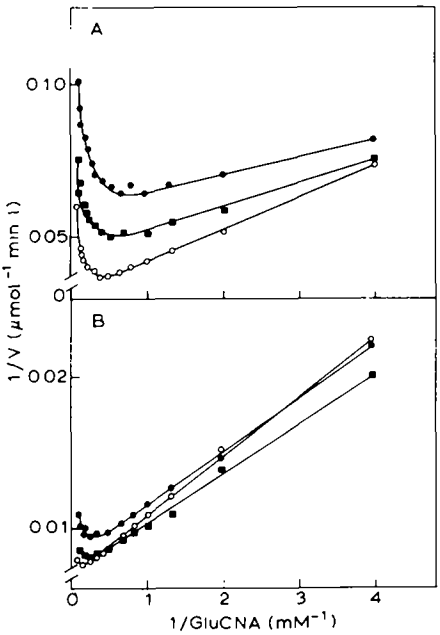


Fig 4 Double-reciprocal plots of activity vs GluCNA in the presence of glycylglycine (●———●, 50 mM, ■———■, 100 mM and ○———○, 200 mM) at pH 6 0 (A) and 8 5 (B) Note the strong inhibition by donor substrate, but also strong activation by acceptor substrate V values were extrapolated to $1/V$ V_1 values were determined at highest GluCNA concentrations Inset table shows the kinetic parameters at 50, 100 and 200 mM glycylglycine Note that the degree of inhibition (V/V_1) is about the same at all concentrations of glycylglycine Note also that glycylglycine acts as an apparent competitive inhibitor by increasing $K_{0\,5}$

A	Glycylglycine (mM)		
	50	100	200
$K_{0\,5}$	0 1	0 15	0 32
V	16	21	31
V_1	10	13	19
V/V_1	1 6	1 6	1 6

B	Glycylglycine (mM)		
	50	100	200
$K_{0\,5}$	0 41	0 44	0 55
V	121	137	141
V_1	91	114	124
V/V_1	1 3	1 2	1 1

ratio of V at 100 mM glycylglycine (transferase) to V in the absence of glycylglycine (hydrolase). The ratio reaches a maximal value at pH 7.5 and a minimal value at pH >10.0, indicating that at physiological pH, transferase reaction was preferred and moreover, that the transferase was optimal in a pH zone where deacylation seemed to be rate limiting.

In view of the objections that might be raised concerning kinetic studies

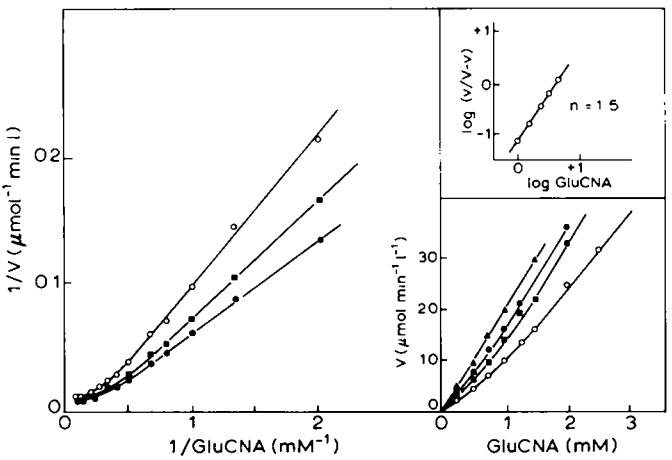


Fig 5 Double-reciprocal plot of activity vs GluCNA at pH 10 5 in the presence of glycylglycine (▲——▲, 5 mM, ●——●, 50 mM, ■——■, 100 mM and ○——○, 200 mM) Note the absence of activation and the presence of apparent competitive inhibition Inset figures show that glycylglycine does not affect the cooperativity of donor substrate binding

done on an enzyme was likely to be attached to detergent micelles, we repeated some experiments with γ-glutamyltransferase in renal brush border membrane Fig. 8 clearly shows that γ-glutamyltransferase in its natural environment behaves exactly as the detergent-purified enzyme. At pH 6.0 and 7.5, activation by donor substrate was observed in the absence of glycylglycine, and the presence of glycylglycine not only stimulated activity but led to donor substrate inhibition.

Discussion

Most kinetic studies that were carried out on γ-glutamyltransferase were done at pH 8.0—8.5 and at low concentration for both donor and acceptor sub-

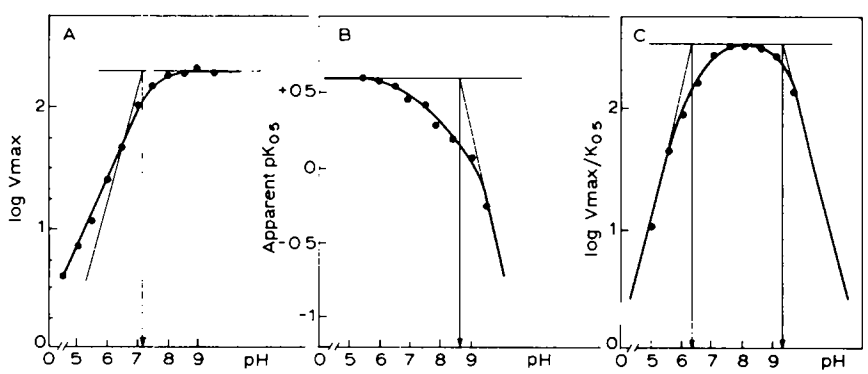
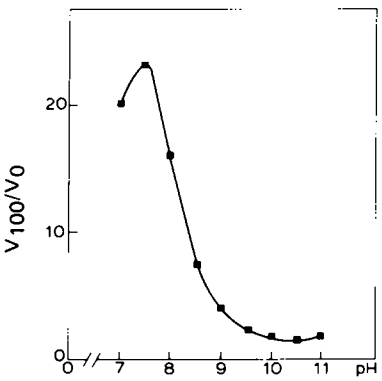


Fig 6A Influence of pH on the maximal velocity of purified γ-glutamyltransferase at 100 mM glycylglycine B Influence of pH on the affinity of purified γ-glutamyltransferase for γ-glutamyl-3-carboxy-4-nitroanilide at 100 mM glycylglycine. C Influence of pH on the ratio of the maximal velocity and $K_{0.5}$ of the purified γ-glutamyltransferase at 100 mM glycylglycine



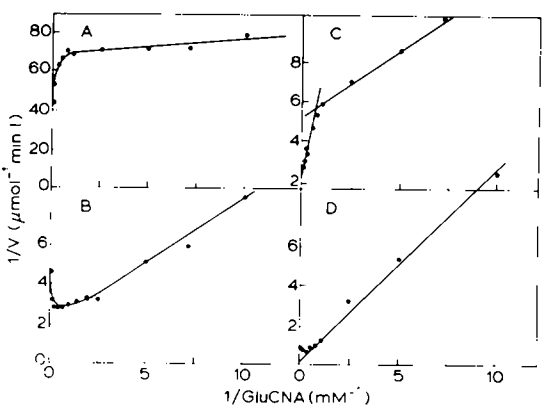


Fig 7 Ratio of transferase activity over hydrolase activity vs pH Ratio of V at 100 mM glycylglycine and V at 0 mM glycylglycine are plotted Note the maximum at pH 7 0—7 5 with a pK of 8 3

Fig 8 Double-reciprocal plot of activity vs GluCNA in the absence and presence of 50 mM glycylglycine for rat kidney brush border membranes Brush border membranes were prepared according to Vannier et al [26] The preparation corresponds to a 20-fold enrichment of a specific marker, alkaline phosphatase, relative to renal cortex homogenate (A) Rate at pH 6 0 in absence of glycylglycine, (B) rate at 6 0 in presence of glycylglycine, (C) rate at pH 7 5 in absence of glycylglycine, (D) rate at pH 7 5 in presence of glycylglycine

strates. They led to the description of a non-sequential ping-pong mechanism [1—8]. Our studies are not contradicting former studies but bring more light to the understanding of the kinetic properties of γ-glutamyltransferase. A careful examination of the enzyme kinetics over a wide range of pH values under conditions of maximal velocity revealed the existence of complex interaction between donor and acceptor sites, which could not be caused by a competing simultaneous autotransfer reaction. Even though the autotransfer reaction was not measured, findings with and without glycylglycine, almost ruled out a significant contribution of an autotransfer under conditions of assay Indeed, binding of an acceptor molecule on the acceptor site induces an inhibition by the donor at high donor concentrations. In the autotransfer reaction, the donor acting as acceptor would necessarily bind to the acceptor site inducing an inhibition instead of an activation. A situation is created in which the same substrate causes stimulation and inhibition of its uptake on binding to the acceptor site. As found with glycylglycine, the stimulatory effect on donor transformation and turnover is such that it should mask the inhibitory effect. Obviously, this is not the case. Data from others indicate that the autotransfer decreases at acidic pH [11]. Contrarily, we have observed that substrate activation is maximal at acidic pH.

A strong argument against autotransfer as the explanation of the activation, is the very different V pH profile in the absence and presence of acceptor. If activation by donor substrate was due to autotransfer the variation of activity with pH should follow that of transferase reaction and it does not.

Therefore, autotransfer, if it occurs at low pH, is not significant. Studies done on human liver γ-glutamyltransferase are in good agreement, since no autotransfer product could be identified at pH <7.0, using chromatography [27]

Our data show a net difference between the hydrolase and transferase activities, especially with respect to optimal pH. The values of acidic pK involved in transferase activity suggest the role of an histidine residue. However, the role of an active histidine was rejected by Elce on the basis of chemical modification [9].

The residue with a pK of 8.6 which controls the hydrolase activity and plays an important role in the apparent affinity of donor substrate as function of pH, could be an α-NH_3 group. Our kinetic data support that of Elce, who recently proposed the active participation of two α-NH_3 groups [9]. The important pH differences observed here, between hydrolase and transferase activities, are reflecting the important changes brought about by interaction of an acceptor molecule on the acceptor site

Another important aspect of the pH study is the difference between acidic and alkaline values both in hydrolase and transferase activity. This suggests a change in rate-limiting step which would explain the identical $K_{0.5}$ variation with pH in both reactions. Deacylation would be rate limiting at low pH and acylation at high pH. This view is supported again by the findings of Elce [9] and Tate [17], which show that a covalent glutamyl enzyme exists at pH 7.5.

Our results suggest the existence of more than one binding site for donor and acceptor. According to Elce [9] and Tate [17], there is one covalent γ-glutamyl/mol enzyme at pH 7.5, which is located on the small subunit of the enzyme. The enzyme being a dimer, the possibility of having another site for donor as well as acceptor substrate is questioned. However, the existence of a latent active site on the large subunit of rat kidney γ-glutamyltransferase was reported recently [18]. This site would be inoperative on the dimeric enzyme under normal conditions It is possible that this site was activated at very high donor substrate concentrations explaining the kinetic behaviour described here. Unfortunately, stoichiometric active site labelling could not be performed because of an insufficient amount of the rabbit liver enzyme.

This study underlines the complex interactions between acceptor and donor substrate binding sites. The only report of such interactions was done by Thompson and Meister [22], in which maleate was shown to influence donor binding.

In view of our observations, it is noteworthy that very similar kinetics were observed with bacterial and mammalian alkaline phosphatases known to have two active sites [23—25].

Conflicting kinetic data reported by different investigators could reflect the molecular structure of the γ-glutamyltransferase used for the kinetic studies. Most studies on the mechanism of this enzyme were done on the so called 'light form', which is obtained as a dimeric protein of the type α-β after a proteolytic treatment. It was shown by Hughey and Curthoys [19] that the Triton X-100-purified enzyme from rat kidney was much heavier than the papain-treated enzyme because of extensive binding to detergent micelles. In the absence of detergent the enzyme aggregates. A proteolytic treatment removes an hydrophobic anchor which permits binding to detergent micelles and lecithin vesicles [19].

The γ-glutamyltransferase used for our study was purified in the presence of Lubrol. It is probable that the high apparent molecular weight is due to molec-

ular association through binding to detergent micelles. It was recently reported that binding of γ-glutamyltransferase to lipid seems to be similar to the binding to plasma membranes, and that the active site of the enzyme in artificial or natural membrane is most probably external to the membrane [20,21]. The natural environment of the enzyme is the lipid matrix of the cell membrane. We have conducted corroborative studies at pH 6.0 and 7.5 with rat kidney brush border membranes. The choice of rat kidney was dictated by the recent studies of Hughey et al. [20] which were done on rat kidney γ-glutamyltransferase, and by the large γ-glutamyltransferase activity of these membranes compared to the liver ones. It is clear that γ-glutamyltransferase in natural membranes exhibits the same properties as that of the detergent-purified enzyme (Fig 9).

Kinetic studies conducted on papain-released γ-glutamyltransferase from rat kidney brush border gave similar results (unpublished data). Thus, the kinetic properties seem to be independent of the molecular form.

Under conditions prevailing in vivo, γ-glutamyltransferase is acting at pH 7.0—7.5 and in the presence of acceptor substrate, where transferase activity is very much favored. The affinity constant $K_{0.5}$ is around 0.1 mM for donor and 2—5 mM acceptor is sufficient to cause donor substrate inhibition. These concentrations are well within physiological limits Moreover, to activate transfer much higher concentrations of acceptor will be required to saturate the second site. γ-Glutamyltransferase would then be regulated both by donor and acceptor substrate. If glutathione is the natural substrate, regulation of γ-glutamyltransferase might be linked to glutathione economy.

Acknowledgements

We wish to thank Mrs. C. Lafaurie and M. Chaussard for their excellent technical assistance. The authors gratefully ackowledge the help of Professor M. Lazdunski in discussions. This work was supported by DGRST grant, No. 78.7.0139.

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