Biochem. J. (1994) 304, 869–876 (Printed in Great Britain)

γ-Glutamyltranspeptidase-catalysed acyl-transfer to the added acceptor does not proceed via the Ping-Pong mechanism

Mikhail Yu. GOLOLOBOV* and Robert C. BATEMAN, Jr.

Department of Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, MS 39406-5043, U.S.A.

Acyl-transfer catalysed by γ-glutamyltranspeptidase from bovine kidney was studied using γ-L- and γ-D-Glu-*p*-nitroanilide as the donor and GlyGly as the acceptor. The transfer of the γ-Glu group to GlyGly was shown to be accompanied by transfer of the γ-Glu group to water (hydrolysis). The results were compared with acyl-transfer catalysed by the representative serine protease, α-chymotrypsin. The main difference between the kinetic mechanism of the acyl-transfer reactions catalysed by these enzymes, which contain an active-site serine and form an acyl-enzyme intermediate but belong to different enzyme classes, was found to consist in the role of the enzyme–donor–acceptor complex. This complex is not formed at any acceptor concentrations in the acyl-transfer reactions catalysed by the serine proteases. In contrast, in the γ-glutamyltranspeptidase-catalysed acyl-transfer the pathway going through the ternary enzyme–donor–acceptor complex formed from the enzyme–acceptor complex becomes the main pathway of the transfer reaction even at moderate acceptor concentrations. As a result, γ-glutamyltranspeptidase catalysis follows a sequential mechanism with random equilibrium addition of the substrates and ordered release of the products. The second distinction concerns the inhibitory effect of the acceptor. In the case of α-chymotrypsin this was the result of true inhibition, i.e. a dead-end formation of the enzyme–acceptor complex. A salt effect caused by the acceptor was the rationale of a similar effect observed in acyl-transfer catalysed by γ-glutamyltranspeptidase.

INTRODUCTION

γ-Glutamyltranspeptidase (γ-GluTP) catalyses the transfer of the γ-Glu group from gluthatione and related donor compounds to a variety of amino acids and short peptides, and is thought to play a key role in glutathione metabolism (Tate and Meister, 1985). This enzyme is reported to catalyse three types of reactions: transfer of the γ-Glu group of the donor to an added acceptor such as amino acids or small peptides; transfer of the γ-Glu group of the donor to water (hydrolysis), and transfer of the γ-Glu group of the donor to another donor molecule (autotransfer) (Allison, 1985; Tate and Meister, 1985). The relative contribution of each of these reactions depends on many factors which are not completely understood. Inhibition studies strongly suggest formation of an acyl-enzyme intermediate in the γ-GluTP catalysis. Phenylmethane sulphonyl fluoride, a reagent that inactivates serine-class proteases, inactivates γ-GluTP (Inoue et al., 1978; Elce, 1980). The glutamine analogues 6-diazo-5-oxo-norleucine and *O*-diazoacetyl-serine inactivate γ-GluTP by attaching covalently and stoichiometrically to the γ-glutamyl site (Tate and Meister, 1977). Studies with 6-diazo-5-oxo-norleucine-labelled γ-GluTP indicate that the covalent attachment of this compound involves an ester bond to an enzyme hydroxyl group (presumably a serine or threonine residue) located in the γ-glutamyl-binding subsite (Tate and Meister, 1978).

Formation of similar acyl-enzyme intermediates is a very well-documented feature of serine proteases (Fastrez and Fersht, 1973; Antonov et al., 1981; Fersht, 1985a,b). Perhaps the most comprehensive study of the kinetic mechanism of the latter enzymes has been performed on α-chymotrypsin. In most serine proteases concomitant transfer and hydrolysis occurs with the acceptor reacting with the acyl-enzyme only (Bender et al., 1964; Berezin et al., 1973; Fersht et al., 1973; Kullmann, 1984; Petkov and Stoineva, 1984; Riechmann and Kasche, 1984, 1985; Schellenberger and Jakubke, 1986, 1991; Bizzozero et al., 1988; Schellenberger et al., 1990, 1991; Gololobov et al., 1990, 1992, 1993). Competitive inhibition of donor consumption by the acceptor, i.e. formation of the enzyme–acceptor complex, occurs only if amino acid β-naphthylamides are the added acceptors (Gololobov et al., 1990). Our initial experiments showed that γ-GluTP-catalysed acyl-transfer could not be interpreted by the model which works for α-chymotrypsin. In this context it was interesting to perform a detailed comparison of the kinetic mechanisms of the two enzymes, γ-GluTP and α-chymotrypsin. Both contain an active-site serine and act according to the acyl-enzyme mechanism, but belong to different classes of enzymes.

EXPERIMENTAL

γ-GluTP from bovine kidney (EC 2.3.2.2), γ-L-Glu-*p*-nitroanilide (Glu*p*NA), GlyGly and [3-(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid (AMPSO) were obtained from Sigma (St. Louis, MO, U.S.A.). D-Glu*p*NA was synthesized as previously described (King and Kidd, 1949; Orlowski and Meister, 1965). Chromatography of the reaction mixtures formed in the course of the reactions was performed on the Whatman No. 1 paper (Fisher Scientific, Norcross, U.S.A.) according to the published method (Orlowski and Meister, 1965; London et al., 1976). Concentration of Glu*p*NA and D-Glu*p*NA in the

Abbreviations used: γ-GluTP; γ-glutamyltranspeptidase; Glu*p*NA, γ-L-Glu-*p*-nitroanilide; D-Glu*p*NA, γ-D-Glu-*p*-nitroanilide; Boc, t-butyloxycarbonyl-; AMPSO, [3-(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid; if not otherwise stated, amino acid residues are of the L-configuration.

* To whom correspondence should be addressed.

chromatography experiments was 5 mM, which was close to the solubility limits of both substrates. Experimental procedures for α-chymotrypsin-catalysed reactions and characteristics of the reagents have been previously described (Gololobov et al., 1990). Kinetic measurements of the γ-GluTP-catalysed reactions were performed using an SF.17MV MicroVolume stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, U.K.). Detection of the *p*-nitroanilide reaction product was performed at 410 nm using a molar absorption coefficient of 8800 $M^{-1} \cdot cm^{-1}$ (Tate and Meister, 1985). The use of a stopped-flow setup for the steady-state measurements allowed us to achieve virtually zero dead-time of the measurements and consumption of both the donor and acceptor being less than 1%, i.e. strictly initial rate conditions were obeyed. The fulfillment of these conditions is extremely important since the rate of the γ-GluTP-catalysed consumption of Glu*p*NA decreased very rapidly after the mixing of the reagents. A complex product inhibition pattern of γ-GluTP from bovine kidney is the most probable rationale of this phenomenon. The rate measurements were made with donor concentrations ranging from 0.50 to 4.5 mM and the acceptor concentration from 0 to 250 mM at pH 9.0 and 25 °C in 0.1 M AMPSO. If necessary, the pH was adjusted to 9.0 after addition of the donor or acceptor to the buffer solution. At each given condition initial rate measurements were repeated five or six times. The S.E.M. of the estimate of the initial rate did not exceed 1%. Values of the Michaelis–Menten parameters were calculated using initial rate measurements obtained at 8–10 donor concentrations at every concentration of the nucleophile. Non-linear regression analysis was performed using KaleidaGraph software (Abelbeck Software, U.S.A.). The values of the reaction rates, $V_{max.}$ and $V_{max.}/K_m$ given in this paper correspond to an enzyme concentration of one unit/ml. One unit of γ-GluTP was defined as that amount of γ-GluTP which being dissolved in 1 ml of 5 mM solution of Glu*p*NA in 0.1 M AMPSO (pH 9.0) produced a reaction rate of 0.1 μM/s at 25 °C. A fresh enzyme stock solution in 0.1 M AMPSO (pH 9.0) was prepared every 3 days and kept at 4 °C. The enzyme activity did not change during this time. Fresh substrate stock solution in 0.1 M AMPSO (pH 9.0) was prepared daily. Under these conditions the difference between initial rates measured with different enzyme and substrate solutions of the same activity and concentration was typically 1–2% and never exceeded 4%.

RESULTS

Lack of autotransfer

Paper chromatography of the reaction mixture showed that in the absence of GlyGly and at a concentration of the donor close to its solubility limit (when occurrence of the autotransfer is expected to be maximal) only two products, glutamic acid and *p*-nitroaniline, formed when either L-Glu*p*NA or D-Glu*p*NA were the donors. After allowing the reaction to proceed to completion the size of the spot corresponding to the initial substrate solution was equal to the size of the spot attributed to glutamic acid (the only product observed), indicating stoichiometric conversion. The kinetics of the reaction studied were found to obey the Michaelis–Menten equation for both D- and L-substrates (Figure 1). Autotransfer should result in deviation from Michaelis–Menten kinetics due to square terms in the rate equation. According to previous work (Thompson and Meister, 1976) the autotransfer is absent when D-Glu*p*NA is a donor. Therefore, γ-GluTP from bovine kidney catalyses the transfer of the donor group to water and the added acceptor but not to another donor molecule.

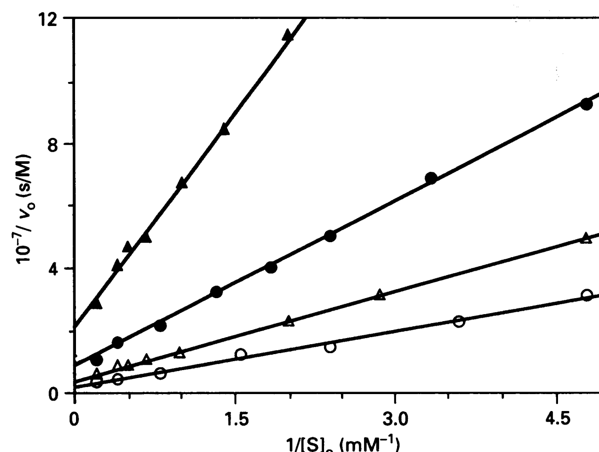


Figure 1 Double-reciprocal plots for γ-GluTP-catalysed release of *p*-nitroaniline from the D- and L-γ-Glu*p*NA with and without GlyGly

The conditions were pH 9.0, 25 °C, 0.1 M AMPSO. Circles denote data obtained with the L-substrate, triangles denote data obtained with the D-substrate. Filled symbols correspond to the reactions without GlyGly, open symbols correspond to the reaction in the presence of 50 mM GlyGly. The straight lines were drawn using the least-squares method. Error bars were omitted because the size of the symbols exceeded the magnitude of the standard errors.

Lack of hydrolysis at high acceptor concentration

In contrast with the reactions occurring in the absence of GlyGly, in the presence of 50 mM GlyGly glutamic acid was not detected in the reaction mixtures using paper chromatography. This means that at GlyGly concentrations higher than 50 mM the hydrolysis of the donor was completely suppressed.

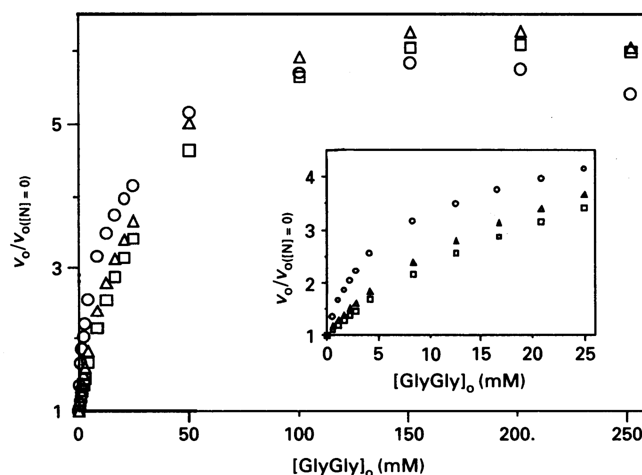


Figure 2 Effect of the GlyGly initial concentration on the change of the initial rate of the γ-GluTP-catalysed consumption of Glu*p*NA

Insert shows the same data at low GlyGly concentrations. The rate at a given GlyGly concentration (v_o) was divided by the rate obtained without GlyGly ($v_{o([N]=0)}$). The conditions were pH 9.0, 25 °C, 0.1 M AMPSO. The Glu*p*NA initial concentration was equal to 0.5 mM (○), 1.75 mM (△) and 2.5 mM (□). Error bars were omitted because the size of the symbols exceeded the magnitude of the standard errors.

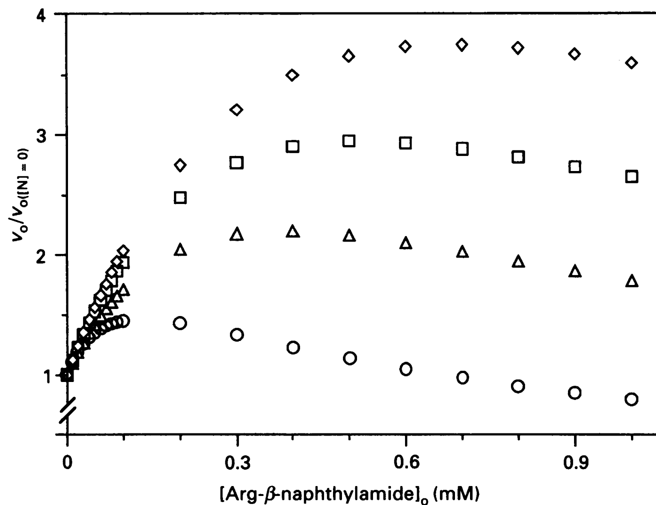


Figure 3 Effect of the Arg-β-naphthylamide initial concentration on the change of the initial rate of the α-chymotrypsin-catalysed consumption of BocMet-p-nitrophenyl ester

The rate at a given Arg-β-naphthylamide concentration (v_o) was divided by the rate obtained without Arg-β-naphthylamide ($v_{o([N]=0)}$). The conditions were pH 8.5, 25 °C, 0.1 M phosphate. The initial concentration of BocMet-p-nitrophenyl ester was equal to 13 mM (○), 38 mM (△), 57 mM (□) and 87 mM (◇). Error bars were omitted because the size of the symbols exceeded the magnitude of the standard errors.

Comparison of γ-GluTP- and α-chymotrypsin-catalysed reactions

If the acceptor interacts with the acyl-enzyme intermediate only, as it does in most α-chymotrypsin-catalysed reactions, $V_{max.}/K_m$ for the donor consumption should not depend on the nucleophile concentration (Bender et al., 1964; Fersht, 1985a,b). Therefore, if α-chymotrypsin and γ-GluTP shared the same kinetic mechanism, lines in Figure 1 corresponding to the data obtained with and without GlyGly would be parallel. However, this was obviously not the case.

The dependence of the initial rate of the donor consumption on the acceptor concentration provided more evidence in favour of a significant difference between γ-GluTP- and α-chymotrypsin-catalysed acyl-transfer (Figures 2 and 3). Arginine-β-naphthylamide was chosen as an acceptor in the α-chymotrypsin-catalysed reactions because amino acid-β-naphthylamides are competitive inhibitors as well as nucleophiles in this case (Gololobov et al., 1990). Glycyl-glycine has been reported to show a similar behaviour in reactions catalysed by γ-GluTP from different sources (London et al., 1976; Stromme and Theodorsen, 1976; Huseby, 1977; Thompson and Meister, 1977; Shaw et al., 1978; PetitClerc et al., 1980; Bagrel et al., 1981) and the same effect of GlyGly was expected with respect to γ-GluTP from bovine kidney. The results at high acceptor concentrations were in accordance with this prediction. Dependencies of $v_o/v_o([N]=0)$ on the concentration of the acceptors had a maximum for both enzymes. This maximum was shifted up when the donor concentration was increased. However, at low acceptor concentration the α-chymotrypsin- and γ-GluTP-catalysed reactions showed dramatic differences. In the α-chymotrypsin-catalysed acyl-transfer the effect of the acceptor on $v_o/v_o([N]=0)$ was more evident at higher donor concentration. In contrast with that, in the γ-GluTP-catalysed reactions the effect of the acceptor on $v_o/v_o([N]=0)$ was more pronounced at lower concentrations of the donor. In addition, γ-GluTP showed an unusual dependence of the apparent K_m for the donor on the acceptor concentration.

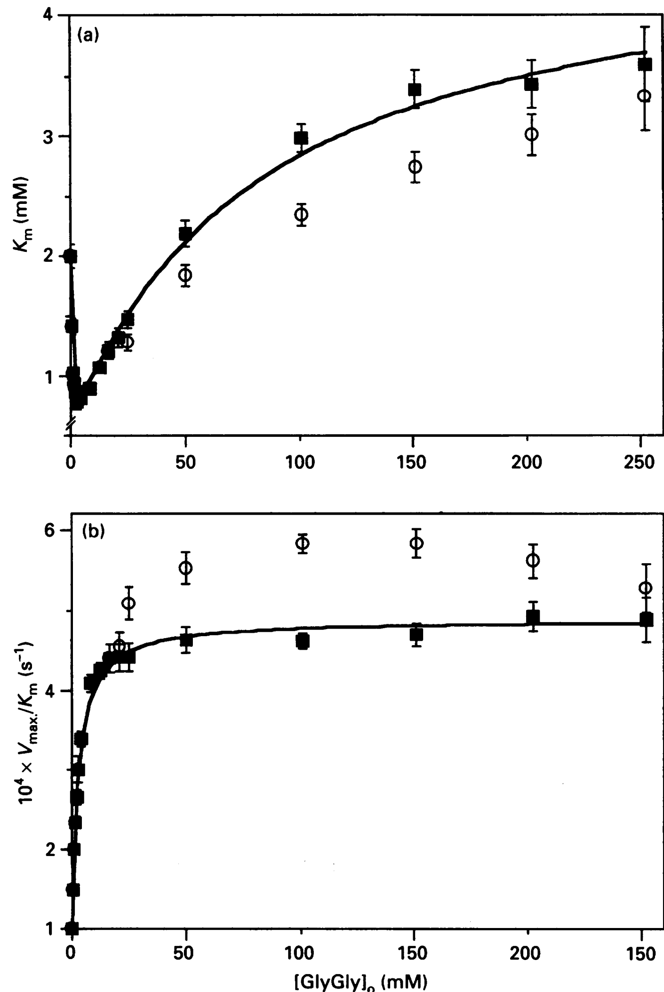


Figure 4 Dependence of the apparent Michaelis–Menten parameters for the donor on the initial concentration of GlyGly for γ-GluTP-catalysed acyl transfer

The conditions were pH 9.0, 25 °C, 0.1 M AMPSO. The lines were drawn using the weighted least-squares method according to eqns (5–7). Bars corresponds to S.E.M.s. If error bars are omitted, the S.E.M.s are less than the size of the symbols. ○ denote experimental values, ■ correspond to the values corrected for the salt effect caused by GlyGly. (**a**) Dependence of K_m. (**b**) Dependence of $V_{max.}/K_m$.

In α-chymotrypsin-catalysed reactions the apparent K_m for the donor increases with the acceptor concentration (Fastrez and Fersht, 1973; Gololobov et al., 1990), while in γ-GluTP-catalysed reactions the apparent K_m for the donor decreased at low concentrations of GlyGly, attained a minimum and increased at high concentrations of the acceptor (Figure 4a). When analysing the dependence of the reaction rate or Michaelis–Menten parameters on the concentration of GlyGly, the concentration of GlyGly was high enough to alter the ionic strength, which could influence the kinetic parameters. Because of that, Figure 4 presents both experimental and corrected (for salt effect) dependencies of the Michaelis–Menten parameters on the concentration of GlyGly. The correction was made according to the data shown in Figure 5. The salt effect was significant at the substrate concentration below K_m and tended to zero when the substrate concentration exceeded K_m. Therefore, the salt affected the apparent $V_{max.}/K_m$ for the donor but not the apparent $V_{max.}$.

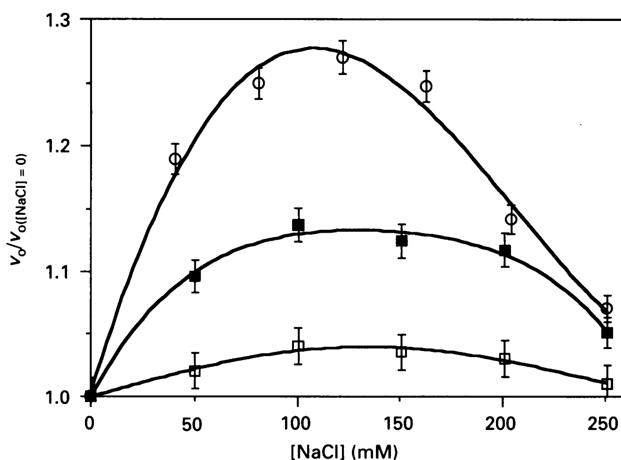


Figure 5 Effect of the NaCl concentration on the initial rate of γ-GluTP-catalysed hydrolysis

The rate without NaCl was recognized as the unit rate. The conditions were pH 9.0, 25 °C, 0.1 M AMPSO. The lines were drawn using the least-squares method according to the polynomial of the 4th degree just to guide the eye and represent no models. The initial Glu*p*NA concentration was equal to 0.5 mM (○), 2.5 mM (■) and 4.5 mM (□).

DISCUSSION

Kinetic model

Figures 1 and 6 show dependencies of the reaction initial rate on the concentration of the substrates in double-reciprocal co-ordinates. If both L-Glu*p*NA and D-Glu*p*NA were the variable substrates, the experimental data could be approximated by straight lines over the whole range of donor concentrations, both in the absence and in the presence of the acceptor (GlyGly). Therefore, the dependence of the rate of donor consumption on the concentration of the donor follows the Michaelis–Menten equation. This fact allows us, for example, to rule out the presence in the reaction of several enzymes with similar activities (e.g. isoforms) as a reason for the unusual kinetic behaviour described above. Besides that, no indications of the existence of the γ-GluTP isoforms are evident in previous studies. As for the dependence of the rate of donor consumption on acceptor concentration, the situation is more complex. The experimental data could not be linearized in double-reciprocal coordinates over the whole range of GlyGly concentrations if GlyGly was the variable substrate. Only when $1/[\mathrm{GlyGly}]_0$ was between 20 M^{-1} and 80 M^{-1} ($[\mathrm{GlyGly}]_0$ was between 12.5 and 50 mM) could the experimental results be approximated by straight lines (Figure 6b). The portion of the dependencies at $1/[\mathrm{GlyGly}]_0 < 20\ M^{-1}$ ($[\mathrm{GlyGly}]_0 > 50$ mM) was a non-linear, convex downward curve. This behaviour usually suggests substrate inhibition by the variable substrate. However, GlyGly has a charged carboxyl group at pH 9.0 and therefore can affect the reaction rate via a salt effect. Figure 5 shows that the ionic strength of the solution did affect the rate of the γ-GluTP-catalysed reactions. After correction for this salt effect the experimental dependence showed no evidence of substrate inhibition (Figure 7). The kinetic model should therefore account for non-Michaelis behaviour with respect to the acceptor concentration over the whole concentration range of the latter and should explain why at higher acceptor concentrations the dependence of the initial rate on the acceptor concentration can still be described by the Michaelis–Menten equation (after correction of the data for the salt effect).

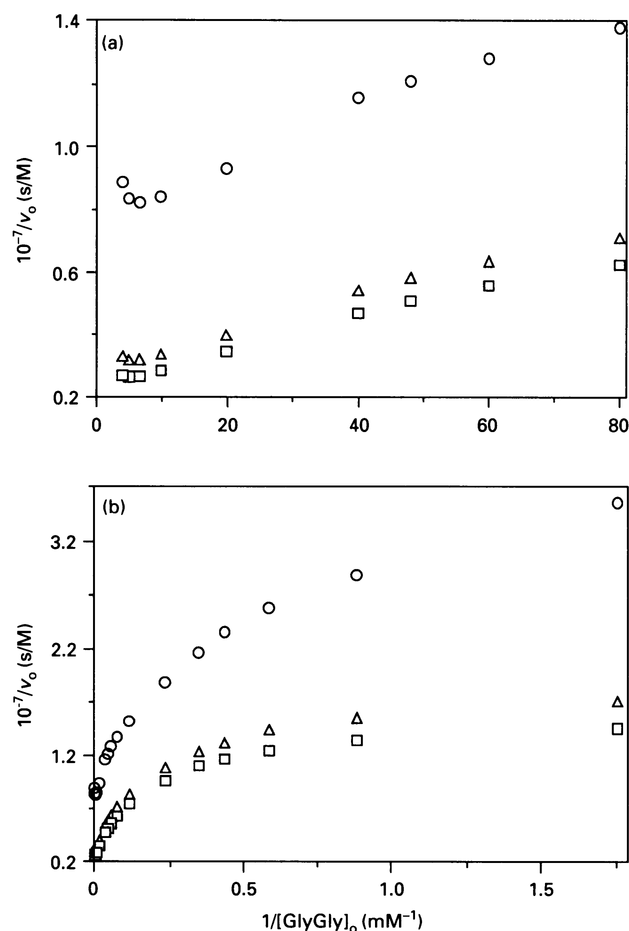


Figure 6 Dependence of the initial rate of γ-GluTP-catalysed reactions on the initial acceptor concentration in double-reciprocal coordinates

The conditions were pH 9.0, 25 °C, 0.1 M AMPSO. The initial Glu*p*NA concentration was equal to 0.5 mM (○), 1.75 mM (△) and 2.5 mM (□). (**a**) and (**b**) present the same experimental data but in different scales.

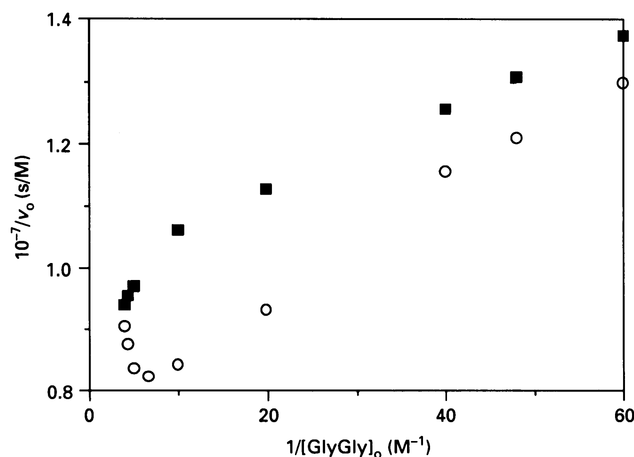
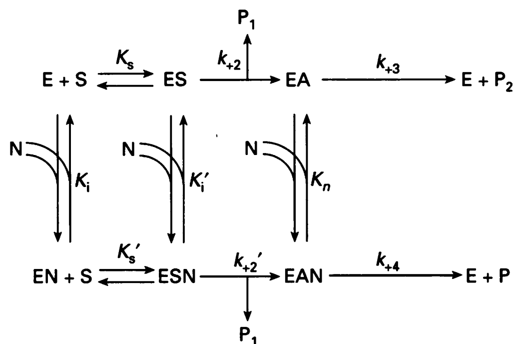


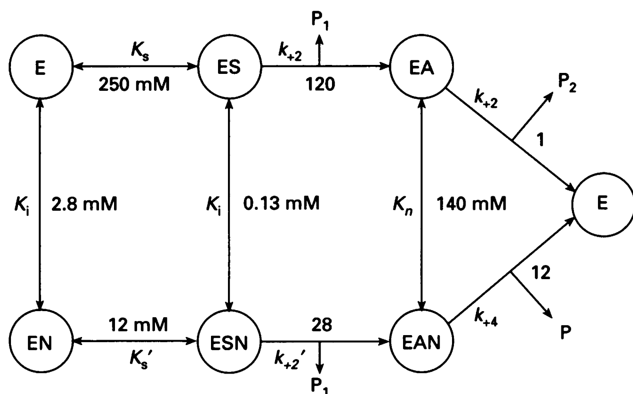
Figure 7 Comparison of γ-GluTP-catalysed reactions with and without correction for the salt effect

The conditions were pH 9.0, 25 °C, 0.1 M AMPSO. ○ denote experimental values, ■ correspond to the values corrected for the salt effect caused by GlyGly.



Scheme 1

Here E represents the enzyme, S denotes the acyl-group donor, N represents the acyl-group acceptor (the nucleophile), P is the product of the transfer of the acyl-group to the acceptor, P_1 and P_2 are the hydrolytic products, ES and EN are the complexes of the enzyme with S and N respectively, ESN is the enzyme-donor-acceptor complex, EA stands for the acyl-enzyme and EAN denotes the acyl-enzyme-acceptor complex. The kinetic constants are indicated for the appropriate reactions in the scheme.



Scheme 2

(Rate constants $k_{+2}k'_{+2}$, k_{+3} and k_{+4} are dimensionless because only their ratio could be evaluated from the experimental data. Because of that k_{+3} was recognized as the unit constant and all other constants were ranked with respect to k_{+3}.)

When trying to interpret our results by a kinetic model, we have to bear in mind that the possible model should include formation of the acyl-enzyme as well as the enzyme–substrate complex (Fersht, 1985a,b). In general, both intermediates, as well as the free enzyme, can bind the acceptor. In this way we come to the model shown in Scheme 1.

A similar model has previously been used (Gololobov et al., 1990, 1992, 1993) to analyse protease-catalysed acyl-transfer. In those works the possibility of the hydrolysis of the EAN complex was assumed. The pathway was proven to occur in some reactions (Riechmann and Kasche, 1984, 1985; Schellenberger and Jakubke, 1986; Gololobov et al., 1990, 1992, 1993). In practice, the hydrolysis of the EAN complex should result in substantial formation of hydrolytic product even at an 'infinite' acceptor concentration. In γ-GluTP catalysis the formation of the hydrolytic product did not occur provided the GlyGly concentration was 50 mM or higher. Therefore, hydrolysis of the EAN complex did not occur. One should also bear in mind that the models with and without the hydrolysis of the EAN complex cannot be discriminated if only the donor consumption is measured. It can be shown that if hydrolysis of the EAN complex occurred, constant k_{+4} in all equations below should be replaced with a sum of two constants: k_{+4} and k_{+5} (Gololobov et al., 1990), where k_{+5} is the rate constant for the hydrolysis of the EAN complex.

Analysis of the kinetic model

Assuming an equilibrium formation of the ES, EN, ESN complexes, the equilibrium between the EA and EAN intermediates and a steady state with respect to EA, the apparent parameters of the Michaelis–Menten equation for the donor consumption depend on the acceptor concentration as follows:

$$V_{\text{max.}} = \frac{k_{+3}\left(1+\frac{k_{+4}[\text{N}]_o}{k_{+3}K_n}\right)\left(1+\frac{k'_{+2}[\text{N}]_o}{k_{+2}K'_i}\right)[\text{E}]_o}{\text{A}+\frac{k_{+3}}{k_{+2}}\text{B}} \tag{1}$$

$$K_m = \frac{\frac{K_s k_{+3}}{k_{+2}}\left(1+\frac{[\text{N}]_o}{K_i}\right)\left(1+\frac{k_{+4}[\text{N}]_o}{k_{+3}K_n}\right)}{\text{A}+\frac{k_{+3}}{k_{+2}}\text{B}} \tag{2}$$

where

$$\text{A} = \left(1+\frac{k'_{+2}[\text{N}]_o}{k_{+2}K'_i}\right)\left(1+\frac{[\text{N}]_o}{K_n}\right) \tag{3}$$

$$\text{B} = \left(1+\frac{k_{+4}[\text{N}]_o}{k_{+3}K_n}\right)\left(1+\frac{[\text{N}]_o}{K'_i}\right) \tag{4}$$

Therefore,

$$\frac{V_{\text{max.}}}{K_m} = \frac{k_{+2}\left(1+\frac{k'_{+2}[\text{N}]_o}{k_{+2}K'_i}\right)[\text{E}]_o}{K_s\left(1+\frac{[\text{N}]_o}{K_i}\right)} \tag{5}$$

[In previous publications (Gololobov et al., 1990, 1993) an error was made in the denominator of the equations for the apparent $V_{\text{max.}}$ and K_m for the donor. This error did not affect the conclusions of those works but became important in the context of this study.]

A combination of $k_{+2}[\text{E}]_o/K_s$ equals the value of $V_{\text{max.}}/K_m$ in the absence of the acceptor. Since the molar concentration of the enzyme was not known, the analysis of the relative changes of $V_{\text{max.}}/K_m$ was preferable. If we denote the apparent $V_{\text{max.}}/K_m$ for the donor in the absence of the acceptor as $(V_{\text{max.}}/K_m)_{([\text{N}]=0)}$ we obtain:

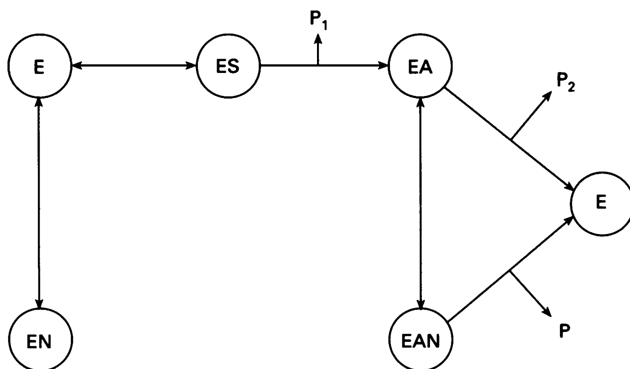
$$\left(\frac{V_{\text{max.}}}{K_m}\right)\bigg/\left(\frac{V_{\text{max.}}}{K_m}\right)_{([\text{N}]=0)} = \frac{1+\frac{k'_{+2}[\text{N}]_o}{k_{+2}K'_i}}{1+\frac{[\text{N}]_o}{K_i}} \tag{6}$$

Eqn. (6) contains only two unknown parameters [K_i and $k'_{+2}/(k_{+2}K'_i)$] and follows from Scheme 1 without any assumptions. Therefore, the estimates of $k'_{+2}/(k_{+2}K'_i)$ obtained from eqn. (6) should be reliable. The following values were obtained (Figure 4b): $k'_{+2}/(k_{+2}K'_i) = 1.73 \pm 0.09\ \text{mM}^{-1}$ and $K_i = 2.8 \pm 0.2$ mM. Using these values we could analyse the most unusual dependence, i.e. the dependence of the apparent K_m on the acceptor concentration. A non-linear weighted regression analysis of the latter dependence (Figure 4a) according to eqns. (2–4)

resulted in the following values of the kinetic parameters: $k_{+4}/(k_{+3}K_n) = 0.14 \pm 0.01\ \text{mM}^{-1}$, $K_n = 140 \pm 40\ \text{mM}$, $k_{+3}/k_{+2} = 0.008 \pm 0.004$, $K_i' = 0.13 \pm 0.06\ \text{mM}$, $K_s k_{+3}/k_{+2} = 1.98 \pm 0.07\ \text{mM}$.

The values of the parameters given above allowed us to calculate all constants denoted in Scheme 1 and perform a complete kinetic description of γ-GluTP catalysis (Scheme 2).

Inspection of Scheme 2 revealed several important features of γ-GluTP catalysis. These include the following: (i) a significant kinetic role of the ESN complex which forms mainly via the EN complex; (ii) free enzyme binds the acceptor much better than the acyl-enzyme; (iii) binding of the donor is very weak and a low value of the Michaelis constant in the absence of the added acceptor is entirely the result of a high value of the acylation constant when compared with the deacylation one; (iv) binding of the donor promotes binding of the acceptor and vice versa; (v) bound acceptor hampers acylation of the enzyme with the bound donor but deacylation of the acyl-enzyme with the bound nucleophile proceeds more than one order of magnitude faster than deacylation of the acyl-enzyme with water. A significant kinetic role of the EAN complex is the most distinctive feature of γ-GluTP catalysis. α-Chymotrypsin-catalysed acyl-transfer and, in general, acyl-transfer reactions catalysed by serine proteases proceed without formation of the ESN complex (Scheme 3).



Scheme 3

Scheme 3 was made using the results of this work and the previously referred studies of α-chymotrypsin, as well as previous studies of trypsin, carboxypeptidases Y and W and alkaline mesentericopeptidase (Seydoux et al., 1969; Riechman and Kasche, 1984, 1985; Shima et al., 1987; Bratovanova et al., 1988; Christensen et al., 1992).

The lack of the ESN complex in the α-chymotrypsin catalysis is a rationale for the very peculiar difference between dependencies of $v_o/v_o([\text{N}] = 0)$ on the acceptor concentration for α-chymotrypsin and γ-GluTP (Figures 3 and 2 respectively). In reactions catalysed by α-chymotrypsin, the effect of $[\text{S}]_o$ on the dependence of $v_o/v_o([\text{N}] = 0)$ on $[\text{N}]_o$ was more pronounced at higher $[\text{S}]_o$. This is in complete accordance with a Ping-Pong mechanism modified with a hydrolytic shunt. For such a mechanism no acceleration must be expected when the donor concentration is lower than the hydrolysis K_m. Under this condition the process is governed by $V_{max.}/K_m$ for the donor, which does not depend on the acceptor concentration for this mechanism. In γ-GlyTP-catalysed reactions, the increase of $[\text{S}]_o$ diminished the effect of $[\text{N}]_o$ on $v_o/v_{o([\text{N}]-0)}$ at low $[\text{N}]_o$ and increased the effect of $[\text{N}]_o$ on $v_o/v_{o([\text{N}]-0)}$ at high $[\text{N}]_o$. The question of whether [S] increased or suppressed the effect of [N] on $v_o/v_{o([\text{N}]-0)}$ entirely depends on the form of the dependence of the apparent K_m on $[\text{N}]_o$. If we denote the apparent K_m for the donor in the absence of the acceptor as $K_{m([\text{N}]-0)}$ the ratio of $v_o/v_{o([\text{N}]-0)}$ is given by eqn. (7):

$$\frac{v_o}{v_{o([\text{N}]-0)}} = \frac{V_{max.}(K_{m([\text{N}]-0)} + [\text{S}])}{V_{max.([\text{N}]-0)}(K_m + [\text{S}])} \tag{7}$$

Therefore, the difference between the two values of $v_o/v_{o([\text{N}]-0)}$ (denoted as D) measured at the same acceptor but different donor concentrations, $[\text{S}]_1$ and $[\text{S}]_2$ ($[\text{S}]_2 > [\text{S}]_1$) is given by the following equation:

$$d = \frac{V_{max.}([\text{S}]_2 - [\text{S}]_1)(K_m - K_{m([N]-0)})}{V_{max.([\text{N}]-0)}(K_m + [\text{S}]_1)(K_m + [\text{S}]_2)} \tag{8}$$

Since $[\text{S}]_2 > [\text{S}]_1$, the sign of D depends on the sign of $K_m - K_{m([\text{N}]-0)}$. In α-chymotrypsin-catalysed reactions $K_m - K_{m([\text{N}]-0)} > 0$ and therefore $D > 0$. In γ-GluTP-catalysed reactions $K_m - K_{m([\text{N}]-0)} < 0$ at low $[\text{N}]_o$ but at higher $[\text{N}]_o K_m - K_{m([\text{N}]-0)} > 0$ (Figure 4a). In accordance to that, $D < 0$ at low acceptor concentrations but $D > 0$ at higher $[\text{N}]_o$.

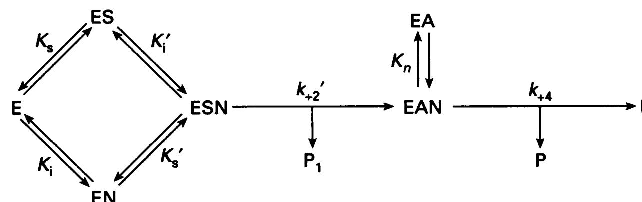
The analysis of the relative contribution of different reaction pathways showed that the acceptor affected not only the rate of the γ-GluTP reactions but, in fact, the reaction mechanism. If we denote the rate of the formation of the product P_1 from the ES complex as v_1 and that from the ESN complex as v_1', the ratio $v_1/(v_1 + v_1')$ reflects the relative contribution of the first pathway. Since $v_1 = k_{+2}[\text{ES}]$, $v_1' = k_{+2}'[\text{ESN}]$ and $[\text{ESN}] = [\text{ES}][\text{N}]_o/k_i'$ the following equation is valid:

$$\frac{v_1}{v_1 + v_1'} = \frac{1}{1 + \dfrac{k_{+2}'}{k_{+2}K_i'}[\text{N}]_o} \tag{9}$$

As shown above $k_{+2}'/(k_{+2}/K_i') = 1.73 \pm 0.09\ \text{mM}^{-1}$. Therefore less than 10 % of the total donor consumption occurs through the ES complex at an acceptor concentration as low as 6 mM. Similarly, if we denote the rate of the formation of the product P_2 from the EA intermediate as v_2 and the rate of formation of the transfer product from the EAN complex as v, the ratio v_2/v reflects the significance of the hydrolytic pathway when compared with the synthetic one. Since $v_2 = k_{+3}[\text{EA}]$, $v = k_{+4}[\text{EAN}]$ and $[\text{EAN}] = [\text{EA}][\text{N}]_o/K_n$ the following equation is valid:

$$\frac{y_2}{v} = \frac{k_{+3}K_n}{k_{+4}[\text{N}]_o} \tag{10}$$

The combination of $k_{+4}/(k_{+3}K_n)$ equals $0.15 \pm 0.01\ \text{mM}^{-1}$. Therefore, if $[\text{N}]_o > 30$–50 mM the relative contribution of the ES $\rightarrow$ EA + P_1 and EA $\rightarrow$ E + P_2 pathways is small and a Ping-Pong mechanism turns into a sequential mechanism with random addition of the substrates and ordered release of the products (Scheme 4).



Scheme 4

The initial rate obeys the following equation:

$$v_o = \frac{k'_{+2}[E]_o[S]_o[N]_o}{(K_s K'_i + K'_s[N]_o) + K'_i\left(1 + \frac{K_n k'_{+2}}{K'_i k_{+4}}\right)[S]_o + \left(1 + \frac{k'_{+2}}{k_{+4}}\right)[S]_o[N]_o} \quad (11)$$

[Eqn. (11) can also be obtained from eqns. (1–4) if both $k_{+4}[N]_o/(k_{+3}K_n)$ and $k'_{+2}[N]/(k_{+2}K'_i) \gg 1$.]

It is reasonable to assume that γ-glutamyltranspeptidase from different sources share the same kinetic mechanism and differences in kinetic behaviour are attributed to different values of the kinetic constants. In this case the model proposed above should account for a number of the results which, at first glance, appear to contradict each other.

Presentation of the experimental data in double-reciprocal coordinates often yields a set of parallel lines (Tate and Meister, 1974; Elce and Broxmeyer, 1976; Karkowski et al., 1976; Stromme and Theodorsen, 1976; Huseby, 1977; Shaw et al., 1977, 1978). This parallelism is generally interpreted as being due to a Ping-Pong mechanism of γ-GluTP catalysis which is correct for the usual two-substrate–two-product Ping-Pong mechanism (Fromm, 1975). However, in addition to the transfer to the added acceptor, γ-GluTP catalyses acyl-group transfer to water. Therefore, the Ping-Pong mechanism has to be modified by a hydrolytic shunt. Transfer and hydrolysis, when occurring simultaneously, should produce curvature in the $1/v_o - 1/[N]_o$ plot since v_o does not tend to zero when $[N]_o$ is described. Figure 6(a) presents dependencies obtained in our experiments. A set of parallel lines in the $1/v_o - 1[N]_o$ coordinates contradicts a modified Ping-Pong mechanism. A simple (non-modified) Ping-Pong mechanism can also be ruled out because it cannot interpret the experimental dependencies of Michaelis–Menten parameters for the donor on the acceptor concentration (Figure 4). However, a sequential random mechanism (Scheme 4) predicts a set of parallel lines in the $1/v_o - 1/[N]_o$ coordinates provided that $K_s K'_i \ll K'_s[N]_o$ or, since $K_s K'_i = K'_s K_i$, $[N]_o > K_i$. The latter condition is obeyed at $[N]_o$ as little as 10–20 mM since $K_i = 2.8$ mM. In this case the apparent $V_{max.}/K_m$ for the acceptor is given by eqn. (12):

$$\frac{V_{max.}}{K_m} = \frac{K_{+4}[E]_0}{K'_i \frac{k_{+4}}{k'_{+2}} + K_n} \quad (12)$$

Figure 6(b) presents the same experimental data as Figure 6(a) but at $1/[GlyGly]_o < 80\ M^{-1}$ ($[GlyGly]_o > 12.5$ mM). This shows that when $1/[GlyGly]_o$ lies between $40\ M^{-1}$ and $80\ M^{-1}$ the results can be approximated by straight parallel lines.

As for dependencies in the $1/v_o - 1/[S]_o$ coordinates, we should bear in mind that a set of parallel lines in these coordinates obtained at different $[N]_o$ means that the apparent $V_{max.}/K_m$ for the donor does not depend on the acceptor concentration. Many papers shows that this is not the case and the apparent $V_{max.}/K_m$ for the donor depends on the concentration of the acceptor and lines in the $1/v_o - 1/[S]_o$ coordinates obtained at different $[N]_o$ are not necessarily parallel (Stromme and Theodorsen, 1976; Thompson and Meister, 1977; PetitClerc et al., 1980; Bagrel et al., 1981; Schiele et al., 1981; Solberg et al., 1981).

The kinetic model of the γ-GluTP catalysis suggested in this work can explain these discrepancies. Figure 4(b) shows that the apparent $V_{max.}/K_m$ for the donor was a constant at $[N]_o > 50$ mM. At lower acceptor concentrations the apparent $V_{max.}/K_m$ for the donor decreased by a factor of 2.5–3. So, presentation of the results in the $1/v_o - 1/[S]_o$ coordinates should produce a set of parallel lines if $[GlyGly]_o > 50$ mM and a set of converging lines at $[GlyGly]_o < 50$ mM. In terms of eqn. (5) this means that both $k'_{+2}[N]_o/(k_{+2}K'_i)$ and $[N]_o/K_i \gg 1$ and (taking into account that $K'_i K_s = K_i K'_s$) $V_{max.}K_m = [E]_o k'_{+2}/K'_s$. In other words, the apparent $V_{max.}/K_m$ for the donor at $[GlyGly]_o > 50$ mM corresponded to the enzyme saturated with the nucleophile. However, if experiments at $[GlyGly]_o < 50$ mM were not performed, this phenomenon would not be noticed. Very few studies have been performed in which the family of the lines in the $1/v_o - 1/[S]_o$ coordinates includes the line obtained at $[N]_o = 0$. Also the values of the kinetic constants for enzymes from different sources are undoubtedly different and depend on the acceptor and donor used. Therefore, the acceptor concentration at which $V_{max.}/K_m$ levels off and converging lines in the $1/v_o - 1/[S]_o$ coordinates into parallel ones may be different.

Now we can go to the very interesting question of the inhibitory effect of the substrates which is reflected, in particular, in the maxima on the dependencies of $v_o/v_{o([N]=0)}$ on the acceptor concentrations (Figures 2 and 3). The model proposed in this work to interpret the γ-GluTP kinetics cannot explain such a dependence. In contrast, Scheme 3 predicts this phenomenon and interprets it as being due to the dead-end formation of the enzyme–acceptor complex. Many previous papers report evidence in favour of the substrate inhibition (Rosalki and Tarlow, 1974; Stromme and Theodorsen, 1976; London et al., 1976; Huseby, 1977; Thompson and Meister, 1977; Shaw et al., 1978; PetitClerc et al., 1980; Bagrel et al., 1981; Schiele et al., 1981; Solberg et al., 1981) which they interpret in terms of Scheme 3. The competitive inhibition constant (K_i) was reported to exceed 150 mM for γ-GluTP from rat kidney (Thompson and Meister, 1977). For enzymes from other sources, the inhibitory effect of GlyGly also becomes evident at GlyGly concentrations exceeding 0.1 M. Similar results were obtained in this work. Our interpretation of this phenomenon, however, differs from that of previous studies in that we take into account that GlyGly has an ionized α-carboxyl termini. In other words, GlyGly is a salt which can affect the reaction as any other salt, e.g. NaCl (Figure 5). Figure 7 shows the comparison of the direct experimental data with the same data corrected for salt effect. This comparison showed that the inhibitory effect of the acceptor could be attributed (at least for reactions catalysed by γ-GluTP from bovine kidney) to the salt effect of this compound. As for the inhibitory effect of Glu*p*NA, published results are often controversial. According to previous studies, the concentration of Glu*p*NA should be at least 3.5 mM to observe this effect. However, there are many papers where an inhibitory effect of Glu*p*NA was not mentioned. In this study we could not find any evidence of inhibition by the donor at Glu*p*NA concentrations up to the solubility limit of 5 mM (Figure 1). The apparent discrepancy between our and certain literature data with this respect is probably the result of the dependence of the effect on the source of the enzyme and the fact that very high concentrations of the donor are necessary to observe this inhibition.

In addition it should be noted that in all previous studies the occurrence of substrate inhibition was stated after the analysis of the data in double-reciprocal coordinates. However, inverting v_o and $[S]_o$ introduces distortion and double-reciprocal plots seriously exaggerate the magnitude, and therefore significance, of the effect. We have to stress that the reason for this distortion consists in the double-reciprocal transformation as such and occurs even in the case of data without random errors. Comparison of Figures 2 and 6(b) showing the same data but in different coordinates presents a good example of such a distortion. Examination of previously published data shows that the effect is usually of the same order of magnitude as shown in

Figure 6. On the other hand, if the double-reciprocal plot shows a lack of substrate inhibition, this phenomenon is certainly absent.

Conclusions

This study presents kinetic evidence in favour of major differences between the kinetic mechanisms of γ-GluTP and serine proteases in spite of the fact that all of these enzymes contain an active-site serine and act with the formation of an acyl-enzyme intermediate. The main difference between reactions catalysed by these enzymes consists in the role of the enzyme–donor–acceptor complex. This complex is not formed in the serine-protease catalysis. In contrast, the main reaction pathway of γ-GluTP-catalysed reactions includes this complex.

We wish to thank Vitas Svedas for valuable advice during work with α-chymotrypsin. This work was supported by the NSF EPSCoR Programme, the State of Mississippi, the University of Southern Mississippi, and NIH Grant DK41892 to R.C.B.

REFERENCES

Allison, R. D. (1985) Methods Enzymol. **113**, 419–437
Antonov, V. K., Ginodman, L. M., Rumsh, L. D., Kapitannikov, Yu. V., Barshevskaya, T. N., Yavashev, L. P., Gurova, A. G. and Volkova, L. I. (1981) Eur. J. Biochem. **117**, 195–200
Bagrel, D., PetitClerc, C., Schiele, F. and Siest, G. (1981) Biochim. Biophys. Acta **658**, 220–231
Bender, M. L., Clement, G. E., Gunter, C. R. and Kezdy, F. J. (1964) J. Am. Chem. Soc. **86**, 3697–3703
Berezin, I. V., Kazanskaya, N. F. and Klyosov, A. A. (1973) FEBS Lett. **15**, 121–124
Bizzozero, S. A., Dutler, H. and Ruckert, P. (1988) Int. J. Pept. Protein Res. **32**, 64–73
Bratovanova, F. K., Stoineva, I. B. and Petkov, D. D. (1988) Tetrahedron **44**, 3633–3637
Christensen, U., Drohse, H. B. and Molgaard, L. (1992) Eur. J. Biochem. **210**, 467–473
Elce, J. S. (1980) Biochem. J. **185**, 473–481
Elce, J. S. and Broxmeyer, B. (1976) Biochem. J. **153**, 223–232
Fastrez, J. and Fersht, A. R. (1973) Biochemistry **12**, 2025–2034
Fersht, A. (1985a) in Enzyme Structure and Mechanism, pp. 195–207, Freeman, New York
Fersht, A. (1985b) in Enzyme Structure and Mechanism, pp. 405–426, Freeman, New York
Fersht, A. R., Blow, D. M. and Fastrez, J. (1973) Biochemistry **12**, 2035–2041
Fromm, H. (1975) in Initial Rate Kinetics, Springer Verlag, New York
Gololobov, M. Yu., Petrauskas, A., Pauliukonis, R., Koschke, V., Borisov, I. L. and Svedas, V. (1990) Biochim. Biophys. Arca **1041**, 71–78
Gololobov, M. Yu., Voyushina, T. L., Stepanov, V. M. and Adlecreutz, P. (1992) Biochim. Biophys. Acta **1160**, 188–192
Gololobv, M. Yu., Stepanov, V. M., Voyushina, T. L. and Adlecreutz, P. (1993) Eur. J. Biochem. **217**, 955–963
Huseby, N.-E. (1977) Biochim. Biophys. Acta **483**, 46–56
Inoue, M., Horiuchi, S. and Morino, Y. (1978) Biochem. Biophys. Res. Commun. **82**, 1183–1188
Karkowski, A. M., Bergamini, M. V. W. and Orlowski, M. (1976) J. Biol. Chem. **251**, 4736–4743
King, F. E. and Kidd, D. A. A. (1949) J. Chem. Soc. 3315–3319
Kullmann, W. (1984) Biochem. J. **220**, 405–416
London, J. W., Shaw, L. M., Fetterolf, D. and Garfinkel, D. (1976) Biochem. J. **157**, 609–617
Orlowski, M. and Meister, A. (1965) J. Biol. Chem. **240**, 338–347
PetitClerc, C., Schiele, F., Bagrel, D., Mahassen, A. and Siest, G. (1980) Clin. Chem. **26**, 1688–1693
Petkov, D. D. and Stoineva, I. (1984) Biochem. Biophys. Res. Commun. **118**, 317–323
Riechmann, L. and Kasche, V. (1984) Biochem. Biophys. Res. Commun. **120**, 686–691
Riechmann, L. and Kasche, V. (1985) Biochim. Biophys. Acta **830**, 164–172
Rosalki, S. B. and Tarlow, D. (1974) Clin. Chem. **20**, 1121–1124
Schiele, F., Artur, Y., Bagrel, D., PetitClerc, C. and Siest, G. (1981) Clin. Chem. Acta **112**, 187–195
Schellenberger, V. and Jakubke, H.-D. (1986) Biochim. Biophys. Acta **869**, 54–60
Schellenberger, V. and Jakubke, H.-D. (1991) Angew. Chem. Int. Ed. Engl. **30**, 1437–1449
Schellenberger, V., Schellenberger, U., Mitin, Y. V. and Jakubke, H.-D. (1990) Eur. J. Biochem. **187**, 163–167
Schellenberger, V., Kosk, M., Jakubke, H.-D. and Aaviksaar, A. (1991) Biochim. Biophys. Acta **1078**, 1–7
Seydoux, F., Yon, J. and Nemethy, G. (1969) Biochim. Biophys. Acta **171**, 145–156
Shaw, L. M., London, J. W., Fetterolf, D. and Garfinkel, D. (1977) Clin. Chem. **23**, 79–85
Shaw, L. M., London, J. W. and Petersen, L. E. (1978) Clin. Chem. **24**, 905–915
Shima, H., Fukuda, M., Tanabe, K., Ito, T. and Kunugi, S. (1987) Bull. Chem. Soc. Jpn. **60**, 1403–1406
Solberg, H. E., Theodorsen, L. and Stromme, J. H. (1981) Clin. Chem. **27**, 303–307
Stromme, J. H. and Theodorsen, L. (1976) Clin. Chem. **22**, 417–421
Tate, S. S. and Meister, A. (1974) J. Biol. Chem. **249**, 7593–7602
Tate, S. S. and Meister, A. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 931–935
Tate, S. S. and Meister, A. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 4806–4809
Tate, S. S. and Meister, A. (1985) Methods Enzymol. **113**, 400–419
Thompson, G. A. and Meister, A. (1976) Biochem. Biophys. Res. Commun. **71**, 32–36
Thompson, G. A. and Meister, A. (1977) J. Biol. Chem. **252**, 6792–6798

Received 17 March 1994/18 July 1994; accepted 21 July 1994