HYDROLYSIS AND TRANSFER REACTIONS CATALYZED BY γ-GLUTAMYL TRANSPEPTIDASE; EVIDENCE FOR SEPARATE SUBSTRATE SITES AND FOR HIGH AFFINITY OF L-CYSTINE

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SUMMARY

γ-Glutamyl transpeptidase was studied with L- and D-γ-glutamyl-p-nitroanilide as γ-glutamyl donors. No autotranspeptidation occurred with the D-γ-glutamyl donor or when the L-γ-glutamyl donor was used at concentrations lower than 10 μM. The K_m values for hydrolysis were 5 and 31 μM for the L- and D-γ-glutamyl donors, respectively; the corresponding V_{max} values were identical. The γ-glutamyl donor site of the enzyme thus exhibits low stereospecificity (but high affinity), while the acceptor site exhibits absolute L-specificity. The K_m value for L-cystine as acceptor is very low (30 μM); the same value was obtained with L- and D-γ-glutamyl donors. The data are consistent with a ping pong mechanism and the existence of separate donor and acceptor enzyme sites. The present findings thus extend the usefulness of γ-glutamyl-p-nitroanilide as a substrate in probing the catalytic behavior of this enzyme.

γ-Glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl moiety of glutathione and of other γ-glutamyl compounds to amino acid and peptide acceptors (reaction (1)). The enzyme also catalyzes the hydrolysis of γ-glutamyl compounds (reaction (2)) and an autotranspeptidation reaction in which the γ-glutamyl compound itself serves as an acceptor of the γ-glutamyl group (reaction (3)). The conditions which

(1) γ-glu-X + acceptor $\rightleftharpoons$ γ-glu-acceptor + HX

(2) γ-glu-X + H_2O $\longrightarrow$ glutamate + HX

(3) γ-glu-X + γ-glu-X $\rightleftharpoons$ γ-glu-γ-glu-X + HX

have been used for study of γ-glutamyl transpeptidase with L-γ-glutamyl-p-nitroanilide as substrate do not permit separate determination of the individual rates of reactions (1), (2), and (3) (1-4), although the contributions of reactions (2) and (3) to the overall rate of p-nitroaniline formation may be reduced by use of high concentrations of donor and

acceptor substrates (3,4). Separate determination of the rates of these reactions can be achieved by less convenient procedures involving separation and determination of the products (3, 5-8). In the present work we have adapted the direct spectrophotometric assay to the separate determination of reactions (1) and (2) by using conditions under which the L-γ-glutamyl donor does not bind to the acceptor site of the enzyme, and also by using a D-γ-glutamyl donor substrate. Thus, we have found that the enzyme has a very high affinity for both the L- and D-isomers of γ-glutamyl p-nitroanilide. The L-isomer has much lower affinity as an acceptor, whereas the D-isomer is inactive as an acceptor. Application of the present approach to study of reaction (1) with L-cystine permitted determination of the true K_m value for this acceptor.

MATERIALS AND METHODS

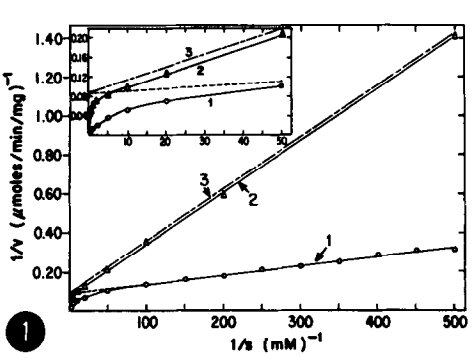
L-γ-Glutamyl p-nitroanilide, glutamate decarboxylase (E. coli) and horseradish peroxidase were obtained from Sigma. L-Cystine was purchased from Calbiochem; D-glutamic acid was obtained from Pierce. D-γ-Glutamyl p-nitroanilide (1) and γ-glutamyl transpeptidase (specific activity = 1360 μmoles/min/mg) (9) were prepared as described. We thank Dr. D. Wellner for providing L-amino acid oxidase and Mr. R. Sekura for D-[1-^{14}C] glutamic acid.

The assays were performed at 37° with a Cary 118 spectrophotometer by measuring the increase in absorbance at 405 nm ($\epsilon = 9900\ M^{-1}\ cm^{-1}$) due to formation of p-nitroaniline. The reaction mixtures contained 57 ng of enzyme, 0.1 M triethanolamine-HCl buffer (pH 8.0, 8.5 or 9.0), 5 μM to 2.5 mM γ-glutamyl p-nitroanilide, and where indicated 0 to 400 μM L-cystine (Vol. = 1.00 ml).

The optical purity of D-γ-glutamyl p-nitroanilide was determined by treating an acid hydrolyzed sample with L-glutamate decarboxylase and measuring the γ-aminobutyric acid formed on a Durrum Model 500 amino acid analyzer. This test showed a 0.8% contamination with the L-isomer, which was confirmed by assay of the unhydrolyzed compound with L-amino acid oxidase (10).

RESULTS

The plot of $1/v$ against $1/s$ with L-γ-glutamyl p-nitroanilide (Fig. 1, curve 1), is linear at substrate concentrations less than 10 μM ($1/s$, 100 mM^{-1}) suggesting that whereas autotranspeptidation predominates at high concentrations, hydrolysis is the sole reaction that occurs in the linear portion of the curve. Amino acid analyses of the products formed confirmed this interpretation; thus, L-γ-glutamyl-L-γ-glutamyl p-nitroanilide but no glutamate was detected when 1 mM substrate was used, while only glu-



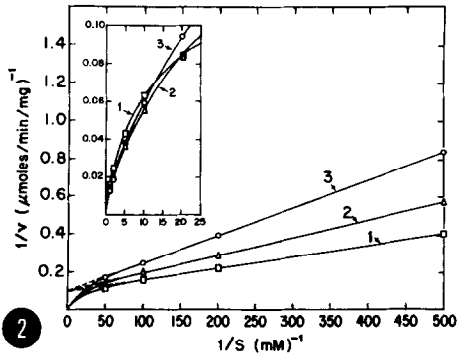


Fig. 1. Effect of γ-glutamyl p-nitroanilide concentrations on activity; reciprocal plots of the data (pH 8.0). Curve 1: L-γ-glutamyl p-nitroanilide; Curve 2: D-γ-glutamyl p-nitroanilide; Curve 3: Data of curve 2 corrected (see the text).

Fig. 2. Effect of pH on activity toward L-γ-glutamyl p-nitroanilide. Curve 1: pH 8.0; Curve 2: pH 8.5; Curve 3: pH 9.0.

tamate was found with 5 μM substrate. Extrapolation of the linear portion of the curve gave K_m and V_{max} values of 5.6 μM and 11.6 μmoles/min/mg, respectively, for the hydrolysis of L-γ-glutamyl p-nitroanilide.

The double reciprocal plot with D-γ-glutamyl p-nitroanilide as donor (Fig. 1, curve 2) becomes linear at concentrations less than 100 μM; however, after correction of these data for 0.8% contamination by the L-enantiomer, the curve is fully linear (curve 3) indicating that D-γ-glutamyl p-nitroanilide does not serve as an acceptor. This is consistent with other data showing that D-amino acids are not acceptors (3, 11, 12), and is also in accord with our finding that no [^{14}C] γ-glutamyl peptides were formed when D-[1-^{14}C] glutamate was added to the reaction mixture. The K_m and V_{max} values for the hydrolysis of D-γ-glutamyl p-nitroanilide are 31 μM and 11.7 μmoles/min/mg, respectively.

Experiments with L-γ-glutamyl p-nitroanilide at higher values of pH gave similar curves (Fig. 2). The K_m increases from 6 μM at pH 8.0 to 16 μM at pH 9.0, while the corresponding V_{max} values remain constant. This result is in accord with previous re-

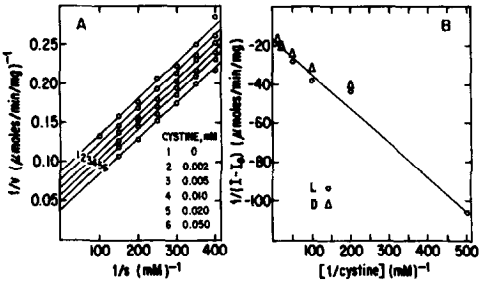


Fig. 3. (A) Reciprocal plots showing the effect of various fixed concentrations of L-cystine on the relation between L-γ-glutamyl p-nitroanilide concentration and initial velocity. (B) Data of (A) replotted for calculation of K_m value for L-cystine (16). [pH 8.0] Values of I were obtained from the ordinate intercepts in (A) at the appropriate concentration of L-cystine; I_o is the value of the intercept in the absence of acceptor. Data were obtained from experiments with L- (open circles) and D- (open triangles) γ-glutamyl p-nitroanilide.

ports that hydrolysis has a lower pH optimum than transpeptidation (pH 8.8) (13, 14) and clarifies a recent observation that the rate of hydrolysis is independent of pH at a substrate concentration of 5 mM (8).

Reciprocal plots of data obtained with L-cystine under conditions that exclude autotranspeptidation lead to a family of parallel lines (Fig. 3A) suggestive of a ping pong mechanism analogous to that observed for transglutaminase (15). Use of a graphic method for calculating kinetic constants described for the latter enzyme (16) based on equations derived by Hsu, et al. (17) gave values of K_m and V_{max} of 30 μM and 36 μmoles/min/mg, respectively, for L-cystine as an acceptor (Fig. 3B). These values were independent of the stereoisomer of γ-glutamyl p-nitroanilide used and indicate an even higher affinity for L-cystine than indicated by previously determined apparent K_m values (18, 19).

DISCUSSION

The present approach seems to have promise for further application to the study of γ-glutamyl transpeptidase. Thus, exclusion of autotranspeptidation reduces the kinetic problem to that of distinguishing comcomitant hydrolysis and transfer (20). The pro-

cedures described here thus extend the usefulness of γ-glutamyl p-nitroanilide as a substrate for studies on this enzyme. It is notable that (a) the affinity of the enzyme for the L- and D-isomers of γ-glutamyl p-nitroanilide is of the same order of magnitude as that of the natural substrate, glutathione (7), (b) the V_{max} values for hydrolysis are the same for both isomers of γ-glutamyl p-nitroanilide, and (c) the K_m value for L-cystine as an acceptor is independent of the optical configuration of the γ-glutamyl donor. The ability of the enzyme to interact effectively with both isomers of the γ-glutamyl donor suggests that it may be feasible to map the active site of γ-glutamyl transpeptidase by a procedure similar to that used previously in the study of glutamine synthetase (21).

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