An Essential Histidine Residue in the Catalytic Mechanism of the Rat Kidney γ-Glutamyl Transpeptidase

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γ-Glutamyl transpeptidase (EC 2.3.2.2) plays a key role in glutathione metabolism by catalyzing the transfer of the γ-glutamyl residue and hydrolysis of glutathione. The functional residues at the active site of the rat kidney γ-glutamyl transpeptidase were investigated by kinetic studies at various pH, the treatment of diethylpyrocarbonate (DEPC), and photooxidation in presence of methylene blue. An ionizable group affecting the enzymatic activity with an apparent p*K*a value of 7.1, which is in the range of p*K*a values for a histidine residue in protein, was obtained by examining the pH-dependence of kinetic parameters. The pH effect on the photoinduced inactivation rate of the enzyme corresponds to that expected for the photooxidation of the free histidine. The involvement of a histidine in the catalytic site of the enzyme was further supported by DEPC modification accompanied by an increase in absorbance at 240 nm, indicating the formation of *N*-carbethoxyhistidine. The histidine located at the position of 382 in the precursor of the enzyme is primarily suspected based on the amino acid sequence alignment of the transpeptidases from various organisms.

Key Words : Gamma-glutamyl transpeptidase, Essential histidine, Kinetic study, Chemical modification, Photo-oxidation

Introduction

γ-Glutamyl transpeptidase (γ-GT) that has been found in various mammalian tissues plays a key role in glutathione metabolism.[1-3] The enzyme has been widely used as a tumor marker in experimental liver carcinogenesis and human cancer. It has been reported that significant changes in the elevated expression of the transpeptidase take place in liver and kidney during oncogenesis.[4,5] Three molecular forms of human γ-GT have been found in normal and renal cell carcinoma tissues.[5] Recent experiments have suggested that the transpeptidases undergo significant post-translational modification *via* proteolytic digestion and glycosylation, which may be different between normal and cancerous tissues.[6,7] While the regulation of γ-GT and its relation to neoplasia is an area of current active research, information regarding its structural and catalytic aspects is little known in spite of the requirement in understanding its physiological function.

The highest activity of γ-GT has been found in the kidney of mammalian tissues.[8] The rat kidney enzyme is an amphipathic heterodimer consisting of two glycosylated subunits. The heavy subunit (H, 51 kDa) contains a membrane spanning N-terminal segment. The light subunit (L, 22 kDa) noncovalently associated with the H subunit has been suggested to contain the active site residues.[9-11] Previous immunological and kinetic experiments have suggested that the two subunits of rat kidney transpeptidase are synthesized as a single common polypeptide precursor (78 kDa).[12] The rat transpeptidase gene has been cloned and well characterized as an attempt to elucidate the mechanism involving its tissue-specific transcription as well as its relation to neoplasia.[13] Also the active site of rat kidney γ-GT was recently investigated using the various ester and amide derivatives of the well-known substrate L-γ-glutamyl-*p*-nitroanilide.[14] However, much less is known about the structural information in the catalytic site of the enzyme.

In the present work, kinetic studies, photooxidation, and chemical modification by diethylpyrocarbonate (DEPC) have been carried out in order to obtain information regarding functional amino acid residues at the catalytic site of rat kidney γ-GT. The results provide biophysical and chemical evidences that a histidine residue is involved in the active site of the enzyme.

Materials and Methods

Materials. γ-GT was isolated from rat kidney using Triton X-100 detergent and converted to a water soluble form by papain treatment as previously described.[15] The specific activity of the apparently homogeneous preparation as judged by native polyacrylamide gel electrophoresis was 393.70 units per mg when assayed as described below. The difference between this preparation and the previous one (488 units/mg) may be due to the different kidney donors. L-γ-glutamyl-*p*-nitroanilide (γ-Glu-PNA), glycylglycine (Gly-Gly), and DEPC were purchased from Sigma Chemical Co. (St. Louis, MO). Methylene blue was obtained from Wako Pure Chemicals (Japan). All other chemicals were of the highest purity commercially available. Cary model 17 spectro-

photometer was used for kinetic studies and spectral experiments.

Enzymatic assay. γ-GT was assayed spectrophotometically using v-Glu-PNA according to the procedure described by Tate and Meister.[16] The reaction mixture for a standard assay contained 2.5 mM γ-Glu-PNA, 20 mM Gly-Gly, and 50 mM Tris-HCl (pH 8.0). One unit of transpeptidase is defined as the amount of enzyme that catalyzes release of 1 mmol of *p*-nitroaniline per minute. Protein concentrations were determined by the method of Lowry *et al.* using bovine serum albumin as a standard protein.[17]

pH effect on the transpeptidase activity. The pH effect on the activity of rat kidney γ-GT was examined by measuring the rate of the *p*-nitroaniline production under the standard condition after incubation for 6 mins at various pH in the pH range of 4.0 to 11.0. The following buffers were utilized at 50 mM concentration for the pH ranges indicated: acetate, pH 4.0-5.5; phosphate, pH 6.0-6.8; Tris, pH 7.0-9.0; carbonate, pH 9.5-11. In order to get information on the functional ionizable residues involved in the active site of the transpeptidase, pH dependent kinetic experiments were carried out in the range of pH 6.0-10.0 within which the enzyme activity is fully reversible. The kinetic parameters for the acceptor substrate, Gly-Gly, at various pH were determined by Double reciprocal plotting and analyzed by the method of Dixon and Webb.[18]

Photooxidation. The photooxidation experiments were performed according to the method described by Rippa and Pontremoli with minor modifications.[19] Methylene blue, at a final concentration of 0.001%, was added to the enzyme solution (0.05 mg/mL) in a test tube, which was put into distilled water in a 2 liter beaker. The water was circulated at 37 °C and the enzyme solution was continuously bubbled with stream of air. The light source was a 300 watt tungsten lamp positioned at 10 cm away from the surface of the test tube. Aliquots were taken at various time and assayed for the enzymatic activity under the standard condition. A control experiment was run in the dark under identical conditions.

Chemical modification. DEPC was freshly diluted with anhydrous ethanol just before use. The concentration of DEPC was spectrophotometically determined by the reaction with L-histidine using a molar extinction coefficient of 3200 $M^{-1}cm^{-1}$ at 240 nm for *N*-carbethoxyhistidine.[20] To the enzyme solution (0.05 mg/mL) was added the ethanolic solution of DEPC (1.0 mM-3.0 mM) and the mixture was incubated at 37 °C. The ethanol concentration did not exceed 5% by volume and was found to have no effect on the activity and stability of enzyme during incubation. At various time intervals during incubation, aliquots were taken and assayed for the residual enzyme activity.

Results

The kinetic studies on the transpeptidase at various pH. The pH profile on the transpeptidase activity revealed a bell shaped curve with an optimum pH of 8.0. (not shown). The result showed that the enzyme activity was fully reversible in the range of pH 6.0 to 10.0 and the enzyme was irreversibly denatured below pH 6.0 or above pH 10.0, which suggested that the activity variation in the pH range of 6.0 to 10.0 was due to the ionizable species affecting the enzymatic activity. In order to obtain information on the essential ionizable groups for the activity of the transpeptidase, a series of kinetic experiments in the range of pH 6.0 to 10.0 were carried out with varying concentration of the acceptor substrate (Gly-Gly). The apparent *K*m and *V*max values determined by Lineweaver-Burk plotting were analyzed according to the method of Dixon and Webb (Fig. 1).[18] A p*K*a value of 7.1 was estimated for an essential ionizable group of the free transpeptidase molecule from the plot of log (*V*m/*K*m) versus pH (upper panel in Fig. 1). The p*K*a value of 7.4 obtained from the plot of log*V*m versus pH (lower panel in Fig. 1) may be accounted for the ionizable residue of an enzyme-substrate complex.[21] The p*K*a values determined by the Dixon and Webb's analysis are in the range of p*K*a value (5.9-7.5) for histidine residues found in biomolecules and comparable to the p*K*a values of 7.3 and 7.5 estimated for an essential histidine of neutral endopeptidase and thermolysin, respectively.[22,23] The result of the pH dependent kinetic experiments, therefore, suggests that the rat kidney γ-GT contains at least one histidine imidazole group in the catalytic site.

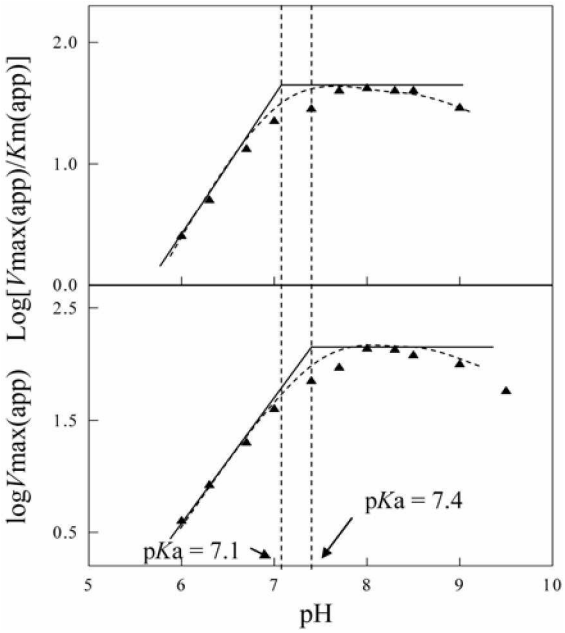

Figure 1. Effect of pH on the enzymatic activity of γ-GT for Gly-Gly: The initial velocities were measured with the varying Gly-Gly concentration at the indicated pH. The resulting kinetic parameters estimated by double-reciprocal plots were plotted by the method of Dixon and Webb.

The p*K*a values of an ionizable residue estimated by the Dixon and Webb's method using the pH dependent kinetic parameters for Gly-Gly were further confirmed by the same treatment with the kinetic parameters for the donor substrate (γ-Glu-PNA) (not shown). The relatively high p*K*a value of 7.1 which is considered to be that of a histidine involved in

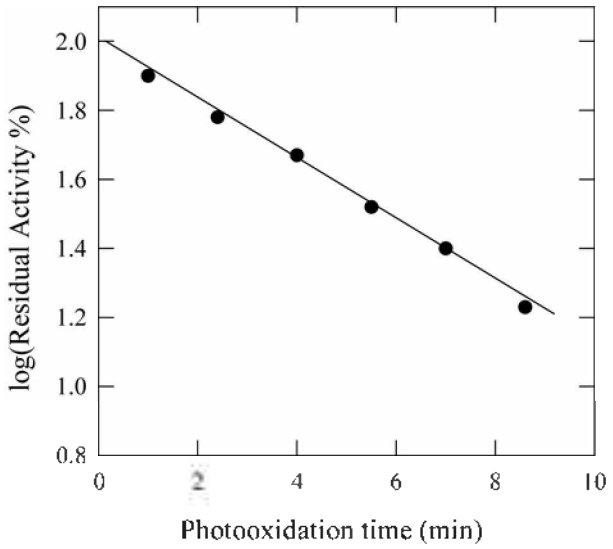

Figure 2. Kinetics of the inactivation of γ-GT by photooxidation in presence of methylene blue.

the enzymatic activity may be explained by the presence of negatively charged group(s) near the ionizable group. The higher p*K*a value (7.4) of the histidine estimated from the plot of log*V*m versus pH may suggest that the ionizable group is at or near the substrate binding site of the enzyme so that the positively charged imidazole group can be stabilized by the carboxylate of substrate.

Photooxidation of γ-GT. The transpeptidase was irreversibly inactivated by photooxidation in the presence of methylene blue as shown in Figure 2. The loss of enzymatic activity follows first-order kinetics down to 80% inactivation. The enzyme solution containing the dye did not lose the activity when kept in the dark as a control experiment. The pH effect of photoinactivation of γ-GT was carried out in the

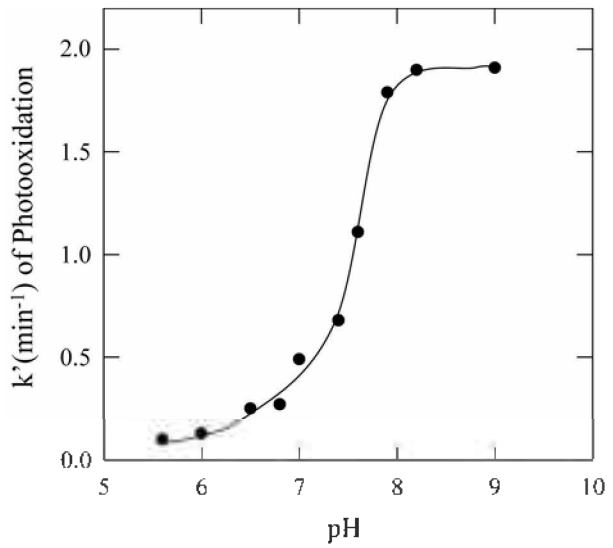

Figure 3. Effect of pH on the photoinactivation rate of the transpeptidase: Apparent first-order rate constants (k') for the loss of enzymatic activity were estimated graphically from semilogarithmic plots similar to Fig. 2.

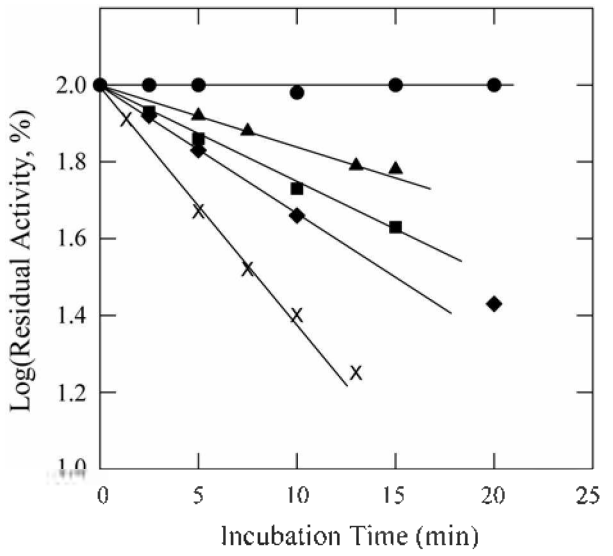

Figure 4. Inactivation of γ-GT by DEPC: The enzyme (0.05 mg/mL) was incubated at concentrations of DEPC [control (●), 1.0 (▲), 1.2 (■), 1.5 (◆), and 2.4 (×) mM] in 50 mM Tris-HCl (pH 7.0) at 37 °C. 10 mL of aliquot from the reaction mixture was taken at the indicated time intervals during incubation and the remaining activity was measured under the standard condition in a final volume of 1 mL.

pH range of 6.0 to 9.0. The dependence of the inactivation rate on the pH of the reaction mixture in Figure 3 showed a pattern similar to that obtained in the cases of the photooxidation of free histidine[19,24] and of the photoinactivation of several enzymes in which histidine residues appeared to be involved in the mechanism of enzymatic reaction.[25,26] The photoinactivation rate of γ-GT was significantly decreased at high ionic strength (not shown). The effect of the ionic strength excludes the possibility of methionine residues as direct participants in the photoinactivation process. The observations from the photoinduced inactivation indicate that histidine residue(s) could be essential for the maintenance of the protein conformation necessary for the catalytic activity or could directly participate in the enzymatic reaction.

Chemical modification by DEPC. Figure 4 shows the DEPC-concentration dependent inactivation of rat kidney γ-GT. The semilogarithmic plot of remaining enzyme activity versus reaction time at various concentrations of DEPC showed a series of straight lines up to first 10 min of incubation. Prolonged incubation beyond 10 min resulted in a deviation from the straight line which is probably due to the significant rate of DEPC hydrolysis under the applied condition. The reaction order with respect to DEPC for the inactivation of the transpeptidase was estimated based on the fact that the apparent first order rate constant (k_{app}) depends on the concentration of DEPC as previously described by Levy *et al.*[27]:

$$k_{app} = k[\text{DEPC}]^n$$
$$\log(k_{app}) = \log k + n\log[\text{DEPC}]$$

Where k is the second-order rate and n is the reaction order

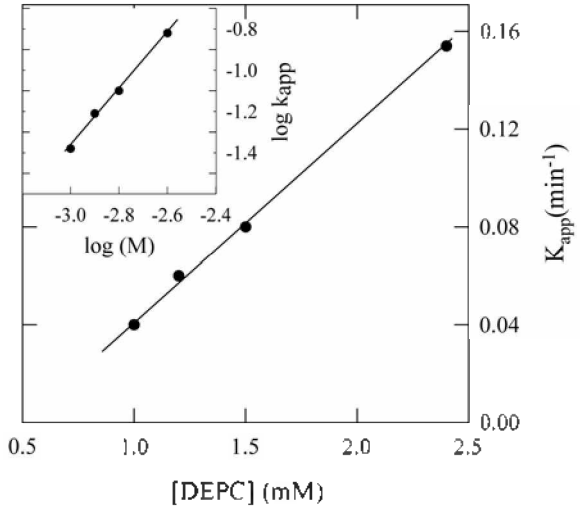

Figure 5. Plot of pseudo first-order rate constant (k_{app}) against DEPC concentrations: Inset is the plot of log (k_{app}) versus log[DEPC]. The pseudo first order rate constants of DEPC inactivation were calculated from the semilogarithmic plot (Fig. 4) of residual activity versus incubation time at various fixed concentration of DEPC.

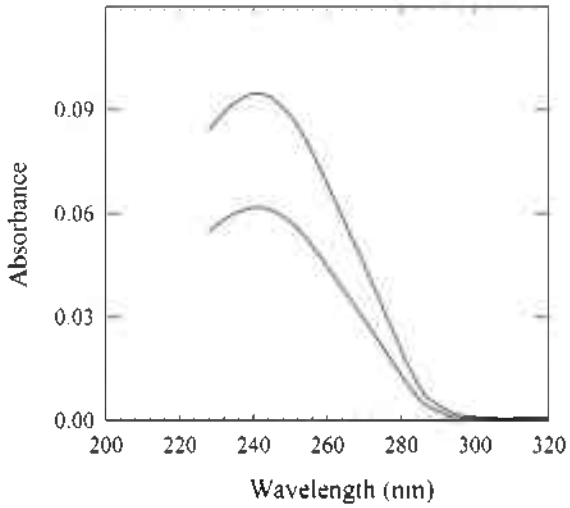

Figure 7. Difference spectra between DEPC-treated and -untreated enzymes: The sample contained 0.2 mL of the enzyme (0.05 mg/mL) and 1.2 mM DEPC in 50 mM Tris-HCl, pH 7.0. The apparent increase in absorbance at 240 nm was recorded after 5 and 10 min of incubation at 37 °C.

of DEPC. The plot of logk_{app} versus log[DEPC] (inset in Fig. 5) gave a straight line with a slope of 1.3, indicating that a reaction of a single residue of the enzyme with one molecule of DEPC could be responsible for the total inactivation of enzyme. DEPC has been successfully used for the chemical modification of histidine(s) essential for the catalytic activity in a number of enzymes.[28-30] The pseudo first-order kinetics of the DEPC inactivation together with the first-order kinetics with respect to DEPC, therefore, suggests that the transpeptidase has a histidine residue that is essential for the enzymatic activity and accessible to DEPC.

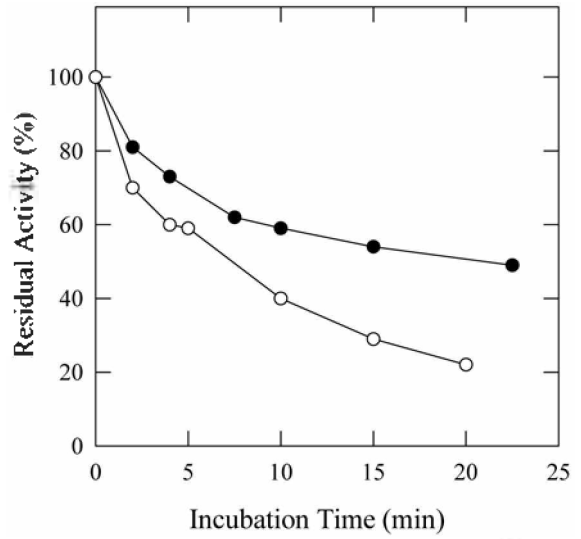

Figure 6. Effect of the substrate Gly-Gly on the chemical modification of γ-GT by DEPC: The enzyme was preincubated in presence (●) or absence (○) of 14.4 mM Gly-Gly in 50 mM Tris-HCl (pH 7.0) before the addition of DEPC at a final concentration of 2 mM.

The effect of substrate on DEPC inactivation of the enzyme was examined by preincubating the substrate, Gly-Gly, and the enzyme before the addition of DEPC (Fig. 6). The enzyme was apparently protected from DEPC inactivation by the substrate. The substrate effect along with the first-order kinetics of the DEPC modification indicates that the inactivation of enzyme by DEPC is due to the modification of a histidine group in the catalytic site of γ-GT.

Although DEPC has a high reactivity toward histidine residue at neutral pH, other nucleophilic groups such as tyrosine, serine, lysine, and cysteine might also be modified with this reagent probably at different pHs.[30,31] The difference spectra (Fig. 7) between enzyme alone and DEPC-modified enzyme gave an apparent increase at 240 nm, indicating the formation of *N*-carbethoxyhistidine.[20] The reaction of the enzyme with DEPC does not result in decrease in the absorbance at 280 nm which could be accounted for the formation of O-carbethoxytyrosine.[32]

Discussion

The results of pH dependent kinetic experiments, photooxidation, and DEPC inactivation of the rat kidney γ-GT cooperatively support the claim that a histidine residue is essential for the enzymatic activity and involved at or near the binding site of acceptor substrate. Since the histidine is in mostly deprotonated state at the optimum pH of enzyme, it is hardly reasonable that Gly-Gly with free carboxylate anion binds to the uncharged imidazole group. The histidine residue, then, would function as a general base catalyst.

Five histidines are found in the light subunit of the rat γ-GT. Two of them are located at the positions of 382 and 504 in its precursor and other three are successively located at the C-terminal region.[33] As an attempt to identify the essential histidine of γ-GT, the amino acid sequences of γ-GTs

Figure 8. The amino acid sequence comparison of γ-GT precursors from various organisms: The N-terminal residue of the L subunit is indicated by ↓. The highly conserved residues are shown in bold. The histidines equivalent to His382 and His504 of the rat γ-GT are marked by an asterisk (*). The sequences of the enzymes were collected from the SWISS-PROT data bank. The abbreviations used are: *E.*, *Escherichai*; *Ps.*, *Pseudomonas*; *B.*, *Bacillus*.; *S.*, *Saccharomyces*.

from various organisms were collected from the SWISS-PROT data bank and aligned using PileUp program (default setting) of the Genetics Computer Group's (GCG) sequence analysis software package. Figure 8 shows the amino acid sequence alignment of γ-GTs from various species. It appears that His382 is completely conserved among the enzymes, suggesting that His382 may be the general base catalyst. It is also noteworthy that His504 is conserved in the enzymes of mammalian species. The present work provides a valuable data for further study such as site directed mutations and X-ray crystallography to understand the active site nature as well as the detailed catalytic mechanism of γ-GT.

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