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PURIFICATION AND SOME PROPERTIES OF γ-GLUTAMYLTRANSFERASE FROM HUMAN LIVER

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Summary

The purification of γ-glutamyltransferase ((γ-glutamyl)-peptide:amino acid γ-glutamyltransferase, EC 2.3.2.2) from normal human liver is described. The procedure includes solubilization of the enzyme from membranes using deoxycholate and Lubrol W, treatment with acetone and butanol, and affinity chromatography on immobilized concanavalin A. Treatment with papain was used to release the enzyme from aggregates of lipid and protein, prior to further purification.

An overall purification of 9400 was achieved and analytical polyacrylamide gel electrophoresis indicated that the final product was homogeneous, and had a molecular weight of 90 000. Gel filtration, however, indicated a molecular weight of 110 000. Two subunits were identified on dodecyl sulfate gel electrophoresis with estimated molecular weights of 47 000 and 22 000.

The kinetic properties studied for the purified enzyme were similar to those found for partially purified (not papain-treated) enzyme, and resembled those of serum γ-glutamyltransferase. The true K_m values for the liver enzyme were estimated to 0.81 mM for γ-glutamyl-*p*-nitroanilide and to 12.4 mM for glycylglycine.

Introduction

γ-Glutamyltransferase ((γ-glutamyl)-peptide:amino acid γ-glutamyltransferase EC 2.3.2.2.) catalyzes the transfer of the γ-glutamyl residue from glutathione and other γ-glutamyl compounds to amino acids or peptides. The enzyme may function in the regulation of glutathione level and amino acid transport through cell membranes (γ-glu cycle), which has been extensively discussed [1,2], but roles in secretion [3] or mercapturic acid formation [4] have also been proposed.

The enzyme activity is high in secretions such as bile [5], seminal fluid [6] and colostrum [7]. Activity is also found in many organs and tissues, where it is associated with membranes. Enzyme activity that is released from membranes by detergents, has been shown to be present in high molecular weight forms [8—10], suggested to result from aggregation of membrane fragments and proteins [8,9,11]. Treatment of such aggregates or membrane preparations with proteolytic enzymes as ficin [12], bromelain [8], trypsin [9] or papain [13], yields, however, a small molecular form of the enzyme.

The highest organ activity is found in kidney, from which several groups have purified the enzyme to an apparently homogeneous state [12,8—10]. The specific activity of the enzyme in normal human liver is low, about 1/10 of that reported in human kidney [10]. Higher specific activities are found in different diseased and intoxicated human livers [14,—16], in neoplastic [17] and in foetal [18] human livers.

Measurements of γ-glutamyltransferase in serum are frequently used as a parameter of hepatobiliary diseases, including alcohol-or drug-induced liver damage [19]. The observed increases in serum enzyme activity are thought to originate in the liver, but the precise mechanisms whereby such increases occur are not known.

As a first step to obtain a means of studying these increases and the underlying mechanisms, a purification and a study of the enzyme from normal human liver was undertaken.

Materials and Methods

L-γ-Glutamyl-*p*-nitroanilide, glycylglycine, α-methyl-D-glucoside, bovine serum albumin (once crystallized), catalase (twice crystallized, from beef liver) and papain (twice crystallized, from Papaya Latex), were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., ovalbumin from Boehringer, Mannheim, W. Germany, and sodium deoxycholate from Merck, Darmstadt, W. Germany. Lubrol W (Cirrasol ALN-WF) was a gift from I.C.I., Oslo, Norway. DEAE-Sephadex A-50 and concanavalin A-Sepharose were bought from Pharmacia Fine Chemicals, Uppsala, Sweden, Ultrogel Aca 34 from LKB Produkter, Bromma, Sweden, and BioGel HTP from BioRad Laboratories, Richmond, U.S.A. Purified goat IgG was a generous gift from E. Bjørklid, Biochemical Section, University of Tromsø.

γ-Glutamyltransferase activity was determined at 37°C in an assay mixture of 4 mM γ-glutamyl-*p*-nitroanilide, 50 mM glycylglycine in 0.1 M Tris buffer (pH 7.6 at 37°C), as described [20] One unit of enzyme activity is the amount of enzyme that converts 1 μmol substrate per min. The activity measurements were done using LKB 8600 Reaction Rate Analyzer, and for the determination of the kinetic parameters a GEMSAEC Fast Analyzer was used as described [21].

Determination of glutaminase activity was carried out at 37°C as described for liver glutaminase [22], using glutamate dehydrogenase to quantitate the amount of glutamate formed [23]. The activity was also measured with 60 mM maleate added to the assay mixture.

Protein was determined by the method of Lowry et al. [24], using albumin as standard.

Polyacrylamide gel electrophoresis was performed according to the standardized produce described by Maurer [25], using gel system la. For preparative electrophoresis, slabs were prepared from the same gel system between two glass plates (10 × 20 cm), separated by a 3 mm thick plastic frame. Dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [26]. Protein samples were incubated 2% dodecyl sulfate, 8 M urea and 5% 2-mercaptoethanol for 5 min at 100° C, and then for at least 30 min at 37° C before electrophoresis.

Molecular weight estimations on polyacrylamide gel electrophoresis of the native and dodecyl sulfate-treated enzyme were based on the retardation coefficients (K_R values) from the Ferguson equation [27] as described by Hedrick and Smith [28], Weber and Osborn [29] and Frank and Rodbard [30]. M_r estimation for the native enzyme was performed in gels of 5-8% acrylamide using albumin, catalase and ovalbumin as standard proteins. Molecular weight estimation of the γ-glutamyltransferase subunits was performed in dodecyl sulfate gels of 9—12% acrylamide, using albumin, IgG and ovalbumin as standard proteins.

Molecular weight estimation of the native enzyme was also performed on a gel filtration column (Ultrogel Aca 34, 2 × 80 cm), calibrated with globular proteins of known molecular weight (IgG, albumin and ovalbumin).

Concentrations during the purification were performed with Amicon Diaflo XM50 Utrafiltration membranes.

Results

Purification procedure

Liver was obtained at autopsy within 30 h after death, the cause of which was considered not to affect the enzymatic activity in the liver. If not used the same day, the liver was cut in slices and frozen at −20° C. All procedures were carried out at 0—4° C unless otherwise stated.

Homogenization. One part of liver was homogenized in four parts of ice-cold 80 mM $MgCl_2$ containing 0.75 mM NaOH in Waring Blendor for 2 min at full speed, and filtered through gauze.

Solubilization. The filtered homogenate was incubated at 37° C for 2 h, then cooled on ice under stirring, and centrifuged for 30 min at 25 000 × *g*. The discarded supernatant contained 7—10% of the homogenate activity. The pellet was homogenized in Waring Blendor in ice-cold 0.1 M Tris buffer (pH 8.5) for 1 min, and deoxycholate and Lubrol W were added to final concentrations of 0.5% each. This suspension was stirred overnight and inactive material was removed by centrifugation at 25 000 × *g* for 30 min.

Acetone precipitation. An equal volume of acetone (precooled to −15° C) was added dropwise under good stirring to the detergent extract over a 30 min period. After centrifugation for 10 min at 15 000 × *g* the pellet was resuspended in 0.1 M Tris buffer)pH 8.5) with 0.5% deoxycholate. This solution was stirred overnight. After centrifugation at 25 000 ×*g* for 30 min, the precipipate was resuspended in 0.1 M Tris buffer (pH 8.5) containing 0.5% deoxycho-

late. This solution was stirred for 2 h, then centrifuged for 30 min at 27 000 × *g*.

Butanol treatment. The combined supernatants were stirred on ice and precooled butanol (−15° C) was added dropwise under good stirring over 30 min. The solution was then stirred for another 30 min, and centrifuged for 30 min at 40 000 × *g*. The butanol phase was sucked off, and precipitates discarded. The water phase was stirred on ice, and deoxycholate was added to a final concentration of 0.2%.

Ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ was added to give a 20% saturated solution. After 30 min the precipitate was removed by centrifugation for 10 min at 12 000 × *g*. The solution was then adjusted to 80% saturation by addition of solid $(NH_4)_2SO_4$, and the precipitate collected after 60 min by centrifugation for 30 min at 20 000 × *g*. The precipitate was dissolved in 0.1 M Tris buffer (pH 8.5) with 0.1% deoxycholate, and dialyzed against 10 l of this buffer overnight.

DEAE-Sephadex A50 I. After equilibrating the column (4.5 × 15 cm) with the dialyzing buffer at room temperature, the diffusate was applied and the column washed with two sample volumes of dialyzing buffer at room temperature. The column was then transferred to 4° C, and eluted with a linear gradient between the dialyzing buffer and a solution containing 0.5 M Tris buffer (pH 9.0), 0.5 M NaCl and 0.1% deoxycholate. The active fractions were collected and concentrated by ultrafiltration. The concentrate was diluted with twice its volume of 0.1 M Tris buffer (pH 8.5), and reconcentrated. The pH of the solution was adjusted to 7.5 with 1 M HCl, $MgCl_2$ was added to a concentration of 80 mM, and the solution was stored on ice overnight. The precipitate was discarded after centrifugation for 15 min at 20 000 × *g*. This removal of deoxycholate was necessary for satisfactory binding of γ-glutamyltransferase to concanavalin A in the next step.

Concanavalin A Sepharose column. The column (4.5 × 8 cm) was equilibrated with 0.1 M Tris buffer (pH 8.0) and 10 mM $MgCl_2$. The enzyme solution was applied, and the column washed with 100 ml of 0.1 M Tris buffer (pH 8.0) and 10 mM $MgCl_2$, then with 50 ml of 10% methylglucoside in 0.1 M Tris buffer (pH 8.0). The column was then maintained at room temperature for 30 min, and the enzyme eluted with 0.5% deoxycholate and 20% methylglucoside in water. The active eluates were collected on ice, and concentrated by ultrafiltration. The concentrate was diluted with twice its volume of 0.1 M Tris buffer (pH 8.0), concentrated again, and the pH was adjusted to 7.3.

Papain treatment. Papain activated with cysteine was added to the solution in the proportions 1 mg papain to 10 mg protein. This solution was kept at room temperature for 24 h, then cooled on ice and fractionated with ammonium sulfate. The fraction from 60 to 90% saturation was collected, and the pellet dissolved in 10 ml 0.1 M tris buffer (pH 8.0).

Gel filtration, Ultrogel Aca 34. The dissolved pellet was then applied to the column (3.5 × 75 cm), which had been equilibrated against 0.1 M Tris buffer (pH 8.0). The column was eluted with Tris buffer and 5-ml fractions collected. Fractions containing γ-glutamyltransferase activity were pooled and concentrated by ultrafiltration.

DEAE-Sephadex A50 II. After applying the sample, the column (2 × 5 cm) which had been equilibrated against 20 mM sodium phosphate buffer (pH 7.0),

was washed with 10 ml of this buffer at room temperature. The column was then maintained at 4° C, and the activity eluted using a linear gradient between the washing buffer and 0.5 M phosphate (pH 6.5) containing 0.3 M NaCl. Fractions of 3 ml were collected, the active fractions were pooled and concentrated. The pH was increased to 7.5, and the sample was dialysed against 2 l 10 mM sodium phosphate buffer (pH 7.5).

Hydroxyapatite column. The column (1 × 3 cm) was equilibrated with dialysis buffer, the sample applied and the column washed with 10 ml of the same buffer. The activity was eluted with 0.1 M phosphate buffer (pH 7.5). The active eluate was concentrated by ultrafiltration, diluted twice with water, reconcentrated, and freeze-dried. Protein material was dissolved in 1 ml distilled water.

Preparative electrophoresis. The polyacrylamide gel plate was prepared as described in Materials and Methods. The protein sample (0.25 ml per gel plate) was applied in wells made in the concentrating gel. The electrophoresis was run for 5 h with constant voltage of 250 V. The gel plate was maintained by cooling at 5—10° C. After electrophoresis, the position of the enzyme was indicated by overlaying the gel with assay mixture. The active zone was rapidly cut out, and the gel was broken up in 0.1 M Tris buffer (pH 8.0). The enzyme was extracted with repeated washings of the gel slurry with distilled water. This solution was freeze-dried, and the material dissolved in a minimum volume of water. A summary of a representative purification is given in Table I. A purification of about 9400-fold was achieved, with an overall yield of 7%. Analytical polyacrylamide gel electrophoresis performed on highly active enzyme preparations showed one protein band (Fig. 1a), regardless of the acrylamide concentration. Gel electrophoresis performed at pH 7.3 [25], also revealed only one protein band (not shown). In dodecyl sulfate gel electrophoresis, two bands were found

TABLE I

SUMMARY OF PURIFICATION SCHEME

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery of activity (%)	Purification factor
Homogenization	3960	7200	160 120	0.045	100	1
Detergent extraction	3200	6480	36 050	0.18	90	4.0
Acetone precipitation	1040	6192	11 680	0.53	86	11.8
Butanol treatment	960	5832	6780	0.86	81	19.1
Ammonium sulfate precipitation, dialysis	280	3744	2 370	1.58	52	35.1
DEAE-Sephadex I	150	3384	1 780	1.90	47	42.2
Concanavalin-A	45	2160	386	5.6	30	124
Papain digestion, ammonium sulfate precipitation	20	1368	107	12.8	19	284
Ultrogel Aca 34	4.8	1080	30.1	35.9	15	797
DEAE Sephadex II	4.5	936	22.8	41.1	13	913
Hydroxyapatite	13.5	792	11.3	70.1	11	1558
Preparative electrophoresis	2.5	508	1.2	423.0	7	9400

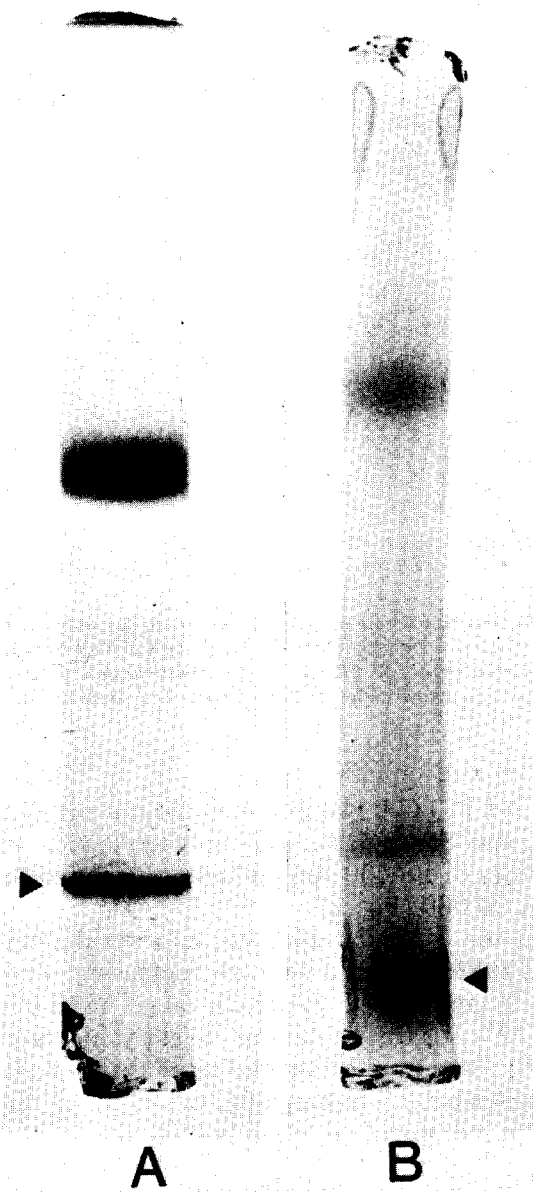

Fig. 1 Polyacrylamide gel electrophoresis of γ-glutamyltransferase. Arrows indicate positions of marker (bromphenol blue). (A) Electrophoresis of 30 μg enzyme protein in 7.5% acrylamide gel, for 3 h at 3 mA. (B) Electrophoresis in 10% acrylamide gel containing dodecyl sulfate of 30 μg enzyme protein for 5 h at 6 mA. The enzyme was incubated in dodecyl sulfate, urea and 2-mercaptoethanol as described in Material and Methods. The protein bands were visible only after prolonged staining.

(Fig. 1b), but only after prolonged staining, even when 30—50 μg enzyme protein was applied.

Molecular weight determination

Using gel filtration on a column calibrated against globular proteins (see Ma-

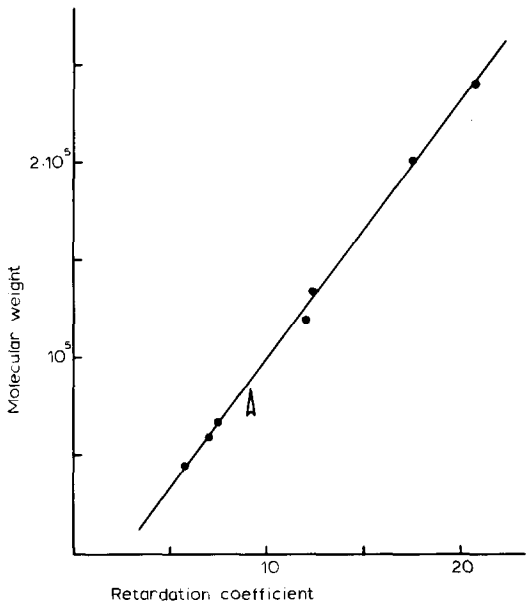

Fig. 2. Estimation of the molecular weight of γ-glutamyltransferase by polyacrylamide gel electrophoresis. The retardation coefficients (K_R), estimated for the different oligomeric forms of the standard proteins (catalase, albumin and ovalbumin) by electrophoresis in gels of 5—8% acrylamide concentrations, are plotted against the corresponding molecular weights. The arrow indicates the K_R value of γ-glutamyltransferase.

terials and Methods), an indicated M_r of 110 000 was obtained for the enzyme.

Using the technique of Hendrick and Smith [28] with polyacrylamide gel electrophoresis, molecular weight values of 88 000—95 000 were estimated for γ-glutamyltransferase, with a mean of 90 000 (Fig. 2).

The apparent molecular weight of the two subunits detected after dodecyl sulfate-polyacrylamide electrophoresis [26], decreased with increasing acrylamide concentration in the gels. Thus, in gels of 9% acrylamide, values of 56 000 and 23 500 were found, whereas in 12% gels, the molecular weight values were estimated to 52 000 and 23 000 (Fig. 3a). By plotting the estimated retardation coefficients (K_R values) vs the molecular weights [30], values of 47 000 and 22 000 were found for the γ-glutamyltransferase subunits (Fig. 3b).

Kinetic measurements

Kinetic studies on serum γ-glutamyltransferase are consistent with a ping-pong mechanism, and the activity was shown to be inhibitid by high concentration of both substrates [21]. A similar inhibition was found by incubating the purified liver enzyme with increasing concentrations of one of the substrates, while the level of the other was kept constant. This is seen as a deflection in the double reciprocal plots (Figs. 4a and 4b). The minima on these plots (i.e. the highest activities) were 6 mM for γ-glutamyl-*p*-nitroanilide at 50 mM glycylglycine (Fig. 4a), and 130 mM for glycylglycine at 4 mM γ-glutamyl-*p*-nitroanilide (Fig. 4b).

In view of the substrate inhibition effects, the true K_m values were estimated

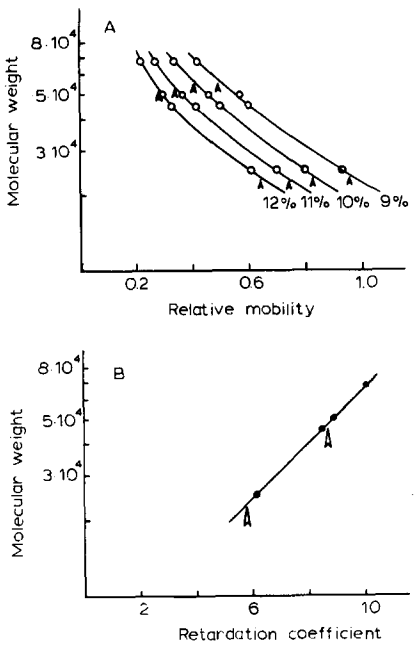

Fig. 3. Estimation of the molecular weights of γ-glutamyltransferase subunits by dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Plots of the relative mobilities of standard proteins (albumin, IgG and ovalbumin) vs their molecular weights, at different acrylamide concentrations (9—12%). Arrows indicate the relative mobilities of the two γ-glutamyltransferase subunits. (B) The retardation coefficients (K_R), estimated for the different standard proteins (albumin, IgG and ovalbumin) by electrophoresis in gels of 9—12% acrylamide concentrations, plotted vs the corresponding molecular weights. Arrows indicate the K_R values for the γ-glutamyltransferase.

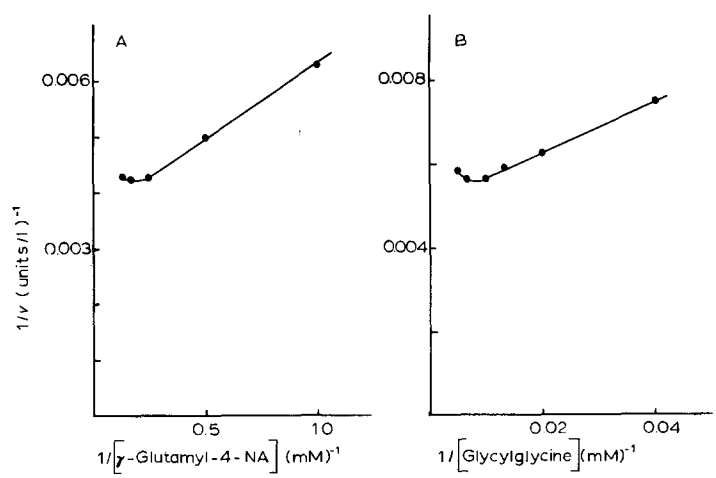

Fig. 4. (A) Effect on the activity of purified γ-glutamyltransferase of various γ-glutamyl-*p*-nitroanilide concentrations (1.0—8.0 mM) at 50 mM glycylglycine. (B) Effect on the activity of purified γ-glutamyltransferase of various glycylglycine concentrations (25—200 mM) at 4 mM γ-glutamyl-*p*-nitroanilide.

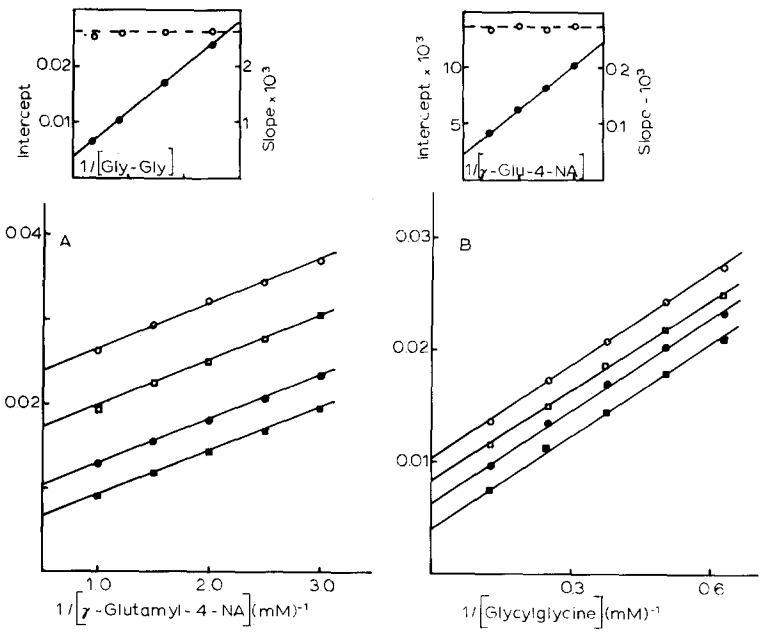

Fig. 5. (A) Effect on the activity of purified γ-glutamyltransferase of various γ-glutamyl-*p*-nitroanilide concentrations (0.2—1.0 mM) at 2 mM (○), 3 mM (□), 6 mM (●) and 12 mM (■) glycylglycine. Inset shows the slopes (stippled curve) and the intercepts vs the reciprocal glycylglycine concentrations. (B) Effect on the activity of purified γ-glutamyltransferase of various glycylglycine concentrations (1.6—8.0 mM) at 0.25 mM (○), 0.33 mM (□), 0.50 mM (●) and 1.0 mM (■) of γ-glutamyl-*p*-nitroanilide. Inset shows the slopes (stippled curve) and the intercepts vs the reciprocal γ-glutamyl-*p*-nitroanilide concentrations.

at highly suboptimal substrate concentrations. The double reciprocal plots shown in Figs. 5a and 5b show families of parallel lines. Secondary plots obtained by plotting the intercepts and slopes of these lines vs the concentration of the non variable substrate, are shown in the insets in Fig. 5, and from these, the true K_m values were calculated. In three experiments, the values were 0.77, 0.78 and 0.88 mM for γ-glutamyl-*p*-nitroanilide (mean 0.81 mM) and 12.0, 12.5 and 12.6 mM for glycylglycine (mean 12.4 mM).

An enzyme preparation purified as far as to the papain step in the purification procedure, showed the same substrate inhibition as did the papain-treated enzyme. The true K_m values were also nearly the same. In two experiments, values of 0.81 and 0.84 mM for γ-glutamyl-*p*-nitroanilide (mean 0.83 mM), and 12.0 and 13.5 mM for glycylglycine (mean 12.8 mM) were found.

The purified enzyme preparation was tested for glutaminase activity, but no activity could be detected, neither with nor without maleate.

Discussion

The activity of γ-glutamyltransferase in normal human liver was solubilized using a combination of deoxycholate and Lubrol W. However, the solubilized enzyme seemed to be present in high molecular weight forms since the enzyme activity eluted on gel filtration as a broad peak starting at the void volume. Moreover, the enzyme activity did not enter polyacrylamide gels on electropho-

resis, even in the presence of deoxycholate or Triton X-100. Formation of aggregates of membrane fragments and proteins are known tendencies of solubilized membrane proteins, particularly on the removal of detergent, and are also reported to occur with kidney γ-glutamyltransferase preparations [8—10].

The described treatment of the human liver γ-glutamyltransferase with papain released all activity from the aggregates, and a low molecular form appeared. Similar observation have been made with the enzyme from kidney [8,9,12,13]. No significant changes in γ-glutamyltransferase activity occurred during incubation of the liver preparation with papain at room temperature for up to 48 h. The loss of activity at this step in the purification procedure (see Table I). occurred during the ammonium sulfate fractionation. In addition, the true K_m values of both substrates as well as the substrate inhibition patterns were also unchanged. Thus, such proteolysis appears to be a mild treatment of γ-glutamyltransferase during purification, but further studies are needed to identify the proteolytic changes.

After a 9400-fold purification, 1.2 mg protein was obtained when starting from a whole liver (1.2 kg). The overall recovery of enzyme activity (7%) seems reasonably high considering the number of steps in the purification procedure. The final preparation had a specific activity somewhat lower than those reported for rat and sheep kidney, but appeared to be homogeneous as judged by analytical polyacrylamide gel electrophoresis.

The molecular weight of the purified enzyme was estimated by polyacrylamide gel elctrophoresis to be 90 000, and by gel filtration to be 110 000. Glycoproteins may behave anomalously in both electrophoresis [25] and in gel filtration [31], and this may account for this discrepancy. However, the value of 90 000 is similar to the values reported for the kidney γ-glutamyltransferase from sheep and man [9,10], while the estimated value for rat kidney γ-glutamyltransferase was 70 000 [8].

The papain-treated liver γ-glutamyltransferase was shown by electrophoresis in the dodecyl sulfate system, to consist of two different subunits, as does the kidney enzyme [8—10]. The molecular weights estimated for the subunits of human liver γ-glutamyltransferase are quite similar to those for the rat kidney enzyme, but somewhat lower than those reported for sheep and human kidney γ-glutamyltransferase [9,10]. However, further studies are needed to establish a reliable molecular weight for the human liver γ-glutamyltransferase, and to establish its quarternary structure.

Rat kidney γ-glutamyltransferase was shown to exhibit phosphate-independent glutaminase activity [8,13], whereas the purified human liver enzyme showed no detectable glutaminase activity. This may indicate a species difference, as the human kidney γ-glutamyltransferase also showed no such activity [10].

Kinetic properties in the form of true K_m values and substrate inhibition patterns for purified enzyme were close to those reported for serum γ-glutamyltransferase [21]. As the activity found in serum is thought to derive mainly from the liver, further comparisons of kinetic and other properties between serum and liver γ-glutamyltransferase, and on the release of enzyme from liver membranes to the circulation, will also provide better understanding of the clinical chemical aspects of the enzyme.

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