Single-chain precursor of renal γ-glutamyl transpeptidase

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The two subunits of γ-glutamyl transpeptidase (EC 2.3.2.2) are derived from a single-chain glycosylated precursor. A small fraction of the propeptide survives proteolytic processing in the rat kidney and has been purified by an immunoaffinity technique. The propeptide contains determinants for both the subunits and its amino acid composition resembles that of the dimeric enzyme. However, the propeptide exhibits less than 2% of the transpeptidase activity shown by the dimeric enzyme.

γ-Glutamyl transpeptidase (Kidney) Brush border membrane Single-chain precursor Immunoaffinity chromatography Proteolytic processing

1. INTRODUCTION

γ-Glutamyl transpeptidase catalyzes the first step in the catabolism of glutathione [1]. In rat kidney, the enzyme is primarily located on the lumenal surface of the proximal tubule microvilli [2,3]. The enzyme is composed of two, non-identical glycosylated subunits [4]. The large subunit (H, M_r 51000) contains the membrane-binding domain at its NH_2-terminus [5]; the small subunit (L, M_r 22000), which possesses the γ-glutamyl binding site [4], is held on the membrane surface via non-covalent interactions with the H subunit. Biosynthesis and processing of transpeptidase were investigated by pulse-chase studies in renal slices [6] and by in vitro translation of its mRNA [7]. These studies showed that the enzyme is initially synthesized as a glycosylated, single-chain precursor (M_r 78000) which is subsequently cleaved to the two subunits. This mode of biosynthesis was later also shown in rat hepatoma [8] and yolk sac tumor cells [9].

Our studies indicated that cleavage of the propeptide occurs after its arrival at the Golgi [6]; later studies have shown that the cleavage in fact begins in the endoplasmic reticulum [7] and significant amounts of the propeptide reach the Golgi where further oligosaccharide and proteolytic processing occurs [10]. We now demonstrate that a small fraction of the propeptide escapes proteolytic cleavage and persists in the kidney. The propeptide, purified by an immunoaffinity technique, exhibits relatively low transpeptidase activity. A preliminary account of this work has appeared [11].

This paper is dedicated to my mentor and friend Professor S.P. Datta whose devotion and ceaseless efforts have been largely responsible for the success of FEBS Letters

Abbreviations: H and L, heavy and light subunits of γ-glutamyl transpeptidase, respectively; IgG, immunoglobulin G

2. MATERIALS AND METHODS

Sepharose CL-4B, protein A-Sepharose CL-4B, and the proteases used in this study were obtained from Sigma. $Na^{125}I$ (17.4 Ci/mg) and L-[^{35}S]methionine (1200 Ci/mmol) were from New England Nuclear. Rat kidney γ-glutamyl transpeptidase (papain-solubilized) was purified as in [12]. Rabbit antiserum against the purified enzyme and the γ-globulin fraction (anti-transpeptidase-IgG) were obtained as described [6]. Rabbit antisera

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directed against the H and L subunits were prepared following separation of the two subunits by SDS-polyacrylamide gel electrophoresis of the SDS-denatured enzyme on 8% cylindrical gels (see [7] for details). The γ-globulin fractions were covalently attached to CNBr-activated Sepharose CL-4B beads as described [13].

Rat kidney slices were labeled with [^{35}S]-methionine and Triton X-100 extracts of the labeled tissue were made as in [6]. Radioiodination of transpeptidase and the propeptide was done according to [14]. Immunoprecipitation (using protein A-Sepharose) and processing of the precipitates were carried out as described previously [7]. SDS-polyacrylamide gel electrophoresis was performed on 10% slab gels using a modified Laemmli buffer system [15]. The gels were stained for protein using Coomassie blue R-250 and the radioactive bands were detected by autoradiography using Kodak XAR-5 film and a DuPont intensifying screen. Amino acid analyses were performed with a Durrum model D500 amino acid analyzer on protein samples after hydrolysis in 6 N HCl at 110°C for 24 h.

3. RESULTS

SDS-gel electrophoresis of the Triton-solubilized γ-glutamyl transpeptidase purified using a column of anti-transpeptidase-IgG-Sepharose indicated the presence of a 78 kDa species in the preparation (fig.1). The enzyme preparation was obtained as follows: brush border membranes from 10 g of rat kidney (male Sprague-Dawley, 250 g) were isolated as described [16]. The membranes were homogenized in 5 ml of 0.01 M Tris-HCl buffer (pH 8) containing 0.15 M NaCl and 1% Triton X-100 (Tris-Triton buffer). After standing on ice for 30 min, the homogenate was centrifuged at 43000 × g for 1 h. The clear supernatant (containing about 2000 units of transpeptidase; see [17] for assay procedure and definition of units) was mixed with anti-transpeptidase-IgG-Sepharose (4 ml of settled slurry) and gently stirred at 25°C for 60 min. The slurry was then poured into a column (1 × 10 cm) and the column washed extensively with Tris-Triton. About 75% of the applied enzyme bound to the IgG-Sepharose. The bound enzyme was eluted with 0.1 M NH_4OH. The eluates (1 ml fractions) were collected in tubes containing

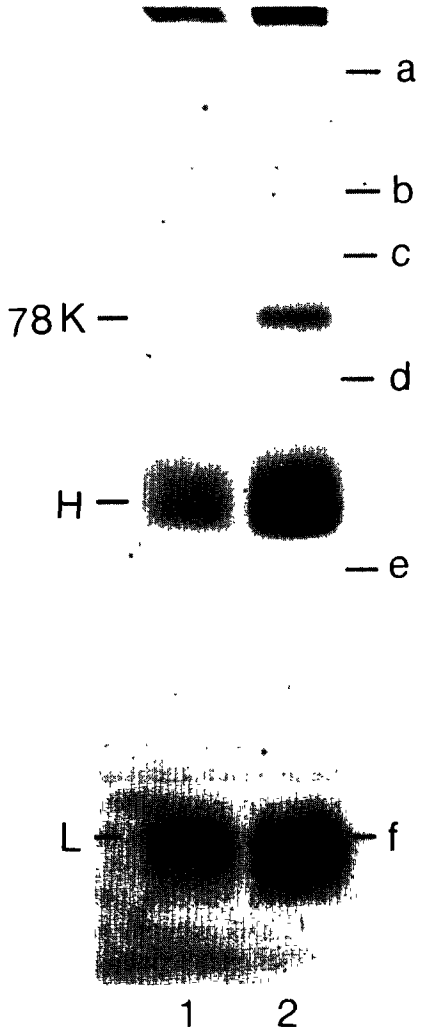

Fig.1. Presence of the 78 kDa species (78K) in Triton-solubilized γ-glutamyl transpeptidase purified by affinity chromatography on an anti-transpeptidase-IgG-Sepharose column. Lanes 1 and 2: two aliquots of the preparation analyzed by SDS-polyacrylamide gel electrophoresis. The gel was stained for proteins. The M_r standards (from Sigma) used in this and subsequent figures are as follows ($M_r \times 10^{-3}$): a, myosin (205); b, β-galactosidase (116); c, phosphorylase b (97.4); d, bovine serum albumin (66); e, ovalbumin (45); and f, carbonic anhydrase (29).

0.1 ml of 1 M Tris-HCl (pH 7). Fractions containing transpeptidase were pooled and the enzyme precipitated by the addition of 10 vols acetone (precooled to −15°C). The precipitate (collected by centrifugation) was dissolved in 0.5 ml of

Table 1

Specificity of the antibodies raised against γ-glutamyl transpeptidase and its subunits[a]

Antibody[b]	Polypeptides immunoprecipitated from:			
	[^{125}I]GGTP	SDS-denatured [^{125}I]GGTP	[^{35}S]Methionine-labeled kidney slice	
			Triton extract	SDS-treated Triton extract
Anti-transpeptidase	H, L	H	78K, H, L	78K, H
Anti-H	none	H	78K	78K, H
Anti-L	none	L	78K	78K, L

[a] Immunoprecipitation using protein A-Sepharose followed by SDS-polyacrylamide gel electrophoresis was used to determine the species that are precipitated by the respective antibody from ^{125}I-labeled transpeptidase ([^{125}I]GGTP), SDS-denatured [^{125}I]GGTP, and either the Triton X-100 extract or the SDS-treated Triton X-100 extract of rat kidney slices pulsed with [^{35}S]methionine. 78K denotes the 78 kDa propeptide of transpeptidase

[b] γ-Globulin fractions of the antisera were used. Antibodies against the two subunits (H and L) were made using SDS-denatured subunits as the antigens

0.01 M Tris-HCl (pH 8) containing 0.2% Triton (yield about 900 units, spec. act. 740 units/mg). Varying amounts of the 78 kDa species were seen on SDS gels in different preparations of the enzyme.

Separation of the 78 kDa species from the dimeric enzyme is based on the finding that the antibodies prepared against the SDS-denatured subunits recognize the propeptide in Triton extracts of the kidney and not the dimeric enzyme (table 1). In a typical purification procedure, the Tris-Triton extract of rat kidney brush border membranes is initially applied to a preimmune γ-globulin-Sepharose column (1 × 5 cm; preimmune serum γ-globulin fraction was prepared from bleedings taken from a non-immunized rabbit and coupled to CNBr-activated Sepharose). The column is eluted with 10 ml Tris-Triton. The eluates (containing all of the transpeptidase activity) are combined and mixed at 25°C for 60 min with anti-H-IgG-Sepharose (about 5 ml of settled slurry). The slurry is then poured into a column (1 × 10 cm) and the column washed extensively with Tris-Triton (the wash contains all of the transpeptidase activity and was used for the purification of the enzyme by chromatography on anti-transpeptidase-IgG-Sepharose as described above). The column is then eluted with 0.1 M NH_4OH (15 ml). The pH of the eluate is adjusted to 8.0 with 2 N acetic acid, the solution dialyzed against 4 l of 1 mM Tris-HCl (pH 8) for 18 h at 4°C, and then lyophilized. The lyophilized sample is dissolved in 0.5 ml of water. Fig.2 (lane 3) shows that this procedure yields essentially homogeneous 78 kDa species. Omission of initial chromatography on the preimmune γ-globulin-Sepharose column results in a preparation containing several other proteins besides the 78 kDa species (fig.2, lane 2). The yield of the propeptide varies from 30 to 40 μg per 10 g kidney.

Anti-H-IgG-Sepharose in the above purification procedure can be replaced by anti-L-IgG-Sepharose; the yields of the 78 kDa species, however, are lower than in the method described above. Fig.3 shows that ^{125}I-labeled propeptide can be precipitated by anti-transpeptidase-IgG and that the immunoprecipitation can be competed out by purified transpeptidase. The amino acid composition of the 78 kDa species resembles that of the Triton-solubilized transpeptidase purified as described above (not shown) and also the published composition of the Triton-solubilized transpeptidase [5]. The 78 kDa species is glycosylated since the ^{125}I-labeled protein binds to concanavalin A-Sepharose (Sigma) and the bound material can be eluted in about 75% yields by α-methylmannoside.

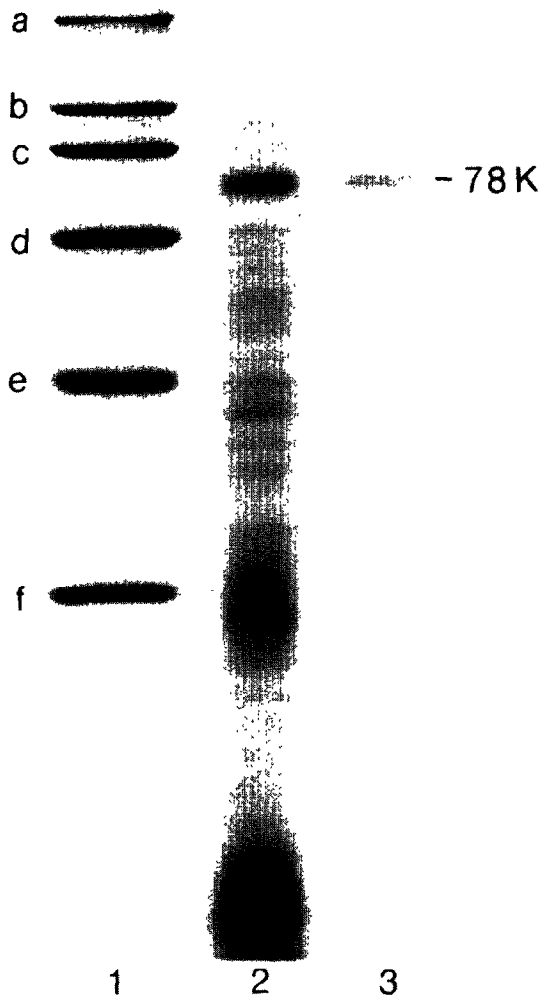

Fig.2. SDS-polyacrylamide gel electrophoresis of the 78 kDa species (78K) purified by immunoaffinity chromatography on an anti-H-IgG-Sepharose column as described in section 2 (lane 3). Lane 2, a preparation in which chromatography on a preimmune γ-globulin-Sepharose column was omitted. The gel was stained for protein.

The propeptide exhibits relatively low transpeptidase activity (specific activity less than 2% that of the dimeric enzyme; table 2). Treatment of the propeptide with chymotrypsin, trypsin, and elastase (propeptide to enzyme ratio, 5:1; incubation at 37°C for 60 min) neither increased its transpeptidase activity nor did these proteases cleave ^{125}I-labeled propeptide into polypeptides resembling

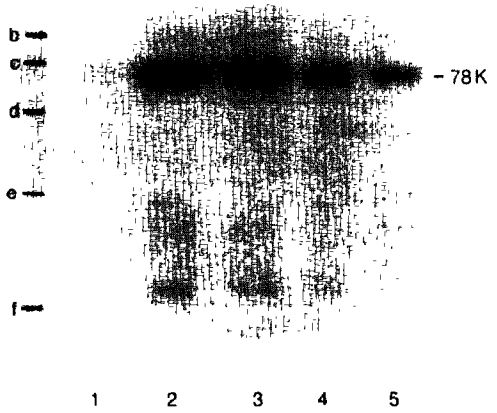

Fig.3. Immunoprecipitation of the 78 kDa species (78K) by anti-transpeptidase-IgG can be competed out by γ-glutamyl transpeptidase. Aliquots of the ^{125}I-labeled 78 kDa species were immunoprecipitated using protein A-Sepharose. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Lanes: 1, preimmune γ-globulin (25 μg); 2–5, anti-transpeptidase-IgG (25 μg per experiment). In lanes 3–5, immunoprecipitation was carried out in the presence of 1, 2, and 4 μg, respectively, of purified γ-glutamyl transpeptidase. The band at approx. 30 kDa is in all likelihood a degradation product of the 78 kDa species.

the H and L subunits. We had previously shown that in vitro translation of rat kidney mRNA in the presence of dog pancreas microsomes results in the synthesis of a 78 kDa, core-glycosylated transpeptidase precursor which is integrated into the cisternae of the microsomal membranes [7]. Upon longer incubation, the 78 kDa species sequestered

Table 2

Transpeptidase activity of γ-glutamyl transpeptidase propeptide (M_r 78000)

Enzyme species	Transpeptidase activity[a] (units/mg)
γ-Glutamyl transpeptidase (HL)	740
Propeptide (78 kDa species)	20 ± 10

[a] Transpeptidase activity was determined in the presence of 1 mM L-γ-glutamyl-*p*-nitroanilide and 20 mM glycylglycine [17]. The activity of different preparations of the propeptide varied from 10 to 30 units/mg

within the microsomal vesicles was cleaved to species corresponding to the two subunits of the enzyme. The radioiodinated 78 kDa species isolated from kidney brush border membranes was, therefore, incubated with dog pancreas microsomes in the absence and in the presence of 0.5% Triton X-100 (to gain access to the cisternal side of the membranes). No cleavage of the ^{125}I-labeled propeptide to species resembling the H and L subunits occurred. Further studies are, however, required since in a separate study it was shown that addition of Triton also inhibited the post-translational cleavage of the 78 kDa species synthesized in vitro from kidney mRNA in the presence of dog pancreas microsomes (unpublished).

4. DISCUSSION

Purification of the γ-glutamyl transpeptidase propeptide (M_r 78000) is based on the observation that the antibodies raised against the SDS-denatured H and L subunits of the enzyme recognize the propeptide but not the dimeric, processed form of the enzyme in Triton extracts of rat kidney. These antibody specificities also indicate that proteolytic cleavage of the propeptide to the dimeric enzyme is most likely accompanied by substantial conformational changes, which may be essential for the formation of a competent active center. The low specific activity exhibited by the propeptide is reminiscent of the classical zymogens of pancreatic enzymes in which case proteolytic processing is essential for the acquirement of the full catalytic potential. On the other hand, proteolytic cleavage of the single-chain precursors of intestinal sucrase-isomaltase and maltase-glucoamylase has little or no effect on their specific activity [18,19]. In the case of sucrase-isomaltase, however, the two subunits exhibit different catalytic functions and presumably are translated from a two-cistron mRNA [20]. In the dimeric γ-glutamyl transpeptidase, the existing data suggest that the active center is located in intersubunit contact regions and probably encompasses domains on both the subunits [21,22]. The possibility that the low activity of the propeptide reflects partial inactivation during the isolation process cannot be entirely ruled out but is not very likely since a technique using similar conditions yields the dimeric enzyme exhibiting specific activity similar to preparations obtained by other methods (table 2 and [17]).

That the isolated 78 kDa species is indeed the single-chain form of transpeptidase is evident from its reactivity with the antibodies raised against the native dimeric enzyme as well as towards the subunit antibodies. Moreover, the amino acid composition of the 78 kDa species is similar to that of the Triton-solubilized transpeptidase. This latter observation suggests that the proteolytic processing of the propeptide in vivo is not accompanied by excission of a significant portion of the polypeptide chain. Our in vitro attempts to cleave the propeptide into subunits resembling the H and L subunits of the enzyme have thus far been unsuccessful. Further work is required on the characterization of the protease(s) involved in the in vivo cleavage of the propeptide.

Presence of the propeptide in the kidney brush border membranes indicates that propeptide cleavage is not essential for the normal intracellular sorting and transport mechanisms which convey the enzyme to its final cellular location. The amount of the propeptide in different batches of the rat kidneys (the brush border fractions) varies from trace (<0.1%) to somewhat higher levels (up to 1% of the total enzyme protein). These estimates are based on the yield of the propeptide compared to the total transpeptidase activity originally present in the brush border membranes. The reasons for this variation and the persistence of the single-chain form of transpeptidase in the kidney are not known. Capraro and Hughey [10] have suggested that incomplete propeptide cleavage in the endoplasmic reticulum could be due either to the competing reactions of glycosylation or might reflect the existence of different but closely related gene products. Such possibilities need investigation. It should be noted that the persistence of the uncleaved form of transpeptidase is not a unique finding. Other such examples are known, a notable one being the porcine and bovine spleen cathepsin D [23]. In porcine spleen, the uncleaved propeptide varies from trace amounts to as much as 10% of the total enzyme protein. Much higher levels of the propeptide are found in the bovine spleen. The availability of the transpeptidase propeptide will allow investigation of a number of problems associated with the processing of this enzyme.

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