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Biochemistry

Subunit structure and isozymic forms of γ-glutamyl transpeptidase*

(glutathione/amino acids/membranes/isoelectric focusing/sialic acid)

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ABSTRACT **γ-Glutamyl transpeptidase is associated with the membranes of a number of epithelial and lymphoid cells. When the enzyme is isolated from rat kidney by a method involving detergent extraction and affinity chromatography, an aggregate of molecular weight greater than 200,000 (heavy form) is obtained. Treatment of the heavy form with bromelain yields a light form of the enzyme (molecular weight of approximately 68,000), which is separable by isoelectric focusing into 12 enzymatically active isozymes which are very similar with respect to catalytic behavior, content of amino acids, hexoses, and aminohexoses, but which differ significantly in sialic acid content. Treatment with neuraminidase converts the acidic isozymes to more basic forms. Each isozyme dissociates in sodium dodecyl sulfate into two nonidentical glycopeptides (molecular weights of 46,000 and 22,000) which can be cross-linked with dimethylsuberimidate to yield a species with an apparent molecular weight of 70,000, which indicates that the isozymes are dimers. Physical and immunological studies indicate that the heavy form of the enzyme contains the dimeric light form as well as other membrane proteins.**

γ-Glutamyl transpeptidase has been known for many years as the enzyme that catalyzes the initial step in the degradative metabolism of glutathione; it transfers the γ-glutamyl moiety to amino acid and peptide acceptors as well as to water. The enzyme, a glycoprotein, is bound to the membranes of a variety of epithelial cells [for recent reviews, see (1–3)] and is found also on the surfaces of lymphoid cells (4). These findings and other considerations have led to the proposal that it is involved in transport (3, 5–8). Although much has been learned about the catalytic properties of γ-glutamyl transpepidase, its purification and characterization have been complicated by the finding that it occurs in many isozymic forms, the nature and significance of which have thus far remained unknown. Recent studies in this laboratory have been directed toward a solution of this problem (1–3, 10–14). We found that the enzyme as usually isolated is an aggregate (heavy form; molecular weight > 200,000) which contains other proteins. A lower molecular weight (light form; molecular weight of approximately 68,000) has been obtained from the heavy form. As shown in the present studies, the light form contains at least 12 isozymes, many of which can be distinguished by their content of sialic acid. All of these isozymes are dimers which can be dissociated into two nonidentical subunits (9). The findings are of significance in relation to further studies on the function of the enzyme and its structural orientation in cell membranes.

EXPERIMENTAL

L-γ-Glutamyl-*p*-nitroanilide, L-γ-glutamyl-*a*-naphthylamide, and Fast Garnet GBC salt were purchased from Sigma. *S*-Acetophenone-glutathione was synthesized as described (10). Ampholines, neuraminidase (*Vibrio comma*), and dimethylsuberimidate were obtained from LKB, Behringwerke, A. G., and Aldrich, respectively.

The heavy form of rat kidney transpeptidase was purified by extraction of the 100,000 × *g* pellet of the kidney homogenate with Lubrol WX, precipitation with acetone, and affinity chromatography on concanavalin A-Sepharose (11). The light form was purified by treatment with bromelain and then by chromatography (11). Both preparations exhibited similar catalytic properties. The heavy form (molecular weight > 200,000) and the light form (molecular weight of 68,000) had specific activities, respectively, of 180 and 1,100 (μmol of *p*-nitroaniline formed/per min/mg of protein). Assays were done with γ-glutamyl-*p*-nitroanilide, *S*-acetophenone-glutathione (10), and L-glutamine + maleate (11, 12).

Polyacrylamide gel electrophoresis was done (15) in 6% gels and 0.05 M Tris-borate buffer (pH 9). The gels were stained for protein (with Coomassie brilliant blue R250), carbohydrates (16), or transpeptidase activity (17). Gel electrophoresis in sodium dodecyl sulfate ($NaDodSO_4$) (18) was done in a buffer consisting of 0.03 M acetic acid, 0.03 M boric acid (adjusted to pH 8.5 with NaOH), and 0.1% $NaDodSO_4$. The proteins were dissociated and their $NaDodSO_4$ complexes formed by heating at 100° for 5 min in 2% 2-mercaptoethanol and 1.5% $NaDodSO_4$. The standard proteins were (subunit molecular weight in parentheses): phosphorylase *a* (100,000), bovine serum albumin (68,000), liver alcohol dehydrogenase (41,000), muscle glyceraldehyde-3-phosphate dehydrogenase (36,000), and human γ-globulin (heavy-chain, 50,000; light-chain, 23,500).

Analytical isoelectric focusing on polyacrylamide gels was done (19) on the following gels (0.5 × 10 cm): 6% acrylamide, 0.15% bis-acrylamide, 0.5% ammonium persulfate, and 2% Ampholines (pH range 5–8). The cathodic chamber (top) contained 2.9% ethanolamine; the anodic chamber contained 0.4% H_2SO_4. A solution (50 μl) containing 10% glycerol and 2% Ampholines was applied to the top of each gel and the gels were subjected to electrofocusing for 10 min. Transpeptidase solutions containing 20% glycerol and 2% Ampholine were applied below the initial Ampholine solution and then subjected to isoelectric focusing (18 hr at 4°). Preparative isoelectric focusing was carried out in an LKB Unifor 7900 electrofocusing apparatus. The column [160 ml containing 1% ampholines (pH 5–8) and 14.5 mg of the light enzyme] was stabilized by a linear gradient of sucrose (5–55% wt/vol). Isoelectric focusing was done for 48 hr at 4°; the voltage was slowly increased to 800 V. Fractions (1 ml) were collected; those containing enzyme were pooled, dialyzed against water, and lyophilized to 1 ml. The samples were each applied to Sephadex G-150 columns (1.5 × 80 cm) and eluted with 0.1 M Tris·HCl buffer (pH 8.0) to remove Ampholines.

Antibodies against the light enzyme were prepared in rabbits by standard methods; the γ-globulin fraction was purified (20).

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

* With the technical assistance of Barbara Nash.

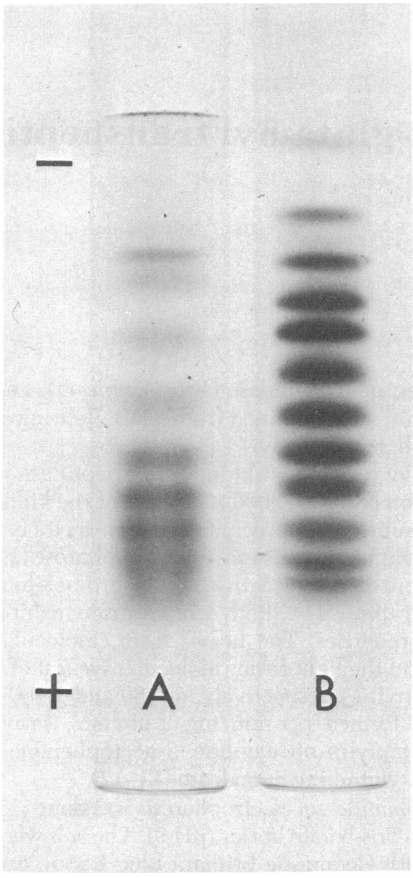

FIG. 1. Electrophoresis and isoelectric focusing of the light form of transpeptidase on polyacrylamide gels. A. Electrophoresis in Tris-borate buffer (pH 9.0) on 6% gels (100 μg of enzyme); B. Isoelectric focusing on 6% gels containing 2% Ampholines, pH range 5–8 (150 μg of enzyme); gels were stained for protein.

RESULTS

Isozymes of the light form of γ-glutamyl transpeptidase

Electrophoresis of the light enzyme on polyacrylamide gels showed considerable heterogeneity (Fig. 1A). All protein bands also stained for carbohydrates and activity. The heavy enzyme, however, did not penetrate a 6% gel under these conditions. Analytical isoelectric focusing of the light form on gels gave 12 protein bands (Fig. 1B) which also stained for activity and carbohydrates. Twelve activity peaks were also seen on preparative isoelectric focusing (Fig. 2). The peaks of activity and protein corresponded well; recovery of activity was 65%. Fractions from the peaks were pooled and freed of Ampholines; on isoelectric focusing on gels, there was some overlap of isozymes, but each fraction contained only the isozymes whose isoelectric points corresponded to the pH regions of the pooled fractions. The composition and specific activities of some of the fractions are shown schematically at the top of Fig. 2 (see also Fig. 3). Fraction VII was most active; lower activities were found in regions of high and low pH probably due to inactivation during isoelectric focusing, but no fractions had activities greater than that of the original enzyme. All isozyme fractions were identical with respect to: (*a*) ratio of catalytic activities (about 1.6) with γ-glutamyl-*p*-nitroanilide and *S*-acetophenone-glutathione (with glycylglycine); (*b*) relative acceptor activities of several L-amino acids and dipeptides; (*c*) effect of maleate on the activity toward L-glutamine; and (*d*) degree of inhibition of L-serine plus borate (21). All of these properties were very similar to those of the light enzyme (11).

Amino acid and carbohydrate composition of the isozymic fractions

The apparent molecular weights of the isozymic fractions were similar (about 68,000) as judged by gel filtration on Sephadex G-150. The amino acid composition of four fractions (IV, VI, X, and XII) was close to that of the light enzyme (11); the hexose and aminohexose contents of these fractions was also similar (about 745 ± 30 and 705 ± 10 nmol/mg of protein, respectively). Significant differences were, however, observed in sialic acid content; thus, relatively low sialic acid content was found in fractions from the high pH region. The light enzyme and fractions IV, VI, X, and XII, contained, respectively, 48, 14, 32, 53, and 61 nmol of sialic acid/per mg of protein. It seems likely that the degree of sialylation accounts, at least in part, for the multiple forms of transpeptidase. Thus, neuraminidase treatment of the light enzyme led to enhanced formation of the more basic isozymes (Fig. 3). Fraction III, which contains the basic isozymes 2 and 3, remained virtually unaffected, whereas such treatment of fraction XI (containing isozymes 10 and 11) gave bands corresponding to isozymes 1 through 11. Neuroaminidase treatment had no effect on catalytic properties.

Subunit composition of γ-glutamyl transpeptidase

Fig. 4 (A–C) shows the results of the electrophoresis in 8% gels in the presence of $NaDodSO_4$. The light form and the isozymes (two are shown in Fig. 4) dissociated into two polypeptide chains with apparent molecular weights, respectively, of 49,000 and 26,000. Dissociation did not require 2-mercaptoethanol. The relative proportion of the two chains (subunits 1 and 2) was similar in all fractions. Treatment of the light enzyme (and of the isozyme fractions) with dimethylsuberimidate (22) prior to dissociation with $NaDodSO_4$, led to a component of molecular weight about 75,000 (Fig. 4E), presumably the undissociated crosslinked form of the enzyme. Both subunits stained for carbohydrate, which decreases binding of $NaDodSO_4$ to protein and thus decreases mobility on $NaDodSO_4$ gels (16). This effect is minimized by increasing gel concentration. Studies with $NaDodSO_4$ gels of increasing concentrations (Fig. 5) gave molecular weight values of 46,000 and 22,000, for the two subunits.

Composition of the heavy form of γ-glutamyl transpeptidase

On $NaDodSO_4$ gels the heavy form gave the two polypeptide chains corresponding to the two subunits of the light enzyme (Fig. 4G), as well as material with higher molecular weight which remained near the top of the gel. The relative concentrations of the two subunits obtained from the heavy enzyme was similar to that observed when the same number of activity units of the light enzyme was run on $NaDodSO_4$ gels. Treatment of the heavy enzyme with dimethylsuberimidate prior to $NaDodSO_4$ gel electrophoresis led to a decrease in the intensity of the two subunit bands and appearance of a new band corresponding to a molecular weight of about 72,000. Thus, the isolated heavy form contains subunits (molecular weights of 46,000 and 22,000) which can be cross-linked. This suggests that the subunits of the light enzyme are not formed by cleavage by bromelain. This conclusion is supported by experiments in which the transpeptidase was precipitated from the heavy form with purified IgG fraction containing antibodies against the light enzyme. The immunoprecipitate (which was enzymatically active) was washed with 1 M NaCl, treated with Na-

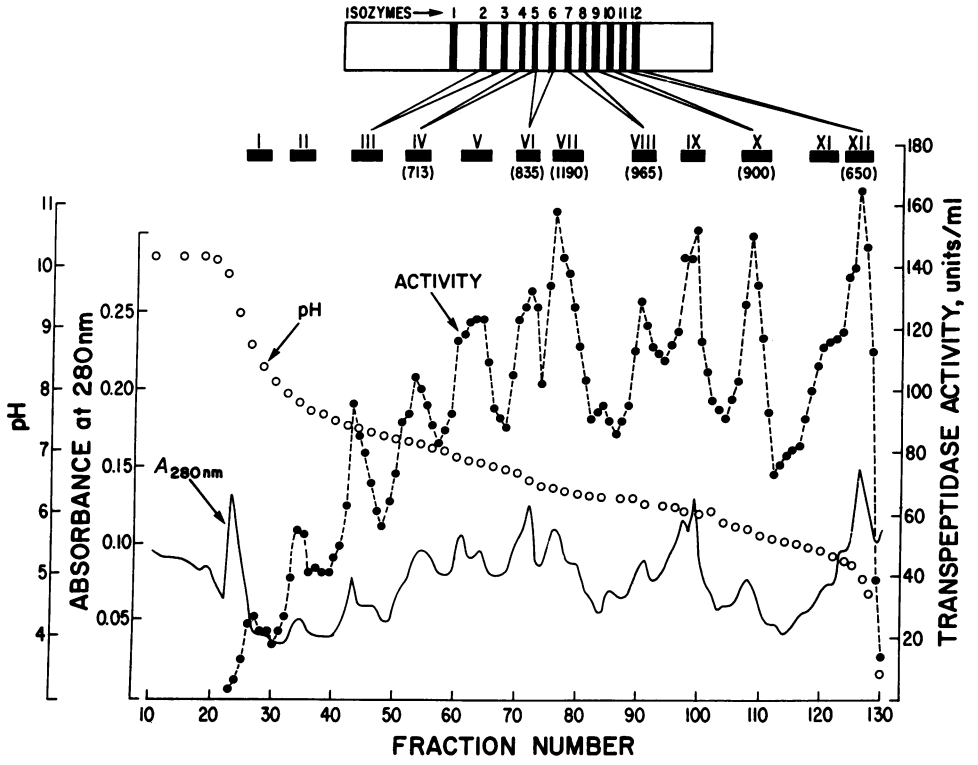

FIG. 2. Separation of the isozymic forms of the light enzyme by isoelectric focusing in a column of Ampholines (pH range 5–8) stabilized by a sucrose gradient. (Enzyme, 14.5 mg; 4°, 48 hr.) Solid line, absorbance at 280 nm; closed circles, transpeptidase (units/ml of fraction); open circles, pH. The bars (top) indicate the fractions pooled; numbers in parentheses are specific activities of pooled fractions. The isozyme composition of some of the pooled fractions as determined by isoelectric focusing on polyacrylamide gels is shown schematically.

$DodSO_4$ and 2-mercaptoethanol, and run on a 12% gel containing 0.1% $NaDodSO_4$. The $NaDodSO_4$ complexes of the heavy and light chains of IgG were run on a separate gel. The immunoprecipitate from the heavy enzyme revealed, in addition to the bands corresponding to the heavy and light chains of IgG, an additional band with an apparent molecular weight of 46,000. Since the molecular weights of the IgG light chain and the subunit-2 of transpeptidase are close (23,000 and 22,000, respectively), a clear separation was not achieved, but broadening of the band in this region indicates the presence of more than one component. The high-molecular-weight material observed in the heavy enzyme on $NaDodSO_4$ gels (Fig. 4G) was not present in the immunoprecipitate, indicating selective precipitation of transpeptidase from this form of the enzyme by anti-IgG.

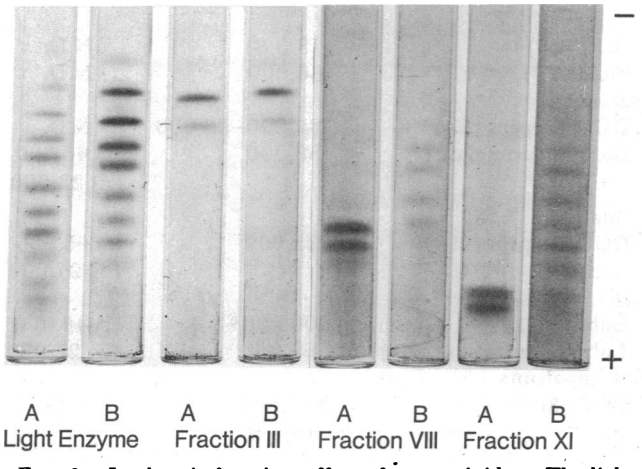

FIG. 3. Isoelectric focusing; effect of neuraminidase. The light enzyme (61 μg), fractions III (15 μg), VIII (19 μg), and XI (20 μg) (see Fig. 2 for fraction identification) were focused in polyacrylamide gels before (A) and after (B) neuraminidase treatment [samples were incubated at 37° for 4 hr in a solution (40 μl) containing 0.1 M Tris-HCl buffer (pH 7.4), 1 mM $CaCl_2$, and 1 activity unit of neuraminidase].

Separation of the subunits of γ-glutamyl transpeptidase

Gel filtration of the light enzyme in 4 M urea (Fig. 6) led to separation of the two subunits, whose identity and purity were confirmed by $NaDodSO_4$ gel studies. The separated subunits were inactive separately or when mixed. They exhibited significant differences in amino acid composition; subunits 1 and 2 contained 30 and 23 nmol of sialic acid per mg, respectively.

DISCUSSION

The isozymic forms of the enzyme seem to be largely due to differences in sialylation which may reflect incomplete posttribosomal processing of this glycoprotein, the structural relationships of the enzyme within the brush border, or the action of endogenous neuraminidases. The $NaDodSO_4$ gel electrophoresis and the crosslinking studies show that (*a*) the enzyme is a dimer consisting of two nonidentical subunits, (*b*) subunit interaction does not involve interchain disulfide bonds, and (*c*) both subunits contain carbohydrates. Gel filtration of the enzyme in urea leads to separation of subunits, which have thus far been found to be enzymatically inactive; attempts to restore activity of the separate or mixed subunits are currently in progress. It would be of interest to learn whether the binding sites for the γ-glutamyl moiety and for the acceptors are located on the same, different, or both subunits. It is notable that several glutamine amidotransferases, e.g., carbamyl phosphate syn-

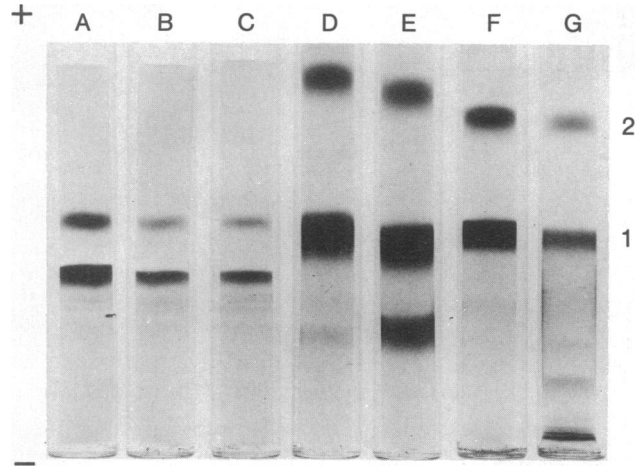

FIG. 4. Polyacrylamide gel electrophoresis in $NaDodSO_4$ (8% gels; 0.1% $NaDodSO_4$). One and two = subunits 1 and 2 respectively. Results are from three separate experiments; (1) gels A, B, and C; (2) D and E; (3) F and G. Gels A, D, and F, are light enzyme; gels B and C are fractions IV and X from Ampholine column (see Fig. 2); gel E is light enzyme treated with dimethylsuberimidate prior to $NaDodSO_4$ treatment; the third band is crosslinked form. Gel G is the heavy enzyme. (Top of gel is at the bottom of Fig. 4).

thetase (23, 24), consist of nonidentical subunits, one of which specifically binds glutamine.

Data on the rat kidney enzyme and other enzyme preparations are summarized in Table 1. The human and ovine kidney enzymes are also dimers, consisting of a larger peptide subunit (molecular weight of 65,000) and a smaller peptide subunit (molecular weight of 25,000). The smaller subunits of rat, human, and ovine kidney enzymes have similar molecular weights (22,000–27,000), while the large subunit of the rat kidney enzyme is much smaller than the larger subunits of the human and ovine enzymes. Possibly the smaller subunits perform similar catalytic functions. There are significant differences between the rat and human enzymes with respect to acceptor specificities and response to maleate (refs. 3, 31; unpublished data obtained in this laboratory); these might be related to the differences in size of the larger subunits. Thus, one may speculate that the smaller subunit has the γ-glutamyl binding site and thus functions as a glutaminase or glutathionase

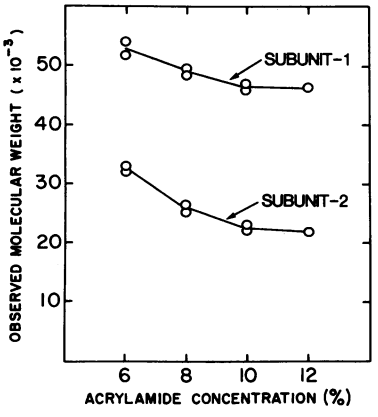

FIG. 5. Effect of polyacrylamide gel concentration on observed molecular weights of subunits 1 and 2 of the light enzyme. Enzyme was electrophoresed on gels of increasing concentration in $NaDodSO_4$; standard proteins were run on similar gels. The observed molecular weights were calculated from standard protein migration curves at each gel concentration.

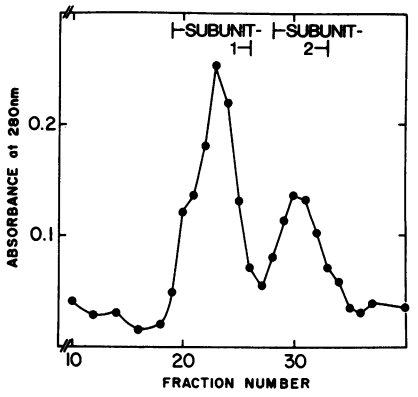

FIG. 6. Separation of the subunits of the light enzyme. Enzyme (6 mg) was applied to a column (1.5 × 85 cm) of Sephadex G-150 equilibrated and developed (at 4°) with 0.02 M Na phosphate buffer (pH 7.2) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and 4 M urea; 1.7 ml per fraction. Aliquots of the peak fractions (23 and 30) were electrophoresed in $NaDodSO_4$-polyacrylamide gels and shown to contain only subunits 1 and 2, respectively.

when separated from the larger subunit, which may contain the acceptor sites; this might account for the differences observed between the acceptor specificities of the rat, on the one hand, and the human and sheep kidney enzymes, on the other. The data suggest that different rat tissues contain different isozyme constellations. Studies on the enzyme from rat seminal vesicles and epididymis (31, 32) show that the former contains isozymes with isoelectric points close to pH 8, while those of the latter are near pH 7; rat kidney has a much wider isozyme spectrum.

Dissociation of the heavy form with $NaDodSO_4$ gave two polypeptide chains with molecular weights close to those ob-

Table 1. Isolation and properties of kidney γ-glutamyl transpeptidase

Enzyme source, procedure* (ref.)	Approximate molecular weight	Subunit molecular weight
Bovine		
DOC (25)	(13.5 S)	N.D.
	(15.1 S)	N.D.
Porcine		
DOC (26)	(8.5 S)	N.D.
Ficin (27)	80,000	N.D.
Human		
DOC, trypsin (28)	80,000–90,000	N.D.
Lubrol, bromelain†	88,000	(i) 65,000
		(ii) 23,000
Ovine		
DOC, trypsin (29)	92,000	(i) 65,000
		(ii) 27,000
Rat		
Lubrol (11)‡	>200,000§	(i) 46,000
Lubrol, bromelain (11)‡	68,000	(ii) 22,000
Rat hepatoma		
DOC (30)	113,000	N.D.
	(5.3 S)	

N.D., not determined.
* DOC, deoxycholate.
† Unpublished data obtained in this laboratory.
‡ Present work.
§ Heavy form of rat kidney enzyme which, in $NaDodSO_4$, yields the two subunits (molecular weight of 46,000 and 22,000, respectively) as well as other higher molecular weight material.

tained by $NaDodSO_4$ dissociation of the light form. $NaDodSO_4$-treatment of the heavy form also gave high-molecular-weight components indicating, as proposed (11), that this enzyme form is an aggregate containing other membrane components. The enzyme can be selectively precipitated from the aggregate by anti-IgG directed against the light form, and the immunoprecipitate dissociates in $NaDodSO_4$ to yield the two polypeptide chains. Thus, the rat kidney transpeptidase in its native form has the two nonidentical subunits present in the light enzyme; these polypeptides are not formed by bromelain cleavage. The data are consistent with the view that the transpeptidase is organized with other proteins within the renal brush border. Studies on the properties of the proteins associated with transpeptidase within the heavy enzyme aggregate would be of importance.

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