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EVIDENCE FOR THE PRESENCE OF A LATENT ACTIVE SITE IN THE LARGE SUBUNIT OF RAT KIDNEY γ-GLUTAMYL TRANSPEPTIDASE

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SUMMARY: γ-Glutamyl transpeptidase (EC 2.3.2.2) of rat kidney is composed of two nonidentical polypeptide chains, the small and large subunits. The active site of this enzyme has previously been shown to be located in the small subunit [Inoue, M., Horiuchi, S. & Morino, Y. (1977) Eur, J. Biochem. 73, 335-342; Tate, S. S. & Meister, A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 931-935] The denaturation of this oligomeric enzyme in 6 M urea, followed by chromatography on a Sephadex G-150, resulted in the separation of the large and small subunits. The removal of urea gave rise to an enzymatically active preparation from the denatured large subunit. Under several renaturation conditions, the small subunit polypeptide chain did not exhibit the enzymatic activity. Upon incubation with 6-diazo-5-oxo-L-[1,2,3,4,5-^{14}C]norleucine, an affinity label for γ-glutamyl transpeptidase, the renatured preparation of the large subunit was covalently labeled with the affinity label with concomitant loss of the enzymatic activity. When the native enzyme was inactivated by the ^{14}C-affinity label, radioactivity was selectively incorporated into the small subunit. These findings indicate that the isolated large subunit possesses an active site which is masked in the native state of the enzyme.

γ-Glutamyl transpeptidase from rat kidney brush border membrane is composed of two nonidentical polypeptide chains, the large subunit and the small subunit (1,2). The affinity labeling of this oligomeric enzyme by 6-diazo-5-oxo-L-norleucine (DON) resulted in the selective covalent labeling of the small subunit (3,4), indicating that the catalytic site of this enzyme resides on the small subunit. However, little has been known about the functional role of the large subunit except that this subunit may be involved in anchoring the membranous enzyme into the renal brush border membrane (1,5). In the course of the study of the subunit-subunit interaction in this oligomeric enzyme, we have found to our surprise that the large subunit, which was isolated from a preparation of γ-glutamyl transpeptidase inactivated by reacting with DON, catalyzed the reaction with L-γ-glutamyl-p-nitroanilide as the substrate.

This communication presents an unequivocal evidence for the presence of

Abbreviations used are: DON, 6-diazo-5-oxo-L-norleucine; SDS, sodium dodecyl-sulfate.

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an active site in the large subunit of γ-glutamyl transpeptidase which remains apparently inoperative in the native state of this oligomeric enzyme.

MATERIALS AND METHODS

Glutathione, L-γ-glutamyl-p-nitroanilide and dithiothreitol were obtained from Sigma Chemical Co. Glyclyglycine was obtained from Protein Research Foundation (Osaka). L-[U-^{14}C]Glutamic acid was purchased from the Radiochemical Centre, Amersham. Other reagents used were of analytical grade. DON was synthesized as described previously (3).

Assay Procedures and Enzyme Preparation

γ-Glutamyl transpeptidase activity was measured with L-γ-glutamyl-p-nitroanilide as the substrate as previously reported (3) according to the method of Orlowski et al. (6) using a High sensitivity spectrophotometer model SM-401 (Union Giken Co. Ltd., Osaka). One unit of the enzyme activity was defined as 1 μmole product/min at 37°C in the presence of 2.5 mM L-γ-glutamyl-p-nitroanilide, 50 mM glycylglycine, 10 mM $MgCl_2$ and 0.1 M Tris-HCl buffer (pH 9.0). Protein was determined with bovine serum alubumin as the standard (7).

γ-Glutamyl transpeptidase was purified from Wistar rat kidney by using papain solubilization (1). The specific activity of the purified enzyme was 2,000 unit/mg. Upon SDS-polyacrylamide gel electrophoresis on 7% gel (8), only two polypeptide chains (the large and small subunit) were detected after staining the gel with Coomassie brilliant blue.

Subunit Separation

The purified enzyme sample thus obtained was denatured in 6 M urea, 1 M propionic acid and 0.1 mM dithiothreitol at 37°C for 2 hr, and chromatographed on a column of Sephadex G-150 (2.5 × 80 cm) previously equilibrated with the same denaturing solution as that used for enzyme denaturation. Fractions of 1.2 ml were collected and analyzed for absorbance at 280 nm of proteins. The subunit separation from [^{14}C]DON-labeled enzyme was performed by the same procedure as mentioned above. Radioactivity was measured on each fraction in a Tri-Carb scintillation spectrometer model 3320 with Bray's solution (9) as scintillant.

Renaturation Procedures

After nonidentical subunits (large and small subunit) of γ-glutamyl transpeptidase were denatured in 6 M urea, 0.2 mM dithiothreitol and 0.05 M Tris-HCl buffer (pH 7.5) at 37°C for 2 hr, they were subjected to renaturation experiment. Renaturation was performed, unless otherwise specified, by dialysis for 8 hr at 4°C against 0.05 M Tris-HCl buffer (pH 7.5) and 0.1 mM dithiothreitol and then further dialysis against the same buffer solution without dithiothreitol for 8 hr at 4°C. The dialysates thus obtained were determined for the enzymatic activity and protein concentrations as described above. The volume of the dialysate was usually 0.2% or less of that of the dialysis buffer.

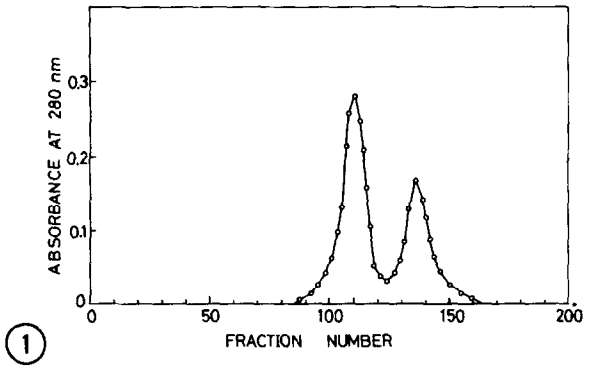
Labeling Experiments

Labeling of the native γ-glutamyl transpeptidase and the renatured large subunit with radioactive DON was performed as reported previously (3). The detailed procedures were described in the legend to Fig. 2.

RESULTS

γ-Glutamyl transpeptidase activity of the large subunit

A pure preparation of rat kidney γ-glutamyl transpeptidase was incubated for 2 hr at 37°C in 6 M urea containing 1 M propionic acid and 0.1 mM dithiothreitol.



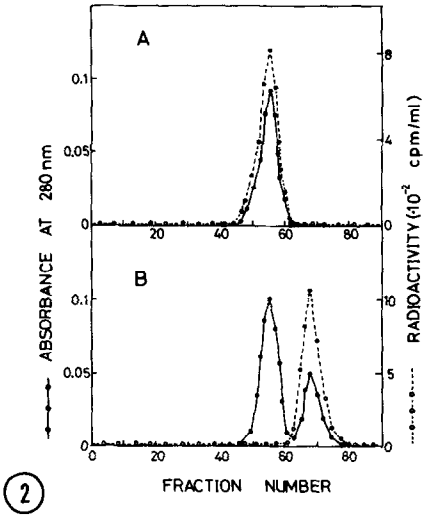


Fig. 1: Subunit separation of γ-glutamyl transpeptidase.
The purified enzyme sample (7.5 mg) was denatured in 6 M urea, 1 M propionic acid and 0.1 mM dithiothreitol at 37°C for 2 hr, and chromatographed on a column of Sephadex G-150 (2.5 × 80 cm) previously equilibrated with the same denaturing solution as that used for the enzyme denaturation. Fractions of 1.2 ml were collected and analyzed for absorbance at 280 nm of proteins.

Fig. 2: Identification of the DON-reactive subunit in both the renatured and native preparations of γ-glutamyl transpeptidase.
A; The renatured preparation (specific activity, 11.3 unit/mg protein) was obtained by dialysis of the denatured large subunit as described under "Materials and Methods" except that 1 mM reduced glutathion was added to the dialysis buffer solution. The renatured preparation thus obtained (1.8 mg) was inactivated with 5 μmole of the radioactive DON (specific radioactivity: 4.25×10^5 cpm/μmol). The reaction mixture contained, in a total volume of 1.0 ml, 2 M sodium maleate and 0.1 M Tris-HCl buffer (pH 7.4). After the inactivation was carried out to the extent that the remaining enzymatic activity was less than 0.1% of the initial activity, the reaction mixture was dialyzed overnight at 4°C against 2 l of 20 mM Tris-HCl buffer (pH 7.2) with three changes of buffer solution. The enzyme solution thus dialyzed was denatured and chromatographed on a column of Sephadex G-150 (1.8 × 60 cm) as described in Materials and Methods. Fractions of 1.0 ml were collected. Radioactivity was measured as described under "Materials and Methods". B; The purified γ-glutamyl transpeptidase (2.5 mg) was inactivated with the radioactive DON (10 μmol) and subjected to subunit separation by the same procedures as described above for Fig. 2A.

The denatured enzyme preparation was passed over a Sephadex G-150 column which was previously equilibrated with the same solution as described above. As shown in Fig. 1, two peak fractions appeared by washing the column with the denaturant-containing solution. Upon SDS-polyacrylamide gel disc electrophoresis, an aliquot from the first peak showed a single protein band migrating at a rate corresponding to that of the large subunit (molecular weight: 46,000) of the enzyme while an aliquot from the second peak exhibited also a single band with

the same mobility as that of the small subunit (molecular weight: 22,000).

An attempt to renature each polypeptide chains thus obtained was made in the following way. The denatured preparations of the large subunit and small subunit were separately subjected to dialysis successively against the solutions containing urea at stepwise decreasing concentrations (3 M, 1.5 M, 0.5 M and, then, 0 M in this order). All solutions contained 50 mM Tris-HCl buffer (pH 7.5) and 0.1 mM dithiothreitol. Since the active site of this enzyme is believed to be located on the small subunit as evidenced by the affinity labeling study (3,4), we expected that only the small subunit preparation should exhibit the enzyme activity after removal of the denaturant. However, the small subunit preparation thus obtained showed only a negligible enzyme activity towards L-γ-glutamyl-p-nitroanilide as the substrate ($<$ 0.001 unit/mg protein) under the present procedure for refolding the polypeptide chains. Subsequently, the addition of an equimolar amount of the renatured preparation of the large subunit to the small subunit sample was found to restore a significant amount of the enzyme activity (1.9 unit/mg protein), which at first appeared to indicate that a cooperation by the large subunit polypeptide chain was requisite for the reconstitution of the active site in the small subunit. However, the renatured preparation of the large subunit alone was found unexpectedly to exhibit a higher enzymatic activity (3.7 unit/mg protein) than that observed in the presence of the small subunit. This finding clearly demonstrates that the renatured preparation of the large subunit itself is enzymatically active. Various procedures for renaturation were examined in an attempt to find most favorable conditions for the restoration of the enzymatic activity of the large subunit as well as the small subunit. Table 1 summarizes the results of these experiments. Thus, the presence of dithiothreitol and glutathione in the dialysis buffer solution appeared to be required for a further recovery of the enzymatic activity of the large subunit polypeptide chain whereas only an insignificant level of the enzyme activity was restored with the small subunit under these conditions.

Affinity labeling of the renatured preparation with radioactive DON

DON is known to label specifically the active site of γ-glutamyl transpeptidase from rat kidney (3,4) and from human kidney (10). To further confirm that the renatured enzymatic activity was really attributed to the large subunit, the affinity labeling technique was utilized. The incubation of the renatured preparation of the large subunit with the radioactive DON resulted in an irreversible inactivation of the enzyme activity (less than 0.1% of the initial activity). The inactivated preparation was dialyzed exhaustively against 20 mM Tris-HCl buffer (pH 7.2), followed by denaturation in 6 M urea containing 1 M

Table 1

Effect of dithiothreitol and reduced glutathione on the renaturation of the large and small subunit. After the large and small subunits were denatured in 6 M urea, 50 mM Tris-HCl buffer (pH 7.5) and 0.1 mM dithiothreitol for 2 hr at 37°C, they were separately transferred to dialysis tubes and then submitted to successive dialysis against the solutions containing urea at stepwise decreading concentrations (3 M, 1.5 M, 0.5 M and, then, 0 M in this order). Each dialysis step required about 6 hours. All dialysis solutions contained 50 mM Tris-HCl buffer (pH 7.5) and the reagents as indicated. Concentrations of these reagents were as follows; dithiothreitol (0.1 mM) and reduced glutathione (1 mM). Dialysis was performed at room temperature. Each value for the renatured specific activity was the mean of triplicate runs in which the final protein concentrations (post-dialysis) of the subunits were within the range of 0.23-0.35 mg/ml.

Renaturation Conditions	Renatured Specific Activity*	
	Large Subunit	Small Subunit
Control	1.7	< 0.001
Dithiothreitol	3.9	< 0.001
Dithiothreitol & Glutathione	13.1	< 0.001

* Specific activity of the renatured enzyme (unit/mg protein)

propionic acid and 0.1 mM dithiothreitol for 2 hr at 37°C. The denatured preparation was dialyzed overnight against 100 volumes of the same denaturant solution, and passed over a Sephadex G-150 column as described earlier. The chromatogram (Fig. 2A) revealed a single protein peak which was also radioactive. Upon electrophoresis in SDS-polyacrylamide gel disc, an aliquot from this peak exhibited a single band with the same relative mobility as that of the large subunit of the enzyme. This finding indicates unequivocally that the renatured enzyme activity was indeed derived from the large subunit but not from the small subunit. The chromatographic pattern of the native enzyme which had been affinity-labeled was also illustrated in Fig. 2B. The radioactivity was exclusively incorporated into the second protein peak which contained the small subunit of the enzyme. This confirms the previous conclusion (3,4) that in the native state of γ-glutamyl transpeptidase, the affinity label reacts only with the active site in the small subunit and, in addition, leads to the contention that the active site of the large subunit is rendered inoperative upon the molecular organization of this oligomeric enzyme into its native state.

DISCUSSION

The present investigation has clearly indicated that the large subunit

polypeptide chain isolated from rat kidney γ-glutamyl transpeptidase is capable of catalyzing the enzymatic decomposition of L-γ-glutamyl-p-nitroanilide. This finding has been an unexpected one since the affinity labeling study of this enzyme (3,4) had unambiguously shown that the active site of this oligomeric enzyme resides on the small subunit but not on the large subunit. Although the data were not shown, the enzymatic reaction catalyzed by the isolated large subunit seemed to be indistinguishable from that catalyzed by the native enzyme with respect to its Km values for both L-γ-glutamyl-p-nitroanilide and glycylglycine, pH-activity profile, etc. Thus, it appears that there is a partial structural homology between the large and small subunit although they represent apparently nonidentical polypeptide chains. Detailed studies are now in progress.

We have not found yet the condition to restore the enzymatic activity of the isolated small subunit. The enzyme preparation employed in the present study was that solubilized from renal brush border membrane by digestion with papain. Hence, it appears possible that the proteolytic modification of the covalent structure of this polypeptide chain might interfere with the natural refolding process necessary for the reconstitution of the active site. Thus, an attempt to regenerate the enzymatic activity of the small subunit isolated from the detergent-solubilized enzyme remains to be done.

In a preliminary experiment, the addition of the isolated small subunit polypeptide chain, though enzymatically inactive by itself, was found to depress the enzymatic activity of the renatured large subunit, indicating that there is a molecular interaction between these two isolated polypeptide chains. This finding supports the contention that the active site in the large subunit is rendered inoperative upon the formation of the native enzyme molecule.

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