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RETENTION OF ENZYMATIC ACTIVITY OF BOVINE ENTEROKINASE AFTER A LIMITED REDUCTION OF DISULFIDE BONDS*

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SUMMARY - A limited reduction of disulfide bonds of bovine enterokinase has been accomplished by incubating the native enzyme with 5 mM dithioerythritol at 25°C for 2 hrs. After alkylation of the partially reduced protein with iodoacetate or iodoacetamide, the modified enzyme retained full enzymatic activity toward trypsinogen and thiobenzyl benzyloxycarbonyl L-lysinate. SDS-gel electrophoresis performed in the absence of mercaptoethanol showed that the heavy and light chains were no longer covalently bound to one another. Since the light chain retained full catalytic activity, intermolecular disulfide bonds holding the heavy and light chains together were non-essential for catalytic activity.

Enterokinase (enteropeptidase, EC3.4.21.9) is a serine proteinase that acts physiologically to convert trypsinogen to trypsin (1,2). The enzyme contains two polypeptide chains-one heavy and one light chain-held together by two or more disulfide bonds (3,4). The serine and histidine residues of the active site are both components of the light chain (5).

We have reported on the purification and properties of bovine duodenal enterokinase (4). The heavy and light chains of the fully reduced and carboxymethylated enzyme were separated under denaturing conditions (3,4). The amino acid composition of the light chain resembled that of bovine trypsin (4). We concluded that the three-dimensional structure of the catalytic chain should be close to that of trypsin. We assumed, therefore, that the isolated light chain may be catalytically active. In our earlier studies, this possibility could not be tested since the two chains were separated as denatured molecules. In this report, we describe a limited reduction of enterokinase under non-denaturing conditions. We show that

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the enzyme after reduction and carboxymethylation retained its enzymatic activity.

METHODS

Enterokinase was purified to homogeneity from bovine duodenal mucosa by the procedure of Liepnieks and Light (4). The peptidase activity of enterokinase was determined after activation of trypsinogen to trypsin. The thiol esterase activity of enterokinase toward thiobenzyl benzyloxycarbonyl L-lysinate was determined by the procedure of Green and Shaw (7). Trypsin activity was estimated by the method of Hummel (6) using tosyl-L-arginine methyl ester as substrate.

A solution of 75 µl (500 µg) of enterokinase in 0.01 M sodium phosphate, 0.2 M NaCl, pH 7.0, was degassed with nitrogen for 15 min. A Hamilton syringe was used to introduce 25 µl of dithioerythritol (3.9 mg/ml) into the reaction mixture (final concentration of dithioerythritol was 5 mM). The reaction mixture was kept at 25°C under a nitrogen atmosphere. At various time periods, 20 µl samples were added to 5 µl of iodoacetate or iodoacetamide at pH 9.0 (final concentration of 20 mM). After a reaction period of 40 min, samples were taken for enzymatic activity measurements and SDS-gel electrophoresis in the absence of mercaptoethanol. The method of Weber and Osborn (8) was followed for the electrophoretic separation on 5% gels at 6 ma/tube.

The mixed disulfide of partially reduced enterokinase and glutathione was prepared by the procedure of Odorzynski and Light (9). Approximately 200 µg of enterokinase was treated with 5 mM dithioerythritol in 0.1 M sodium phosphate buffer, containing 0.2 M sodium chloride, pH 7.0, for 2 hrs, at 25°C, under a nitrogen atmosphere. A 50 µl sample was diluted with 0.45 ml of 0.2 M oxidized glutathione in 0.2 M Tris, pH 9.0, and stirred overnight at 25°C. The mixture was dialyzed exhaustively against distilled water.

RESULTS AND DISCUSSION

The rate of hydrolysis of the synthetic thiol ester substrate, thiobenzyl benzyloxycarbonyl L-lysinate, by enterokinase is presented in Figure 1. The activity was linear with enzyme concentration from 5 to 140 nanograms. The thiol ester substrate permitted a direct assay of enterokinase and the assay detected as little as 3.2×10^{-11} M of enzyme. Figure 2 presents a double reciprical plot for the hydrolysis of thiobenzyl benzyloxycarbonyl L-lysinate by enterokinase. A Km of 0.033 mM and a kcat of 87 s^{-1} were found for the substrate at 25°C. The kinetic parameters were close to the values found by Green and Shaw (7) for the hydrolysis of the same substrate by bovine β-trypsin (Km of 0.05 mM and a kcat of 75 s^{-1}). The use of thiobenzyl benzyloxycarbonyl L-lysinate as a substrate provided

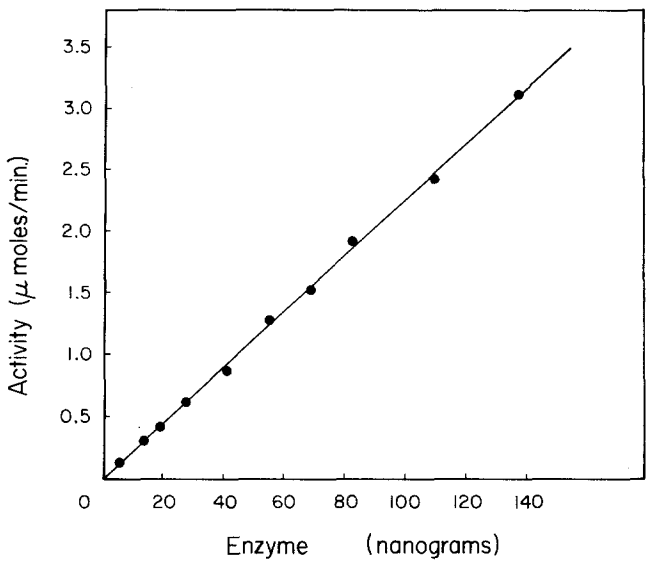

Figure 1. The hydrolysis of thiobenzyl benzyloxycarbonyl L-lysinate by enterokinase at 25°C. The reaction mixture contained 900 μl of 0.1 M Tris, pH 8.0, 100 μl of thiol ester substrate (final concentration of 1×10^{-3} M), 2 μl of 1.3×10^{-1} M 5,5' - dithiobis (2-nitrobenzoic acid) in dimethyl formamide, and enzyme as indicated. The absorbancy at 412 nm was monitored continuously.

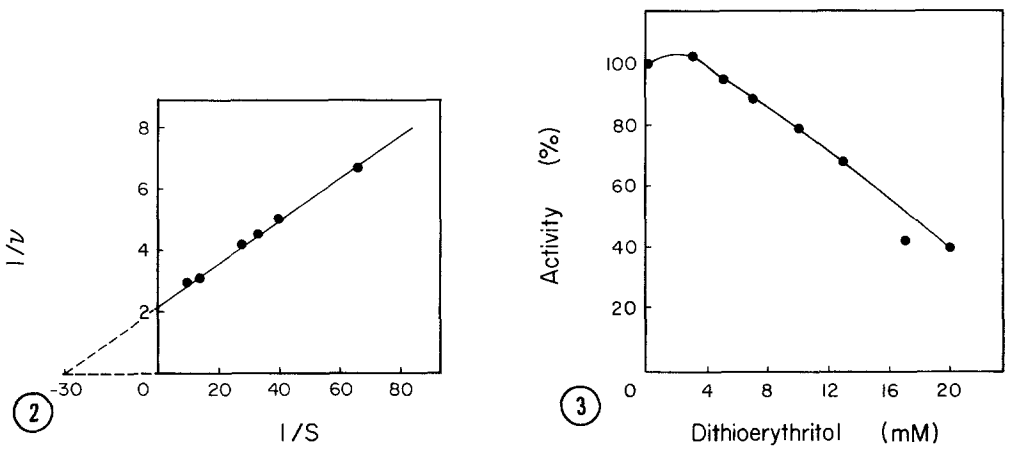

Figure 2. A double reciprocal plot for the hydrolysis of thiobenzyl benzyloxycarbonyl L-lysinate by enterokinase at pH 8.0 and 25°C. Reaction mixtures contained 14 nanograms per ml of enzyme and 0.01 to 0.1 mM substrate. Other conditions were the same as in Fig. 1.

Figure 3. The effect of dithioerythritol reduction of enterokinase on enzymatic activity. The reaction mixture contained 20 nanograms per ml of native enzyme in 0.01 M sodium phosphate buffer, pH 7.0, 0.2 M sodium

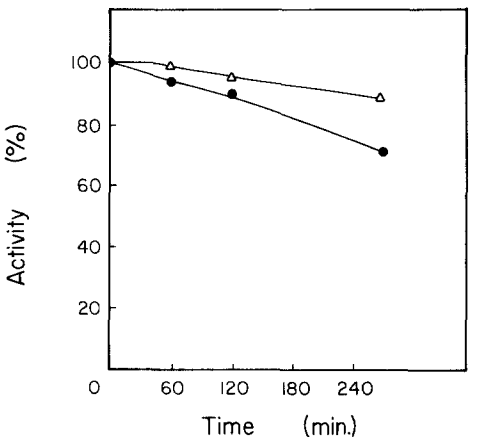

Figure 4. The effect of limited reduction and carboxymethylation of enterokinase on thiol esterase (Δ) and peptidase (•) activities.

a highly sensitive and direct measure of enzymatic activity in the chemical modifications described below.

The effect on enzyme activity of the treatment of native enterokinase with varying amounts of dithioerythritol is shown in Figure 3. A progressive decrease in the activity of the enzyme is seen when the concentration of dithioerythritol is greater than 5 mM. A complete retention of enzyme activity was observed with 5 mM dithioerythritol after an incubation at 25°C for 2 hrs.

In a separate experiment, enterokinase was again reduced with 5 mM dithioerythritol for 2 hrs. but the newly formed sulfydryls were converted to the stable carboxymethyl derivative with iodoacetate. Figure 4 shows that the thiol esterase and peptidase activities remained essentially constant for the first 120 min; thereafter, the peptidase activity decreased more rapidly. When the reduction was monitored with SDS-gel electrophoresis (Fig.5), about 50% of the intact enterokinase was still present after 1 hr. However, a complete separation of the carboxymethylated light and heavy chains of

Figure 3 (continued).
chloride, dithioerythritol as indicated, at 25°, for 2 hrs. The pH was lowered to 5.0 and the activity determined by activation of 4 μM trypsinogen for 30 min. at 35°.

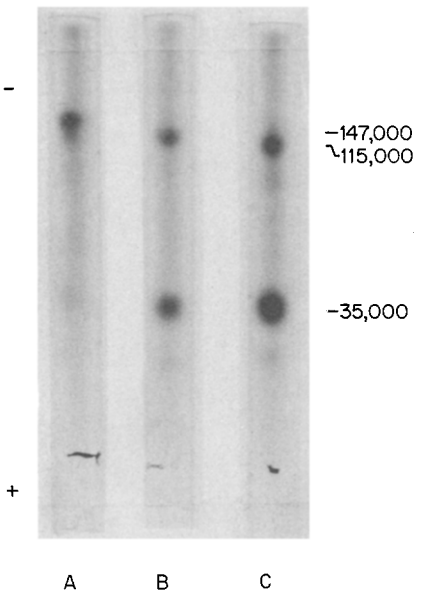

Figure 5. SDS-gel electrophoresis of enterokinase before and after limited reduction with 5 mM dithioerythritol. A) native enterokinase in the absence of mercaptoethanol; B) native enterokinase in the presence of 1% mercaptoethanol; and C) native enterokinase after reduction with 5 mM dithioerythritol for 2 hrs. and carboxymethylation with 20 nM iodoacetate.

enterokinase took place in 2 hrs. The sulfhydryls were also alkylated with iodoacetamide or converted to a mixed disulfide derivative of glutathione. These derivatives were 80 to 90% active. We conclude from these studies that the light chain of enterokinase was fully active whether present as the S-carboxymethyl, S-carboxyamidomethyl, or as the mixed disulfide of glutathione. Neither the size of the derivative nor its charge seemed to influence the enzymatic activity.

The retention of enzymatic activity of partially reduced enterokinase showed that extensive cleavage of intramolecular disulfide bonds did not take place. Apparently, the intramolecular disulfides were protected from the reducing agent by the globular structure of the light chain. However, it is possible that a few intramolecular disulfides that were nonessential for activity could have been cleaved since our methods of analysis would not have detected such changes.

These studies clearly show that it is not necessary to have the heavy and light chains linked by disulfide bonds for enterokinase to be active. Unfortunately, these studies do not clarify the function of the heavy chain. Since enterokinase is a membrane-bound enzyme, the heavy chain may contain a region that serves to anchor the enzyme in the membrane. It is also possible that the heavy chain increases the stability of the light chain; preliminary attempts to separate the two chains on gel filtration chromatography were unsuccessful. The system clearly requires further study before the role of the heavy chain is understood.

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