The Preparation and Properties of the Catalytic Subunit of Bovine Enterokinase

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A limited reduction of the disulfide bonds of bovine enterokinase (enteropeptidase, EC 3.4.21.9) was accomplished with 50 mM dithioerythritol, at pH 9.0, and at 4 °C. The conditions separated the heavy and light subunits quantitatively with improved reliability when compared to the conditions used previously (Savithri, N-tosyl-L-arginine methyl ester and N-tosyl-L-lysine were alkylated with iodoacetate and then resolved on Sephadex G-150. Amino acid analyses and the incorporation of [14C]carboxymethylcysteine residues showed that 3.1 carboxymethylcysteine residues were in the catalytic subunit and 8.9 in the heavy subunit. The catalytic subunit had normal catalytic activity toward N-benzoyl-DL-arginine methyl ester, enhanced activity toward N-tosyl-L-arginine methyl ester and N-tosyl-L-lysine methyl ester, and lower activity toward N-benzoyl-DL-arginine p-nitroanilide. The catalytic subunit retained the restricted specificity of intact enterokinase, but the rate of activation of trypsinogen was much slower. It is likely that the limited reduction of the disulfide bonds of the catalytic subunit altered the interaction of protein substrates with the specificity site.

Enterokinase (enteropeptidase, EC 3.4.21.9) plays a key role in mammalian digestion as the physiological activator of trypsinogen (Kunitz, 1939; Yamashina, 1956; Maroux et al., 1971). Highly purified preparations have been obtained from the intestines of the pig (Maroux et al., 1971; Baratti et al., 1973), cow (Anderson et al., 1977; Liepnieks and Light, 1979), and human (Grant and Hermon-Taylor, 1976). The enzyme is a serine proteinase (Baratti et al., 1973; Liepnieks and Light, 1979) and the active site is part of the light chain (catalytic chain) (Baratti et al., 1973; Liepnieks and Light, 1979).

The principal properties of bovine enterokinase that are important for the studies reported below are: 1) the remarkable specificity toward the tetra-aspartyl lysyl sequence of the activation peptide of trypsinogen (Yamashina, 1956; Maroux et al., 1971; Liepnieks and Light, 1979); 2) the catalytic efficiency for the activation of trypsinogen which is 2000-fold greater than with bovine trypsin (Maroux et al., 1971; and 3) the size of the intact enzyme (M, = 150,000) with a heavy (M, = 115,000) and light (M, = 35,000) chain linked by a disulfide bond (Liepnieks and Light, 1979).

In preliminary experiments, we described experimental conditions that limited the reduction of disulfide bonds of native enterokinase to the disulfide bond linking the heavy and catalytic subunits (Savithri and Light, 1980). We showed that the S-alkylated catalytic subunit in the reaction mixture retained enzymatic activity. The limited reduction of disulfide bonds provided a unique opportunity to study further the properties of the isolated catalytic subunit. In this report we describe the purification of the catalytic subunit and the enzymatic properties toward both small synthetic substrates and protein substrates. We also report the extent of reduction that occurred in both subunits and the number of disulfide bonds that link the two subunits.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

The disulfide bonds of globular proteins are usually found in the interior of the molecule where these bridges stabilize the folded structure (Lu, 1977; Richardson, 1981). Generally, the disulfide bonds resist chemical modification because of steric constraints and general inaccessibility. The observation that enterokinase undergoes a limited reduction suggests that the reactive disulfide bonds are located on the surface of the protein and exposed to the reagent.

We could not estimate the number of disulfide bonds linking the two subunits because the isolated heavy subunit has 9 carboxymethylcysteine residues and the catalytic subunit three. We were unable to find conditions that would limit the extent of the reduction to a single disulfide bond and still accomplish the separation of the two subunits. Nevertheless, only two possibilities for the disulfide distribution connecting the subunits are reasonable. One possibility is that one disulfide linking the catalytic and heavy subunits and an intramolecular disulfide of the catalytic subunit were reduced. A second possibility is that three disulfides link the subunits. We eliminate a third possibility of two intermolecular disulfide bonds.

1 Portions of this paper (including "Experimental Procedures," "Results," Table I, and Figs. 1-6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1116, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
properties of the catalytic subunit must be the loss of the cysteine residue, and enterokinase does not contain cysteine (Liepnieks and Light, 1979). We hope that the elucidation of the amino acid sequence of the catalytic subunit sometime in the future will provide a more rational basis for deciding between the two possibilities.

We examined the properties of the catalytic subunit toward synthetic substrates and proteins. The specificity was restricted to lysine and arginine residues and the catalytic efficiency toward ester substrates was the same or greater than with the intact enzyme. Titration with an active-site reagent was normal suggesting a retention of the geometry of the groups comprising the active site and the specificity pocket. However, we observed significant differences with protein substrates and proteinase inhibitors. Protein substrates were hydrolyzed with the same restricted specificity of the intact enzyme but at a much slower rate. The catalytic subunit was inhibited by both PTI and STI. It should be recalled that the native enzyme was only inhibited by PTI (Liepnieks and Light, 1979). The inhibition of the catalytic subunit by STI was unexpected and represents a new property of the subunit.

The most likely explanation for the different enzymatic properties of the catalytic subunit must be the loss of the heavy subunit. A likely possibility is that protein substrates and proteinase inhibitors have multiple subsite interactions with regions on both the catalytic and heavy subunits. The absence of the heavy subunit would decrease the number of subsite interactions and alter the specificity of binding. An alternative possibility is that the loss of the heavy subunit causes a small conformational change in the catalytic subunit, altering the subsite binding of proteins. The inhibition by STI suggests that the subsite binding now present is incapable of distinguishing one proteinase inhibitor (PTI) from another (STI). The reduction of the disulfides alters the properties of the catalytic subunit sufficiently for the subunit to become more like trypsin itself.

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REFERENCES

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Catalytic Subunit of Enterokinase

Supplementary Material:

The preparation and properties of the catalytic subunit of enterokinase

Experimental procedures and results

Enterokinase was purified from the commercial sample by the procedure of Fauser and Lehtinen (1973). The assay procedure and the substrate used were the same as described previously (Fauser and Lehtinen, 1973).

Enzymatic activity

L. coli enterokinase activity was determined with the method of Fauser and Lehtinen (1973). Detection of the enzyme was performed with a sheep anti-enterokinase immune serum. The assay mixture was incubated for 10 min, followed by adding 2% trichloroacetic acid to precipitate the protein, and 50 mg hydrolyzing the substrate. The absorption of the supernatant was measured at 235 nm in a Spectra-Fluorimeter.

The catalytic subunit of enterokinase was purified from the commercial sample by the procedure of Fauser and Lehtinen (1973). The enzyme was incubated with 2% trichloroacetic acid to precipitate the protein, and 50 mg hydrolyzing the substrate. The absorption of the supernatant was measured at 235 nm in a Spectra-Fluorimeter.

Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Catalytic Subunit</th>
<th>Enterokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-Arg-OMe</td>
<td>16.5</td>
<td>21.6</td>
</tr>
<tr>
<td>Leu-Arg-OMe</td>
<td>10.2</td>
<td>13.7</td>
</tr>
<tr>
<td>Leu-Arg-OMe</td>
<td>9.4</td>
<td>12.4</td>
</tr>
<tr>
<td>Arg-OMe</td>
<td>18.5</td>
<td>24.0</td>
</tr>
<tr>
<td>Arg-OMe</td>
<td>6.4</td>
<td>9.0</td>
</tr>
</tbody>
</table>

The specific activity of the catalytic subunit was 21.6 pmol/mg/min, while the specific activity of the enterokinase was 24.0 pmol/mg/min.

The catalytic subunit and enterokinase preparations were subjected to SDS-PAGE and analyzed by the method of Laemmli (1970). The molecular weight of the catalytic subunit was estimated to be 100,000 daltons.

RESULTS

The experimental conditions for the limited reduction of enterokinase differed from those used earlier (Fauser and Lehtinen, 1973). The temperature was lowered from 37°C to 25°C and the pH was raised from 7.0 to 8.2. The conditions were selected to minimize denaturation of the enzyme and to maintain the potential for denaturation. The substrate was quantitatively released after 2 h of reduction (Fig. 1). In addition to reducing mixture to prevent proteolysis and to recover the heavy subunit in an active trypsin-like form. The reduction was performed by adding the activity of the protease inhibitor and the enzyme was present in a 1:1 molar ratio.

The procedure of Fauser and Lehtinen (1973) was followed for performing SDS-polyacrylamide gel electrophoresis. Samples of enterokinase were analyzed using a buffer containing 0.1 M Tris-HCl, 6.8 M guanidine- HCl, and 0.1 M sodium dodecyl sulfate (SDS).

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The catalytic subunit preparations were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by the method of Laemmli (1970). The molecular weight of the catalytic subunit was estimated to be 100,000 daltons.

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Catalytic Subunit of Enterokinase

**Figure 2.** Gel filtration of partially reduced enterokinase. A 2 mg sample was applied to a Sephadex G-150 column (0.6x60 cm) equilibrated with 0.02 M Tris, 0.02 M CaCl₂, pH 7.8. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h at 5°C. The conditions were the same as in Fig. 1 except PTI was omitted. Absorbance at 280 nm (- - -), enterokinase activity toward Bz-Arg-OMe (--------), enterokinase activity toward Bz-Arg-OEt (-------------).

**Figure 3.** The enzymatic activity of partially reduced enterokinase. The conditions of reduction and alkylation were the same as in Fig. 1 and alkylation were the same as in Fig. 2. Enzymatic activity toward Bz-Arg-OEt (-------------), toward trypsinogen (- - -).

**Figure 4.** The rate of formation of SCM-trypsin from the hydrolysis of SCM-trypsinogen by the catalytic subunit. The catalytic subunit (0.07 μM) was incubated with SCM-trypsinogen (14 μM) in 0.01 M Tris, 0.02 M CaCl₂, pH 8.0, at 37°C. At timed intervals, samples were boiled for 3 min in the presence of SDS and mercaptoethanol and separated on SDS-polyacrylamide gel electrophoresis. The scanned gel was scanned using the peaks representing the SCM-trypsinogen (---) and SCM-trypsinogen (-----) areas. The peak area after 5 h of hydrolysis was 10% of the zero time value.

**Figure 5.** The inhibition of the catalytic subunit with PTI. The catalytic subunit (30 μM), or enterokinase (25 μM), and PTI (10 to 150 μM) were incubated in 0.1 M veronal, 0.02 M CaCl₂, pH 8.0, for 30 min. The conditions were the same as in Fig. 1 except that the activity was determined by titration with C6G-Br-OMe. Catalytic subunit (--------), native enterokinase (-----).

**Figure 6.** The inhibition of the catalytic subunit with STI. The conditions were the same as in Fig. 5. Catalytic subunit (--------), native enterokinase (-----).