The Identification of the Histidine Residue at the Active Center of Chymotrypsin*

Eng Bee Ong, Elliott Shaw,† and Guenther Schoellmann

From the Department of Biochemistry, Tulane University School of Medicine, New Orleans 12, Louisiana

(Received for publication, August 7, 1964)

In order to determine the structural features of the active centers of enzymes, a new approach was begun in this laboratory which makes use of substrate-like molecules to carry reactive groupings into the active center region. Thus, for chymotrypsin, the chloromethyl ketone derived from N-tosyl-L-phenylalanine (α-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone) was chosen and shown to inactivate chymotrypsin in a specific, stoichiometric manner and to provide the first direct evidence for the involvement of histidine in the catalytic process of that enzyme (1, 2), a role that had been deduced by other workers from a variety of evidence (3). The covalent attachment of the inhibitor to the enzyme is a key feature of the inactivation and was necessary to permit a subsequent degradation that could establish the location in the primary structure of chymotrypsin of the altered amino acid residue.† The use of TPCK-14C facilitated the structural study. Since chymotrypsin has 2 histidine residues and since both of these are in the B chain of the enzyme, the approach selected was to isolate the B chain from the inhibited enzyme before proteolytic digestion in the hope of reducing the complexity of resultant peptide mixtures. It was possible to obtain from peptic digests of the B chain an inhibitor-bound decapetide the composition of which agreed adequately with information accruing from studies on the primary sequence of chymotrypsinogen (5) and permitted identification of the active center histidine. This paper gives in detail the results of this study, which have already been published in preliminary form (6).

It was realized that this approach to the investigation of the active centers of enzymes would be of general value, and it is of considerable interest that its application to trypsin has also met with success. The chloromethyl ketone derived from Nα-tosyl-L-lysine effectively inactivates trypsin by a reaction at its active center (7) that is both stoichiometric and specific.

EXPERIMENTAL PROCEDURE

Enzymes—α-Chymotrypsin (three times crystallized), trypsin (twice crystallized, lyophilized, salt-free), and peapain (twice crystallized) were purchased from Worthington Biochemical Corporation, Freehold, New Jersey. Column Chromatographic Techniques—Sephadex G-25 and Sephadex G-50 (medium mesh) were commercial preparations obtained from Pharmacia, Uppsala, Sweden. Dowex 1-X2 (200 to 400 mesh) and Dowex 50-X2 (200 to 400 mesh) were purchased from Bio-Rad Laboratories, Richmond, California, and the J. T. Baker Chemical Company, Phillipsburg, New Jersey, respectively. DEAE-cellulose was a product of Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York.

Ninhydrin determinations were carried out according to the procedure of Moore and Stein (8). Radioactivity was measured in a windowless gas flow Geiger counter. Volatile buffers were prepared according to Holeysovsky et al. (9).

Amino Acid Analyses—Quantitative amino acid analyses were performed with a Beckman/Spinco automatic amino acid analyzer, model MS, according to the procedure of Spackman, Stein, and Moore (10). Hydrolysates (22-hour) were prepared according to the procedure described (2).

Electrophoresis and Paper Chromatography—High voltage paper electrophoresis was performed with a high voltage electrophorator from Gilson Medical Electronics, Middleton, Wisconsin. For all paper chromatographic work, Whatman No. 1 paper was used.

RESULTS

Preparation of Chymotrypsin-TPCK and Isolation of Modified B Chain—Fully active chymotrypsin (2.50 g, 100 μmoles) in 0.005 M sodium phosphate buffer (5 liters), pH 7.0, was incubated at 37°C with an 8-fold molar excess of TPCK-14C added as a methanolic solution (4.0 mg per ml). The completely inactivated enzyme solution was dialyzed against distilled water at 4°C with frequent changes of water and then lyophilized. Chymotrypsin-TPCK (mol. wt. 25,300) so prepared and used for subsequent degradation had a specific activity of 1.4 × 106 c.p.m. per μmole; amino acid analysis showed a loss of 1 histidine residue as expected (2). The disulfide bonds of the inhibited enzyme were reduced with sodium selenite and cupric ions according to the procedure of Pechère et al. (11), and the S-sulfo-B chain was purified according to the procedure of Van Hoang et al. (12). Fig. 1 shows one of the elution patterns. The modified B chain (Peak 2) is well separated from the C chain (Peak 1). As expected, the radioactivity in the sample appeared chiefly in the modified B chain. The fractions from each peak were pooled, dialyzed, and lyo-
The results of the amino acid analyses of modified B chain are included in Table I for comparison with those reported. The analyses showed the presence of 1.1 histidine residues in the modified B chain as compared to 2.0 residues in the unmodified B chain. The identity of the B chain was also confirmed by an NH2-terminal determination with the dinitrofluorobenzene method (12). The cross-contamination indicated by the presence of radioactivity in the C chain is in agreement with the reported results.

Preliminary Purification and Analysis of Peptic Hydrolysate of Modified B Chain—The desired result of proteolytic digestion of the B chain was to obtain peptides containing 10 to 15 amino acid residues by means of a specific cleavage. In order to select the enzyme and the conditions for the hydrolysis, three enzymes (pepsin, trypsin, and chymotrypsin) which are sufficiently specific in their action for a controlled degradation of protein were tried in preliminary experiments, with the use of gel filtration for analysis of the hydrolysate produced. It was found that trypsin gave digests from which much of the radioactivity precipitated on adjustment to 0.2 N with acetic acid. Radioactivity of chymotryptic digests appeared close to the hold-up volume of the Sephadex eluates. Both enzymes thus gave an impression that little proteolysis had occurred. However, peptic digests remained fully soluble and indicated a more complete digestion; consequently, peptic digestion was chosen for degradation of the modified B chain.

A 0.1% solution of the modified B chain in 0.2 N HCl was adjusted to pH 1.8 with 0.1 N HCl and equilibrated in a water bath maintained at 37 ± 0.1° prior to the addition of an aliquot of freshly prepared 0.05% pepsin solution in 0.2 N HCl (enzyme to substrate ratio, 1:25 by weight). The digestion was carried out for 12 hours, maintained at pH 1.8, and stirred occasionally. The reaction mixture was then lyophilized. The mixture after lyophilization was dissolved in 0.2 N acetic acid and centrifuged, and the entire supernatant fraction was subjected to gel filtration on Sephadex G-50 columns. The results are shown in Fig. 2.
Fig. 3. Purification of TPCK ^14C peptide on Dowex 50 X2. The sample was peptide Fraction S-I (Fig. 2); column size, 1.0 × 150 cm; column temperature, 32 ± 0.1 °C; fraction size, 10 ml; and flow rate, 60 ml per hour. From alternate fractions, 0.2-ml and 0.4-ml portions were determined for radioactivity and ninhydrin color, respectively.

Fig. 4. Chromatography of major TPCK peptide on DEAE-Sephadex A-25. The sample was the major TPCK peptide peak from Dowex 50 (Fig. 3); column size, 0.6 × 50 cm; fraction size, 2.8 ml; and flow rate, 10 ml per hour. Portions (0.05 ml each) from alternate fractions were taken for radioactivity assay. For the linear gradient, 200 ml of each buffer were used. Fraction S-III) and consisted of about 13% of the total weight applied to the column; however, the radioactivity yield was 94%.

Peptide Fraction S-I (1 mg) was also submitted to “fingerprinting” analysis by paper chromatography in a solvent system consisting of 1 butanol, acetic acid, and water (14:18:4.3 by volume), followed by high voltage electrophoresis in a pyridine-acetate buffer, pH 3.6, at 35 volts per cm for 75 minutes (13). About three major radioactive spots together with about seven minor spots were located either along the origin or in the anodic region, suggesting that the peptides were acidic.

The Sephadex-purified radioactive Fraction S-I, which had been shown to be heterogeneous, was now further purified by the successive use of cation and anion exchange columns. The basis for the choice of the two types of ion exchangers was that they function under vastly different conditions.

Purification and Analysis of Sephadex-purified Radioactive Peptides—Chromatographic fractionation of Sephadex Fraction S-I on Dowex 50-X2 gave the elution pattern shown in Fig. 3, which reveals that the major portion of the radioactivity emerged with the first buffer (pH 2.5, 0.1 M pyridine-formate). The state of purity of the radioactive peaks was checked by the peptide mapping method of Ingram (14). The ninhydrin-positive spots of the developed chromatograms were punched out and scanned. It was found that all of the peaks, with the exception of the breakthrough peak, contained relatively simple mixtures since the number of spots averaged not more than four per chromatogram. Of the four spots, two of them (nonradioactive) were consistently noted in every peptide map.

The major TPCK peptide fraction, which contained 35% of the total radioactivity recovered in the eluate and corresponded to a ninhydrin-positive peak, was shown, upon peptide mapping, to give three ninhydrin-positive spots; two of them contained estimated relative radioactivities of 30 and 70%. Both radioactive spots traveled in the direction of the anode.

Final Purification and Analysis of Major TPCK Peptide
**Figure 5.** Location of the active center histidine of \( \alpha \)-chymotrypsin. A section of the internal disulfide region of the \( \beta \) chain containing both histidine residues is shown. The histidine residue alkylated by TPCK is *circled*. The sequences shown are those in which current structural studies are in agreement (5,17).

DEAE-Sephadex A-25 was found to be capable of resolving the major peptide into three seemingly pure components by means of a linear gradient as shown in Fig. 4. The distribution of radioactivity of the three peptides was 20, 60, and 20%, respectively. To determine the amino acid composition and the purity of these peptides, an aliquot from each was analyzed quantitatively for amino acids. The major Peptide DS-II had the composition shown in Table II. The presence of a histidine derivative was indicated by the stoichiometric amount of TPCK-\( ^{14} \)C in the peptide. The quantitative agreement of radioactivity and integral ratios of amino acid residues testified to the purity of this peptide, which was clearly a decapptide. A comparison of the results of amino acid analysis of Peptides DS-I, DS-II, and DS-III revealed that Peptides DS-II and DS-III had the same amino acid composition, whereas Peptide DS-I differed from them by having an additional valine residue.

The NH\(_2\)-terminal residue of the DS-II decapptide was determined by the dinitrofluorobenzene method; identification of the dinitrophenylamino acid was accomplished by means of thin layer chromatography on silica gel in the solvent system chloroform-benzyl alcohol-acetic acid (70:30:3) (16). Alanine was found to be the NH\(_2\)-terminal residue. For a standard, stannylprolylglycine was carried through the entire procedure. Dinitrophenylalanine was also used as a marker in the chromatographic step.

**Table II**

<table>
<thead>
<tr>
<th>Residue</th>
<th>Amount</th>
<th>No. of residues</th>
<th>Keil et al. (15), Peptide 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.100</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.190</td>
<td>1.80</td>
<td>1.88</td>
</tr>
<tr>
<td>Serine</td>
<td>0.106</td>
<td>1.06</td>
<td>0.91</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.112</td>
<td>1.12</td>
<td>1.10</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.105</td>
<td>1.05</td>
<td>1.00</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.091a</td>
<td>0.91</td>
<td>0.94</td>
</tr>
<tr>
<td>Valine</td>
<td>0.105</td>
<td>1.05</td>
<td>1.00</td>
</tr>
<tr>
<td>Histidine</td>
<td>0</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>(^{14} )C-Labeled inhibitor</td>
<td>0.105b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \) Corrected for 80% re-formation of cysteine from S-sulfo-cysteine during acid hydrolysis.

\( ^{b} \) Based on specific activity of TPCK-\( ^{14} \)C.

**DISCUSSION**

The isolation of a histidine-containing peptide from the active center of \( \alpha \)-chymotrypsin has been simplified in the present work by the preliminary separation of the \( S \)-sulfo derivative of the modified \( B \) chain. This eliminated more than two-fifths of the protein and resulted in the formation of a less complex mixture of peptides after proteolytic digestion. Unfortunately, however, the modified \( S \)-sulfo-\( B \) chain, like the \( B \) chain of \( S \)-sulfochymotrypsinogen itself (17), was largely unattacked by trypsin. Hydrolysis by pepsin did occur, however, and gave results that were fortunate in several respects. The cleavages were such that the radioactive peptides were of intermediate chain length and hence could be readily separated as a group from smaller fragments. A 7-fold purification was accomplished merely by gel filtration. It was also fortunate that the bulky inhibitor residue did not alter the sites of peptic cleavage. Hence a fragment was obtained which proved to be identical in composition with the one already isolated from the unmodified enzyme, thus facilitating identification of the histidine residue which had been labeled.

In the purification of the labeled peptides, all of which contained an \( S \) sulfo-cysteine residue, the sequential use of anionic and cationic exchange columns proved profitable, as has been pointed out by Schroeder et al. (18).

The cationic exchange chromatogram shown in Fig. 3 is indeed complex, but only one major, radioactive fraction was obtained. It contained 35% of the total radioactivity eluted from the column and was well resolved from adjacent peptides. Further purification of the peak on DEAE-Sephadex (Fig. 4) provided a radioactive decapptide, DS-II, the analysis of which indicated that a pure TPCK decapptide had been obtained.

The NH\(_2\)-terminal residue was found to be alanine. Among the products from a peptic digestion of chymotrypsinogen, Keil et al. (15) found a histidine-containing decapptide (Peptide 17, Table II). As can be seen from Table II, we have isolated a labeled peptide of exactly the same amino acid composition, except for the absence of free histidine, from TPCK-\( ^{14} \)C-inhibited chymotrypsin. The data strongly suggest that it is this histidine that has been labeled and hence is derived from the active site of the enzyme.

Recently, tentative primary structures for chymotrypsinogen have been proposed by Keil, Prusik, and Sorm (5) and by Hartley (17). Although these structures differ in a number of details, there is agreement on the existence of a loop in the \( B \) chain formed by an intrachain disulfide bond, which brings into juxtaposition the 2 histidine residues of the enzyme. This section of the molecule is shown in Fig. 5 with the sequences in which there is current agreement (5,17). The composition of the decapptide is shown with 2 threonines and 1 serine (rather than 2 serines and 1 threonine (5)) because of the results of this paper and the agreement with the earlier finding of Keil et al. (15) and of Hartley (17). The presence of NH\(_2\)-terminal...
alanine is in agreement with the supposition that the labeled peptide originated from this portion of the molecule. Consequently, the histidine residue at the active center of chymotrypsin that reacts with TPCK is the one nearer the COOH terminus of the B chain.

Although only the composition of the major radioactive peak has been discussed, other radioactive peaks were analyzed as well in order to determine whether or not the second histidine residue in chymotrypsin had been alkylated to a small extent. Thus, Fraction 70 from the Dowex 50 column (Fig. 3) was investigated with results that indicated that it too contained the same histidine residue (19). No evidence was found to suggest alkylation at the second histidine residue. However, the proximity of the two histidines may mean that both play a role in catalysis, as has already been suggested for chymotrypsin (6) as well as for trypsin (20).

**SUMMARY**

A procedure has been developed for the isolation of histidine-containing peptides from the active center of chymotrypsin. Chymotrypsin was inactivated with radioactive chloromethyl ketone derived from N-tosyl-L-phenylalanine (TPCK), and the disulfide bonds of the inhibited enzyme were cleaved by sulfitolysis. The modified S-sulfo-B chain was isolated chromatographically and found to contain most of the bound radioactivity. Analysis revealed loss of about 1 histidine residue as in the case of the inhibited enzyme itself. This chain was digested with pepsin, and the peptide mixture was purified successively on columns of Sephadex G-50, Dowex-50, and diethylaminoethyl-Sephadex. The major radioactive peptide thus obtained was shown to be a histidine-containing decapeptide, the amino acid composition and NH₂-terminal residue of which agreed uniquely with the region of one of the histidine residues in the primary sequence of the enzyme. This residue was thus identified as being directly involved in the catalytic process.

**REFERENCES**

The Identification of the Histidine Residue at the Active Center of Chymotrypsin
Eng Bee Ong, Elliott Shaw and Guenther Schoellmann


Access the most updated version of this article at http://www.jbc.org/content/240/2/694.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/240/2/694.citation.full.html#ref-list-1