Amino Acid Sequences Involving the Histidine Residues of Porcine Trypsin*

Richard A. Smith and Irvin E. Liener

From the Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55101

SUMMARY

Peptides containing the 4 histidine residues of porcine trypsin have been isolated and their sequences determined. Two of these peptides, isolated from the enzyme which had been oxidized with performic acid, were found to be connected by a disulfide bond in the unmodified enzyme. The sequence

Asn-Ser-Gly-Ser*-His-Phe-Cys-Gly-Gly-Ser-Leu
A1a-Ala-Ala-His-Cys-Tyr-Lys

is identical with that found in the region of the active center of bovine trypsin, which includes histidine-29 and histidine-46, except that a serine residue (asterisk) replaces a tyrosine residue in the bovine enzyme.

The sequence of one of the two other histidine-containing peptides bears some similarity to the sequence surrounding histidine-79 of bovine trypsin. The lack of structural homology in this region of the molecule between bovine trypsin and bovine chymotrypsin would suggest, however, that this histidine residue is of little functional importance. The 4th histidine residue of porcine trypsin (for which there is no equivalent in the bovine enzyme) appears to fill a deletion which had been postulated as being necessary in order to demonstrate the structural homology between bovine trypsin and bovine chymotrypsin.

Present evidence indicates that the most important chemical feature of the active site of such animal proteinases as trypsin, chymotrypsin, and elastase is the occurrence of a serine and a histidine residue placed in juxtaposition to one another by a folding of the molecule (see review by Dixon (1)). Recent determinations of the complete amino acid sequence of bovine trypsinogen (2, 3) and bovine chymotrypsinogen (4) have revealed a striking degree of homology in the region of the molecule comprising the active site of these two enzymes, thus suggesting that these two enzymes are most likely derived from a common evolutionary precursor (2, 5). If such a hypothetical ancestral gene did in fact exist, one would expect that the same enzyme from different species of animals (so-called "heteroenzymes" (6)) would exhibit an even higher degree of structural homology in order to account for the characteristic differences in specificity between trypsin and chymotrypsin. A comparison of the primary structures of such heteroenzymes could yield important clues as to the chemical nature of the sites responsible for this difference in specificity.

We have previously reported the isolation of a radioactive heptadecapeptide from porcine trypsin which had been inactivated with diisopropyl phosphofluoridate (7). This peptide contained a sequence of 13 amino acids which was identical with the sequence in the vicinity of the active serine residue of bovine trypsin. In an extension of these studies, we now wish to report the sequences surrounding the 4 histidine residues of porcine trypsin, and to compare these with the known sequences involving the 3 histidine residues of bovine trypsin and the 2 histidine residues of chymotrypsin.

EXPERIMENTAL PROCEDURE

Preparation of Enzyme Digests

Sequential Digestion of Oxidized Porcine Trypsin with Trypsin and Pepsin—Crystalline porcine trypsin, prepared by the method of Travis and Liener (8), was oxidized with performic acid in the manner described by Dixon, Kaufman, and Neurath (9). A 0.5% solution of oxidized trypsin in 0.001 M HCl was adjusted to pH 8 with triethylamine, and crystalline bovine trypsin (Worthington) was added to give an enzyme to substrate ratio of 1:40. A pH of 8 was maintained in an autotitrator (Radiometer) with 0.15 M triethylamine as the titrating agent. After 6 hours at 37°C, the digestion was terminated by lowering the pH to 3 with 1 N HCl. In order to concentrate the digest, the latter was lyophilized, and the pH of a 1% solution of this material was adjusted to 3. Upon standing at 0°C for 5 hours, a copious precipitate formed which was collected by centrifugation. This acid-insoluble fraction, representing 10 to 15% of the
original protein, was found to contain most of the histidine. A suspension of this insoluble fraction (5 mg per ml) was adjusted to pH 2, and crystalline pepsin (Worthington) was added to give an enzyme to substrate ratio of 1:30. This pH was maintained at 37° for 20 hours with the autotitrator through the intermittent addition of 0.1 N HCl. A small amount of insoluble material which remained was removed by centrifugation, and the supernatant solution was lyophilized. Peptides isolated from this digest will hereafter be referred to as "TP-peptides."

Sequential Digestion of Intact Porcine Trypsin—To a 0.5% solution of porcine trypsin in 5% formic acid was added 1.4% of its weight of crystalline pepsin. After standing at 37° for 20 hours, the digest was clarified by centrifugation and lyophilized. Peptides isolated from this digest will be designated as "P-peptides."

Isolation of Histidine Peptides

Preliminary fractionation of each digest was carried out on columns of Dowex 50-X2 (Aminex, 200 to 325 mesh, Bio-Rad) with the pyridine-acetic acid buffer systems described by Schroeder et al. (11). Up to 1 g of digest was chromatographed on columns measuring 2 × 150 cm; smaller loads, not exceeding 250 mg, were chromatographed on columns 100 or 150 cm long with a diameter of 0.9 cm. All columns were jacketed, and the temperature of the circulating water was maintained at 39°. With a diameter of 0.9 cm. All columns were jacketed, and the temperature of the circulating water was maintained at 39°. All aliquots (200 ul) from every second or third tube of the effluent stream was monitored in a continuous fashion with a Technicon AutoAnalyzer (16). Peptides which had been subjected to successive chromatography on Dowex 50 and Dowex 1 invariably proved to be homogeneous when examined by high voltage paper electrophoresis at pH 3.7 and 6.4. Stoichiometry of the amino acid composition was presumed to be a further indication of the homogeneity of peptides isolated in this manner.

Techniques Used for Sequence Analysis

**Amino Acid Analysis**

All amino acid analyses were performed at 30° on a Spinco model 120 amino acid analyzer by the procedure of Spackman, Stein, and Moore (17).

**NH2-terminal Sequence**

The technique of subtractive Edman degradation (18) was used for the stepwise analysis of amino acids from the amino-terminal end of peptides. In cases when the results of a degradation were in doubt, the phenylthiohydantoin derivative was subjected to alkaline hydrolysis (19) and the free amino acid was identified on the amino acid analyzer. Where greater sensitivity was desired, the Edman degradation was combined with the use of 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl reagent) for the identification of amino terminal residues (20).

**COOH-terminal Residues**

Peptides, dissolved in 0.1 M pyridine, pH 7.8, were treated with crystalline preparations of carboxypeptidase A or B (Worthington) at enzyme to substrate levels ranging from 1:20 to 1:100 at 37°. Aliquots of the digest were removed at various intervals of time for direct analysis of free amino acids on the analyzer.

**Partial Acid Hydrolysis**

Dilute acid hydrolysis was used to effect the preferential release of aspartic acid in certain peptides (21). The peptide was dissolved in 1 ml of 0.03 M HCl, and aliquots of this solution were transferred to a series of test tubes. The tubes were sealed under vacuum and kept at 110° for various periods up to 40 hours. The contents of each tube were analyzed directly on the analyzer for free amino acids. The possibility that small peptides might exhibit chromatographic behavior identical with that of free amino acids cannot be ruled out, however.

**Preparation and Digestion of TLCK-treated Trypsin**

Porcine trypsin (50 mg) was inactivated by treatment with TLCK (Cyclo Chemical, Lot F-1794) as described by Shaw, Maes-Guia, and Cohen (22). Oxidation and subsequent digestion with trypsin and pepsin were performed as already described for the preparation of the TP-peptides except that 0.1 M pyridine, pH 7.8, and 5% formic acid were used for digestion with trypsin and pepsin, respectively. A control digest of porcine trypsin which had not been treated with TLCK was prepared in the same way. Aliquots of each digest were examined by the peptide mapping technique of Katz et al. (12). Both ninhydrin and Pauly reagents were used in the visualization of the spots.

**RESULTS**

*Isolation and Sequence of Histidine Peptides from Tryptic-Peptic Digest of Oxidized Porcine Trypsin*

**Fig. 1 shows the distribution of peptides when a tryptic-peptic digest of oxidized porcine trypsin was chromatographed on Dowex 1-X2 (AG1-X2, 200 to 400 mesh, Bio-Rad) starting buffers having a pH of 8.3 or 9.3, followed by gradient elution with increasing concentrations of acetic acid (11) or formic acid (14). Peptides in individual tubes of the effluent were analyzed by reaction with ninhydrin (15), or, in some cases, the effluent stream was monitored in a continuous fashion with a Technicon AutoAnalyzer (16). Peptides which had been subjected to successive chromatography on Dowex 50 and Dowex 1 invariably proved to be homogeneous when examined by high voltage paper electrophoresis at pH 3.7 and 6.4. Stoichiometry of the amino acid composition was presumed to be a further indication of the homogeneity of peptides isolated in this manner.

**The abbreviations used are:**

TLCK, 1-1-chloro-3-tosylamido-7-amino-2-heptanone (tosyl lysylchloromethyl ketone); GLx, glutamyl or glutaminyl residue; Aex, asparyl or aspartamyl residue.
Peptide TP-la to four stages of Edman degradation are shown (Fig. 2C). Their composition and the results of subjecting the peptides to the following partial sequence: Asx-Ser-Gly-Ser-His (Ser, Gly, Leu, Phe, CySOJ-Gly-Gly-Leu. The composition of Peptide TP-1b, a hexapeptide, is consistent with the NH2-terminal sequence of Peptide TP-1, from which it must have been derived through chymotryptic cleavage of the Phe-CySOJ-H bond. Peptide TP-2: Ile-Ile-Thr-His-Pro-Asx-Phe—The values shown in Table I for the composition of this peptide were obtained after 72 hours of hydrolysis. The usual 22 hours of hydrolysis gave the same values except that only 1 isoleucine residue was released under these conditions. Six stages of the Edman degradation established the sequence Ile-Ile-Thr-His-Pro-Asx-Phe. A small portion of the dipeptide that remained at the end of the fifth stage of the degradation migrated as a neutral peptide when subjected to electrophoresis at pH 3.7, indicating that asparagine rather than aspartic acid was a component of this peptide. When 0.25 μmole of Peptide TP-2 was digested with carboxypeptidase A (enzyme to substrate ratio, 1:40), only phenylalanine (0.25 μmole) was released after 24 hours. Peptide TP-3: Ala-Ala-His-CySOJ-H-Tyr-Lys—This peptide proved to be particularly resistant to Edman degradation beyond the first stage, which indicated alanine to be NH2-terminal. When 0.23 μmole of this peptide was treated with carboxypeptidase B (enzyme to substrate ratio, 1:40) for 14 hours, 0.23 μmole of lysine was released. Subsequent treatment of this peptide...
TABLE I

Data pertaining to composition and sequence of TP-peptides produced by sequential digestion of oxidized porcine trypsin by trypsin and pepsin.

Each column shows the composition of each peptide before and after successive stages of Edman degradation. Values shown are based on the molar ratios of amino acids obtained after 22 hours of hydrolysis with the exception of Peptide TP-2, for which a period of 72 hours was used to effect the complete hydrolysis of isoleucine. Residues to the nearest whole integer are shown in parentheses. Values in boldface type are considered to represent a significant loss from the previous stage of degradation.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide TP 1</th>
<th>Peptide TP-1a</th>
<th>Peptide TP-2</th>
<th>Peptide TP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 0</td>
<td>Stage 1</td>
<td>Stage 2</td>
<td>Stage 3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.08(1)</td>
<td>0.27</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>2.03(3)</td>
<td>3.00</td>
<td>2.24</td>
<td>2.29</td>
</tr>
<tr>
<td>Proline</td>
<td>2.77(3)</td>
<td>3.00</td>
<td>2.12</td>
<td>2.38</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.98(1)</td>
<td>1.00</td>
<td>0.96</td>
<td>0.98</td>
</tr>
<tr>
<td>Cyteic acid</td>
<td>1.07(1)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.02</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.06(1)</td>
<td>0.85</td>
<td>0.96</td>
<td>1.17</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.08(1)</td>
<td>1.20</td>
<td>0.84</td>
<td>0.97</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.00(1)</td>
<td>0.93</td>
<td>1.00</td>
<td>0.98</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.12(1)</td>
<td>0.96</td>
<td>1.05</td>
<td>0.97</td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Chromatography on Dowex 50 and electrophoretic examination of peptides from unmodified porcine trypsin digested with pepsin, trypsin, and chymotrypsin. Load, 1 g of digest; column size, 2 X 150 cm; flow rate, 80 ml per hour; 10 ml per tube. See the legend to Fig. 1 for composition of buffers used for elution and other explanatory notes. The gradients that were used here, however, were linear (24) and were produced with two 800-ml buffer vessels.

digest with carboxypeptidase A (enzyme to substrate ratio, 1:40) effected the release of 0.22 pmole of tyrosine in 18 hours. The COOH-terminal sequence of Peptide TP-3 is thus Tyr-Lys. The peptide that remained after treatment with the carboxypeptidases was separated from the free amino acids by passage through a column (1.6 X 45 cm) of Bio-Gel P2 (Bio-Rad) with the use of 0.1 M pyridine-acetic acid buffer, pH 6.5, for elution. This peptide had the composition His, 0.86; Ala, 2.00; CySOH, 0.97. Unlike the original peptide, this partially degraded derivative could not be carried through two stages of the Edman procedure, and the following data were obtained: Stage 1: His, 0.73; Ala, 1.19; CySOH, 1.00. Stage 2: His, 0.73; Ala, 0.62; CySOH, 1.00. These results permit the assignment of 2 alanine residues at the NH2-terminal end of Peptide TP-3. The peptide remaining after two stages of Edman degradation was treated with the dansyl chloride and hydrolyzed with acid (20). The dansyl derivative thus produced was identified as histidine by chromatography on silica gel with solvent A of Seiler and Wiechmann (23). This evidence placed histidine after the 2 alanine residues and thus established the complete sequence of Peptide TP-3 as shown above.

Isolation and Sequence of Histidine Peptides from Peptic-Tryptic-Chymotryptic Digest of Porcine Trypsin

In order to elucidate more fully the sequence of those histidine peptides located in the proximity of disulfide bonds, unmodified porcine trypsin was subjected to the combined action of pepsin, trypsin, and chymotrypsin. When this digest was chromatographed on Dowex 50, five Pauly-positive peptides were detected in the effluent (Fig. 3). Only one of these, however, Peptide PTC-5, was found to contain a cystine residue based on the formation of cysteic acid after oxidation. This peptide was further purified by chromatography on Dowex 1 as shown in Fig. 4.

Fig. 4. Chromatography of Peptide PTC-5 on Dowex 1. Column size, 0.9 X 150 cm; flow rate, 40 ml per hour; 4 ml per tube. Elution was performed with a concave gradient (24) produced by passing 250 ml of 0.5 N acetic acid into 500 ml of N-ethylmorpholine buffer, pH 9.3. The effluent was monitored with an Auto-Analyzer (16). Only the curve obtained after alkaline hydrolysis of the peptide is shown.
Peptide PTC-5: Cys-Gly-Gly-Ser-Leu Ala-Ala-His-Cys——From the composition of this peptide (His, 0.80; Ser, 0.83; Gly, 2.00; Ala, 2.04; Cys, 1.50; Leu, 0.81) it may be concluded that it contains a portion of the sequences found in both peptides TP-1 and TP-3. After oxidation with performic acid (25), two new peptides, PTC-5a and PTC-5b, were produced, which could be separated by high voltage paper electrophoresis at pH 3.7. These were eluted from paper and subjected to amino acid analysis and sequence studies.

The composition of Peptide PTC-5a (Ser, 1.05; Gly, 2.10; Leu, 0.83; CysSOH, 0.86) was identical with that of Peptide TP-1a, which, it will be recalled, had the sequence CysSOH—Gly—Gly—Ser—Leu. Its identity with Peptide TP-1a was verified by the fact that a cysteic acid residue was lost after one Edman degradation and leucine was released after digestion with carboxypeptidase A.

Peptide PTC-5b proved to be a tetrapeptide (His, 0.88; Ala, 2.00; CysSOH, 0.97), which lost 1 alanine residue after one stage of Edman degradation. This peptide must, therefore, represent the first four amino acids of Peptide TP-3, Ala-Ala-His-CysSOH.

The evidence which leads to the reconstruction of a sequence in which Peptides TP-1 and TP-3 are linked by a disulfide bridge is summarized in Fig. 5.

**Isolation and Sequence of Histidine Peptide from Pepit Digest of Porcine Trypsin**

A peptide which accounted for the fourth histidine residue of porcine trypsin was isolated from a peptic digest of the unmodified enzyme. Of the five Pauly-positive peptides detected in the effluent from Dowex 50 (Fig. 6), only one of these, Peptide P-1, differed in composition from the other histidine peptides which had already been isolated. When the fractions containing this peptide were pooled and chromatographed on Dowex 1, however, two histidine peptides were obtained, denoted as P-1a and P-1b in Fig. 7. The composition of these peptides before and after successive stages of the Edman degradation is recorded in Table II.

**Fig. 5.** Amino acid sequence of two histidine peptides of porcine trypsin (TP-1 and TP-3) which are connected by a disulfide bridge. —, NH<sub>2</sub>-terminal analysis by Edman degradation; —, action of carboxypeptidase A or B.

**Fig. 6.** Chromatography on Dowex 50 and electrophoretic examination of peptides from unmodified porcine trypsin digested with pepsin. Conditions of chromatography were the same as those for Fig. 3 except that linear gradients were produced with 500-ml buffer vessels.

**Table II**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide P-1a</th>
<th>Peptide P-1b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 0</td>
<td>Stage 1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.94(2)</td>
<td>1.95</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.92(1)</td>
<td>1.00</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.00(1)</td>
<td>0.62</td>
</tr>
<tr>
<td>Valine</td>
<td>0.90(1)</td>
<td>1.03</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.12(1)</td>
<td>1.00</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.88(1)</td>
<td>0.92</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.03(1)</td>
<td>1.05</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>

* Value not obtained because of malfunction of analyzer.

Peptide P-1a: Gly-Glx-His-Asx-Asx—Ile—The first two stages of the Edman degradation showed Gly-Glx to be the aminoterminal amino acid of this peptide. Degradation of this peptide beyond the second step yielded results which were difficult to interpret and hence are not shown here. Digestion with carboxypeptidase A (enzyme to substrate ratio, 1:20) for 24 hours released 20% of the isoleucine but no other amino acids. Thus, the following partial sequence could be postulated at this point: Gly-Glx (His, Asx) Ile.

Because of the presence of aspartic acid (or asparagine) residues in Peptide P-1a, dilute acid hydrolysis was used to effect the preferential cleavage of bonds involving this amino acid (21). The rates at which the various amino acids were released from this peptide when hydrolyzed with 0.03 N HCl at 110° are shown in Fig. 8.

In order to interpret these data, consideration was given to the fact that aspartic acid is preferentially liberated from a peptide by cleavage on both sides of aspartic acid residues (21). It thus becomes possible to deduce, from the relative rates at which amino acids are released, which amino acids are located between 2 aspartic acid residues, or are NH<sub>2</sub>-terminal or COOH-terminal amino acids followed or preceded by aspartic acid,
Amino Acid Sequences Involving Histidines of Porcine Trypsin
Vol. 242, No. 18

Identification of Histidine Residue of Porcine Trypsin That Reacts with TLCK

Porcine trypsin which had been inactivated with TLCK was oxidized and digested with trypsin and pepsin. In Fig. 9 a peptide map of this digest is compared with that of porcine trypsin prepared under identical conditions but not treated with TLCK. In the latter case three of the Pauly-positive spots were identified as Peptides TP-1, -2, and -3 by comparison with authentic samples of those peptides treated under the same conditions. Peptide TP-3 was absent, however, in the digest of the TLCK-treated trypsin. Since histidine residues in which the nitrogens of the imidazole ring have been substituted do not react with reagents of the Pauly type (29, 30), it may be concluded that TLCK must have reacted with the histidine residue of peptide TP-3.

DISCUSSION

The inactivation of bovine trypsin by TLCK (22, 26) and bovine chymotrypsin by L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (31) has been shown to involve the alkylation of histidine 16 of trypsin and histidine 57 of chymotrypsin. Both of these histidine residues are located in a homologous sequence of amino acids which is characterized by a second histidine residue, the 2 histidines being brought together by a disulfide bridge.

Fig. 8 shows that aspartic acid and isoleucine were released most rapidly and at approximately the same rate, whereas histidine, glycine, and glutamic acid were liberated at appreciably slower rates. These experimental observations are in accord with Sequence a.

Peptide P-1b: Gly-Glx-His-Asx-Asx-Ile Val-Leu—The composition of this peptide would suggest its structure to be identical with that of Peptide P-1a elongated by the two amino acids valine and leucine. This conclusion was supported by the fact that three stages of Edman degradation showed Gly-Glx-His to be the amino-terminal sequence of Peptide P-1b. Treatment with carboxypeptidase A (enzyme to substrate ratio, 1:15) for 21 hours effected the quantitative release of leucine and 20% of the valine, hence establishing Val-Leu as the COOH-terminal sequence of Peptide P-1b.

Edman degradation of the peptides TP-1, TP-2, and TP-3 are in accord with the postulated sequences. The expected rates of release were determined from the positions of the Pauly-positive spots in the peptide maps (30). The results of Edman degradation of peptide TP-3 are shown in Table 1. The expected rates of release of aspartic acid, isoleucine, histidine, and glutamic acid were confirmed by the presence of the corresponding spots at the expected times of appearance.

**Table 1**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Expected Rates of Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-1</td>
<td>Asp, Ile &gt; Gly, His, Glu</td>
</tr>
<tr>
<td>TP-2</td>
<td>Asp &gt; Ile, His &gt; Gly, Glu</td>
</tr>
<tr>
<td>TP-3</td>
<td>Asp &gt; Ile, His, Gly, Glu</td>
</tr>
</tbody>
</table>

Fig. 10. Comparison of relevant homologous sequences of bovine trypsin, porcine trypsin, and bovine chymotrypsin. The sequences of bovine trypsin and bovine chymotrypsin have been aligned and numbered in accordance with Walsh and Neurath (2) and Hartley et al. (5). Identical residues have been underlined.
It is evident from Fig. 10A that the sequence around the TLCK-reactive histidine residue of porcine trypsin is identical with that of bovine trypsin with the exception that tyrosine-28 of the bovine enzyme is replaced by serine in the porcine enzyme. A single base mutation in the triplet codon could account for the interchange of tyrosine and serine. As far as structural considerations are concerned, the substitution of serine for tyrosine may be classified as a “favorable” interchange (32) and hence should not lead to any gross distortion of the tertiary structure of the enzyme in this region of the molecule.

Based on studies on the reaction of cyanide fluoride with the tyrosine residues of bovine trypsin, Hachimori et al. (33) suggest that tyrosine-28 determines the specificity of trypsin by virtue of its ability to form a hydrogen bond with the basic side chain of lysine or arginine. In chymotrypsin tyrosine-28 is replaced by phenylalanine-39 (Fig. 10A), which these authors believe may be the site of hydrophobic interaction with the phenylalanine or tyrosine side chain which characterizes the substrates preferentially cleaved by chymotrypsin. Since tyrosine-28 of bovine trypsin is replaced by a serine residue in porcine trypsin, it is obvious that tyrosine per se cannot be responsible for the specificity of trypsin. In this connection, the recent report by Geratz (34) that p-aminophenylpyruvic acid is a more potent inhibitor of trypsin than p-aminobenzamidine may be cited. It was suggested that this increase in inhibitory strength might be attributed to a hydrogen bonding between the keto group of the pyruvic moiety and the hydroxyl group of either a tyrosine or a serine residue. The latter would presumably be located near an anionic site to which substrates of trypsin bind electrostatically through their positive charge (35). Our results are therefore consistent with the possibility that a tyrosine or a serine residue may be an important component of the specificity site of trypsin.

When the structure of Peptide TP-2 of porcine trypsin is compared with the sequence surrounding the third histidine residue of bovine trypsin, only a limited degree of structural similarity may be noted (Fig. 10B). Of the seven amino acids found in Peptide TP-2, three of these—iso-leucine, histidine, and proline—may be aligned with identical amino acids in bovine trypsin; the tyrosine-phenylalanine interchange at position 82 may be the outcome of a single base change. Two of the three remaining amino acids represent unfavorable interchanges (polar-hydrophobic: serine-iso-leucine and threonine-valine). This stretch of 20 amino acids which bears very little structural homology to bovine trypsin (5). The lack of homology between trypsin and chymotrypsin in this region of the molecule is particularly evident in the limited sequence shown in Fig. 10B. There is very little reason to believe, therefore, that this particular histidine residue of porcine and bovine trypsin plays any significant role in the catalytic property of these enzymes.

Since bovine trypsin contains only 3 histidine residues, a direct comparison with the sequence of the fourth histidine peptide of porcine trypsin (P-1b) is not possible. There is, however, a rather marked similarity between the sequence of Peptide P-1b and the sequence of residues 57 through 63 of bovine trypsin if one assumes histidine to fill a gap which Hartley et al. (5) have shown between glutamic acid-58 and aspartic acid-59 (Fig. 10C). Although no assignment of amide groups has been made for Peptide P-1b, it may be assumed that these groups are most likely located in the same position as in the bovine trypsin sequence. This would mean that five amino acid residues are identical and, furthermore, that both the valine-asparagine and leucine-valine interchanges are possible through a change of one nucleotide base in each case. Residues 69 through 76 of bovine chymotrypsin occur at the same point in the linear sequence of the enzyme as residues 57 through 63 of bovine trypsin. It will be noted that in this sequence a phenylalanine residue in position 71 coincides with the position assigned to the histidine residue in the sequence for porcine trypsin. Present evidence does not permit any definite conclusion regarding the significance of this homology in this region of the molecule.

REFERENCES
