The Chemical Structure of Tryptophanase from Escherichia coli

I. ISOLATION AND STRUCTURE OF A PYRIDOXYL DECAPEPTIDE FROM BOROHYDRIDE-REDUCED HOLOTRYPTOPHANASE*

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SUMMARY

Following reduction with NaBH₄, carboxymethylation, and chymotryptic digestion, a decapeptide containing the N⁴-
pyridoxyllysine residue was isolated from the tryptophanase of Escherichia coli by the consecutive use of Dowex 1 column chromatography, paper chromatography, and high voltage paper electrophoresis. The primary structure of this peptide was determined to be

\[
\text{Ser-Ala-Lys-Lys-Asp-Ala-Met-Val-Pro-Met} \quad \text{Pxy}
\]

Its structure differs from those found for the corresponding peptides from other enzymes so far studied.

EXPERIMENTAL PROCEDURE

Materials

Apo- and holotryptophanase were prepared by the procedures previously described (1, 8). Guanidine hydrochloride was recrystallized twice from methanol. Iodoacetic acid was recrystallized twice from hot chloroform. Pyridine and phenylisothiocyanate (Eastman) were redistilled. Other chemicals used in peptide purification were reagent grade; for the Edman degradation procedures, spectrophotometric grade chemicals were used without further purification.

1-Chloro-3-tosylamido-7-amino-2-heptanone-treated chymotrypsin was the gift of Dr. F. H. Carpenter. Diisopropyl fluorophosphate-treated carboxypeptidase A, leucine aminopeptidase, and trypsin were purchased from Worthington. Thermolysin was a gift from Dr. H. Matsubara. \(\epsilon\)-N-Pyridoxyllysine was synthesized according to the method of Dempsey and Snell (4) by Dr. W. Dowhan of this laboratory.

Methods

Preparation of Reduced Carboxymethylated Tryptophanase—Holotryptophanase was reduced under conditions described previously (2) with sodium borohydride to convert the linkage between pyridoxal-5'-P and apoenzyme to an acid-stable secondary amine; the reduced protein was then carboxymethylated with iodoacetic acid in the presence of 6 M guanidine hydrochloride and 0.05 M dithiothreitol according to the previous procedures (2).

Chymotryptic Digestion—About 300 mg of reduced carboxymethylated holotryptophanase was suspended in 15 ml of 1% NH₄HCO₃ solution and 3 mg of 1-chloro-3-tosylamido-7-amino-2-heptanone-chymotrypsin was added. The digestion was carried out at 25° for 15 hours with a second addition of 3 mg of 1-chloro-3-tosylamido-7-amino-2-heptanone-chymotrypsin after 3 hours. The digest was lyophilized.

Dowex Chromatography of Peptide Digests—The lyophilized chymotryptic digest was dissolved in 8 ml of Buffer A (2% pyridine-0.01% acetic acid (\(\rho\)/\(\rho\), pH 7.3) and chromatographed at 25° on a column (9.4 × 110 cm) prepared from Dowex AG1-X2 (200 to 400 mesh, acetate form) previously equilibrated with Buffer A. The column was eluted at 25° and a flow rate of 20 ml per hour at decreasing pH as follows (5): (a) 700 ml of Buffer A, (b) a linear gradient between 1000 ml of Buffer A and 1000 ml of 2% pyridine-1% acetic acid, and (c) a linear gradient between 500 ml of 1% acetic acid and 500 ml of 5% acetic acid.

Fractions (7 ml) were collected and monitored for ninhydrin color values (6) without alkaline hydrolysis, and for their absorbance at 325 μm. The latter measurement detects those
fractons containing the covalently bound pyridoxyl group. Pyridoxyl peptides are also readily located on paper chromatograms by their strong fluorescence in ultraviolet light.

**Amino Acid Analysis**—After hydrolysis of samples with 6 N HCl for 20 hours at 105°, amino acid analyses were performed by the method of Spackman, Stein, and Moore (7) with an automatic amino acid analyzer (Beckman model 120C).

**Determination of Amino Acid Sequence of Peptide**—The following procedures were employed for the determination of amino acid sequence of the peptide.

(a) Digestions with carboxypeptidase A were carried out as described by Freidank-Conrat, Harris, and Levy (8). Lecine aminopeptidase was used under the conditions described by Hill et al. (9). The liberated amino acids were determined with the amino acid analyzer.

(b) For digestion with thermolysin, 0.3 μmole of peptide was treated with 0.07 mg of thermolysin for 2 hours in 0.01 M Tris-Cl buffer, pH 8.0, in the presence of 0.002 M CaCl₂ at 40° (10). The small fragments obtained were separated by paper chromatography.

(c) For tryptic digestion, 0.07 μmole of the peptide was treated with 0.1 mg of trypsin in 0.005 M Tris-Cl buffer, pH 8.0, at 40° for 15 hours. The products were separated by high voltage paper electrophoresis.

(d) For tritium labeling of the COOH-terminal amino acid, peptide (0.05 μmole) was dissolved in 0.2 ml of T₂O (100 mCi), 0.4 ml of pyridine, and 0.1 ml of acetic anhydride and kept at room temperature overnight (11). After removal of T₂O by evaporation in vacuum, the residue was hydrolyzed with 6 N HCl at 105° for 20 hours. The hydrolysate was chromatographed in duplicate on Whatman No. 3MM paper, one sheet being exposed to performic acid vapor (12) before development.

(e) Edman degradation was carried out as described by Light and Greenberg (14) or Konigsberg and Hill (15). The liberated phenylthiohydantoin derivatives were identified by comparison with authentic standards after paper chromatography with the solvent system of Sjöquist (16) and Edman and Sjöquist (17). In some cases, thin layer chromatography on silica gel (Eastman chromatogram sheets No. 6060) with chloroform as solvent was used (18). Phenylthiohydantoin-amino acids were located by the iodonitroprussid method (16). Subtractive analysis for amino acids was also carried out to confirm the identity of the terminal residue removed at each degradation step (19, 20).

**RESULTS**

**Isolation of Pxy-peptide from Chymotryptic Digests**

The elution pattern obtained upon chromatography of the chymotryptic digest over Dowex AG1-X2 (Fig. 1) showed most of the Pxy-peptides (absorbing at 325 μm) in Fractions 280 to 315; small amounts were also present in Fractions 465 to 470. The former fractions were lyophilized, then applied as a band on Whatman No. 3MM paper and developed with Solvent A (1-butanol-acetic acid-pyridine-water, 15:3:10:12 (21)) for 15 to 20 hours at 25°. Two fluorescent bands were detected on the paper chromatogram (Fig. 2A). They were cut out and stitched separately to another paper which was subjected to paper electrophoresis at pH 6.3 (22), and 2000 volts for 1 hour. Both fluorescent bands showed identical mobility not only at pH 6.3 (Fig. 2B) but also after elution and subsequent electrophoresis at pH 3.7 (23) and pH 1.9 (24). Only one ninhydrin-positive spot was present on the latter electrophoretograms. Such results are common in peptides containing methionine, where partial oxidation to the sulfoxide occurs during paper chromatography without change in electrophoretic mobility. From these results and the identical amino acid analysis (next section), we concluded that the two peptides were identical except for the oxidation state of methionine. Methionine sulfoxide is known to reduce to methionine during HCl hydrolysis. The absorption spectrum of the purified fluorescent peptide (Fig. 3) shows the expected absorption maximum at 325 μm of the pyridoxylamine residue; absorbance at 253 μm results principally from contaminating pyridine. Recovery of this peptide was calculated from the absorbance at 253 μm to be about 15% of that present in the initial chymotryptic digest.

**Amino Acid Composition of Purified Pxy-peptides**

The amino acid composition of two independently prepared samples of the purified fluorescent peptide are shown in Columns 1-7. Chemical Structure of Tryptophanase from E. coli. I

FIG. 1. Chromatography of the chymotryptic digest of NaBH₄-reduced and carboxymethylated holotryptophanase on Dowex AG1-X2 acetate (see "Methods" for details). ---, fractions exhibiting absorption at 325 μm; --, the absorbance at 570 μm following reaction of 0.3 ml of the effluent with ninhydrin without exhibiting absorption at 325 μm; F, the absorbance at 570 μm.

FIG. 2. A, separation of fluorescent (hatched) from nonfluorescent peptides by paper chromatography. Both types of peptides were ninhydrin-reactive. Conditions are described in the text. B, high voltage paper electrophoresis of the two fluorescent bands from A (see text for details).
Both of the fluorescent bands obtained from paper chromatograms showed this same amino acid composition. For identification of the ε-N-Pxy-lysine residues, the following methods were employed.

**Amino Acid Analysis**—In the usual analysis (9-cm column, pH 5.28) Pxy-lysine was eluted at almost the same position as histidine. To distinguish them, the ratio of the signal height at 570 mμ to that at 440 mμ at the position corresponding to histidine was calculated. For histidine this value is 4.3 to 4.5; for synthetic ε-N-Pxy-lysine it is 3.6 to 3.8. Both acid hydrolysates and leucine aminopeptidase digests of this peptide gave the value 3.6 to 3.9.

**High Voltage Electrophoresis**—On high voltage paper electrophoresis at pH 3.7 and 6.5 an acid hydrolysate of the fluorescent peptide (Fig. 4) shows ninhydrin-reactive zones that migrate identically with standards of lysine, neutral amino acids, aspartic acid, ε-N-Pxy-lysine. The latter spot was fluorescent, showed a blue color with 2,6-dichloroquinone chloride, a yellow color with Pauly reagent, and on elution from paper absorbed at 325 mμ. None of the zones migrated as histidine or gave the color of histidine with the Pauly reagent. These data demonstrate the presence of both lysine and ε-N-Pxy-lysine and the absence of histidine in hydrolysates of this peptide.

**End Group Analysis**

Digestion with leucine aminopeptidase (5 hours, 40°) liberated serine (0.3 residue) and alanine (0.2 residue) indicating that the NH₂-terminal sequence was Ser-Ala-. The presence of serine as the NH₂-terminal amino acid was shown more conclusively by use of the Edman degradation (Table I).

Carboxypeptidase A (5 hours, 40°) released methionine (0.5 residue); COOH-terminal methionine was confirmed by the selective tritium labeling method, which showed that methionine was the only amino acid which showed substantial incorporation of tritium (Fig. 5).

![Fig. 3. Spectrum of the purified peptide containing the ε-N-Pxy-lysine residue. About 0.04 μmole of peptide was dissolved in 1 ml of 0.05 M potassium phosphate buffer, pH 7.0. High absorbance values at 253 mμ are caused by contaminating pyridine.](image)

![Fig. 4. Comparative migratory characteristics of (1) synthetic ε-N-Pxy-lysine, (2) an acid hydrolysate of the purified Pxy-peptide (0.06 μmole), and (3) a mixture of neutral (alanine (Ala), methionine (Met)), acidic (glutamic acid (Glu), aspartic acid (Asp)), and basic (lysine (Lys), histidine (His), arginine (Arg)) amino acids following electrophoresis for 60 min at 2000 volts and pH 3.7 (A) or pH 6.5 (B). Hatched zones are fluorescent; dotted zones are positive to Pauly reagent; all zones are ninhydrin-reactive (see text for other details).](image)

| Amino acid composition of fluorescent decapeptide and results of its degradation by Edman procedure |

Italicized values in parentheses are idealized from the experimental values which immediately precede them. Experimental values which decrease significantly during a given reaction cycle are italicized.

| Amino acid | Sample 1 | Sample 2, degradative cycle |
|------------|----------|-----------------------------|
| | 0 | 1st | 2nd | 3rd | 4th | 5th |
| Serine | 1.05 (1) | 0.90 (1) | 0.80 | 0.14 | + | + | + |
| Alanine | 2.11 (2) | 2.00 (2) | 2.08 (2) | 1.48 (1) | 1.28 (1) | 1.35 (1) | 1.18 (1) |
| Lysine | 1.11 (1) | 1.06 (1) | 0.61 (1) | 0.95 (1)a | 0.81 | 0.23 |
| ε-N-Pxy-lysine | 0.88 (1) | 0.73 (1) | 0.81 (1) | 1.26 (1)a | 0.87 (1) | 0.40 |
| Aspartic acid | 1.98 (1) | 1.05 (1) | 1.09 (1) | 1.49 (1) | 1.15 (1) | 1.05 (1) | 0.61 |
| Methionine | 1.89 (2) | 1.62 (2) | 2.00 (2) | 1.70 (2) | 1.88 (2) | 1.70 (2) | 1.63 (2) |
| Valine | 1.00 (1) | 0.95 (1) | 1.06 (1) | 1.05 (1) | 1.05 (1) | 1.02 (1) | 0.97 (1) |
| Proline | 1.08 (1) | 0.96 (1) | 1.10 (1) | 0.96 (1) | 1.11 (1) | 1.04 (1) | 1.08 (1) |
| Phenylthiodyantoin-amino acid identified | | | | | | |

| Sample 2, degradative cycle |
|-----------------------------|
| 0 | 1st | 2nd | 3rd | 4th | 5th |
| Serine | 1.05 (1) | 0.90 (1) | 0.80 | 0.14 | + | + | + |
| Alanine | 2.11 (2) | 2.00 (2) | 2.08 (2) | 1.48 (1) | 1.28 (1) | 1.35 (1) | 1.18 (1) |
| Lysine | 1.11 (1) | 1.06 (1) | 0.61 (1) | 0.95 (1)a | 0.81 | 0.23 |
| ε-N-Pxy-lysine | 0.88 (1) | 0.73 (1) | 0.81 (1) | 1.26 (1)a | 0.87 (1) | 0.40 |
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| Valine | 1.00 (1) | 0.95 (1) | 1.06 (1) | 1.05 (1) | 1.05 (1) | 1.02 (1) | 0.97 (1) |
| Proline | 1.08 (1) | 0.96 (1) | 1.10 (1) | 0.96 (1) | 1.11 (1) | 1.04 (1) | 1.08 (1) |
| Phenylthiodyantoin-amino acid identified | | | | | | |

* These values are not too reliable because of irregular base-line of the analyzer.

**TABLE I**

*Not analyzed.*
**Edman Degradation of Fluorescent Peptide**

Table I shows the results of amino acid analysis of the peptide remaining after each of five sequential Edman degradations. Subtractive analysis following further degradation was ambiguous, but alanine (6th), methionine (7th), and valine (8th) were identified as their phenylthiohydantoin derivatives after the designated reaction cycle. These results indicated that the amino acid sequence of this peptide was

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Ser-Ala-Lys-Lys-Asp-Ala-Met-Val-Pro-Met
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The sequence of the last four amino acids was confirmed by the following experiments.

**Thermolysin Treatment of Fluorescent Peptide**

A peptide map of a small portion of the thermolysin digest showed two major ninhydrin-reactive products (Fig. 6) only one of which was fluorescent. The remainder of the digest was purified by paper chromatography and the amino acid composition of each peptide was determined. Peptide 1 (Th-I) contained methionine, 1.56 (2); valine, 1.00 (1); and proline, 1.17 (1). Peptide 2 (Th-II) contained lysine, 0.98 (1); Pxy-lysine, 0.86 (1); aspartic acid, 0.85 (1); serine, 0.71 (1); and alanine, 1.71 (2); and obviously corresponds to the first 6 amino acid residues of the decapptide. Carboxypeptidase A released alanine (0.4) and aspartic acid (0.2), confirming the expected COOH-terminal sequence of this peptide as -Asp-Ala. Digestion of Th-I with carboxypeptidase A released methionine (0.8); leucine aminopeptidase also released methionine (0.75), showing that both its NH₂- and COOH-terminal amino acids are methionine. Application of the Edman method released only phenylthiohydantoin-methionine in the first reaction cycle. Analysis of amino acids following the second cycle (valine, 0.86; proline, 1.08 (1); methionine, 0.92 (1)) showed that valine had been removed, while the third step (valine, 0.18; proline, 0.41; methionine, 1.00) removed proline and yielded free methionine in the unhydrolyzed solution. These data establish the sequence of Th-I as Met-Val-Pro-Met.

**Trypsin Digestion of Fluorescent Decapeptide**

To confirm the presence of the unusual linkage in the decapptide, it was subjected to tryptic digestion, and the digests were analyzed by paper electrophoresis at pH 3.7 and 2000 volts for 60 min (Fig. 7). Three ninhydrin-reactive
TABLE II
Comparative sequences of pyridoxyl peptides isolated from partial digests of NaBH₄-reduced pyridoxal-dependent enzymes

| Enzyme                                      | Sequence                          | Reference |
|---------------------------------------------|-----------------------------------|-----------|
| Aspartate aminotransferase                  | Ser-Lys-Asn-Phe                   | 29        |
| Extramitochondrial                          |                                   |           |
|                                              | Pxy                               |           |
| Mitochondrial                               | Ala-Lys-Asn-Met                   | 29        |
|                                              | Pxy                               |           |
| Glutamate decarboxylase                     | Ser-Ile-Ser-Ala-Gly-His-Lys-Phe    | 30        |
|                                              | Pxy                               |           |
| Phosphorylase                               | Met-Lys-Phe-Met                    | a         |
|                                              | Pxy                               |           |
| Pyridoxamine pyruvate transaminase           | Val-Thr-Gly-Pro-Asp-Lys-Cys-Leu    | c         |
|                                              | Pxy                               |           |
| Tryptophanase                               | Ser-Ala-Lys-Asp-Ala-Met-Val-Pro-Met| d         |
|                                              | Pxy                               |           |

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**DISCUSSION**

In all pyridoxal-dependent enzymes so far examined (e.g., phosphorylase (25), aspartate aminotransferase (26), glutamic decarboxylase (27), serine transhydroxymethylase (28), pyridoxal alanine aminotransferase (4), etc.) azomethine linkage is formed between coenzyme and the ε-amino group of a definite lysine residue on the apoenzyme which on reduction with sodium borohydride yields an amine bond which is stable to acid and enzymatic hydrolysis. Because of its fluorescence and absorption at 325 to 330 μm, detection of the pyridoxyl peptide in such digests is relatively simple, a circumstance that greatly facilitates its isolation. Such peptides have now been isolated from several reduced pyridoxal-5'-P proteins; their structures are compared in Table II. In every case the sequence of amino acids surrounding the ε-N-Pxy-lysine residue differs markedly. Tryptophanase is the only enzyme found so far to contain the sequence²

-Lys-Lys-

Pxy

In space-filling models of this decapeptide, the 5'-phosphate group of the pyridoxyl-5'-P residue is in a position which permits its ready interaction with the ε-amino group of the adjacent lysine residue, thus indicating that this is probably one of the points through which combination of coenzyme to apoenzyme occurs. It is also possible that this group might, under some circumstances, participate in formation of an aldamine structure (25, 32). Although indirect evidence has implicated a histidine residue as one of the catalytic groups of the active site of both tryptophanase (3) and aspartate aminotransferase (33), neither of the Pxy-peptides obtained from these enzymes contains histidine. Such a residue might, however, be far removed on the same polypeptide chain. To elucidate the relationship of structure to the mechanism of action of these enzymes will require characterization of much longer segments of the peptide chain, an operation

² A similar sequence, reported earlier for phosphorylase, was shown to be erroneous; ε-N-Pxy-lysine tends to break down in part to lysine during acid hydrolysis (31).
now under way in this laboratory, and probably a determination of its three-dimensional structure as well.

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