The Amino Acid Sequence of $\Delta^5$-3-Ketosteroid Isomerase of Pseudomonas testosteroni*

(Received for publication, June 28, 1971)

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SUMMARY

The primary structure of the identical subunits of crystalline $\Delta^5$-3-ketosteroid isomerase (EC 5.3.3.1) of Pseudomonas testosteroni has been determined by standard procedures to be the following: Met-Asn-Thr-Pro-Glu-His-Met-Thr-Ala-Val-Val-Gln-Arg-Tyr-Val-Ala-Ala-Leu-Asn-Ala-Gly-Asn-Leu-Asn-Gly-Ile-Val-Ala-Leu-Phe-Ala-Asp-Asn-Ala-Thr-Val-Glu-Asn-Pro-Val-Gly-Ser-Glu-Pro-Arg-Ser-Gly-Thr-Ala-Ala-Ile-Arg-Glu-Phe-Tyr-Ala-Asn-Ser-Leu-Lys-Leu-Pro-Leu-Ala-Val-Glu-Leu-Thr-Gln-Glu-Val-Arg-Ala-Val-Ala-Asn-Gln-Ala-Ala-Phe-Ala-Val-Ser-Ala-Leu-Phe-Glu-Asp-Ala-Thr-Val-Glu-Arg-Lys-Thr-Val-Ala-Val-AIe-Asp-His-Phe-Arg-Phe-Asn-Gly-Ala-Ala-Gly-Lys-Val-Ala-Val-Ser-Met-Arg-Ala-Leu-Phe-Glu-Asp-Ala-Thr-Val-Glu-Arg-Lys-Thr-Val-Ala-Val-AIe-Asp-His-Phe-Arg-Phe-Asn-Gly-Ala-Ala-Gly-Lys-Asn-Ile-His-Ala-Val-Glu-Ala. Thus, each subunit consists of a single polypeptide chain of 125 amino acid residues. The subunit molecular weight, calculated from the amino acid sequence, is 13,394 or 33% of the ultracentrifugally determined molecular weight of the intact isomerase.

This paper describes the complete amino acid sequence of the identical subunits of crystalline $\Delta^5$-3-ketosteroid isomerase (EC 5.3.3.1) of Pseudomonas testosteroni. This steroid-induced enzyme catalyzes the conversion of a number of $\Delta^5(3)$ and $\Delta^8(10)$-3-ketosteroids to the corresponding $\Delta^4$-3-ketosteroids, and its action is exemplified by the formation of $\Delta^8$-androstene-3,17-dione (Structure II) from $\Delta^5$-androstene-3,17-dione (Structure I).

The enzyme is of special interest from a mechanistic viewpoint (1). It has an extraordinarily high molecular turnover number of $17.6 \times 10^6$ min$^{-1}$ at 25°C and pH 7.0, with $\Delta^5$-androstene-3,17-dione as substrate, and is therefore probably the most active catalyst known. The isomerase promotes a direct intramolecular cis,cis, diaxial proton transfer from C-4 to C-6. Ultraviolet absorption and fluorescence spectroscopy, as well as isotope exchange experiments, favor the participation of an enolic intermediate in the enzymatic reaction (1-3).

* This study was supported by Grants AM 07422 and GM 1183 from the National Institutes of Health and by the Gustavus and Louise Pfeiffer Foundation of New York.

EXPERIMENTAL PROCEDURE

Crystalline $\Delta^5$-3-ketosteroid isomerase was isolated from P. testosteroni as previously described (6). Protein measurements are based on an $A_{280}$ of 0.413 per mg per ml (6). Three times crystallized $\alpha$-chymotrypsin was obtained from Worthington, as were twice crystallized elastase, papain, and pepsin, trypsinf treated with L-tosylamido-2-phenylethyl chloromethyl ketone, and carboxypeptidases A and B treated with diisopropylfluorophosphosphate. Three times crystallized thermolysin was supplied by Calbiochem. The analytical grade anion exchange resin, Dowex 1-X2, was a product of Bio-Rad Corporation, Richmond, California. Chromobeads A, a special form of Dowex 50, of fine and carefully graded particle size, was obtained from Technicon, Ardsley, New York. Hydrazine sulfate (J. T. Baker Chemical Company, Phillipsburg, New Jersey) and 95% hydrazine (Eastman) were used for hydrazinium reactions. 1-Dimethylaminonaphthalene-5-sulfonyl chloride was supplied by Pierce. Phenylisothiocyanate (Eastman) was distilled before use. Pyridine (Fisher, infrared spectrally analyzed grade) was distilled from ninhydrin.

I

II
Amino Acid Analyses—Samples were hydrolyzed in vacuo in glass-distilled constant boiling HCl at 105°, usually for 20 hours. Analyses were performed on the Spinco model 120C amino acid analyzer by an accelerated version of the methods of Spackman, Stein, and Moore (7). Norleucine standard (Technicon) was added to the amino acid calibration mixture (Beckman).

Paper Chromatography and Electrophoresis—Partition chromatography and high voltage electrophoresis on either Whatman No. 1 or 3MM filter paper were used for examination of the purity of peptides and for peptide purification. Paper chromatography was run either in butanol-pyridine-acetic acid-water, 75:50:15:60 (v/v), hereafter referred to as Solvent 1, or in butanol-acetic acid-water, 200:30:75 (v/v), hereafter referred to as Solvent 2. Electrophoresis was run at 2000 or 3000 volts on a Savant FP 30 water-cooled plate at 25-30°, in pyridine acetate buffers of either pH 4.75 or pH 6.5 (1.25% pyridine tiritated to the appropriate pH with glacial acetic acid).

In analytical paper chromatography and electrophoresis runs the peptides were located by staining the entire paper with ninhydrin. In preparative purification procedures, side bands were cut out and stained and the peptides were eluted from the unstained portion of the paper, except as indicated.

Chymotryptic Digestion of Isomerase—Crystalline isomerase (39.6 mg), having a specific activity of 54,000 units per mg, was dissolved in 0.05 M potassium phosphate buffer, pH 7.0, dialyzed against distilled water, heated with stirring at 100° for 10 min, and then cooled in an ice bath. To the resulting 11.7 ml of milky suspension were added 1.3 ml of 10% NH₄HCO₃. Initially and after 3 hours, 1.25% (by weight) portions of chymotrypsin (dissolved in 0.001 M HCl) were added. The digest was magnetically stirred and was maintained at 37° for 44 hours, with the addition of 5 ml of 1% NH₄HCO₃ at 3 hours. The digestion mixture was lyophilized repeatedly from H₂O to remove NH₄HCO₃.

Chromatographic Separation of Chymotryptic Peptides—The lyophilized chymotryptic digest was suspended in 0.3 M pyridine hydrochloride, pH 2.2, and centrifuged to remove insoluble material which was purified separately (see below). The supernatant fraction was subjected to chromatography in four portions on a column (0.9 x 29 cm) of the Beckman Spinco model 120C amino acid analyzer, which was packed to a height of 20 cm with Chromobeads A, maintained at 54.5°, and equilibrated with starting buffer. The column was developed at a flow rate of 90 ml per hour with 495 ml of pyridine-acetic acid buffer, pH 2.2, dialyzed against distilled water, heated with stirring at 100° for 10 min, and then cooled in an ice bath. To the resulting 11.7 ml of milky suspension were added 1.3 ml of 10% NH₄HCO₃. Initially and after 3 hours, 1.25% (by weight) portions of chymotrypsin (dissolved in 0.001 M HCl) were added. The digest was magnetically stirred and was maintained at 37° for 44 hours, with the addition of 5 ml of 1% NH₄HCO₃ at 3 hours. The digestion mixture was lyophilized repeatedly from H₂O to remove NH₄HCO₃.

Further Purification of Chymotryptic Peptides—Fractions corresponding to peaks on the recorder chart of the split stream column chromatography were examined for purity by partition chromatography on Whatman No. 3MM paper in Solvent 1. Those fractions which were found to contain multiple ninhydrin-positive components were purified by paper chromatography in the same solvent system. The 24 chymotryptic peptides that were isolated are designated as C-1 to C-24 according to the order of their occurrence in the final sequence.

The portion of the chymotryptic digest which was insoluble in 0.3 M pyridine hydrochloride at pH 2.2 contained undigested protein and one important chymotryptic peptide (C-6). This residue was dissolved in 1.5 ml of 50% acetic acid and dialyzed against two 12.5-ml portions of the same solvent for a total of 20 hours. The outer fluid from the dialysis was pooled and dried under vacuum. The residue was dissolved in pyridine-collidine-acetic acid buffer (160 ml of pyridine, 10 ml of collidine, and 1.1 ml of acetic acid per liter), pH 8.1, (8) and applied to a column (1.0 x 19 cm) of Dowex AG 1-X2 (100 to 200 mesh). The column was developed with 10 ml of each of the following solvents: pyridine-collidine-acetic acid buffer, pH 8.1; 0.2 M acetic acid; 0.4 M acetic acid; 1.0 M acetic acid; and, finally, 50% acetic acid. Fractions of 5 ml were collected, and an aliquot was taken from each fraction for hydrolysis and amino acid analysis.

Enzymic Hydrolysis of Peptides—Thermolysin digests were performed at 40° in 0.2 M ammonium acetate buffer, pH 8.2, with an enzyme to substrate ratio of 3 to 10% (by weight). Papain digests, in 0.2 M pyridine acetate buffer of pH 5.45 (0.01 M 2-mercaptoethanol), proceeded at 37° with 2 to 5% enzyme for 20 to 90 min. Hydrolysis with pepsin was carried out in 0.01 M HCl at 25° for 18 hours, with 5% enzyme. Trypsin and chymotryptic hydrolysis of peptides was performed in 0.02 M NH₄HCO₃ at 40°. A single chelate digest was run at 43° in 0.2 M ammonium acetate (pH 8.2) with 10% (by weight) chelate. All digest fractions were carried out with magnetic stirring, in tubes which were flushed with nitrogen and stoppered.

Sequence Studies—The subtractive method (9) of Edman degradation (10) was used most extensively in determination of amino-terminal residues and internal sequences of the peptides. Carboxyl-terminal residues were determined by hydrazinolysis (11) and by the use of carboxypeptidases A and B. Hydrolysis with carboxypeptidase B (2 to 10%) was performed in 0.02 M NH₄HCO₃ at 25° for 2 to 20 hours. Carboxypeptidase A digests were carried out in 0.2 M N-ethylmorpholine acetate, pH 7.6, at 4° for 4 to 24 hours, with enzyme to substrate ratios ranging from 3 to 10%. Carboxypeptidase digests were dried in a stream of nitrogen, dissolved in 0.01 M HCl, and applied directly to the columns of the amino acid analyzer. In some cases, the use of carboxypeptidase A was preceded by dansylation (12) of the peptide. In this procedure, residual peptides after carboxy-
peptidase digestion are not ninhydrin-positive and therefore do not interfere with the analysis of the released amino acids.

RESULTS AND DISCUSSION

Carboxyl terminal Residue of Isomerase

Hydrazinolysis of the intact protein yielded only alanine as the carboxyl-terminal residue. The yield was 200%, based on a molecular weight of 40,800 (4).

Composition of Chymotryptic Peptides

The elution pattern of the chymotryptic digest is shown in Fig. 1. All of the 24 chymotryptic peptides that were isolated required further purification, except C-4, C-19, C-20, C-22, and C-23. An additional peptide, C-6, was recovered in the 50% acetic acid eluate from the AG 1-X2 chromatography of the pH 2.2 precipitate of the chymotryptic digest. Table I contains the amino acid compositions of the chymotryptic peptides.

Isolation of New Tryptic Peptides T-6 and T-7

The tryptic digest and the purification of all but two of the tryptic peptides have been previously described (5). These peptides have been renumbered according to their sequence in the molecule, and will be designated by their new numbers in this paper. Table II gives both old and new designations. Two additional tryptic peptides (T-6 and T-7) have been isolated from precipitated or insoluble fractions obtained at the conclusion of the tryptic digest. The precipitate present at the end of the digest (5) was dissolved in 2 ml of 50% acetic acid and dialyzed against two 14-ml portions of the same solvent for a total of 24 hours. The 28 ml of outer fluid were concentrated in a stream of nitrogen at 25°. T-7 was then isolated by gel filtration on a column (0.9 x 10 cm) of Sephadex G-75 in 50% acetic acid, and detected by ninhydrin assay (13) after alkaline pH 2.2 precipitate of the chymotryptic digest. Table I contains the amino acid compositions of the chymotryptic peptides.

### Table I

**Amino acid composition of chymotryptic peptides of Δ^3-3-ketosteroid isomerase**

| Amino acid     | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 | C-8 | C-9 | C-10 | C-11 | C-12 |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|
| Aspartic acid  | 1.2 (1) |       |     |     |     | 2.8 (3) |     |     |     | 1.0 (1) |      |      |
| Threonine      | 0.8 (1) | 0.8 (1) | 0.6 (1) |     |     |     | 1.9 (2) |     |     |     |      |      |
| Serine         |     |     |     |     |     |     | 1.8 (2) |     |     |     |      |      |
| Glutamic acid  | 1.1 (1) | 1.1 (1) | 1.0 (1) |     |     |     | 2.2 (2) | 1.1 (1) | 1.1 (1) | 1.1 (1) |      |      |
| Proline        | 0.8 (1) |     |     |     |     |     | 1.8 (2) |     |     |     | 1.0 (1) | 1.2 (1) |
| Glycine        |     | 2.0 (2) |     |     |     |     | 2.0 (2) |     |     |     |      |      |
| Alanine        | 1.1 (1) | 1.1 (1) |     |     | 1.9 (2) | 2.0 (2) |     |     | 1.8 (2) | 2.0 (2) | 1.0 (1) | 1.1 (1) |
| Valine         |     | 2.0 (2) | 1.9 (2) |     | 1.1 (1) | 1.2 (1) | 2.3 (2) |     |     |     |      |      |
| Methionine     | 1.0 (1) |     |     |     |     |     |     |     |     |     |      |      |
| Isoleucine     |     |     |     |     |     |     |     |     |     |     | 0.9 (1) | 1.0 (1) |
| Leucine        | 1.0 (1) | 1.0 (1) |     |     |     |     |     |     |     |     |      | 1.0 (1) |
| Tyrosine       | 0.9 (1) |     |     |     |     |     |     |     |     |     |      | 1.1 (1) |
| Phenylalanine  |     |     |     |     |     |     |     |     |     |     | 1.1 (1) | 2.9 (3) |
| Lysine         |     |     |     |     |     |     |     |     |     |     | 0.9 (1) | 1.0 (2) |
| Histidine      | 0.7 (1) |       |     |     |     |     |     |     |     |     |      | 0.7 (1) |
| Arginine       | 1.0 (1) | 1.0 (1) |     |     |     |     |     |     |     |     |      | 1.0 (1) |
| Total residues | 6 | 7 | 5 | 2 | 4 | 12 | 16 | 6 | 7 | 4 | 8 | 4 |

| Amino acid     | C-13 | C-14 | C-15 | C-16 | C-17 | C-18 | C-19 | C-20 | C-21 | C-22 | C-23 | C-24 |
|----------------|------|------|------|------|------|------|------|------|------|------|------|------|
| Aspartic acid  | 1.1 (1) |     |     |     |     |     |     |     |     |     |     | 1.0 (1) |
| Threonine      | 0.8 (1) |     |     |     |     |     |     |     |     |     |      |      |
| Serine         | 0.7 (1) |     |     |     |     |     |     |     |     |     |      |      |
| Glutamic acid  | 1.1 (1) | 3.1 (3) | 1.0 (1) |     |     | 1.0 (1) | 1.0 (1) |     |     | 1.0 (1) | 1.0 (1) |      |
| Proline        |     |     |     |     |     | 1.0 (1) | 1.0 (1) |     |     | 1.0 (1) |      |      |
| Glycine        |     |     |     |     | 1.2 (1) |     |     |     |     | 2.3 (2) |     |      |
| Alanine        | 0.9 (1) | 3.9 (4) | 2.1 (2) | 1.0 (1) |     |     |     |     |     | 1.0 (1) | 1.0 (1) |      |
| Valine         | 1.2 (1) | 2.3 (2) |     |     | 1.2 (1) | 2.3 (2) |     |     | 1.7 (2) |     |      |      |
| Methionine     | 1.0 (1) |     |     |     |     |     |     |     |     |     |      | 0.9 (1) |
| Isoleucine     |     |     |     |     |     |     |     |     |     |     | 1.0 (1) | 1.0 (1) |
| Leucine        | 1.0 (1) |     |     |     |     |     |     |     |     |     |      | 1.0 (1) |
| Tyrosine       | 0.9 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) |     |     |     |     |     |     |     |      |
| Phenylalanine  |     |     |     |     | 0.7 (1) | 1.0 (1) |     |     | 1.1 (1) |     | 1.0 (1) | 1.0 (1) |
| Lysine         |     |     |     |     | 0.7 (1) | 1.0 (1) |     |     | 1.0 (1) | 0.9 (1) |      |      |
| Arginine       | 0.9 (1) | 0.9 (1) |     | 1.0 (1) |     |     |     |     |     |     |      | 1.0 (1) |
| Total residues | 4 | 13 | 4 | 2 | 4 | 2 | 13 | 2 | 9 | 3 | 4 | 9 |

* Values obtained after 72 hours of hydrolysis.
TABLE II

Amino acid composition of tryptic peptides of Δ^2-3-ketosteroid isomerase

| Amino Acid  | T-1 (13) | T-2 (12) | T-3 (11) | T-4 (10) | T-5 (9) | T-6 (8) | T-7 (7) | T-8 (6) | T-9 (5) | T-10 (4) | T-11 (3) | T-12 (2) | T-13 (1) | Total residues
|-------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|----------|
| Aspartic acid | 1.0 (1) | 0.5 (6) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 12
| Threonine    | 2.0 (2) | 1.0 (1) | 1.0 (1) | 0.9 (1) | 1.0 (1) | 0.9 (1) | 0.8 (1) | 1.0 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 7
| Serine       | 1.0 (1) | 0.8 (1) | 1.0 (1) | 1.0 (1) | 1.1 (1) | 0.9 (1) | 1.0 (1) | 0.9 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 5
| Glutamic acid | 2.2 (2) | 2.1 (2) | 3.9 (4) | 1.1 (1) | 3.1 (3) | 3.2 (3) | 3.2 (3) | 3.2 (3) | 3.2 (3) | 3.2 (3) | 3.2 (3) | 3.2 (3) | 3.2 (3) | 12
| Proline      | 1.1 (1) | 2.0 (2) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 7
| Glycine      | 3.0 (3) | 1.0 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 5
| Alanine      | 1.0 (1) | 5.8 (6) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 2.1 (2) | 21
| Methionine   | 2.0 (2) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 14
| Isoleucine   | 0.7 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 3
| Leucine      | 2.9 (3) | 3.9 (4) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 1.1 (1) | 8
| Tyrosine     | 1.1 (1) | 1.1 (1) | 1.0 (1) | 1.1 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 1.2 (1) | 3
| Phenylalanine | 1.2 (1) | 0.9 (1) | 1.1 (1) | 1.1 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 0.8 (1) | 8
| Lysine       | 0.8 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 4
| Histidine    | 0.7 (1) | 0.9 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 1.3 (1) | 3
| Arginine     | 0.8 (1) | 1.1 (1) | 1.0 (1) | 1.0 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 0.9 (1) | 7
| Total residues | 13 32 7 20 8 12 19 11 10 6 5 6 6 125

* Peptide numbers in parentheses are those assigned in a previous publication (5). Tryptic peptides have been renumbered according to their positions in the sequence.

† Total residues include only those present in distinct tryptic peptides. Thus, T-4 (the peptide containing both T-5 and T-6), T-9, and T-10 are not included.

‡ Values obtained after 72 hours of hydrolysis.

The tryptic digest was taken up in 10% formic acid (5) and subjected to gel filtration on a column (1.1 X 53 cm) of Sephadex G-25F in 0.02 M NaHCO₃. This procedure yielded another tryptic peptide, T-6, in pure form. Table II summarizes the amino acid compositions of all of the tryptic peptides, including those previously reported (5).

Amino Acid Sequence of Tryptic Peptides

The methods used are indicated in the sequence given for each peptide.¹

Peptide T-1 (Residues 1 through 13)

| Composition: | 2.0 1.0 2.0 1.1 2.2 0.7 |
| Step 1: | 1.0 1.0 1.8 0.9 2.2 0.6 |
| Step 2: | 0.8 0.4 1.8 1.1 2.1 0.7 |
| Step 3: | 0.8 1.3 1.2 2.1 0.8 |
| Step 4: | 0.8 1.4 0.5 2.1 0.8 |
| Step 5: | 1.2 0.4 1.7 0.7 |

¹ The following nomenclature is used throughout. T and C represent peptides obtained by the use of trypsin and chymotrypsin, respectively. These peptides are numbered according to their positions in the sequence. Pep, Pap, E, and Th refer to the products of hydrolysis of tryptic and chymotryptic peptides with pepsin, papain, elastase, and thermolysin, respectively. Arrows to the left and right represent the use of carboxypeptidase A or B and Edman degradation, respectively. Double underlining indicates hydrazinolysis. Step 1, Step 2, etc. refer to the results of Edman degradation, and the amino acid removed at each step is indicated in boldface type.

Peptide T-1 (continued)

| Composition: | 1.0 1.9 0.8 |
| Step 1: | 1.0 2.2 0.7 |
| Step 2: | 1.0 1.9 0.7 |
| Step 3: | 1.0 2.0 0.8 |
| Step 4: | 0.9 2.1 0.9 |
| Step 5: | 0.9 2.0 0.8 |

These values were obtained after 72 hours of hydrolysis. T-1 has previously been shown to be the amino-terminal tryptic peptide since the amino-terminal sequence of the isomerase is Met-Asn-Thr-Pro (5), which is not found in any other tryptic peptide. Arginine is placed at the carboxyl terminus of T-1, in accordance with the specificity of trypsin.

Partial acid hydrolysis of T-1 (6 M HCl, 100°, 10 min), followed by peptide mapping of the digest (electrophoresis, pH 4.7; partition chromatography, Solvent 1) and elution of the ninhydrin-positive spots in 0.1 N HCl, yielded two major fragments in yields of 20 to 30%.

T-1-A-1 (Residues 2 through 6)

| Composition: | 0.9 0.9 1.2 1.0 |

T-1-A-2 (Residues 8 through 13)

| Composition: | 0.6 0.8 0.9 1.2 1.0 |

The low value for valine is due to incomplete hydrolysis of a valyl-valine peptide bond under the conditions used. T-1 was
FIG. 2. Summary of data establishing the sequence of residues 14 through 45. Tryptic peptide T-2 and the fragments obtained by hydrolysis of T-2 with thermolysin, pepsin, chymotrypsin, and papain are shown above the line of the sequence. Corresponding hydrolyzed with chymotrypsin (10% by weight) for 2 hours at 40°. The resulting fragments were separated by partition chromatography in Solvent 2.

T-1-C-1 (Residues 1 through 7)

Met—Asn—Thr—Pro—Glu—His—Met

Composition: 1.7 1.1 1.0 1.0 1.1 0.8

Hydrazinolysis (after performic acid oxidation (15)) : methionine sulfone, 0.4.

Carboxypeptidase A (16 hours): methionine, 0.7; histidine, 0.2.

Electrophoresis, pH 4.7: neutral, therefore contains glutamic acid, not glutamine.

T-1-C-2 (Residues 8 through 13)

Thr—(Ala, Val, Val, Gin)—Arg

Composition: 0.8 1.0 2.0 1.1 1.0

Step 1: 0.1 0.9 2.0 1.1 1.0

These values were obtained after 72 hours of hydrolysis. Electrophoresis, pH 6.5: basic, therefore contains glutamine.

Peptide T-2 (Residues 14 through 45)

Tyr—(Asx, Thr, Ser, Glx, Pro)—Thr—Val—Leu—Gly—Val—Val—Gly—Val—Gly—Val

Composition: 2.0 1.5 1.8 1.0

Step 1: 2.1 1.1 1.9 1.0

Step 2: 2.0 0.5 1.9 1.1

Step 3: 2.0 0.5 1.5 1.1

Hydrazinolysis: glycine, 0.5.

T-2-Pep-2 (Residues 19 through 25)

Asx—Ala—Gly—(Asx, Leu, Asx)—Gly

Composition: 2.9 1.3 1.8 1.0

Step 1: 2.1 1.1 1.9 1.0

Step 2: 2.0 0.5 1.9 1.1

Step 3: 2.0 0.5 1.5 1.1

Values for valine and isoleucine in the composition of T-2-Pep-3 and after Steps 1 and 2 are those obtained after 68 hours of hydrolysis.

T-2-Pep-3 (Residues 26 through 32)

Ile—Val—Ala—Leu

Composition: 0.6 0.7 1.1 1.0

Step 1: 0.1 0.9 1.0 1.1

Step 2: 0.2 0.9 1.1

Step 3: 0.0 1.0

Carboxypeptidase B (20 hours): threonine, 0.3.

T-2-Pep-4 (Residues 33 through 35)

Phe—(Ala, Asx, Asx, Asx)—Thr

Composition: 0.9 2.0 2.4 0.6

Step 1: 0.2 1.9 2.4 0.7

Carboxypeptidase A (16 hours): threonine, 0.3.

T-2-Pep-5 (Residues 36 through 45)

Val—Glx—Asx—Pro—(Val, Gly, Ser, Glx, Pro)—Arg

Composition: 2.0 2.1 1.0 2.0 1.2 0.9 0.9

Step 1: 1.2 1.0 1.0 1.8 1.1 0.9

Step 2: 1.1 1.3 0.9 1.9 1.1 0.8

Step 3: 0.9 1.3 0.5 1.6 1.0 0.8

Step 4: 0.9 1.1 0.4 1.0 1.0 0.7

The values for arginine were not determined in Steps 1 through 4, since this residue has been shown by carboxypeptidase treatment to occupy the carboxyl-terminal position of T-2. Hydrolysis of T-2 by chymotrypsin (3% by weight) for 4 hours at 40°, followed by partition chromatography of the digest in Solvent 1, yielded only one fragment in pure form.
**T-2-C-1 (Residues 31 through 55)**

\[ \text{Ala-Asx-Asx-Ala-(Thr, Val, Glx, Asx, Pro),} \]

Composition: 1.9 3.0
Step 1: \(1.2 \ 2.7\)
Step 2: \(1.0 \ 2.0\)
Step 3: \(1.0 \ 1.4\)
Step 4: \(0.5 \ 1.4\)

**T-2-C-1 (continued)**

\[ \text{Val, Gly, Ser, Glx, Pro)–Arg} \]

Composition: 1.2 1.2
Step 1: 1.2 1.1
Step 2: 1.1 1.0
Step 3: 1.3 1.1
Step 4: 1.2 1.0

Arginine was not determined in Steps 1 through 4 since it is known to be at the carboxyl terminus of T-2. T-2-C-1 includes the portion of T-2 represented by T-2-Pep-5 as well as 5 of the 6 residues comprising T-2-Pep-4. Thus, it is apparent that T-2-Pep-4 is adjacent to T-2-Pep-5, the carboxyl-terminal peptic fragment of T-2. T-2 was hydrolyzed with papain (2% by weight) for 20 min at \(37^\circ\). Partition chromatography in Solvent 2 yielded two fragments in pure form.

**T-2-Pep-1 (Residues 53 through 54)**

\[ \text{(Asx, Ala, Thr, Val, Glx, Asx, Pro, Val)–Gly} \]

Composition: 2.3 1.1 0.8 1.6 1.2 0.7 1.0

These values were obtained after 72 hours of hydrolysis. Carboxypeptidase A (16 hours): glycine, 0.12.

**T-2-Pep-2 (Residues 42 through 55)**

\[ \text{Ser–Glu–Pro–Arg} \]

Composition: 0.9 1.1 1.0 0.9
Step 1: 0.1 1.0 1.0 0.9
Step 2: 0.3 1.1 0.9

Electrophoresis, pH 6.5; neutral, therefore contains glutamic acid.

Thermolysin (3% by weight) was used to hydrolyze T-2 in a 3-hour reaction at \(40^\circ\). Ion exchange chromatography on a column of Chromobeads A yielded the elution pattern shown in Fig. 3A. Peptides Th-1 and Th-3 were resolved by gel filtration through a fine grade of Sephadex G-25. Th-2 was subjected to the same procedure followed by partition chromatography in Solvent 2.

**T-2-Th-1 (Residues 14 through 17)**

\[ \text{Val, Ala, Ala)} \]

Composition: 1.0 1.1 1.9

This peptide contains the only tyrosine residue of T-2, and therefore is derived from the amino terminus of T-2.

**T-2-Th-2 (Residues 18 through 22)**

\[ \text{Leu–(Asn, Ala, Gly, Asn)–Thr} \]

Composition: 1.0 2.0 1.0 1.0
Step 1: 0.3 1.9 0.9 1.2

Electrophoresis, pH 6.5: neutral, therefore contains asparagine.

**T-2-Th-3 (Residues 23 through 25)**

\[ \text{Leu–Asn–Gly} \]

Composition: 0.9 1.1 1.0
Step 1: 0.2 0.9 1.0
Step 2: 0.0 1.0

Electrophoresis, pH 6.5: neutral, therefore contains asparagine.

**T-2-Th-4 (Residues 30 through 45)**

\[ \text{Phe–(Ala, Asx, Asx, Ala)–Thr} \]

Composition: 2.1 3.2 0.9
Step 1: 0.7 2.1 3.2
Step 2: 0.2 1.9 3.3
Step 3: 1.0

Arginine, previously shown to be carboxyl-terminal in T-2, was not determined in Step 1. T-2-Th-4 was subjected to further hydrolysis with thermolysin (6.5% by weight) in a 16-hour digest at \(40^\circ\). Two fragments were resolved on a column (0.9 \(\times\) 17 cm) of Chromobeads A in 0.2 M pyridine acetate, pH 3.1.

**T-2-Th-4 (continued)**

\[ \text{Phe–(Ala, Asx, Asx, Ala)–Thr} \]

Composition: 0.6 2.0 2.2
Step 1: 0.0 1.6 2.3
Step 2: 1.1 1.8
Step 3: 1.0 1.0

Electrophoresis, pH 6.5: acidic.

Electrophoresis (pH 6.5) of residue after Step 2: acidic.

Electrophoresis (pH 6.5) of residue after Step 3: neutral, there-
fore the residue removed in Step 3 was aspartic acid, and the residual tripeptide contains asparagine.

Carboxypeptidase A (22 hours): threonine, 0.5.

T-2-Th-4 B (Residues 36 through 45)

(Val, Glu, Asx, Pro, Val, Gly, Ser, Glu, Pro)–Arg

Composition: 2.1 1.9 1.1 1.8 1.0 0.8 0.7

Electrophoresis, pH 6.5: acidic.

Since studies on a corresponding chymotryptic peptide, C-7, (see text below) have shown the Asx residue in this portion of the sequence to be asparagine, both Glx residues in the arginine-containing acidic peptide, T-2-Th-4 B, must be glutamic acid.

Peptide T-8 (Residues 49 through 53)

Ser–Gly–Thr–Ala–Ala–Ile–Arg

Composition: 2.1 1.0 0.8 2.1 1.0 1.0

Step 1: 0.1 0.9 0.8 2.1 1.0 1.0

Step 2: 0.5 0.7 2.0 0.9 1.0 1.0

Step 3: 0.4 0.4 2.1 1.0

Step 4: 0.4 0.4 1.7 1.0

Carboxypeptidase B (1 hour): arginine, 0.3.

Carboxypeptidase A (3 hours): isoleucine, 0.8; alanine, 0.5.

Peptide T-4 (Residues 53 through 72)

Glx–(Phe, Tyr, Ala, Asx, Ser, Leu, Lys, Leu, Pro, Glx)

Composition: 3.9 0.9 1.1 2.0 1.1 0.9 0.9 0.9

Peptide T-4 (continued)

Leu, Ala, Val, Glx, Leu, Thr, Glx, Glx, Glx, Val, Arg

Composition: 1.9 0.9 0.9

The amino-terminal residue of T-4 has previously been shown to be glutamine or glutamic acid (5). The amino acid composition of T-4 is equivalent to the sum of those of T-3 and T-9. Thus, T-4 serves as an overlap for these two smaller tryptic fragments.

Peptide T-5 (Residues 53 through 60)

Glx–Phe–Tyr–Ala–Asx–Ser–Leu–Lys

Composition: 1.0 1.0 1.0 1.0 1.0 0.9 0.9 0.9

Step 1: 0.3 1.0 1.0 1.1 1.1 0.8 1.1

Step 2: 0.2 0.2 0.9 1.1 1.0 1.0 1.0

Step 3: 0.3 1.1 1.0 0.9 1.0

Step 4: 0.6 1.0 0.9 1.1

Step 5: 0.4 0.6 0.9 1.0

Electrophoresis, pH 6.5: neutral.

Carboxypeptidase B (20 hours): arginine, 0.4.

The evidence for the structure of T-7 is summarized in Fig. 4. T-7 was hydrolyzed with thermolysin (6.5% by weight) for 18 hours at 40°. The resulting peptides were resolved by gradient elution from a column (0.9 x 17 cm) of Chromobeads A, with pyridine acetate buffers (Fig. 3B).

T-7-Th-1 (Residues 73 through 77)

Ala–Val–Ala–(Asx, Glx)–[Ala–Ala–(Phe–Ala)

(Phe–Thr)–Val–Ser]–(Phe–Glu)–Tyr–Gln–Gly–Arg

Composition: 0.9 1.1 1.0 1.0 1.0 0.9

Step 1: 0.9 1.0 1.0 1.0 1.0 0.9

Step 2: 0.9 1.0 1.0 1.0 1.0 0.9

Step 3: 0.9 1.0 1.0 1.0 1.0 0.9

Carboxypeptidase B (20 hours): arginine, 0.8.

Hydrazinolysis of residual peptide: valine, 0.7.

Electrophoresis, pH 6.5: acidic.

T-6 was hydrolyzed with chymotrypsin (5% by weight) for 40 hours at 40°. The products were separated on a column (0.9 x 17 cm) of Chromobeads A, in pyridine acetate buffers.

T-6-C-1 (Residues 61 through 67)

Leu–(Pro, Leu, Ala, Val, Glu, Leu)

Composition: 2.6 0.9 1.1 1.2 1.3

Step 1: 0.2 1.9 1.1

Step 2: 1.2 1.0 0.8

Electrophoresis, pH 6.5: neutral, therefore one Glx residue is glutamic acid and the other is glutamine. Electrophoresis, pH 6.5, of the residual peptide after Step 2: neutral, therefore the 3rd residue in T-6-C-2 is glutamic acid, and the 2nd residue is glutamine.

Peptide T-7 (Residues 58 through 61)

Ala–Val–Ala–(Asx, Glx)–[Ala–Ala–(Phe–Ala)

(Phe–Thr)–Val–Ser]–(Phe–Glu)–Tyr–Gln–Gly–Arg

Composition: 0.9 1.1 1.0 1.0 1.0 0.9

Step 1: 0.9 1.0 1.0 1.0 1.0 0.9

Step 2: 0.9 1.0 1.0 1.0 1.0 0.9

Step 3: 0.9 1.0 1.0 1.0 1.0 0.9

Carboxypeptidase B (20 hours): arginine, 0.4.

The evidence for the structure of T-7 is summarized in Fig. 4. T-7 was hydrolyzed with thermolysin (6.5% by weight) for 18 hours at 40°. The resulting peptides were resolved by gradient elution from a column (0.9 x 17 cm) of Chromobeads A, with pyridine acetate buffers (Fig. 3B).

T-7-Th-1 (Residues 73 through 77)

Ala–Val–Ala–(Asx, Glx)

Composition: 1.8 1.1 1.0 1.1

T-7-Th-2 (Residues 78 through 79)

Ala–Ala

This fraction contained only alanine after acid hydrolysis. However, amino acid analysis of the unhydrolyzed material yielded no free alanine. Since one step of Edman degradation, followed by direct amino acid analysis of the residue, yielded alanine, T-7 Th 2 must be a dipeptide.

T-7-Th-3 (Residues 80 through 81)

Phe–Ala

Composition: 0.9 1.1

Step 1: 0.0 1.0
FIG. 4. Summary of data establishing the sequence of residues 73 through 102. Tryptic Peptides T-7 and T-8 and the fragments obtained therefrom by further degradation with elastase and thermolysin are designated above the line of the sequence. Shown below the line of sequence are the corresponding chymotryptic peptides of the isomerase, as well as the tryptic and peptic fragments of C-19.

**T-7-Th-4 (Residues 82 through 83)**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| Phe-Thr | 0.9 1.0     | 0.0 1.0|        |

**T-7-Th-5 (Residues 84 through 85)**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| Val-Ser | 1.2 0.8     | 0.0 1.0|        |

**T-7-Th-6 (Residues 86 through 87)**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| Phe-Glu | 0.9 1.1     | 0.0 1.0|        |

The residue after Step 1 was analyzed directly and found to be glutamic acid.

**T-7-Th-7 (Residues 88 through 91)**

| Peptide | Composition | Step 1 | Step 2 | Step 3 |
|---------|-------------|--------|--------|--------|
| Tyr-Glu-Gly-Arg | 0.7 1.2 1.3 0.9 | 0.2 1.0 1.1 0.7 | 0.4 1.0 1.0 |        |

Electrophoresis, pH 6.5: basic, therefore contains glutamine.

**T-7-E-1 (Residues 73 (74) through 77 (78))**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| (Ala, Val, Ala, Asn, Gln) | 2.1 1.0 0.9 1.1 | 0.9 1.1 |        |

Electrophoresis, pH 6.5: neutral, therefore contains asparagine and glutamine.

**T-7-E-2 (Residues 79 through 84)**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| (Ala, Phe, Ala, Phe, Thr, Val) | 2.2 1.8 0.8 1.0 |        |        |

Peptide T-8 (Residues 92 through 102)

| Peptide | Composition | Step 1 | Step 2 | Step 3 |
|---------|-------------|--------|--------|--------|
| Lys-Thr-(Val-Val-Ala-Pro)(Ile-Asp-His) | 0.8 0.8 2.2 1.0 1.0 0.9 1.0 | 0.4 0.9 2.2 | 1.1 0.9 1.0 1.1 0.9 | 0.9 |

**Peptide T-8 (continued)**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| (Phe)-Arg | 1.0 1.0 | 0.9 1.0 | 0.9 1.0 |

These values were obtained after 72 hours of hydrolysis.

**T-8-Th-1 (Residues 92 through 95)**

| Peptide | Composition | Step 1 | Step 2 | Step 3 |
|---------|-------------|--------|--------|--------|
| Lys-Thr- | 0.9 1.1 |        |        |        |

**T-8-Th-2 (Residues 94 through 97)**

| Peptide | Composition | Step 1 | Step 2 | Step 3 |
|---------|-------------|--------|--------|--------|
| Val-Val-Ala-Pro | 2.2 0.9 0.9 | 1.1 0.9 1.1 | 0.4 1.0 1.0 | 0.0 1.0 |

These values were obtained after 72 hours of hydrolysis.

**T-8-Th-3 (Residues 98 through 100)**

| Peptide | Composition | Step 1 | Step 2 | Step 3 |
|---------|-------------|--------|--------|--------|
| Ile-Asp-His | 1.0 1.1 0.9 | 0.0 1.1 0.9 | 0.0 1.0 | 0.0 1.0 |

Electrophoresis, pH 4.7: neutral, therefore contains aspartic acid.

**Peptide T-9 (Residue 92)**

| Peptide | Composition | Step 1 | Step 2 |
|---------|-------------|--------|--------|
| Lysine | 1.0 1.0 |        |        |
Primary Structure of Δ^3-3-Ketosteroid Isomerase

**Peptide T-10 (Residues 93 through 102)**

Thr-(Val, Val, Ala, Pro, Ile, Asx, His, Phe)-Arg

Composition: 1.0 2.1 1.2 1.2 1.1 1.1 1.3 1.1 1.0
Step 1: 0.2 1.3 1.3 1.3 1.3 1.3 1.0 1.1 1.0

Arginine must be at the carboxyl terminus, in accordance with the specificity of trypsin.

**Peptide T-11 (Residues 103 through 108)**

Phe-Asn-Gly-Ala-Gly-Lys

Composition: 0.9 1.0 2.0 1.0 1.0 1.0
Step 1: 0.1 0.9 2.0 1.0 0.8
Step 2: 0.3 2.0 1.0 0.6

Carboxypeptidase B (24 hours): lysine, 0.8.
Hydrazinolysis of residual peptide: glycine, 0.5.
Carboxypeptidase A (18 hours): glycine, 0.8; alanine, 0.6.
Electrophoresis (pH 4.7): basic, therefore contains asparagine.

**Peptide T-12 (Residues 109 through 113)**

Val-Val-Ser-Ala-Arg

Composition: 1.6 0.9 0.8 0.8 1.0
Step 1: 1.0 0.9 1.0 1.0
Step 2: 0.2 0.9 0.9 1.0
Step 3: 0.4 0.9 1.0

Arginine is located at the carboxyl terminus, according to the specificity of trypsin.

**Peptide T-13 (Residues 114 through 119)**

Ala-Leu-Phe-Gly-Glu-Lys

Composition: 0.9 1.0 0.9 1.0 1.0 1.0
Step 1: 0.1 1.0 1.0 1.0 1.1
Step 2: 0.3 1.0 1.1 1.1
Step 3: 0.3 0.1 1.1 1.3
Step 4: 0.2 0.3 0.5 1.0

The placement of lysine at the carboxyl terminus of T-13 is based on the specificity of trypsin.
Electrophoresis (pH 6.5): neutral, therefore contains glutamic acid.

**Peptide T-14 (Residues 120 through 125)**

Asx-Ile-His-Ala-Gly-Ala

Composition: 0.9 0.9 0.9 2.0 1.0
Step 1: 0.2 0.9 0.9 2.0 1.0
Step 2: 0.2 0.9 0.9 2.0 1.0
Step 3: 0.3 1.8 1.0
Step 4: 0.4 1.5 1.0

Carboxypeptidase A (24 hours): alanine, 0.7; glycine, 0.2.
Electrophoresis, pH 4.7: basic, therefore contains asparagine.

**Amino Acid Sequence of Chymotryptic Peptides**

**Peptide C-1 (Residues 68 through 101)**

(Asx, Thr, Pro, Glx, His, Met)

Composition: 1.2 0.8 0.8 1.1 0.7 1.0

**Peptide C-2 (Residues 8 through 14)**

Thr-Ala-Val-Val-(Glx, Arg)-Tyr

Composition: 0.8 1.1 2.0 1.1 1.0 0.9
Step 1: 0.4 1.0 1.7 1.2 1.0 1.0
Step 2: 0.5 1.5 1.0 1.0 0.8
Step 3: 0.4 1.2 1.0 0.8 0.8
Step 4: 0.6 1.0 0.9 0.7

The values are those obtained after 72 hours of hydrolysis.
Hydrazinolysis: tyrosine, 0.40.

**Peptide C-3 (Residues 8 through 12)**

Thr-Ala-(Val, Val, Glx)

Composition: 0.7 1.1 1.9 1.0
Step 1: 0.1 1.0 1.9 1.1

These values were obtained after 72 hours of hydrolysis.
Carboxypeptidase A (24 hours): valine, 1.1; glutamine, 0.5.

**Peptide C-4 (Residues 13 through 14)**

Arg-Tyr

Composition: 1.0 1.0
Step 1: 0.0 1.0

**Peptide C-5 (Residues 15 through 18)**

Val-Ala-Asx-Leu

Composition: 1.1 1.9 1.0
Step 1: 0.3 1.9 1.1

Hydrazinolysis: leucine, 0.6.

**Peptide C-6 (Residues 19 through 30)**

Asx-Ala-(Gly, Asx, Leu, Asx, Gly, Ile, Val)

Composition: 2.8 2.0 2.0 1.9 0.9 1.2
Step 1: 2.1 1.8 2.2 1.9 0.9 1.1
Step 2: 2.0 1.3 2.0 1.7 0.9 1.1

**Peptide C-6 (continued)**

Val-Gly

Composition: 1.1
Step 1: 1.0
Step 2: 1.0

These values were obtained after 72 hours of hydrolysis.
Hydrazinolysis: phenylalanine, 0.2.
Carboxypeptidase A (4 hours): phenylalanine, 1.0; leucine, 1.0; alanine, 0.5.

**Peptide C-7 (Residues 31 through 48)**

Ala-Asx-(Asx, Ala, Thr, Val, Glx)](Asx-Pro--

Composition: 1.9 3.0 1.9 2.3 2.2 1.8
Step 1: 1.2 2.8 1.8 2.0 2.1 1.7
Step 2: 1.3 2.2 1.7 1.9 2.0 1.6

**Peptide C-7 (continued)**

Val-Gly)Ser,Glx,Pro,Arg,Ser,Gly-Thr

Composition: 2.0 1.8 1.0
Step 1: 2.0 1.8 1.0
Step 2: 2.0 1.7 0.9

Hydrazinolysis: threonine, 0.5.
Hydrolysis of C-7 with papain (5% by weight) for 14 hours at 37°C, followed by partition chromatography of the digest in Solvent 2, yielded two fragments in pure form.

**C-7-Pap-1 (Residues 31 through 37)**

\[ \text{Ala–Asx–(Asx, Ala, Thr, Val, Glx)} \]

Composition: 1.8 2.0 1.1 1.2 1.0

**C-7-Pap-2 (Residues 38 through 41)**

\[ \text{Asn–Pro–Val–Gly} \]

Composition: 1.0 0.8 1.0 1.1

Step 1: 0.3 1.0 0.9 1.1

Step 2: 0.2 0.9 1.0

Step 3: 0.0 1.0

Electrophoresis, pH 6.5: neutral, therefore contains asparagine.

**Peptide C-8 (Residues 49 through 54)**

\[ \text{Ala–(Ala, Ile, Arg, Glx, Phe)} \]

Composition: 1.8 1.0 1.0 1.1 1.1

Step 1: 1.1 0.9 0.9 1.0 1.0

**Peptide C-9 (Residues 49 through 55)**

\[ \text{Ala–(Ala, Ile, Arg, Glu–Phe–Tyr)} \]

Composition: 2.0 1.0 1.0 1.1 0.9

Step 1: 1.2 1.0 0.8 1.0 0.9

Hydrazinolysis: tyrosine, 0.3.

Carboxypeptidase A (24 hours): tyrosine, 0.9; phenylalanine, 0.8; glutamic acid, 0.2.

**Peptide C-10 (Residues 56 through 59)**

\[ \text{Ala–Asn–Ser–Leu} \]

Composition: 1.0 1.0 0.9 1.1

Step 1: 0.6 1.0 0.9 1.0

Hydrazinolysis: leucine, 0.6.

Carboxypeptidase A (24 hours): leucine, 0.5.

Hydrazinolysis of residual peptide: serine, 0.2.

Electrophoresis, pH 6.5: neutral, therefore contains asparagine.

**Peptide C-11 (Residues 60 through 67)**

\[ \text{Lys–Leu–(Pro, Leu, Ala, Val, Glx)–Leu} \]

Composition: 0.7 2.9 1.0 1.1 1.1 1.1

Step 1: 0.1 3.0 0.8 1.0 1.2 1.1

Step 2: 2.1 0.8 1.0 1.0 1.1

Hydrazinolysis: leucine, 0.3.

**Peptide C-12 (Residues 60 through 63)**

\[ \text{Lys–Leu–Pro–Leu} \]

Composition: 0.9 1.9 1.2

Step 1: 0.1 2.0 1.0

Step 2: 1.2 0.9

Step 3: 1.0 0.0

**Peptide C-13 (Residues 64 through 67)**

\[ \text{Ala–Val–Glu–Leu} \]

Composition: 0.0 1.2 1.0 1.0

Step 1: 0.3 1.0 1.0 1.0

Step 2: 0.4 1.0 1.0

Step 3: 0.0 0.0 1.0

Electrophoresis, pH 6.5: acidic, therefore contains glutamic acid.

Carboxypeptidase A (24 hours): leucine, 0.8; glutamic acid, 0.2.

**Peptide C-14 (Residues 68 through 80)**

\[ \text{Thr–Glu–(Glx, Val, Arg, Ala, Val)} \]

Composition: 0.8 3.1 2.3 0.9 3.9

Step 1: 0.4 3.1 2.2 0.9 3.8

Step 2: 0.2 2.5 2.1 0.8 3.7

**Peptide C-14 (continued)**

\[ \text{Ala, Asx, Glx, Ala–Ala–Phe} \]

Composition: 1.1 0.9

Step 1: 1.1 0.9

Step 2: 1.0 0.8

Hydrazinolysis: phenylalanine, 0.6.

Carboxypeptidase A (44 hours): phenylalanine, 0.7; alanine, 0.7.

**Peptide C-15 (Residues 77 through 80)**

\[ \text{Glx–(Ala, Ala, Phe)} \]

Composition: 1.0 2.1 1.0

Step 1: 0.3 2.1 0.9

**Peptide C-16 (Residues 81 through 83)**

\[ \text{Ala–Phe} \]

Composition: 1.0 1.0

Step 1: 0.0 1.0

**Peptide C-17 (Residues 83 through 86)**

\[ \text{Thr–Val–Ser–Phe} \]

Composition: 0.9 1.2 1.0 1.0

Step 1: 0.1 1.1 0.9

Step 2: 0.4 1.1 0.9

Hydrazinolysis: phenylalanine, 0.4.

**Peptide C-18 (Residues 87 through 88)**

\[ \text{Glu–Tyr} \]

Composition: 0.0 1.0

Step 1: 0.0 1.0

Carboxypeptidase A (16 hours): tyrosine, 1.0; glutamic acid, 1.0.

**Peptide C-19 (Residues 89 through 101)**

\[ \text{Glx–(Ala, Glx, Ala)–Arg–Lys–Thr–Val–(Val, Ala)–Pro–(Ile, Asp, His)–Phe} \]

Composition: 1.0 1.2 0.8 0.7 1.0 2.3 1.1

**Peptide C-19 (continued)**

\[ \text{Pro–(Ile, Asp, His)–Phe} \]

Composition: 0.9 1.0 1.1 0.7 1.0

Carboxypeptidase A (1½ hours): phenylalanine, 1.0.
The evidence for the structure of C-19 is summarized in Fig. 4. Hydrolysis of C-19 with trypsin (5% by weight) for 16 hours at 40°, followed by purification of the products by gel filtration through a column (0.7 x 110 cm) of Sephadex G-25F in 50% acetic acid, yielded two fragments, C-19 T-1 and C-19 T-2.

C-19-T-1 (Residues 89 through 92)  
(Glx, Gly)–Arg–Lys  
Composition: 1.0 1.1 1.1 0.7  
Hydrazinolysis: lysine, 1.0.

C-19-T-2 (Residues 93 through 101)  
Thr–Val–(Val, Ala, Pro, Ile, Asx, His)–Phe  
Composition: 0.7 2.0 1.0 1.1 0.9 1.0 0.8 0.8  
Step 1: 0.3 2.0 1.1 0.9 1.0 0.8 0.9

These values were obtained after 66 hours of hydrolysis. Hydrolysis of C-19 with pepsin (10% by weight) for 18 hours at 25° yielded two fragments which were separated by ion exchange chromatography on AG 1-X2 in pyridine-collidine-acetic acid buffers.

C-19-Pep-1 (Residues 89 through 96)  
(Glx Gly)–Arg–Lys–Thr–Val–(Val, Ala)  
Composition: 1.0 1.2 0.9 0.9 0.9 2.0 1.1  
Step 1: 0.5 2.0 1.1 0.8 1.0  
Step 2: 1.5 1.0 1.0 0.8 1.0 0.7 0.8

Methionine was determined as the sulfone, after performic acid oxidation (15).

C-19-Pep-2 (Residues 97 through 101)  
Pro–(Ile, Asp, His)–Phe  
Composition: 1.0 1.0 1.0 0.9 1.0  
Step 1: 0.5 0.9 1.0 0.8

Electrophoresis, pH 4.7: neutral, therefore contains aspartic acid.

Peptide C-20 (Residues 102 through 103)  
Arg–Phe  
Composition: 1.0 1.0  
Step 1: 0.0 1.0

Peptide C-21 (Residues 104 through 112)  
(Asx, Gly, Ala, Gly, Lys, Val, Val, Ser, Met)  
Composition: 0.7 2.3 1.2 1.1 1.7 1.1 0.9

Peptide C-22 (Residues 113 through 115)  
Arg–Ala–Leu  
Composition: 1.0 1.0 1.0  
Step 1: 0.4 1.0 1.0

Step 2: 0.0 1.0

Fig. 5. Complete amino acid sequence of the identical subunits of Δ3-3-ketosteroid isomerase of *Pseudomonas testosteroni*. The tryptic peptides are shown above the sequence and the chymotryptic peptides are shown below.
Peptide C-M (Residues 113 through 116)

Composition: 1.0 1.0 1.0 1.0

Step 1: 0.2 1.0 1.0 1.0
Step 2: 0.4 1.0 1.0
Step 3: 0.0 1.0

Peptide C-M (Residues 117 through 125)

Gly-Glx-(Lys, Asx, Ile, His, Ala, Gly)-Ala

Composition: 2.1 1.1 1.0 1.0 0.9 0.9 2.1

Step 1: 1.3 1.0 0.9 1.0 0.9 0.9 2.0
Step 2: 1.1 0.3 0.8 1.0 0.8 0.7 2.0

Hydrazinolysis: alanine, 0.8.

Complete Sequence

The complete amino acid sequence of the identical subunits of P. testosteroni Δ^1-3-ketosteroid isomerase, deduced primarily from the structure of, and overlaps among, the tryptic and chymotryptic peptides, is shown in Fig. 5.

Comments

Comparison of the amino acid composition with the sum of the residues found in the amino acid sequence of each subunit has led to the suggestion that there are three subunits per molecule of weight 40,800 (4). The latter determination is based on an ultracentrifugation measurement carried out on a small sample by the Archibald approach to equilibrium method (4). There are also three binding sites per mole for steroids as measured by spectrophotometric titration and fluorescence-quenching measurements (2). The calculated molecular weight of the polypeptide chain determined by sequence analysis is 13,394, which is 33% of the ultracentrifugally determined molecular weight. Hydrazinolysis released 3.0 residues of alanine and Edman degradation yielded a minimum of 1.8 residues of methionine as the phenylthiohydantoin (5) per unit of molecular weight of 40,800. All of these findings are consistent with the presence of three polypeptide chains per molecule. The structural work provides solid evidence that the chains are identical. However, all of the evidence cited for the presence of three subunits per molecule is dependent upon the accuracy of the ultracentrifugally determined molecular weight.

Since it has been postulated that histidine may have a role in the mechanism of action of this enzyme, and since at least one histidine residue has been shown to be essential (1), it is of interest to note that 3 histidine residues are present in the subunit polypeptide chain, 1 near the amino terminus (residue 6), 1 near the carboxyl terminus (residue 122), and the 3rd toward the central portion of the chain (residue 100). It has also been suggested that tyrosine may be involved in the active center of the enzyme (2). Three residues of this amino acid are present in positions 14, 55, and 88.

A rather high incidence of clustering among the hydrophobic residues in the chain may also be noted. Such groupings are found in positions 7 to 12, 14 to 31, 54 to 59, 61 to 65, 73 to 86, 93 to 98, and 103 to 107. However, it is not known what significance these hydrophobic clusters might have with regard to either the three-dimensional structure or the function of the enzyme, although the steroidal substrates are highly hydrophobic, and the enzyme has the unusual property of being soluble in high concentrations of ethanol (6).

Acknowledgment—We are grateful to Dr. S. H. Moolgavkar for carrying out some of the carboxypeptidase degradations of the tryptic peptides.

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*J. Biol. Chem.* 1971, 246:7514-7525.

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