Tryptophan Synthetase $\beta_2$ Subunit

PRIMARY STRUCTURE OF THE PYRIDOXYL PEPTIDE FROM THE ESCHERICHIA COLI ENZYME*

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SUMMARY

Radioactivity incorporated into an $\epsilon$-N-5'-phosphopyridoxal-5'-phosphate residue of the $\beta$ chain by reduction of the $\beta_2$ subunit of the enzyme with tritiated sodium borohydride appears in two tryptic peptides. These pyridoxyl peptides differ by only a single arginyl residue. As expected, tryptic digests of normal protein contain two peptides that together make up the pyridoxyl peptide region. The primary structure in this region was determined to be: Arg-Glu-Asp-Leu-Leu-His-Gly-Gly-Ala-His-Lys(Pxy)-Thr-Asn-Gln-Val-Leu-Gly-Gln-Ala-Leu-Leu-Ala-Lys.

Tryptophan synthetase (EC 4.2.1.20) from Escherichia coli has been the subject of many genetic and biochemical studies (1–3). The native enzyme is composed of four polypeptide chains, $\alpha_2\beta_2$. The molecule dissociates into $\alpha$ and $\beta_2$ subunits. Although the subunits exhibit maximal enzymatic activity only when complexed together, each in the dissociated form retains a vestige of catalytic activity in one of the two tryptophan synthetase half-reactions, the reversible aldolysis of indole 3-glycerolphosphate to indole and n-glyceraldehyde 3-phosphate (ac subunit), or the condensation of indole and L-serine to form tryptophan (1, 3). The primary structure of the $\alpha$ chain has been determined (4) and the identification of residues essential for enzymatic activity is under way (2, 5). The $\beta_2$ subunit, or the condensation of indole and L-serine to form tryptophan (1, 3). The primary structure of the $\alpha$ chain has been determined (4) and the identification of residues essential for enzymatic activity is under way (2, 5). The $\beta_2$ subunit has been determined (6) and some characteristics of its active site region were known (3), but till now no sequence information has been available for the $\beta$ chain of the enzyme.

The $\beta_2$ subunit contains 2 molecules of tightly bound pyridoxal-5'-P per dimer (6). As with other pyridoxal-5'-P proteins (7–9), the cofactor can be covalently attached to the $\epsilon$-amino group of a lysine residue by reduction with sodium borohydride (3, 6). This cofactor remains attached during tryptic digestion and purification of the peptides, providing a convenient marker for the lysyl residue entering into Schiff base formation with the pyridoxal-5'-P in the resting enzyme (7, 8). Detection of the pyridoxyl peptide can be made easier by the use of tritiated borohydride in the reduction step, as will be shown.

Although some of the residues of the pyridoxyl peptide must, in the intact protein, be situated near the cofactor, it is probably not precise to call this an “active site peptide.” In this paper and the succeeding one (10) on the analogous peptide from the $\beta_2$ subunit of Pseudomonas putida (tryptophan synthetase) we will use the term pyridoxyl peptide (8) to signify a peptide containing an $\epsilon$-N-5'-phosphopyridoxyllysine.

When trypsin is used for proteolysis of borohydride-reduced protein, one of its normal cleavage sites will be missing because of the modification of a lysine residue. Ordering of amino acids in the pyridoxyl peptide can be greatly facilitated by recognition of the two peptides in a digest of normal protein which go to make up the pyridoxyl peptide in the reduced digest. This was shown in an earlier study of bovine liver glutamate dehydrogenase (11), and is confirmed in this and the P. putida (10) studies.

EXPERIMENTAL PROCEDURE

The $\beta_2$ subunit of tryptophan synthetase was purified from an extract of E. coli trpA2F/trpA2 cells (12) by a procedure described previously (6). Pyridoxal-5'-P was from Sigma and tritiated sodium borohydride, 200 mCi per mmole, from New England Nuclear. Trypsin, twice crystallized salt free or the same treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone, were products of Worthington. Both, as were the disopropyl fluorophosphate-treated carboxypeptidase A and B preparations. Aminopeptidase M was obtained from Rohl GMBH in Darmstadt, but is available in the United States through Henley and Company, Inc., New York. Thermolysin obtained as a gift from Dr. S. Endo was recrystallized three times by his procedure (13). $\epsilon$-N-Pyridoxyllysine and $\epsilon$-N-5'-phosphopyridoxyllysine were gifts of Dr. E. H. Fischer. Collidine was redistilled. Other solvents for peptide separation and Edman degradation were spectroquality. Phenylisothiocyanate was from Eastman.

Preparation of Labeled and Unlabeled Performic Acid-Oxidized $\beta_2$ Subunits—$\beta_2$ Protein was reduced by the addition of a minute amount (<0.1 mg) of tritiated borohydride to 10 mg of protein at 25° in 1 ml of 0.1 M potassium phosphate, pH 7.3, containing 1 mm 2-mercaptoethanol and 20 µM pyridoxal-5'-P. The yellow color disappeared in less than 1 min. After 3 min, the reaction mixture was dialyzed against several changes of deionized water until the dialysate lacked radioactivity, then combined with 4

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times as much protein reduced in the usual way (6) with unlabeled borohydride. The combined samples were then oxidized with performic acid by the method of Hirs (14). No loss of tritium occurred in this process. The oxidized protein was dialyzed against deionized water to remove excess formic and performic acids, then lyophilized. Radioactivity in protein and peptides was determined in a Packard liquid scintillation counter with a conventional dioxane-naphthalene-based scintillant mixture.

Tryptic Digestion—Weighed samples of lyophilized, performic acid-oxidized protein were dissolved in 1 to 2 ml of deionized water and brought to pH 8.5 to 9.0 with 0.1 N NaOH. One per cent (w/w) of trypsin dissolved in a small volume of 1 mM HCl was added and digestion carried out at 37° for 90 min. The digest was maintained at pH 8.0 to 9.0 by intermittent additions of 0.1 N NaOH during the first 30 min. Digests done with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin were indistinguishable from those made with untreated enzyme. Upon acidification of the digest to pH 3.0 with 1 N HCl and chilling to 4°, a small amount of insoluble “core” appeared. This was usually removed by centrifugation but could be added to the cation exchange column without affecting the yield or purification of the peptides of interest.

Ion Exchange Chromatography of Tryptic Digests—The first separation was accomplished on a column, 0.6 × 100 cm, of the spherical cation exchange resin “P” (Technicon Corporation), monitored with a Technicon peptide analyzer. After each use the resin was regenerated with 2 N pyridine, then equilibrated with 0.15 M pyridine-acetate buffer, pH 3.1 (12 ml of pyridine, 209 ml of glacial acetic acid per liter). The column was eluted by means of a gradient prepared in a nine-chambered Varigrad as recommended in the Technicon peptide analyzer Instruction Manual except that the first chamber contained 0.15 M pyridine-acetate, pH 3.1, instead of 0.20 M pyridine-acetate at the same pH. After about 80% of the gradient had been used the eluent was changed to 2 N pyridine. Early experiments monitored by the ninhydrin reaction both alkali-hydrolyzed and unhydrolyzed samples. Later only the unhydrolyzed sample was taken to conserve material. The digest from more than 100 mg of protein could be applied to the P resin column without evidence of overloading.

After fractions were evaporated over NaOH and H2SO4 in an evacuated desiccator at 50°, 2 to 5% of each was chromatographed on Whatman No. 1 paper in 2-butanol-formic acid-water (80:9:21) and stained first with ninhydrin, then by a chlorine-tolidine method for ninhydrin-negative material (15). The desired fractions were then dissolved in pH 8.3 collidine-pyridine-acetate buffer (16) and applied to a column, 0.6 × 70 cm, of Dowex 1-X2 (>400 mesh) prepared as described by Schroeder et al. (16). A gradient was generated with 90-ml volumes in the nine-chambered Varigrad, beginning with the pH 8.3 collidine-pyridine-acetate buffer in Chambers 1 and 2 and having the following amounts of 2 N acetic acid diluting the same buffer in succeeding chambers: 2.25, 4.5, 9.0, 22.5, and 45 ml. The last two chambers each contained 90 ml of 2 N acetic acid. Again, a portion of the column effluent was monitored without alkaline hydrolysis in the Technicon peptide analyzer.

Sequential Edman Degradation of Peptides—The method of Gray (17) was used, with the NH2-terminal amino acid being determined by dansylation of a 2- to 4-nmole aliquot after each step. Dansyl-amino acids1 were identified by thin layer chromatography (18) on polyamide sheets (Gallard-Schlessinger Corp., Carle Place, New York).

Enzymatic Digestions of Peptides—Carboxypeptidase A or B digestion was carried out with a standard method (19). The amino acids released were determined by placing the reaction mixture, or an aliquot of it, directly on the amino acid analyzer column. Aminopeptidase M digestion was carried out for 2 hours at 37° in 5 mM Tris-Cl buffer, pH 7.9, made 2.5 mM with sodium borohydride-reduced β subunit are designated T-11, etc. Thermolytic peptides are prefixed with TH and a peptide obtained after thermolysis digestion of carboxypeptidase B-treated material is designated CPTB.
Table I

Amino acid content of peptides purified from E. coli tryptophan synthetase β₂ subunit

<table>
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<tr>
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<td>Aspartic acid</td>
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<td>0.91</td>
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<td>1.00</td>
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<td>0</td>
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<td>Glutamic acid</td>
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<td>0</td>
<td>0</td>
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<td>Leucine</td>
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<td>4.67</td>
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<td>2.18</td>
<td>0.93</td>
<td>1.92</td>
<td>1.19</td>
<td>1.10</td>
<td>0.77</td>
<td>0</td>
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</table>

⁹ Values given are normalized to lysine (1.00) unless noted otherwise. Values less than 0.2 residues are recorded as 0.

ᵇ Authentic samples of G-N-pyridoxyllysine and histidine do not separate on the short column of the amino acid analyzer. The values shown represent an arbitrary apportionment of the ninhydrin-positive material eluting at this position.

c Uncorrected for destruction or incomplete release.

d Threonine was taken as 1.00. Recovered in 74% yield from a thermolysin digest of 380 nmoles of T-11.

e Glutamic acid was taken as 1.00. Recovered in 65% yield from a thermolysin digest of 380 nmoles of T-11.

f Recovered in 25% yield (after two column chromatographies and one paper chromatography) from a thermolysin digest of 380 nmoles of T-11.

g Aspartic acid was taken as 1.00. Recovered in 39% yield from a thermolysin digest of 67 nmoles of T-23.

h Recovered in 60% yield (approximately) from a thermolysin digest of 300 nmoles of T-23.

i Alanine was taken as 1.00. Recovered in 40% yield from a carboxypeptidase R followed by thermolysin digest of 180 nmoles of T-23.

MgCl₂. The reaction was terminated either by freezing or by application to the amino acid analyzer. Thermolysin digestion was done for 3 hours at 40° in 40 mM Tris-Cl, pH 7.9, containing 15 mM CaCl₂.

Paper Electrophoresis—High voltage electrophoresis was performed on Whatman No. 3MM paper under 2000 volts. Peptides were identified by ninhydrin followed by a histidine-specific stain (20).

Amino Acid Analysis—Peptides were hydrolyzed 18 to 24 hours in 6 N HCl at 110° in vacuo. Amino acids were determined with a Beckman 120C amino acid analyzer with standard techniques.

RESULTS

Purification of Labeled Peptides—Na⁺¹⁴-Labeled, performic acid-oxidized β₂ protein, 46 mg, was digested with trypsin and subjected to cation exchange chromatography (Fig. 1). Most of the radioactivity appeared in two major peaks. (Fractions 24 to 26 and 36 to 39 contained 34 and 37% of the tritium placed on the column.) Although there were some minor peaks of radioactivity, these were not pursued further. The fractions in each major peak were combined and chromatographed on Dowex 1. Most of the radioactivity from the first peak emerged in one fraction (Fraction 23, Fig. 24). On paper chromatography two peptides were seen in this fraction (Rf 0.03 and
0.16). Only the slower moving peptide contained radioactivity. It was eluted from paper and analyzed for amino acid content (Pxy-1, Table I). The second radioactive region from Fig. 1 was chromatographed similarly by anion exchange and also revealed a single radioactive peak (Fractions 22 to 23, Fig. 2B). This peak contained only one peptide (Rf = 0.03) by paper chromatography. Amino acid analysis (Table I) showed that this peptide (Pxy-2) differed from Pxy-1 by only a single arginyl residue. On each ion exchange column the recovery of tritium was greater than 70%. The specific radioactivities of Pxy-1 and Pxy-2 were essentially identical (800 to 850 cpm per nmole), and not very different from that of the undigested protein (950 cpm per nmole, assuming a molecular weight of 44,000 for the β chain).

**Purification of T-11 and T-23—**Fig. 3 presents the cation exchange chromatogram of a tryptic digest of 98 mg of performic acid-oxidized β2 protein. In this and many other similar experiments two peptides were seen that were absent in digests of the reduced β2 subunit. These appear in Fractions 14 to 15 (Rf = 0.25) and 39 to 40 (Rf = 0.03) of Fig. 3. Fig. 4 shows the anion exchange chromatograms of fractions from these two regions. The new peptide in Fractions 14 to 15 (T-11) was not retained by the Dowex 1 column (Fractions 1 to 2, Fig. 4, A and B). It was sometimes accompanied by a ninhydrin-negative peptide having a greater Rf in paper chromatography. The second new peptide, designated T-23, was nicely separated from contaminating peptides (Fractions 21 to 22, Fig. 4, C and D). Peptides T-11 and T-23 were recovered in yields of 27 to 40% in several experiments. The compositions of T-11 and T-23 are given in Table I. It can be seen that the sum of the two equals peptide Pxy-1.

![Graph](http://www.jbc.org/)

**Table II**

Amino acid sequence of primary and secondary peptides derived from E. coli tryptophan synthetase β2 subunit

The arrow (→) designates residues positioned by the dansyl-Edman procedure. The lysyl residue indicated by the asterisk is actually ε-N-phosphopyridoxyllysine. Ascertainment of the amide residues is described in the text.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Pxy-1</td>
<td>Gly Asp Leu His Gly Gly Ala His Lys Thr Aen Gln Val Leu Gly Gln Ala Leu Ala Lys</td>
</tr>
<tr>
<td>Pxy-2</td>
<td>Arg Gly Asp Leu His Gly Gly Ala His Lys Thr Aen Gln Val Leu Gly Gln Ala Leu Ala Lys</td>
</tr>
<tr>
<td>T-22</td>
<td>Gly Asp Leu His Gly Gly Ala His Lys Thr Aen Gln Val Leu Gly Gln Ala Leu Ala Lys</td>
</tr>
<tr>
<td>T-23-TH1</td>
<td>Gly Asp Leu</td>
</tr>
<tr>
<td>T-23-TH2</td>
<td>Leu His Gly Gly Ala His Lys</td>
</tr>
<tr>
<td>T-23-C8TH2</td>
<td>Leu His Gly Gly Ala</td>
</tr>
<tr>
<td>T-11</td>
<td>Thr Aen Gln Val Leu Gly Gln Ala Leu Ala Lys</td>
</tr>
<tr>
<td>T-11-TH1</td>
<td>Thr Aen Gln</td>
</tr>
<tr>
<td>T-11-TH2</td>
<td>Val Leu Gly Gln Ala Leu</td>
</tr>
<tr>
<td>T-11-TH3</td>
<td>Leu Ala Lys</td>
</tr>
</tbody>
</table>
The method we have used, labeling with NaBaH₄, appears to
various means have been used to identify peptides containing
tide contains 1 asparaginyl and 2 glutaminyl residues.
Table II for T-11. Thermolysin cleavage followed by ion ex-
lished results.
of detecting such peptides.
us to be especially useful when limited amounts of material are
available. In our case the method appeared quite specific even
in the face of an irregular trypsin cleavage at the NH₂ terminus
and free arginine is also definitely present in the digest.2 Per-
fectly the presence of the strongly negatively charged cofactor
on the central lysine residue in the pyridoxal peptide protects
against further trypsin digestion when the initial cleavage occurs
between rather than distal to a pair of basic residues, such as
Lys–Arg or Arg–Arg. In any case we feel it highly unlikely that
the presence of two labeled peptides, Psy-1 and Psy-2, in the
reduced protein digest indicated molecular heterogeneity of the
β chain of the β₂ dimer, especially since the same situation has
been found with the β chain of the analogous P. putida enzyme
(10). Any such heterogeneity should have been apparent in
earlier studies of the electrophoretic behavior of fully reduced
and hybrid (half-reduced, half-normal) β₂ molecules (21).
In several attempts we detected no significant differences be-
tween the peptide maps of trypptic digests of reduced and non-
reduced β₂ subunit other than the peptides described in this
paper. Such differences as may be seen in Figs. 1 and 3 merely
represent variation in column behavior and chromatographic Rₖ values, and the use of a larger sample in the experiment of
Fig. 3. A comparison of the pyridoxal-P-binding region of
tryptophan synthetase with that of other pyridoxal-P-proteins
will be deferred to the companion paper (10) which presents the
sequence of an analogous region in P. putida. It should be noted
that an extensive series of well mapped missense mutations
affecting the enzymatic activity of the E. coli protein (22) opens
up the possibility of determining the functionally essential
residues in this region by a combination of genetic and protein
sequence analyses.

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DANSYLATION OF PSY-1 AND PSY-2—Dansylation of Psy-1 gave evidence of a single NH₂-terminal residue, glutamic acid or glutamine. The arginyl residue of Psy-2 was found to be NH₂-terminal by dansylation and by subtractive Edman degradation. Dansylation of the portion of Psy-2 remaining after a single Edman degradation step showed the same NH₂ terminus as Psy 1, glutamic acid or glutamine.

Sequence Analysis of T-23 and T-11—Analysis by dansylation showed glutamic acid or glutamine at the NH₂ terminus of T-23 and threonine at the NH₂ terminus of T-11. Sequential degradation of T-23 with dansylations of aliquots after each step and a subtractive analysis after steps 5 and 7 gave the sequence shown in Table II. Thermolysin digestion released the basic peptic, T-23-TI2, Leu-His-Gly-Ala-His-Lys, and one acidic peptide, T-23-TI1, Glu-Asp-Leu. These were easily separable by ion exchange chromatography. Brief digestion of T23 with carboxypeptidase B followed by thermolysin yielded one slightly basic fragment (T-23-CBTH2, Table II) and the acidic fragment seen previously, Glu-Asp-Leu. These two peptides were separated from free lysine and histidine by paper electrophoresis, eluted from the paper, and subjected to the dansyl-Edman procedure to confirm the sequence found for T-23. Finally, T-23 was digested with aminopeptidase M. On amino acid analysis, 1 molar equivalent of glutamic acid and 1 of aspartic acid were found, showing the absence of amide residues in this decapeptide.

Ten sequential steps of Edman degradation coupled with
dansyl end group analysis established the sequence shown in Table II for T-11. Thermolysin cleavage followed by ion exchange chromatography produced two neutral peptides (T-11-
TH1 and T-11-TH2) and one basic fragment (T-11-TH3) whose compositions are also given in Table II. Aminopeptidase M
digestion released neither aspartic nor glutamic acids, but the predicted amount of ninhydrin-positive material was eluted in
the serine region of the chromatogram, confirming that the
peptide contains 1 asparaginyl and 2 glutaminyl residues.

DISCUSSION
By now the technique of covalently attaching pyridoxal-P to
a lysine residue by borohydride reduction is well established. Various means have been used to identify peptides containing
the attached cofactor, such as absorption at 325 nm (8), fluores-
cence (7–9, 11), and a diagonal electrophoresis technique (9).
The method we have used, labeling with NaDBH₄, appears to
us to be especially useful when limited amounts of material are
available. In our case the method appeared quite specific when
in the face of a regular trypsin cleavage at the NH₂ terminus
of the peptide. The appearance of two new peptides in trypsin
digests of unreduced protein, representing the proximal and
distal halves of the tritiated peptide in the reduced protein digest,
confirms an earlier finding (11) that blocking an otherwise
susceptible trypsin cleavage point represents yet another means
of detecting such peptides.

At present we can offer no firm explanation for our failure to
observe two forms of the proximal "half-peptide." The amount
of T-23 found seemed comparable to the amount of T-11 present,
and free arginine is also definitely present in the digest.² Per-
haps the presence of the strongly negatively charged cofactor on

² R. Fluri, L. E. Jackson, W. E. Lee, and I. P. Crawford, unpub-
lished results.
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