Arginine Decarboxylase from Escherichia coli

IV. STRUCTURE OF THE PYRIDOXAL PHOSPHATE BINDING SITE*

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SUMMARY

A total of three pyridoxyl-peptides were isolated from the inducible arginine decarboxylase of Escherichia coli B following reduction with NaBH₄ and proteolysis with trypsin or chymotrypsin. Sodium borohydride reduces the Schiff base formed between pyridoxal-5'-P and the ε-amino group of a lysyl residue of the protein. The sequence analysis of the three peptides is consistent with a unique pyridoxal-P binding site which has the structure Ala-Thr-His-Ser-Thr-His-(P-Pxy)Lys-Leu-Leu-Asn-Ala-Leu-Ser-Gln-Ala-Ser-Tyr. This sequence has several residues (including a histidyl residue adjacent to P-pyridoxyl lysine) which are identical with or very similar to the corresponding residues of glutamate decarboxylase from E. coli W; their possible participation in catalysis is discussed.

Data are presented which demonstrate the power of a "differential" technique for the isolation of pyridoxyl-peptides; this approach, similar to the "diagonal" procedure of Brown and Hartley (Biochem. J., 89, 59P (1963)) is based on the altered elution of these peptides from ion exchange resins after treatment with alkaline phosphatase.

With the introduction of NaBH₄ as a means for covalently attaching the pyridoxal-P residue to the polypeptide chain of pyridoxal-P-dependent enzymes (1) by reduction of the Schiff base formed between the coenzyme and a unique lysyl residue on the protein and the recent adaptation of the "diagonal" technique of Brown and Hartley (2) to the isolation of P-pyridoxyl-peptides (3), information about the nature of pyridoxal-P binding sites has become readily accessible. Partial sequences are now known for the pyridoxal-P sites of phosphorylase (4), glutamate decarboxylase (3), tryptophanase (5), aspartate aminotransferase (6), tryptophan synthetase,1 and pyridoxamine pyruvate transaminase,2 an enzyme which acts on pyridoxal but does not contain pyridoxal-P.

The extent to which those residues near the lysine which binds pyridoxal-P are involved in the active site or contribute to the specific binding of the coenzyme is not known; the first step of enzymic catalysis is in fact the displacement of the ε-amino group of the lysyl residue by the substrate to form the substrate-pyridoxal-P Schiff base. In the P-Pxy⁺-peptides examined thus far, no obvious homologies which could potentially be correlated with function have been detected. However, these enzymes catalyze reactions (phosphorolysis of glycogen, decarboxylation, β-elimination and replacement, transamination, and the transaminase half-reaction in which pyridoxal is a substrate rather than a coenzyme) which have different mechanisms, and would probably be expected to have rather different active sites. We present here the amino acid sequence of a segment of the pyridoxal-P binding site of arginine decarboxylase (7-9), which catalyzes a reaction analogous to that of glutamate decarboxylase except for its substrate specificity.

EXPERIMENTAL PROCEDURE

Materials—Arginine decarboxylase was purified by the procedure of Boeker, Fischer, and Snell (9); protein determinations were performed in a Beckman DU spectrophotometer using an extinction coefficient, E₂₈₀ = 15.7 (7). Enzymic activity was assayed in 0.2 M sodium acetate buffer, pH 5.2, containing 0.15 M L-[U-1⁴C]arginine and 0.05 mm pyridoxal-P, using the technique of Morris and Pardee (10).

Chymotrypsin, alkaline phosphatase, diisopropylfluorophosphate-treated carboxypeptidase A, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were obtained from Worthington, L-[U-¹⁴C]arginine from New England Nuclear, and dansyl-Cl from Calbiochem. Dowex 1 and 50 (Bio-Rad) were previously treated according to Schroeder (11, 12) and the N-ethylmaleimide, α-picoline, and pyridine used in the elution buffers were redistilled. Sequanal grade phenylisothiocyanate and pyridine, and reagent grade trifluoroacetic acid (all from Pierce Chemical Company, Rockford, Illinois) were used in the Edman degradations.

Isolation of Pxy-peptides—Pxy-peptides were isolated from...

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1 J. P. Crawford, personal communication.

2 The abbreviations used are: P-Pxy and Pxy⁺, the phosphorylated and nonphosphorylated derivatives of the pyridoxyl moiety; dansyl-Cl, dimethylaminonaphthalenesulfonfonyl chloride.
both chymotryptic and tryp tic digests of arginine decarboxylase; all chromatographic and lyophilization steps were performed in the dark to minimize photodestruction of the pyridoxyl peptides. In each case, 8 ml of a 2% solution of NaBH₄ was added in two 4-ml portions to 740 mg of enzyme in approximately 40 ml of 0.01 M sodium phosphate, pH 7.0; NaBH₄ reduction destroys about 98% of the activity of arginine decarboxylase (7). After 15 min, the solution was centrifuged briefly to break up the foam, made 8 M in urea, allowed to stand for 15 min, and dialyzed exhaustively against distilled water. The chymotryptic (1:100, w/w; 5 hours) and tryptic (1:50, w/w; 21 hours) digestions were carried out at room temperature and pH 8.0, and maintained at this pH with a Radiometer TTT1 autotitrator; the resulting solutions were frozen and lyophilized.

The chymotryptic digest was dissolved in the pH 9.4 buffer of Schroeder (12), centrifuged to remove a very small amount of insoluble material, and applied to a column (1.0 x 100 cm) of Dowex 1-X2. The column (Fig. 1A) was developed at 38°C according to Schroeder (12) except that the gradient was expanded to include a pH 7.4 buffer. Fractions of 1.8 ml were collected and aliquots were analyzed with ninhydrin after alkaline hydrolysis (13). Fluorescence was determined in a Farrand spectrofluorometer following dilution in 0.5 M sodium phosphate buffer, pH 7.0. The fluorescent material eluting at pH 5.6 was pooled, lyophilized, dissolved in 1 ml of distilled water, adjusted to pH 8.0 with NaOH, and treated with 0.05 mg of alkaline phosphatase for 16 hours at room temperature. The pH was then adjusted to 10 with NaOH and the solution was applied to a column (0.6 x 60 cm) of Dowex 1-X2 which was developed as before but with appropriately reduced volumes of elution buffers (Fig. 1B). Since the phosphate group of the P-Pxy-peptide had been removed by alkaline phosphatase, the fluorescent peak now eluted at pH 7.4. This material was pooled, lyophilized, and dissolved in 1 ml of distilled water. Although the Pxy-peptide was now sufficiently pure for sequence analysis (see "Results"), the final traces of impurities were removed by chromatography on a column (0.6 x 60 cm) of Dowex 50-X2 as described by Schroeder (11). The Pxy-peptide eluted at pH 4.4 (Fig. 1C) and was pooled, lyophilized, and dissolved in 1 ml of distilled water.

Chromatography of the tryp tic digest of arginine decarboxylase on a column (1.0 x 100 cm) of Dowex 1-X2 produced two fluorescent peaks (Fig. 2A) which were then individually chromatographed on columns (0.6 x 60 cm) of Dowex 50-X2 (Figs. 2B and 3A), treated as before with alkaline phosphatase, and rechromatographed on columns (0.6 x 60 cm) of Dowex 1-X2 (Figs. 2C and 3B).

Sequence Analysis—Edman degradations were performed on a micro scale using the procedure of Gray (14) except that the phenylthiocarbamyl-amino acids were extracted with ethyl acetate. Samples for amino acid analysis were hydrolyzed in twice distilling water.
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Fig. 3. Isolation of an 11-residue Pxy-peptide from a tryptic digest of arginine decarboxylase. A, the fluorescent peak eluting at pH 5.5 in Fig. 2A was chromatographed on Dowex 50. B, the fluorescent peak emerging at pH 3.5 was treated with alkaline phosphatase and rechromatographed on Dowex 1. The composition of this peptide is shown in Table II. The fluorescent peak eluting at pH 3.7 in A was further purified and shown to be identical with the 17-residue Pxy-peptide of Fig. 2A.

TABLE I

<table>
<thead>
<tr>
<th>Isolation of tripeptide</th>
</tr>
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<tbody>
<tr>
<td>Residue</td>
</tr>
<tr>
<td>e-Pxy-lys</td>
</tr>
<tr>
<td>Leu</td>
</tr>
<tr>
<td>Asn</td>
</tr>
<tr>
<td>Ala</td>
</tr>
<tr>
<td>Thr</td>
</tr>
<tr>
<td>Ser</td>
</tr>
<tr>
<td>Gly</td>
</tr>
<tr>
<td>Others</td>
</tr>
</tbody>
</table>

The dansyl-Cl procedure of Gray (17) was used to determine amino-terminal residues. Dansyl amino acids were separated by thin layer chromatography on Silica Gel G with chloroform-ethanol-acetic acid (38:4:3) for acidic and neutral amino acids and the 1-butanol-pyridine-acetic acid-water (30:20:6:24) for histidine and e-Pxy-lysine (18).

Carboxypeptidase digestions (1:100 molar ratio) were carried out in 0.2 M N-ethylmorpholine acetate, pH 8.5, with noreleucine added as an internal standard; separate incubations were performed for each time point. One milliliter of 0.2 M sodium citrate, pH 2.2, was added to each sample to stop the reaction and amino acid analysis was begun immediately to minimize the cyclization of glutamine to pyrrolidone-carboxylic acid. Asparagine and glutamine were separated from serine, but not from each other, by performing the first part of the analysis at 30° instead of 55°.

RESULTS

Isolation of Pxy-peptides—As shown in Figs. 1 to 3, a single tripeptide was isolated from the chymotryptic digest of arginine decarboxylase (Table I), and two peptides, 11 and 17 residues long, from the tryptic digest (Tables II and III). As will be shown in the next section, the tripeptide was included in the 11-residue peptide, and, in turn, this peptide was wholly included in

tilled 5.7 N HCl at 108° for 16 to 20 hours in sealed, evacuated tubes. Analyses were performed on a Beckman model 120C amino acid analyzer using the system described by Spackman, Stein, and Moore (15) for acidic and neutral amino acids and the system described by Strausbauch and Fischer (3) for basic amino acids including e-Pxy-lysine. The values given for e-Pxy-lysine have not been corrected for destruction during acid hydrolysis; the recovery of e-Pxy-lysine from the dipeptide (e-Pxy)Lys-Phe, and e-Pxy-lysine (18).

The results are shown in Table II. The values for the acidic and neutral amino acids are given in Table II. The values for the amino-terminal residues of both the tripeptide and the 11-residue peptide were found to be alanine. The stoichiometry of the tripeptide was found to be Ala-Lys-Leu, and of the 17-residue peptide, Ala-Lys-Leu-Pxy-lysine. Attempts to measure the yield of fluorescent material after each purification step were not sufficiently reproducible to provide useful information. Based on the amino acid analyses of the pure peptides, the over-all yield was 25% (2.3 μmoles) for the tripeptide, 17% (1.5 μmoles) for the 11-residue peptide, and 2% (0.2 μmole) for the 11-residue peptide. The average yield of each step is therefore about 60%, which is reasonable in view of the sensitivity of P-Pxy-peptides to light (16) and lyophilyzation (3).

Neither of the tryptic peptides contains arginine or lysine, probably as a result of non-specific side or the prolonged digestion (21 hours at 1:50, w/w). A single peptide, containing one arginine and 31 other residues, was isolated from a 5-hour tryptic digestion at 1:100, w/w. However, the composition of this peptide was not appreciably altered after 7 cycles of the Edman degradation and coupling the product with dansyl-Cl; thin layer chromatography on Silica Gel G with chloroform-ethanol-acetic acid (38:4:3) showed that the resulting dansyl-peptide cochromatographed with dansyl leucyl leucine rather than with dansyl-leucine.

Sequence of Pyridoxal-P Binding Site—Using dansyl-Cl, the NH2-terminal residues of both the tripeptide and the 11-residue peptide were found to be Pxy-lysine, and that of the 17-residue peptide was found to be alanine. The stoichiometry of the tripeptide, Pxy-Lys, Leu (Table I), was confirmed by carrying out 1 cycle of the Edman degradation and coupling the product with dansyl-Cl; thin layer chromatography on Silica Gel G with chloroform-ethanol-acetic acid (38:4:3) showed that the resulting dansyl-peptide cochromatographed with dansyl leucyl leucine rather than with dansyl-leucine.
Subtractive Edman degradations were carried out on the 11-residue peptide until the supply was exhausted; the results (Table II) show that the NH$_2$-terminal sequence is the same as that of the tripeptide and establish the next 3 residues unambiguously. Data for the 17-residue peptide are shown in Table III. After 6 cycles, the composition of this peptide corresponded exactly to that of the 11-residue peptide, and the next residue (Cycle 7) was Pxy-lysine, as expected. In order to conserve material, no analysis was performed after Cycles 8 through 11; the composition after Cycle 12 was consistent with a loss of about 50% of each of the expected residues, namely, asparagine (1), alanine (1), and leucine (3). The 13th residue was clearly serine; the composition of the remaining peptide was essentially unchanged after Cycles 14 and 15. It is possible, therefore, that the 14th residue is a glutamine which underwent cyclization to pyrrolidone-carboxylic acid.

The apparent sequence in the vicinity of the pyridoxal-P binding site of arginine decarboxylase is shown in Table IV, together with previously published reports of the isolation of Pxy-peptides. Details of the digestion and separation procedure are given under "Experimental Procedure"; the amino acid analyzer system used separates asparagine and glutamine from serine but not from one another. Leucine was also released at a rate intermediate between that of alanine and asparagine plus glutamine; no other residue, in particular no aspartic or glutamic acid, was released.

**TABLE III**

<table>
<thead>
<tr>
<th>Residue</th>
<th>Composition after cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-Pxy-lys</td>
<td>0.78 0.78 0.83 0.83 0.81 0.90 0.16 0.16</td>
</tr>
<tr>
<td>His</td>
<td>1.97 1.93 1.21 1.14 1.00 0.38 0.18 0.16</td>
</tr>
<tr>
<td>Asx</td>
<td>1.09 1.10 1.17 1.12 1.31 1.30 1.32 0.83 0.83 0.78 0.76</td>
</tr>
<tr>
<td>Glx</td>
<td>1.13 1.11 1.13 1.08 1.19 1.21 1.27 (1.16) (1.16) (1.16)</td>
</tr>
<tr>
<td>Thr</td>
<td>1.97 1.64 1.14 1.05 0.46 0.43 0.40 0.76 0.78 0.67</td>
</tr>
<tr>
<td>Ser</td>
<td>2.82 2.67 2.84 2.60 2.18 2.34 2.52 2.65 1.47 1.55</td>
</tr>
<tr>
<td>Ala</td>
<td>3.02 1.86 2.14 2.10 2.14 2.15 2.16 1.51 1.48 1.42</td>
</tr>
<tr>
<td>Leu</td>
<td>3.00 3.00 3.00 3.00 3.00 3.00 3.00 3.00</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.80 0.77 0.77 0.77 0.72 0.71 0.73 0.27 0.44 0.37</td>
</tr>
</tbody>
</table>

a In addition to those shown, 1.06 residues of NH$_4$, 0.21 residue of glycine, and 0.04 residue of the other amino acids were found.

b The data shown in Columns 12 to 14 were obtained in a second, later degradation.

c An essentially identical composition was also obtained after cycle 15.

Shown by dansylation to be NH$_2$-terminal; the 2nd residue is therefore threonine.

Fig. 4. Digestion of the 17-residue Pxy-peptide with carboxypeptidase. Details of the digestion and separation procedure are given under "Experimental Procedure"; the amino acid analyzer system used separates asparagine and glutamine from serine but not from one another. Leucine was also released at a rate intermediate between that of alanine and asparagine plus glutamine; no other residue, in particular no aspartic or glutamic acid, was released.
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**Table IV**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> arginine decarboxylase</td>
<td>Pxy Ala-Thr-His-Ser-Thr-His-Lys-Leu-Asn-Ala-Leu-Ser-Gln-Ala-Ser-Tyr</td>
</tr>
<tr>
<td><em>Escherichia coli</em> glutamate decarboxylase</td>
<td>Pxy Ser-Ile-Ser-Ala-Ser-Gly-His-Lys-Phe</td>
</tr>
<tr>
<td><em>Escherichia coli</em> tryptophanase</td>
<td>Pxy Ser-Ala-Lys-Lys-Asp-Ala-Met-Val-Pro-Met</td>
</tr>
<tr>
<td>Pig heart aspartate aminotransferase</td>
<td>Ala-Ser-Lys-Asn-Phe</td>
</tr>
<tr>
<td>Extramitochondrial (6)</td>
<td>Pxy Ala-Lys-Asn-Met</td>
</tr>
<tr>
<td>Mitochondrial (6)</td>
<td>Pxy Val-Thr-Gly-Pro-Asp-Lys-Cys-Leu</td>
</tr>
<tr>
<td><em>Pseudomonas</em> pyridoxamine pyruvate transaminase</td>
<td>Pxy Ala-Ser-Gly-Thr-Gly-Asp-Met-Lys-Phe-Met-Gly-Arg-Thr-Leu</td>
</tr>
</tbody>
</table>

with sequences from other enzymes which are pyridoxal-P-dependent or use pyridoxal as a substrate. Since glutamate and arginine decarboxylases have quite different structural characteristics (Table V) but catalyze the same type of reaction, it is possible that the similarities in their Pxy-peptides represent catalytically important features. Of the 8 corresponding residues known for each enzyme, 3 are identical (Ser, His, Lys) and 2 represent conservative substitutions (Thr-Ser and Leu-Phe) in terms of both function and the genetic code. No other pair of peptides in Table IV shows this much homology. In an attempt to gain further insight into the significance of these similarities, the structure of the Pxy-peptide of lysine decarboxylase from *Escherichia coli* B is currently being determined.4

A pattern which emerges from the peptide sequences shown in Table IV is the occurrence of a histidine residue immediately adjacent to Pxy-lysine in the two decarboxylases and the γ chain of tryptophan synthetase. As pointed out by Strausbauch and Fischer (3), this residue could either participate in a proton transfer reaction or, by ion pair formation, provide a binding site for the carboxyl group of the substrate or pyridoxal-P itself. A common feature of the mechanism of both α-decarboxylation and β-replacement reactions (for recent reviews, see Boeker and Snell (23) and Snell and DiMari (24)) is the requirement for specific protonation of the substrate α-carbon in the Schiff base intermediate formed just prior to the release of products. If, as in the case in nonenzyme pyridoxal catalyzed α-decarboxylation reactions (23) and the β-decarboxylation catalyzed by aspartate β-decarboxylase (for a recent review, see Tate and Meister (28)), this protonation is not specific; transamination can occur, leading to an inactive, pyridoxamine-P form of the enzyme. Of the peptides shown in Table IV, only the first three are from enzymes which have a mechanism requiring this specific protonation step. Other pyridoxal-P dependent enzymes in this class catalyze aldol cleavage, racemization, and γ-replacement reactions, but not

4 D. Sabo, unpublished results.
γ-elimination reactions; structures of Pxy-peptides from such enzymes are not yet available.

Kalyankar and Snell (25) have pointed out that ion pair formation between a positively charged group and the α-carboxyl would probably inhibit decarboxylation by reducing the negative charge on the leaving group; this role for a histidine residue thus appears to be unlikely. On the other hand, ion pair formation between the phosphate group of pyridoxal-P and a positively charged residue might very well contribute to the binding of the coenzyme; it may be noteworthy that there is a basic residue sufficiently close to the pyridoxal-P to fulfill this role in each of the peptides shown in Table IV except for aspartate transaminase, in which only 4 residues are known, and pyridoxamine pyruvate transaminase, in which such ion pair formation could not occur. Of course, binding of the coenzyme most certainly involves multiple interactions with other side chains far removed in terms of primary structure from the lysyl residue to which pyridoxal-5’-P is attached.

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