THE CHYMOTRYPTIC PHOSPHOPYRIDOXYL PEPTIDE OF DOPA DECARBOXYLASE FROM PIG KIDNEY

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Summary. The amino acid sequence of the coenzyme-binding site of DOPA-decarboxylase from pig kidney has been determined. A sample of enzyme was reduced with $NaBH_4$, aminoethylated and then digested with chymotrypsin. A single phosphopyridoxyl peptide was isolated and its sequence proved to be: Asn-Phe-Asn-Pro-His-Lys(Pxy)-Trp. Sequence homologies at the active site of various pyridoxalphosphate enzymes, and particularly of the bacterial decarboxylase, are discussed ,with some emphasis on the constant presence of a histidine residue adjacent to the phosphopyridoxyl-lysine.

Since the discovery that pyridoxyl-P serves as an irreversible bound label if its Schiff base linkage to a lysine side chain is first reduced with $NaBH_4$(1) the primary structure of the pyridoxyl-peptides has been determined for several B_6-dependent enzymes with the hope that discovery of possible similarities between these structures could prove useful in suggesting general considerations on the structure-function relationships of these enzymes.

The enzymes so far examined from this point of view are representative of the various type of reactions requiring pyridoxal-P as a cofactor and include, in addition to phosphorylase (1,2), the isozymes of aspartate aminotransferase (3-6), various decarboxylases (7-10), tryptophanase and tryptophan synthetase (11-14), pyridoxamine-pyruvate transaminase (see (9)), D-serine dehydratase

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(15) and serine transhydroxymethylase (16).

In particular the sequences of the phosphopyridoxyl peptides of four decarboxylases purified from E.coli have been determined (7-10) and this represents the only example known for pyridoxal-P enzymes which are from the same source and which catalyse a single type of reaction.

In order to assess the significance of the homology found in the sequences of these bacterial enzymes it seemed of some importance the determination of the active-site sequence of a DOPA-decarboxylase which is purified from a different source, i.e., a mammalian one, and with a different substrates specificity, i.e. aromatic amino acids.

Materials and methods

L-DOPA decarboxylase was purified from pig kidney as previously described (17). Chymotrypsin, E.coli alkaline phosphatase and diisopropyl fluorophosphate-treated carboxy-peptidase A were obtained from Worthington. Hog kidney aminopeptidase was from P.L. Biochemicals and iminopeptidase (prolidase) from Miles. DOPA decarboxylase (about 10 mg/ml in 0.1M phosphate buffer, pH 6.8) was reduced by dialysis against 5 mM $NaBH_4$ in the same buffer at 4°C for 30 min, followed by extensive dialysis against distilled water and then freeze-dried.

This and subsequent operations were performed in the dark to minimize photodestruction of the chromophore. The reduced enzyme was aminoethylated according to Cole (18), extensively dialyzed against distilled water and then freeze-dried. A 250 mg portion of reduced and aminoethylated DOPA decarboxylase was digested with 5 mg of chymotrypsin for 8 h at 37°C in 0.1M ammonium bicarbonate. Digestion was stopped by acidification with acetic acid (final concentration 10% by volume); preliminary fractionation of the peptide mixture was performed by gel filtration through a Sephadex G-25 (fine) column (4x130 cm) eluted with 10% acetic acid.

The effluent was monitored for transmittance at 254 nm (Uvicord I, LKB) and for absorbance at 325 nm. Further purification of the phosphopyridoxyl peptide was accomplished by ion-exchange chromatography on Chromobead P (Technicon) resin according to the procedure of Bossa et al. (19). The effluent was monitored by a combination of the following techniques: transmittance at 280 nm (Uvicord II, LKB), absorbance at 325 nm, analytical thin layer chromatography on cellulose plates developed in 1-butanol-acetic acid-water-pyridine (15:3:12:10, by volume) examined under U.V.

light for localization of the blue fluorescent spots typical of pyridoxyl peptides.

Final purification was achieved by a second ion exchange chromatography after removal of the phosphate group of the phosphopyridoxyl peptides with alkaline phosphatase according to the technique described by Strausbauch and Fischer (7). Amino acid compositionswere determined using an LKB 3201 instrument (single column system) after hydrolysis with 6 N HCl at 110°C for 24 h in tubes thoroughly flushed with nitrogen before evacuation and sealing. Peptide sequence was established using the dansyl-Edman technique with identification of dansyl-amino acids on polyamide plates (20).

Carboxypeptidase A digestions of the peptide were performed according to Ambler (21).

Aminopeptidase digestions were performed at 37°C in 0.1M Tris/HCl, pH 8.0 with an enzyme: substrate ratio of 5 μg/25 nmol, with or without the addition of prolidase (30 μg/25 nmol) and 10 mM (final) $MnCl_2$.

Amino acids released were determined quantitatively on the amino acid analyzer.

RESULTS AND DISCUSSION

The elution pattern from Sephadex G-25 of the peptide mixture obtained after chymotryptic digestion of reduced aminoethylated DOPA-decarboxylase is shown in fig. 1. Several fractions with the absorbance characteristic of phosphopyridoxyl peptides were obtained; only the major one, indicated by a solid bar, could be successfully subjected to further purification procedures, which consisted in a column chromatography on Chromobead P resin; tubes containing material absorbing at 325 nm were pooled and lyophilized (fig. 2, A, solid bar). This material, after treatment with alkaline phosphatase, was rechromatographed on the same column under identical conditions (fig. 2, B) as outlined under Materials and Methods.

The amino acid analysis of the purified peptide is: Asp, 1.7; Pro, 0.8; Phe, 1.0; His, 1.1; Lys (Pxy), 1.0. The presence of 1 mol of Trp per mol of peptide was revealed by the U.V. spectrum of the peptide. Peptide sequence was determined by the dansyl-Edman procedure and found to be: Asx-Phe-Asx-Pro-His-Lys(Pxy). The presence of the tryptophan residue after the pyridoxyl lysine, at the C-terminus of the peptide was determined by carboxypeptidase A

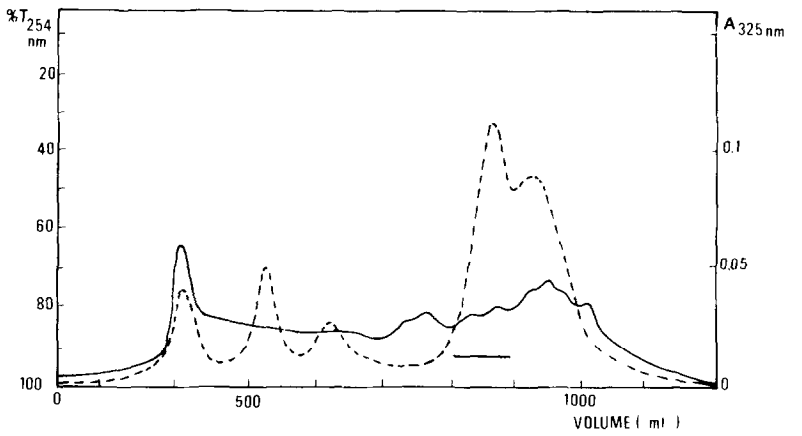

Figure 1 - Fractionation on Sephadex G-25 of the peptide mixture after chymotryptic digestion of reduced, aminoethylated DOPA-decarboxylase. Transmittance (254 nm), ———— and absorbance (325 nm), ..., profile. The bar indicate the fraction pooled.

digestion of an aliquot of the material.

Extensive aminopeptidase digestion released equimolar amounts of asparagine and phenylalanine while digestion with aminopeptidase plus prolidase released: Asp (0.4), Asn (1.6), Pro (1.0), His (1.0), Lys (0.5) and Trp (0.5) (moles of aminoacid released per mole of peptide). Since the Asn released is higher than unity, it may be inferred that both Asx residues are asparagine; the small amount of aspartic acid should be considered as coming from partial deamidation of the peptide. The whole of these results indicates the following sequence around the lysine residue that participates in binding of the coenzyme in DOPA-decarboxylase: Asn-Phe-Asn-Pro-His-Lys(Pxy)-Trp.

This sequence is reported in Table I together with those of phosphopyridoxyl peptides of the bacterial decarboxylases so far examined. Comparison of the latter sequences has already been attempted; for example the predominance of charged and hydroxylated residues on the amino aide has been observed (9). This observation is confirmed by the data hereby reported for DOPA decarboxylase; moreover, the presence of a tryptophan residue, not found in any

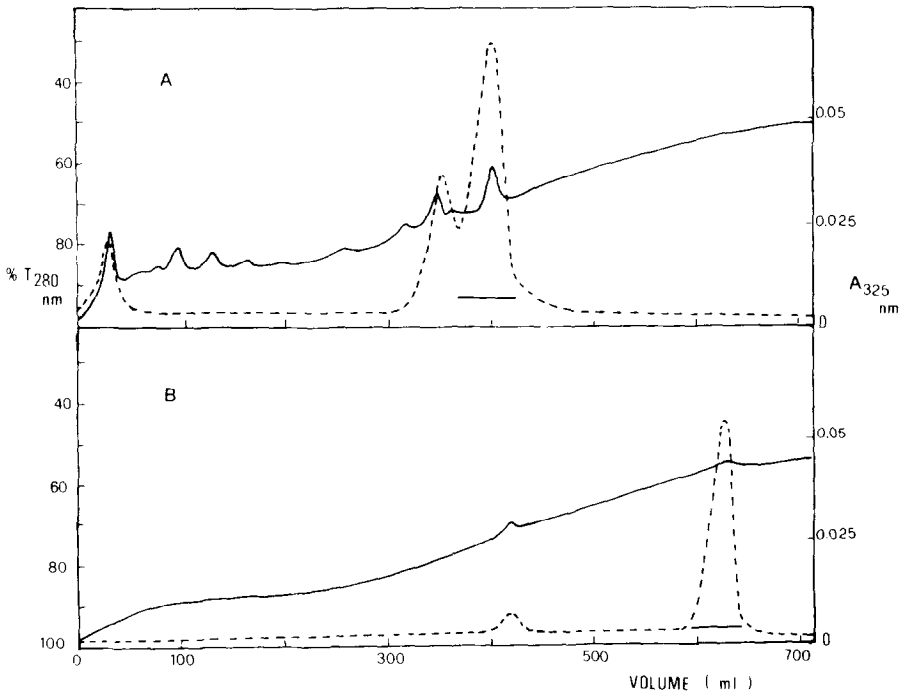

Figure 2 - Isolation of the pyridoxyl-peptide from the Sephadex G-25 fraction. A, chromatography on Chromobead P resin. The major fraction absorbing at 325 nm, was treated with alkaline phosphatese and then rechromatographed (B) under identical experimental conditions. Transmittance (280 nm), ——— and absorbance (325 nm), ..., profile. Bars indicate the fractions pooled.

other decarboxylase, adjacent to the carboxylic side of pyridoxyl-lysine deserves some attention, considering the specificity for aromatic aminoacid substrates of this enzyme.

A finding of particular interest in DOPA-decarboxylase is the occurrence of a histidine residue immediately adjacent to the aminic side of pyridoxyl-lysine as it has been found in the four bacterial decarboxylases (7-10), in the β chain of bacterial tryptophan sinthetases (12,13) and, more recently, in the mammalian serine transhydroxymethylase (16). This finding should not be considered fortuitous, in view of the different catalytic function and the various origin of the enzymes. It has already been suggested (8) that this residue could play a catalytic role in enzymes

TABLE I

Amino acid sequences of pyridoxyl peptides from coenzyme-binding sites of various amino acid decarboxylases.

Enzyme and reference	Source	Sequence
L-DOPA decarboxylase	Pig kidney	Asn-Phe-Asn-Pro-His-Lys(Pxy)-Trp
Arginine decarboxylase (8)	E.coli	Ala-Thr-His-Ser-Thr-His-Lys(Pxy)-Leu-Leu-Asn-Ala-Leu-Ser--Gln-Ala-Ser-Tyr
Lysine decarboxylase (9)	E.coli	Val-Ile-Tyr-Glu-Thr-Glu-Ser-Thr-His-Lys(Pxy)-Leu-Leu-Ala-Ala-Phe
Ornithine decarboxylase (10)	E.coli	Val-His-Lys(Pxy)-Gln-Gln-Ala-Gly-Gln
Glutamate decarboxylase (7)	E.coli	Ser-Ile-Ser-Ala-Ser-Gly-His-Lys(Pxy)-Phe

that share, as common feature, the requirement for specific protonation of the substrate α-carbon in the Shiff base intermediate formed just prior to the release of the product. Alternatively,the histidine residue could provide a binding site for the coenzyme by ion pair formation with the phosphate groups, as observed in space-filling models or for the carboxyl group of the substrate (7,8,11, 15).

In this respect it should be noticed that the presence of group at the active site with a pK of 6.7 has been inferred from a study of the variation of the dissociation constant of thiosemicarbazide for DOPA decarboxylase as function of pH (22).

It is important to remember that recently in different pyridoxal-dependent enzymes the protonation of the cofactor has been shown to proceed stereospecifically and with the same absolute stereochemistry. This regularity suggests similar regularity in the geometry of coenzyme binding, a fact that could be interpreted as evidence for the evolution of this entire family of enzymes from a common ancestor (23). The homologies found between the sequence around the coenzyme binding lysine of DOPA decarboxylase and those of other pyridoxal-P enzymes increase the experimental basis for this hypothesis.

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