The Pyridoxal-Binding Site in Pyridoxamine–Pyruvate Transaminase

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The enzyme–substrate complex formed between pyridoxamine–pyruvate transaminase (EC 2.6.1.30) and pyridoxal was reduced with NaBH₄. After carboxymethylation and tryptic digestion, pyridoxyl-lysine-containing peptides were isolated by a combination of Sephadex and Dowex 50 chromatography. Analysis of these peptides shows the structure around the pyridoxal-binding lysine residues to be Ala-Asp-Ile-Tyr-Val-Thr-Gly-Pro-Asx-Lys(Pxy)-Cys-Leu(Pro₂, Gly₂, Ala₂, Met)(Thr, Leu₂)Gly-Val-Ser-Glu-Arg. This structure differs from those found for the corresponding peptides from pyridoxal phosphate-dependent enzymes.

Pyridoxamine–pyruvate transaminase (EC 2.6.1.30) catalyses reaction (1) and is unique among transaminases so far studied in not containing pyridoxal phosphate.

Mechanistic investigations (Ayling & Snell, 1968) show that the substrates pyridoxal and pyridoxamine play the same role in reaction (1) as the more tightly bound coenzymes pyridoxal phosphate and pyridoxamine phosphate play in transamination reactions such as reaction (2), which is catalysed by aspartate aminotransferase (EC 2.6.1.1).

noteworthy because it forms part of the active site of the enzyme, and the corresponding sequences from several pyridoxal phosphate-dependent enzymes are available for comparison.

Materials and Methods

Chemicals

Iodoacetic acid was recrystallized from light petroleum (b.p. 60–70°C) before use. Pyridine was refluxed with either ninhydrin or phthalic anhydride, then distilled. Phenyl isothiocyanate was redistilled under vacuum.

Indeed, reaction (1) may be considered a model for the half-reactions (2a) and (2b), catalysed by the coenzyme-dependent transaminases.

In the intermediate enzyme–pyridoxal complex formed during reaction (1), the formyl group of pyridoxal forms an azomethine linkage to an ϵ -amino group of lysine; borohydride reduces this bond to form a stable inactive pyridoxyl-protein, from which *N*⁶-pyridoxyl-lysine can be isolated by acid hydrolysis (Dempsey & Snell, 1963). The present study reports the isolation of pyridoxyl-peptides from enzymic digests of the pyridoxyl-protein and the partial amino acid sequence around the pyridoxyl-lysine residue. This portion of the peptide chain is especially

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Lys(Pxy), *N*⁶-pyridoxyl-lysine; Cm-Cys, carboxymethylcysteine.

Purification of pyridoxamine–pyruvate transaminase

The purification procedure of Wada & Snell (1962) was used with a few modifications. *Pseudomonas* MA-1 was cultured as described by Ayling & Snell (1968) in pyridoxine-containing media. The expense of this medium, along with losses incurred by the multiple modification of the enzyme, limited the amount of protein available for structural analysis. The enzyme was crystallized three times from 50%-saturated (NH₄)₂SO₄ solutions at 4°C.

Analytical procedures

Most amino acids were analysed on a Beckman 120B amino acid analyser. During acid hydrolysis pyridoxyl-lysine is partially (about 30%) converted into lysine. Tryptophan and *N*⁶-pyridoxyl-lysine were

determined spectrally. Paper electrophoresis was done at either pH 3.5 or pH 6.5. Peptides were located by observing fluorescence of the pyridoxyl residue, by radioautography of the [^{14}C]carboxymethyl group with Kodak No-Screen X-ray film, or with ninhydrin as described by Heilmann *et al.* (1957).

Dowex 50W-X2 columns (0.9 cm $\times$ 48 cm) were eluted at 29 ml/h with a linear gradient of 250 ml each of: (A), 16 ml of pyridine plus 400 ml of acetic acid, made up to 1 litre with distilled water (final pH 2.8); (B), 100 ml of pyridine plus 69 ml of acetic acid plus 450 ml of distilled water (final pH 5.0); (C) 600 ml of pyridine plus 100 ml of acetic acid plus 300 ml of distilled water (final pH 5.7) (Schroeder *et al.*, 1962).

N-Termini were identified by the dansyl chloride method (Gray & Hartley, 1963), and chromatography on polyamide sheets (Woods & Wang, 1967) with 90% (v/v) formic acid/water (3:200, v/v), benzene/acetic acid (9:1, v/v) and *n*-heptane/butan-1-ol/acetic acid (3:3:1, by vol.) as solvents. For dansylarginine, dansylhistidine or monodansyllysine, a 3:1 (v/v) mixture of ethanol and pyridine/acetate buffer [pyridine/acetic acid/water (1:2:250, by vol.), pH 4.4] was used as one solvent. Dansyl-*N*⁶-pyridoxyl-lysine was partially decomposed to α -dansyl-lysine during hydrolysis.

Sequential degradation was by the dansyl-Edman method of Gray (1967). For identification of phenylthiohydantoins, butyl acetate extracts were dried and hydrolysed in 0.1 M-NaOH as described by Van Orden & Carpenter (1964), and then applied to the amino acid analyser.

Digestion of peptides

For chymotryptic digestion, peptide was dissolved in 0.5 M-Tris/HCl/0.02 M-CaCl₂ buffer, pH 7.9. Chymotrypsin in 1 mM-HCl was added and digestion done at 26°C for 60–80 min.

For carboxypeptidase digestion, 2 μ l of carboxypeptidase A suspension or 30 μ l of carboxypeptidase B was dissolved in 0.1 ml of 10% LiCl. Portions (10–20 μ l) of these solutions were added to the peptide after it was dissolved in 0.1 ml of either 0.05 M-sodium barbital/0.20 M-NaCl/HCl buffer, pH 7.5, or 0.025 M-Tris/HCl/0.5 M-NaCl buffer, pH 7.5. After 5–300 min at 37°C, this digestion was terminated by adding sodium citrate buffer, pH 2.2, in which the sample was to be applied to the amino acid analyser. Blanks (omitting substrate) were always run.

Preparation and tryptic digestion of the protein derivative

About 275 mg (1.83 μ mol) of transaminase was dialysed against 0.02 M-potassium phosphate buffer, pH 7.0. Sufficient pyridoxal (7.0 μ mol) was added to saturate the two binding sites per molecule of enzyme

as monitored by measuring the A_{415} as described by Dempsey & Snell (1963). Reduction of the azomethine linkage was carried out with 0.04 M-NaBH₄ (28 μ mol) and followed to completion by monitoring the decrease in A_{415} . Only 0.4% of the enzyme activity remained after reduction. Acetic acid was used to decompose excess NaBH₄. Freeze-dried reduced enzyme dissolved in 20 ml of deionized 9.8 M-urea was added to NH₄HCO₃ (158 mg) in sufficient 1 M-NH₃ to adjust the pH to 8.13. After 6 h at 25°C, 1.16 mg of dithiothreitol was added and incubation continued for 40 min. Iodo[^{14}C]acetate (5 mg; 120 μ Ci) in 0.2 ml of 0.2 M-NaOH was added followed by 1.0 ml of 0.2 M-HCl to adjust the pH to 8. After 40 min, an additional 1.16 mg of dithiothreitol was added, followed by an excess (26 mg) of unlabelled iodoacetate in 1.0 ml of 0.2 M-NaOH and then 0.5 ml of 0.2 M-HCl. After 2 h the reaction was terminated with 0.2 ml of 2-mercaptoethanol and the mixture dialysed against 0.1 M-ammonium acetate, pH 8.5. The protein derivative, which was precipitated during dialysis, was freeze-dried. The dry protein was dissolved in 20 ml of water at pH 11.6, but 0.2 M-HCl was added without delay to bring the pH to 9.5, at which point the solution became turbid. Then 4.4 mg of 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK)-treated trypsin in 1 mM-HCl/5 mM-CaCl₂ was added. The turbidity disappeared quickly. After 350 min at 29°C an additional 4.4 mg of trypsin was added. After a total of 630 min, when the pH had become constant (pH 8.3), the digest was freeze-dried.

Purification of tryptic peptides

The tryptic digest was dissolved in 40% (v/v) acetic acid with enough HCl to lower the pH to 2.2. The digest was placed on a column (2 cm $\times$ 190 cm) of Sephadex G-50 (fine) and eluted with 10% acetic acid. Peptides that contained most (90%) of the *N*⁶-pyridoxyl-lysine (determined by measuring A_{322}) were in fraction T-II, which also contained one-third of the radioactivity. Fraction T-II was resolved into fractions T-II-1 to T-II-8 by passage over Dowex 50W-X2, and each of these fractions was passed through Sephadex G-25 in 10% acetic acid. The bulk of the 322 nm-absorbing material (58%) and radioactivity (64%) of fraction T-II was divided equally between the two major fractions T-II-3 and T-II-5, each of which had a constant ratio of radioactivity/ A_{280} throughout the single peak seen on Sephadex chromatography; each had a single *N*-terminus. The remainder of the 322 nm-absorbing material and radioactivity was contained in several minor fractions, some of which were due to incomplete carboxymethylation, whereas the others were probably derived from the unique pyridoxyl sequence by amide loss or other side reactions, including extraneous enzymic cleavage.

Results and Discussion

Peptide T-II-3 gave *N*-terminal alanine by the dansyl method. No other α -dansyl-amino acids were detected. Mixed carboxypeptidase A and B released 2.1 leucine residues, 1.1 threonine residue and smaller amounts of alanine (0.5) and methionine (0.7), suggesting a *C*-terminus of (Ala, Met)(Thr, Leu₂).

A chymotryptic digest of peptide T-II-3, chromatographed on Dowex 50W-X2, gave two major fractions, the first of which contained about 60% of the radioactivity. This fraction was chromatographed on a column (2cm×45cm) of Sephadex G-25 (fine) to resolve a radioactive peptide T-II-3 C1 and a non-radioactive peptide T-II-3 C2; the second fraction from Dowex 50 on similar purification yielded one peptide designated T-II-3 C3. No other peptides were found in amounts large enough to be purified. The amino acid compositions of these chymotryptic peptides (hereafter referred to as 3 C1–3 C3) are shown in Table 1.

Peptide 3 C2 is from the *N*-terminus of its parent peptide T-II-3, since it was the only peptide with *N*-terminal alanine. Peptide 3 C1 arose from the *C*-terminus of peptide T-II-3, since its digestion with carboxypeptidase released leucine and threonine in the ratio 2.3, along with smaller amounts of alanine and methionine. Peptide 3 C3 was derived from peptide 3 C1, since both contain the unique pyridoxyl-lysine and carboxymethylcysteine residues. This relationship was supported by dansyl analysis

and by carboxypeptidase A+B digestion, which showed peptide 3 C3 to have *N*-terminal valine and *C*-terminal CmCys-Leu. Dansyl-Edman analysis revealed peptide 3 C1 to be Val-Thr-Gly-Pro-Asx-Lys(Pxy)-CmCys-. (Dansyl-*N*⁶-pyridoxyl-lysine was not observed directly for the sixth residue, but a faint spot corresponding to its breakdown product α -dansyl-lysine was observed.)

The known specificity of chymotrypsin allows the structure of peptide 3 C2 to be written as Ala(Asx, Ile)Tyr, and, since peptides 3 C2 and 3 C1 account for the entire composition of peptide T-II-3, the latter must have the partial structure Ala-(Asx, Ile)-Tyr-Val-Thr-Gly-Pro-Asx-Lys(Pxy)-CmCys-Leu-(Pro₂, Gly₂, Ala₂, Met)(Thr, Leu₂).

Peptide T-II-5 appears to contain the sequence of peptide T-II-3 with five additional residues at the *C*-terminus. Dansyl-Edman degradation and carboxypeptidase digestion revealed the sequence Ala-Asx-Ile-Tyr-Val-Thr-Gly-Pro-Asx-Lys(Pxy)-CmCys-Leu-(Pro₂, Gly₂, Ala₂, Met)(Thr, Leu₂)(Ser, -Glx, Gly, Val)-Arg. This peptide was digested with chymotrypsin and fractionated on a column (2cm×196cm) of Sephadex G-25. The first peptide T-II-5 C1 was identical with peptide 3 C1 according to its composition and the amino acids released by carboxypeptidase A digestion. Peptide T-II-5 C2 had the same composition as peptide 3 C2, and they were assumed to be identical. It released only 1mol.prop. of isoleucine and 1mol.prop. of tyrosine on digestion with carboxypeptidase A. Electrophoresis of peptide

Table 1. *Amino acid compositions of various peptides*
Numbers in parentheses are the integral numbers assumed in the structure finally proposed.

Amino acid	Peptide	Amino acid composition (mol/mol of peptide)				
		T-II-3	T-II-5	T-II-3 C1	T-II-3 C2	T-II-3 C3
CmCys	...	0.6 (1)	0.8 (1)			0.4 (1)
½CyS		0 (0)	0 (0)	0.5 (1)		
Asp		2.0 (2)	2.3 (2)	1.1 (1)	1.0 (1)	1.0 (1)
Thr		1.7 (2)	1.9 (2)	1.7 (2)		0.9 (1)
Ser		0.5 (0)	1.3 (1)			
Glu		0.6 (0)	1.4 (1)			
Pro		2.9 (3)	3.3 (3)	3.0 (3)		1.2 (1)
Gly		2.9 (3)	3.7 (4)	3.0 (3)		1.4 (1)
Ala		3.0 (3)	3.3 (3)	1.5 (2)	1.1 (1)	
Val		1.3 (1)	2.3 (2)	1.0 (1)		0.9 (1)
Met		0.6 (1)	0.7 (1)	0.4 (1)		
Ile		1.2 (1)	1.3 (1)		1.0 (1)	
Leu		2.9 (3)	3.3 (3)	2.8 (3)	0.3	1.1 (1)
Tyr		0.8 (1)	1.0 (1)		1.0 (1)	
Lys		0.3*	0.3*	Trace*		0.2*
Lys(Pxy)		0.8*(1)	1.2 (1)	1.3 (1)		0.6 (1)
Arg		0 (0)	0.8 (1)			

* *N*⁶-Pyridoxyl-lysine is partially converted into lysine during acid hydrolysis. The intact molecule emerged in the position of histidine.

T-II-5 C2 at pH 6.4 showed that it was anionic and therefore contained an aspartic residue rather than an asparagine group.

Peptide fraction T-II-5 C3 was located with phenanthrenequinone reagent (Itano, 1966). The fractions containing arginine residues were pooled and purified electrophoretically at pH 3.5. One peptide resolved (T-II-5 C3a) had the composition: Ser, 0.8; Glx, 1.0; Gly, 1.0; Val, 1.1; Arg, 1.0; plus contaminants Asp, 0.1; Ala, 0.2; Met, 0.3. Dansyl-Edman degradation gave the sequence Gly-Val-Ser-Glu-Arg. The carboxylic form of the glutamic residue was established by electrophoresis (pH 6.4) (Offord, 1966) of the neutral Glu-Arg dipeptide obtained after partial Edman degradation, as well as by the electrophoresis of intact peptide T-II-5 C3a.

The partial structure of peptide T-II-5 combined with that of peptide T-II-3a allows the primary structure around the active centre to be drawn as Ala-Asp-Ile-Tyr-Val-Thr-Gly-Pro-Asx-Lys(Pxy)-Cys-Leu-(Pro₂, Gly₂, Ala₂, Met)(Thr, Leu₂)-Gly-Val-Ser-Glu-Arg.

The primary structures of pyridoxyl-peptides derived from a variety of pyridoxal phosphate-dependent enzymes have now been determined. The presence of a cysteine residue adjacent to the pyridoxyl-lysine residue is a unique feature of the peptide from pyridoxamine-pyruvate transaminase, and is noteworthy in view of the report (Fujioka & Snell, 1965) that this enzyme contains one essential highly reactive thiol group per active site which is protected from reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) by the presence of pyridoxal or its analogues.

It is striking that, although important homologies occur in the structure of the peptides from four decarboxylases (Strausbach & Fischer, 1970; Boeker *et al.*, 1971; Sabo & Fischer, 1972; Applebaum *et al.*, 1975), no homologies occur in the three transaminases so far studied (Morino & Watanabe, 1969; Ovchinnikov *et al.*, 1973; the present work) or in D-serine dehydratase (Huang & Snell, 1972), tryptophanase (Kagamiyama *et al.*, 1972) or tryptophan synthetase (Fluri *et al.*, 1971), even though all six of these enzymes act by initially labilizing an α -H atom of their substrates (Snell & DiMari, 1970). Such sequences, of course, represent only part of each active site, and residues that catalyse common

mechanistic features of these reactions may be far removed in sequence but held in necessary proximity by appropriate folding of the peptide chain.

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