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AMINO ACID SEQUENCE AROUND THE PYRIDOXAL 5'-PHOSPHATE BINDING SITES OF 6-PHOSPHOGLUCONATE DEHYDROGENASE *

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Summary

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate: $NADP^+$ 2-oxidoreductase(decarboxylating), EC 1.1.1.44) from *Candida utilis* is inhibited by reaction with pyridoxal 5'-phosphate. The aldehydic group of this compound forms a Schiff base with the ϵ-amino group of a lysine residue: reduction of this enamine with tritiated borohydride can label this amino acid.

Two tryptic peptides, TS_2 and TS_3, have been isolated from the labelled protein and found to have the following amino acid sequences:

TS_2: Ile-Leu-Asx-Glx-Ala-Gly-Gly-Lys(P-Pxy)-Gly-Glx-Thr-Lys
TS_3: Thr-Val-Ser-Lys(P-Pxy)-Val-Asp-His-Phe-Ile-(Glx,Asx,Glx)-Ala-Lys

where Lys(P-Pxy) indicates the modified lysine residue. The similarities between the amino acid sequences around the pyridoxal phosphate binding lysines of 38 peptides, obtained from enzymes which have pyridoxal phosphate as cofactor or inhibitor, are discussed and a prediction is made on the presence of reverse turns in these peptides.

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* Supplementary data to this article are deposited with and can be obtained from Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/163/69183/657 (1981) ●●●—●●●. The supplementary information includes: one table containing the amino acid sequences of peptides labelled with pyridoxal phosphate.

Abbreviations: Lys-(Pxy), N^6-(phosphopyridoxyl)-L-lysine; TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone.

Introduction

Since the discovery that pyridoxal 5'-phosphate is an inhibitor of several enzymes [1,2], besides acting as a cofactor for some others, a growing number of amino acid sequences of pyridoxal phosphate-binding peptides have been described [3].

At present, pyridoxal phosphate appears to be the best known reagent for specifically labelling lysine residues in proteins. The aldehydic group of pyridoxal phosphate forms an aldimine linkage with the ϵ-amino group of lysine residues; the reduction of this Schiff base makes the inhibition irreversible and introduces a permanent marker into the protein.

6-Phosphogluconate dehydrogenase from *Candida utilis* is one of the first enzymes shown to be inhibited by pyridoxal phosphate [1]. We now report the amino acid sequences of two peptides from the enzyme labelled with this compound and discuss the similarities between them and other peptides, also labelled with pyridoxal phosphate, from other enzymes which bind pyridoxal phosphate as a cofactor or as an inhibitor.

Materials and Methods

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate: $NADP^+$ 2-oxidoreductase(decarboxylating), EC 1.1.1.44) from *C. utilis* was prepared and assayed as previously described [4]. The crystals of enzyme were collected by centrifugation and dissolved in distilled water; the protein solution was subjected to gel filtration on a column (1.2 × 20 cm) of Sephadex G-25 equilibrated in 50 mM triethanolamine-HCl buffer, pH 7.5/1 mM EDTA.

TPCK trypsin, carboxypeptidase and thermolysin were purchased from Worthington Biochemicals. Pyridoxal phosphate was obtained from Sigma Chemical Co., and dissolved immediately before use.

50 mg enzyme (1 μmol of subunit) were treated with 2 μmol pyridoxal phosphate in 50 mM triethanolamine-HCl buffer, pH 7.5 (final volume: 10 ml). After 20 min of incubation at room temperature, ^{3}H-labelled sodium borohydride (720 μCi/μmol, Radiochemical Centre, Amersham) was added; the protein was freed from excess of reagents by gel filtration in the above mentioned triethanolamine buffer and the fractions were analysed for protein concentration and radioactivity. The amount of pyridoxal phosphate bound irreversibly to each enzyme subunit was determined spectrophotometrically from the value of the absorbance at 325 nm, assuming a molar extinction coefficient of 9.710 for the reduced pyridoxal phosphate derivative. Alkylation of the enzyme thiol groups was performed following the method of Harris and Perham [5], with slight modifications.

Trypsin digestion of the pyridoxal phosphate-treated and carboxymethylated protein, the separation and purification of the resulting peptides, amino acid and sequential analyses were carried out as previously described [6]. Fractions containing ^{3}H were detected with a Packard liquid scintillation counter.

Peptide maps were prepared as described by Harris and Perham [5]. Specific color reactions were performed according to Easley [7]. Carboxypeptidase and thermolysin digestion of peptides were performed by standard techniques

[8,9]. Amide content of peptides was estimated on the basis of their electrophoretic mobilities at pH 6.5 and their molecular weight [10]. Peptide mobility was relative to aspartic acid taken as 1.

Results

Upon treatment of native 6-phosphogluconate dehydrogenase with pyridoxal phosphate at pH 7.5 and reduction with NaB^3H_4, an enzyme preparation showing 25% residual activity and 1.1 residues of pyridoxal phosphate/enzyme subunit is obtained.

As a preliminary step in the identification of the reactive lysines, the pyridoxal phosphate-treated enzyme, reduced with ^{3}H-labelled borohydride and carboxymethylated, was digested with TPCK trypsin and then fingerprinted. Peptide maps were submitted to autoradiography and examined under ultraviolet light: as shown in Fig. 1 three labelled, blue fluorescent spots can be evidenced. From their electrophoretic mobility, two peptides behave as neutral and a third as an acidic one.

Large scale purification of the labelled peptides was achieved by ion-exchange column chromatography. The elution profile from a PA-35 column of a tryptic digest of pyridoxal phosphate-treated 6-phosphogluconate dehydrogenase shows three ^{3}H-labelled peaks absorbing at 325 nm (Fig. 2). Peak TS_1 was subjected to both electrophoresis and chromatography and proved to consist of a family of very faintly labelled peptides which were not further analysed.

High voltage electrophoresis at pH 6.5 (60 V/cm, 40 min) allowed to separate from peak TS_2 a radioactive and fluorescent band, which has been further submitted to electrophoresis at pH 2.2 (80 V/cm, 25 min) and to descending chromatography, using acetic acid/*n*-butanol/water/pyridine (3 : 15 : 12 : 10)

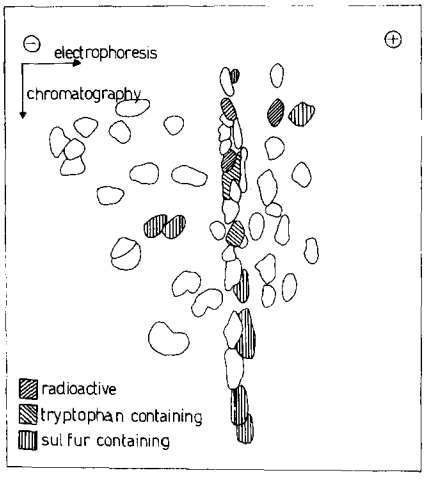


Fig. 1. Peptide map of a tryptic digest of 6-phosphogluconate dehydrogenase, treated with pyridoxal phosphate, reduced with ^{3}H-labelled borohydride and carboxymethylated. Electrophoresis: pH 6.5, 60 V/cm, 40 min. Chromatography: acetic acid/*n*-butanol/water/pyridine (3 : 15 : 12 : 10).

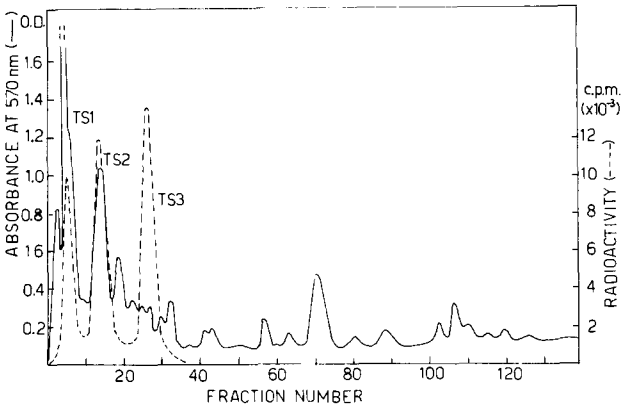


Fig. 2. Elution profile of tryptic peptides from 50 mg 6-phosphogluconate dehydrogenase treated with pyridoxal phosphate, reduced with ^{3}H-labelled borohydride and carboxymethylated. Protein digest was dissolved in 1.5 M acetic acid and chromatographed at 50°C on a PA-35 column (0.9 × 25 cm) with a linear gradient obtained by 250 ml of 0.05 M pyridine-acetate buffer, pH 3.1, in the mixer and 250 ml of 2.0 M pyridine-acetate buffer, pH 5.0, in the reservoir. Fractions of 2.7 ml were collected with a flow rate of 29 ml/h. The fractions were analysed for radioactive content (right ordinate) and absorbance at 570 nm (left ordinate), after alkaline hydrolysys and reaction with ninhydrin.

as a solvent. A second pyridoxal phosphate-peptide has been isolated from peak TS_3 with the same purification procedures. The amino acid composition, N-terminus, specific radioactivity and electrophoretic mobility at pH 6.5 of both peptides are reported in Table I. Peptide TS_2 consists of 12 residues, with iso-

TABLE I

AMINO ACID COMPOSITION AND OTHER CHARACTERISTICS OF TRYPTIC PEPTIDES FROM PYRIDOXAL PHOSPHATE-LABELLED 6-PHOSPHOGLUCONATE DEHYDROGENASE

Values are expressed as mol/mol peptide.

Amino acid	Peptide	
	TS_2	TS_3
Asp	1.08	2.09
Thr *	0.97	0.93
Ser *		0.98
Glu	1.99	1.97
Gly	2.99	
Ala	1.05	1.09
Val		1.81
Ile	0.82	0.94
Leu	0.88	
Phe		0.99
Lys	0.88	1.04
His		0.90
Lys(P-Pxy)	0.60	0.80
N-terminal	Ile	Thr
Specific radioactivity **	870	1230
Electrophoretic mobility	0	—0.26

* Not corrected for destruction.

** Values are expressed as cpm/nmol peptide.

leucine at N-terminal end, while peptide TS_3 is 14 residues long with threonine as the N-terminal.

The isolation of only two peptides in appreciable amount (15% final yield) indicates that the reaction between pyridoxal phosphate and 6-phosphogluconate dehydrogenase from *C. utilis* takes place mainly at two lysines, although other lysine residues appear to be capable of limited reaction.

On the basis of the electrophoretic mobility at pH 6.5 [10] and assuming a net charge of —0.3 for ε-pyridoxyl-lysine, TS_2 peptide contains two amide groups, whereas TS_3 shows only one.

Eight cycles of dansyl-Edman degradation allowed to sequence the N-terminal portion of TS_2 peptide. Carboxypeptidase digestion yielded lysine, threonine and glutamic acid or glutamine, not distinguishable from glutamic acid in our conditions of amino acid analysis, in a molar ratio of 1 : 0.9 : 0.6, showing the C-terminal sequence as Glx-Thr-Lys.

The sequence of TS_2 pyridoxyl peptide was thus established as the following:

Ile-Leu-Asx-Glx-Ala-Gly-Gly-Lys(P-Pxy)-Gly-Glx-Thr-Lys
→ → → → → → → → ← ← ←

where Lys(P-Pxy) indicates the modified lysine residue.

In order to elucidate the complete sequence, TS_3 peptide was divided in two aliquots: the first was submitted to seven cycles of dansyl-Edman degradation. The resulting sequence was the following:

Thr-Val-Ser-Lys(P-Pxy)-Val-Asp-His-Phe

The second aliquot of the peptide was treated with thermolysin and the digest was submitted to high voltage electrophoresis at pH 6.5 and paper chro-

TABLE II

AMINO ACID COMPOSITION AND OTHER CHARACTERISTICS OF TERMOLYTIC PEPTIDES FROM PEPTIDE TS_3

Values are expressed as mol/mol of peptide.

Amino acid	Peptide			
	TL_1	TL_2	TL_3	TL_4
Asp		1.08	1.08	
Thr *	0.87			
Ser *	1.16			
Glu			1.93	
Ala			1.08	1.07
Val	0.99	0.96		
Ile			0.78	
Phe		1.00		
Lys			1.10	0.93
His		0.96		
Lys(P-Pxy)	0.98			
N-terminal	Thr	Val	Ile	Ala
Electrophoretic mobility	+0.36	0	—0.28	+0.66

* Not corrected for destruction.

matography as described before. Four peptides, TL_1, TL_2, TL_3, and TL_4 were purified and analysed. Table II shows their amino acid composition, N-terminal residue and electrophoretic mobility. From the reported data the conclusion can be drawn that TL_1 comes from the N-terminus of the pyridoxyl peptide; TL_2 can be situated in the central portion, because of its sequence, while TL_3 contains the C-terminal lysine. TL_4, which contains only Ala and Lys, is a small peptide coming from TL_3.

From the electrophoretic mobility of TL_3, the net charge of this peptide is −0.1 [10]: this result confirms that two, of the three acidic residues, are present as free carboxyl groups.

All the above results allowed us to establish the final sequence:

$$\overrightarrow{\text{Thr-Val-Ser-Lys(P-Pxy)-Val-Asp-His-Phe-Ile-(Glx,Asx,Glx)-Ala-Lys}}$$

In the peptide map (Fig. 1) a third fluorescent and radioactive spot is shown: the chromatographic mobility of this spot is very similar to that of the acidic peptide identified as TS_3. We were unable to isolate a second neutral peptide, but from the similar chromatographic behaviour we suppose that another acidic peptide is present in the amide form.

Discussion

Amino acid sequence around specific lysine residues involved in the aldimine linkage with pyridoxal phosphate has been worked out for several enzymes on which pyridoxal phosphate acts as a cofactor or as an inhibitor. A considerable amount of data are now available to compare the structure of pyridoxal phosphate binding sites of different proteins which share the common feature of specifically binding this compound.

Together with the peptides from 6-phosphogluconate dehydrogenase described in the present paper, we report in the data stored in the BBA Data Bank (see footnote on title page) the amino acid sequence around the enamine forming lysines of 36 more peptides obtained from enzymes which are dependent on, or are inhibited by, pyridoxal phosphate (Table III). In spite of the different catalytic functions and origin of such enzymes, the peptides reported in this data display some striking similarities as well as some differences. In our discussion we shall compare the sequences of peptides obtained from enzymes in which pyridoxal phosphate is directly involved in the catalytic activity (the first 14 lines of Table III) with those from enzymes which are inhibited by pyridoxal phosphate (the last 23 lines).

A first general feature of almost all of them is the fact that they are particularly rich in non-polar amino acids, as visualized in Fig. 3. An hydrophobic environment for the pyridoxal phosphate binding site in some pyridoxal phosphate-dependent or inhibited enzymes has already been suggested [11,26,31]; such an environment could play a role in addressing or binding pyridoxal phosphate to this part of the proteins.

A second similarity is the presence of hydroxylated residues (serine or threonine) at a definite distance (3 or 4 amino acid residues apart) from the labelled lysines (empty circles, Fig. 4). Most of these residues are on the N-terminal

TABLE III

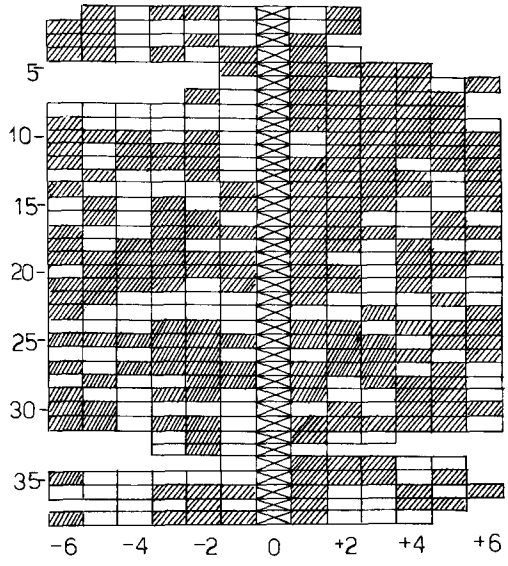
Enzymes whose amino acid sequences around the pyridoxal phosphate-labelled lysines are reported in data held in the BBA Data Bank.

Peptide number	Enzyme	Source	Ref.
1	Pyridoxamine-pyruvate aminotransferase	P. MA1	11
2	Serine transhydroxymethylase	Rabbit liver	12
3	Glutamate decarboxylase	*E. coli*	13
4	Serine dehydratase	*E. coli*	14
5	Serine dehydratase	*E. coli*	15
6	Serine dehydratase	*E. coli*	15
7	Ornithine decarboxylase	*E. coli*	16
8	Lysine decarboxylase	*E. coli*	11
9	Arginine decarboxylase	*E. coli*	17
10	Tryptophanase	*E. coli*	18
11	Tryptophan synthase	*E. coli*	19
12	Tryptophan synthase	*Ps. putida*	20
13	Aspartate aminotransferase	Pig heart	21—23
14	Aspartate aminotransferase	Pig heart	21—24
15	Phosphorylase	Rabbit muscle	25
16	Glyceraldehyde 3-phosphate dehydrogenase	Rabbit muscle	26
17	Glyceraldehyde 3-phosphate dehydrogenase	Rabbit muscle	26
18	Alcohol dehydrogenase	Horse liver	27
19	Aldolase	Rabbit muscle	28
20	Phospholypase A_2	Snake venom	29
21	Phospholypase A_2	Snake venom	29
22	Phospholypase A_2	Snake venom	29
23	Phospholypase A_2	Snake venom	29
24	Aspartate transcarbamylase	*E. coli*	30
25	Glutamate dehydrogenase	Bovine liver	31
26	Glutamate dehydrogenase	Bovine liver	32
27	Glutamate dehydrogenase (NADP)	*N. crassa*	33
28	Glutamate dehydrogenase (NAD)	*N. crassa*	34
29	Glutamate dehydrogenase (NAD)	*N. crassa*	34
30	Glutamate dehydrogenase (NAD)	*N. crassa*	34
31	Glutamate dehydrogenase (NAD)	*N. crassa*	34
32	Glutamate dehydrogenase (NAD)	*N. crassa*	34
33	Glutamate dehydrogenase (NAD)	*N. crassa*	34
34	Malate dehydrogenase	Porcine heart	35
35	Ribonuclease A	Bovine pancreas	36,37
36	Ribonuclease A	Bovine pancreas	36
37	6-Phosphogluconate dehydrogenase	*C. utilis*	
38	6-Phosphogluconate dehydrogenase	*C. utilis*	

side; this applies particularly to the enzymes which have pyridoxal phosphate as coenzyme.

A last observation can be made: while the peptides from pyridoxal phosphate-dependent enzymes contain very few (3.3%) arginine or lysine residues (filled circles, Fig. 4) and in half of these peptides a histidine residue precedes the labelled lysine, the peptides of the pyridoxal phosphate-inhibited enzymes contain a higher percent (11.4%) of basic amino acid and only three of them contain a histidine residue.

From the above observations, summarized in Fig. 4, the most common feature of the majority (30 over 34) of the peptides is the presence of a basic or/and a hydroxylated residue at a well defined distance from the labelled lysine.



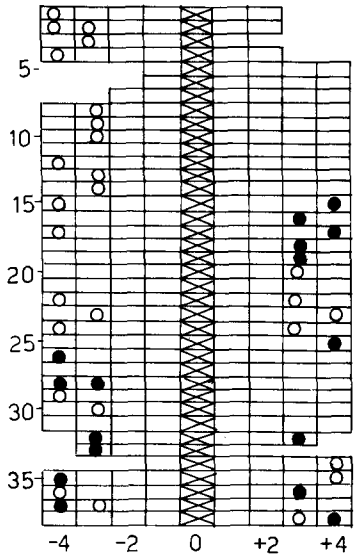


Fig. 3. Non-polar amino acid residues around the pyridoxal phosphate-labelled lysine in the peptides reported in data stored in the BBA Data Bank. Each line represents a peptide and each rectangle an amino acid residue. The pyridoxal phosphate-labelled lysine is marked 0. The shadowed areas indicate the positions of non-polar residues.

Fig. 4. Basic and hydroxylated amino acid residues positioned 3 or 4 residues far away from the pyridoxal phosphate-labelled lysine in the peptides reported in stored data. (See legend of Fig. 3) ○, serine or threonine residues; ●, lysine or arginine residues.

Early reports that pyridoxal is a less effective inhibitor than pyridoxal phosphate [1] pointed to a critical role of the phosphate anion in the inhibition mechanism. Indeed for some enzymes it has been shown, on the basis of kinetic analyses, that the inactivation by pyridoxal phosphate is due to an affinity labelling at an anion binding site, with the formation of a detectable, non-covalent complex, which precedes the formation of the Schiff base [37—40].

It has been suggested that pyridoxal phosphate bridges such a phosphate binding site with the ϵ-amino group of an adjacent lysyl residue positioned at a given distance [41]. The site involved in the binding of the phosphate moiety of pyridoxal phosphate can be considered a general anion binding site: indeed pyridoxal phosphate and pyridoxamine phosphate can be displaced by polyhydroxyanions from some enzymes tested [26,40,41].

A phosphate ion can be bound to a protein through ionic and/or hydrogen bonds: the basic and hydroxylated amino acid residues present in almost all the listed sequences can form these bonds. It is very tempting to postulate that the above residues are primarily involved in constructing the anion (phosphate) binding site and consequently direct the aldehydic group of pyridoxal phosphate toward a nearby ϵ-amino group of a lysine residue.

A first support to this hypothesis comes from the observation that, from studies on the three-dimensional structure of glyceraldehyde 3-phosphate dehydrogenase, it has been shown that inorganic phosphate binds to threonine

208, while lysine 212 forms a Schiff base with the aldehydic group of pyridoxal phosphate [42,43].

So far, the inhibition of some enzymes by pyridoxal phosphate has been generally ascribed to the modification of a lysine residue which was considered essential to the catalytic activity. We suggest that this lysine was not essential for catalytic activity, but it could only play the role of keeping the pyridoxal phosphate in such a position that a phosphate (or anion) binding site was unavailable for a natural anionic substrate. According to this hypothesis, interest should be focused on the basic and/or hydroxylated amino acid residues near the labelled lysine, rather than on this residue itself. Of course this hypothesis requires further kinetic and crystallographic support in order to be demonstrated.

The knowledge of the three-dimensional structure of proteins containing the peptides listed in Table III could be useful to test our hypothesis, but it is available only for five of them, namely peptides 16, 17, 18, 35 and 36 [44—46]. However, it is possible to predict the secondary structure of proteins from their amino acid sequences [47], and this has been done for at least two of the proteins examined, namely aldolase [48] and glutamate dehydrogenase [49]. For the other enzymes discussed here, the shortness of the known sequences around the labelled lysine allowed us to predict only the presence of reverse turns. Assignment of reverse turns were made where $pt \geqslant 1 \cdot 10^{-4}$ [47].

In Fig. 5 we indicate with bars the tetrapeptides which can be predicted to form reverse turns. The heavily marked bar in the peptide number 16 indicates a reverse turn detected by X-ray crystallography. As apparent from Fig. 5, 20

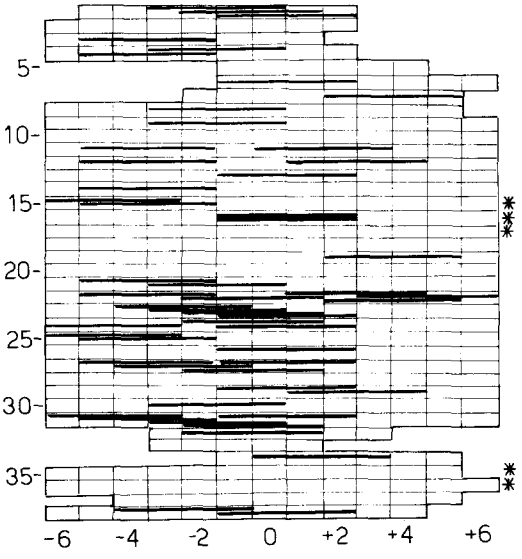


Fig. 5. Tetrapeptides predicted to form reverse turns. The tetrapeptides which can be predicted to form reverse turns are indicated by a bar. The peptides whose three-dimensional structure is established by X-ray crystallographic studies are indicated by an asterisk and were not subjected to the calculations for the prediction of reverse turn. The heavily marked bar at peptide number 16 shows the position of a reverse turn detected by X-ray crystallography.

out of the 38 peptides contain at least one tetrapeptide which includes the labelled lysine and can be predicted to form a reverse turn. X-ray crystallography has indicated that three other peptides (namely the peptides 17, 18 and 35) do not form reverse turns but are placed in a loop of the polypeptide chain.

Too little information is available at present to further speculate on the secondary structure of these peptides, but it is interesting to recall that it has been suggested [50] that while there is not a phosphate binding site in proteins which is both common and unique, there is a group of proteins which bind a phosphoryl or a pyrophosphoryl moiety in a loop of the polypeptide chain.

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