BLOCKED α-AMINO GROUPS IN PEPTIDES DUE TO DIKETOPIPERAZINE FORMATION

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1. Introduction

An unexpected type of blockage of α-amino groups in tryptic and chymotryptic peptides was found during structural analysis of yeast alcohol dehydrogenase. Two peptides derived from internal regions of the protein were partially cyclized by diketopiperazine formation, involving aspartyl α-carboxyl groups liberated during β-aspartyl shifts. In at least one of the peptides this shift was accompanied by a desamidation. Corresponding fragments without diketopiperazine formation were also recovered.

This reaction is suggested to be of general occurrence in peptides or proteins derived from certain amino acid sequences, especially from A↓B-Asn-C (C preferably Gly), when cleaved at the arrow. Diketopiperazine formation may therefore be a regular explanation to the presence of some blocked peptides. A summary of these results has previsouly been given [1].

2. Materials and methods

Yeast alcohol dehydrogenase (Boehringer Mannheim GmbH, Germany) was dialyzed against distilled water, lyophilized, dissolved (10 mg/ml) in 6 M guanidine–HCl, 0.1 M Tris–HCl, 2 mM EDTA, pH 8.1, reduced with dithiothreitol (0.5 μmole/mg protein) for 2 hr at 37°C and carboxymethylated with iodo [2-^{14}C] acetate (1.5 μmole/mg protein) for 2 hr at room temperature. Reagents were removed by dialysis against distilled water. Enzymatic digestions (enzyme to substrate ratio of 1:100, w/w) were performed at 37°C for 4 hr in 0.1 M ammonium bicarbonate with TPCK-trypsin or α-chymotrypsin treated with TLCK [2]. Peptides were purified by exclusion chromatography on Sephadex G-50 fine (Pharmacia, Uppsala, Sweden), high-voltage paper electrophoresis and paper chromatography, as previously described [3]. Compositions were determined with a Beckman 120 B amino acid analyzer fitted with a high-sensitivity chart. End-groups were identified by the dansyl method [4] using thin-layer chromatography on polyamide sheets [5] as previously reported [6]. The dansyl–Edman method [7, 8] was used for sequential degradation.

3. Results

3.1. *Preparation of peptides*

The blocked peptide TA and a corresponding fragment with a free N-terminus (peptide TB) were recovered from the tryptic digest. They eluted at a volume of about 1250 ml from a 5 × 90 cm column of Sephadex G-50 fine. The blocked peptide CA and the corresponding free fragment (peptide CB) were obtained from the chymotryptic digest (at an elution volume of 1400 ml from the same column). TA and CA were conspicuous by low electrophoretic mobilities at acidic pH due to the absence of charged α-amino groups. Data for these peptides and secondarily produced fragments are given in table 1. Overlapping fragments were purified by similar methods after different proteolytic treatments of the carboxymethylated protein, as indicated below.

Table 1
Data for diketopiperazine-blocked peptides and secondarily produced fragments from carboxymethylated yeast alcohol dehydrogenase.

Peptide	TA	TA1	TA2	TA1a	TA1b	CA	CA1	CA2
Recovery %	13	44	42	35	45	4	20	30
Electrophoretic mobility at pH 6.5 [9]	0	0.38	−0.33	0.68	0	0.82	0.70	0.67
Composition								
Cys(CM)	– –	– –	– –	– –	– –	1.8 (2)	0.8 (1)	0.9 (1)
Asp	1.1 (1)	1.0 (1)	– –	1.0 (1)	– –	1.0 (1)	1.1 (1)	– –
Thr	1.7 (2)	1.7 (2)	– –	– –	1.9 (2)	0.4 –	– –	– –
Ser	– –	– –	– –	– –	– –	1.0 (1)	1.1 (1)	– –
Glu	– –	– –	– –	– –	– –	1.2 (1)	– –	1.0 (1)
Pro	0.9 (1)	– –	0.9 (1)	– –	– –	– –	– –	– –
Gly	3.2 (3)	1.2 (1)	2.1 (2)	1.1 (1)	– –	0.9 (1)	1.2 (1)	– –
Ala	3.2 (3)	1.0 (1)	2.1 (2)	0.9 (1)	– –	0.9 (1)	– –	0.9 (1)
Val	1.5 (2)	0.9 (1)	0.9 (1)	– –	1.1 (1)	– –	– –	– –
Met	0.8 (1)	– –	0.8 (1)	– –	– –	0.8 (1)	0.8 (1)	– –
Leu	0.9 (1)	0.9 (1)	– –	– –	1.0 (1)	0.9 (1)	0.8 (1)	– –
Tyr	– –	– –	– –	– –	– –	0.8 (1)	– –	1.0 (1)
Lys	1.0 (1)	– –	1.0 (1)	– –	– –	– –	– –	– –
Total	15	7	8	3	4	10	6	4
N-terminus	None	None	Val	Asp Ala	Thr	None	None	Ala

For compositions, hydrolyses were perfomed in 6 N HCl (containing 1 ‰ mercaptoethanol) at 110°C for 24 hr. Values given are molar ratios without corrections for destruction, incomplete hydrolysis or impurities. Recovery of peptides is based on the total amount of protein digested, recovery of secondary fragments on the amount of parent peptide.

3.2. *Structural analysis*

3.2.1. Peptides TA and TB

These two neutral peptides have identical compositions. Their structures were determined as summarized in table 2. After digestion with TLCK-chymotrypsin two fragments were obtained from either peptide (TA1 and TA2 from TA, TB1 and TB2 from TB, table 2) and purified by paper electrophoresis. The fragments account for the total compositions of the parent peptides as shown for TA and its products in table 1, and the terminal residues give the order of the fragments in the original peptides. TA and TA1 could not be sequentially degraded.

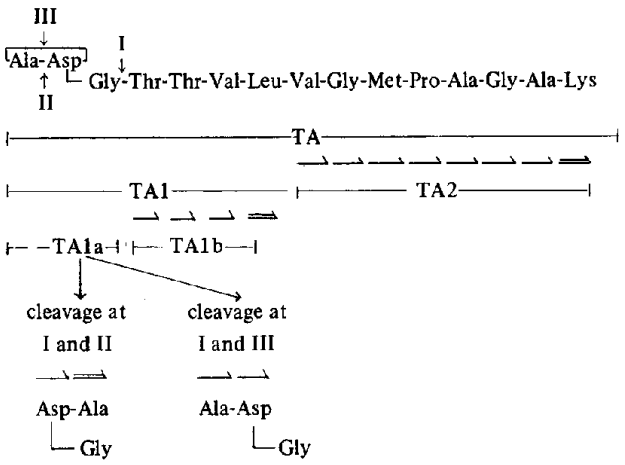
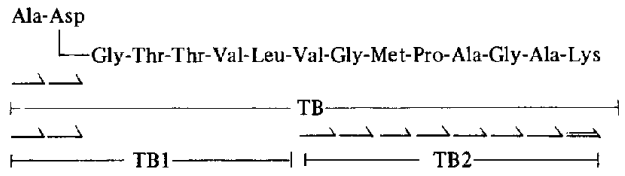
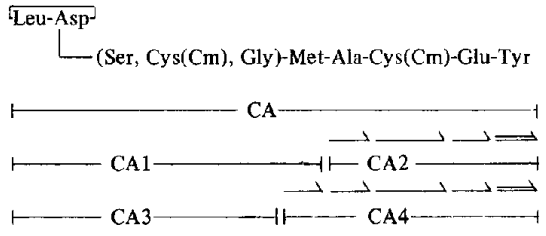
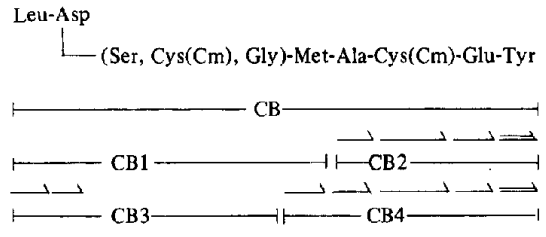
TA1 was submitted to partial acid hydrolysis at room temperature for 24 hr in 300 µl of 9.3 N HCl. Three peptides were produced and purified by paper electrophoresis. Two of these were acidic, identical in composition and did not separate (TA1a), and the third (TA1b) was neutral.

As shown in table 1, TA1a and TA1b account for the total composition of TA1 but both fractions have free α-amino groups. The blocked N-terminus of TA1 was therefore liberated during the partial hydrolysis. The order of the fragments in the original peptide TA1 is evident from their terminal residues (table 2) and from the structure of peptide TB1. The glycine residue in TA1a (table 1) could not be detected by Edman degradation, nor by carboxypeptidase A digestion. It is therefore deduced to be linked to the aspartic acid residue by a β-aspartyl bond. Acid hydrolysis of the peptide bonds indicated in table 2 liberates the blocked α-amino group of TA1 and yields the two tripeptides TA1a. A diketopiperazine ring thus explains the absence of a free N-terminus in TA and TA1. These results are supported by the structure of TB and by mass spectrometric analysis of TA1 (Jörnvall and Ohlsson, unpublished results).

The compositions and electrophoretic mobilities

Table 2

Amino acid sequences of diketopiperazine-blocked peptides and corresponding fragments with free N-terminal residues.

Peptide	Sequence
TA	
TB	
CA	
CB	

—› Indicates sequence analysed by the dansyl–Edman method.
⟹ Indicates residue proven to be C-terminal by recovery of the dansyl-derivative at this stage even without hydrolysis after dansylation. Marked arrows indicate bonds cleaved by partial acid hydrolysis of peptide TA1.

at pH 6.5 of TB1 and TB2 were identical to those of TA1 and TA2, respectively. The first two residues of TB and TB1 could be revealed by the dansyl–Edman method (table 2) but further steps yielded no results. TB is thus derived from the same region of the protein as TA and has a β-linkage between Asp and Gly but is not cyclized to the diketopiperazine-blocked derivative.

3.2.2. Peptides CA and CB

These two peptides have identical compositions. Redigestion with TLCK-chymotrypsin yields two acidic peptides in each case (CA1 and CA2 from CA, CB1 and CB2 from CB, Table 2). These also occur in low yield in the original chymotryptic digest. CA1 and CB1 have identical compositions. The former is blocked like CA, while the first two residues of CB and CB1 may be revealed by the dansyl–Edman method (table 2), although further steps give no results. CA2 and CB2 are identical (table 2). Digestion of CA or CB with thermolysin also yields two acidic peptides in each case (CA3, CA4, CB3 and CB4, table 2).

Peptides CA and CB are therefore derived from the same region of the protein. CA is blocked in a manner similar to TA, while CB, like TB, has a β-aspartyl linkage at position two (table 2).

3.3. *Overlapping fragments*

The N-terminal region of TA/TB was detected in a chymotryptic peptide, which by the dansyl–Edman method gave the sequence Val-Arg-Ala-Asx-. Further sequential degradation was repeatedly unsuccessful. Digestion with trypsin yielded the basic dipeptide Val-Arg and an acidic fragment identical to peptide TB1. Hence, the structure of the chymotryptic peptide is

Val-Arg-Ala-Asp
└Gly-Thr-Thr-Val-Leu

which proves that TA and TB are internally derived.

The N-terminal region of CA/CB was overlapped by a cyanogen bromide fragment, 23 residues long, with N-terminal glycine and C-terminal homoserine. After digestion with chymotrypsin or trypsin a fragment identical to CB1 but with C-terminal homoserine was recovered showing that CA and CB are internally derived.

The internal origin of peptides TA and CA exclude that they constitute the explanation of the absence of free N-terminal residues [10, 11] in the whole protein, in agreement with other results [12]. Internally derived peptides may thus be blocked by cyclizations involving more than one residue.

4. Discussion

4.1. *Diketopiperazine formation*

Peptide TA is produced by tryptic cleavage of an Arg–Ala bond. The originally present sequence in the intact protein is deduced to be -Arg-Ala-Asn-Gly, as a basic peptide with this structure was identified from a peptic digest of the protein. A tryptic peptide with the N-terminal sequence Ala-Asn-Gly- and the same composition as TA has also been reported [11]. Hence, peptide TA is obtained by a tryptic cleavage together with a desamidative β-aspartyl shift of the glycyl residue and ring closure between the liberated α-amino group of Ala and α-carboxyl group of Asp, to produce the diketopiperazine ring. The cyclization is not quantitative, as judged by the presence of peptide TB (in an amount about equal to that of TA), but the β-aspartyl shift seems to be almost so (present in TA, TB and the overlapping chymotryptic fragment).

Desamidative β-aspartyl shifts are well known, especially with glycine, in natural [13] and synthetic peptides [14–19]. A substituent (amide or ester) on the β-carboxyl group seems to be a prerequisite which is compatible with the sequence Asn-Gly in the original protein. Basic pH, which was used for the tryptic and chymotryptic digestions, facilitates the shift [14–19]. Significantly, it was not observed in the peptic digest carried out under acidic conditions.

The ring closure accompanying the β-aspartyl shift is previously not reported in tryptic or chymotryptic peptides. It is presumably generated when the peptide is formed, either at the enzymatic cleavage or at the β-aspartyl shift, since the non-cyclized peptides seem to be stable. No tendency to loss of N-terminal residues was thus observed in peptides TB or CB on storage or secondary enzymatic digestions and following purifications. In case of peptides CA/CB the original structure in the protein in unknown but an Asn-Gly or Asn-Ser sequence is probable (table 2). The latter

may also participate in β-aspartyl shifts [18]. The mechanism for the cyclization in peptide CA is therefore likely to be the same as in the case of TA.

Terminal α-amino groups in tryptic and chymotryptic peptides may thus be blocked by diketopiperazine formation. This is suggested to be of general occurrence in suitable peptides. These include peptides produced at basic pH and derived from regions in a protein where an asparagine residue occupies position two after a bond cleaved, and where residue three is glycine or perhaps serine. The sequence restrictions and the blocked nature of the products may explain why such peptides have apparently not previously been identified.

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