A METHOD OF DISTINGUISHING BETWEEN ASPARTIC ACID AND ASPARAGINE AND BETWEEN GLUTAMIC ACID AND GLUTAMINE DURING SEQUENCE ANALYSIS BY THE DANSYL-EDMAN PROCEDURE

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1. Introduction

The dansyl-Edman procedure is widely used for sequence analysis of peptides due to its high sensitivity and rapidity [1,2]. The method suffers from the major disadvantage that, under the conditions required to liberate N-terminal Dns-amino acids from peptides, complete hydrolysis of the amide side chains of asparagine and glutamine occurs, thus making it impossible to distinguish between aspartic acid and asparagine or between glutamic acid and glutamine. One solution to the problem is to measure the mobility of the intact peptide on electrophoresis at pH 6.5 relative to the mobility of aspartic acid; for peptides not containing histidine, a good correlation exists between mobility, molecular weight and net ionic charge [3]. This method, however, only provides an unambiguous assignment of acid and amide side chains if a single such residue occurs in the peptide or alternatively if all such residues are either acids or amides. This restriction can be removed by measuring the mobility of the peptide after each step of Edman degradation, but this is very wasteful of time and material.

Clearly, the most satisfactory approach would be to modify side chain carboxyl groups before sequence analysis in such a way that the modification is retained under the relatively extreme conditions required to hydrolyse dansylated peptides. The reports by Rosenthal and Atassi [4,5] that treatment with diborane [6–8] leads to specific reduction of carboxyl groups in peptides and proteins led us to investigate the use of this reagent for the purpose outlined above. We report here the results of reduction and sequence analysis of several peptides containing acid and amide side chains.

2. Materials and methods

The peptides used were obtained mainly from digests of aspartate aminotransferase with pepsin [9], trypsin [10] and thermolysin [11]. The bee venom peptides mellitin [12] and peptide 401 [13] were gifts from Dr R. A. Shipolini. Peptides Asp–Phe and Glu–Ala–Ala were obtained from Cyclo Chemical, Los Angeles, California, U.S.A., as was α-amino-δ-hydroxyvaleric acid. Homoserine was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. and diborane (1 M) in tetrahydrofuran from Ventron Corp., Beverly, Mass., U.S.A.

Reduction of peptides was carried out essentially as previously described [4,5]. The dried peptide (50–100 nmol) was dissolved or suspended in water

(100 μl) at 0°C and trifluoroacetic acid added (approx. 10% by vol). The mixture was then immediately lyophilized. To the dried peptide-trifluoroacetate was added diborane (1 M) in tetrahydrofuran (150 μl) and the sample maintained at 0°C for 2 hr. Solvent and remaining diborane were removed in a stream of N_2 after which methanol (250 μl) acidified with trifluoroacetic acid was added and the mixture maintained for 1.5 hr at room temperature. The methanol was removed by evaporation and the residue treated twice more with acidified methanol in the same way.

Sequence analysis was carried out by the dansyl-Edman method [2], identification of Dns-amino acids being achieved by t.l.c. on polyamide plates using the solvent systems described by Woods and Wang [14] and by Crowshaw et al. [15]. Qualitative amino acid analysis by complete dansylation of peptide hydrolysates was done as previously described [10].

3. Results

Reduction of aspartic acid and glutamic acid residues located internally in peptides gives rise to homoserine and α-amino-δ-hydroxyvaleric acid respectively. Dns-derivatives of these amino acids were prepared and their behaviour on t.l.c. on polyamide sheets, using the usual solvent systems [14,15], was compared with that of the Dns-derivatives of aspartic acid, glutamic acid, serine and threonine. All six derivatives were well resolved and no difficulty was experienced in identifying Dns-homoserine or Dns-α-amino-δ-hydroxyvaleric acid in the presence of the other Dns-amino acids.

Samples of the synthetic peptides Asp–Phe and Glu–Ala–Ala were reduced, hydrolysed, and the hydrolysates completely dansylated. Analysis of the hydrolysate of reduced Asp–Phe showed mainly homoserine with a trace of aspartic acid. (No phenylalanine was detected. This residue was presumably reduced to the corresponding aminoalcohol [4] and no attempt was made to characterize the derivative). Analysis of reduced Glu–Ala–Ala showed α-amino-δ-hydroxyvaleric acid, a trace of glutamic acid, and alanine. Samples of the bee venom peptides mellitin and peptide 401 were similarly reduced and hydrolysed. Neither of these peptides contains aspartic or glutamic acids, but mellitin contains glutamine and a C-terminal glutamine amide whereas peptide 401 contains asparagine and a C-terminal asparagine amide. Analysis of the hydrolysates of the reduced peptides showed complete absence of homoserine or of α-amino-δ-hydroxyvaleric acid whereas aspartic acid (in the case of peptide 401) and glutamic acid (in the case of mellitin) were present in the expected amounts.

Having established that reduction with diborane gave nearly complete conversion of acid side chains to the corresponding alcohols but left amide side chains unchanged, the technique was applied to various peptides obtained from aspartate aminotransferase by digestion with proteolytic enzymes [9–11]. After reduction, sequence analysis was carried out by the dansyl-Edman procedure [2]. Since the amino acid sequences of these peptides were known, except for assignment of some acid and amide side chains, complete analyses were not carried out, and frequently Dns-derivatives were identified only at those positions where residues of interest were known to occur. The results are summarized in table 1 and amplified below.

Peptide TAE-6 [10]. The amino acid sequence was Glx–Val–Glu–Tyr. Mobility measurements [16] indicated that the N-terminal residue was glutamine. On sequence analysis after reduction, residues 1 and 3 were identified as glutamic and α-amino-δ-hydroxyvaleric acids respectively, thus confirming the sequence of the original peptide as Gln–Val–Glu–Tyr.

Peptide TM-8 [10]. The sequence was Tyr–Trp–Asx–Thr–Glx–Lys–Arg and mobility data [16] indicated that residues 3 and 5 were both acids. This was confirmed for residue 3 by sequencing after reduction.

Peptide RP-32 [9]. The sequence was Tyr–Asx–Glx–Arg–Val–Gly–Asn–Leu. Mobility data [16] indicated that one of residues 2 and 3 was an acid and the other an amide. Sequencing after reduction showed that residue 2 was asparagine and residue 3 glutamic acid.

Peptide RP-8′. This peptide was derived from RP-8 [9] by cleavage with chymotrypsin and had the sequence Glx–Glx; mobility data [16] showed that one residue was glutamic acid and the other glutamine. The results in table 1 indicate that the N-terminal residue was glutamine.

Peptide TL-42′. This was derived from TL-42 [11]

Table 1
Partial sequences of peptides after reduction with diborane

Peptide	Residue found at the given position after reduction and sequencing by the dansyl-Edman procedure							
	1	2	3	4	5	6	7	8
TAE-6	Glu	Val	AHV	–				
TM-8	Tyr	–	Hse	–	–	–	–	
RP-32	–	Asp	AHV	–	–	–	–	–
RP-8′	Glu	–						
TL-42′	AHV	Asp	–	–	–	–		
RP-12′	AHV	Asp	–	–	–	–	–	
RP-4	–	–	Thr	Hse	Hse	–	–	–
TAE-15	Ala	–	Asp	–	Thr	Gly	Thr	Hse

Homoserine (Hse) and α-amino-δ-hydroxyvaleric acid (AHV) were usually accompanied by trace amounts of aspartic and glutamic acids respectively. Residues found as Hse and HVA originated from aspartic and glutamic acids, whereas residues found as Asp and Glu originated from asparagine and glutamine as explained in the text. Usually, residues not of interest in the present context were not identified (indicated by –).

by cleavage with chymotrypsin and had the sequence Glx–Asx–Ala–Pro–Glx–Phe; mobility data [16] showed that there were two acid and one amide side chains. Reduction and sequencing showed residues 1 and 2 to be glutamic acid and asparagine respectively; hence residue 5 is also glutamic acid.

Peptide RP-12′. This was derived from RP-12 [9] by digestion with chymotrypsin and had the sequence Glx–Asx–His–Asx–Gly–Val–Phe. The results in table 1 show that residues 1 and 2 are glutamic acid and asparagine respectively. The peptide was neutral at pH 6.5 [16]; hence residue 4 must also be asparagine.

Peptide RP-4 [9]. The sequence was Tyr–Arg–Thr–Asx–Asx–Cm Cys–Glx–Pro–Trp–Val–Leu. After reduction and sequencing, both residues 4 and 5 were shown to be aspartic acid.

Peptide TAE-15 [10]. This peptide had the sequence Ala–His–Asx–Pro–Thr–Gly–Thr–Asx–Pro–Thr–Pro–Glx–Glx–Trp–Lys. Reduction and sequencing identified residues 3 and 8 as asparagine and aspartic acid respectively; it was not possible to determine the sequence as far as residues 12 and 13.

It is clear from these results that the method described provides a reliable procedure for the assignment of acid and amide side chains in peptides. The results obtained were consistent with data from mobility measurements and in all cases agreed with amide assignments reported by Ovchinnikov et al. [17]; these were obtained by application of the direct Edman method. It is of particular interest that, for some of the peptides in table 2, mobility data alone provided ambiguous amide assignments (e.g. RP-32, RP-8′, TL-42′, RP-12′); in all these cases reduction and sequence analysis gave clear identifications.

The reduction procedure is simple and can be carried out on a large number of samples at the same time. The method has, however, certain limitations which should be pointed out. Generally, C-terminal residues will be converted to amino alcohols and it will probably not be possible to identify these as the Dns-derivatives by the normal procedures. C-terminal asparagine and glutamine, after reduction and hydrolysis will yield β-amino-γ-hydroxybutyric acid and γ-amino-δ-hydroxyvaleric acid respectively, the Dns-derivatives of which may not be resolved from those of the other hydroxy amino acids. Hence in general previous information about the C-terminal residue may be necessary before complete sequence analysis of a reduced peptide. A more serious point concerns the yield of peptide after reduction. Quantitative analysis of hydrolysates of mellitin before and after reduction showed that in the latter case the yield of each amino acid was approx. 50% compared with the

yield from a non-reduced sample. This must be taken to show that partial reduction of peptide bonds occurs, contrary to findings previously reported [4]. The reduction is not accompanied by peptide bond cleavage, since no new N-terminal residues appear. Partial peptide bond reduction, although still allowing sequence analysis to proceed on that fraction of the molecules which are not reduced, will obviously result in rapid decrease in yield of the new N-terminal as sequencing is continued. This effect probably accounts for the failure to sequence completely some of the peptides in table 1. In contrast to the partial reduction of peptide bonds, amide side chains did not appear to be affected by diborane; this is consistent with the reported slower reduction of primary amides compared with secondary and tertiary [8].

Experiments are now in progress to find conditions for the reduction under which essentially complete modification of carboxyl groups is retained, but peptide bonds are not affected. This may be possible by changes in time of reaction, temperature or solvent.

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