THE NUMBER AND NATURE OF α,β-UNSATURATED AMINO ACIDS IN NISIN

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1. Introduction

Identical COOH-terminal sequences, viz. dehydroalanyllysine, have recently been established for the peptide antibiotics nisin [1] and subtilin [2]. The release of one equivalent of pyruvyllysine upon treatment of nisin at 100°C for 10 minutes with hydrogen chloride in glacial acetic acid [1] indicated the presence of one residue of the α,β-unsaturated amino acid. However, carboxymethylcysteine was found in excess of one residue in the hydrolysate of the addition product of mercaptoacetamide to nisin. The excess of carboxymethylcysteine over 1 residue did not result from β-elimination of lanthionine. The test for sulfhydryl groups with maleimide was negative; the hydrolysate of the nisin-mercaptan addition product was free of cystine and contained the same number of lanthionine residues as nisin. Oxygen had to be excluded carefully in order to determine the presence of 2 residues of dehydroalanine and 1 residue of β-methyldehydroalanine in the antibiotic.

2. Methods and results

Nisin which showed 1.2 amino acid residues in an asymmetric peak at the effluent volume of carboxymethylcysteine [1] on the chromatogram of the 60-cm column of the amino acid analyzer [3] was subjected to cyanogen bromide cleavage in 60% formic acid (10-fold excess of reagent over methionine, 0°C, 24 hours) [4] with the result that two fragments were obtained after separation by gel chromatography on a Sephadex G-25 column and countercurrent distribution. One of the fragments contained the H_2N-terminal isoleucine, the other the COOH-terminal lysine of nisin [1]. Amino acid analysis showed the COOH-terminal fragment to have 0.4 residues of S-carboxymethylcysteine and also that it had lost 0.6 residues of lysine. The H_2N-terminal fragment contained 1.3 residues of an amino acid in an *asymmetric* peak at the effluent volume of S-carboxymethylcysteine.

The S-carboxymethylcysteine peak of the COOH-terminal fragment was *symmetric*. The sum of the amino acids in each fragment added up to the number of residues present in nisin [1] with the exception of the loss in lysine.

These data indicated that the conditions under which the addition of mercaptan was carried out did not sufficiently exclude oxygen and that the oxidation of sulfhydryl groups was accompanied by the formation of peroxide which in turn oxidized unsaturated amino acids.

Nisin was therefore dissolved in degassed water and the solution adjusted to 25% in ethanol. Methylmercaptoacetate was used in the addition since it can be purified more readily and does not seem to be subject to the same rapid autoxidation as mercaptoacetamide. The reaction vessel was evacuated and flushed several times with nitrogen before being sealed under vacuum. The pH of the solution was 4.5. The reaction was allowed to proceed for 13 days after which time the reaction mixture was lyophilized and passed over a Sephadex G-25 column to remove excess reagent.

The amino acid analysis of this material showed the presence of 2.3 residues of amino acids at the effluent volume of S-carboxymethylcysteine; the peak was again asymmetric.

This material was subjected to cleavage with cyanogen bromide to give two fragments as described above. The COOH-terminal fragment was now found to contain 1 residue of S-carboxymethylcysteine as well as its full lysine complement.

The H_2N-terminal fragment contained 1.8 residues of amino acids at the effluent volume of S-carboxymethylcysteine but this peak was also asymmetric.

Hydrolysates of both fragments were subjected to electrophoresis in pyridine acetate buffer of pH 6.5. With the exception of aspartic acid, S-carboxymethylcysteine is easily separated from the other amino acids found in the hydrolysate.

Before desulfurization with Raney nickel the isolated thioether amino acids were passed over the 60-cm column of an amino acid analyzer [3] in order to ascertain the absence of other amino acids.

Only alanine was found as the result of desulfurization of S-carboxymethylcysteine from the COOH-terminal fragment. However, equal amounts of alanine and α-aminobutyric acid were identified as products of the desulfurization of the amino acids from the H_2N-terminal fragment. The precursor of aminobutyric acid must have been β-methyl-S-carboxymethylcysteine which in turn was formed by mercaptan addition to *β-methyldehydroalanine.* This is borne out by the observation that the two acids are partially separated on the 150-cm column of the amino acid analyzer [5] and that the effluent volume of the material isolated from nisin corresponds to that of synthetic β-methyl-S-carboxymethylcysteine prepared from methylmercaptoacetate and the azlactone of N-benzoyl methyldehydroalanine.

The same observations pertain to the addition of mercaptan to fragments of nisin obtained by cleavage with cyanogen bromide [4] at 0°C.

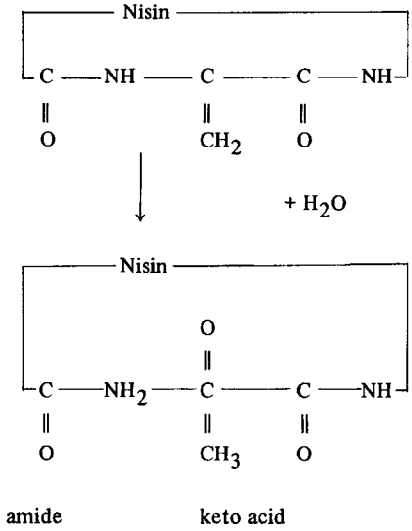
The decrease in the numbers of residues of ammonia and the change in the values of the extinction coefficients at 250 mμ (table 1) support the distribution of α,β-unsaturated amino acids indicated above.

3. Discussion

The α,β-unsaturated amino acids are possibly the result of biosynthetic steps in which peptide bonds are formed from aminoacyl amides and keto acids. The significance of α,β-unsaturated amino acids for various types of biological activity has already been demonstrated [1].

The presence of α,β-unsaturated amino acids in nisin imposes a high degree of chemical reactivity upon this peptide molecule which is expressed in: (a) the ready loss of hydroalanyllysine in the form of pyruvyllysine [1], (b) the multicomponent system of commercially available nisin, (c) the increase in the number of residues of S-carboxymethylcysteine and/or β-methyl-S-carboxymethylcysteine in the hydrolysates of fragments of nisin as opposed to the values determined for these amino acids in the hydrolysate of the immediate reaction product of the mercaptan addition.

The α,β-unsaturated amino acids of nisin react with water with the formation of amide and keto acid. This reaction is reversible:



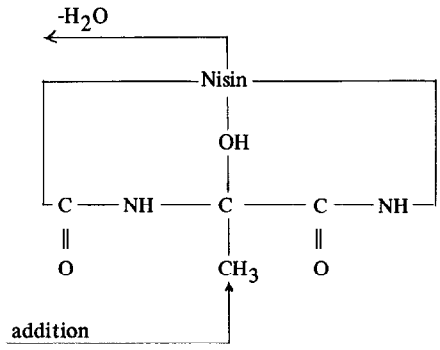
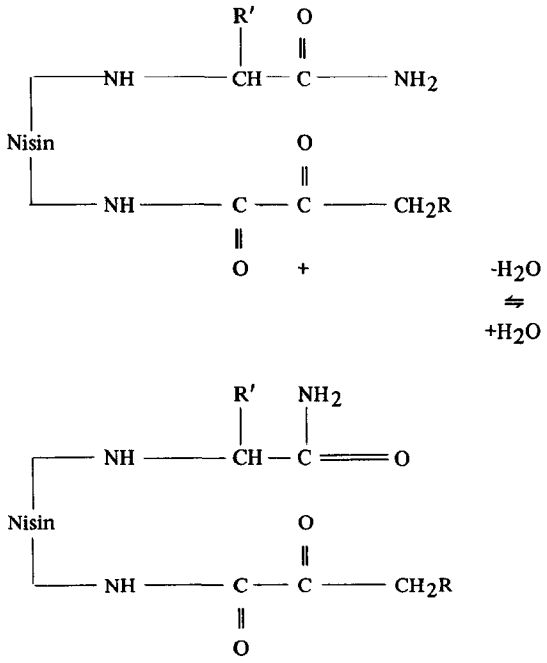


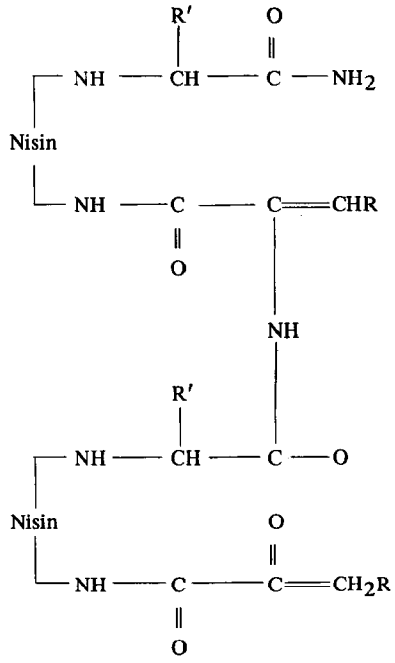
Table 1
Numbers of residues of ammonia and values of extinction coefficients in fragments of nisin before and after mercaptan addition.

Fragment	Ammonia		ϵ250 mμ	
	before mercaptan	after addition	before mercaptan	after addition
H_2N-terminal	3 *	1.3	5990	583
COOH-terminal	1	0.1	3250	393

* The additional residue of ammonia is contributed by an amide group present in nisin.

and may take place either intramolecularly (cf. above mechanism) or intermolecularly (cf. mechanism below). In the course of the intermolecular reaction products of higher molecular weight are formed (e.g. dimers, R = H or CH_3; R′ = side chain of an amino acid present in nisin):





The high molecular weight components are removed during the separation and isolation of fragments of the cyanogen bromide cleavage and the amino acid analytical data show no longer fractional values (vide supra, the residue numbers for S-carboxymethylcysteine and β-methyl-S-carboxymethylcysteine).

Mercaptan addition products of monomers of nisin always yield low molecular weight fragments. The cross-linkages in polymers of nisin, however, seem to occur in positions such that low molecular weight fragments are rarely formed upon treatment with cyanogen bromide. Since these polymers do not necessarily contain the maximum possible number of unsaturations, lower than theoretical values are initially found for S-carboxymethylcysteine and β-methyl-S-carboxymethylcysteine.

This view is supported by the observation that the increase in S-carboxymethylcysteines in fragments from samples with extensive oxidative cleavage of unsaturations is twice that of samples from additions under the exclusion of oxygen.

We have earlier demonstrated that the molecular weight of nisin is 3500 [1]. Molecular weights in multiples of 3500 have been reported repeatedly. The dissociation of nisin of "molecular weight" 7200 to a product of molecular weight 3500 in boiling 1 N hydrochloric acid [6] must be interpreted as cleavage of a dimer in which two monomeric units were linked via an α,β-unsaturated amino acid.

The natural occurrence of α,β-unsaturated amino acids is not necessarily restricted to peptides isolated from microorganisms. A careful search might well reveal their presence in proteins isolated from higher organisms. Enzymes with covalently bound keto acids from various sources have recently been isolated. Pyruvic acid, for instance, is present in D-proline reductase [7]. These keto acids may well be derived from α,β-unsaturated amino acids.

Studies are now under way to elucidate the complete structure of nisin.

References

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