Selective Cleavage of the Phosphoamide Bond in Adenylyl-(5$’$$\rightarrow$N)-Peptides

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Abstract

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Selective Cleavage of the Phosphoamide Bond in Adenylyl-(5 →N)-Peptides

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Continuing the study of nucleophilic substitution reactions at the phosphorus atom [1,2] in nucleotide peptides of the phosphoamide type, we investigated the interaction of the latter with hydroxylamine. It turned out that in this case, as in those studied by us earlier [2,3], cleavage of the phosphoamide bond occurs only under conditions in which protonation of the amide nitrogen can take place. Hydroxylamine displaces the amino acid (peptide) from adenylyl-(5 →N)-amino acid (peptide) at pH 4.5, apparently forming O-adenylylhydroxylamine [4]. At neutral and alkaline* pH values hydroxylamine does not react with nucleotidyl-(5 →N)-amino acids (peptides).

[scheme: adenylyl-(5 → N)-amino acid (peptide) + hydroxylamine → $NH_2CHCO\ldots + AMP{-}ONH_2$]

The objects of the study were synthetically obtained adenylyl-(5 →N)-amino acids and adenylyl-(5 →N)-peptides [5]. Upon treatment of an aqueous solution of the corresponding compound with hydroxylamine (pH 4.5, 1 hr, 37°), liberation of the amino acid or peptide occurred; these were determined by quantitative paper chromatography using the ninhydrin method [6]. On the basis of these data the percentage cleavage of the phosphoamide bond was determined (see Table 1). Under analogous conditions, but in the absence of hydroxylamine, the phosphoamide bond in the compounds studied is not cleaved (see also [3]). As follows from Table 1, by this method one can determine from 65 to 75% of the amino acid (peptide) entering into the nucleotide peptide.

It also follows from the data of Table 1 that under the action of hydroxylamine, even under such mild conditions, the phosphoamide bond is cleaved equally readily both in amino-acid (I-III) and in peptide (IV, V) derivatives of adenylic acid; cleavage of peptide bonds in compounds IV and V was not observed. In this connection it proved possible to develop a method for determining the N-terminal amino acid in nucleotidyl-(5 →N)-peptides, using the dinitrophenylation reaction of the peptide liberated after cleavage of the phosphoamide bond, followed by hydrolysis of the DNP**-peptide to the DNP-acid. After appropriate treatment of compounds IV and V, DNP-phenylalanine was identified by paper chromatography and UV spectroscopy.

* If an ester of nucleotidyl-(5 →N)-amino acid was introduced into the reaction, then at pH 12 with hydroxylamine its hydroxamate is formed.
** DNP = dinitrophenyl.

Thus, hydroxylamine apparently can be used not only for the determination of nucleotide amino acids with ester (O-aminoacyl-t-RNA*) or anhydride (aminoacyl adenylates) nucleotide-peptide bonds, but also of nucleotide peptides of the phosphoamide type (7). The difference is that, in the case studied, the reaction proceeds at acidic pH values and is determined not by the hydroxamic acid,

Table 1

Determination of amino-acid residues in adenylyl-(5 →N)-amino acids (peptides) with hydroxylamine

Compound studied	R_f in system 1	Amino acid (peptide) formed	R_f in system 1	Cleavage of the P–N bond, %
I. Adenylyl-(5 →N)-phenylalanine, methyl ester	0.30	Methyl ester of phenylalanine	0.71	70
II. Adenylyl-(5 →N)-leucine, methyl ester	0.35	Methyl ester of leucine	0.69	75
III. Adenylyl-(5 →N)-phenylalanine	0.30	Phenylalanine	0.61	65
IV. Adenylyl-(5 →N)-phenylalanylglycine, methyl ester	0.33	Methyl ester of phenylalanylglycine	0.80	72
V. Adenylyl-(5 →N)-phenylalanylvalylglycine, methyl ester	0.37	Methyl ester of phenylalanylvalylglycine	0.75	67

but by an amino acid or peptide with a free amino group. The use of dinitrophenylation after cleavage of the phosphoamide bond by hydroxylamine makes it possible to determine the amino acid bound in the nucleotide peptide directly to phosphorus (the N-terminal amino acid).

Experimental Part

The systems used in the chromatographic study were:

1. *n*-Butanol–water–acetic acid (4 : 5 : 1).
2. *n*-Butanol–butyl acetate–1% ammonia (1 : 2 : 3).
3. Isoamyl alcohol–*n*-butanol–ethanol–0.1 N potassium biphthalate (30 : 30 : 11 : 45)**.
4. Isopropanol–NH_3–H_2O (7 : 1 : 2).

For chromatography, Leningrad paper of grade B was used. Values of R_f are given for ascending chromatograms.

Substances absorbing in the UV were detected on chromatograms with Brumberg' s chemoscope. Compounds with a free amino group were visualized with ninhydrin. The hydroxamates of amino-acid derivatives of AMP were visualized on paper with a solution of ferric chloride.

Preparation of the hydroxylamine solution. 6.95 g of $NH_2OH \cdot HCl$ are dissolved in 45 ml of 2 N NaOH; then, using a potentiometer, the solution is carefully brought to the required pH by addition of 4 N NaOH or 4 N HCl, after which the total volume of the solution is brought to 50 ml with distilled water.

Reaction of adenylyl-(5 →N)-phenylalanine (III) and of the methyl esters of adenylyl-(5 →N)-phenylalanine (I) and -leucine (II) with hydroxylamine at different pH values. 3-4 mg of the compound studied are dissolved in 1 ml of distilled water; 0.1 ml of this solution is taken, and 0.1 ml of freshly prepared hydroxylamine solution, adjusted to the required pH–4.5; 7.3; 12 (see above)–is added to it. To control samples, respectively, 0.1 ml each of acetate (pH 4.5), phosphate (pH 7.3) buffer solutions and 0.1 N NaOH (pH 12) are added. The reaction mixtures and control samples are left in a thermostat at 37° for 1 h, then quantitatively applied as a 2-cm strip to chromatographic paper and chromatographed in system 1. The results obtained are presented in Table 2.

* t-RNA–transfer ribonucleic acid.
** For chromatography in this system, the paper is previously impregnated with a 0.1 N solution of potassium biphthalate.

The amount of amino acids liberated as a result of the reaction of compounds I–III with hydroxylamine at pH 4.5 is determined quantitatively, and the percentage cleavage of the P–N bond is calculated relative to the amount of starting material in the control sample (at pH 4.5 without NH_2OH), which is determined by the formula* $0.339 \cdot \Delta_{260}$ (see Table 1).

Table 2

Products detected in the reaction of compounds I–III with NH_2OH at different pH values

Starting compounds	pH	Detected compounds after incubation with hydroxylamine	R_f in system 1	Detected compounds after incubation without hydroxylamine	R_f in systems 1	R_f in systems 4
I	4.5	Methyl ester of phenylalanine	0.71	I	0.30	0.56
I	7.3	I	0.30	I	0.30	0.56
I	12	Hydroxamate I	0.17	III	0.30	0.30
II	4.5	Methyl ester of leucine	0.69	II	0.35	0.59
II	7.3	II	0.35	II	0.35	0.59
II	12	Hydroxamate II	0.20	II *	0.32	0.33
III	4.5	Phenylalanine	0.61	III	0.30	0.30
III	7.3	III	0.30	III	0.30	0.30
III	12	III	0.30	III	0.30	0.30

* II –adenylyl-(5 →N)-leucine.

Reaction of methyl esters of adenylyl-(5 →N)-phenylalanylglycine (IV) and -phenylalanylvalylglycine (V) with hydroxylamine at pH 4.5.

The reaction of IV and V with NH_2OH at pH 4.5 is carried out as described above for I–III. On chromatography in system 1, the methyl esters of phenylalanylglycine (R_f 0.80) and phenylalanylvalylglycine (R_f 0.75), respectively, are detected; these were determined quantitatively with the aid of calibration curves constructed for the esters of the indicated peptides (for the percentage cleavage of the phosphoamide bond, see Table 1).

Determination of the N-terminal amino acid in adenylyl-(5 →N)-peptides (amino acids).

After treatment of compounds I, III–V with hydroxylamine (see above), the reaction mixture is applied as a 2-cm band to paper and chromatographed in system 1. The zones of the paper that are colored by treatment with ninhydrin and correspond to the distribution levels of control samples of peptide esters (amino acids) are cut out, eluted with 50% methanol, and evaporated in vacuo. To the residue are added 0.5 ml of a 2% $NaHCO_3$ solution and 0.5 ml of a 5% solution of fluorodinitrobenzene in alcohol; the mixture is shaken for 5 h (pH 8 –9). The excess fluorodinitrobenzene is extracted with ether, the latter being separated with a pipette. The aqueous layer is acidified to pH 2, extracted with ether, and the extract is evaporated in vacuo. The residue is hydrolyzed with 6N HCl (16 h, 100°), evaporated in vacuo, extracted with ether, and the ether solution is applied to a chromatogram. For all the compounds studied, DNP-phenylalanine (R_f 0.73), λ_{max} 357 m (in 1% NH_3), is detected in system 2. After ether extraction of the hydrolysates of the DNP peptides, the residue is dissolved in water and chromatographed in system 1. In the case of IV glycine is detected (R_f 0.15), and in the case of V–glycine and valine (R_f 0.30).

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* The coefficient 0.339 was calculated for adenylic acid for a volume of 5 ml (50% methanol).

Note: Figure translations are in progress. See original paper for figures.