UNUSUAL RESULT OF MODIFICATION OF ANEMONOTOXIN RTX-III BY MALONALDEHYDE

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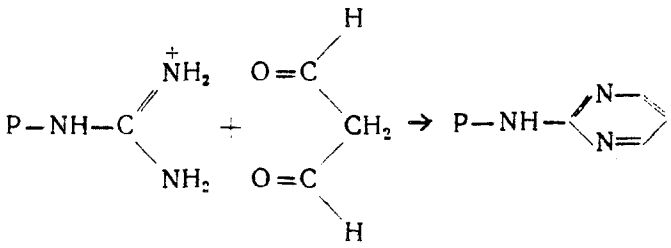
The modification of the arginine residues of neurotoxin RTX-III from Radianthus macrodactylus with malonaldehyde led to the formation of a derivative with a maximum in the UV spectrum at 340 nm in place of the expected N^{δ}-(pyrimidin-2-yl)ornithine with absorption at 315 nm. On the basis of chemical transformations and the results of absorption spectroscopy, it has been concluded that the product obtained was an intermediate of the reaction – a Schiff's base. It has been shown that the stabilization of the reactive intermediate is due to the influence of steric factors. The further conversion of the toxin derivative taking place in neutral and alkaline media has been investigated; it is connected with a rearrangement of the malonaldehyde residue of the enol-enamine type.

The method of the selective chemical modification of arginine residues in proteins with malonaldehyde (MA) was first proposed by King [1]. The reaction takes place in 10 N HCl, and tetraethoxypropane is used as the source of MA. The study of the reaction of MA with arginine made by King showed that its final product was N^{δ}-(pyrimidine-2-yl)ornithine (POr) having a characteristic maximum in the UV spectrum at 315 nm.



No reaction intermediates were detected among the modification products nor was the occurrence of a side reaction with the amino groups.

The aim of the present work was to elucidate the functional role of the arginine residues of anemonotoxin RTX-III from Radianthus macrodactylus. The polypeptide chain of this toxin includes 48 amino acid residues and contains three disulfide bonds. The two arginine residues of RTX-III occupy positions 13 and 45 [2]. The material of this paper has been published previously in the form of theses [3].

RTX-III readily took part in a reaction with a fivefold molar excess of MA, as a result of which a product was formed with a characteristic maximum in the UV at 340 nm. The toxicity of this product, purified by chromatography on SP-Sephadex C-25 in a gradient of ammonium acetate buffer was four times less than that of the native RTX-III (Fig. 1). In control, the toxin kept under the reaction conditions for an hour retained its activity completely. In the spectrum of the modified protein the maximum at 315 nm corresponding to POr was completely absent. A variation of the time of incubation from 0.25 to 1.5 h or of the excess of MA in the reaction mixture of from 5- to 200-fold did not lead to the appearance of POr among the reaction products. Previously [4], in order to prevent side reactions of proteins with MA, it was proposed to add to the reaction mixture after the completion of modification

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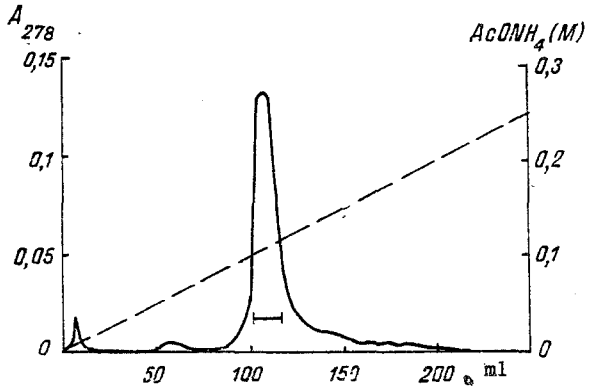


Fig. 1. Purification by ion-exchange chromatography of RTX-III modified with malonaldehyde. Column of SP-Sephadex C-25 (10 × 80 mm) equilibrated with 10 mM ammonium acetate, pH 5.0. Linear gradient of ammonium acetate of from 10 to 250 mM, pH 5.0. Rate of elution 8.0 ml/min.

an excess of guanidine hydrochloride. We used this method, but the neutralization of the MA residues had no effect on the final result of modification.

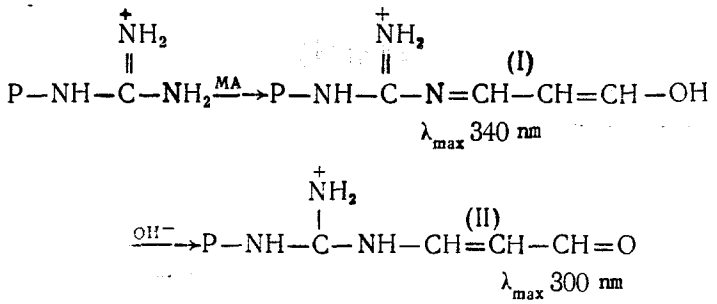
A study of the pH stability of the modification product showed that in solutions with a neutral pH it was slowly converted into a compound with absorption in the UV at 300 nm. In an alkaline medium a considerable acceleration of this process was observed, which, in the final account, led to the complete disappearance of the maximum at 340 nm. In a solution with pH 10 about 8 h was necessary for this. At acid pH values, the adduct of the RTX-III with MA was relatively stable: on storage in a solution with pH 3 at 5°C slight changes in its UV spectrum did not appear in under a week. It must be mentioned that POr is completely stable in solutions at both acid and alkaline pH values [1, 4].

According to the results of amino acid analysis, the degree of modification of the RTX-III was 0.3, although chromatography on SP-Sephadex C-25 and reversed-phase HPLC showed that the reaction product was free from traces of unmodified protein. The low value of the degree of modification suggested that the reaction product was unstable under the conditions of acid hydrolysis. We assumed that the reaction of MA with RTX-III did not proceed to completion but stopped at the stage of formation of an intermediate. In this case, the group labile under the conditions of acid hydrolysis is the azomethine grouping of a Schiff base formed by the guanidino group of the arginine residue and one of the two aldehyde groups of the MA.

The capacity of β-dicarbonyl compounds for keto-enol tautomerism is well known. For MA, the tendency to enolization is expressed particularly strongly, which is due to the great lability of its α-hydrogen atoms (pK_α = 5.0) and the ease of formation of an intermediate resonance-stabilized carbanion [5]. In view of the fact that keto-enol transformations are catalyzed both by bases and by acids, it may be expected that under the conditions of modification the MA is largely enolized. This was shown by the presence of intense absorption in the 340 nm region which we observed in the MA obtained by the hydrolysis of tetraethoxypropane in 10 N HCl. This absorption band was absent from the spectrum of MA under the conditions of a neutral medium [6]. It was not present, either, in the spectrum of a solution in 10 N HCl of glutaraldehyde, which is not a β-aldehyde. The condensation of the guanidino group with the enolic form of MA in the first stage of the reaction should lead to the formation of a Schiff's base (I) having a structure isoelectronic with MA. This explains the presence in the spectrum of the modification product of a maximum at 340 nm (see scheme on following page).

The presence of a base probably catalyzes the rearrangement of the initial enol (I) into the enamine (II), which is accompanied by the appearance of a maximum in the UV at 300 nm. The low rate at which this transformation takes place is characteristic for reactions with the participation of α-hydrogen atoms [5].

An attempt to perform the reverse transformation by adding HCl was unsuccessful: even two days after the acidification of the solution to pH 1 the spectrum of the sample under



investigation remained unchanged. The impossibility of the occurrence of the reverse reaction is probably explained by the fact that the substituted nitrogen atom is simultaneously part of the resonance cation formed on the protonation of the guanidino group. Under these conditions part of the electron cloud is split off from it, which prevents the rearrangement of the enamine into an enol.

For an additional proof of the presence of a carbonyl function in the modified protein we studied its interaction with hydroxylamine, 2,4-dinitrophenylhydrazine, and sodium tetratritioborate. The treatment of both forms of the modified protein with hydroxylamine at pH 6.0 led to the disappearance of the maximum at 340 and 300 nm and to the appearance of absorption at 328 nm which showed the formation of an oxime.

The reaction with 2,4-dinitrophenylhydrazine [7] was carried out with the two forms of the modified protein in 1 N HCl, which eliminated the possibility of a rearrangement of the enol into an enamine during the treatment. Since 2,4-dinitrophenylhydrazine can be sorbed by the protein, after the end of the reaction it was carefully removed by chromatography on Polikhrom and SP-Sephadex C-25. The modified toxin in the initial form (enol) bound the 2,4-dinitrophenylhydrazine to an insignificant degree, while treatment of the protein that had first been kept in an alkaline medium led to a considerable inclusion of the reagent. In the spectrum of the modified toxin, together with a decrease in the absorption at 300 nm, a broad maximum appeared in the 430-440 nm region corresponding to a 2,4-dinitrophenylhydrazone. The different capacities of the two forms of modified protein for reacting with 2,4-dinitrophenylhydrazine confirmed the correctness of the assumption of the occurrence of a rearrangement of the enol (I) into the enamine (II), since only the latter can react with 2,4-dinitrophenylhydrazine.

Reduction of the modified toxin with sodium tetratritioborate (pH 9) led to the inclusion of tritium, to the disappearance of the maximum at 300 nm, and to the appearance of absorption in the 250-260 nm region, on the background of which the usual maximum at 280 nm became unclear.

The results given indicate the presence in the modified toxin of an aldehyde group the appearance of which can be connected only with the cessation of the modification reaction at the stage of an intermediate without the subsequent closure of the pyrimidine ring.

The treatment with MA of the toxin after oxidative cleavage of the three disulfide bonds by performic acid led to the formation of POr with λ_{max} 315 nm. This shows that the stabilization of the active intermediate was due to the steric hindrance existing in the microenvironment of the arginine residues.

It must be mentioned that the proofs given are of indirect nature, but the specific nature of the material investigated – M 5280, abundance of peptide and functional groups – did not permit the use of the usual set of spectral methods employed for establishing the structures of organic compounds. A stricter more detailed analysis of the UV spectra obtained requires the existence of model compounds the synthesis of which is, in our opinion, extremely problematical. In view of this, we considered it necessary to limit ourselves to the use of the chemical approach and of absorption spectroscopy. In addition, a further investigation of the reaction described, while not promising great discoveries, would lead us in a direction away from our main purpose – the study of the structural-functional interrelationship of the anemonotoxins.

On the general theoretical level, the reaction of MA with RTX-III shows that the interaction of low-molecular-mass reagents with proteins and peptides may take place with considerable departures from the usual course of organic reactions.

EXPERIMENTAL

UV spectra were taken on a Cary 219 UV spectrophotometer (Varian) in 0.01 M ammonium acetate, pH 5.0. The toxin RTX-III was isolated by the method of Romey et al. [9].

Amino acid analysis was carried out after the hydrolysis of a sample of the protein (5-10 nmole) in 6 N HCl (200 µl) at 110°C for 24 h in evacuated and sealed ampuls.

Modification of RTX-III with Malonaldehyde. RTX-III (5 mg, 1 µmole) was dissolved in 1 ml of 10 N HCl cooled to 0°C, and 0.2 ml of a freshly-prepared solution of 1,1,3,3-tetraethoxypropane (5 µl in 1 ml of 10 N HCl) was added, which corresponded to a fivefold molar excess with respect to the protein. The reaction mixture was incubated at 4°C with stirring for 20 min and was deposited on a column of Polikhrom (10 × 120 mm) for the rapid elimination of the reagents. The column was washed with ice water, and the protein was eluted with 50% ethanol. The modified toxin was purified by chromatography on a column of SP-Sephadex C-25 (10 × 80 mm) equilibrated with 0.01 M ammonium acetate, pH 5.0, in a linear gradient of ammonium acetate of from 0.01 to 0.25 M, pH 5.0. The rate of elution was 0.8 ml/min. UV spectrum: $\lambda_{max}^{H_2O}$, nm: 278, 340 (log ε 4.03; 4.06).

Reaction with Hydroxylamine. The toxin (0.5 mg, 0.1 µmole) was incubated in 1 ml of 0.1 M hydroxylamine, pH 6.0, for 6 h, after which it was desalted on a column of Polikhrom (10 × 20 mm).

Reaction with 2,4-Dinitrophenylhydrazine. The toxin (1 mg, 0.2 µmole) was dissolved in 1 ml of water, the solution was treated with 1 ml of 5 mM 2,4-dinitrophenylhydrazine in 2 N HCl (25-molar excess), and the mixture was incubated with stirring for 2 h, after which it was desalted on a column of Polikhrom (8 × 40 mm). The protein was eluted with 50% ethanol and the resulting solution was evaporated and deposited on a column of SP-Sephadex C-25 (10 × 40 mm) equilibrated with 0.01 M ammonium acetate buffer, pH 4.5. The reagent residues were eluted with the same buffer, after which the protein was eluted with 0.3 M ammonium acetate, pH 6.

Reduction with Sodium Tetratritioborate. The protein (0.5 mg, 0.1 µmole) in 0.5 ml of 0.05 M sodium borate buffer, pH 9, was treated with 200 µl of a solution of NaB^3H_4 (40 MBq) in the same buffer. After incubation in an ice bath for 15 min, 1 mg of sodium tetrahydroborate was added and the mixture was incubated for 30 min and was acidified with 0.5 ml of a 1 N solution of HCl, and the protein was desalted on a column of Polikhrom (8 × 40 ml).

SUMMARY

The modification of anemonotoxin RTX-III with malonaldehyde leads to the formation of a relatively stable reaction intermediate. The reason for the cessation of the reaction at the intermediate stage is steric hindrance.

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