MECHANISM OF THE INACTIVATION OF THE ANTIBIOTIC POLYMYXIN M

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We have previously [1, 2] found that when polymyxin M is thermostatted in a 0.1 N solution of ammonia for 3.5 hr at 37° C the antibiotic is almost completely inactivated. The results of a detailed study of this phenomenon show that the inactivated antibiotic, unlike active polymyxin M, contains free α-amino groups of α, γ-DABA* [1]. The suggestion was put forward that under these conditions $N^{\alpha} \rightarrow N^{\gamma}$ transacylation of the residues adjacent to the α, γ-DABA amino acids or the fatty acid ($N^{\alpha} \rightarrow N^{\gamma}$ migration) took place [3, 4]. The existence of $N^{\alpha} \rightarrow N^{\gamma}$ migration was shown with a model peptide α-caprylyl-α,γ-DABA [5]. Subsequently [6], in a series of peptides of α, γ-DABA both $N^{\alpha} \rightarrow N^{\gamma} \rightarrow N^{\alpha}$ migration taking place in alkaline or acid solutions was detected by electrophoresis.

The present paper gives the results of a quantitative study of $N^{\alpha} \rightleftarrows N^{\gamma}$ migration of peptides of α, γ-DABA and polymyxin M. These results are necessary to explain the mechanism of the inactivation of the antibiotics of the polymyxin series. The method of study used was the complete dinitrophenylation of the peptide with its subsequent hydrolysis and the determination of the amount of α-DNP-, γ-DNP-, and α, γ-DABA in an automatic amino acid analyzer.

Experimental

Starting materials. The investigation was carried out with a sample of polymyxin M sulfate (batch No. 8, Kiev Medicinal Preparations Factory) with an activity of 11 000 units/mg. The peptides α-pel-DABA and γ-pel-DABA, the picrates of the peptides α-glyc-DABA and γ-glyc-DABA, and also the picrates of the diketopiperazines glyc-DABA and DABA-DABA were obtained in the Institute of Organic Chemistry of the Academy of Sciences of the Czechoslovakian SSR (Prague) by K. Podushka and one of us [6]. The standard α-DNP-DABA and γ-DNP-DABA were synthesized in the Laboratory of the Chemistry of Proteins, Nucleotides, and Antibiotics, Moscow State University. The peptide B1 was isolated from the butanolic fraction [7] of the partial hydrolysis of DNP-polymyxin M by its separation on a column of Sephadex G-25 (fine), and the peptide EF-1 from the partial hydrolyzate of inactive polymyxin M [3] by extraction with ether.

Conditions for obtaining the products of $N^{\alpha} \rightleftarrows N^{\gamma}$ transacylation. A weighed sample of the peptide (1-2 mg) was dissolved in 2 ml of 0.1 N ammonia and thermostatted at 37° C for 4 hr. Then the solution was evaporated to dryness in a rotary evaporator at 35° C.

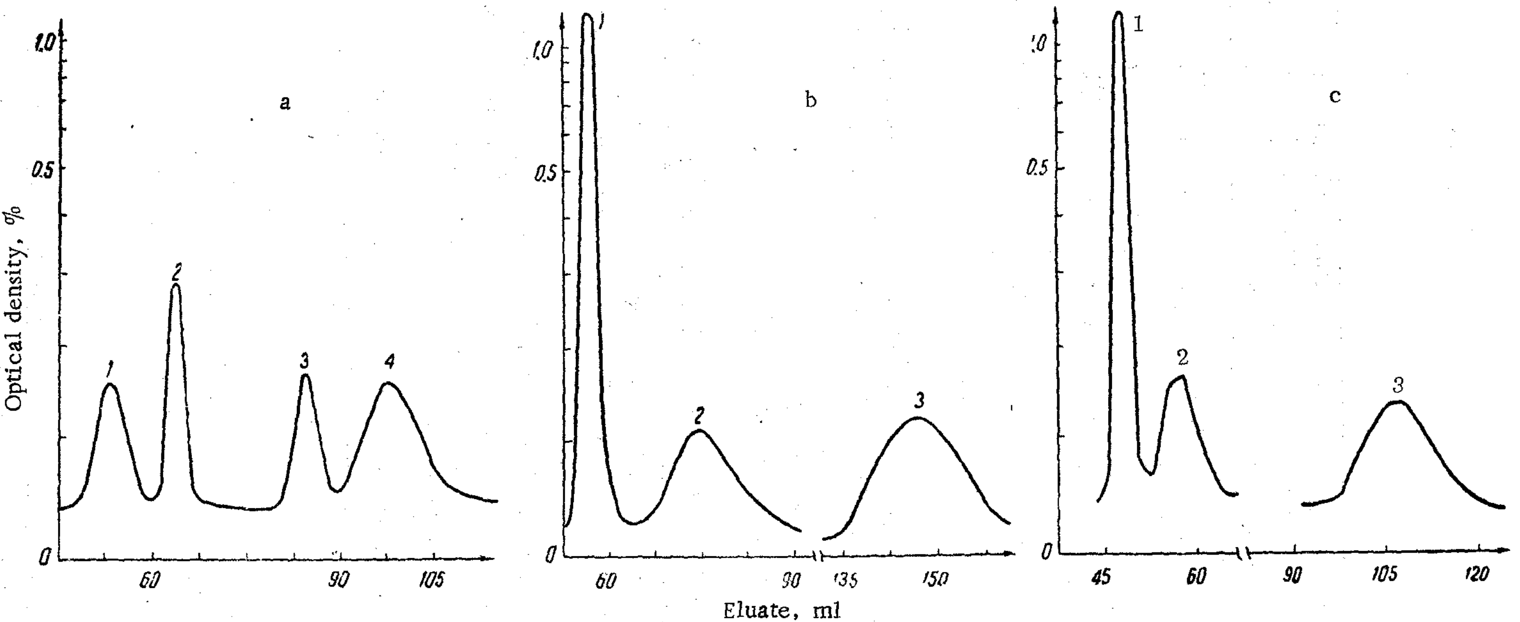
Dinitrophenylation. A weighed sample of 1-2 mg of the peptide was dissolved in a mixture of 0.5 ml of alcohol and 0.5 ml of carbonate buffer with pH 9.5 [8], and 0.5 ml of an alcoholic solution of FDNB (10 mg/ml) was added. The mixture was kept for 1 hr at room temperature and then for 1.4 hr at 37° C. The completeness of dinitrophenylation was checked by paper electrophoresis. After the end of the reaction, the solution was dried in vacuum.

Complete hydrolysis of the DNP derivatives. The hydrolysis of the DNP derivatives was carried out in sealed tubes in 2 ml of 6 N hydrochloric acid for 16 hr at 105°-108° C. The hydrolyzate was repeatedly evaporated in vacuum with water and the residue was dissolved in 2 ml of sodium citrate buffer at pH 2.2 and was used for investigation on an automatic amino acid analyzer.

Conditions of electrophoresis. Electrophoresis was carried out in an instrument working on the humid chamber principle [9] as described previously [10]. Whatman No. 1, and 3 MM chromatographic paper was used, and electrophoresis was carried out with 1 N CH_3COOH (600 V, 70 min) and a mixture of 85% HCOOH- 98% CH_3COOH- H_2O (28 : 20 : 52 by volume) [11] (300 V, 3-4 hr). The free peptides were detected by spraying the electrophoregrams with a 0.2% solution of ninhydrin in acetone.

Separation and quantitative determination of α-DNP- and ω-DNP-isomers of the diamino acids. This process was carried out on a Nitachi (Japan) KLA-2 automatic amino acid analyzer by Stein and Moore's method [12, 13]. Separation

* The following abbreviations are used in this paper: pel = pelargonic acid; α, γ-DABA = α, γ-diaminobutyric acid; DNP = 2, 4-dinitrophenyl-; FDNB = 1-fluoro-2, 4-dinitrobenzene; glycyl-DABA = glycyldiaminobutyryldiketopiperazine; DABA-DABA = diketopiperazine from α, γ-DABA; peptide EF-1 = peptide from the partial hydrolyzate of DNP-polymyxin M (inactivated [3]).



Separation of the basic amino acids and their mono-DNP derivatives on a Hitachi KLA-2 analyzer (column 15 cm, 50° C): a) 0.36 N Na^+ citrate buffer, pH 5.26: 1) α-DNP-DABA; 2) α,γ-DABA; 3) NH_3; 4) γ-DNP-DABA. b) 0.36 N Na^+ citrate buffer, pH 5.26: 1) lysine; 2) α-DNP-lysine; 3) ε-DNP-lysine. c) 0.7 N Na^+ citrate buffer, pH 5.28; 1) ornithine; 2) α-DNP-ornithine; 3) δ-DNP-ornithine.

was effected on a column (0.9 × 15 cm) of the resin Amberlite CG-120, type III (400 mesh). Elution was performed with 0.36 N sodium citrate buffer with pH 5.26 at 50 ± 1° C with a buffer feed rate of 30 ml/hr. In the separation of α-DNP- and β-DNP-ornithines, 0.7 N sodium citrate buffer with pH 5.28 was used. The neutral amino acids were analyzed on a column (0.9 × 50 cm) at 50° C with a mixed buffer 3.25–4.25 after 0.5 hr (figure).

Table 1

Quantitative Analysis of Peptides kept for Four Days at 37° C in 0.1 N NH_4OH*

Compound investigated	Number of amino acid residues					
	α-DNP-DABA		γ-DNP-DABA		α,γ-DABA	
	μmole	%	μmole	%	μmole	%
α-pel-DABA	0.940	56.5	0.655	39.3	0.071	4.2
γ-pel-DABA	1.120	59.4	0.633	33.5	0.133	7.1
α-glyc-DABA	—**	—	1.05	—	—	—
	2.479***	76.8	0.386	11.7	0.429	13.1
γ-glyc-DABA	1.383**	92	—	—	0.12	8
	0.561***	81.7	0.067	9.9	0.058	8.5
glyc-DABA (cyclic)	0.575	26.1	0.195	8.9	1.43	65
DABA-DABA (cyclic)	0.252	14.1	0.678	38	0.855	47.9

*The analysis of the peptides on the amino acid analyzer was carried out after their complete dinitrophenylation and acid hydrolysis under standard conditions (see Experimental Section).

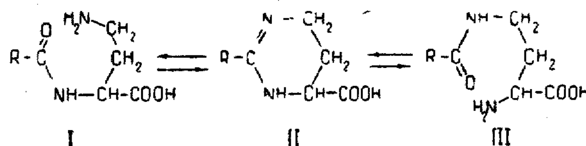
**After thermostatting in 0.1 N NaOH.

***After thermostatting under the given conditions.

Determination of the micromolar coefficients of α-DNP- and γ-DNP-DABA. An accurately weighed amount of analytically pure α-DNP- and γ-DNP-DABA was dissolved in sodium citrate buffer with pH 2.2 in a measuring flask. An aliquot part was deposited on a 15-cm column, and the micromolar coefficients C for α-DNP- and γ-DNP-DABA were obtained as the result of three successive analyses as 6.61 and 12.64, respectively. The extinctions were measured at 570 mμ.

Percentage degradation. On acid hydrolysis, the α-DNP- and γ-DNP-DABA were determined by analyzing in an automatic amino acid analyzer aliquot parts of accurately measured solutions of the DNP-amino acids before and after hydrolysis. By this procedure the percentage degradation of α-DNP-DABA was found to be 16.6% and of γ-DNP-DABA 10.5% (from literature data [14], for γ-DNP-DABA 10-11%).

It is known [5, 6] that $N^{\alpha} \rightleftarrows N^{\gamma}$ migration forms a mixture of two peptides, one at the α-amino group of DABA (I) and the other at the γ-amino group (III). A third substance is an intermediate (II) which, as may be assumed, has an amidine grouping.



The presence of these three substances was shown in the peptides of α, γ-DABA by paper electrophoresis in comparison with synthetic samples [6]. The determination of the amount of migration products by the ninhydrin method does not give accurate results because of the difficulties connected with separation of the α- and γ-isomers due to the closeness of their R_f values and electrophoretic mobilities. Moreover, the velocities of the reactions of ninhydrin with the α- and γ-amino groups differ, which may also lead to errors.

For the quantitative determination of the α- and γ-isomeric peptides of α, γ-DABA we used the dinitrophenylation method [8, 15]. FDNB accurately registers the α- or γ-amino group of a peptide present in the reaction mixture.

Table 2 Results of the Quantitative Analysis of Active and Inactive DNP-Polymyxin M and Peptides and of Their Partial Hydrolyzates

Compound investigated	number of amino acid residues														
	leucine			threonine			γ-DNP-DABA			α-DNP-DABA			α,γ-DABA		
	μmole	residues	%	μmole	residues	%	μmole	residues	%	μmole	residues	%	μmole	residues	%
Peptide B1	0.488	1	25.3	0.457	0.9	23.7	0.566	1.1	29.3	—	—	—	0.418	0.9	21.7
Peptide EF-1	—	—	—	—	—	—	0.540	—	56.0	0.388	—	35.1	0.085	—	8.8
Active DNP-polymyxin M	0.115	1	10.3	0.305	2.7	27.2	0.596	5.2	53.2	—	—	—	0.105	0.9	9.4
Inactivated DNP-polymyxin M	0.134	1	10.59	0.331	2.47	16.17	0.304	2.27	24.04	0.341	2.54	26.94	0.155	1.16	12.26

From the content of α-DNP- and γ-DNP-derivatives of α,γ-DABA obtained as a result of acid hydrolysis of the peptide it is possible to estimate the amount of isomeric peptide and, thereby, easily to obtain a quantitative evaluation of $N^{\alpha} \rightleftarrows N^{\gamma}$ migration.

It was necessary to solve the problem of the separation and quantitative determination of the α- and γ-DNP derivatives of α,γ-DABA. In the literature only a few, and these not very effective, chromatographic and electrophoretic methods for the separation of α-DNP- and ω-DNP-derivatives of diamino acids have been described [3, 14, 16, 17]. The most complete separation of the isomers is achieved by paper electrophoresis at pH 9.1 [16]. However, the analysis takes a long time (17 hr) and the adsorption of the DNP derivatives on the paper requires the introduction of additional correction factors.

We resolved to effect the separation and quantitative determination of the α- and γ-DNP isomers of α,γ-DABA on an automatic amino acid analyzer. The analysis was carried out on a column (0.9 × 15 cm) filled with the resin Amberlite CG-120 (type III) using 0.36 N sodium citrate buffer with pH 5.26. The separation of the α-DNP and γ-DNP isomers of α,γ-DABA is based on their different solubilities and basicities. The figure (a) shows the elution curves for α-DNP- and γ-DNP-DABA, α,γ-DABA, and NH_3. As can be seen from the figure, the peak of α-DNP-DABA issues first (106 min) and that of γ-DNP-DABA last (195 min). The whole analysis takes 3.5 hr. The clear separation of the pairs α-DNP-DABA and α,γ-DABA, and NH_3 and γ-DNP-DABA enabled them to be accurately identified and quantitatively determined by their absorption at 570 mμ. The micromolar coefficient C for α-DNP-DABA is 6.61 and for γ-DNP-DABA 12.64; C for α,γ-DABA has been determined previously at 13.06. α-DNP-lysine, ε-DNP-lysine, and lysine itself are well separated under similar conditions [see figure (b)]. Ornithine and its isomeric DNP derivatives are satisfactorily separated in 0.7 N sodium citrate buffer with pH 5.28 [see figure (c)]. In the analysis, the neutral amino acids, FDNB and dinitrophenol issue from the column during the first 50–65 min.

For the quantitative evaluation of $N^{\alpha} \rightleftarrows N^{\gamma}$ migration, the α- and γ-peptides of α,γ-DABA were kept for 4 days at 37° C in 0.1 N ammonia. The course of the transacylation was observed by means of electrophoresis in 1 N CH_3COOH. After 4 days, the peptides had been dinitrophenylated. The completeness of the dinitrophenylation was checked by electrophoresis in a mixture of 85% HCOOH, 98% CH_3COOH, and H_2O (28 : 20 : 52, ml). Then, without separating the residual FDNB, the DNP-peptides were hydrolyzed and the resulting hydrolyzates were investigated in the amino acid analyzer. The results of the analysis taking the percentage degradation of the α- and γ-DNP-DABA into account are given in Tables 1–3.

It can be seen from the tables that transacylation takes place both with α- and γ-peptides, the α-peptides giving considerably more of the isomeric peptide (~ 56.5% in the case of α-pel-DABA and ~ 76.8% in the case of α-glyc-DABA) than the γ-peptide (~ 33.5% in the case of γ-pel-DABA and ~ 9.9% in the case of γ-glyc-DABA). Attention is attracted by the fact that from 4.2 to 13.1% of free α,γ-DABA is found in the hydrolyzates of all the peptides kept in 0.1 N ammonia and treated with FDNB. In the control hydrolyzates of α-DNP- and γ-DNP-DABA no free α,γ-DABA was found. No free

α, γ-DABA was found, either, in the hydrolyzate of completely dinitrophenylated α-glyc-DABA (cf. Table 1). However, in the analogous hydrolyzate of γ-glyc-DABA about 7% of free α, γ-DABA was found. Its presence may be due to the formation during the dinitrophenylation process of an intermediate substance (II) (see scheme) which, as we have succeeded in showing, does not react with FDNB, or by the degradation of the peptide with the liberation of free α, γ-DABA. The intermediate (II) when treated with FDNB gives neither α, γ-DABA was found in this hydrolyzate. Consequently, we consider it possible to estimate the amount of intermediate (II) formed during "Inactivation" in the reaction mixture from the amount of free α, γ-DABA (cf. Table 3). Moreover, it can be seen from Table 3 that under the given conditions of "inactivation" the equilibrium is displaced in the direction of the formation of a greater amount of γ-peptide. This displacement can be explained by the lower basicity of the α-amino group of α, γ-DABA as compared with the γ-amino group and also by possible steric hindrance to the attack of the ....—C(=O)—NH(γ)···· bond of the α-amino group.

Table 3

Results of $N^{\alpha} \rightleftarrows N^{\gamma}$ Migration in Peptides of α, γ-DABA*

Compound investigated	Amount of substance, %		
	initial peptide	isomeric peptide or percentage transacylation	intermediate compound
α-pel-DABA	39.3	56.5	4.2
γ-pel-DABA	59.4	33.5	7.1
α-glyc-DABA	11.7	76.8	13.1
γ-glyc-DABA	81.7	9.9	8.4
peptide EF-1	56	35.1	8.9
polymyxin M** (indicated)	44.77	50.22	5.01

*The peptides were kept for 4 days at 37° C in 0.1N NH_4OH.

**The mean values of five parallel experiments. Only the amount (μ mole) of α-DNP- and γ-DNP-DABA and the excess of α, γ-DABA as compared with active polymyxin M were taken into account for the calculation.

Thus, it has been shown that under the conditions of the inactivation of polymyxin M the α- and γ-isomers of N-pel-DABA form an equilibrium mixture containing about 58% of the γ-isomer and 36% of the α-isomer; in the case of the peptides of N-glyc-DABA, the mixture contains about 79% of the γ-isomer and 10% of the α-isomer.

The method of quantitatively determining the α- and γ-DNP derivatives of α, γ-DABA proposed in the present paper has enabled a quantitative evaluation of the $N^{\alpha} \rightarrow N^{\gamma}$ migration in the antibiotic polymyxin M to be carried out under conditions causing its inactivation. The quantitative ratio of α-DNP-DABA and γ-DNP-DABA in inactivated polymyxin M has also been determined previously [17]. However, the degree of degradation of the α- and γ-DNP-DABA and their losses on paper electrophoresis and elution were not then taken into account, nor were the quantitative ratios of all the amino acids present in the antibiotic determined.

A complete amino acid analysis of active DNP-polymyxin M has shown that leucine, threonine, α, γ-DABA, and γ-DNP-DABA are present in the proportion 1.0 : 2.7 : 0.9 : 5.2, respectively, which agrees with information on its structure [17, 18]. The following amino acid ratios have been obtained for inactivated polymyxin M: leuc : threo : α, γ-DABA : α-DNP-DABA : γ-DNP-DABA = 1.0 : 2.5 : 1.2 : 2.5 : 2.3. Thus, in inactivated polymyxin M, $N^{\alpha} \rightarrow N^{\gamma}$ migration has taken place to the extent of approximately 50%. It is interesting to note that a similar magnitude of the transacylation process is found in the peptide EF-1 (cf. Table 3). It is important to note also that, in the case of inactivated DNP-polymyxin M, an excess (~ 5%) of free α, γ-DABA appears as compared with active DNP-polymyxin M. This fact leads to the idea that, as in the peptides considered, a small amount of substances of structure II is formed in the molecule of polymyxin M on inactivation, as has been predicted previously [3]. The ring formation does not change the total charge and this does not enable the molecule of the intermediate to be separated from the initial antibiotic by electrophoresis.

Although when polymyxin M is inactivated $N^{\alpha} \rightarrow N^{\gamma}$ migration takes place to the extent of approximately 50% and the inactivated polymyxin M has 2.5 free α-amino groups and 2.3 free γ-amino groups (cf. Table 2), it is difficult to assume that only 2.5 free γ-amino groups take part in $N^{\alpha} \rightarrow N^{\gamma}$ migration. Evidently, the transacylation took place with all five free γ-amino groups of polymyxin M, although not to equal extents, the total giving about 50%. In addition to the change in the secondary structure and the optical activity caused by the prolonged action of an alkaline medium, the appearance in each molecule of polymyxin M of free α-amino groups of α, γ-DABA and the intermediate substance (II) causes a marked change in its microbiological properties. This fact requires further careful study.

The use of an amino acid analyzer for the quantitative determination of the α- and γ-DNP derivatives of α, γ-DABA also enabled us to establish the quantitative amino acid composition of the peptide B1 isolated from a partial hydrolyzate of DNP-polymyxin M (active) (see Table 2). It is interesting that it has been possible to follow by the

same method the isomerization of the glyc-DABA and DABA-DABA diketopiperidines described previously [6]. It was shown that under the conditions of the inactivation of polymyxin M, DABA-DABA forms by ammonolysis 53% of α-DABA-pyrrolidone and 12% of α-aminopyrrolidone, and no $N^{\alpha} \rightarrow N^{\gamma}$ migration takes place in the diketopiperazine under these conditions. Under the same conditions glyc-DABA forms 71% of α-glyc.-pyrrolidone. Only 8% of the initial diketopiperazine remains.

The quantitative study of $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in various peptides of α, γ-DABA will be continued in the future.

Summary

1. A method for the separation and quantitative determination of α- and ω-DNP derivatives of α, γ-DABA, ornithine, and lysine has been proposed.

2. A quantitative evaluation of the $N^{\alpha} \rightleftarrows N^{\gamma}$ migration in four peptides of α, γ-DABA has been carried out.

3. The degree of migration in inactive polymyxin M and the DNP-peptides from a partial hydrolyzate of the inactivated antibiotic has been determined.

4. The amino acid composition of the DNP peptide B1 from a partial hydrolyzate of active DNP-polymyxin M has been established.

5. The quantitative aspect of the conversion of DABA-DABA and glyc-DABA diketopiperazines has been studied.

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