THE CHANNELS IN THE GRAMICIDIN S WITH UREA CRYSTAL STRUCTURE: NEW MECHANISM OF TRANSMEMBRANE IONIC TRANSPORT

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1. Introduction

Cyclic decapeptide antibiotic gramicidin S c[-(Val-Orn-Leu-D-Phe-Pro)$_2$-] was isolated by C.F. Gause and M.G. Branzhnikova in 1944 [1]. The objects of the antibiotic action are the cellular membranes, which in its presence stop their function as a penetration barrier for substances and ions. There are facts which testify the absence of specific protein receptors of gramicidin S, that is, its interaction with the membrane lipid components [2].

From the time of the antibiotic isolation many authors were advancing various models of its molecule conformation based on the common principles of the peptide chain folding [3], as well as on semi-empirical calculations [4-7]. Most widespread became the β-model suggested by Hodgkin-Oughton [3] according to which the molecule consists of two antiparallel β-chains, joined by proline bridges, and is stabilized by four cross-cyclic H-bonds. This model was supported by the investigations of the conformation states of gramicidin S and its N, N' diacetyl derivative in different media by physicochemical, mostly spectral, methods [8]. G.N. Tishchenko and co-workers [9-12] obtained a series of crystals of heavy-atom derivatives of gramicidin S with two independent molecules in the asymmetric part of the unit cell. However, interpretation of the Patterson maps was unsuccessful, probably due to the uniform alternation of the gramicidin S molecules and heavy atoms connected with them, that in combination with the high symmetry of the unit cell and the great number of the atoms in it made the maps most complicated to interpret. Attempts were made to use the heavy-atom method to solve the structure [7-13]. Nevertheless, up to 1978 the X-ray structure investigations of gramicidin S were unsuccessful.

In 1978 an announcement was published in *Nature* about the obtaining of the gramicidin S structure model at the Physics Department of York

University, England [14]. The progress was conditioned, on the one hand, by successful crystallization of gramicidin S –one molecule in the basic part of the unit cell with space group $P3_121$, $a = 25.8$, $c = 21.49$ Å– from the alcohol solution in presence of urea and HCl and, on the other hand, by the creation in York of a very powerful version of the direct method program MULTAN-78 [15]. The structure model obtained was refined using 4902 reflections in the sphere of about 1 Å and with the least-squares program with fast Fourier-transform algorithm [15] to R-factor 0.25. However, since the crystals were unstable and in the course of data collection the authors had to use several samples, the quality of the experimental data was not very high. The standard deviations for the C atoms of the cycle were equal to 0.04 Å. The positions of a series of atoms were undefined. Good-quality crystals were grown at the Institute of Crystallography, Academy of Sciences of the USSR, that, in combination with the methods and programs for X-ray structure analysis developed in the above-mentioned Institute made the investigation successful.

2. *Experimental*

The crystals of the gramicidin S complex with urea, suitable for X-ray structure analysis were grown from water-alcohol solution of the antibiotic in the form of the hydrochloride in the presence of HCl and urea. The unit cell parameters $a = 26.0$, $c = 21.5$ A were close to those given in [15]; space group $P3_121$. As the crystals were unstable in the open air, they were packed in quartz capillary in the presence of the mother liquid. X-ray measurements were carried out on a Syntex $P2_1$ diffractometer at room temperature using $CuK\alpha_1$ radiation, $\vartheta - 2\vartheta$ scan technique, scan angle 0.32-0.45°, 4918 reflections, $d_{min} \geq 0.98$ Å. For the structure investigation 4028 reflections were used. The starting model [15] included 81 atoms of gramicidin S molecule. By the automatic sequential approximation method [16] the positions of the next 5 peaks were revealed, which were interpreted as two water and one urea molecules. The final synthesis contained a lot of extra peaks, which were quite difficult to interpret. Sequential isotropic–anisotropic refinement of the atoms localized, the difference map calculation, the new peaks selection according to the crystal-chemical criteria with an account of possible statistical occupation of some positions by water molecules and by the terminal C atoms of some side chain etc., allowed us to determine the parameters of 18 more atoms, including the atoms of the solvents– water and alcohol. But the Cl^- ions, whose presence may be expected taking into consideration the crystallization conditions, were not revealed. Besides the C, N and O atoms, 59 H atoms were localized in the series of subsequent

difference maps, and their positional parameters were refined. The positions of 26 more H atoms were calculated geometrically. All the H atoms were refined isotropically. The final R-factor was 6.2%.

In the course of the investigation of the fine features of the structure the least squares refinement of the extinction parameter was most helpful: in addition to the reduction of the R-factor, especially for reflections with small sin ϑ/λ, the quality of electron density function distribution became considerably higher. For this structure the blocked full-matrix "cascade" least-square procedure realized in AREN–system [17] was especially useful. The essence of this procedure is as follows. Before the refinement process the program calculates and puts down F_{calc} over all the atoms of the structure in the computer memory. Owing to the limitations of the computer memory the refinement procedure was separated into steps with up to 170 atomic parameters being refined simultaneously at each step. At the beginning of each step from the previous F_{calc} the contributions of the refined atoms are subtracted and those refined at the previous stage, are added. Then, during the iterative process only the values depending on the atoms refined at the given stage are calculated. Such a procedure allows one to refine structures with up to 430 independent atoms in anisotropic approximation on an ordinary PC-AT/XT within reasonable calculation time.

All the calculations were carried out using the AREN program system on EC-1045 and ARENPC on PC-AT [18].

3. Results and discussion

The conformation of the gramicidin S molecule (Figure 1, Table 1) is similar in general outline to the "pleated sheet" structure suggested by Hodgkin and Oughton in 1953 [3], but differs from the latter by the twisting of the molecular cycle, similar to the one realized in the β-sheets of the protein molecules –the torsion angle between the mean planes of half-rings of the main chain CA(Orn2) ... CA(Orn7) and CA(Orn7) .... CA(Orn2) is 40.9 Å:

The gramicidin S molecule in crystals possesses a 2-fold axis; its 30-membered cycle, made of the antiparallel peptide chains, has a roughly rectangular shape about 4.8x13.6 Å in size. The maximum size of the molecule, namely, the distance between terminal atoms CZ of the Phe radicals is equal to 22.9 A, CG atoms of the Pro-cycles are at 16.9 Å distance from each other, smaller rectangle sides being created by the atoms of the Phe and Pro residues of the main chain.

As the planes of the Phe 4 and Pro 5 rings are roughly coplanar (the torsion angle is 26.9 °), it is possible to speak about a stacking interaction bet-

Table 1. Conformational angles (degrees) [19] for gramicidin S molecule.

Angle				Val1	Orn2	Leu3	Phe4	Pro5	Val6	Orn7	Leu8	Phe9	Pro10
C'	N	CA	C'	–118	–109	–123	61	–79	–125	–105	–140	57	–94
N	CA	C'	N	156	132	92	–125	–4	154	136	120	–134	11
CA	C'	N	CA	172	171	–178	–175	–173	174	179	–172	–177	–172
N	CA	CB1	CG1A	–28	–172	–59	174	13	58	–64	–73	175	39
N	CA	CB1	CG2A	167									
N	CA	CB2	CG1B	60					–67				
N	CA	CB2	CG2B	–44									
CA	CB	CG	CD1		175	–158	–75	–9		174	–67	–87	–37
CA	CB	CG	CD2A			93	107				174	89	
CA	CB	CG	CD2B			–60							
CB	CG	CD1	CE1				–179					176	
CB	CG	CD1	N					2					21
CB	CG	CD1	NE		–173					–178			
CB	CG	CD1	CE2				178					–179	
CG	CD1	CE1	CZ				–1					2	
CG	CD2	CE2	CZ				–5					4	
CG	CD	N	CA					7					3
CD	N	CA	C'					107					94

ween them. The coplanarity between the Phe 9 and Pro 10 is noticeably broken, the torsion angle being 44°, apparently, at the expense of the formation of the intramolecular H-bond between the terminal N atom of the Orn 2 and carbonyl O atom of the Phe 4 2.75(2)Å. The symmetric intramolecular H-bond does not appear as both O and N atoms of the Phe 9 make H-bonds with N and O atoms of the urea molecule (Table 2).

A specific feature of the molecule is the location of the extended side chains of the ornitine residues on the one side of the molecular cycle in the form of peculiar "legs-tentacles". One of these legs is "fastened" by intramolecular H-bond, as stated above, the other one is free (Fig. 1,b). The distance between the terminal NE atoms of the ornitine "tails" is 5.65 Å.

The hydrophobic side chains of the Val and Leu residues are oriented in the direction opposite to the ornitine side chains of the molecular cycle. There is statistical disorder in the orientation of the Val 1 and Leu 3 residues side chains. In the first one, the CB, CG1 and CG2 atoms occupy with

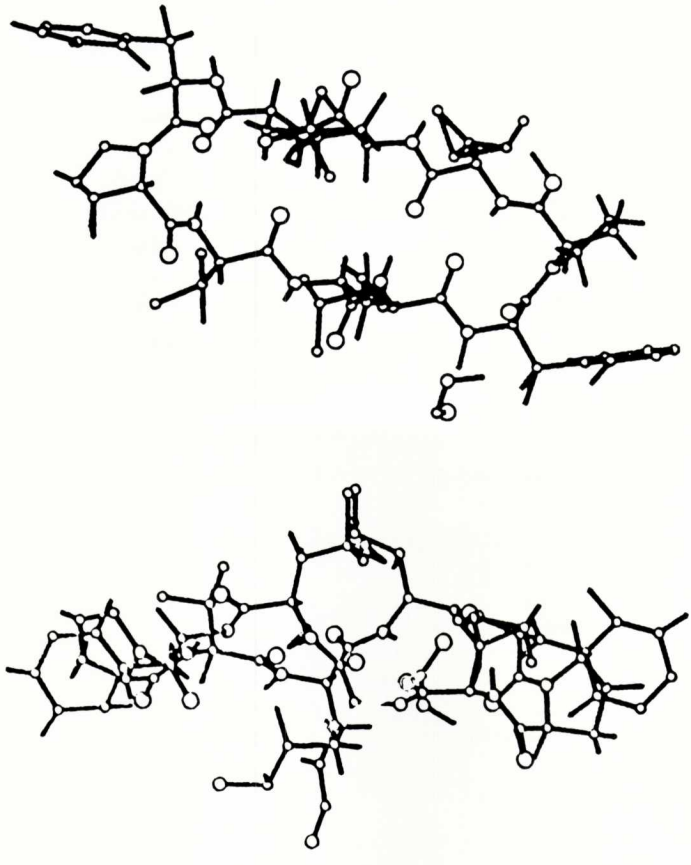

Fig. 1. Gramicidin S molecule conformation in crystal, a) view along the molecular pseudoaxis 2; b) view perpendicular to that axis, the ornitine "tails" are seen.

equal probabilities two different positions A and B, in the second one the CD1 and CD2 atoms are also situated in two positions A and B with probabilities 0.7 and 0.3, respectively, the positions of the CD1A and CD2B coincide. It is known [20,21] that for the aliphatic side chains (in our case Val, Orn, Leu) the torsion angle around the bond CA-CB (χ^1) may be 60, 180, and –60°, the last value being preferable, as the CG atom in this case is situated between the small N and H atoms. The least probable value is 60°. In gramicidin S molecule the angle χ^1 is close to preferable value –60° (see table 1) for all the aliphatic side chains except Orn 2 and position 1 of Val 1. For Orn 2 this angle value deviated from 180° towards negative values (–172°) owing to steric influence of the C=O group. In other words, this radical is in trans-orientation, the others in the gauche one. The ornitine

"tails" acquire the stretch conformation; the χ^2 and χ^3 parameters are close to 180°, the main orientation of the Leu 2 and Leu 8 radicals is trans, the χ^2 angle is nearly equal to 180°.

For the Phe residues three conformations of the side chain are possible [22] with the χ^1 (N CA CB CG) angle values close to 60, 180 and –60°, though the conformation with $\chi = 60°$ is seldom realized. The χ^{21} (CA CB CG CD1) and χ^{22} (CA CB CG CD2) angles are close to 90 and –90°, respectively. The phenyl ring plane is nearly perpendicular to CA CB CG plane. The Phe radicals in the gramicidin molecule are characterized by the χ^1 angles close to 180°, χ^{21} and χ^{22} to 90 and –90°, respectively. The Pro 5 and Pro 10 residues in the molecule have identical conformation (see Table 1), namely, C_s–C^β-exo (envelope, CB in the bent corner, CB and CG on the both sides of the plane of the cycle with symmetry C_s) [23, 24].

There is one urea molecule $OC(NH_2)_2$ for every gramicidin molecule in the structure, which occupies a special position on the crystallographic

Table 2. H-bonds A...B (r ≤ 3.2Å) in the gramicidin S structure.

N	Atom A	Atom B	Position B Sym	TZ	r, A	N	Atom A	Atom B	Position B Sym	TZ	r, A
1	NVal1	OLeu8	1	0	3.19	21	OW4	OW10	1	0	2.93
2	NLeu8	OVal1	1	0	2.93	22	OW5	OW11	1	0	2.48
3	NVal6	OLeu3	1	0	3.27	23	OW5	OW17	1	0	3.10
4	NLeu3	OVal6	1	0	2.82	24	OW7	OW17	1	0	2.80
5	NEOrn2	OPhe4	1	0	2.75	25	OW8	OW12	1	0	2.57
6	NPhe9	OUrea	1	0	2.81	26	OW11	OW19	1	0	2.99
7	NUrea	OPhe9	1	0	2.99	27	OW12	OW16	1	0	2.40
8	NPhe9	OUrea	5	–1	2.81	28	OPro10	OW2	5	0	2.77
9	OPhe9	OW6	1	0	2.96	29	NEOrn7	OW7	3	–1	3.07
10	NEOrn2	OW10	1	0	3.19	30	NEOrn2	OW7	2	0	3.11
11	NEOrn2	OW14	1	0	2.68	31	NPhe4	OW2	5	0	2.85
12	NOrn7	OW1	1	0	2.92	32	OPro5	OW1	4	–1	2.75
13	OOrn7	OW8	1	0	2.70	33	OW3	OW5	6	0	2.98
14	OOrn7	OW10	1	0	3.15	34	OW4	OW14	3	–1	2.74
15	OPro5	OW4	1	0	3.15	35	OW4	OW10	4	–1	2.93
16	OPro5	OW18	1	0	2.98	36	OW4	OPro5	4	–1	3.15
17	OW2	OW13	1	0	2.98	37	OW6	OW12	5	–1	2.69
18	OW2	OW20	1	0	3.07	38	OW9	OW18	4	–1	2.80
19	OW3	OW5	1	0	2.97	39	OW11	OW12	4	–1	2.98
20	OW4	OW14	1	0	2.74	40	NOrn2	OOrn2	5	0	3.03
						41	NEOrn7	OW10	1	0	3.15

Note. TZ is the translation along axes z. The numbers of symmetry transformations are given in the "Sym" column, namely,
1) x, y, z; 2) –y, x–y, 1/3+z; 3) y–x, –x, 2/3+z;
4) x–y, –y, 2/3–z; 5) –x, y–x, 1/3–z; 6) y, x, –z.

two-fold axis and is connected with the antibiotic molecule by two H-bonds, namely OU ... NPhe 9 2.81 Å and Ou ... OPhe 9 2.99 Å. The geometry of this molecule is usual. On one antibiotic molecule fall 20 water molecules, the positions of three of them are fully occupied, the rest of them with a probability 0.56-0.20. One of the water positions (OW2) is 2/3 occupied by the water molecules and 1/3 occupied by O atom of the alcohol molecule. The atom C1 of the alcohol molecule is also localized. The water molecules OW are H-bonded with antibiotic molecules and with other water molecules (see table 2). Some of the OW positions with the occupation less than 1 can not be filled simultaneously, i.e., they belong to different gramicidin S molecules in one channel or even to different channels.

There is a complicated system of the intra- and intermolecular H-bonds, with and without participation of water, alcohol and urea molecules (see table 2). First of all this is the four cross-cyclic H-bonds, which stabilize the elongated conformation of the molecular cycle. These bonds are not equal, namely, the "central" ones are shorter (2.82 and 2.93 Å) and the "peripheral" ones longer (3.27 and 3.19 Å), the latter being not quite parallel to each other. The conformation of the molecule is influenced to a smaller extent by the intramolecular H-bond between terminal NE atom of the Orn 2 residue and O atom of the Phe 4 residue 2.75 Å long, and also by two intermolecular H-bonds with the urea. These bonds were mentioned earlier. Note that only one intermolecular H-bond is realized directly between the atoms of the neighbouring gramicidin molecules, the water molecules take part in the rest of them.

The conformational parameters φ and ω (see Table 1) of the equal residues are close to each other, for the Val, Orn, Leu their values correspond to the "pleated sheet" structure with the antiparallel chains (β-structure), for which $\varphi = -139^\circ$, $\psi = 135^\circ$, $\omega = -178^\circ$ [25]. The angles φ and ψ for the Pro and Phe residues differ from typical ones for the ordinary regular structures. The ω angle is close to 180° for all the residues, that corresponds to the trans-peptide bond. The greatest deviations from this value have the angles for residues 1,2,8 and 10, $\Delta\omega$ being equal to 8,9,8 and 8°, respectively, that would be explained by the influence of the strongly pronounced statistical disordering of the Val 1 residue side chain and of H-bond N1 and one atom of Phe 9 with O urea.

The bond lengths in the trans-peptide groups of all the residues are close to the standard values [26]. The bond lengths in the side chains in some cases significantly differ from the usual value of 1.54 Å, due to large thermal vibrations of the terminal atoms and, especially, statistical disorder of some of them. The full picture of the bond angles of the backbone distribution is close to that expected for peptide chains [26]. As for the short intra- and intermolecular contacts, it is possible to say that they are absent in the struc-

ture, especially taking into account the modern ideas about the values of the Van der Waals radii [27].

Of the greatest interest is the packing of the gramicidin S, urea and water molecules in the structure. The gramicidin S molecules, collected around 3_1 axis according to the law of the left-handed double helix, form the channels (figure 2), whose outside hydrophobic surface is made of uncharged side radicals, the inside surface is made of the main chain atoms, mainly, of the O and N atoms.

The ornitine "tails" with the N atoms at the ends are turned inside the channel (figure 2,a). The inner diameter of the channel can change on account of the conformational change of these "tails". The channel is filled by the water molecules. The geometrical characteristics of the channel are: the external diameter 30-35 Å, the internal one (without ornitine "tails") about 12 Å, the channel diameter, limited by the terminal N atoms of the ornitine residues, as was already mentioned above, may change from about 3.3 to 6.2 Å. Thus, the ions and particles of rather large sizes may pass through the

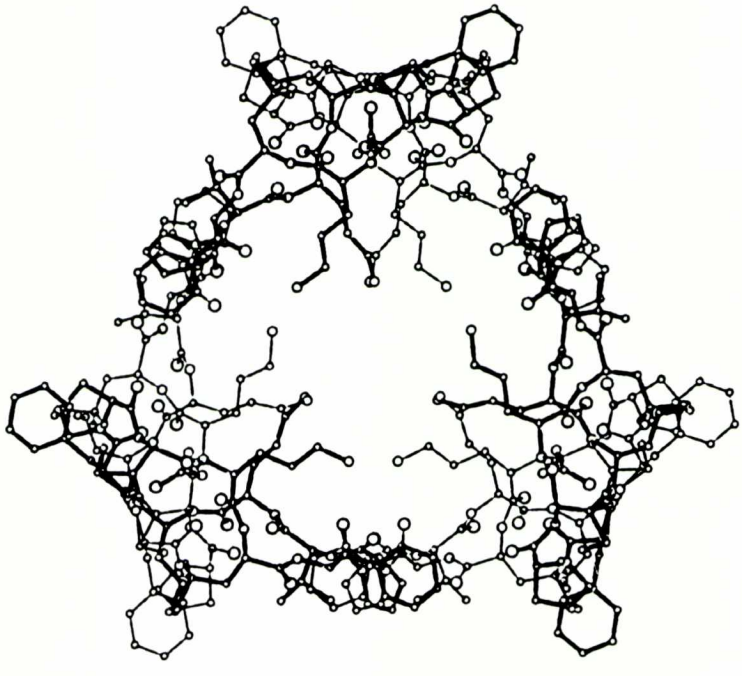

Fig. 2,a. Gramicidin S molecules in the structure, collecting around 3_1 axis, according to the law of the left-handed double helix, form the channels: view of the channel along the c-axis, the ornitine tails which define the diameter and polarity of the interior of the channel, are seen, in the channel there are also water molecules.

channel. The ions and particles can be transported across the membrane under the action of the transmembrane potential. A very compact packing of the gramicidin S channels in the crystal structure should be noted.

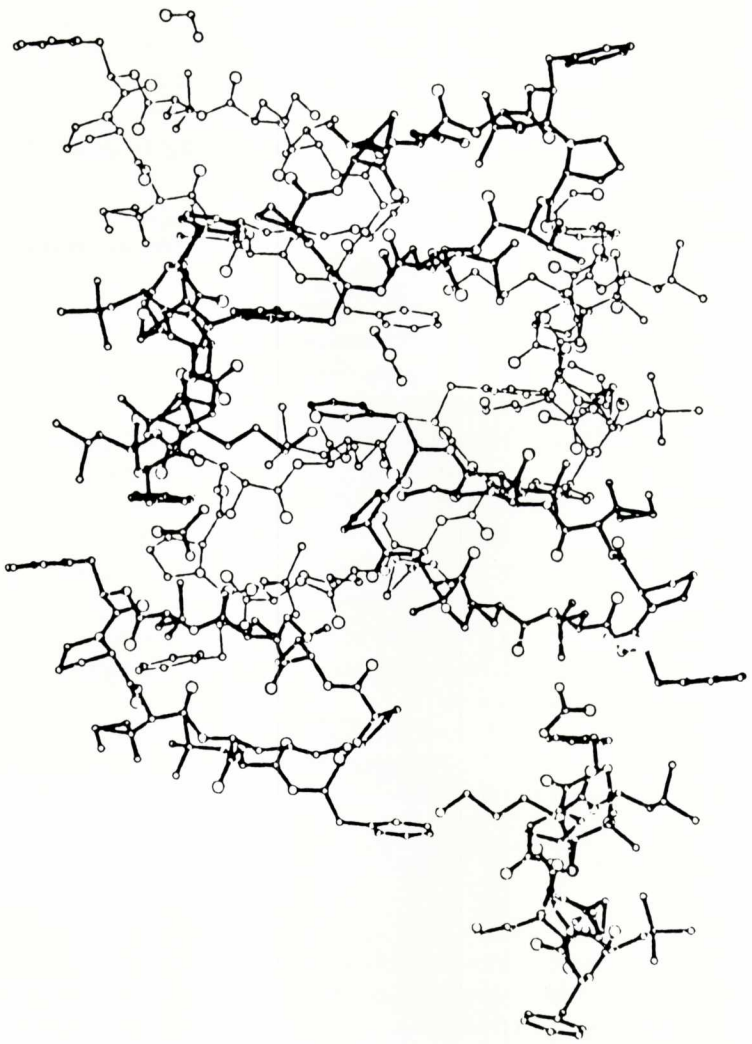

Fig. 2,b. Gramicidin S molecules in the structure, collecting around 3_1 axis, according to the law of the left-handed double helix, form the channels: view of the channel perpendicular to the c-axis. The side chains forming a hydrophobic exterior of the channel as well as the urea molecules are seen.

An important feature of the structure investigated is the absence of Cl^- ions and, consequently, of the positive charge on the terminal NE atoms of the ornitine residues. The gramicidin S crystal studied in this work was obtained from a solution of gramicidin S hydrochloride upon addition of small amounts of hydrochloric acid and urea. Therefore, both the presence of Cl^- ions in the mother liquid and their absence in the crystals formed is beyond doubt, though gramicidin S usually exists in the form of a salt with the positive charge at the terminal N atoms of the Orn residues and the compulsory counter-ion presence, for example, Cl^- ion. We can think of three reasons for that extremely unusual situation. First, it is an energetic gain at the expense of the channels formation, accompanied by the transition from four-covalent one-charged state of the NE atoms of the ornitine residues to the three-covalent and by the active participation of these atoms in the H-bonds (see Table 2). Second, it is the energetic gain at the expense of a very close packing of the channels arisen. Third, it is the binding of the Cl^- ions by the urea molecules in the mother liquid.

It should be noted, that the mechanism of the ion transport effected by gramicidin S has remained unclear up to now. It was mentioned in [2] that the distance between the NE atoms of the two ornitine residues in the gramicidin S molecule, established by the spectral methods, is similar to the distance between the negative charges on the phosphate groups in the phospholipide membranes. The possibility of the creation of the gramicidin S channels was noted by the authors of [28-30], who studied the action of the antibiotic on the mytochondrial membranes, though no assumptions were made about the structure of these channels. According to the data of these authors the channels let pass some cations, for example, K^+ and Na^+, but the penetrating anions, as phosphate and acetate, intensify the gramicidin S action on the membranes.

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Abstract

The crystal structure of the membrane-active antibiotic-cyclopeptide gramicidin S complex with urea was determined by X-ray structure analysis. The gramicidin S molecule possesses an antiparallel β-structure, its slightly twisted 30-membered cycle has a roughly rectangular shape about 4.8 x 13.6 Å in size, the maximum size of the molecule is 22.9 Å. The extended side chains of the Orn residues are located on one side of the molecular cycle in the form of peculiar "legs-tentacles". One of these legs is "fastened" by the intramolecular H-bond to the O atom of the nearest Phe 4 residue, the other one

is free. There is one urea molecule and twenty water molecules per one antibiotic molecule structure. The positions of three water molecules are fully occupied, the others are occupied with the probability of 0.56-0.20. One of the "water" positions is 2/3 occupied by water, and 1/3 occupied by alcohol. There is a complicated H-bond system in the structure: intra- and intermolecular, with and without the participation of water, alcohol and urea molecules. The gramicidin S molecules collected around 3_1 axis according to the law of the left-handed double spiral form channels, whose outside hydrophobic surface is made of the main chain atoms, mainly, of O and N atoms and of ornitine "tails" with uncharged NE atoms at the ends. Due to a change in the conformation of these "tails", the inner diameter of the channel filled by water molecules may change from 3.4 to 6.3 Å, the inner diameter (without ornitine "tails") is about 12.7 Å. Thus the ions and particles rather large in size may pass through the channel. The gramicidin channels are discovered and described for the first time. The channels in the crystal structure are closely packed under the hexagonal law. The Cl^- ions, that are present in large amounts in the mother solution, are absent in crystals. That is in accordance with the absence of charges on the terminal NE atoms of the Orn residues. This unusual phenomenon is explained in the paper.

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