THE MECHANISM OF ACTION OF MYXOVALARGIN A, A PEPTIDE ANTIBIOTIC FROM *MYXOCOCCUS FULVUS*†

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Myxovalargin A has two modes of action. At low concentrations (below 1 μg/ml) it inhibits bacterial protein synthesis specifically and instantaneously. *In vitro* experiments suggest that it interferes with the binding of aminoacyl tRNA to the A site of the ribosome. At higher concentrations (above 5 μg/ml), or upon prolonged incubation, the antibiotic damages cell membranes. This leads to secondary effects, like decreased O_2 consumption or instant break down of RNA synthesis, and may be the reason for the irreversibility of the antibiotic action. The membrane effect is not restricted to prokaryotes and may explain the high toxicity of the compound for higher organisms.

Myxovalargin A is one of three chemically related peptide antibiotics isolated from the culture supernatant of the myxobacterium, *Myxococcus fulvus* strain Mx f65. It is a linear peptide with a molecular weight of about 1,700 consisting of 14 amino acids, 3-methylbutyric acid at the amino end, and agmatine at the carboxyl terminal[1,2]. Among the amino acids, there are several unusual species, like α,β-dehydrovaline, α,β-dehydroisoleucine, *N*-methylalanine, and β-tyrosine. The exact amino acid sequence is still under investigation. Because of its many hydrophobic side chains, the antibiotic is rather lipophilic. Of the three peptide antibiotics produced, myxovalargin A is produced in excess. All experiments described in this article have been performed with this compound.

Myxovalargin is active mainly against Gram-positive bacteria (MIC 0.3~5 μg/ml). It is a specific inhibitor of prokaryotic protein synthesis. In *Staphylococcus aureus* protein synthesis is inhibited immediately upon addition of the antibiotic, while the syntheses of the other cellular macromolecules respond only 10 to 15 minutes later[1]. At higher concentrations, however, myxovalargin is also toxic for animals (LD_{50} for mice 10 mg/kg, sc).

In this article we report on experiments with *in vitro* protein synthesis, and on an effect of myxovalargin on membranes which could be the basis for its toxicity for animal cells.

Materials and Methods

Organisms

Bacillus subtilis and *Candida albicans* were from our culture collection. *Escherichia coli* NF 118 was kindly provided by Dr. U. SCHAIRER of this institute.

Influence of Myxovalargin on Viability of Bacteria

B. subtilis cells were suspended in Cas acid medium: Casamino Acids (Difco) 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.03%, $(NH_4)_2SO_4$ 0.06%, phosphate buffer 1 mM, pH 7.4, standard trace elements and vitamin solution. The final cell density was 3.1×10^8/ml (optical density at 623 nm, or OD_{623}, 0.5). The suspension was then divided into several parallels to which different quantities of

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myxovalargin were added. After shaking for 20 minutes at 30°C, the cultures were diluted 1: 10^5, and 0.1 ml samples were plated on EBS-agar (peptone from casein, tryptically digested (Merck, Darmstadt) 0.5%, Proteose peptone (Difco) 0.5%, peptone from meat (Merck) 0.1%, yeast extract (Difco) 0.1%, agar 1.5%, pH 7.0) to determine the number of the colony forming units (cfu).

Protein and RNA Synthesis *In Vivo*

Protein and RNA synthesis in *B. subtilis* were studied in Cas acid medium. Either [*U*-^{14}C]tyrosine (0.1 μCi/ml; specific activity: 10 Ci/mol, from Amersham International, Braunschweig, FRG), or 8.5 μM uracil + 0.1 μCi [2-^{14}C]uracil/ml (specific activity: 58 Ci/mol) were added. The cell density was 1.3×10^8/ml (OD_{623} 0.2). The cultures were incubated at 35°C under shaking. Samples of 0.5 ml were pipetted into 2.5 ml of cold 2.5% perchloric acid (PCA), the precipitates were collected on glass fiber filters (Whatman GF/B) and washed with 2×5 ml of PCA and 1×5 ml of 95% ethanol. After drying, the radioactivity was determined in a Packard Tri Carb scintillation spectrometer. Myxovalargin was added after 15 minutes of incubation at a concentration of 1.5 μg/ml.

Protein Synthesis *In Vitro*

Protein synthesis was studied *in vitro* with a preparation from *E. coli* strain NF 118[3]. Poly-U (80 μg/ml; from Boehringer, Mannheim) was used as the template, and 7.5 μM phenylalanine + 2.5 μCi/ml of [*U*-^{14}C]phenylalanine (specific activity 496 Ci/mol) were added. The total volume of the test was 100 μl. The reaction was started by adding the S 30 extract from desintegrated *E. coli* (supernatant from the centrifugation step at $30{,}000\times g$). The mixture was incubated for 25 minutes at 37°C and then poured into 2 ml of cold 5% trichloroacetic acid (TCA). The precipitate was collected on a membrane filter (cellulose nitrate, 0.45 μm pore size; from Sartorius, Göttingen) and washed with 2×5 ml of TCA. Radioactivity was determined as above. To measure the kinetics of the formation of poly-phenylalanine, a test with the same concentration as above was performed. Aliquots were taken after different times and treated as above. After 6 minutes the sample was divided. To one part, myxovalargin (31 μg/ml final concentration) was added; the second part was the control.

Fractionation of the S 30 Extract

S 30 extract was centrifuged for 2.5 hours at $250{,}000\times g$ in buffer 1 (see below) in a Beckman ultra centrifuge equipped with a Ti60 rotor. After centrifugation the upper half of the supernatant was withdrawn and used as "S 100" fraction. The lower half was discarded. The pellet was washed once in buffer 1 and then suspended in the same buffer[4].

Amino Acid-tRNA Synthetase

Amino acid-tRNA synthetase was measured according to FUJIMOTO *et al.*[5]. [^{14}C]Phe-tRNA and f-[*methyl*-^{14}C]Met-tRNA were prepared and isolated as described by RAVEL and SHOREY[6]. The final pellets were examined for free amino acids by TLC.

Tests for Binding of [^{3}H]GTP and [^{14}C]Phe-tRNA

Tests for binding of [^{3}H]GTP and [^{14}C]Phe-tRNA to S 100 were done according to RAVEL and SHOREY[6].

Binding of [^{14}C]Phe-tRNA to Ribosomes

Binding of [^{14}C]Phe-tRNA to ribosomes was performed as described by NIRENBERG and LEDER[8]. For binding of f-Met-tRNA, AUG instead of poly-U was used.

Puromycin Reaction

The puromycin reaction was tested according to the procedure of BROT *et al.*[9]. In a second experiment washed (1 M NH_4Cl) ribosomes were used to study binding and puromycin reaction with acetyl-[^{14}C]Phe-tRNA using the procedure of WURMBACH and NIERHAUS[10] with the exception that S 100 was used instead of elongation factor G.

Preincubation of Ribosomes with Myxovalargin

0.1 ml of the ribosomal fraction was incubated with 31 μg myxovalargin/ml for 5 minutes at 37°C. After centrifugation for 2.5 hours at $250{,}000\times g$, the supernatant was carefully removed. The pellet

was suspended in buffer 2. The puromycin reaction was tested with these ribosomes.

Buffers

Buffer 1: 10 mM Tris-HCl, pH 7.5+10 mM $MgCl_2$.

Buffer 2: 100 mM Tris-HCl, pH 7.7+10 mM $MgCl_2$+20 mM β-mercaptoethanol[6].

Buffer 3: 50 mM Tris-HCl, pH 7.6+80 mM KCl+80 mM NH_4Cl+10 mM $MgCl_2$+1 mM dithiothreitol (DTT).

Cell-free Eukaryotic Protein Synthesis

The wheat germ system for protein synthesis was prepared and assayed according to ROBERTS and PATTERSON[11] and DAVIES *et al.*[12]. The rabbit reticulocyte lysate was purchased from Amersham International, Braunschweig. The test was performed as described by PELHAM and JACKSON[13].

Release of UV-absorbing Material from *B. subtilis*

The experimental procedure of CHOU and POGELL[14] was adopted. Log-phase cells were washed with 0.05 M phosphate buffer pH 7.2 containing 0.05 M sucrose, and were then suspended in the same medium to a cell density of 5.2×10^8/ml (OD_{623} 0.8). This suspension was divided into 4 aliquots, to which myxovalargin was added at different concentrations. The suspensions were incubated at 35°C under shaking. At intervals, the OD_{623} of the suspension and, after removal of the cells by centrifugation, the absorption of the supernatant at 280 nm were determined.

Release of UV-absorbing Material from Erythrocytes

Human erythrocytes were obtained from the blood bank and prepared for the experiment as described by STECK and KANT[15]. They were suspended in buffer to a cell density of 1.5×10^8/ml and incubated at room temp after addition of myxovalargin. Release of material absorbing at 280 or 570 nm, respectively, was measured after removal of the cells.

Effect of Myxovalargin on O_2-consumption

B. subtilis was suspended in Cas acid medium to a cell density of 4×10^8/ml (OD_{623} 0.63). O_2-consumption was measured at 35°C in presence and absence of myxovalargin using an oxygen measuring cell (Radiometer, Leverkusen). *C. albicans* was suspended in Mycophil medium (Phytone peptone (BBL) 1%, glucose 1%) to a cell density of 9.1×10^7/ml (OD_{623} 1.25). O_2-consumption was measured at 30°C.

Protein Concentrations

Protein concentrations were determined according to BRADFORD[16].

Results

Effects on Viability of Bacteria

When added to cultures of *B. subtilis*, low concentrations of myxovalargin led to a drastic decrease in cfu. In the range of the minimal inhibitory concentration (MIC 0.6 μg/ml) the number of viable cells remained constant over 20 minutes.

Effects on Prokaryotic Protein Synthesis

As with *S. aureus*[1], incorporation of tyrosine into TCA insoluble material stopped immediately when myxovalargin was added to a culture of *B. subtilis*. Using an *E. coli* system for studying protein synthesis *in vitro*, the incorporation of radioactive phenylalanine into TCA precipitable material was found to be strongly inhibited by the antibiotic. The concentration of myxovalargin required to inhibit the reaction by 50% was about 1 μg/ml. At 10 μg/ml, the system was completely blocked (Fig. 1). The synthesis of poly-Phe was only slightly inhibited when myxovalargin was added after the polymerization reaction had been started (Fig. 2).

Fig. 1. Effect of different concentrations of myxovalargin on cell-free protein synthesis using an *Escherichia coli* system, measured as incorporation of [^{14}C]Phe into TCA insoluble material.

The antibiotic was added at the beginning of the reaction.

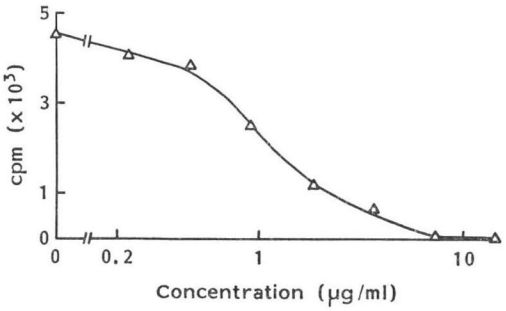
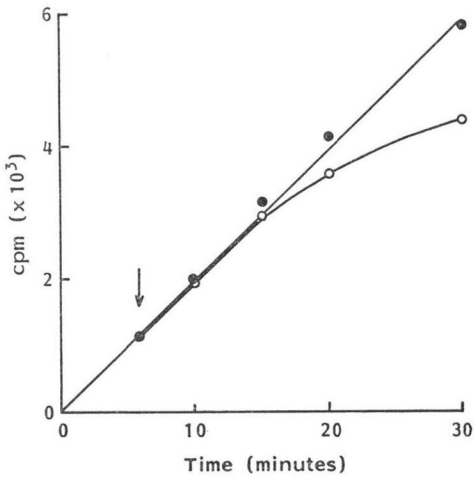


Fig. 2. Kinetics of cell-free protein synthesis (*Escherichia coli* system).

Myxovalargin (31 μg/ml) was added 6 minutes after the reaction had been started (arrow).

● Control without myxovalargin.



Consecutively, several intermediary steps of protein synthesis were tested. The formation of aminoacyl tRNA was not inhibited by myxovalargin. Binding of GTP to S 100 was also not influenced. Myxovalargin interfered, however, with the binding of Phe-tRNA to ribosomes (30~54% inhibition, Table 1), while the binding of f-Met-tRNA was not influenced. This was particularly evident when the complete system was used. The puromycin reaction was strongly inhibited, especially when myxovalargin was added at the very beginning of the experiment (Table 2). The puromycin reaction with f-Met-tRNA was inhibited to the same degree whether the ribosomes were preincubated with myxovalargin or the antibiotic was added directly to the reaction mixture (Table 2). Table 3 shows the interaction of acetyl[^{14}C]Phe-tRNA with washed ribosomes. Myxovalargin inhibited the binding of this tRNA species strongly when the P site was occupied by deacylated tRNA. On the other hand, when no deacylated tRNA was present so that acetyl-Phe-tRNA would bind preferentially to the P site, only a weak inhibition was observed. The puromycin reaction was blocked by myxovalargin irrespective of a preincubation with deacylated tRNA.

Effects on Eukaryotic Protein Synthesis

Much higher concentrations of the antibiotic were required to inhibit *in vitro* protein synthesis in eukaryotic systems. Of the two systems tested, the rabbit reticulocyte system was inhibited to 66% with 303 μg myxovalargin/ml, whereas the wheat germ system was inhibited to only 27% with 267 μg/ml.

Effects on RNA Synthesis

RNA-synthesis in *B. subtilis* was blocked only about 10 minutes after addition of small, but growth-inhibiting amounts of myxovalargin (1.5 μg/ml). When higher concentrations were used, however, the synthesis of RNA was inhibited immediately upon addition of the antibiotic (Fig. 3).

Release of UV Absorbing Material from Cells

In presence of myxovalargin, cell suspensions of *B. subtilis* in buffer showed a decrease in OD_{623},

Table 1. Effect of myxovalargin on the binding of [^{14}C]Phe-tRNA or f-[^{14}C]Met-tRNA to *Escherichia coli* ribosomes.

Reaction mixture*	Radioactivity bound to nitrocellulose filters** (cpm)	
	Without myxovalargin (=100%)	With 31 μg myxovalargin/ml (% residual activity)
Ribosomes+[^{14}C]Phe-tRNA+poly-U	498	349 (70%)
Ribosomes+[^{14}C]Phe-tRNA+poly-U+GTP	790	418 (53%)
Ribosomes+[^{14}C]Phe-tRNA+poly-U+S 100	439	203 (46%)
Ribosomes+[^{14}C]Phe-tRNA+poly-U+S 100+GTP	1,875	533 (28%)
Poly-U+GTP+[^{14}C]Phe-tRNA (control)	38	35
Ribosomes+f-[^{14}C]Met-tRNA+AUG+GTP	608	653 (107%)
Ribosomes+f-[^{14}C]Met-tRNA+AUG+GTP+S 100	793	866 (109%)
AUG+f-[^{14}C]Met-tRNA (control)	36	nt

* Complete reaction mixture (100 μl): Buffer 3; 108 μg ribosomal fraction (by protein); 50 μg S 100 fraction (by protein); 6,400 cpm [^{14}C]Phe-tRNA or 11,200 cpm f-[^{14}C]Met-tRNA; 100 μmol GTP; 4 μg poly-U or 10 μg AUG. Incubation was for 10 minutes at 30°C. Background values without template are subtracted.

** Pore size 0.45 μm.

nt: Not tested.

Table 2. Effect of myxovalargin on the puromycin reaction with *Escherichia coli* ribosomes.

	Radioactivity extractable with EtOAc after addition of			
	Poly U+[^{14}C]Phe-tRNA (cpm)		AUG+f-[^{14}C]Met-tRNA (cpm)	
	Without myxovalargin	With myxovalargin	Without myxovalargin	With myxovalargin
Experiment A	1,224 (100%)	459 (38%)	1,963 (100%)	455 (23%)
Experiment A′			1,552 (100%)	251 (16%)
Experiment B	1,020 (100%)	805 (78%)	1,625 (100%)	484 (29%)

In all three experiments, the test volume was 200 μl.

Experiment A: Buffer 3 containing 12 mM β-mercaptoethanol instead of DTT+ribosomes (420 μg by protein)+myxovalargin (31 μg/ml)+poly-U (4 μg) and [^{14}C]Phe-tRNA (6,400 cpm) or +AUG (10 μg) and f-[^{14}C]Met-tRNA (11,200 cpm). After 5 minutes at 23°C, S 100 (160 μg by protein) and GTP (400 μmol) were added. After 5 minutes incubation at 37°C, puromycin (200 μmol) was added. Final incubation was for 30 minutes at 0°C.

Experiment A′: As in A, but with ribosomes that were first preincubated with myxovalargin, then sedimented by centrifugation and resuspended in mixtures without myxovalargin.

Experiment B: Buffer 3+ribosomes+poly-U and [^{14}C]Phe-tRNA or +AUG and f-[^{14}C]Met-tRNA for 5 minutes at 23°C. After addition of S 100+GTP, incubation for 5 minutes at 37°C. Then addition of myxovalargin and, after 1 minute at 23°C, of puromycin and final incubation for 30 minutes at 0°C.

and the UV absorption of the supernatant increased concomitantly. The degree and rate of these changes depended on the myxovalargin concentration applied (Fig. 4). *C. albicans*, on the other hand, showed no such effects. Human erythrocytes released material absorbing at 280 or 570 nm, respectively, when myxovalargin was added to the suspension (Fig. 5).

Effects on O_2-consumption

Myxovalargin inhibited O_2-consumption of *B. subtilis*. Rate and degree of the inhibition depended on the concentration of the antibiotic (Figs. 6 and 7). *C. albicans* was insensitive to myxoval-

Table 3. Effect of myxovalargin on the binding of acetyl[^{14}C]Phe-tRNA to washed ribosomes of *Escherichia coli* and on the puromycin reaction.

Preincubation with deacyl tRNA		Acetyl-Phe-tRNA bound to ribosomes (cpm)		Puromycin reaction (cpm)			
				Presence of S 100		Absence of S 100	
		Without myxovalargin	With myxovalargin*	Without myxovalargin	With myxovalargin*	Without myxovalargin	With myxovalargin*
Experiment 1	−	325 (100%)	265 (82%)	317 (100%)	77 (24%)	274	0
	+	270 (100%)	130 (48%)	373 (100%)	45 (12%)	131	0
Experiment 2	−	361 (100%)	312 (86%)	193	0	119 (100%)	37 (31%)
	+	452 (100%)	135 (30%)	237	0	51	44

* 28 μg/ml.

Fig. 3. Effect of myxovalargin on RNA-synthesis in *Bacillus subtilis*, measured as incorporation of [2-^{14}C]uracil into perchloric acid insoluble material.

○ With 1.5 μg myxovalargin per ml, △ with 20 μg myxovalargin per ml, ● control without myxovalargin. Myxovalargin was added at the time indicated by the arrow.

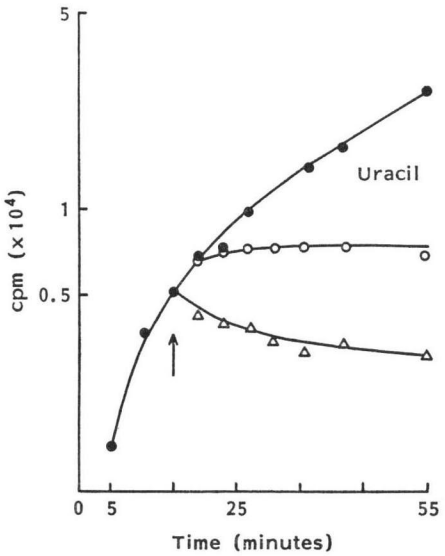
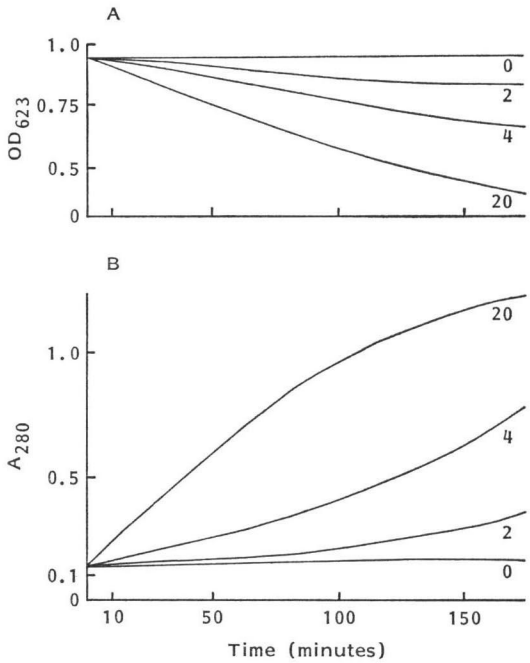


Fig. 4. Effect of myxovalargin on permeability of *Bacillus subtilis* cells.

A: Decrease of optical density (measured at 623 nm); B: increase of UV absorbing material in the supernatant (measured at 280 nm), both in presence of different concentrations of myxovalargin as indicated at the right end of the curves (μg/ml).



argin in this test, too.

Discussion

Our earlier experiments with *S. aureus* indicate that myxovalargin stops protein synthesis immediately and specifically when applied at low concentrations, while RNA, DNA and cell wall syntheses slow down only about 15 minutes later[1]. The experiments with *B. subtilis* confirm the results. At high concentrations, however, RNA synthesis also was interrupted instantly upon addition of the antibiotic (Fig. 3). In this case, inhibition of protein synthesis could obviously not be the reason for the block in RNA synthesis.

Protein synthesis in a cell-free system from *E. coli* was efficiently inhibited by myxovalargin (Fig. 1).

Fig. 5. Effect of myxovalargin on permeability of human erythrocytes.

A: Increase of UV absorbing material (measured at 280 nm) in the supernatant, B: increase of material absorbing at 570 nm.

○ With 2.6 μg myxovalargin/ml; △ with 18.6 μg/ml; ● control without myxovalargin.

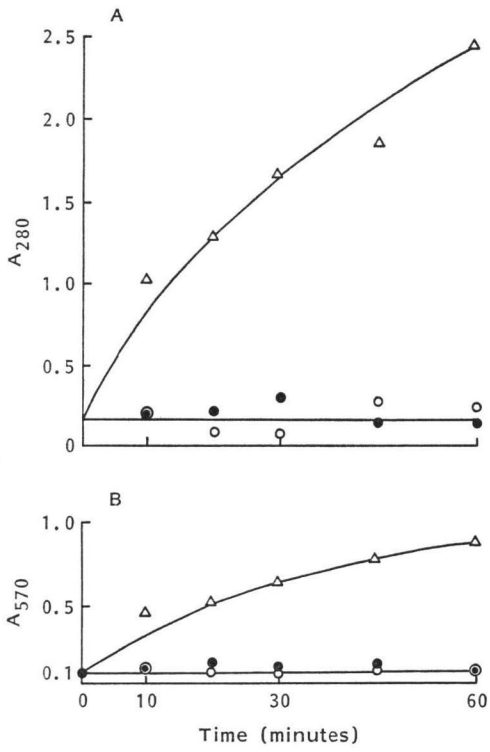
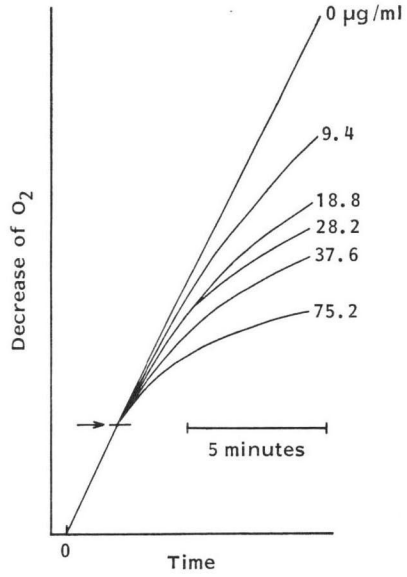


Fig. 6. Effect of myxovalargin on O_2-consumption by cells of *Bacillus subtilis*.

The curves show the kinetics of inhibition at different concentrations of the antibiotic, which was added at the time indicated by the arrow.



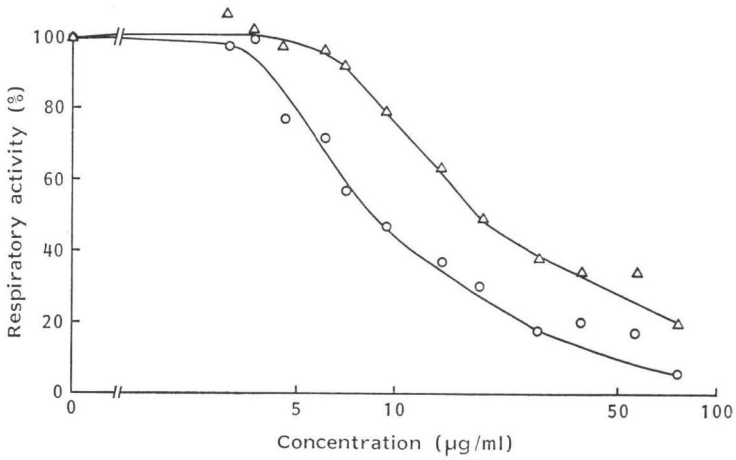
Inhibition occurred only if myxovalargin was present before the reaction had started (Fig. 2). This indicated that the block is at an early step of protein synthesis. Myxovalargin did not act upon the reactions of the S 100 fraction since neither aminoacyl-tRNA synthesis nor the binding of GTP to S 100 components were influenced.

Non-enzymatic binding of Phe-tRNA to ribosomes was reduced only by 30~54%, but enzymatic binding (with S 100 and GTP added) was strongly inhibited (Table 1). These results were confirmed by binding studies with f-Met-tRNA (Table 2) and acetyl-Phe-tRNA (Table 3). The binding of f-Met-tRNA, with and without S 100, was not influenced by myxovalargin. The binding of acetyl-Phe-tRNA was essentially inhibited only when its preferred binding site, the P site, was blocked by deacylated tRNA[10]. This suggests that myxovalargin acts at the A site of the ribosome.

The inhibition of the puromycin reaction was independent of a preincubation of the ribosomes with deacylated tRNA (Table 3). This shows that transpeptidation is blocked due to inhibition of the binding of aminoacyl tRNA to the ribosomes rather than inhibition of the translocation step. Table 2 also shows that good inhibition occurred only when myxovalargin was present from the very beginning, especially when a poly-U template and Phe-tRNA were used. This is in accordance with the results in Fig. 2. That myxovalargin is bound to the ribosomes themselves could be seen when ribosomes were incubated in buffer with myxovalargin and then removed from the incubation buffer by centrifugation. These ribosomes were strongly inhibited in the puromycin reaction (Table 2). Myxovalargin seems thus to interfere with one of the first steps of protein synthesis or with monosomes only[17]. A similar mechanism is known for the antibiotics lincomycin[18] and spectinomycin[19].

In vitro protein synthesis in two eukaryotic systems was not affected at concentrations at which the *E. coli* system was completely blocked. At very high concentrations, there was a partial inhibition of eukaryotic protein synthesis.

Fig. 7. Effect of myxovalargin on the rate of O_2-consumption by *Bacillus subtilis* 3.6 (△) and 7.2 (○) minutes after addition of myxovalargin.



From this, and from the high toxicity of myxovalargin for mice[1], it must be concluded that the antibiotic has a second effect besides inhibition of protein synthesis. As it is known that many peptide antibiotics act on membranes[20], myxovalargin was tested for membrane activity. Rapid loss of optical density of cell suspensions of *B. subtilis* in presence of myxovalargin and release of UV absorbing material into the medium indicated that at higher concentrations the antibiotic indeed interacted with the bacterial membrane. Even at low concentrations damage of the cell membrane became apparent after prolonged incubation (Fig. 4).

The concentrations of myxovalargin required to inhibit O_2-uptake of *B. subtilis* (Figs. 6 and 7) were much higher than those needed for inhibition of protein synthesis, showed a similar concentration dependence as the increase in permeability, and the effect became apparent only after several minutes. Thus, interference with O_2-consumption can not be the reason for the inhibition of protein synthesis. On the other hand, the block in protein synthesis and the following interruption of other cell activities could not be the reason for the lower O_2-uptake, as O_2-consumption was not affected at all at low concentrations at which protein synthesis was already blocked. Rather the reduction of O_2-uptake must be explained by damage of the bacterial membrane. The same effect may be responsible for the fact that myxovalargin is bactericidal even at low concentrations. The instant interruption of RNA synthesis by higher myxovalargin concentrations may equally be due to this membrane effect.

Myxovalargin seems to act on the membranes of erythrocytes in a similar manner as on bacterial membranes (Fig. 5). This may be the reason for the high toxicity of the antibiotic for mice in spite of its negligible activity in eukaryotic *in vitro* protein synthesis. Another source of toxicity could be an effect of myxovalargin on protein synthesis in mitochondria, which has not yet been tested. The resistance of *Candida* and of various molds must be due to impermeability to the antibiotic.

There are clear differences between the action of myxovalargin and that of peptide antibiotics with a primary membrane activity, *e.g.* polymyxin. The latter antibiotic disrupts the (outer) membrane of Gram-negative bacteria[20,21]. Its effect on O_2-consumption is very fast, even at low concentrations, and DNA and RNA synthesis stop very soon and both at the same time[21]. Furthermore, polymyxin has no effect on protein synthesis *in vitro*[22].

Acknowledgments

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