High-Ionic Strength Interference of Ribosomal Inhibition Produced by Aminoglycoside Antibiotics†

Irma Marín, José P. Abad, Dionisio Ureña, and Ricardo Amils*

Centro de Biología Molecular (CSIC-UAM) "Severo Ochoa", Universidad Autónoma de Madrid, Cantoblanco, Madrid 28049, Spain

Received August 14, 1995; Revised Manuscript Received October 9, 1995⊗

ABSTRACT: A protein synthesis cell-free system capable of performing with similar efficiencies in different ionic conditions has been developed for the halotolerant marine bacterium *Vibrio costicola*. The system has been used to test the effect of ionic strength on the interference produced by thirty translation inhibitors with different structural, functional, and domain specificities. In general, at high ionic strengths, the inhibition of protein synthesis produced by polycationic antibiotics like the aminoglycosides is much less pronounced than the inhibition obtained at low ionic strengths, while non-aminoglycosidic antibiotics show similar inhibitory activities at both high and low ionic conditions. These results strongly suggest that competition between polycationic antibiotics and cations at high concentrations in the media is responsible for the lack of inhibition by aminoglycoside antibiotics at high ionic strengths, rather than a lack of binding sites.

Since the discovery that aminoglycoside antibiotics do not interfere with protein synthesis in cell-free systems from extreme halophilic archaebacteria (Amils et al., 1989, 1993; Sanz et al., 1993), considerable efforts have been devoted to proving whether this lack of inhibition was due to the absence of structural features responsible for the antibiotics binding or to competition between the cationic nature of the inhibitors and the extreme ionic conditions required for the halophilic ribosomes to function. Experiments performed at suboptimal ionic conditions suggested that cationic competition rather than a lack of binding structures was the most likely explanation for the characteristic insensitivity exhibited by extreme halophilic ribosomes to aminoglycosidic antibiotics (Sanz et al., 1993). However, it seemed necessary to determine this by using a cell-free protein synthesis system with a well-established antibiotic inhibitory pattern that could, at the same time, perform in a wide range of cationic concentrations.

Vibrio costicola, a marine bacterium that is capable of growing at different salt concentrations and has ribosomes that can synthesize proteins at rather high ionic strengths (Widro et al., 1975; Kamekura & Kushner, 1984), was selected for the study of the effect of ionic strength on the inhibition produced by translational antibiotics. The protein synthesis cell-free system devised by Kamekura and Kushner (1984) for the study of the halotolerant characteristics of *V. costicola* ribosomes was modified to allow it to perform at different ionic strengths. *V. costicola* protein synthesis inhibition with thirty antibiotics, with different structural characteristics and specificities, has been tested at different ionic conditions. The results obtained indicate that, at high ionic strengths, the inhibition produced by aminoglycosides is very low when compared to the inhibition obtained at low ionic strengths, while other antibiotics exhibit similar inhibitory values at different ionic conditions.

MATERIALS AND METHODS

Bacterial Cultures

V. costicola NCBM 701 was grown in 1% proteose peptone (Difco), 1% tryptone (Difco), 1 M NaCl, and 0.1% yeast extract (Difco) (pH 7) at 30 °C until middle exponential phase. Cells were harvested by centrifugation at low speed and stored at −70 °C. *Escherichia coli* MRE600 was grown as previously described (Amils et al., 1979).

Preparation of Cellular Extracts and Ribosomes

Frozen *V. costicola* cells were thawed on a prechilled mortar, ground with twice their wet weight of alumina (Serva) for 20 min, and extracted with 1 mL of buffer BK [120 mM NH_4Cl, 20 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 3 mM and spermidine, 10 mM Tris-HCl (pH 7.5)] per gram of wet cells. RNase-free DNase I (Sigma) was added to the suspension to achieve a final concentration of 2 μg/mL and the mixture incubated at 4 °C for 20 min. The solution was centrifuged at 26500*g* for 10 min. The supernatant was centrifuged at 60000*g* for 25 min to eliminate cell debris and intact cells. The clear supernatant was centrifuged overnight at 100000*g*. The pellet was resuspended in buffer BK and the mixture stored in small aliquots at −70 °C. Two-thirds of the upper part of the supernatant was dialyzed against buffer BK overnight and stored in small aliquots at −70 °C. Ribosomes and soluble factors for *E. coli* were prepared as previously described (Amils et al., 1979).

In Vitro Protein Synthesis

V. costicola System. (a) Low-Ionic Strength Incubations. The reaction mixture contained in a final volume of 60 μL

† This work was supported by the Spanish Interministerial Commission for Science and Technology Grant PB-92-0129 (DGICYT), EC Grant Bio 92-CT93-0274, and an institutional grant from the Fundación Ramón Areces.

* Corresponding author: Ricardo Amils, Centro de Biología Molecular, Edificio de Biológicas, Universidad Autónoma de Madrid, Cantoblanco, Madrid 28049, Spain. Phone: 34-1-3978077. Fax: 34-1-3978344.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1995.

0006-2960/95/0434-16519$09.00/0

consisted of 35 mM Tris-HCl (pH 7.7), 4 mM $MgAc_2$, 160 mM NH_4Cl, 5 mM 2-mercaptoethanol, 4 mM spermidine, 15 μg of polyuridilic acid [(poly(U)], 1.7 mM ATP, 0.3 mM GTP, 6.25 mM phosphoenolpyruvate (PEP), 20 μM phenylalanine, 2 μg of [^{14}C]phenylalanine (Amersham, 504 mCi/mmol), 20 μg of tRNA, 2 μg of pyruvate kinase, 24 pmol of 70S, and the optimal amount of S-100 fraction optimized for each preparation.

(b) High-Ionic Strength Incubations. The experiments performed at high ionic strengths were done using conditions identical to those described for low ionic strengths, except that the monovalent cation concentration was increased by addition of $(NH_4)_2SO_4$ up to a 500 mM final concentration. Some experiments were performed at 250 mM ammonium sulfate (medium ionic strength). Incubations in all the conditions were done at 30 °C for 30 min. The polymerization was stopped by addition of 2 mL of 5% cold trichloroacetic acid (TCA). The samples were incubated for 15 min at 90 °C and filtered through GFC filters (Wathman) at 4 °C. Each filter was then washed three times with 5% TCA and once with 2 mL of ethanol. The radioactivity retained in the filters was measured by a liquid scintillation counter. Antibiotics were added at different concentrations from stock solutions. The percentage of inhibition produced by the different antibiotics was obtained by comparison with the appropriate controls containing the correspondent solvents used for the preparation of the antibiotics.

V. costicola/E. coli Hybrid System. Some of the experiments were performed with a hybrid system obtained by the complementation of *V. costicola* ribosomes with *E. coli* soluble factors. The ionic conditions were those described for the *V. costicola* system.

Control Systems. The poly(U)-directed poly(phenylalanine) syntheses described by Amils et al. (1979) and Jimenez et al. (1975) were used for *E. coli* and *Saccharomyces cerevisiae,* respectively.

Antibiotics

The antibiotics used in this work are protein synthesis inhibitors with bacterial (group I), eukaryotic (group II), or universal (able to inhibit bacterial and eukaryotic systems, group III) domain specificities. These antibiotics were obtained from the following sources: althiomycin, amicetin, neamine, and sparsomycin from Upjohn; anisomycin and carbomycin from Pfizer; anthelmycin, hygromycin B, and tobramycin from Eli Lilly; blasticidin S from Nobuo Tanaka; cryptopleurine from Chemasea Manufacturing Pty.; cycloheximide, fusidic acid, gentamycin, kanamycin, neomycin, streptovitacin A, tetracycline, and toxin T2 from Sigma; edeine from Pharmaceutical Works, Polfa; paromomycin, griseoviridin, and streptimidone from Park Davis; pretazettine and puromycin from Serva; ribostamycin and α-sarcin from the Michigan Department of Public Health, Lansing; thiostrepton from Squibb; tubulosine from Boehringer Mannheim; and tylophorine from Dr. E. Gellert.

RESULTS AND DISCUSSION

It has been reported that the *V. costicola* translational cell-free system is capable of performing at high ionic strengths, depending on the salt concentration present in the growth media (Widro et al., 1975; Kamekura & Kushner, 1984). In principle, the *V. costicola* protein synthesis system facilitates the study of the effect of ionic strength on the interference produced by translational inhibitors. The basic conditions described by Kamekura and Kushner (1984) have been modified to allow the study of protein synthesis inhibition at ionic conditions close to those required for extreme halophilic ribosomes (Sanz et al., 1988).

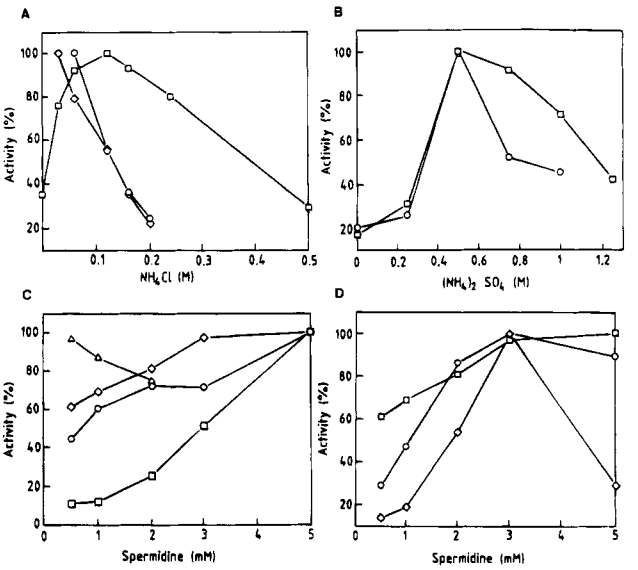

FIGURE 1: Optimization of the *V. costicola* protein synthesis cell-free system. (A) Effect of NH_4Cl: □, *V. costicola*; ○, heterologous *V. costicola/E. coli* system; and ◇, *E. coli* system. (B) Effect of ammonium sulfate: □, no NH_4Cl addition; and ○, 160 mM NH_4Cl. (C) Effect of different concentrations of magnesium in the spermidine optimization: □, no Mg^{2+}; ○, 2.5 mM Mg^{2+}; ◇, 5 mM Mg^{2+}; and △, 10 mM Mg^{2+}. (D) Effect of different concentrations of spermidine: □, homologous *V. costicola* system; ○, hybrid *V. costicola/E. coli* system and ◇, *E. coli* system. 100% activities correspond to polymerization of 374 and 390 pmol of phenylalanine for the homologous and heterologous *V. costicola* systems, respectively, and to 480 pmol for the *E. coli* system.

Optimization of a V. costicola Protein Synthesis Cell-Free System

Ribosomes from *V. costicola* cells grown in the presence of 1 M NaCl were tested for their translational efficiency using a poly(U)-directed poly(phenylalanine) synthesis system. The following optimizations were made.

(i) Monovalent Cations. Figure 1A shows the effect of different NH_4Cl concentrations on the polymerization efficiency of *V. costicola* ribosomes. Optimal polymerization was obtained at 160 mM NH_4Cl, a value close to the internal concentration of Cl^- measured for *V. costicola* grown at 1 M NaCl (Kamekura & Kushner, 1984). Higher concentrations of this salt show the characteristic inhibitory effect of the Cl^- anion. The similar inhibitory effect produced by Cl^- in the *E. coli* (c/c) and the hybrid *V. costicola* system (*V. costicola* ribosomes complemented with *E. coli* S-100 fraction) suggests that the interactions of the elongation factors with the ribosomes are the translational features which are most sensitive to the damaging Cl^- anion. This conclusion does not exclude other possible inhibitory effects produced by this anion, such as its well-documented interference with messenger binding to the ribosomes (Choquet et al., 1989).

It has been reported that *V. costicola* ribosomes were able to perform protein synthesis at high salt concentrations if the presence of Cl^- was avoided by the use of salts from

organic acids (Kamekura & Kushner, 1984). The effect of the addition of ammonium glutamate in the absence or in the presence of a constant concentration of 160 mM NH_4Cl was studied. Although protein synthesis can be achieved at relatively high ionic strengths, 0.2 M salt, the results obtained are somewhat different from those reported by Kamekura and Kushner using sodium glutamate and an S30 ribosomal fraction, in which optimal activity was reached at 0.4 M sodium glutamate. At the concentration range required for our comparative antibiotic sensitivity experiments, very low activity was obtained, 20% activity at 1 M ammonium glutamate (data not shown).

In order to achieve ionic conditions on the same order of magnitude as those required for extreme halophilic systems, we tested the effect of ammonium sulfate on the *V. costicola* system. The results are shown in Figure 1B. Optimal polymerization was obtained at 0.5 M ammonium sulfate regardless of the presence or absence of 160 mM ammonium chloride in the reaction mixture. In the absence of NH_4Cl, significant polymerization was achieved at high concentrations of ammonium sulfate, 70% at 1 M salt concentration. Sodium sulfate cannot replace the requirements for ammonium sulfate (data not shown).

One of the most intriguing results obtained with extreme halophilic ribosomes was the absolute requirement of ammonium sulfate for adequate translational efficiency (Sanz et al., 1988). The results obtained with *V. costicola* indicate that ammonium sulfate provides an adequate *in vitro* functional structure for ribosomes exposed to high ionic strengths in the cytoplasm. We already know that the concentrations of ammonia and sulfate are very low in extreme halophilic archaea and in *V. costicola*; thus, an intracellular component with physicochemical properties similar to those of this macromolecule-ordering salt is likely to have physiological implications (Sanz et al., 1988). It is obvious from the analysis of the results that the macromolecular interactions involved in the protein synthesis of halotolerant ribosomes are dependent not only on the cations but also on the nature of the counteranions. A similar situation is found in the protein synthesis and reconstitution experiments of halophilic ribosomes (Sanz at al., 1988; Sanchez et al., 1990). These observations make the analysis of effects produced by the different components much more complicated.

(ii) Divalent Cations and Spermidine. The *V. costicola* protein synthesis system is not as dependent on critical concentrations of divalent cations as other previously described cell-free systems. Figure 1C shows the combined effect of different Mg^{2+} and spermidine concentrations on the efficiency of polymerization of the *V. costicola* system. Some divalent cations are required for the protein synthesis system because in the absence of Mg^{2+} and spermidine there is very little poly(phenylalanine) synthesis. The spermidine effect is only noticeable in the absence of Mg^{2+}. This insensitivity, which has only been observed in protein synthesis cell-free systems from microorganisms with a high content of monovalent cations in the cytoplasm, might be related to the reliance of the ribosomal functional structure on components other than the classical divalent cations to prevent its competition with the variable concentration of monovalent cations needed to overcome the osmotic pressure.

Figure 1D shows the differential effect of spermidine to the homologous *E. coli* (c/c) and *V. costicola* (v/v) systems at low Mg^{2+} concentrations (5 mM). Also, the characteristics of the hybrid system using *V. costicola* ribosomes and *E. coli* elongation factors (v/c system) are displayed. The hybrid system seems to be more sensitive to divalent cations than the homologous *V. costicola* system since it exhibits a spermidine dependence profile at low Mg^{2+} concentrations similar to that of the *E. coli* system.

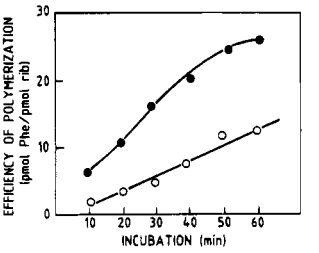

FIGURE 2: Kinetics of the poly(phenylalanine)synthesis in different ionic conditions. The conditions for the protein synthesis were those described in the Materials and Methods. ●, low ionic conditions; and ○, high ionic conditions.

Using the optimal ionic conditions described in Materials and Methods, a linear polymerization is observed at least in the case of 1 h incubation (Figure 2). The average efficiencies of polymerization obtained at low and high ionic conditions are 15.6 and 5.8 pmol of phenylalanine per picomole of ribosomes, respectively, after incubation for 30 min at 30 °C. The efficiency of the hybrid system was similar to the homologous one at the same ionic conditions.

Inhibition of V. costicola Protein Synthesis at Different Ionic Conditions

The availability of an efficient protein synthesis system for *V. costicola* capable of performing at different ionic strengths made the comparative study of the inhibition produced by antibiotics at different ionic conditions feasible. The ionic conditions selected to test the inhibitory effect produced by different antibiotics were the following: (i) low ionic strength and 160 mM NH_4Cl, similar to the conditions used for other bacterial and eukaryotic systems, and (ii) high ionic strength and 160 mM NH_4Cl + 0.5 M $(NH_4)_2SO_4$, on the same order of magnitude as the ionic conditions used for extreme halophilic cell-free systems. Some experiments were done at intermediate ionic strengths, 160 mM NH_4Cl + 0.25 M $(NH_4)_2SO_4$, in order to detect a possible transition between low and high ionic conditions.

The inhibitory effects displayed by the different antibiotics tested are shown in Table 1. Some of the comparative inhibitory curves for representative antibiotics are shown in Figures 3 and 4. The results have been grouped according to the phylogenetic specificity of the functional markers and compared to the inhibition produced in selected reference systems: *Haloferax mediterranei* (extreme halophilic system), *E. coli* (reference bacterial system), and *S. cerevisiae* (reference eucarial system).

Bacterial-Targeted Antibiotics (Group I Inhibitors). The sensitivity patterns obtained with 11 specific inhibitors of bacterial ribosomes can be divided into two groups. The first group comprises inhibitors having different functional specificities and chemical structures which show no significant differences in sensitivity at either ionic condition or at different antibiotic concentrations: althiomycin, carbomycin (Figure 3), griseoviridin, and thiostrepton (Figure 3). The secondgroup corresponds to most of the aminoglycoside

Table 1: Sensitivity of *V. costicola* Protein Synthesis Systems to Antibiotics[a]

	V. costicola			controls		
	without $(NH_4)_2SO_4$	250 mM $(NH_4)_2SO_4$	500 mM $(NH_4)_2SO_4$	*Hf. med*	*E. coli*	*S. cer*
Group I						
althiomycin	++ (3×10^{-7})	++	++ (2×10^{-6})	±	++	−
carbomycin A	++ (8×10^{-6})	++	++ (10^{-5})	+	++	−
griseoviridin	++ (10^{-6})	++	++ (2×10^{-6})	−	++	−
thiostrepton	++ (3×10^{-6})	++	++ (1.2×10^{-6})	+	++	−
aminoglycosides						
gentamycin	+ (3×10^{-7})	±	± (3×10^{-4})	−	++	±
kanamycin	++ (1.1×10^{-7})	++	− (4×10^{-4})	−	++	±
neamine	+ (4×10^{-7})	±	± (2×10^{-4})	−	++	±
neomycin	++ (4×10^{-6})	++	− (10−3)	−	++	±
paromomycin	++ (9×10^{-6})	+	± ($>10^{-3}$)	−	++	±
ribostamycin	++ (4×10^{-6})	++	± (2.5×10^{-4})	−	++	−
tobramycin	++ (2×10^{-6})	++	++ (9×10^{-6})	−	++	+
Group II						
anisomycin	−	−	−	+	−	++
cicloheximide	−	−	−	−	−	++
cryptopleurine	−	−	−	±	−	++
streptimidone	−	−	−	−	−	++
streptovitacin A	−	−	−	−	−	++
pretazettine	−	−	−	−	−	++
α-sarcin	−	−	−	−	±	++
toxin T-2	−	−	−	−	−	++
tylophorine	−	−	−	−	−	++
tubulosine	−	−	−	−	−	++
Group III						
amicetin	+ (6×10^{-7})	++	+ (1.2×10^{-4})	±	++	+
anthelmycin	++ (10^{-7})	++	++ (3×10^{-6})	±	++	++
blasticidin S	++ (3.5×10^{-6})	++	++ 1.8×10^{-6})	+	++	++
edeine	++ (2×10^{-6})	++	++ (7×10^{-7})	−	++	++
fusidic acid	++ (6×10^{-7})	++	++ 1.3×10^{-7})	−	++	++
hygromycin B	++ (9×10^{-7})	++	± (10^{-4})	−	++	++
puromycin	+++ (8×10^{-7})	+++	+++ (2.5×10^{-6})	++	++	++
sparsomycin	++ (8×10^{-6})	++	++ (10^{-4})	++	++	++
tetracycline	++ (3×10^{-7})	++	+++ (2×10^{-6})	±	++	++

[a] Group I, inhibitors of bacterial ribosomes; group II, inhibitors of eukaryotic ribosomes; and group III, inhibitors without domain specificity. The antibiotic inhibitory effect was tested in a poly(U)-directed poly(phenylalanine) synthesis as dscribed in Materials and Methods. The values in the table correspond to the following criteria: −, no inhibition at the maximal concentration of antibiotic used or if inhibition is obtained only at concentration higher than 10^{-4} M; ±, 50% inhibitory effect obtained at concentrations 2 orders of magnitude higher than the bacterial (*E. coli)* or eukaryotic *(S. cerevisiae*) reference systems; +, 50% inhibitory effect obtained 1 order of magnitude higher than the reference systems; + +, similar sensitivity to the reference systems; + + +, higher sensitivity than the reference systems. The molar concentrations at which there is 50% inhibition for the different antibiotics are shown in parentheses; $>10^{-3}$ indicates that, at this concentration, the 50% inhibition has not been obtained. *Hf. med, Haloferax mediterranei; S. cer, S. cerevisiae.*

antibiotics tested in this work, which show significant differences in sensitivity depending on the ionic conditions of the translational cell-free system: gentamycin, kanamycin (Figure 4), neomycin (Figure 4), paromomycin, and ribostamycin (Figure 4). Tobramycin shows a much less pronounced difference between both ionic conditions. The current lack of knowledge about the molecular bases of the interference produced by this antibiotic prevents us from being able to suggest an appropriate explanation for the special behavior of this antibiotic, but this result clearly indicates that no competition is established between the amino groups of this antibiotic and the cations present in the reaction mixture, suggesting that for tobramicin these amino groups might not be relevant to its binding to the ribosomes.

Eukaryotic-Targeted Antibiotics (Group II Inhibitors). As expected, none of the antibiotics tested in this group show any inhibitory effect on the *V. costicola* ribosomal system due to its lack of binding sites for these eukaryotic inhibitors. These results underline the high level of specificity exhibited by these ribosomal functional effectors. No difference in sensitivity was detected for any of the 10 antibiotics of this group at the different ionic conditions used for the assay.

Universal Antibiotics (Group III Inhibitors). Eight out of nine inhibitors of this group do not show any difference in sensitivity at either, low or high, ionic condition: anthelmycine, amicetine, blasticidine S, edeine, fusidic acid (Figure 3), puromycin (Figure 3), sparsomycin, and tetracycline. Only one inhibitor of this group, higromycin B, which is related to the aminoglycoside group of antibiotics due to its sugar structure, shows differential inhibitory curves at both ionic conditions (Figure 4).

It is noteworthy that puromycin produces a more efficient inhibition of *V. costicola* protein synthesis at both ionic conditions than of the *E. coli* and *S. cerevisiae* reference systems. This result agrees with the oversensitivity exhibited by extreme halophilic ribosomes to this antibiotic (Sanz et al., 1988), suggesting that the affinity of this important effector for the peptidyltransferase center is higher for ribosomes exposed to high concentrations of monovalent cations in the cytoplasm, probably as a consequence of the physicochemical characteristics of its binding site.

It is clear from the results presented that the increase of ionic strength mainly affects the inhibition produced by the polycationic aminoglycosides, while the rest of the antibiotics do not show any significant changes in inhibition at different

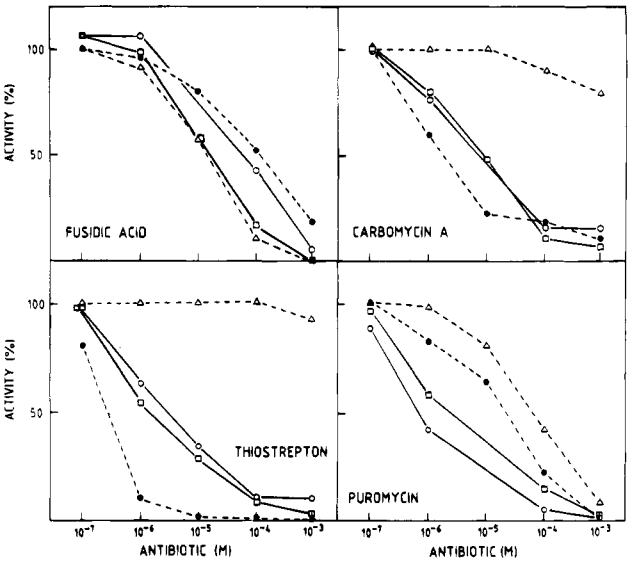

FIGURE 3: Inhibition produced by non-aminoglycosidic antibiotics in different ionic conditions. The conditions for the protein synthesis were as described in Materials and Methods. *V. costicola*: ○, low salt; and □, high salt. Controls: ●, *E. coli*; and △, *S. cerevisiae*. 100% activities correspond to polymerization of 374 pmol of phenylalanine for the *V. costicola* high-salt concentration system, 139 pmol for the *V. costicola* low-salt concentration system, 480 pmol for the *E. coli* system, and 358 pmol for the *S. cerevisiae* system.

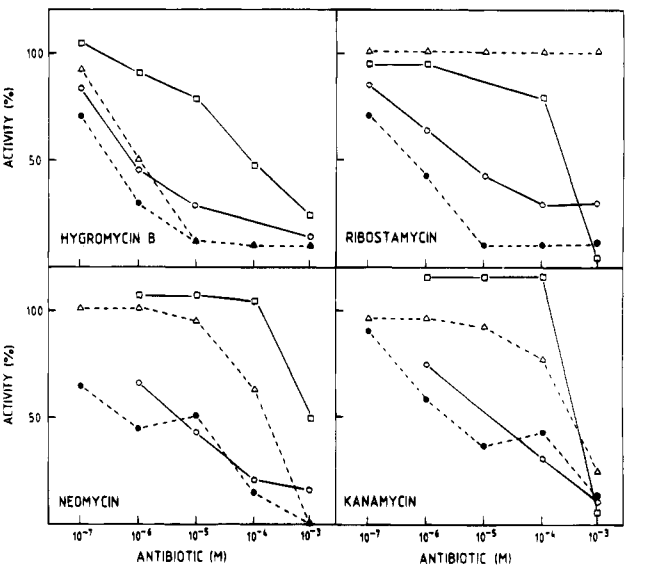

FIGURE 4: Inhibition produced by aminoglycosidic antibiotics in different ionic conditions. The conditions for the protein synthesis are those described in Materials and Methods. *V. costicola*: ○, low salt; □, high salt. Controls: ●, *E. coli*; and △, *S. cerevisiae*. 100% activities for the different systems were those stated in the caption to Figure 3.

ionic conditions. If the fact that some of the unaffected antibiotics (edeine, thiostreptone, and tobramycine) exhibit charged groups in their structures is taken into consideration, it has to be concluded that these cationic structures are not directly involved in the mode of action of the antibiotics on the ribosomes, because the high concentrations of cations present in the reaction mixture do not affect their affinity for the ribosome.

Most of the inhibition curves obtained at intermediate ionic strengths are similar to those obtained at low ionic strengths, indicating that the decrease in inhibition observed for the aminoglycoside antibiotics appears at rather high concentrations.

The comparative inhibitory results obtained using ammonium sulfate lead us to conclude that competition between polycationic antibiotics and the monovalent cations present in the halophilic protein systems rather than the lack of a binding site has to be considered in the negative inhibitory values obtained with aminoglycoside antibiotics in extreme halophilic ribosomal systems.

REFERENCES

Amils, R., Matthews, E. A., & Cantor, C. R. (1979) *Methods Enzymol. 59*, 449–461.

Amils, R., Ramirez, L., Sanz, J. L., Marín, I., Pisabarro, G., & Ureña, D. (1989) *Can. J. Microbiol. 35*, 141–147.

Amils, R., Cammarano, P., & Londei, P. (1993) in *The Biochemistry of Archaea* (Kates, M., Kushner, D. J. and Matheson, A. T., Eds.) pp 393–432, Elsevier: Amsterdam.

Choquet, C. G., Kamekura, M., & Kushner, D. J. (1989) *J. Bacteriol. 171*, 880–886.

Jimenez, A., Sanchez, L., & Vazquez, D. (1975) *Biochim. Biophys. Acta 383*, 427–434.

Kamekura, M., & Kushner, D. J. (1984) *J. Bacteriol. 160*, 385–390.

Sanchez, E., Ureña, D., Amils, R., & Londei, P. (1990) *Biochemistry 29*, 9256–9261.

Sanz, J. L., Marín, I., Balboa, M. A., Ureña, D., & Amils, R. (1988) *Biochemistry 27*, 8194–8199.

Sanz, J. L., Marín, I., Ureña, D., & Amils, R. (1993) *Can. J. Microbiol. 39*, 311–317.

Widro, R., Kogut, M., & Kushner, D. J. (1975) *FEBS Lett. 60*, 210–215.

BI951908I