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Biochemical and Kinetic Characteristics of the Interaction of the Antitumor Antibiotic Sparsomycin with Prokaryotic and Eukaryotic Ribosomes†

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ABSTRACT: Using ^{125}I-labeled phenol–alanine sparsomycin, an analogue of sparsomycin having higher biological activity than the unmodified antibiotic, we studied the requirements and the characteristics of its interaction with the ribosome. The drug does not bind to either isolated ribosomal subunits or reconstituted whole ribosomes. For sparsomycin binding to 70S and 80S ribosomes, the occupation of the peptidyl-transferase P-site by an N-blocked aminoacyl-tRNA is a definitive requirement. The sparsomycin analogue binds to bacterial and yeast ribosomes with K_a values of around 10^6 M^{-1} and 0.6×10^6 M^{-1}, respectively, but its affinity is probably affected by the character of the peptidyl-tRNA bound to the P-site. Chloramphenicol, lincomycin, and 16-atom ring macrolides compete with sparsomycin for binding to bacterial ribosomes, but streptogramins and 14-atom ring macrolides do not. Considering the reported low affinity of puromycin for bacterial ribosomes, this antibiotic is also a surprisingly good competitor of sparsomycin binding to these particles. In the case of yeast ribosomes, blasticidin is a relatively good competitor of sparsomycin interaction, but anisomycin, trichodermin, and narciclasin are not. As expected, puromycin is a poor competitor of the binding in this case. The results from competition studies carried out with different sparsomycin analogues reveal, in some cases, a discrepancy between the drug ribosomal affinity and its biological effects. This suggests that some intermediate step, perhaps a ribosomal conformational change, is required for the inhibition to take place.

Sparsomycin[1] (Figure 1) is a broad-spectrum antibiotic that inhibits protein synthesis by interacting with the ribosome at the peptidyl transferase center, blocking peptide bond formation [see Ottenheijm et al. (1986) for a review on sparsomycin]. Lately, the preparation of sparsomycin derivatives considerably more active than the original drug (van den Broek et al., 1987, 1989)—some of which have produced promising results in ongoing clinical tests (Zylicz, 1988)—has stirred up new interest in this drug as an antitumor agent. Sparsomycin is also of considerable interest from an even more basic point of view since its strong inhibitory activity on all cell types, including the highly resistant archaebacteria (Cammarano et al., 1985), indicates the existance of a highly conserved target for drug action in all ribosomes. In fact, sparsomycin has been extensively used as a tool in ribosome and protein synthesis studies (Ottenheijm et al., 1986). The drug has been found to induce intriguing effects on the ribosomal particle, confirming a close interrelation between the A- and P-sites in the peptidyl transferase center. Thus, sparsomycin blocks the

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[1] Abbreviations: sparsomycin, *N*-[1-(hydroxymethyl)-2-[[(methylthio)methyl]sulfinyl]ethyl]-3-(1,2,3,4-tetrahydro-6-methyl-2,4-dioxo-5-pyrimidinyl)-2-propenamide; phenol–alanine sparsomycin, derivative in which the (methylthio)methyl group has been replaced by 4-hydroxyphenyl and the hydroxymethyl group has been replaced by methyl, i.e., *N*-[1-methyl-2-[(4-hydroxyphenyl)sulfinyl]ethyl]-3-(1,2,3,4-tetrahydro-6-methyl-2,4-dioxo-5-pyrimidinyl)-2-propenamide.

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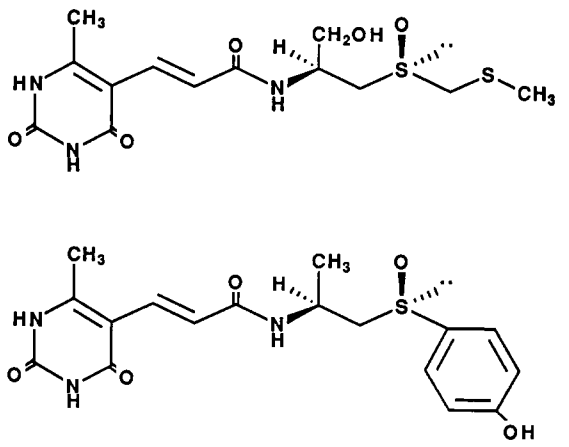


FIGURE 1: Sparsomycin (top structure) and phenol–alanine sparsomycin (bottom structure).

binding of nonacetylated substrates to the ribosomal A-site (Pestka, 1969) but strongly stimulates the interaction of N-blocked substrates (Monro et al., 1969) to a site that seems not to be identical with the P-site (Perez-Gosalbez et al., 1983). On the other hand, sparsomycin seems to inhibit peptide bond formation only when an N-blocked aminoacyl-tRNA is sitting on the P-site (Busiello & Di Girolamo, 1973; Smith, 1973), suggesting one of two things: either the interaction site for the drug is open only in these conditions or the drug is able to bind to ribosomes without any inhibitory effect until the P-site is occupied. Also, the reported "preincubation effect" of the drug on ribosomes, which stimulates its inhibitory action in an in vitro system (Coutsogeorgopoulos et al., 1975), seems to indicate the induction of a time-dependent conformational change that might permanently inactivate the ribosomal particle.

Similarly, the effect of sparsomycin on the interaction of other peptidtyl transferase inhibitors with the ribosome has been studied in detail, leading also to some controversial results. For example, it has been shown that sparsomycin has no effect on the binding of chloramphenicol and erythromycin to free ribosomes, suggesting the existence of independent, nonoverlapping sites (Fernandez-Muñoz et al., 1971). However, these two antibiotics inhibit the sparsomycin-dependent binding of substrates to the P-site (Monro et al., 1969), indicating, in this case, some functional relationship among their ribosomal binding sites. It has also been shown that sparsomycin is able to inhibit the binding of chloramphenicol when polysomes instead of free ribosomes are used in the test (Contreras & Vazquez, 1977). This apparent contradiction in the experimental data may simply be the result of a shortage of information on the interaction of sparsomycin with the ribosome, the gathering of which has been so far constrained by a lack of labeled drug to carry out studies on the direct drug–ribosome interaction.

The development of a total synthesis for sparsomycin (Helquist & Shekhani, 1979; Liskamp et al., 1981; Ottenheijm et al., 1981) has allowed a large number of drug analogues to be prepared (Lazaro et al., 1987; van den Broek et al., 1987, 1989). Extensive structure–activity relationship studies of the drug have been performed that, in addition to pinpointing the functionally important parts of the molecule, have indicated the existence of a hydrophobic region in the peptidyltransferase center, which plays a role in the interaction of some of the more active sparsomycin derivatives with the ribosome (van den Broek et al., 1989; Ballesta & Lazaro, 1990). One of the newly synthesized sparsomycin derivatives, phenol–alanine sparsomycin[1] (Figure 1), can easily be radioactively labeled by iodination. Using this labeled derivative, we previously showed that sparsomycin, contrary to a very appealing proposal (Flynn & Ash, 1983), does not bind covalently to ribosomes (Lazaro et al., 1991). In this report, we describe a detailed analysis of the sparsomycin interaction with the ribosome using the same compound.

Materials and Methods

Strains. *Escherichia coli* MRE600 and *Saccharomyces cerevisiae* Y166 have been used throughout this work.

Ribosomes. Bacterial and yeast cells from mid-log-phase cultures growing in rich medium were broken by grinding with alumina and sea sand, respectively, and recovered according to standard procedures (Staehelin & Maglott, 1971). Ribosomal subunits were obtained by sucrose gradient centrifugation on zonal rotors as previously reported (Eikenberry et al., 1970). A molar extinction coefficient at 260 nm of 4.8×10^{-7} M^{-1} cm^{-1} and a molecular weight of 2.7×10^6 daltons have been considered for the 70S particle.

Synthesis of Sparsomycin Derivatives. Phenol–alanine sparsomycin (Figure 1) was synthesized from 6-methyluracil and L-alanine as described previously (van den Broek et al., 1987). The synthesis of other sparsomycin derivatives was published earlier (van den Broek et al., 1987, 1989). Purity of the compounds was checked by HPLC and TLC.

Preparation of ^{125}I-Labeled Phenol–Alanine Sparsomycin. Iodination, with either ^{125}I or nonradioactive iodine, was carried out with chloramin T. A very mild iodination procedure was employed to avoid drug degradation as described previously (Tejedor & Ballesta, 1982). The iodinated derivatives are readily separated from the unmodified sparsomycin by TLC with chloroform–methanol (80:20) as a solvent. Purity of the preparations was checked by TLC and HPLC. Radioactive derivatives of very high specific radioactivity (>100 Ci/mmol) were obtained. When needed, the labeled derivative was mixed with the nonradioactive compound to obtain the required final specific activity for each experiment.

Binding of Sparsomycin to Ribosomes. Fifteen picomoles of ribosomes (15 pmol of 50S and 25 pmol of 30S in the case of ribosomal subunits) in 50 μL of either SB buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 6 mM β-mercaptoethanol) or poly(Phe) mixture (50 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$, 90 mM KCl, 15 mM β-mercaptoethanol, 1 mM GTP, 10 mM ATP, 0.2 mg/mL poly(uridylic acid), 1.5 mg/mL tRNA, 2.5 mg/mL phosphoenolpyruvate, 20 μg/mL pyruvate kinase, 30 μM phenylalanine, and 10 μL of S-100 fraction) was incubated at 37 °C for 30 min with the indicated concentration of drug. A fixed amount of ^{125}I-labeled phenol–alanine sparsomycin (50000 cpm) was present in every sample and increasing amounts of nonradioactive I-labeled phenol–alanine sparsomycin were added to obtain the indicated final concentration. After incubation the samples were diluted with 1 mL of SB buffer and filtered through nitrocellulose filters. After being washed twice with the same buffer, the samples were counted in a γ counter.

Competition of Sparsomycin Binding by Antibiotics. Ribosomes (0.1 μM), in the poly(Phe) mixture indicated above, were incubated at 37 °C for 20 min, and then 0.1 μM ^{125}I-labeled phenol–alanine sparsomycin and increasing concentrations (0.01–1000 μM) of the competing antibiotic were added at the same time and incubated for 30 min at the same temperature. Binding was detected by filtration as described previously.

Binding of Phe-tRNA and N-Acetyl-Phe-tRNA. Binding was performed in 50-μL samples containing 30 mM Tris-HCl, pH 7.4, 30 mM KCl, 50 mM NH_4Cl, and either 10 or 15 mM

Table I: Binding of ^{125}I-Labeled Phenol–Alanine Sparsomycin to Ribosomal Particles[a]

particle	conditions	binding to ribosomes from *E. coli* (cpm)	binding to ribosomes from *S. cerevisiae* (cpm)
ribosome	SB	13725	7402
large subunit	SB	595	
small subunit	SB	559	
large + small subunit	SB	636	1890
ribosome	poly(Phe)	13239	
ribosome	poly(Phe) + TC	14307	
large subunit	poly(Phe)	814	
small subunit	poly(Phe)	556	
large + small subunit	poly(Phe)	9315	12089
large + small subunit	S100	1800	
large + small subunit	tRNA + poly(U)	1272	

[a] The particles (0.3 μM) were incubated with labeled antibiotic, 1 μM (50000 cpm) either in buffer (SB conditions) or in poly(U)-dependent poly(phenylalanine) synthesis [poly(Phe) conditions] as described under Materials and Methods. In the second case the incubation mixture was either complete [poly(Phe) conditions] or contained only the S100 fraction (S100 conditions) and only tRNA and poly(uridylic acid) [tRNA + poly(U) conditions]. The requirement of protein synthesis was tested by adding 0.1 mM tetracycline (TC) to one sample after a previous 5-min incubation to allow the start of the polymerization process. The results correspond to the average value of two different experiments carried out with duplicated samples.

Table II: Effect of Aminoacyl-tRNA on the Binding of Phenol–Alanine Sparsomycin to Ribosomes[a]

Mg concn (mM)	inhibitor of tRNA binding (μM)	binding of ^{125}I-phenol–Ala sparsomycin (cpm)	binding of *N*-acetyl-Phe-tRNA (cpm)
	(A) Effect of *N*-Acetyl-Phe-tRNA		
10		10045	3973
10	100	10466	3596
15		7962	6909
15	100	10184	3368
	(B) Effect of Phe-tRNA		
10		1698	5559
10	1	996	843
15		970	7575
15	1	1319	3725

[a] 50S and 30S subunits were incubated in 30 mM Tris-HCl, pH 7.4, 50 mM NH_4Cl, 30 mM KCl, and the indicated $MgCl_2$ concentration in the presence of either *N*-acetyl-aminoacyl-tRNA or aminoacyl-tRNA for 20 min as indicated under Materials and Methods. When indicated, tetracycline (in the case of *N*-acetyl-aminoacyl-tRNA) and edeine (in the case of aminoacyl-tRNA) were added to the sample. Then the ^{125}I-labeled phenol–alanine sparsomycin was added and incubation was prolonged for an additional 20 min. The results correspond to the average value of two different experiments carried out with duplicated samples.

$MgCl_2$, 10000 cpm of either Phe-tRNA or *N*-acetyl-Phe-tRNA (specific activity 500 cpm/pmol) and 5 μg poly(uridylic acid). The samples were incubated at 37 °C for 30 min and after dilution with 1 mL of buffer were filtered through nitrocellulose filters, washed with buffer, and counted.

Biological Tests. The synthesis of poly(phenylalanine) and the puromycin reaction were performed as previously described (Van den Broek et al., 1987).

Computer Programs. The kinetic parameters have been calculated by using the EBDA program of G. A. McPherson (McPherson, 1985).

Results

Biological Characteristics of Phenol–Alanine Sparsomycin. Phenol–alanine sparsomycin is a derivative of sparsomycin carrying a hydroxyphenyl group instead of the CH_2-S-CH_3 group attached to the sulfoxide end of the molecule (Figure 1). Like other sparsomycin derivatives with hydrophobic groups at the same position (van den Broek et al., 1989), this compound has a higher inhibitory activity (ED_{50} = 2.4 μM) than the unmodified drug (ED_{50} = 8.5 μM) when tested in an *Escherichia coli* poly(U)-dependent polypeptide synthesis system. Iodination slightly affects the activity of the derivative, increasing the ED_{50} to 5.6 μM, but it is still more active than unmodified sparsomycin (van den Broek et al., 1987, 1989). Sparsomycin fully competes with this derivative for binding to the ribosomes (Table IV), indicating that both compounds bind to the same site in the particles.

Requirements for Sparsomycin Binding to Ribosomal Particles. The binding of the drug to the different ribosomal particles was tested by using ^{125}-labeled phenol–alanine sparsomycin (Table I). Binding is detected only to undissociated 70S and 80S particles. The mere reassociation of ribosomal subunits did not result in binding of the drug to the formed ribosome. Binding is, however, detected when the particles are incubated under the conditions of poly(phenylalanine) synthesis. The complete system is required since the presence of either the S100 supernatant fraction or the tRNA and poly(U) alone have no stimulating effect on the binding. Protein synthesis is, however, not required since inhibition of the system with tetracycline did not affect the induced capacity of the reassociated particles to bind sparsomycin.

These results suggest that interaction of sparsomycin with the ribosome could require a previous binding of aminoacyl-tRNA, and therefore, this step was analyzed in more detail. The binding of the drug to reassociated particles was performed in the presence of either *N*-acetyl-aminoacyl-tRNA (Table IIA) or aminoacyl-tRNA (Table IIB). It was found that at 10 mM $MgCl_2$, when the tRNA is mainly bound in the P-site (insensitive to tetracycline), only *N*-acetyl-aminoacyl-tRNA has an stimulating effect on drug binding. Additional binding of tRNA to the A-site by increasing the magnesium concentration does not stimulate the binding of sparsomycin; on the contrary, a clear inhibition is observed. This is abolished when the interaction of the tRNA molecule at the A-site is blocked by the presence of tetracycline. Edeine, which blocks the binding of tRNA at the P-site (Szer & Kurylo-Borowska, 1970, 1972; Obrig et al., 1971), has a negative effect on the low sparsomycin interaction that takes place in the presence of Phe-tRNA (Table II). In fact, edeine is able to block the interaction of sparsomycin to the ribosomes in the presence of N-blocked aminoacyl-tRNA only when it is present at the beginning of the reaction and inhibits the interaction of the tRNA with the ribosome (Figure 2). Once the tRNA–mRNA–ribosome complex is formed and has become edeine resistant (Szer & Kurylo-Borowska, 1972), the addition of the drug had little effect on the sparsomycin binding to the ribosomal particle (Figure 2).

These results indicate that the presence of an N-acetylated aminoacyl-tRNA molecule at the P-site of the ribosome is a requirement for the binding of sparsomycin to the ribosome. Similar requirements were found for the binding of sparsomycin to yeast ribosomes (data not shown).

Kinetics of Sparsomycin Binding to Ribosomes. By using labeled phenol–alanine sparsomycin, the kinetic parameters of the binding process to either bacterial or yeast ribosomes were determined (Table III) from Scatchard plots of the binding data, which in all cases were linear (not shown). The results obtained by using free bacterial and yeast ribosomes

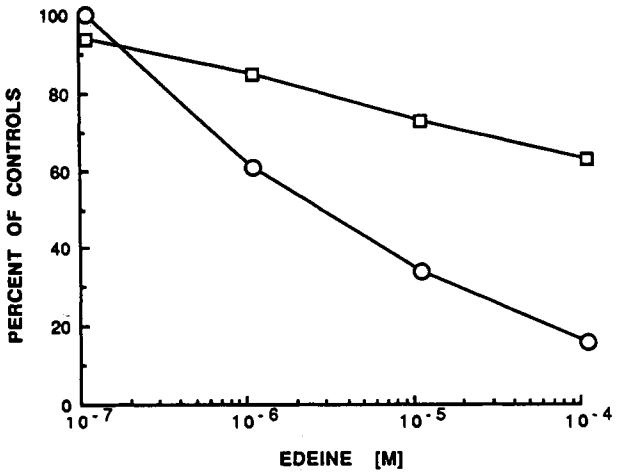


FIGURE 2: Inhibition of ^{125}I-labeled phenol–alanine sparsomycin binding to ribosomes by edeine. Ribosomes in the presence of *N*-acetyl-Phe-tRNA and poly(uridylic acid) were incubated with ^{125}I-labeled phenol–alanine sparsomycin in the conditions indicated under Materials and Methods, and edeine was added either at the beginning of the incubation (○) or after 20 min of preincubation (□). The samples were than incubated for 30 min, diluted, and filtered as indicated.

Table III: Kinetic Constants of ^{125}I-Labeled Phenol–Alanine Sparsomycin Binding to Ribosomes[a]

particle	binding conditions	K_a (μM)	ν
70S	standard buffer	0.30	0.17
		28.00	>1.00
70S	poly(Phe)	0.30	0.17
		1.10	0.38
50S + 30S	poly(Phe)	1.60	0.73
80S	standard buffer	0.16	0.11
80S	poly(Phe)	0.66	1.00

[a] Binding was performed under the conditions indicated under Materials and Methods. Dissociation constants (K_a) and number of binding sites (ν) were obtained from Scatchard plots of the resulting data. When biphasic plots were obtained, the constants for the two straight lines are presented.

in standard buffer indicated that only a fraction of the particles (10–20%) are able to bind the drug with high affinity [K_a = (0.16–0.3) × 10^6 M^{-1}]. The rest of the particles either did not bind the drug (80S ribosomes) or bound the antibiotic with a much lower affinity (70S ribosomes, K_a = 28 × 10^6 M^{-1}). This binding heterogeneity of the ribosome preparations might be due to a variable extent of P-site occupation by peptidyl-tRNA. In order to overcome this variability, the experiments were repeated under conditions of poly(phenylalanine) synthesis that would force P-site occupation. In these conditions, practically all the 80S ribosomes bind sparsomycin at a single site with a K_a value of 0.66 × 10^6 M^{-1}. Under the same conditions, however, the bacterial ribosomes still bound the drug in a biphasic manner with two different K_a values (0.3 × 10^6 M^{-1} and 1.1 × 10^6 M^{-1}). Moreover, saturation of the ribosomes is not achieved and only 38% of the particles are able to bind the drug. Since this heterogeneity could be due to the presence of different components of the protein synthesis machinery still bound to the particles, we tested the binding to reconstituted ribosomes obtained from purified 50S and 30S subunits under conditions of poly(phenylalanine) synthesis. In these conditions a unique high-affinity binding site was found having a K_a value of 1.6 × 10^6 M^{-1}.

Competition of Sparsomycin Binding to Ribosomes by Other Antibiotics. A number of antibiotics have been shown to interact with the peptidyltransferase center of the ribosome, blocking in this way the protein synthesis process (Vazquez, 1979; Gale et al., 1981). Studies on the effect of sparsomycin on the interaction of some of these radioactively labeled compounds (Ottenheijm et al., 1986) have revealed that in some cases there is a competition for binding to the ribosome. This suggests the existence of closely overlapping sites. Alternative explanations, like the existence of allosteric effects, cannot be excluded, and further analysis of the effect of these drugs on the binding of labeled sparsomycin analogues might shed light on this point.

Table IV: Competition of Sparsomycin Ribosomal Binding by Other Antibiotics[a]

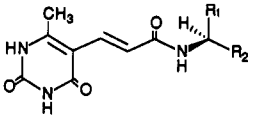
antibiotic	inhibition of ^{125}I-labeled phenol–alanine sparsomycin (ED_{50} values) 70S (μM)	80S (μM)
sparsomycin	0.2	0.5
puromycin	3.0	50
lincomycin	3.0	
chloramphenicol	1.7	
tylosin[b]	1.0	
streptogramin A	>100	
streptogramin B	inactive	
erythromycin	inactive	
blasticydin		20
anisomycin		>100
trichodermin		>100
narciclasin		>100

[a] Ribosomes and ^{125}I-labeled phenol–alanine sparsomycin were used at 0.1 μM in the conditions indicated under Materials and Methods. The concentration of the competing antibiotic that reduces the binding of the labeled compound to 50% (ED_{50}) is shown. [b] Only 50% of the bound sparsomycin derivative could be competed by tylosin.

Table IV shows the results of this study. The higher concentrations of unmodified sparsomycin required to observe competition for the binding of ^{125}I-labeled phenol–alanine sparsomycin to the ribosomes confirms the higher affinity of this derivative for the ribosomes and is consistent, as well, with its higher biological activity. This difference is more evident in the case of the eukaryotic ribosomes. Other bacterial-specific peptidyltransferase inhibitors, chloramphenicol, lincomycin, and tylosin, competed reasonably well with the labeled compound, but streptogramin A and B and erythromycin did not affect the interaction of the sparsomycin derivative. In the yeast ribosomes, only blasticidin S, which is supposed to have a mode of action similar to that of sparsomycin (Vazquez, 1979), competed reasonably well with sparsomycin; the other peptidyltransferase inhibitors were poor competitors of sparsomycin binding.

Competition with Different Sparsomycin Analogues. An extensive number of sparsomycin analogues have been synthesized and tested in different functional assays (Lazaro et al., 1987; van den Broek et al., 1987, 1989). These studies have established detailed structure–function relationships for the sparsomycin molecule (Ballesta & Lazaro, 1990), and data on the relative ribosomal affinity of these analogues will lead to a more detailed understanding of the drug–ribosome interaction. Table V shows the results of competition studies carried out with some representative sparsomycin derivatives, together with previously reported data on their biological activity (van den Broek et al., 1987, 1989). As expected, the affinities of the compounds—defined by the concentration of the compound that produces 50% inhibition of ^{125}I-labeled phenol–alanine sparsomycin binding (ED_{50}) in a competition assay—parallel quite well to their biological activities. Some results suggest, however, that the relationship between drug binding and its biological effects is not straightforward. Thus, compound 5, in which the sulfoxide function of sparsomycin

Table V: Activity of Sparsomycin Analogues



analogue	R_1	R_2	chirality	activity[a] ED_{50} (analogue)/ED_{50} (sparsomycin) poly(Phe) synthesis	puromycin reaction	binding competition
sparsomycin	CH_2OH	$CH_2S(O)CH_2SCH_3$	*ScRs*	1.0	1.0	1.0
2	CH_2OH	$CH_2S(O)CH_2SCH_3$	*ScSs*	250.0	132.0	100.0
3	CH_2OH	$CH_2S(O)CH_2SCH_3$	*RcSs*	250.0	1000.0	500.0
4	CH_2OH	$CH_2S(O)CH_2SCH_3$	*RcRs*	250.0	400.0	280.0
5	CH_2OH	$CH_2SCH_2SCH_3$	*Sc*	230.0	71.5	21.2
6	CH_2OH	$CH_2SCH_2SCH_3$	*Rc*	250.0	1000.0	500.0
7	H	$CH_2S(O)CH_2SCH_3$	*Rs*/*Ss*	11.7	4.0	24.0
8	CH_3	$CH_2S(O)CH_2SCH_3$	*ScRs*	0.5	2.5	0.5
9	$CH(CH_3)CH_2CH_3$	$CH_2S(O)CH_2SCH_3$	*ScRs*	1.4	6.2	0.5
10	CH_2OH	$CH_2S(O)(CH_2)_2CH_3$	*ScRs*	16.6	2.2	0.8
11	CH_2OH	$CH_2S(O)(CH_2)_5CH_3$	*ScRs*	0.8	1.8	0.5
12	CH_2OH	$CH_2S(O)(CH_2)_6CH_3$	*ScRs*	0.4	<0.1	0.4
13	CH_3	$CH_2S(O)C_6H_5$-4OH	*ScRs*	0.3	2.3	0.5
14	CH_3	$CH_2S(O)C_6H_5$-4OH	*ScSs*	250.0	11.7	17.0
15	CH_3	$CH_2S(O)(CH_2)_2CH_3$	*ScRs*	15.0	4.0	2.0
16	CH_3	$CH_2S(O)(CH_2)_2CH_3$	*ScSs*	500.0	40.0	37.5

[a] The activity and binding results have been normalized by dividing them by the values obtained with unmodified sparsomycin, which are 8.5 mM, 0.1 mM, and 0.2 mM for the poly(U)-dependent system, the puromycin reaction, and the binding competition assay, respectively. The poly(U)-dependent assay and the puromycin data have been previously reported (van den Broek et al., 1987, 1989), and the binding data have been obtained in this work as indicated under Materials and Methods.

has been replaced by a sulfur atom, was practically inactive in the poly(phenylalanine) assay and considerably less active than sparsomycin in the puromycin reaction, but it was able to bind to the ribosome with relatively high affinity. The modification introduced in derivative 14 had similar effects. Moreover, compound 10 bound very strongly to the ribosome but was a poorer inhibitor than sparsomycin, especially in the polymerization test.

Discussion

The results clearly indicate that ribosomes do not bind sparsomycin with high affinity unless the P-site is occupied by an N-blocked aminoacyl-tRNA molecule. Neither free tRNA nor unblocked aminoacyl-tRNA is able to stimulate the drug interaction. The presence of an apolar group seems to be required for the opening of an otherwise unaccessible peptidyltransferase center domain, which seems to be neccesary for the high-affinity binding of the drug. In this sense it is interesting to note that our previous results indicate the existence of a hydrophobic domain at the peptidyltransferase center, very close to the sparsomycin binding site, that might be responsible for the strong ribosomal affinity for some of the hydrophobic sparsomycin derivatives (van den Broek et al., 1989).

The requirement of an N-blocked aminoacyl-tRNA at the P-site for sparsomycin binding explains the insensitivity to the drug of the first peptide bond formation in eukaryotic systems (Busiello & Di Girolamo, 1973; Smith, 1973) since the presence of unblocked Met-tRNA at the P-site would not allow the interaction of the antibiotic. Only after the first bond has been formed and the resulting dipeptidyl-tRNA has been translocated to the P-site is sparsomycin able to bind and block the formation of the peptide bonds.

The three-sites model of the ribosome also proposes the existence of two different types of A-site, the i-form when the P-site is occupied by the initiator tRNA at the initiation and the E-site is empty and the e-form during the rest of the translation process (Rheinberger et al., 1990). It has been reported that some antibiotics are able to inhibit only ribosomes with the e-form but not those with the i-form (Hausner et al., 1988). We are of the opinoin that sparsomycin acts differently since the only requirement for binding is the blocking of the α-amino group of the substrate bound at the P-site regardless of the state of the E-site; thus, the equivalent insensitivity to sparsomycin of the first peptide bond formation in eukaryotes has not been documented in bacterial systems, which initiate with formylated Met-tRNA.

The increase in the number of high-affinity binding sites obtained from kinetic experiments when binding was carried out under polymerization conditions confirms the previous results. The capacity of yeast ribosomes to bind the drug with high affinity increases from less than 0.2 to 1 molecule per particle. In bacterial preparations the results are less clear-cut and suggest the existence of a heterogeneous population of particles; in standard buffer, about 15% of the particles in the original preparation bind the drug with a K_a value of 0.30×10^6 M^{-1}. When binding is carried out under protein synthesis conditions, the number of sites binding with a K_a value of 0.30×10^6 M^{-1} remains constant. A similar number of new sites appear that bind the drug with slightly less affinity ($K_a = 1.1 \times 10^6$ M^{-1}), but about 50% of the particles still interact with the drug with very low affinity. The reason for this low affinity is not obvious, but it seems to be related to the presence of some substrates or supernatant factors bound to the particles as indicated by the observation that ribosomes reconstituted from purified ribosomal subunits bind the drug in a homogeneous way with a high affinity constant.

It is interesting that a small fraction of particles present in bacterial and yeast ribosomal preparations show an affinity for the drug slightly higher than the one found when they were tested under poly(Phe) conditions. This difference suggests that part of the "native" ribosomes are better suited for interaction with the drug than those produced artificially in the poly(phenylalanine) synthesis system, probably due to the

presence of bound peptidyl-tRNA. In fact, it would not be surprising if the affinity of the ribosome for sparsomycin were affected by the character of the peptidyl-tRNA occupying the P-site.

The results from competition studies using other peptidyltransferase inhibitors agree in general with data previously reported (Vazquez, 1979; Gale et al., 1981). They confirm that chloramphenicol and sparsomycin indeed compete for binding to the ribosome, and previous data indicating that sparsomycin has no effect on the chloramphenicol interaction probably indicate that ribosomes with a free P-site were used and these are unable to bind sparsomycin. In fact, the extent of competition reported varies depending on the source of ribosomes (Fernandez-Muños et al., 1971; Contreras & Vazquez, 1977). Also, lincomycin and tylosin are both reasonably good competitors of sparsomycin, but erythromycin and streptogramin A and B are not. The clear-cut difference between erythromycin and tylosin confirms the different effects caused by the interaction of these two drugs with the ribosome. They are typical representatives of the 14-atom and 16-atom lactone ring macrolide groups, which compete for binding to the ribosome and have similar structural components in their ribosomal interaction sites (Cundliffe, 1990). In the case of yeast ribosomes, only blasticidin, an antibiotic reported to have a similar mode of action (Vazquez, 1979), competes partially with sparsomycin.

The results of competition studies obtained with puromycin in the case of bacterial ribosomes are unexpected. Although the reported data strongly suggest overlapping binding sites for puromycin and sparsomycin (Coutsogeorgopoulos et al., 1975), the low affinity of puromycin for the ribosomes (Fernandez-Muñoz & Vazquez, 1973) made it reasonable to expect relatively high ED_{50} values, like in the case of yeast particles; the strong competition obtained in *E. coli* ribosomes (ED_{50} = 1.0 μM) is highly surprising. The results were confirmed with different preparations of labeled drug and ribosomes. There is no obvious interpretation of these results. However, a detailed study of the puromycin interaction with different ribosomal particles has not yet been published, and considering the changes that take place in the A-site during protein synthesis (Rheinberger et al., 1990), it would not be surprising that the affinity of the ribosomes for this drug is also affected by the type of substrate occupying the P-site as in the case of sparsomycin.

In general, the competition capacity of the different sparsomycin derivatives tested parallels quite well with their previously reported inhibitory activity (van den Broek et al., 1987, 1989) although some discrepancies are interesting. It seems that an increase or a decrease in the affinity of some derivative does not always mean a parallel effect on their inhibitory activity, and in addition, the effect is not similar in the different biological tests used. These data suggest that the inhibitory effect of the drug might not take place simply by direct physical contact with its binding site. Probably, some later step has to occur to inhibit the ribosome activity, and this step is differently affected as a consequence of the alterations in the molecular structure of some analogues. In fact, a conformational change of the ribosome upon sparsomycin interaction has been proposed to explain the "preincubation" effect reported to occur on the puromycin reaction inhibition (Coutsogeorgopoulos et al., 1975). It has been suggested that the sulfoxide group in the antibiotic molecule might be involved in this effect (Lee & Vince, 1978). Alternatively, the appearance of an additional binding site for the new structural elements in the antibiotic molecule could perhaps explain the dissociation of the sparsomycin analogue interaction and its biological effects. This new binding site might be closely linked to the original one to account for the competition effect but would not be implicated in the inhibition. This interpretation was previously proposed to account for the dramatic increase in activity of some of the hydrophobic derivatives of sparsomycin (van den Broek et al., 1989).

Finally, all these results confirm the interest of sparsomycin as a basic tool in protein synthesis and ribosome structure investigation. It would be of the greatest importance to reveal the structure of the drug binding site, as this seems to be located in a critical region of the peptidyltransferase center.

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Motion of Aromatic Side Chains, Picosecond Fluorescence, and Internal Energy Transfer in *Escherichia coli* Thioredoxin Studied by Site-Directed Mutagenesis, Time-Resolved Fluorescence Spectroscopy, and Molecular Dynamics Simulations†

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ABSTRACT: We have determined the picosecond fluorescence of the four aromatic amino acid residues (W28, W31, Y49, and Y70) in wild-type *Escherichia coli* thioredoxin (wt Trx) and a mutant Trx with W31 replaced by phenylalanine, Trx-W28-W31F. The internal motions of the four aromatic side chains were also analyzed. We examined the possibility of using internal energy transfer from tyrosine to tryptophan as a measure of long-range distances. The major features of the lifetime distribution of tryptophan fluorescence were unchanged in the W31F mutation, indicating that the environment of W28 is similar in both wt Trx and Trx-W28-W31F. However, the mutation of W31F changed the mobility of W28, situated close to the active-site disulfide/dithiol, but not the mobility of two tyrosines, Y49 and Y70, situated on the other side of the molecule. The mobility of the two tyrosine residues increased upon reduction of the active-site disulfide, indicating a looser structure with reduction. This increased motion could also be seen from molecular dynamics simulations. The change in energy transfer rates, as judged by tyrosine fluorescence lifetimes, was in agreement with energy transfer rates calculated from the molecular dynamics simulations. The anisotropy of tryptophan and tyrosine fluorescence could be separated in three parts: (I) overall rotation of the protein (10^{-9} s), (II) internal mobility of side chains (10^{-10} s), and (III) a very fast relaxation (10^{-12} s). We can only experimentally detect this very fast relaxation when the internal motion is not present.

To understand the action of enzymes, it is important to study their dynamics. From molecular dynamics simulations, it is known that proteins vibrate around an average structure, but during catalysis by an enzyme it is often necessary that larger rearrangements occur. Thioredoxin from *Escherichia coli* is a suitable model protein for studying side-chain rearrangements since it contains a redox-active disulfide in the active site.

All thioredoxins have a common active disulfide ring W31-C-G-P-C-K36 (Holmgren, 1985). *E. coli* Trx[1] contains 108 amino acid residues, a single disulfide bridge (C32–C35), two tyrosines (Y49, Y70), and two tryptophans (W28, W31). The molecule is globular and has five β-pleated sheet strands and four α-helixes. The active center disulfide bridge is located in a loop positioned at the C-terminal end of a β-strand (β_2) and followed by an α-helix (α_2). The active center is close to Y70 and both the tryptophans (Figure 1). The disulfide bridge involved in the redox action of the thioredoxin is exposed in the protein.

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[1] Abbreviations: Trx, Thioredoxin; wt, wild-type; Trx-W28-W31F, Trx with tryptophan 28 exchanged for phenylalanine; Trx-S_2, oxidized Trx; Trx-$(SH)_2$, reduced Trx; MD, molecular dynamics; DTT, dithiothreitol.

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