RIBOSOMAL AMBIGUITY (*ram*) MUTATIONS FACILITATE DIHYDROSTREPTOMYCIN BINDING TO RIBOSOMES

August BÖCK, Angelika PETZET and Wolfgang PIEPERSBERG
Lehrstuhl für Mikrobiologie der Universität Regensburg, 8400 Regensburg, Germany

Received 18 April 1979
Revised version received 5 June 1979

1. Introduction

The antibiotic streptomycin binds to *Escherichia coli* 70S ribosomes or 30S subunits in 1 : 1 stoichiometric ratio (for review see ref. 1). One-step mutations in the structural gene for ribosomal protein S12 render strains either high-level resistant ($strA^R$) to the drug or they lead to streptomycin-dependent growth ($strA^D$) (for review see ref. 2, 3); ribosomes from these streptomycin-resistant or -dependent cells do not bind the drug in concentrations which promote specific binding to sensitive ribosomes [1]. This effect of S12 mutations on the streptomycin phenotype is paralleled by an influence on translational fidelity: both $strA^R$ and $strA^D$ mutations increase the fidelity of protein synthesis [2].

The opposite effect is brought about by the ribosomal ambiguity (*ram*) mutations in proteins S4 [4] or S5 [5,6]. They increase ambiguity of translation when present alone or in combination with mutations in the gene for protein S12, both of the $strA^R$ or $strA^D$ type [5–7]. In addition, they revert from streptomycin-dependence to -independence [8–12] and they decrease the level of streptomycin-resistance in $strA^R$ strains [4,13]. As an explanation for this pleiotropic effect of *ram* mutations on the streptomycin phenotype we have postulated recently that S4 or S5 mutations of the *ram* type facilitate streptomycin binding. In an otherwise wild-type ribosome this would lead to the streptomycin hypersensitivity first detected by Rosset and Gorini for S4 *ram* mutants [4] and later also demonstrated for S5 *ram* strains [13]. In combination with a $strA^R$ or $strA^D$ mutation the *ram* mutations would promote the formation of a binding site with intermediate affinity which results in the partial resistance observed. The data presented in this communication show that *ram* mutant ribosomes, indeed, have a higher affinity for dihydrostreptomycin than ram^+ strains.

2. Materials and methods

2.1. *Strains and growth conditions*

All organisms (for characteristics see ref. 13) except strains d10 and d1023 were grown at 37°C in the modified salt solution P [14] supplemented with 0.4% glucose, 0.2% ammonium sulfate and 40 μg L-arginine per ml. d10 and d1023 were cultivated in rich medium [13]. The streptomycin requirement of strain d10, which is streptomycin-dependent [12], was by-passed by supplementing the medium with 3% ethanol. At an A_{420} of about 1.5 growth of cultures was stopped by chilling; the cells were collected by sedimentation at 8000 × *g* (10 min), washed once in TMNSH buffer (10 mM Tris–Cl, pH 7.5; 10 mM magnesium acetate, 100 mM ammonium chloride, 6 mM 2-mercaptoethanol), sedimented again and stored frozen at −80°C until use.

2.2. *Preparation of ribosomes and ribosomal subunits*

The cells were suspended in TMNSH buffer containing 2 μg DNase (RNase-free; Boehringer Mannheim) per ml and broken by passage through a French press cell at 12 000 psi. S30 extracts were obtained after centrifugation at 12 000 × *g* (10 min) and 30 000 × *g* (30 min). 70S ribosomes were obtained by layering

4-ml portions of the S30 extract on 4 ml 30% sucrose solution made up in TMNSH and by centrifuging for 150 min at 50 000 rev./min in a 75 Ti rotor. The pellet was resuspended in TMNSH and the ribosomal suspension was dialysed overnight against TMNSH. After a further centrifugation at 30 000 × *g* (10 min), ribosomes were incubated for 10 min at 37°C and then used for equilibrium dialysis.

Ribosomal subunits were isolated by preparation of an S30 extract in 10 mM potassium phosphate pH 7.5, 1 mM magnesium acetate, 6 mM 2-mercaptoethanol and by separation of 80 to 100 A_{260} units of it by centrifugation through 10–30% linear sucrose gradients made up in the same buffer in a SW 27 rotor at 24 000 × *g* for 15 h. 30S and 50S peak material of the gradients was collected, the magnesium concentration was adjusted to 10 mM and the subunits were sedimented in a 60 Ti rotor by centrifugation at 42 000 rev./min for 15 h. They were resuspended in TMNSH buffer, dialysed for at least 6 h against TMNSH, freed from aggregates by centrifugation at 30 000 × *g* (10 min), activated by a 10 min incubation at 37°C and then used for equilibrium dialysis.

2.3. *Equilibrium dialysis*

Equilibrium dialysis was carried out in 200 μl dialysis chambers separated by a Visking dialysis membrane (Type 36/32). Prior to use, membranes were boiled twice 5 min each in 1 mM EDTA. Each dialysis experiment was done with 12 concentration steps of [^{3}H]dihydrostreptomycin. For ribosomes and 30S subunits having a wild-type S12 protein the concentration range was from 3.35 to 0.36 μM of the antibiotic, for ribosomes containing an S12 of the streptomycin-resistant or -dependent type, the concentration was varied from 13.4 to 0.42 μM (total input into the ribosome-free chamber). The other chamber was filled with 200 μl of suspensions of 70S (15 A_{260}/ml) or 30S (5 A_{260}/ml). 1 A_{260} unit of 30S was taken to correspond to 74.1 pmol, 1 A_{260} of 70S to correspond to 25.2 pmol [19]. Dialysis was carried out for 16 h at 4°C on a rotating drum. From each chamber three times 25 μl were drawn with a syringe and pipetted into 5 ml Aqualuma scintillation fluid (Lumac Systems AG, Basel). Radioactivity was measured by counting each sample twice for 10 min. Data were then plotted according to Scatchard [15]. Control experiments were performed which ruled out any quenching effects of the amount of ribosomes used on [^{3}H]dihydrostreptomycin radioactivity.

2.4. *Chemicals*

[^{3}H]dihydrostreptomycin sesquisulfate was bought from Amersham Buchler. The specific radioactivity was 3.0 Ci/mmol taking a molecular weight of 1461. For calculating the mol of antibiotic from the cpm values it was assumed that under the conditions of the experiments complete dissociation of the sesquisulfate takes place.

3. Results and discussion

Figure 1 shows an example of an equilibrium dialysis experiment of 30S subunits and 70S ribosomes from strains which contain a wild-type S12 protein but a mutant *ram* S4 protein (strain T90/N422-1) or two mutant *ram* proteins, S4 and S5 (strain d/T-7). Clearly, *ram* ribosomes or 30S subunits exhibit an increased affinity for the antibiotic in comparison to wild-type ribosomes (strain T/BM-24).

Figure 2 illustrates another example, namely binding of dihydrostreptomycin to ribosomes which contain an S12 protein of the streptomycin-resistant phenotype alone (T/Sm 1-31), or in combination with an altered S4 ambiguity protein. The presence of

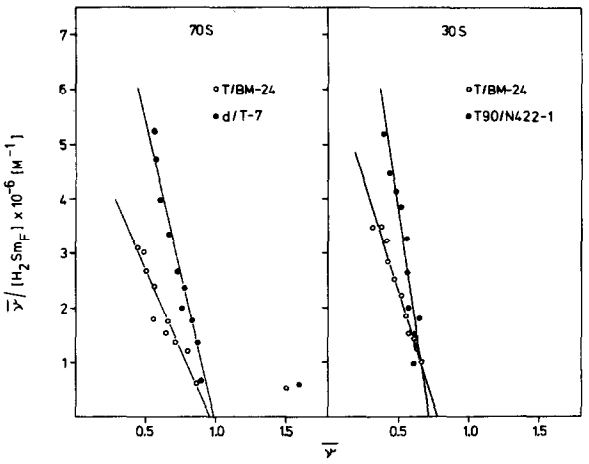

Fig.1. Scatchard plots of the interaction of [^{3}H]dihydrostreptomycin with 70S ribosomes from the wild type (T/BM-24) and a S4/S5 double *ram* mutant (d/T-7) and of 30S ribosomes from the wild type and an S4 ambiguity mutant (T90/N422-1). $\bar{\nu}$: moles dihydrostreptomycin bound per mole of 70S ribosome or 30S particle; [H_2Sm_F]: Concentration of unbound dihydrostreptomycin.

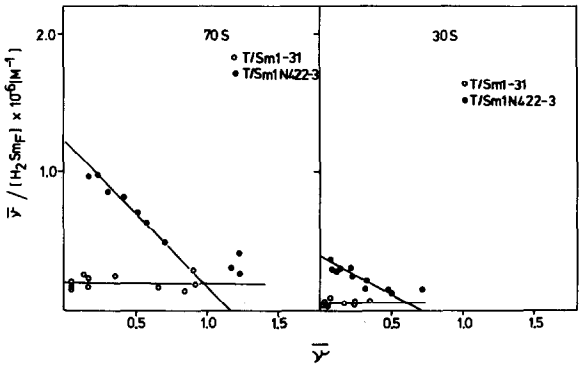

Fig.2. Scatchard plots of dihydrostreptomycin binding to 70S and 30S particles of a streptomycin-resistant ($strA^R$) strain (T/Sm 1-31) and of its derivative containing an S4 *ram* mutation (strain T/Sm 1N422-3).

the altered S12 prevents specific binding of [^{3}H]-dihydrostreptomycin; the additional presence of a ribosomal ambiguity mutation in protein S4 restores binding again, though with a much lower affinity constant (note the difference in scale in relation to fig.1).

The collected data are listed in table 1. The first four strains possess a wild-type S12 protein, but contain one or two mutant *ram* proteins [13]. In all experiments performed both 70S ribosomes and 30S subunits reproducibly exhibited higher association constants for dihydrostreptomycin than those from the wild-type.

The next four strains all harbour an altered S12 protein with the amino acid exchange characteristic for *strA*40 mutations [13]. In the range of the dihydrostreptomycin concentration employed specific binding to either 30S or 70S ribosomes was not

Table 1
Association constants of 70S ribosomes and 30S subunits for dihydrostreptomycin

Strain	Mutated ribosomal protein	Particle	$K_A \times 10^{-6}$ (M^{-1})	
T/BM-24	None	70S	7.6	(5)[a]
		30S	9.8	(4)
T90/N422-1	S4	70S	14.3	(3)
		30S	15.2	(2)
T90/d1023-4	S5	70S	12.7	(3)
		30S	17.2	(2)
d/T-7	S4 + S5	70S	15.2	(3)
		30S	17.4	(1)
T/Sm1-31	$S12^R$	70S	No specific	
		30S	binding	
T/Sm1N422-3	$S12^R$ + S4	70S	0.98	(2)
		30S	0.59	(2)
T/Sm1N421-2	$S12^R$ + S5	70S	0.80	(2)
		30S	0.46	(2)
T/Sm1S4S5-4	$S12^R$ + S4 + S5	70S	0.89	(3)
		30S	0.45	(2)
d10	$S12^D$	70S	(0.18)[b]	(1)
d1023	$S12^D$S5	70S	1.45	(1)

[a] Number in parentheses gives the number of dialysis experiments performed with 12 concentration values each

[b] Slope difficult to determine due to very low binding; the given value is the maximal one which can be derived from the experimental data

$S12^R$, $S12^D$ denote S12 alterations leading to a streptomycin-resistant or streptomycin-dependent phenotype

K_A: association constant was calculated from the slope of the Scatchard plots

detected. The additional presence of an altered S4 and/or S5 protein, however, promotes binding again with an affinity between 1/10 to 1/20 that of the control strains (with the ambiguity mutation alone). In contrast to the first four strains listed in the table, 30S subunits of these mutants show a lower affinity for the antibiotic than the corresponding 70S ribosomes. It must be emphasized in this connection, however, that in contrast to the results obtained with 70S those observed with 30S subunits were more variable. As this was not observed with subunits of the upper four strains it might be a specific effect of the simultaneous presence of S12 and S4/S5 mutant proteins in the ribosome.

The last two strains listed are d10 which is streptomycin-dependent [12] and d1023 which contains the same S12 mutation as d10 and in addition an S5 mutation suppressing streptomycin-dependence leading to low level streptomycin resistance [12,16]. d10 was grown in the presence of 3% ethanol to avoid exposure of ribosomes to streptomycin prior to equilibrium dialysis. Under this condition growth of d10 was not clearly exponential, but control streaks indicated that the cells had maintained their streptomycin-dependent phenotype.

Table 1 shows that d10 70S ribosomes have a very low affinity for dihydrostreptomycin and that inclusion of the S5 mutation drastically increases the association constant. (At the moment, we cannot say how ethanol in the growth medium affects the formation of the streptomycin site; a detailed investigation on this subject, especially in phenotypically masked strains, is in progress.)

The results demonstrate that binding of dihydrostreptomycin to bacterial ribosomes is not only affected by alterations of ribosomal protein S12 but also by those in the fidelity proteins S4 and S5.

(i) S4 and/or S5 alterations facilitate binding of dihydrostreptomycin.

(ii) When present on streptomycin-resistant or -dependent ribosomes the altered S4 and/or S5 proteins revert the inhibition of dihydrostreptomycin binding caused by the respective S12 mutation, most probably by 'opening' of the normal binding site and not by creating an entirely new one.

It has been reported that streptomycin binding requires the presence of protein S12 and that the drug binds to a site formed by proteins S3 and S5 [17] or directly to 16S rRNA [19]. One possible explanation of our binding data could be that neither S12, S4, S5 are constituent proteins of the binding site but that the latter may be shielded by alterations in protein S12 [19] and reformed by altered S4 or S5 proteins.

With these findings several of the pleiotropic effects of fidelity mutations on the streptomycin phenotype can be explained:

(i) The streptomycin hypersensitivity of S4 [4] and S5 [13] *ram* mutants originally was thought to result from the fact that the increased misreading level can be raised to a lethal extent by lower concentrations of streptomycin than those necessary to kill a wild-type cell [2,4,7]. The results described above, however, rather provide evidence that the higher affinity of *ram* ribosomes for dihydrostreptomycin might at least contribute to the enhanced sensitivity.

(ii) The intermediate streptomycin-resistance of strains in which streptomycin-dependence is suppressed by S4 or S5 mutations [8–12] or in which a streptomycin-resistance marker is genetically combined with a *ram* mutation [4,13] might simply reflect the fact that the different S12/S4 or S12/S5 mutant protein combinations might form binding sites with different affinities. The changes in concentrations of streptomycin necessary to derestrict restriction of misreading in such strains could also be a function of such differences.

The association constants are not proportionally correlated with the level of streptomycin resistance in all cases, because the S4/S5 double *ram* mutants are more sensitive than either of the single *ram* mutants [13], but they obviously do not differ in affinity constants of their ribosomes (table 1). Factors which might be involved could be a difference between the in vivo and in vitro behaviour of S4/S5 double *ram* mutant ribosomes or alterations in streptomycin uptake.

There is an extremely good correlation now between the effect of mutations in ribosomal protein S4, S5 and S12 on dihydrostreptomycin binding and translational fidelity. Mutations which facilitate streptomycin binding increase misreading, those which impair it concomitantly decrease misreading. It is

tempting to speculate that the affinity of the ribosome for dihydrostreptomycin is a direct expression of the affinity of the mRNA primed ribosomal A-site for non-cognate aminoacyl-tRNA.

Acknowledgements

We are greatly indebted to E. Holler and R. Jaenicke for help with equipment and for advice and to H. G. Wittmann for providing strains. We thank K. Nierhaus for critically reading the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

[1] Pestka, S. (1977) in: Molecular Mechanisms of Protein Synthesis (Weissbach, H. and Pestka, S. eds) Academic Press, New York, p. 468.
[2] Gorini, L. (1974) in: Ribosomes, (Nomura, M., Tissières A. and Lengyel, P. eds) Cold Spring Harbor Laboratory, p. 791.
[3] Stöffler, G. and Wittman, H. G. (1977) in: Molecular Mechanisms of Protein Synthesis (Weissbach, H. and Pestka, S. eds) Academic Press, New York, p. 117.
[4] Rosset, R. and Gorini, L. (1969) J. Mol. Biol. 39, 95–112.
[5] Piepersberg, W., Böck, A. and Wittmann, H. G. (1975) Mol. Gen. Genet. 140, 91–100.
[6] Cabezon, T., Herzog, A., De Wilde, M., Villarroel, R. and Bollen, A. (1976) Mol. Gen. Genet. 144, 59–62.
[7] Biswas, D. K. and Gorini, L. (1972) J. Mol. Biol. 64, 119–134.
[8] Deusser, E., Stöffler, G., Wittmann, H. G. and Apirion, D. (1970) Mol. Gen. Genet. 109, 298–302.
[9] Birge, E. A. and Kurland, C. G. (1969) Science 166, 1282–1284.
[10] Stöffler, G., Deusser, E., Wittmann, H. G. and Apirion, D. (1971) Mol. Gen. Genet. 111, 334–341.
[11] Kreider, G. and Brownstein, B. L. (1971) J. Mol. Biol. 61, 135–142.
[12] Hasenbank, R., Guthrie, Ch., Stöffler, G., Wittmann, H. G., Rosen, L. and Apirion, D. (1973) Mol. Gen. Genet. 127, 1–18.
[13] Piepersberg, W., Noseda, V. and Böck, A. (1979) Mol. Gen. Genet. 171, 23–34.
[14] Fraenkel, D. G. and Neidhardt, F. C. (1961) Biochim. Biophys. Acta 53, 96–110.
[15] Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–670.
[16] Piepersberg, W., Böck, A., Yaguchi, M. and Wittmann, H. G. (1975) Mol. Gen. Genet. 143, 43–52.
[17] Schreiner, G. and Nierhaus, K. H. (1973) J. Mol. Biol. 81, 71–82.
[18] Biswas, D. K. and Gorini, L. (1972) Proc. Natl. Acad. Sci. USA 69, 2141–2144.
[19] Van Holde, K. E. and Hill, W. E. (1974) in: Ribosomes (Nomura, M., Tissères, A. and Lengyel, P. eds) Cold Spring Harbor Laboratory, p. 53.