BIOCHEMICAL CHARACTERIZATION OF RIBOSOMES OF KASUGAMYCIN-DEPENDENT MUTANTS OF *ESCHERICHIA COLI*

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1. Introduction

The ribosomal initiation process can be investigated using the antibiotic kasugamycin (Ksg), which inhibits the binding of fMet-tRNA$_f$ to the ribosome [1]. Strains resistant to Ksg due to a mutation at the *KsgA** locus [2] have lost the activity of a methylase which acts on two adjacent adenosines near the 3′-end of the 16 S RNA [3]. Though resistance is a function of the 30 S subunit, it is only manifested in the presence of 50 S subunits [4]. Methylation of these adenosines near the 3′-end of the 16 S is apparently universal to all organisms, but its functional significance is not known for certain. The presence of the methyl groups facilitates subunit association [5].

As an additional probe of this ribosomal neighbourhood, Ksg-dependent mutants have been isolated and characterized [6]. A number of these mutants are Ksg-resistant as well as dependent, yet (as found by genetic analysis) do not have a *KsgA* mutation. Mutational alterations in 50 S proteins have been shown in some of the mutants to be responsible for the mutant phenotype [6,7], possibly an in vivo indication of the above-cited in vitro effect of 50 S subunits on 30 S phenotype [4].

It was considered worthwhile to analyze the ribosomes of these Ksg-dependent mutants in vitro. The mutants selected had a variety of phenotypes, and were tested for the methylation of the 16 S RNA, and for presence or absence of methylase. The effect of Ksg on fMet-tRNA$_f$ binding and on a natural message-directed polypeptide synthesizing system was also determined, to see if Ksg-dependence could be demonstrated in vitro.

The conclusions from this work were:

(i) No mutant showed an in vitro dependence on Ksg for polypeptide synthesis or fMet-tRNA$_f$ binding;
(ii) Irrespective of in vivo phenotype, ribosomes of *KsgA* strains were Ksg-resistant in vitro whilst ribosomes of *KsgA** strains were Ksg-resistant in vitro;
(iii) As expected, all *KsgA* strains had ribosomes that were not methylated at the substrate adenosines of the 16 S RNA;
(iv) All strains with a mutational alteration in ribosomal protein S9 (as determined by two-dimensional gels) were hypersensitive to Ksg in vitro (though resistant and dependent in vivo). The ribosomes of no other strain were hypersensitive to Ksg.

2. Materials and methods

Ksg-dependent mutants, designated MV, were isolated and characterized as in [6]. After growth in rich medium to late log or early stationary phase, cells were washed and frozen at −80°C until use. Transduction experiments to test for presence of the *KsgA* mutation were done using phage P1 as in [6].

Ribosomes, ribosomal subunits, ribosomal wash and S100 fraction were isolated according to [4]. The methylase activity assay has been described [8]. 30 pmol unmethylated ribosomes from strain TPR 201 [3] were present and either 15 μl ribosomal wash or 15 μl S100 fraction were checked for methylase activity. The methylation assay was performed with purified methylase [8]. ~14 pmol 30 S ribosomes were used in each assay. The specific activity of the [^{3}H]methyl-*S*-adenosylmethionine used was

*Corrigendum: *KsgA* should read *ksgA* throughout

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780 cpm/pmol. MS2 RNA-dependent binding of fMet-tRNA in the absence and presence of kasugamycin was done as in [4]. The polypeptide synthesis experiments were done according to [9].

3. Results

3.1. *Methylase*

Using ribosomes from a *KsgA* strain as substrate, MV mutants were tested for presence of methylase activity in the S100 and the ribosomal wash (methylase is normally found in both [8]). As shown in table 1, there was agreement with the genetic data. Strains that were $KsgA^+$ had methylase activity; *KsgA* strains did not have methylase activity. In vivo, there was some variation in degree of Ksg-resistance conferred by *KsgA* mutations, but no corresponding variation in levels of residual methylase activity were measurable in vitro.

3.2. *Methylation of 16 S RNA*

Washed ribosomes of MV mutants were tested as substrate for purified methylase. Ribosomes from all strains that were *KsgA* had ribosomes with 16 S RNA which could be methylated in vitro (table 1). Ribosomes from strains that were $KsgA^+$, and so had methylase, could not be methylated in vitro by purified methylase (table 1).

3.3. *Inhibition by Ksg*

Inhibition of ribosomes of a sensitive strain by Ksg has been shown to be due to inhibition of fMet-$tRNA_f$ binding to the ribosome [1,4]. For this reason, the effect of addition of Ksg on fMet-$tRNA_f$ binding to ribosomes of MV mutants was tested to see if dependence or stimulation could be demonstrated in the same fashion. The effect of Ksg on polypeptide synthesis was also investigated. A like conclusion was reached in both cases, that over a range of antibiotic concentrations no dependence on or stimulation by Ksg was observed (table 2). This remained true even when other parameters, such as temperature and $[Mg^{2+}]$, were varied.

It was also found that irrespective of the level of in vivo Ksg-resistance of a strain, in vitro resistance of its ribosomes depended solely on the presence or absence of a *KsgA* mutation in the strain (representative results are shown in table 2). If the strain was *KsgA*, then its ribosomes were about as resistant as previously characterized Ksg-resistant *KsgA* strains. If the strain was $KsgA^+$, its ribosomes were sensitive in vitro.

An interesting observation was that all strains which had been found by two-dimensional polyacrylamide gel electrophoresis to possess mutational alterations in ribosomal protein S9 ([6], E. R. D., unpublished) had ribosomes which were hypersensitive to Ksg. The ribosomes of no other strain showed this hypersensitivity.

4. Discussion

The observed presence or absence of methylase, and corresponding methylation status of the target adenosine moieties in the 16 S RNA, fully agrees with the genetically determined status of the *KsgA* locus.

Table 1
Methylase activity and methylation of 16 S rRNA of the MV mutants

Strain[a]	*ksgA* genotype[a]	Methylase activity in ribosomal wash[b]	Methylase activity in S100 fraction[b]	Methylation of 30 S ribosomes[c]
L44	+	16 000	4000	500
MV1	–	500	500	38 000
MV2	–	500	500	33 000
MV9	+	22 500	7000	600
MV10	–	400	500	37 000
MV11	+	19 500	10 000	600
MV17	–	500	600	30 000
MV21	+	21 500	6000	500

[a] See [6] for isolation and characterization of the strains
[b] Methylase activity in the ribosomal wash and in the S100 fraction is given in cpm (see section 2)
[c] Methylation of 30 S ribosomes is expressed in cpm (see section 2)

Table 2
Effect of kasugamycin on fMet-tRNA binding and polypeptide synthesis

Strain	Altered protein[a]	In vivo phenotype[b]	Percentage inhibition by Ksg[c]	
			fMet-tRNA binding	Polypeptide synthesis
L44		S	35	35
MV1		D	10	10
MV2	S18,L11	D/S	12	5
MV9	S9,S18	D/R	60	n.d.
MV10		D/R/S	11	10
MV11		D	40	20
MV17		D/S	5	n.d.
MV21	S9	D	75	60

[a] Proteins were analyzed by two-dimensional gel electrophoresis [6]
[b] The in vivo phenotype of the mutants was analyzed [6], at several temperatures and with different amounts of Ksg: S, sensitive; R, resistant; D, dependent
[c] MS2 RNA-dependent binding of fMet-tRNA and MS2 RNA dependent polypeptide synthesis have been performed with various amounts of Ksg and at 25°C, 37°C and 44°C. The % inhibition in this table was measured at 37°C in the absence and presence of 0.05 mg Ksg/ml (cf. section 2); n.d., not done

However, these results suggest that whilst Ksg inhibition of ribosomes of a sensitive strain occurs because of inhibition of fMet-tRNA$_f$ binding, inhibition of a Ksg-dependent strain by the absence of Ksg arises at another point in ribosome function. Since dependence on, or even stimulation by antibiotic was also not observed in a natural message-primed polypeptide synthesizing system, Ksg-dependence must be due to some subtle aspect of translational machinery function that has not yet been duplicated in vitro.

This work also showed that, unless a strain was *KsgA*, its ribosomes were sensitive to Ksg in vitro even if the strain was several times more resistant in vivo than wildtype. Explanations of these results in terms of uptake/permeability mutations cannot apply to these mutants because:

(i) Uptake/permeability mutants can explain resistance but not dependence;
(ii) Genetic studies [6] have shown the involvement of ribosomal mutations in the phenotype of these mutants.

As in the case of dependence, resistance in *KsgA*$^+$ Ksg-dependent strains must arise from a subtlety of protein synthesis that was not reproduced in these experiments.

The hypersensitivity of ribosomes of all mutants with alterations in protein S9 is strong evidence that:

(i) S9 is in the ribosomal neighbourhood responsible for Ksg and/or fMet-tRNA$_f$ binding, or subunit interaction;
(ii) The mutational alterations observed in protein S9 are involved in the Ksg phenotype (there is no such evidence in the case of mutants with S13 or L14 altered).

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