CHANGES IN ACCESSIBILITY TO CHEMICAL MODIFICATION OF *ESCHERICHIA COLI* RIBOSOMES INDUCED BY STREPTOMYCIN

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1. Introduction

The interaction of streptomycin (Sm)* with the ribosome has been studied by a variety of methods, including an analysis of thermodynamic properties [1,2], spin-labeling [3], hydrogen–tritium exchange [4], and thermal denaturation [5]. Chemical modification offers a direct method of probing ribosome structure and has been to show specific structural changes in ribosomes [6,7]. Modification with the reagent kethoxal (3-ethoxy-1,1-dihydroxy-2-butanone) offers a particularly useful probe of ribosome structure, since it binds to both ribosomal RNA [8,9] and ribosomal protein [8,10] and changes in both components could be simultaneously monitored.

We have investigated the accessibility of ribosomes to chemical modification with kethoxal in the presence and absence of Sm. In this paper, significant changes in availability for chemical modification are reported with prebinding of 70S ribosomes with Sm. Stimulation of kethoxal binding was found in both RNA and protein ribosomal fractions and in both 16S and 23S RNAs. The results suggest a substantial rearrangement of the ribosome structure with the presence of Sm.

2. Materials and methods

2.1. *Chemicals*

[^{3}H]kethoxal (spec. act. 8.9 mCi/mmol) was from New England Nuclear. Test of purity was as previously described [8]. Streptomycin sulfate was generously provided by Dr H. D. Brown of Merck Sharp & Dohme Research Laboratories.

Solutions for ribosome isolations were as follows: Solution I (10 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM β-mercaptoethanol). For ribosome reaction with kethoxal and subsequent procedures, Solution I was the same except Tris–borate (Solution Ia) was substituted for Tris–HCl. Solution II was the same as I except $MgCl_2$ was 0.3 mM and Solution II as I except NH_4Cl was 0.35 M.

2.2. *Ribosome preparation*

Ribosomes were prepared from *E. coli* strain Q13 RNase I$^-$ (mid-logarithmic cells), purchased from General Biochemicals and *E. coli* MRE-600 Str^R RNase I$^-$ kindly supplied by Dr Julian Davies. Preparation of 70S ribosomes was according to Traub et al. [11]. After centrifugation at high and low speed for several cycles, ribosomes were twice layered over 30% sucrose in Solution III and centrifuged to a pellet. They were washed once through Solution Ia and resuspended in the same buffer for reaction with kethoxal.

2.3. *Reaction conditions*

The reaction mixture (usually 0.1 ml), containing 40–80 A_{260} ribosomes in Solution Ia, was preincubated at 37°C with antibiotic for 15 min (see text for concentrations used), [^{3}H]kethoxal (1×10^4 μmol/μmol ribosomes) added, and incubation continued for 60 min. After incubation, ribosomes were twice

* *Abbreviations:* streptomycin, Sm; ribonuclease, RNase.

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layered over 30% sucrose in Solution Ia and centrifuged to remove unbound kethoxal. Particles were then used for determination of bound kethoxal to ribosomes and for further extractions of protein and RNA.

2.4. *Extraction of proteins and disc electrophoresis separation*

Ribosomal proteins for gel electrophoresis were extracted by the method of Spitnik-Elson [12]. Urea and LiCl were added to ribosomes in Solution Ia to a final concentration of 4 M and 2 M, respectively. After 48 hr at 0°C, RNA was separated by centrifugation at 15 000 rev/min. The supernatant was dialyzed against 6 M urea–20 mM Tris–borate, pH 8.0, 6 mM β-mercaptoethanol for gel electrophoresis. Disc electrophoresis in polyacrylamide gels was similar to the modification [13] of the Reisfeld et al. [14] technique. Gels were fixed and stained in 7.0% acetic acid with Amido Schwarz after electrophoresis. Gels were scanned for optical density at 600 nm sliced for radioactive counting. Gel slices were dried at 50°C, hydrolyzed in 0.1 ml–0.5 ml 30% H_2O_2 at 50°C 1–6 hr and counted in 10 ml Aquasol.

2.5. *Determination of [^{3}H]kethoxal bound to total protein fractions*

[^{3}H]kethoxal binding to total ribosomal protein was determined either by counting aliquots of LiCl-urea supernatant fractions or by counting the protein phase of a phenol extraction of ribosomes, with 10 ml Aquasol. Both methods gave nearly identical results. Average deviations of several determinations were usually within 10%.

2.6. *RNA extractions*

RNA was extracted by the phenol method and ethanol precipitation. Tris–borate buffer was used in all extraction procedures. RNA from LiCl-urea protein separations was also used for determination of bound kethoxal. Separation of 23S and 16S RNAs was by centrifugation with a 5–30% sucrose gradient in 0.01 M Tris–acetate pH 7.0, 0.1 M potassium acetate. Centrifugation was at 24 000 rev/min for 16 hr. After centrifugation, the centrifuge tube contents were fractionated, the UV absorbance determined, and samples counted (generally 0.5 ml in 10 ml Aquasol).

3. Results

3.1. *Stimulation of kethoxal binding to ribosomes by Sm*

Kethoxal is a dicarbonyl compound that reacts with guanidino groups and is specific for single-stranded guanine residues in RNA [15–17] and arginine in protein [18]. Under appropriate buffer conditions (Tris–borate or Tris–acetate) the reagents binds in a stable manner to both RNA and protein in ribosomes. Preincubation of ribosomes with Sm induces a stimulation of kethoxal binding to ribosomes. Fig.1 shows the kinetics of kethoxal labeling of ribosomes with

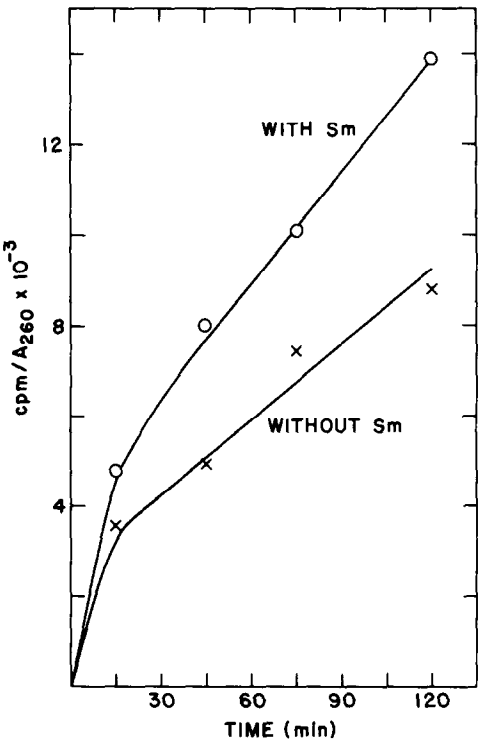

Fig.1. The time course of reaction of 70S ribosomes with [^{3}H]kethoxal, incubated with Sm (450 Sm/ribosome) and without Sm. The reaction conditions were as follows: 92 A_{260} 70S ribosomes in solution Ia (0.19 ml) was incubated at 37°C with 1.0 μmol Sm for 15 min, [^{3}H]kethoxal (11 μmol) was added and aliquots were removed at various times. Incubation conditions for the control were identical except for the absence of Sm. The reaction was stopped by freezing aliquots at −70°C. Removal of unbound [^{3}H] kethoxal was by centrifugation over sucrose.

Table 1
[^{3}H]Kethoxal bound to ribosomes and ribosomal components in the presence of streptomycin

E. coli	Sm/ribosome (ratio)	Kethoxal bound with Sm/without Sm (ratio)			
		Ribosomes	23S RNA	16S RNA	Protein
Q13	330	1.17	1.33	1.10	1.12
Q13	5	1.04	1.02	0.98	1.15
MRE-600 Str^R	400	1.02	1.03	1.02	0.97

and without Sm. The labeling pattern is biphasic. Sm changes the initial rate of labeling and the total number of kethoxal molecules bound. After 120 min incubation, there are 72 mol kethoxal bound to 70S ribosomes as compared to 112 mol bound with Sm present.

3.2. *RNA and protein changes*

To determine which ribosomal components account for the increased reagent binding in the presence of Sm, RNA and protein were separated from reacted 70S ribosomes after removal of unbound reagent and analyzed for bound kethoxal. Ribosomal RNA was separated from protein by LiCl-urea and bound kethoxal determined (table 1). In addition to increased reagent bound to proteins, increased binding to 23S and 16S RNAs was found at high Sm/ribosome ratios. A typical sucrose gradient RNA separation is shown in fig.2. Stimulation of binding was prominent in 23S RNA and , to a lesser extent, in 16S RNA and was observed at high concentrations of Sm/ribosome (table 1). Polyacrylamide gel electrophoresis of 50S

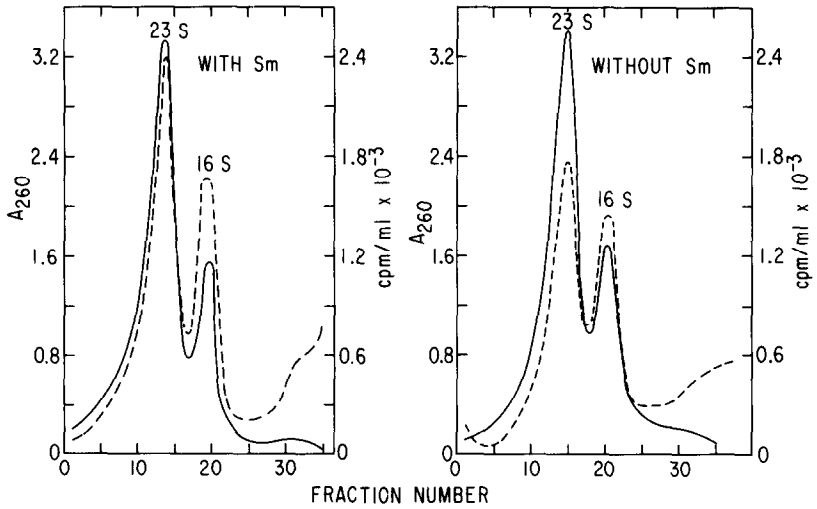

Fig.2. Separation of 23S and 16S RNAs from [^{3}H]kethoxal reacted 70S ribosomes. Left drawing shows the sucrose gradient separation of RNA from 70S ribosomes reacted in the presence of Sm (330 Sm/ribosomes) and the right, in the absence of Sm. The reaction mixture with Sm (0.16 ml) contained 29 A_{260} 70S ribosomes, 0.23 μmol Sm and 7.2 μmol kethoxal. (——) A_{260}; (– – –) [^{3}H] cpm.

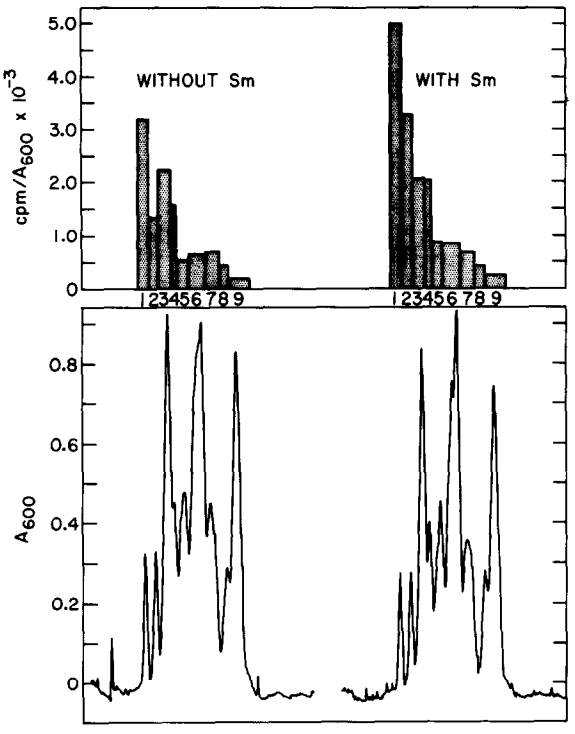

Fig.3. Polyacrylamide gel disc electrophoresis of 50S ribosomal proteins from [^{3}H]kethoxal reacted 70S ribosomes, with and without the presence of Sm. Proteins were extracted by LiCl-urea, dialyzed and run on 7.5% gels. After electrophoresis, gels were stained with Amido Schwarz in 7% acetic acid, scanned at 600 mm, sliced and counted. A) Right histogram represents relative value of cpm/A_{600} of gel fractions sliced; right gel scan is at 600 nm. With Sm (400 Sm/ribosome) present during reaction. B) Left histogram and gel scan represent reaction without Sm.

proteins from 70S reacted ribosomes (fig.3) showed a significant increase in the specific activity of at least 3 proteins bands (bands 1, 2 and 5) analyzed from ribosomes reacted in the presence of Sm.

Reaction of 70S ribosomes from Sm resistant cells showed that Sm has little effect on the overall binding of kethoxal to 70S ribosomes. Table 1 shows no significant increase in binding to ribosomal RNA or protein fractions at a molar ratio of 400Sm/ribosome.

3.3. *Reaction at high salt and high Mg^{++}*

It has recently been demonstrated that Sm binds to 50S subunits as well as to 30S subunits [19] and to ribosomal RNA (20). At high concentrations of salt (250 mM NH_4Cl), Sm binding to 50S subunits, 16S and 23S RNAs is reduced, whereas binding to 30S subunits is unchanged [19]. Reaction of 70S ribosomes with kethoxal at 250 mM NH_4Cl in the presence of Sm was performed to determine if antibiotic induced binding of the reagent could be abolished or greatly reduced. Stimulation of kethoxal binding to ribosomes at 250 mM NH_4Cl was still present, including binding to the 23S RNA (table 2).

At 20 mM Mg^{++}, however, Sm did not appear to stimulate kethoxal binding to ribosomes as at the lower Mg^{++}concentration. Table 2 shows that kethoxal labeling of ribosomes at 20 mM Mg^{++} does not appear to change with the presence of Sm.

3.4. *Binding of kethoxal to poly AUG in the presence of Sm*

In order to exclude the possibility that increased reaction is due to a stimulation of the primary

Table 2
Binding of [^{3}H]Kethoxal to ribosomes with and without streptomycin at high salt of high Mg^{++}

Sm/ribosome (ratio)	NH_4Cl (mM)	$MgCl_2$ (mM)	Kethoxal bound with Sm/without Sm (ratio)			
			Ribosomes	23S RNA	16S RNA	Protein
400	250	10	1.18	1.19	1.07	1.12
500	30	20	1.02	–	–	–

chemical reaction, and not the assumed increase in the available number of sites, synthetic polynucleotide poly AUG was incubated with Sm (0.1 mg/mg poly AUG) and kethoxal, with the same incubation conditions used for ribosome reactions. The ratio of bound kethoxal with Sm to binding without Sm was 0.84, showing no enhancement but a decrease in kethoxal binding in the presence of antibiotic.

We therefore, think it unlikely that there is a stimulation of the primary reaction or a formation of crosslinks between the RNA components of the ribosome and Sm that may have reacted with kethoxal.

4. Discussion

The present work clearly shows that Sm can enhance the reaction of ribosomes with kethoxal. The increased kethoxal binding occurs with RNA and protein components and with ribosomal subunits when 70S ribosomes are reacted. The initial rate of reaction of ribosomes with kethoxal, and the total number of reaction sites is increased by the presence of Sm. Presumably this is due to unfolding of ribosomes induced by Sm.

The major Sm-induced increase in reactable sites that we can detect occur at high ratios of Sm/ribosome. It is of interest that effects of Sm on thermal denaturation of ribosomes [5] and spin-labeling [3] are observed at high molar ratios of antibiotic.

Are the Sm-induced changes in accessibility to modification by kethoxal specific? Two observations appear to indicate that this is probably so. The stimulation is not abolished by reaction at 0.25 M salt and Sm resistant ribosomes do not show the changes in reagent binding found with sensitive ribosomes. We must exercise caution in this conclusion, however. Ribosomes may aggregate with the presence of Sm and the possibility exists that ribosomes from Sm resistant cells aggregate differently from sensitive ribosomes.

The gel electrophoresis protein data show changes in reaction of several different 50S proteins from 70S reacted ribosomes. This result, together with the data showing the induced binding of kethoxal to 23S RNA, is particularly significant, since this shows involvement of 50S conformational changes in 70S ribosomes with Sm. The stimulation to 23S RNA is not abolished by high salt (0.25 M). At this salt concentration, Sm binding to 50S subunits is substantially reduced, whereas binding to 30S subunits is unaffected [19]. These data suggest that Sm-induced changes in binding of kethoxal to both subunits of 70S ribosomes, and presumed conformational change, is primarily due to the binding of Sm to the 30S and not the 50S subunit.

Recently we have found that other aminoglycoside antibiotics such as kanamycin and tobramycin also stimulate ribosome chemical reactivity (unpublished results). We are currently exploring whether the changes in ribosome modification are specific for each antibiotic.

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