RIBOSOMAL DIMERS PRODUCED BY DIMERS OF AURINTRICARBOXYLIC ACID

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1. Introduction

Aurintricarboxylic acid (ATA) inhibits protein synthesis in various cell-free systems most likely by interacting with 30 S ribosomal proteins [1]. The blockage is specific for the binding of messenger-RNA to the ribosome [2–4]. The binding of ATA to the ribosome itself does not depend on the presence of mRNA.

In *Escherichia coli* considerable knowledge exists concerning the primary structure of the 16 S RNA and the 21 different proteins present in the small ribosomal subunit [6–8] and the topology of these proteins within the 30 S particle has been electron-microscopically studied [9–12]. The functional roles of some of the individual proteins have been deduced from reconstitution experiments in vitro or from analyses of various mutant ribosomes resistant to ribosomal inhibitors. Thus the way to correlate certain defined translational functions with relevant regions of the ribosomal images visible in the electron microscope has been explored.

The present study was undertaken in order to visualize directly the binding of ATA to ribosomes and their subunits using dimers of the drug.

2. Materials and methods

2.1. *Preparation of ribosomes and ribosomal subunits*

70 S ribosomes were prepared from exponentially growing cells of *Bacillus subtilis* strain 60015 (try^-, met^-) [13] by two consecutive centrifugations through a 50% glycerol cushion in buffer containing 10 mM Tris–HCl, pH 7.8, 50 mM NH_4Cl, 10 mM Mg-acetate, and 6 mM 2-mercaptoethanol. The centrifugations were for 3 h at 260 000 × *g* and 4°C. Subunits were obtained by zonal centrifugation on a 10–30% glycerol gradient in the above buffer containing 1 mM instead of 10 mM Mg-acetate. Purified ribosomes and subunits were stored frozen in 10 mM Mg^{2+}-buffer at −70°C.

2.2. *Gradient centrifugation*

Samples, 0.1 ml, containing 65 μg ribosomes (1 A_{260} unit) were centrifuged on 10–30% linear sucrose gradients in the above buffer in a SW 41 rotor (Beckman) for 3.5 h at 200 000 × *g* and 5°C. The absorbance patterns of the gradients were monitored at 260 nm. *S* values of the peak fractions were calculated [14] using values of Z_0 −10 and a ribosomal density *d* 1.6.

2.3. *Synthesis of ATA-dimers*

Two equivalents of ATA-ammonium salt (100 mg, 211 μmol) and one equivalent of 1,4-butanedioldiglycidyl ether (20 μl) were mixed in 2.5 ml H_2O [15]. The pH was adjusted and maintained at 11.5 with 1 M KOH and the mixture was shaken at room temperature for 20 h. The reaction mixture was then applied onto a column (2 × 70 cm) containing Sephadex G-25 pre-swollen and equilibrated with H_2O. The dimers were eluted prior to the unreacted (monomeric) ATA. Fractions containing ATA were easily detectable by the red colour of the drug. Fractions containing the dimers were pooled and rechromatographed on the same column. The resultant purified dimers were collected and freeze-dried.

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2.4. *Electron microscopy of ribosomes*

Electron micrographs were taken at an operating voltage of 80 kV in a Simens Elmiscope 101 equipped with a liquid nitrogen cold finger. Samples were prepared from ribosome solutions containing 40–65 μg ribosomes/ml. They were negatively stained in 2% uranyl acetate [10,11,16].

3. Results

ATA was dimerized with the bisepoxyd 1,4-butane-dioldiglycidyl ether as linker molecule according to the reaction shown in fig.1. During the 20 h incubation period, at pH 11.5, approx. 40% ATA was converted into dimers. They were separated from the unreacted ATA and the ATA-oxirane ether monomer by gel filtration on Sephadex G-25.

Purified 70 S ribosomes were incubated with a 10-fold molar excess of ATA-dimers, and the reaction mixture was analyzed on a linear sucrose gradient. As can be seen from the absorbance pattern (fig.2) a fraction of about 30% of the applied ribosomes sedimented with *S* values greater than that of the 70 S ribosomes. A defined peak was found in the 85 S region of the gradient. This indicated that the addition of ATA-dimers to ribosomes caused formation of ribosome-dimers and more complex aggregates. When the ribosome samples were inspected under the electron microscope directly after ATA-dimer addition (fig.3) the above result was visually confirmed. In

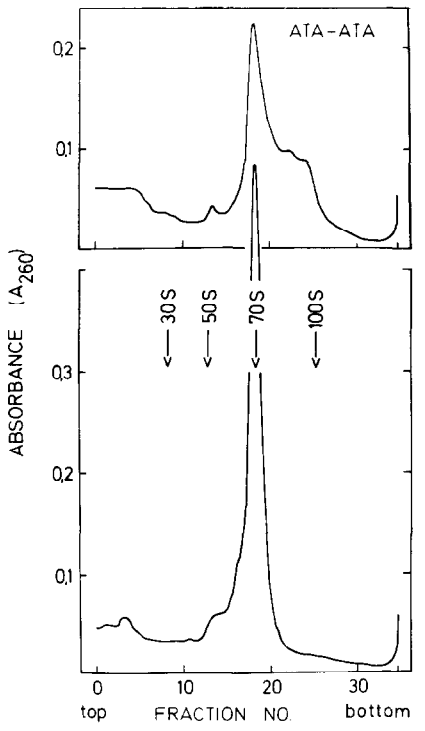

Fig.2. Sedimentation profile of the 70 S ribosomes after treatment with ATA-dimers. 65 μg (25 pmol) ribosomes were treated with 250 pmol ATA-dimers and layered onto a 10–30% linear sucrose gradient. Centrifugation was in a SW 41 rotor (Beckman) for 210 min at 40 000 rev/min and 5°C. The A_{260} absorbance pattern was recorded. *S* values were calculated as described by McEven [14]. Top: 70 S ribosomes after addition of ATA-dimers; bottom: 70 S ribosomes after addition of an equimolar amount of ATA-monomer.

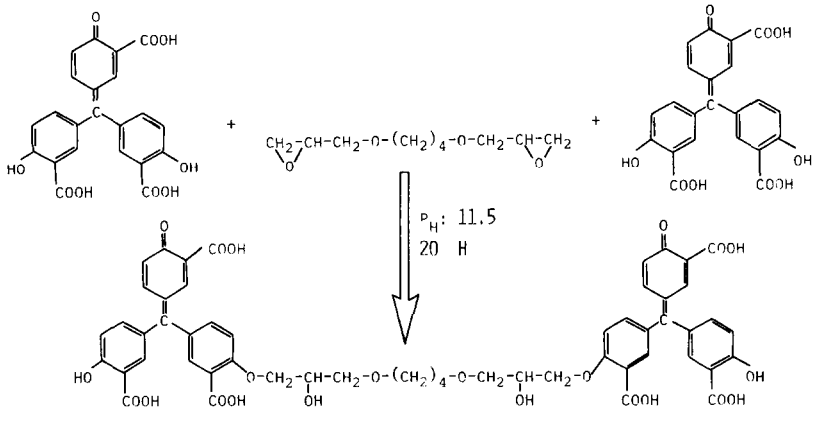

Fig.1. Schematic illustration of the reaction to synthesize dimers of aurintricarboxylic acid. ATA was incubated for 20 h at room temperature and pH 11.5. The dimers were isolated by gel filtration.

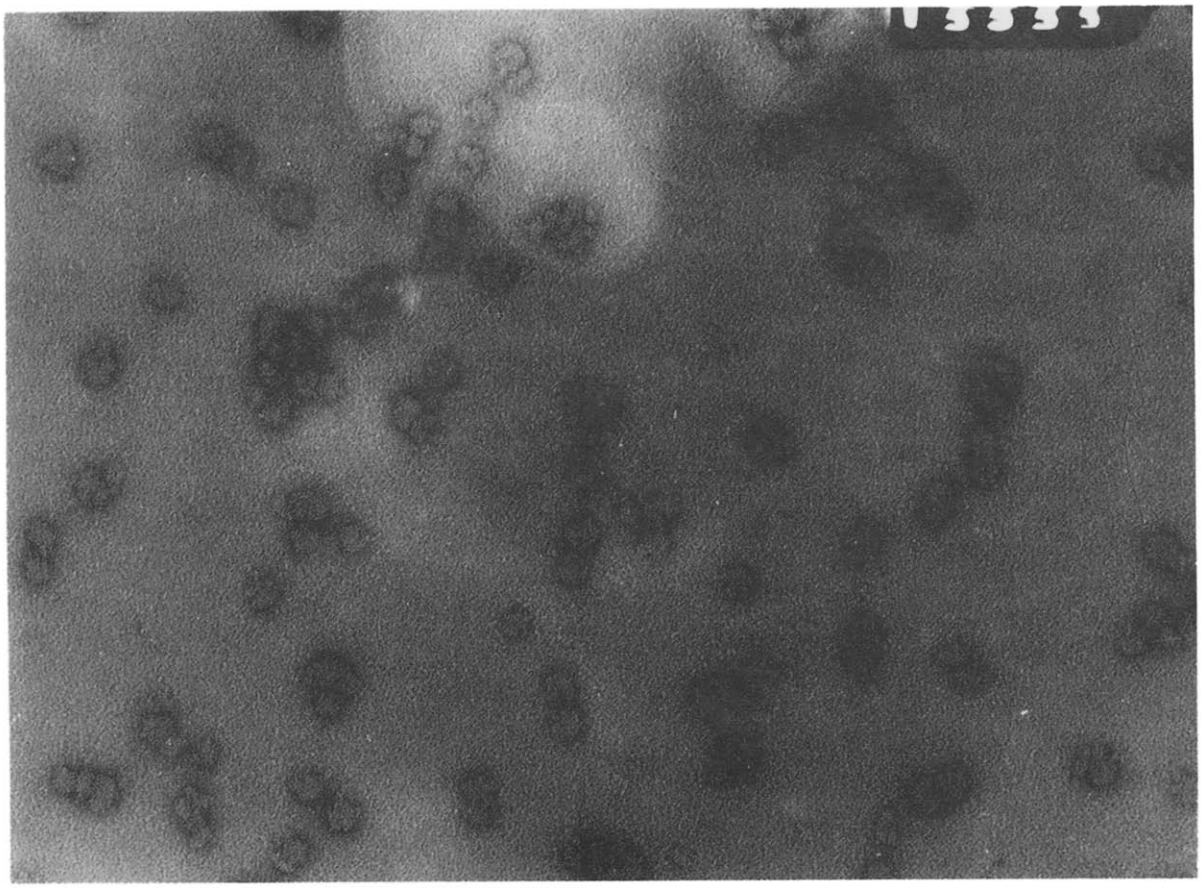

Fig.3. Electron micrograph of 70 S ribosomes negatively stained with 2% uranyl acetate after treatment with ATA-dimers. Note the presence of dimers and, to much less extent, higher aggregates.

addition to undefined aggregates a pronounced number of dimers is visible. The limited solubility of the drug and its ability to chelate Mg^{2+} did not allow us to increase its concentration and possible convert more 70 S ribosome monomers into dimers.

Similar results were obtained when purified 30 S ribosomal subunits were treated with ATA-dimers. In this case a defined peak was visible sedimenting at 50 S (fig.4) which is most likely a dimeric form of the 30 S subunit. Only minor amounts of multiple aggregates were present. The electron microscopy of the 30 S subunits after treatment with ATA-dimers demonstrated the predominant formation of dimers of the 30 S ribosomal subunit. They apparently were linked together in such a way that their concave region of the surface is facing one another (fig.5). In contrast to the 30 S subunits no significant complex formation was observed when 50 S subunits were tested (data not shown).

4. Discussion

As described above, we found that binding of ATA-dimers to the 30 S ribosomal subunits of *Bacillus subtilis* was more pronounced than to the 70 S ribosomes. Its binding to the 50 S subunits was negligible. These results indicate that the binding site(s) specific for ATA-dimers is (are) located on the surface of the small subunit but not on the large subunits as is

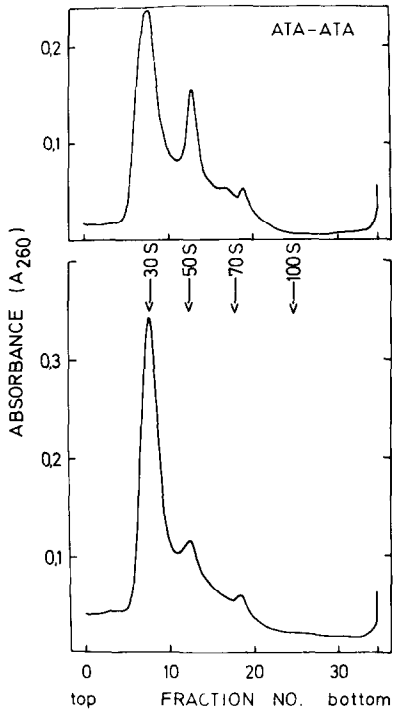

Fig.4. Sedimentation profile of 30 S ribosomal subunits after treatment with ATA-dimers. For details see fig.2.

similarly the case with the monomeric form of ATA. A major ATA binding region is apparently in the area of the small subunit where the large subunit attaches to form a 70 S complex. This would explain why the affinity for ATA-dimers of the 30 S subunit is higher than that for the 70 S ribosomes. In both cases, however, dimers were formed. This may indicate that there exists more than one site that binds ATA molecules, one being located in the region of the 30 S subunit that is buried inside when a 70 S ribosome is formed and a second site (or sites) remaining exposed even after the 50 S subunit is associated. Judging from the electronmicrographs obtained with the 30 S subunits after treatment with ATA-dimers it appears that

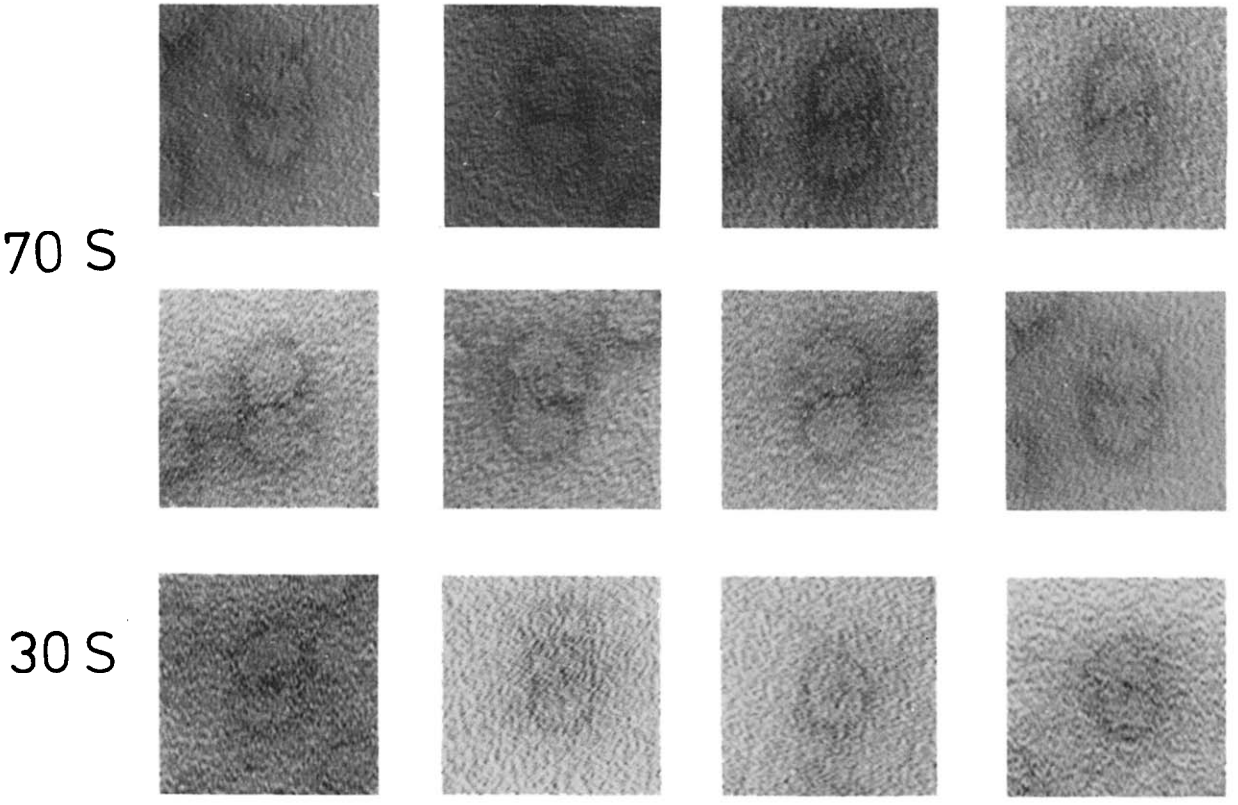

Fig.5. Selected immages from electron micrographs of 70 S ribosomes and 30 S subunits after treatment with ATA-dimers. Negative contrast with 2% uranyl acetate.

at least one of the ATA-binding sites is located on the inside of the concave shaped part of the small ribosomal subunit. Since the linker molecule is relatively short (11 Å), the binding site is most likely on the ribosomal surface or close to it.

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