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Studies on the function of two adjacent N^6, N^6-dimethyladenosines near the 3' end of 16 S ribosomal RNA of Escherichia coli. IV. The effect of the methylgroups on ribosomal subunit interaction

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ABSTRACT

The effect of the presence or absence of the methylgroups of the $m_2^6Am_2^6A$ sequence near the 3' end of 16S rRNA of *Escherichia coli* on the interaction of the ribosomal subunits has been studied, using wild-type (methylated) and mutant (unmethylated) ribosomes. Subunit exchange experiments and competitive association experiments show a strong preference of the 50S subunit for association with methylated 30S subunits. The results indicate that the equilibrium constant of the reaction 70S $\rightleftarrows$ 30S + 50S is dependent on the methylgroups; mutant 30S·50S couples are less stable than wild-type 30S·50S couples. It is postulated that the methylgroups also stimulate the interaction between 30S subunits and initiation factor IF-3.

INTRODUCTION

A great variety of organisms, prokaryotes as well as eukaryotes, contain the methylated sequence $m_2^6Am_2^6A$ in the 3' terminal part of the ribosomal RNA of the small subunit of the ribosome (1-5). A mutant of *Escherichia coli*, resistant to the antibiotic kasugamycin, lacks the four methylgroups on these neighbouring adenosines (6). It was shown that the mutant does not produce active methylase, the enzyme responsible for methylation of the adjacent adenosines (7).

Structurally wild-type and mutant ribosomes only differ with respect to the presence of the methylgroups. Functionally two differences between wild-type and mutant ribosomes have been described. In the first place the lack of the methylgroups renders the mutant resistant to kasugamycin, an inhibitor of initiation of protein synthesis (6-8). Although, *in vitro* mutant 30S subunits are as sensitive to the drug as wild-type 30S subunits, when tested for the binding of fMet-tRNA, the addition of 50S subunits from either mutant or wild-type strain brings about the resistance to kasugamycin (8). In the second place, mutant 30S subunits require more initiation factor IF-3 for optimal binding of fMet-tRNA than do wild-type 30S subunits, provided that neither initiation factor IF-1 nor 50S subunits are present (9).

These findings and the fact that the methylated adenosines were localized at that region of the 30S subunit that interacts with the 50S subunit (10), prompted us to study the interaction between 30S subunits and 50S subunits in more detail. This paper describes further studies on the differences between wild-type and mutant ribosomes, caused by the respective presence and absence of the methylgroups.

MATERIALS AND METHODS

Materials. Compare paper I of this series (8) for the growth of bacteria and for the isolation of ribosomes, and ribosomal subunits. The purification of methylase is described in paper III of this series (11). *E. coli* strain PR7 and its kasugamycin resistant derivative TPR201 were a gift of Dr. J.E. Davies. Cloacin DF13 was a gift of Dr. F.K. De Graaf.

Subunit exchange reaction. The reaction mixture with a final volume of 0.25 ml contained 50 mM Tris-HCl, pH 7.5, 6 mM $MgAc_2$, 50 mM NH_4Cl, 6 mM 2-mercaptoethanol, 3 OD_{260} units 70S ribosomes (65 pmol) and 1 OD_{260} unit 30S subunits (65 pmol). The mixtures were incubated for 30 min at 37°C. These are optimal conditions for subunit exchange (18). After cooling the reaction mixtures were analyzed on 17 ml 15-30% linear sucrose gradients in the same incubation buffer. The gradients were centrifuged in a Spinco L2-65 ultracentrifuge for 17 h at 19,000 rev/min in a SW27 rotor. After centrifugation the gradients were monitored continuously at 260 nm through a Gilford spectrophotometer and fractions of 15 drops were collected. The fractions were tested for the presence of 30S mutant subunits, in dissociated and associated state.

Assay for mutant 30S subunits. The assay mixture with a final volume of 0.1 ml contained 90 mM Tris-HCl, pH 7.5, 3 mM $MgAc_2$, 50 mM NH_4Cl, 6 mM 2-mercaptoethanol, 0.02 mM (methyl-^{3}H)AdoMet (780 cpm/pmol), 5 pmol purified methylase and 0.05 ml samples of each fraction. The mixtures were incubated for 30 min at 37°C, cooled and 10 µg Bovine Serum Albumin was added. After precipitation with 10% TCA (trichloroacetic acid) the mixtures were filtered on nitrocellulose filters (Selectron BA85), washed with 10% TCA and dried. Radioactivity was determined in a liquid scintillation counter.

Association reaction. The association reactions were done under the same salt conditions as the exchange reactions. Each incubation contained 2 OD_{260} units wild-type 50S subunits (65 pmol). The amount of 30S subunits is indicated in the figure legend. After 30 min incubation at 37°C, the

mixtures were cooled and analyzed on sucrose gradients in exactly the same way as the mixtures of the exchange reaction. In case of the competitive association experiments, the fractions of the gradient were essayed for the presence of mutant 30S subunits.

Preparation of methylated and control mutant 30S subunits. The incubation mixture contained in a 250 µl volume 6 OD_{260} units mutant 30S particles, 50 mM Tris-HCl, pH 7.5, 4 mM $MgAc_2$, 50 mM NH_4Cl, 6 mM 2-mercaptoethanol, 0.2 mM AdoMet and 40 pmol methylase (1.4 units). After 30 min incubation at 37°C, the mixture was cooled and layered on a 15% sucrose cushion containing standard buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgAc_2$, 60 mM NH_4Cl and 6 mM 2-mercaptoethanol). After 6 h centrifugation at 50,000 rev/min with a 50 Ti rotor in a Spinco L2-65 the pellet was resuspended in standard buffer. In the case of control mutant 30S subunits S-adenosylmethionine (AdoMet) was omitted, to prevent methylation.

Cleavage by cloacin DF13. Reaction mixtures contained 10 mM Tris-HCl, pH 7.5, 10 mM $MgAc_2$, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol, 4 OD_{260} units 70S ribosomes (88 pmol) and 0.02 µg activated cloacin (12), in a final volume of 0.05 ml. The mixtures were incubated at 37°C during the times indicated in figure legends. After cooling they were analyzed by means of gel electrophoresis as described by Baan *et al.* (13). The extent of cleavage was calculated using the 5S ribosomal RNA peak in the electropherogram as an internal standard.

RESULTS

In general ribosomal subunits can be divided in two classes with respect to their association properties: those forming "loose" and those forming "tight" couples (14). "Loose" couples are distinguishable from "tight" couples because they fall apart in subunits during sucrose gradient centrifugation at 6 mM Mg^{2+} (14) and the occurrence of three separate zones, 70S, 50S and 30S, during sedimentation in sucrose gradients of *E. coli* ribosomes is indicative of ribosomal heterogeneity (15). The dissociation constant of the reversible equilibrium 70S $\rightleftarrows$ 30S + 50S has been found to be 0.9×10^{-9} M for "tight" couples (16) and 0.7×10^{-6} M for "loose" couples (17).

Figure 1 shows that when equimolar amounts of 30S and 50S subunits are mixed and centrifuged at 6 mM Mg^{2+} on sucrose gradients, mutant and wild-type 30S particles generate the same amount of "tight" couples. Hence, according to this criterium, the methylgroups on the adenosines have no in-

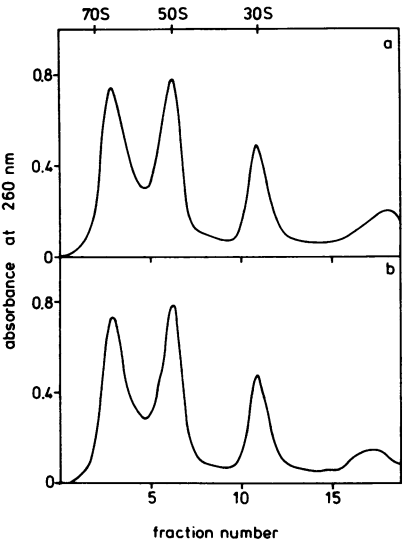

Figure 1. Association experiments. Incubation of 30S and 50S particles results in the formation of 70S couples. The conditions of the incubations are described in the Materials and Methods. Panels A and B show the absorbance profile at 260 nm of the sucrose gradients.
Panel A. Incubation of 65 pmol wild-type 50S subunits with 65 pmol wild-type 30S subunits.
Panel B. Incubation of 65 pmol wild-type 50S subunits with 65 pmol mutant 30S subunits.

fluence on the association of the subunits (compare also paper II of this series (9)). However, if the absence of the methylgroups would only result in a relative small change in the stability of the "tight" couples (*e.g.* by increasing the dissociation constant to 10^{-8} M) one could hardly expect to find a difference in sedimentation on sucrose gradients at these concentrations of particles (2×10^{-7} M). A more sensitive method to study subunit interaction is to measure the exchange of subunits with 70S ribosomes (14). When "tight" couples consisting of mutant 30S and 50S subunits (the origin of the 50S subunits is irrelevant) were less stable than "tight" couples consisting of wild-type 30S and 50S subunits, then incubation and centrifugation of mutant "tight" couples with wild-type 30S subunits would deprive these couples of mutant 30S subunits and would yield a peak of 30S subunits enriched in mutant particles. On the other hand when wild-type couples would be incubated with mutant 30S particles, most of the mutant particles would be expected to sediment at the 30S position. The presence of mutant 30S subunits, either in "tight" couples or as free particles, can be assayed in the fractions of the gradients, by their ability to incorporate radioactive methylgroups from S-adenosylmethionine when purified methylase is added (compare Materials and Methods and paper III (11) of this series).

Figure 2 shows the results of two exchange experiments. In Figure 2A wild-type "tight" couples were incubated with mutant 30S subunits. After in-

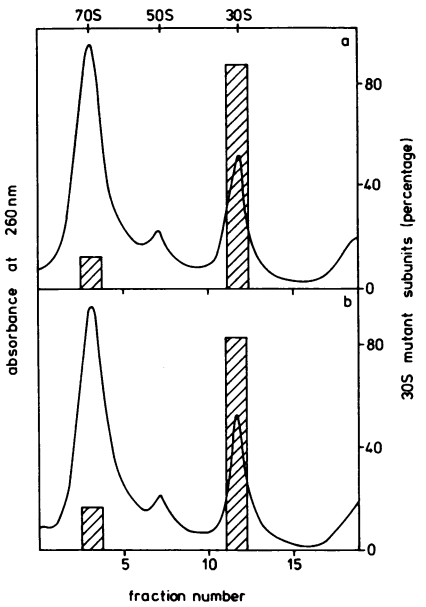

Figure 2. Subunit exchange experiments. Mixtures containing equimolar amounts of 70S ribosomes of one type (mutant or wild-type) and 30S subunits of the other type are incubated and analysed on sucrose gradients, as described in the Materials and Methods. Panels A and B show the absorbance profile at 260 nm of the sucrose gradients. The height of the hatched blocks indicates the percentage of mutant 30S particles present under the 70S peak and under the 30S peak (100% equals the total amount of mutant 30S subunits per mixture).
Panel A. Incubation of wild-type 70S ribosomes and mutant 30S subunits.
Panel B. Incubation of mutant 70S ribosomes and wild-type 30S subunits.

cubation, centrifugation and analysis of the fractions of the gradient, it turned out that only 12% of all mutant 30S particles is found in "tight" couples. In Figure 2B mutant "tight" couples were incubated with wild-type 30S subunits. After analysis of the gradient only 17% of all mutant 30S particles is found in "tight" couples, whereas 83% is found at the 30S position.

An additional experiment is shown in Figure 3. As we have seen from Figure 1, less than half of the isolated subunits are able to form "tight" couples. The deficiency is in the 30S particles and not in the 50S particles, because an increase in the amount of 30S particles drives almost all 50S particles to the 70S position (results not shown). When 50S particles are incubated with an excess of both wild-type and mutant 30S particles (Figure 3), most of the 50S particles are found in "tight" couples after gradient centrifugation. However, after analysis of the gradient fractions it appears that virtually all these "tight" couples contain wild-type 30S subunits, while the mutant 30S subunits stay at the 30S position.

To ascertain that this difference between wild-type and mutant ribosomes is caused by the respective presence and absence of the methylgroups, a control experiment was done with methylated mutant 30S subunits and control mutant 30S subunits. Methylated mutant 30S subunits are prepaired by treat-

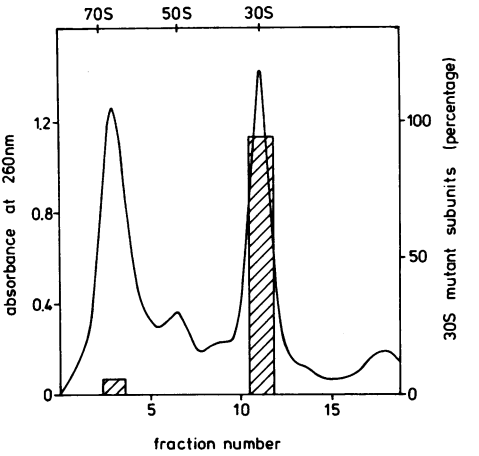

Figure 3. Competitive association experiment.
Incubation of 65 pmol wild-type 50S subunits with both 100 pmol wild-type 30S subunits and 100 pmol mutant 30S subunits results in the formation of 70S couples. The conditions of the incubation are described in the Materials and Methods. The absorbance profile at 260 nm of the sucrose gradient is shown. The height of the hatched blocks indicates the percentage of mutant 30S particles present under the 70S peak and under the 30S peak (100% equals the total amount of mutant 30S subunits per mixture),

ing mutant 30S particles with purified methylase and S-adenosylmethionine, resulting in complete methylation of the adenosines (11). Control mutant 30S particles went through the same treatment with the omission of S-adenosylmethionine. Methylated and control mutant particles were incubated with 50S wild-type subunits (like the experiment of Figure 3) and centrifuged on a sucrose gradient. Analysis of the gradient shows that nearly all the 30S particles under the 70S peak were methylated mutant 30S subunits, whereas the 30S peak contains most of the control mutant 30S subunits (results not shown, compare Figure 3).

These results show that mutant 30S·50S couples are less stable than wild-type 30S·50S couples. From these data it is difficult to quantitate the difference in stability of these couples in terms of dissociation constants and more elaborate experiments are required. As a point of interest it should be mentioned that initiation factor IF-1 which is known to increase the rate constants of the equilibrium 70S $\rightleftarrows$ 30S + 50S (18-20), does not affect the outcome of the experiment presented in Figure 2 (data not shown).

A further indication of the effect of the methylation of adenosines on

the interaction of the subunits was obtained through a different approach. The bacteriocins colicin E3 and cloacin DF13 cut the 16S rRNA at 49 nucleotides from the 3' end only when the 30S particle is complexed with a 50S particle (21,22). Assuming that the stability of mutant ribosomal couples would be smaller than the stability of wild-type ribosomal couples, the rate at which the 16S rRNA is cleaved by the bacteriocin could consequently also be smaller. As Figure 4 shows this turned out to be the case. When mutant and wild-type couples are incubated with a small amount of cloacin DF13 during different periods of time, there is a substantial decrease in the rate of cleavage of mutant couples compared with wild-type couples.

DISCUSSION

This paper describes further experiments on the function of the methylgroups of the sequence $m_2^6Am_2^6A$ found in the 3' terminal part of 16S rRNA (compare the first three papers of this series (8,9,11)). Several studies locate the 3' end of 16S rRNA (23) and in particular the dimethylated adenosines (10) in that region of the 30S particle which is supposed to interact with the 50S particle. Furthermore, the binding of fMet-tRNA to 30S subunits from the kasugamycin resistant strain (*i.e.* those lacking the methylgroups) becomes only then resistant to kasugamycin when 50S subunits are present (8).

This paper demonstrates directly that the methylgroups affect ribosomal subunit interaction. It is very likely that the forces that hold the two subunits together require a multitude of interactions, like hydrogen bonding, hydrophobic and ionic interactions (14). A specific suggestion of such an interaction is the proposed basepairing between the 3' ends of 16S and

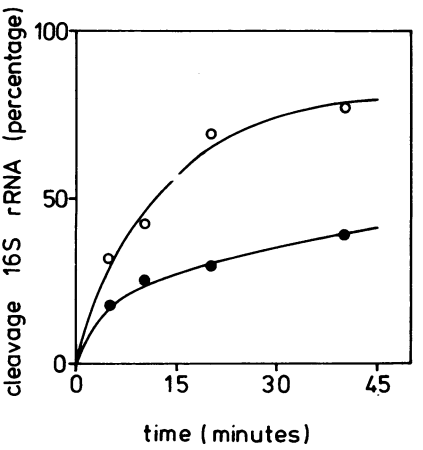

Figure 4. Cleavage by cloacin DF13. Wild-type 70S ribosomes and mutant 70S ribosomes were incubated with cloacin during the indicated times, as described in the Materials and Methods. The extent of cleavage of 16S RNA was calculated relative to the 5S RNA peak. 100% means complete cleavage of 16S rRNA. Cleavage of wild-type 70S ribosomes (percentage) O—O; cleavage of mutant 70S ribosomes (percentage) ●—●.

23S ribosomal RNA (24). In this model initiation factor IF-3 (also anti-association factor (18-20)), which can be cross-linked to the 3' end of 16S rRNA and to the 3' end of 23S rRNA (24) would destabilize 30S·50S interactions and might favour 30S·mRNA binding (25). In this connection it is interesting that the only difference between methylated and unmethylated ribosomes in protein synthesis, that we could find, was a higher IF-3 requirement for optimal binding of fMet-tRNA by mutant 30S subunits, programmed with MS2 phage RNA, as compared with wild-type 30S subunits (9).

These findings and the fact that *in vitro* methylation of mutant 30S subunits is inhibited by 50S subunits and by initiation factor IF-3 (11) suggests that the methylgroups may also have a stabilizing effect on the interaction of 30S subunits with initiation factor IF-3. The absence of the methylgroups destabilizes the interaction with the 50S subunit and the interaction with IF-3, which would result in a higher requirement for IF-3 as far as the binding of fMet-tRNA is concerned. Although we have not been able to show a difference in IF-3 dependent dissociation between wild-type and mutant ribosomes (9), the methods used, were probably not enough sophisticated to reveal such a difference and more work needs to be done.

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