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Ribosome structure: Localization of N^6,N^6-dimethyladenosine by electron microscopy of a ribosome–antibody complex

(immune electron microscopy/kasugamycin/ribosome topography/antibodies against nucleosides/minor nucleosides)

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ABSTRACT **Antibodies to the minor nucleoside N^6,N^6-dimethyladenosine have been used to map a unique location of the nucleoside in the small subunit of the *Escherichia coli* ribosome. Antibodies were induced in rabbits by a nucleoside-bovine albumin conjugate and shown to be highly specific for the dimethyladenosine hapten. The antibodies were shown to interact with 30S ribosomal subunits from strain PR7, but not with subunits from its mutant strain TPR201, which is resistant to kasugamycin and lacks the two successive residues of dimethyladenosine normally found near the 3′-end of *E. coli* 16S ribosomal RNA. Electron micrographs of strain PR7 subunits, crosslinked by single IgG molecules, show a single binding site on the surface of the ribosome. This binding site is consistent with observations relating the 3′-end of the ribosomal RNA, binding of initiation factor IF-3 and messenger RNA, and mapping of specific ribosomal proteins.**

The 30S ribosomal subunit of the *Escherichia coli* ribosome is made up of 21 different proteins and one molecule of RNA. Much effort has been devoted to determination of the structures of the individual components (reviewed in refs. 1–3); more recently, attention has been directed toward defining the overall structure and interrelationship of the various components of the ribosome using techniques such as reconstitution (4) chemical crosslinking (e.g., ref. 5), energy transfer between protein pairs (6), and electron microscopy. Through electron microscopic observation of ribosomal subunits crosslinked by individual antibodies to a specific protein component, Lake and his colleagues (7–9) and Tischendorf *et al.* (10–12) have independently localized many of the proteins of the 30S subunit.

Although most of the nucleotide sequence of *E. coli* 16S ribosomal RNA is known (3), relatively less is known about its structural disposition and function within the small ribosomal subunit. Some conclusions can be reached by determination of nucleotide sequences protected by individual proteins or capable of being crosslinked to individual proteins (reviewed in ref. 13); e.g., the 3′-end of the RNA, postulated (14) to be involved in mRNA binding, has been crosslinked to the initiation factor IF-3 (15).

The occurrence of two successive residues of N^6,N^6-dimethyladenosine (m_2^6Ado) 24 and 25 residues from the 3′-end of the 16S RNA (3) and nowhere else in the molecule, together with our experience in the induction and purification of antibodies against nucleosides (16), suggested that we investigate the use of antibodies against m_2^6Ado as an immunochemical probe of ribosome structure. A vital control in these experiments has been the availability of a kasugamycin-resistant mutant of *E. coli*, which differs from the normal sensitive strain in lacking the methylase responsible for modification of the adenosine residues to m_2^6Ado (17). In this paper we present results showing the specificity of the antibodies and their exploitation in the localization of this modified nucleoside on the surface of the ribosomal subunit. A preliminary report of some of this work has been presented (18).

Abbreviation: m_2^6Ado, N^6,N^6-dimethyladenosine.

MATERIALS AND METHODS

Procedures used in the synthesis and characterization of nucleoside-protein conjugates (19), immunization, blood collection and serum preparation, Ouchterlony gel-diffusion analysis, and in the preparation of nucleoside-agarose affinity adsorbents have been described (16). Briefly, toepads of young adult male rabbits were injected with a 1% solution of bovine serum albumin-dimethyladenosine conjugate emulsified with 9 volumes of complete Freund's adjuvant. Thirteen days later 0.5 mg of the conjugate was injected subcutaneously, and blood was collected weekly from day 21. Antibodies were purified from serum by precipitation from 40% saturated ammonium sulfate solution, followed by passage through a column of DEAE-cellulose overlaid with carboxymethyl-cellulose [in order to remove ribonuclease (20)]. Proteins in the mixture were analyzed by polyacrylamide gel electrophoresis at pH 8.9 in 7.5% gels (21).

Binding of radioactive nucleoside to antibody was measured by a membrane filter assay similar to that of Humayun and Jacob (22). Millipore type HA 0.45 μm filters were used, and [^{3}H]dimethyladenosine was prepared by Nova Chemicals, Rosemead, CA. Radioactive adenosine was purchased from Amersham/Searle. Unlabeled nucleosides were commercial products.

E. coli strains PR7 and TPR201 (a kasugamycin-resistant mutant of PR7 lacking m_2^6Ado in its ribosomal RNA) were the kind gift of Dr. Julian Davies. Bacteria were cultured in Difco antibiotic no. 3 medium and ribosomes prepared as described (23) except that the final NH_4Cl wash was omitted. Ribosomal subunits were separated by sucrose density gradient centrifugation (Spinco SW 25.2 rotor, 25,000 rpm for 12 hr, 5–20% sucrose wt/vol). Subunits were concentrated from gradient fractions by ethanol/$MgCl_2$ precipitation (24) and were resuspended and stored at −60° in buffer I (10 mM magnesium acetate/10 mM Tris·HCl, pH 7.5/30 mM NH_4Cl/6 mM 2-mercaptoethanol) or suspended for immediate use in buffer II (1 mM $MgCl_2$/10 mM Tris·HCl, pH 7.8/200 mM NH_4Cl). Ribosomes labeled with ^{32}P were prepared from bacteria grown in Difco antibiotic no. 3 medium supplemented with up to 0.1 mCi/ml of ^{32}P as orthophosphate.

Prior to antibody binding, ribosomal subunits were "activated" for 5 min at 37° (25) in buffer II. Antibodies in buffer II were added, and the mixture was incubated for 2 min at 37° followed by at least 30 min at 0°. Reaction mixtures were analyzed by sucrose density gradient centrifugation (Spinco SW 27.1 rotor, 26,000 rpm for 12 hr, 5–20% sucrose wt/vol in buffer II). Ribosome–anti-m_2^6Ado complexes were precipitated by addition, to the equivalence point, of anti-rabbit globulins (Miles-Yeda) that had been purified by precipitation from 40%

saturated ammonium sulfate and passage through DEAE-cellulose and carboxymethyl-cellulose (20).

For electron microscopy, reaction mixtures (100–150 μl) of "activated" ribosomal subunits and antibodies in buffer II were freed of unreacted globulins by passage through a 1 × 20 cm column of Sepharose 6B (8). Samples were prepared for microscopy by negative staining with 1% uranyl acetate using the method of Valentine *et al.* (26) as modified by Lake and Kahan (9). Electron micrographs were obtained with a Hitachi HU-11A microscope at 75 kV and a magnification of 40,000.

RESULTS

Antibody Production and Characterization. The bovine albumin-dimethyladenosine conjugate used in immunization was characterized spectrally (16) and calculated to contain 21 mol of nucleoside per mol of albumin. A total of six rabbits were immunized with this preparation; all showed a strong response within 2 weeks of the booster injection. Preliminary immunodiffusion analysis sometimes showed weak precipitation of bovine albumin or nucleoside conjugates of bovine albumin. But, in all cases, conjugates of dimethyladenosine and rabbit albumin gave strong precipitin lines; N^6-monomethyladenosine conjugates were less reactive, and an adenosine-rabbit albumin conjugate was not precipitated by sera from early bleedings. (Some late bleedings produced sera that weakly precipitated the adenosine conjugate.) Rabbit albumin alone was never precipitated.

Specificity of the antibodies was further examined in ligand binding experiments; [^{3}H]dimethyladenosine binding was measured in the presence of varying amounts of related nucleosides, as shown in Fig. 1. Various other nucleosides and derivatives were also tested, and the data are summarized in Table 1. These results were interpreted to indicate a strong antihapten response, with only a weak recognition of the parent nucleoside adenosine by those antibodies in the population that tightly bound dimethyladenosine.

As a final measure of specificity, several preparations were compared in the direct binding of radioactive adenosine and dimethyladenosine. Serum or the globulin fraction of the serum used bound 0.002 mol of adenosine per mol of dimethyladenosine bound (in separate experiments). Passage of the globulin fraction through DEAE-cellulose and carboxymethyl-cellulose had no effect on this ratio. This fraction was passed through an agarose-adenosine affinity column, and the earliest (unretarded) fraction was compared with the tightly bound antibodies (eluted with 10% acetic acid): adenosine binding in the unretarded fraction was undetectable (less than 0.05% of the dimethyladenosine bound) while the retained fraction of antibodies bound 0.025 mol of adenosine per mol of dimethyladenosine.

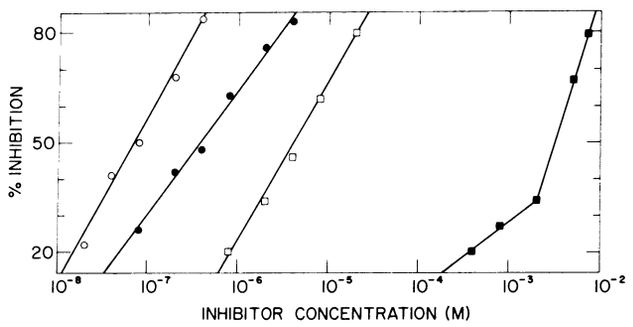

FIG. 1. Effect of inhibitors on [^{3}H]dimethyladenosine binding by antibodies. Reaction mixtures (250 μl) contained radioactive dimethyladenosine (5.5×10^{-8} M, 0.1 μCi/ml), unlabeled nucleoside as indicated, and antibody (0.1–0.4 μl of serum or the equivalent) in 0.14 M NaCl/0.01 M Tris·HCl buffer, pH 7.2. Samples were incubated 60 min at 37° followed by 60 min at 0°, and 200-μl portions were then rapidly passed through Millipore 25 mm type HA filters (previously wet with 2 ml of buffer). The filters were immediately washed with three 2-ml portions of buffer and ^{3}H was measured by scintillation counting. Inhibitors used were: (○) N^6-dimethyladenosine; (●) N^6-dimethyladenine; (□) N^6-monomethyladenosine; (■) adenosine.

Table 1. Inhibition of binding of [^{3}H]dimethyladenosine

Inhibitor used	Concentration at 50% inhibition (M)
N^6-Dimethyladenosine	7.5×10^{-8}
N^6-Dimethyladenine	3.5×10^{-7}
N^6-Dimethyl-AMP	6.5×10^{-7}
α-*N*-Acetyllysine-N^6-dimethyladenosine morpholidate*	8×10^{-8}
N^6-Monomethyladenosine	4×10^{-6}
N^6-Isopentenyladenosine	4×10^{-4}
Puromycin	1×10^{-7}
Puromycin aminonucleoside	7×10^{-8}
Adenosine	2×10^{-3}

* Ref. 15.

Polyacrylamide gel electrophoresis of the antibody preparations showed a broad single band corresponding to 7S gamma globulins; contamination by albumin and IgM fractions was barely detectable, and estimated at less than 1%.

Interaction of Antibodies with Ribosomes. Antibody precipitation of ^{32}P-labeled 30S ribosomal subunits from *E. coli* strain PR7 (containing dimethyladenosine) and its kasugamycin-resistant, dimethyladenosine-free mutant strain TPR201 was compared. Antidimethyladenosine antibodies alone precipitated neither type of ribosome significantly (less than 1%). Addition of goat antibodies directed against rabbit globulins caused precipitation of up to 86% of the ^{32}P of PR7 ribosomes, while about 25% of the ^{32}P of TPR201 ribosomes was precipitated. As a control, nonspecific rabbit gamma globulin was substituted for antidimethyladenosine antibodies; precipitation of ^{32}P of either PR7 or TPR201 ribosomal subunits with anti-rabbit globulin was equivalent at about 10% of the ^{32}P present. In all cases above, addition of 0.1 mg of adenosine per ml to the reaction mixtures had no effect on the precipitation of ^{32}P-labeled ribosomal subunits.

Precipitation of 70S ribosomes by the same double-antibody technique was not conclusive; almost 40% of the ^{32}P of PR7 ribosomes was precipitated with antidimethyladenosine antibodies, but in control experiments 30% of the TPR201 ^{32}P was precipitated with the antibodies, and 26% of either preparation of ribosomes was precipitated with nonspecific rabbit globulins. Sucrose density gradient centrifugation of 70S preparations (in the absence of added antibodies) showed a low level (about 5%) of ribosomes dissociated into 30S and 50S components.

Mixtures of antidimethyladenosine antibodies and ribosomal subunits (at subunit concentrations of 1–2 μM) were fractionated by sedimentation through sucrose gradients. The extent of antibody–subunit interaction was determined by measurement of the reduction in absorbance of the 30S peak, and the formation of a more rapidly sedimenting shoulder or peak presumed to contain subunits crosslinked by antibody molecules. In several experiments either purified 30S subunits or a mixture of 30S and 50S subunits was reacted with 0.1–2 equivalents of antibody-combining site. Small subunits from

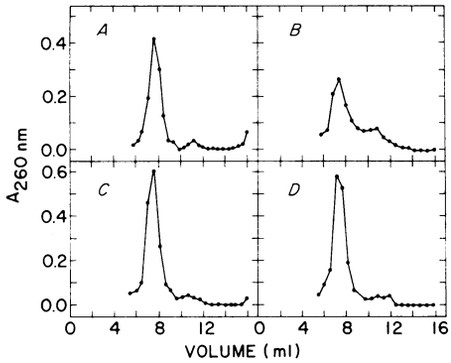

FIG. 2. Sucrose gradient centrifugation of ribosomal subunit-antibody mixtures. Reaction and centrifugation methods are detailed in *Materials and Methods*. Sedimentation was left to right. In each instance, ribosomal subunits were 1 μM. (*A*) PR7 small subunits, no added antibody; (*B*) PR7 small subunits, 2 μM antibody-combining sites; (*C*) TPR201 small subunits, no added antibody; (*D*) TPR201 small subunits, 2 μM antibody-combining sites.

strain PR7 (containing dimethyladenosine) showed consistent interaction with antibody. The extent of interaction increased with added antibody up to about 0.6 equivalent, and was then approximately constant up to the highest concentration tested. In contrast, no significant interactions with antibody were observed with 50S subunits from either strain or 30S subunits from strain TPR201. Fig. 2 illustrates one such experiment, in which two equivalents of antibody-combining sites were added per mol of 30S subunit. Reduction of the PR7 30S peak (Fig. 2*B*) was about 40%, while the TPR 201 peak was not significantly altered (Fig. 2*D*). Absorption measurements (at 280 and 260 nm) of fractions near the top of gradients *B* and *D* (data not shown) were characteristic only of added antibody; no evidence of ribosome degradation was ever seen.

Electron Microscopy of Antibody-Ribosomal Subunit Complexes. Antidimethyladenosine antibodies were incubated with an equivalent of 30S ribosomal subunits and unreacted globulins were removed by gel filtration. Electron microscopy of preparations from *E. coli* TPR201 (lacking m_2^6Ado) showed only rare pairs of 30S subunits which could have been interpreted as antibody-crosslinked. In contrast, ribosomes from strain PR7 were frequently seen in pairs, which we have interpreted to be antibody-linked. About 250 such pairs have been examined, and results are illustrated in Fig. 3.

Electron micrographs of small ribosomal subunits of *E. coli* give characteristic images that represent different orientations of the particle about its long axis; in our micrographs all of the views described by Lake (27) could be clearly identified. About 90% of the PR7 ribosomal subunits that appeared to be antibody-linked were joined near the level of the partition dividing the subunit into its upper one-third and lower two-thirds portions (27); the remaining 10% appeared linked at a level distinctly different from the partition.

Antibody-linked pairs showing all of the characteristic views of the ribosomal subunit were examined to further localize the binding site. Subunits in the quasisymmetric view showed antibody attached at the shoulder (e.g., Fig. 3*B*, frames 3, 4, and 6). Subunits in an intermediate view, characterized by a cleft between the platform and the upper one-third portion of the subunit, appeared linked at the platform rather than the upper one-third (e.g., frames 1, 7, 8, and 9). Subunits in the asymmetric view showed attachment at either the convex (e.g., frames 2 and 5) or concave (e.g., frames 5, 10, and 11) side at the level of the partition. These observations are consistent with a single binding site, located on the platform at the level of the junction with the lower two-thirds and in a region away from the periphery of the subunit when seen in the asymmetric view. This interpretation is illustrated in Fig. 4. The resolution we have attained with this staining technique is not sufficient to distinguish between a binding site on the inside and one on the outside surface of the platform.

DISCUSSION

In order to confidently interpret the structural significance of electron micrographs of antibody-linked ribosomal dimers, it is necessary to clearly determine the specificity of the antibody preparation. This is particularly true in the present case, since the target in the ribosomal subunit is a modification of one of the common nucleosides which is itself present in several hundred residues. Moreover, the initial immunogen was a synthetic product and the linkage of the dimethyladenosine hapten, via the 2′ and 3′ positions of the ribose, is significantly unlike the internucleotide linkage in which dimethyladenosine occurs in ribosomal RNA. Antibodies to such haptens have frequently been reported to crossreact with other nucleosides (refs. 16 and 28 and refs. therein).

Our results show the antibody preparations used to be highly specific for dimethyladenosine. First, the preparations show a very low capacity for binding of adenosine, and this activity can be further reduced or eliminated by adsorption on an adenosine-agarose column. Second, the antibodies that strongly bind dimethyladenosine show very inefficient interference by adenosine; Fig. 1 shows that about 10^5 times as high a concentration of adenosine as dimethyladenosine is needed to halve the binding of radioactive hapten. A significant crossreaction or interference could be expected from N^6-monomethyladenosine, but this nucleoside appears absent from 16S ribosomal RNA in *E. coli* (3). The binding data of Table 1 also show the modified base to be dominant in hapten interactions, since free base, nucleoside, nucleoside derivatives, and the 5′-monophosphate are relatively similar in their action. Thus, antibody molecules should selectively bind exposed dimethyladenosine residues in an RNA molecule also containing adenosine.

Interaction of antibody with ribosomal subunits can be interpreted at several levels. Double-antibody precipitation experiments show almost quantitative interaction of antibodies with ribosomal subunits containing dimethyladenosine (PR7) and little interaction with subunits that lack the modified nucleoside (TPR201). The slight precipitation of TPR201 ribosomes appears in part nonspecific, since nonimmune globulins cause some background precipitation, and in part due to genuine crossreaction with some component of the ribosome. Such slight crossreaction is consistent with the infrequent crosslinking of TPR201 subunits seen in electron microscopy and could also help explain the approximately 10% of PR7 subunits seen to be bound at a site inconsistent with that proposed in Fig. 4.

Sedimentation in sucrose gradients also indicates that a significant interaction occurs between antibodies and PR7 subunits. The observed reduction in the 30S peak (e.g., Fig. 2*B*) is consistent with that expected if the ribosome concentration in the reaction mixture and the dissociation constant of the ribosome–antibody complex are similar. Moreover, the time needed to obtain separation by sedimentation is sufficient to permit significant dissociation of the complexes formed. Such dissociation may explain the lack of a distinct faster sedimenting peak in Fig. 2*B*. Altered sedimentation of only PR7 small subunits indicates that the interaction with antibodies depends on the presence of dimethyladenosine in the ribosomal subunit, and further indicates that the dimethyladenosine in the subunit

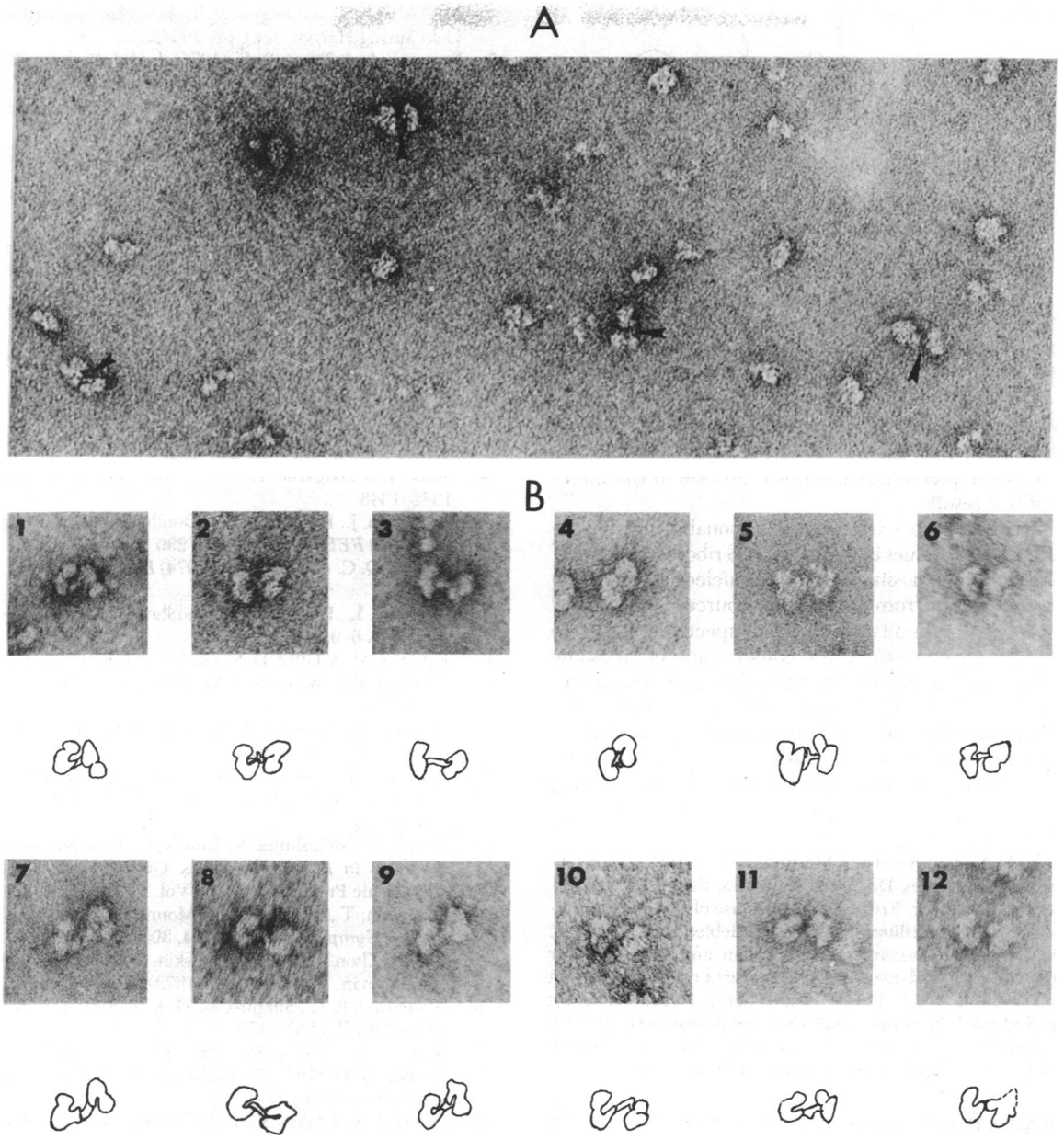

FIG. 3. Electron micrographs of PR7 small subunits reacted with antidimethyladenosine antibodies. (*A*) A field (×217,000) showing individual small subunits and subunit pairs linked by single antibody molecules (arrows). (*B*) A gallery of antibody-linked subunit pairs at higher magnification (×254,000); below each frame is an interpretive drawing.

must be available for interaction, and not buried deep in the ribosome structure.

Electron microscopy of antibody–ribosome mixtures shows a significant number of crosslinked PR7 subunits pairs, almost all of which are consistent with a unique binding site on the surface of the 30S subunit. Figs. 3 and 4 summarize the binding observations and our interpretations of them with respect to the model proposed by Lake (27).

This interpretation appears consistent with present knowledge of 30S subunit topography and function. The 3′-end of the RNA has been implicated in mRNA binding (14) and a complex of fragments of R17 mRNA and the 3′-end of *E. coli* 16S rRNA has been isolated and characterized (29). The 3′-end of the ribosomal RNA has also been crosslinked to the initiation factor IF-3 (15) which promotes binding of mRNA to the small subunit. IF-3 has been chemically crosslinked to ribosomal proteins S1, S11, S12, S13, S19, and S21 (30) and to protein S7 (15). Lake and Kahan (9) have localized determinants of proteins S11, S13, and S19 of this group to sites on the subunit platform or on the platform side of the upper portion of the subunit. The model of Tischendorf *et al.* (12) does not include a platform, but their localization of proteins S7, S11, S12, S13, S19, and S21 includes in each case one or more binding sites on the smaller portion of the ribosomal subunit. Thus, proteins associated with IF-3, and therefore with mRNA binding and the 3′-end of 16S ribosomal RNA, are consistently localized together on the ribosomal subunit. Crosslinking of the 3′-end of the ribosomal RNA to IF-3 and the results reported here independently localize the 3′-terminal section of RNA in the same area and support the suggestion that the upper platform side of the subunit is the site of the initiation of protein synthesis (9).

The proposed localization of dimethyladenosine is on the face of the ribosomal subunit proposed (27) to be in contact with the 50S subunit in the intact 70S ribosome. Our inability to effi-

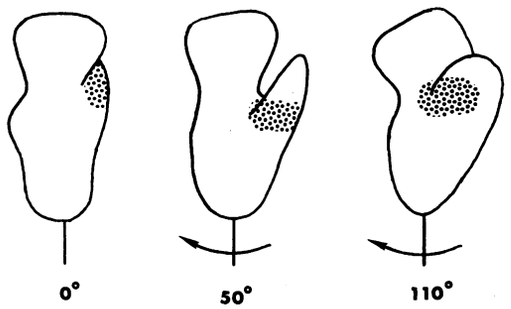

FIG. 4. Localization of dimethyladenosine in the small ribosomal subunit of *E. coli*. The shaded area of the model adapted from Lake (27) indicates the binding site of antidimethyladenosine, as determined by electron microscopy. For details see Fig. 3 and *text*.

ciently precipitate 70S ribosomes in double-antibody experiments is consistent with the model, but the high background levels of ribosome precipitation require caution in the interpretation of this result.

We believe our data are sufficient to reasonably localize the dimethyladenosine residues in the *E. coli* 30S ribosomal subunit. Since the dimethyladenosine-containing nucleotide sequence is found in ribosomes from a variety of sources (31), similar experiments with subunits from other species could give meaningful results concerning the conservation of ribosome structure and the function of dimethyladenosine. The results also suggest that other well-characterized and/or purified antibodies to modified nucleoside haptens could be used in the study of the localization and function of the nucleoside components in a variety of macromolecules or macromolecular complexes.

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