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Magnesium-Dependent Interaction of 30S Ribosomal Subunits with Antibodies to N^6,N^6-Dimethyladenosine†

Samuel M. Politz‡ and Dohn G. Glitz*

ABSTRACT: The modified nucleoside N^6,N^6-dimethyladenosine occurs in *Escherichia coli* 16S ribosomal RNA only in two successive positions near its 3′ end. Antibodies directed against dimethyladenosine were induced with a nucleoside–albumin conjugate. As measured by second antibody precipitation of immune complexes, antidimethyladenosine antibodies bound 30S ribosomal subunits, ribosomal core particles, and ribosomal RNA which contain dimethyladenosine but showed little cross-reactivity with RNA or ribosomal subunits from a kasugamycin-resistant mutant which lacks dimethyladenosine. Antibody binding to ribosomal subunits was strongly influenced by the concentration of magnesium ion in the reaction medium and by the prior treatment of the subunits. Functionally active 30S subunits showed a striking binding optimum at 2–4 mM Mg^{2+}; this optimum disappeared if the subunits were inactivated by dialysis against low concentrations of magnesium ion. Instead, the inactivated subunits showed a gradual increase in antibody binding as the magnesium ion concentration was raised to 20 mM; binding of 16S ribosomal RNA or subribosomal core particles from 30S subunits gave qualitatively similar curves, with no evidence of a low [Mg^{2+}] optimum. The stability of antibody–subunit complexes was also found to depend upon subunit conformation and magnesium ion concentration; the half-life of an inactivated subunit–antibody complex (15 mM Mg^{2+}) averaged 130 min, while active subunit–antibody complexes (3 mM Mg^{2+}) had an average half-life of 70 min. More of the immune complexes with inactivated subunits were found to survive sucrose gradient sedimentation (relative to active subunits), and the concentration of subunits needed to halve antibody binding of [^{3}H]-N^6,N^6-dimethyladenosine was lower with inactivated subunits. The results suggest that the antibody binding optimum seen with active subunits at 2–4 mM Mg^{2+} represents a dynamic aspect of the three-dimensional ribosomal subunit structure; a site near the 3′ end of the RNA is involved, and both the availability of the modified nucleoside to an antibody probe and the stability of the resulting complexes are involved.

The 30S subunit of the *Escherichia coli* ribosome is a complex of 21 proteins and one molecule of RNA. The physical structure of the subunit and its components and the relationships of structure to subunit function have become major areas of research [reviewed by Brimacombe et al. (1978), Cox (1977), and Kurland (1977)]. Work from several laboratories has resulted in the localization of most of the subunit proteins and in the delineation of some of the structural and functional roles of the ribosomal RNA.

Shine & Dalgarno (1974) called particular attention to a nucleotide sequence near the 3′ end of the 16S ribosomal RNA that binds mRNA during the initiation of protein synthesis. The chemical cross-linking of the 3′ end of the RNA to initiation factor IF-3 (Van Duin et al., 1975) and the immunoelectron microscopic localization of the 3′ terminus (Olson & Glitz, 1979) within an initiation neighborhood (Kurland, 1977; Lake, 1978) further implicate this region of the RNA in the initiation step. A modified nucleoside, N^6,N^6-dimethyladenosine (m_2^6Ado),[1] occurs 24 and 25 residues from the 3′ end of the 16S RNA and nowhere else in the molecule (Brosius et al., 1978; Carbon et al., 1978, 1979). We have prepared and characterized antibodies to dimethyladenosine, and, following the techniques pioneered by Lake (Lake et al., 1974; Lake & Kahan, 1975; Lake, 1978) and in the Berlin laboratories (Tischendorf et al., 1974a,b, 1975), we have localized the nucleoside by electron microscopy of an antibody–subunit complex (Politz & Glitz, 1977). This localization is consistent with results which have identified ribosomal components that can interact with IF-3 (Czernilofsky et al., 1975; Heimark et al., 1976; Van Duin et al., 1976) and with the immunoelectron microscopic localization of the derivatized 3′ terminus (Olson & Glitz, 1979; Shatsky et al., 1979).

During the course of the characterization of ribosome–anti-m_2^6Ado interactions, we noted a significant variation in the ability of antibodies to bind different subunit preparations. The variations appeared at least partly related to methods of subunit isolation and storage. In part due to stimulation by conversations with Dr. Pallaiah Thammana and the results of Thammana & Cantor (1978), we have now examined antibody binding as a function of magnesium ion concentration and ribosome conformation. The extent of apparent anti-

† From the Department of Biological Chemistry, UCLA School of Medicine, and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received February 27, 1980.* This work was supported by National Science Foundation Research Grant PCM 77-14872 and by U.S. Public Health Service Training Grant GM 1531.

‡ Present address: Thimann Laboratories, University of California, Santa Cruz, CA 95064.

[1] Abbreviations used: m_2^6Ado, N^6,N^6-dimethyladenosine; anti-m_2^6Ado, antibodies to N^6,N^6-dimethyladenosine; rRNA, ribosomal ribonucleic acid.

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m_2^6Ado interaction with ribosomal subunits depends greatly on the methods through which the subunit–antibody interactions are quantitated. Moreover, the Mg^{2+} dependence changes qualitatively if subunits are functionally inactivated by dialysis against buffers containing low concentrations of Mg^{2+}. Antibody binding of inactivated 30S subunits, 16S RNA, subribosomal core particles, and free N^6,N^6-dimethyladenosine all differs markedly from that seen with activated subunit preparations. Our results are consistent with extensive literature suggesting that the *E coli* 30S subunit can exist in at least two functionally different conformations (Zamir et al., 1971, 1974; Hermoso et al., 1976; Allen & Wong, 1979) and that the conformation of 16S RNA may be important in determining these states (Hochkeppel et al., 1976; Hochkeppel & Craven, 1977; Hogan & Noller, 1978; Thammana et al., 1979). But our data do not support the conclusion of Thammana & Cantor (1978) that m_2^6Ado is hidden in the active form of the subunit. Instead, we suggest that a less stable complex is formed between antibodies and active 30S subunits.

Materials and Methods

Ribosome and RNA Preparation. E. coli strains PR7 and TPR201, a kasugamycin-resistant mutant of PR7 lacking N^6,N^6-dimethyladenosine in its 16S rRNA (Helser et al., 1972), were the kind gift of Dr. Julian Davies. Bacteria were cultured in Difco antibiotic no. 3 medium (unlabeled cells) or 1% tryptone and 0.5% NaCl, supplemented with up to 10 mCi/L ^{32}P as orthophosphate (ICN). In some cases the low phosphate medium of Landy et al. (1967) was used to obtain ribosomes of higher specific activity. Cells were harvested in late log phase and were broken open by grinding with alumina (Type 305, Sigma Chemical Co.) in buffer I (20 mM Tris-HCl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 30 mM NH_4Cl, pH 7.8) as described by Traub et al. (1971).

Ribosomes were isolated from lysates by differential centrifugation in buffer I. Ribosomal subunits were separated by sucrose gradient centrifugation [Spinco SW25.2 rotor, 25 000 rpm for 12 h, 5–20% w/v ultrapure sucrose (Schwarz/Mann) in dissociation buffer (1 mM $MgCl_2$, 10 mM Tris-HCl, 200 mM NH_4Cl, and 6 mM 2-mercaptoethanol, pH 7.8]. Subunits were concentrated from gradient fractions by ethanol/$MgCl_2$ precipitation (Staehelin et al., 1969) and were resuspended in dissociation buffer for immediate use in antibody or tRNA binding experiments. Small subunits were repurified by a second identical sucrose gradient step if analytical sucrose gradients showed significant amounts of large subunit contamination. Ribosomal core particles were prepared according to Traub et al. (1971). RNA was prepared from purified 30S subunits and occasionally from unfractionated 70S ribosomes by phenol extraction (Traub et al., 1971); if necessary, 16S RNA was purified by sedimentation in sucrose gradients (Spinco SW25.2 rotor, 22 500 rpm for 21 h, 5–20% sucrose w/v in buffer I).

Poly(uridylic acid)-stimulated phenylalanyl-tRNA (Phe-tRNA) binding to 30S subunits was measured by the assay of Nirenberg & Leder (1964) using a modification of the conditions of assay 1 of Zamir et al. (1971) to avoid ribosome activation in the course of the assay. [^{14}C]Phenylalanyl-tRNA was purchased from New England Nuclear Corp. When desired, ribosomes were activated by incubation for up to 30 min at 37 °C (Zamir et al., 1971, 1974). Inactivated 30S subunits were generated by dialysis at 0 °C for at least 18 h against a buffer containing 10 mM Tris-HCl, pH 7.8, 200 mM NH_4Cl, and 0.5 or 1.0 mM $MgCl_2$ (Zamir et al., 1971, 1974).

Immunological Techniques. Procedures used in the synthesis and characterization of nucleoside–protein conjugates (Erlanger & Beiser, 1964), immunization, blood collection, and serum preparation, and in the preparation of nucleoside–agarose affinity adsorbents have been described (Eichler & Glitz, 1974; Politz & Glitz, 1977). Antibodies were purified from rabbit serum by precipitation from 40% saturated ammonium sulfate solution, followed by passage through a column of DEAE-cellulose overlaid with carboxylmethylcellulose [in order to remove ribonuclease (Palacios et al., 1972)], or by gel filtration on Ultrogel AcA 22 (LKB). Most preparations were further purified by passage through a column of adenosine–agarose (Eichler & Glitz, 1974). Characterization of the antibodies used here has been described by Politz & Glitz (1977).

Measurement of Antibody–Subunit Interaction. (*a*) *Second Antibody Precipitation: Anti-m_2^6Ado in Excess.* Complexes of antibodies with radiolabeled subunits were quantitated by precipitation with a second antibody, goat antirabbit γ-globulin. Typically, ^{32}P-labeled 30S subunits [4 pmol, (3–20) × 10^3 cpm] in 190 μL of buffer containing 10 mM Tris-HCl, pH 7.8, 200 mM NH_4Cl, and a concentration of $MgCl_2$ varying from 0.5 to 40 mM were mixed with 60 μL of anti-dimethyladenosine globulins (30 pmol of dimethyladenosine binding capacity, 100 μg of protein) dissolved in the same buffer. After at least 2 h at 0 °C, goat antirabbit γ-globulin (Calbiochem, 100 μL in buffer), in an amount sufficient to precipitate 100 μg of rabbit IgG, was added to each reaction mixture. After at least 1 h at 0 °C, each sample was diluted with 2 mL of cold buffer and immediately filtered under vacuum through a prewet Whatman GFA glass fiber filter. The tubes were rinsed, the filters were quickly washed with three 2-mL portions of cold buffer, and ^{32}P retained on the filters was then measured in a Beckman scintillation counter in 3a70B scintillation cocktail (Research Products International). Similar conditions were used to measure precipitation of ^{32}P-labeled 16S ribosomal RNA, [^3H]-N^6,N^6-dimethyladenosine, (Moravek Biochemicals, City of Industry, CA), or ^{32}P-labeled ribosomal core particles. In control experiments, nonimmune rabbit IgG was substituted for anti-m_2^6Ado.

(*b*) *Inhibition of Binding: Second Antibody Precipitation.* Complex formation between anti-m_2^6Ado and an approximately equivalent level of radioactive m_2^6Ado or ribosomal subunits was measured in the presence of nonradioactive competitors by using second antibody precipitation. Reaction mixtures (250 μL) included up to 15 pmol each of radioligand and anti-m_2^6Ado (measured as nucleoside binding capacity), sufficient nonimmune rabbit γ-globulin to bring the IgG level to 100 μg, and competitor. Other conditions were as described above. In the absence of added inhibitor, 30–50% of the total radioactivity was recovered in the precipitate.

(*c*) *Dissociation of Antibody–Subunit Complexes: Sepharose–Protein A Adsorption.* Reaction mixtures containing 40 pmol (6 × 10^5 cpm) of ^{32}P-labeled ribosomal subunits and 80 pmol of anti-m_2^6Ado (400 μg of IgG) or an equivalent level of nonimmune IgG were incubated at 4 °C overnight in 200 μL of buffer (10 mM Tris-HCl, pH 7.8, 200 mM NH_4Cl, and 3.0 or 15 mM $MgCl_2$). Dissociation of the complexes (at 0 °C) was initiated by addition of 800 μL of 1.25 mM m_2^6Ado in buffer. Samples of 100 μL were withdrawn at specified times and mixed with 400 μL of a suspension of *Staphylococcus aureus* Protein A–Sepharose CL 4B (Pharmacia). After exactly 4 min, the suspension was diluted with 2 mL of cold buffer and immediately filtered under vacuum through a prewet Whatman GFA glass fiber filter. Each filter was

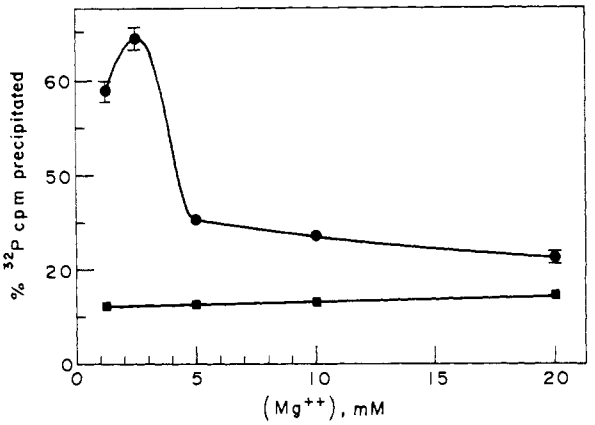

FIGURE 1: Second antibody precipitation of 30S ribosomal subunits with antidimethyladenosine antibodies. Subunits were from *E. coli* strain PR7 (●) or its m_2^6Ado-free mutant TPR201 (■). No correction for nonspecific precipitation with nonimmune globulins has been made.

quickly washed with five 2-mL portion of cold buffer, and ^{32}P retained on the filter was measured as described above. In control experiments nonimmune rabbit IgG replaced the anti-m_2^6Ado.

(*d*) *Sucrose Gradient Sedimentation.* Complexes of anti-m_2^6Ado and ribosomal subunits were measured by sedimentation as described in Figure 4.

Results

Second Antibody Precipitation of 30S Ribosomal Subunits. In order to measure the direct interaction of subunits with antibodies to N^6,N^6-dimethyladenosine, complexes formed with radioactive subunits and an excess of antibodies (produced in rabbits) were precipitated with goat antirabbit γ-globulins. In preliminary experiments subunit and anti-m_2^6Ado levels were optimized to attain maximal precipitation of PR7 subunits (containing m_2^6Ado) relative to TPR201 subunits (free of m_2^6Ado) and minimal precipitation of either type of subunit with nonimmune rabbit γ-globulins. Under optimal conditions up to 70% of the ^{32}P of PR7 subunits was precipitated, with less than 10% nonspecific precipitation in controls.

This technique was used to examine the magnesium ion dependence of subunit binding by anti-m_2^6Ado; results are shown in Figure 1. The pronounced optimum in precipitation of PR7 subunits, seen at 2–4 mM Mg^{2+}, was observed in each of nine separate subunit preparations. In contrast, precipitation of m_2^6Ado-free TPR201 subunits was low and nearly independent of Mg^{2+} concentration in each instance. In parallel experiments [^{3}H]m_2^6Ado was used in place of ribosomal subunits. In several experiments the amount of nucleoside in the precipitate ranged from 38 to 45% of the added radioactivity and was independent of magnesium ion concentration over the range 0.5–50 mM.

Effect of Subunit Inactivation on Antibody Binding. Prolonged dialysis against buffers containing low concentrations of magnesium ion is known to functionally inactivate 30S ribosomal subunits (Zamir et al., 1971, 1974). The precipitation of inactivated PR7 subunits is compared to that of active (as isolated) subunits in Figure 2. Only activated subunits showed the binding peak at 2–4 mM Mg^{2+}, while precipitation of inactivated subunits increased gradually as the magnesium ion concentration was increased. At 15–20 mM Mg^{2+} both active and inactivated subunits were usually precipitated at equivalent levels. Precipitation of inactivated subunits at all magnesium concentrations tested was much less than the precipitation of active subunits at the optimal (3 mM) magnesium ion concentration. Reactivation of dialyzed subunits,

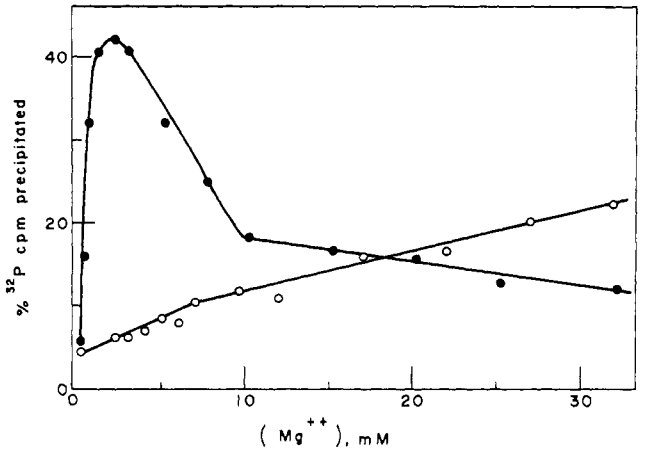

FIGURE 2: Effect of ribosome inactivation on second antibody precipitation of 30S subunits by antidimethyladenosine antibodies. Subunits from *E. coli* strain PR7 which were active (●) or inactivated by dialysis against buffer containing 0.5 mM $MgCl_2$ (○). Values are corrected for nonspecific precipitation with nonimmune globulins.

by incubation at 37 °C in 20 mM Mg^{2+}, restored the peak at 2–4 mM Mg^{2+}, although complete restoration was not always attained.

For assurance that the dialysis procedure actually inactivated the 30S subunits, the poly(uridylic acid)-stimulated binding of [^{14}C]phenylalanyl-tRNA^{Phe} was measured by using unlabeled subunits prepared and treated exactly as described for ^{32}P-labeled subunits. Under the conditions used, subunits "as isolated" showed 85% of maximal activity. Inactivation by dialysis against 1 mM Mg^{2+} buffer reduced this level to 30–35% of the maximum; reactivation by 5-min incubation with 20 mM Mg^{2+} at 37 °C then gave a threefold stimulation to the maximal (100%) level of 2.6 pmol of [^{14}C]phenylalanyl-tRNA^{Phe} bound per 20 pmol of ribosomal subunits. Zamir et al. (1971) showed similar levels of tRNA-Phe binding by activated 30S subunits (2–3 pmol of tRNA per ~30 pmol of ribosomal subunits).

Inhibition of Antibody Binding. The experiments above show that, under conditions of antibody excess, the extent of subunit binding depends on both the functional state of the ribosomal subunit and the Mg^{2+} concentration. Competitive binding experiments, under conditions near antibody and radioantigen equivalence, were used to investigate the relative affinities of anti-m_2^6Ado binding to active vs. inactivated 30S subunits at different Mg^{2+} concentrations.

First, varying amounts of unlabeled N^6,N^6-dimethyladenosine were used to inhibit the binding of equivalent quantities of [^{3}H]dimethyladenosine or ^{32}P-labeled ribosomal subunits, as measured by second antibody precipitation. Fifty percent inhibition of [^{3}H]dimethyladenosine binding was seen at 5×10^{-8} M dimethyladenosine whether the magnesium ion concentration was 3 or 20 mM. Similarly, 50% inhibition of subunit binding was seen at 3×10^{-8} M dimethyladenosine if the radioligand was active ribosomes at 3 mM Mg^{2+} or inactivated ribosomes at 20 mM Mg^{2+}. Thus, antibody populations with similar affinities for dimethyladenosine are involved in the binding of nucleoside or ribosomal subunits, regardless of whether conditions are optimized for the interaction with active subunits (3 mM Mg^{2+}) or inactivated subunits (20 mM Mg^{2+}).

Second, antibody precipitation of [^{3}H]dimethyladenosine was measured in the presence of competing levels of unlabeled subunits or nucleoside. The results are shown in Figure 3. The midpoint of each inhibition curve appears independent of the magnesium ion concentration of 3 mM (panel A) or

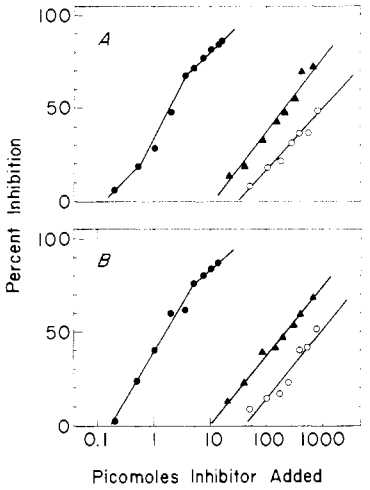

FIGURE 3: Inhibition of [^{3}H]dimethyladenosine binding by 30S ribosomal subunits and nucleoside. Second antibody precipitation was used to measure radioligand binding at 3 mM Mg^{2+} (panel A and at 20 mM Mg^{2+} (panel B). Inhibitors used were (●) N^6,N^6-dimethyladenosine, (○) active 30S ribosomal subunits from strain PR7, and (▲) inactivated 30S ribosomal subunits. All values are corrected for nonspecific precipitation with nonimmune rabbit globulins.

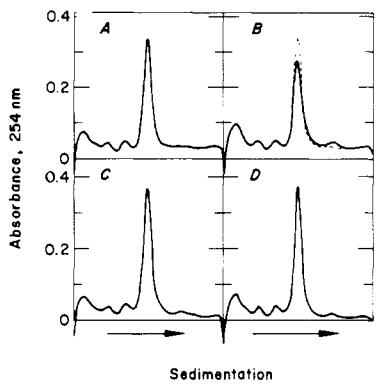

FIGURE 4: Sedimentation of complexes of ribosomal subunits and antibody. Each reaction mixture contained 27 pmol of 30S subunits and serum globulins as indicated below in a total volume of 73 μL of dissociation buffer. After 30 min at 0 °C, 40-μL portions were layered on 4.4-mL gradients of 5–20% sucrose in dissociation buffer and centrifuged for 105 min at 50 000 rpm in a Spinco SW50.1 rotor. An Isco gradient fractionator and a LDC UV monitor were used to obtain the tracings. A: PR7 subunits plus 54 μg of nonimmune globulins. B: PR7 subunits plus 54 μg of antidimethyladenosine globulins (2 dimethyladenosine-combining equivalents). The dashed line is a tracing of pattern A. C: TPR201 subunits plus 54 μg of nonimmune globulins. D: TPR201 subunits plus 54 μg of antidimethyladenosine globulins.

20 mM (panel B); in each instance, 50% inhibition by dimethyladenosine occurs at 1.5 pmol/250-μL assay, compared to 200 pmol for inactivated 30S subunits and 10^3 pmol for active subunits. Thus, regardless of the magnesium ion level, a higher concentration of active subunits (relative to inactivated subunits) is needed to displace the radioligand, suggesting that the inactive subunit–antibody complex is the more stable.

Sedimentation of Antibody–Ribosomal Subunit Complexes. The interaction of ribosomal subunits with antibodies is often studied by sucrose gradient sedimentation [e.g., Stöffler et al. (1973), Lake et al. (1974), Politz & Glitz (1977), Thammana & Cantor (1978), and Keren-Zur et al. (1979)]; complex formation is judged by reduction of the 30S subunit peak, balanced by formation of a faster sedimenting peak or shoulder containing immunoglobulin cross-linked subunit dimers. Figure 4 shows such an experiment, in which formation of complex is seen with PR7 subunits but not with TPR201

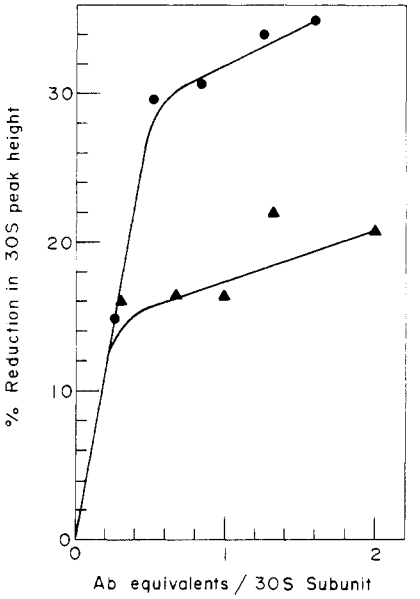

FIGURE 5: Sedimentation analysis of complex formation between antidimethyladenosine and 30S subunits from *E. coli* PR7. Ribosomal subunits (54 pmol) were incubated with nonimmune rabbit globulins and antidimethyladenosine (total protein 108 μg) and centrifuged as in Figure 5. (▲) PR7 subunits as isolated (active); (●) PR7 subunits inactivated by dialysis.

subunits. Figure 5 shows the extent of complex formed between a fixed amount of active or inactivated subunits and different quantities of anti-m_2^6Ado. At saturation, about twice as many inactivated subunits are complexed, relative to active subunits.

Rate of Dissociation of Antibody–Subunit Complexes. The sedimentation experiments described above detect only those complexes sufficiently stable to allow separation by 1.5–2 h of centrifugation. The rates of dissociation of anti-m_2^6Ado complexes of active and inactivated subunits were measured to help evaluate this effect. Complexes of antibody with ^{32}P-labeled 30S subunits were formed by overnight incubation of reaction mixtures; dissociation was initiated by dilution with excess nonradioactive m_2^6Ado. Separation of antibody-bound subunits by precipitation with a second antibody was not fully effective, since the time needed to efficiently precipitate anti-m_2^6Ado complexes was comparable to the half-lives being measured. Adsorption of anti-m_2^6Ado–subunit complexes to immobilized *S. aureus* protein A (which binds the F_c portion of IgG molecules) was much more rapid, although the background level of apparent ^{32}P-labeled subunit binding by nonimmune IgG was almost twice that seen in the second antibody technique. Dissociation of active subunit complexes at 3 mM Mg^{2+} (the optimum from Figure 1) and inactivated subunit complexes at 15 mM Mg^{2+} (also optimal) is shown in Figure 6. In all such experiments, inactivated 30S subunits were found to form the more stable complexes with anti-m_2^6Ado.

Interaction of Ribosomal RNA and Ribosomal Cores with Antibodies. Second antibody precipitation was used to quantitate the interaction of anti-m_2^6Ado with 16S ribosomal RNA or with core particles prepared by centrifugation in CsCl gradients. Precipitation of ^{32}P-labeled 16S rRNA from strain TPR201 (m_2^6Ado free) was inefficient (2% of added ^{32}P) and independent of magnesium ion concentration over the range 0–100 mM Mg^{2+}. In contrast, [^{32}P]rRNA from strain PR7 was precipitated to a significant extent; under the conditions used, 5% of the added ^{32}P was precipitated in the absence of added Mg^{2+}, with a nearly monotonic increase to a plateau of 45% precipitation at 60–100 mM Mg^{2+}. Second antibody

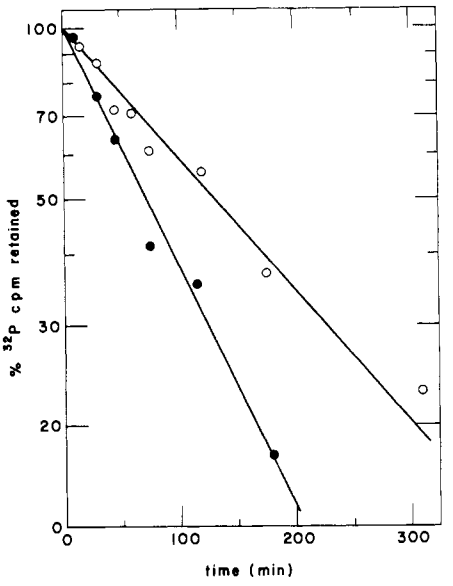

FIGURE 6: Dissociation of subunit–antibody complexes. Complex dissociation was initiated at 0 min by dilution with 4 volumes of 1.25 mM m_2^6Ado in the appropriate buffer, and antibody-bound ^{32}P was separated by adsorption to Protein A–Sepharose. (●) Active PR7 subunits, 3 mM Mg^{2+}; (○) inactivated PR7 subunits, 15 mM Mg^{2+}. Values are corrected for nonspecific precipitation with nonimmune globulins.

precipitation of ribosomal core particles from PR7 subunits resulted in curves similar to those seen with inactivated PR7 subunits. In no instance was evidence for a binding peak at 2–4 mM Mg^{2+} ever seen with rRNA or core particles.

Discussion

The experiments reported here confirm and extend earlier conclusions (Politz & Glitz, 1977; Thammana & Cantor, 1978) that the interaction of dimethyladenosine-specific immunoglobulins with ribosomal subunits requires (minimally) the presence of dimethyladenosine in the ribosomal RNA. The effects of magnesium ion concentration and the state of subunit activation on the antibody interaction require further consideration. Since the interaction of antibodies with free [^{3}H]-N^6,N^6-dimethyladenosine is independent of variations in Mg^{2+} concentration, it is unlikely that the magnesium ion is affecting antibody structure or stability. Instead, the Mg^{2+} effect is most reasonably explained in terms of the structure of the ribosomal subunit. The availability of ribosomal m_2^6Ado for antibody binding may depend on subunit conformation, as suggested by Thammana & Cantor (1978) and Stöffler et al. (1980), and/or the stability of subunit–antibody complexes may differ depending on the conformation of the subunit. Our results emphasize the latter interpretation.

An alternative view, that an increasing Mg^{2+} concentration permits subunit aggregation and thus an apparent increase in antibody binding, is not consistent with either the results of Thammana & Cantor (1978) or the sharp maximum of active subunit precipitation seen at 2–4 mM Mg^{2+}. Gualerzi et al. (1973) report that dimers of 30S particles formed at high Mg^{2+} levels are blocked from binding the initiation factor IF-3. Although dimerization of 30S subunits might limit the accessibility of the dimethyladenosine and thus explain the reduction in antibody binding seen from 4–20 mM Mg^{2+} (Figures 1 and 2), neither the low level of binding at 0.5 mM Mg^{2+} nor the sharp increase in binding at 0.5–3 mM Mg^{2+} is consistent with such an interpretation. The Mg^{2+} concentration that is optimal for antibody binding to active 30S subunits (2–4 mM) is also within the range reported for important Mg^{2+}-dependent functional changes in 30S subunits including tRNA binding (Zamir et al., 1971), 30S–50S subunit association (Hapke & Noll, 1976), and factor-dependent initiation complex formation (Noll & Noll, 1974). Thus, the variable reactivity of the dimethyladenosine in the ribosomal subunit may be a manifestation of functionally significant conformational changes.

Sedimentation of antibody–subunit complexes through sucrose gradients, with the same antibody preparation used here, led Thammana & Cantor (1978) to the conclusion that only inactivated subunits bind antibody, while the m_2^6Ado is buried in the activated 30S subunit. Although our results in sedimentation experiments are similar (Figures 4 and 5), our interpretation is different. Competitive binding experiments indicate that m_2^6Ado in either active or inactivated subunits is exposed and can bind antibody but that binding inhibition by active subunits is effective only at higher subunit concentration (Figure 3). This result indicates that antibody affinity for active subunits is less than that for inactivated subunits. Yet m_2^6Ado availability, as measured by second antibody precipitation, is potentially highest with activated subunits, so long as an excess of antibody is present to shift the equilibrium toward complex formation (Figure 2). The dissociation of an active subunit–anti-m_2^6Ado complex, formed under optimal binding conditions of 3 mM Mg^{2+}, is about twice as rapid as dissociation of a complex formed (at 15 mM Mg^{2+}) with inactivated subunits (Figure 6, 70 vs. 130 min). The sedimentation experiments of Thammana & Cantor (1978) required 4.5 h, sufficient for most active subunit–antibody dimers to dissociate and not be detected. Moreover, their complexes were formed in 5 mM Mg^{2+}, a level higher than the optimum for complex formation with active subunits. Our gradients (Figure 4) were run for 105 min; dissociation was sufficient to reduce the amount of complex seen with active subunits, but complex was still detectable as a separate faster sedimenting peak. [Figures 4 and 5 also demonstrate complex formation more clearly than our earlier result which involved sedimentation for 12 h (Politz & Glitz, 1977; Figure 2).]

The second antibody precipiation experiments (Figures 1 and 2) required only a few minutes to separate antibody-bound and free ribosomal subunits and thus allowed observation of equally specific but more transient complexes.

Our interpretation is reasonably consistent with the results of Hogan & Noller (1978), who found that although the dimethyladenosine-containing oligonucleotide was more reactive to kethoxal modification in inactivated 30S subunits, it was less affected by inactivation than most (~80%) of the kethoxal reactive sites, and no oligonucleotide was made less kethoxal reactive by inactivation. It is similarly compatible with the cross-linking results of Wollenzien et al. (1979) and Thammana et al. (1979), which imply a pairing of the 3′ and 5′ ends of 16S rRNA in inactivated subunits or isolated RNA but not in active subunits. Thus, the environment of the m_2^6Ado residues is agreed to differ in active vs. inactivated subunits, regardless of the interpretation of the antibody binding data. Moreover, localization by electron microscopy of dimethyladenosine (Politz & Glitz, 1977; Stöffler et al., 1980) and the 3′ end of the RNA (Olson & Glitz, 1979; Shatsky et al., 1979) to the region of the 30S subunit proposed to be the site of initiation (Lake, 1978) makes the effects we seen interesting from the viewpoint of ribosome function.

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