Journal of Supramolecular Structure 10:443–455 (1979)
Biological Recognition and Assembly 1–13

The Accessibility of Antigenic Determinants of Ribosomal Protein S4 In Situ

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Antibodies to Escherichia coli ribosomal protein S4 react with S4 in subribosomal particles, eg, the complex of 16S RNA with S4, S7, S8, S15, S16, S17, and S19 and the RI* reconstitution intermediate, but they do not react with intact 30S subunits. Antibodies were isolated by three different methods from antisera obtained during the immunization of eight rabbits. Some of these antibody preparations, which contained contaminant antibodies directed against other ribosomal proteins, reacted with subunits, but this reaction was not affected by removal of the anti-S4 antibody population. Other antibody preparations did not react with subunits. It is concluded that the antigenic determinants of S4 are accessible in some protein deficient subribosomal particles but not in intact 30S subunits.

Key words: ribosomes, 30S subunit structure, immunochemistry

Antibodies to individual ribosomal proteins have been utilized to delineate the functional roles and structural locations of these proteins in the intact subunit. These studies have generally assumed that each of the proteins studied has exposed antigenic determinants [1, 2] and that the observed functional inhibition or antibody binding could be ascribed to the protein which had been chosen as the immunogen [3]. Ribosomal protein S4 has been assigned a role in several functional sites on the basis of inhibition of activity by antibody putatively specific for S4 [1] and has been assigned locations on the 30S subunit surface by immunoelectron microscopy [4, 5]. In the case of S4, however, the reactivity of anti-S4 antibody with subunits was originally described as weak, and demonstrable only in certain assays [2]. Subsequently it was found that the replacement of Escherichia coli S4 with its functional homolog from Bacillus stearothermophilus only partly inhibited the reaction of anti-E coli S4 with hybrid 30S subunits, although no cross-reaction could be demonstrated with the purified proteins [4]. Therefore, we felt it important to test the hypothesis that S4 was, in fact, not available to antibody in the intact subunit and that the observed functional inhibition and map locations were the result

Received April 4, 1979; accepted April 18, 1979.

0091-7419/79/1004-0443$02.60

of the reaction of minor amounts of contaminating antibodies directed against other ribosomal proteins. In order to test the greatest possible variety of antibody populations, we have immunized eight rabbits, collected serum throughout the course of long immunization programs (up to 32 weeks of immunization), and purified anti-S4 IgG preparations by three different procedures. We have tested these preparations under a variety of reaction conditions and found that none of these antibody preparations gave detectable reaction with S4 in the intact 30S subunit. Thus the functional and structural roles ascribed to S4 must be considered unproven until an S4-specific reaction of antibody with subunits can be convincingly demonstrated.

MATERIALS AND METHODS

Preparation of Ribosomes, Ribosomal Proteins, and rRNA

Isolation of E coli K12 (strain PR-C10) 70S ribosomes, zonal separation of 30S and 50S subunits, and purification of 30S ribosomal proteins (r-proteins) were done as described by Held et al [6]. 16S RNA was prepared by phenol extraction of 70S ribosomes according to Traub et al [7]. Then 12.5 mg of 5S, 16S, and 23S rRNA was separated by centrifugation on linear 15–30% sucrose gradients in SSC-EDTA (0.15 M NaCl, 0.015 M Na citrate, 0.01 M ethylenediaminetetracetic acid, pH 7.0) for 2.25 h at 50,000 rpm in a Beckman VTi50 rotor at 4°C. The pooled 16S RNA was precipitated with 2 volumes ethanol at −20°C, dialyzed against 0.03 M Tris (pH 7.4), and stored at −70°C. Salt-washed 30S subunits were prepared according to the method described by Staehelin and Maglott [8]. Alternatively, 70S ribosomes in TMA I (0.03 M NH_4Cl, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.3, 0.001 M dithiothreitol [DTT]) were brought to 1 M NH_4Cl with 4 M NH_4Cl, then pelleted by centrifugation. Subunits were isolated from these washed ribosomes.

Reconstitution, Isolation, and Analysis of 30S Ribosomal Subunits and Reconstitution Intermediates

Reconstitution of 30S subunits from TP30 or the 21 purified proteins was done according to Held et al [6]. Subribosomal particles were prepared by mixing 1 volume of the desired mixture of pure proteins in TRI buffer (1.0 M KCl, 0.02 M $MgCl_2$, 0.03 M Tris, pH 7.4, 0.001 M DTT) with 2 volumes of 16S RNA in TRO buffer (0.02 M $MgCl_2$, 0.03 M Tris, pH 7.4, 0.001 M DTT) and incubating 1 h at 40°C. Reconstitution was done at a final RNA concentration of 800–1,600 pmoles/ml with a (1.5–2)-fold excess of protein. Reconstituted particles were purified by centrifugation through 15–30% sucrose gradients in TMA I buffer for 75 min at 50,000 rpm in a VTi50 rotor at 4°C. The purified particles were pelleted (10 h at 40,000 rpm, Beckman SW50.1 rotor) from the sucrose-TMA I solution, redissolved in TMA I, and incubated 10 min at 40°C before use. Generally the reconstitution intermediates were prepared just prior to analysis. Poly-U-directed [^{14}C] phenylalanine incorporation activity of 30S subunits was assayed as described by Held and Nomura [9]. Two-dimensional polyacrylamide gel electrophoresis was done by a modification of the method described by Howard and Traut [10]. The size of the first-dimension gel was decreased to 1.5 mm × 7 cm and 80 pmoles of RNase-digested subunits were used per gel. Electrophoresis was for 2.5 h at 80 V in the first dimension and 6 h at 120 V in the second dimension (both at 24°C).

Antisera Production

Antisera to E coli 30S subunit protein S4 were prepared by immunization of New Zealand white rabbits with emulsions prepared by mixing equal volumes of 0.25–1.0 mg/ml S4 in 0.15 M NaCl, 0.5% phenol, and Freund's complete adjuvant (Perrin's modification, Calbiochem). Biweekly injections of 0.25–1.0 mg antigen were administered subcutaneously at 4–8 sites. Blood was collected from ear veins at least seven days after the most recent immunization. Immunization was repeated at varying intervals for up to one year.

Double-Immunodiffusion

Sera were tested for antibody by double-immunodiffusion in 1% agar gels against 4×10^{-6} M antigen, as previously described [11]. Antisera were tested directly and after (5–10)-fold concentration for the presence of antibodies to heterologous 30S ribosomal subunit proteins, as previously described [12].

Preparation of IgG Fractions

The total immunoglobulin G fraction of specific rabbit antisera was partially purified by ion-exchange chromatography on DEAE-Sephadex A50 [13] using a 0–0.5 M NaCl gradient in 0.02 M phosphate buffer (pH 7.2) to elute bound IgG, ammonium sulfate precipitation of all IgG-containing fractions, and gel filtration on Sephacryl S200 (Pharmacia). Alternatively, the IgG fractions were prepared by affinity chromatography on Protein A–Sepharose CL-4B (Pharmacia) and gel filtration. Serum (5 ml) was applied at 4°C to a 0.9-cm × 6-cm Protein A–Sepharose CL-4B column equilibrated with saline-phosphate (SP) buffer (0.15 M NaCl, 0.075 M potassium phosphate, pH 7.15) and recycled over the column for 1–2 h at a flow rate of 0.2–0.3 ml/min. The unbound serum proteins were eluted from the column with SP buffer. Bound IgG was eluted with 0.1 M glycine-HCl (pH 3.0). The neutralized IgG was further purified by gel filtration and concentrated by ammonium sulfate precipitation.

Immunoabsorption Affinity Purification of S4-Specific IgG

S4 (3 mg) in 6 M urea, 0.10 M potassium phosphate buffer pH (7.0) was reacted with a 10-ml slurry of the N-hydroxysuccinimide ester of succinylated aminoalkyl Bio-gel A (Affigel-10, Biorad) for 18 h at 4°C. The coupling reaction was terminated with 1 M methylamine phosphate (pH 9). The gel was washed extensively with cycles of Buffer I (0.20 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris, pH 7.4, at 24°C) and desorption buffer (0.20 M KCl, 0.10 M Na citrate, pH 2.2) ending with Buffer I. Total IgG (5–20 mg) in Buffer I was applied to a 10-ml column of S4-Affigel and cycled over the column for 12 h at 0.2 ml/min and 4°C. Unbound IgG was eluted with Buffer I. Bound, S4-specific IgG was eluted with desorption buffer. IgG-containing fractions were pooled and neutralized with solid Tris (Sigma). Carrier nonimmune IgG was added and the IgG concentrated by precipitation with 50% saturated $(NH_4)_2SO_4$.

Quantitative Immunoprecipitation

Quantitative immunoprecipitation analysis of AS4 IgG was done generally according to the procedure outlined by Mauer [14]. A constant amount (200–400 μg) of purified

IgG was added to a series of tubes containing increasing amounts of S4 (0–10 μg). The reaction was done in reconstitution buffer in 50 to 100-μl volumes. Samples were incubated for 30 min at 37°C followed by 48–96 h at 4°C with occasional vortexing. The precipitates were centrifuged at 4°C in a Beckman Microfuge B for 1.5 min. The supernatants were removed and the precipitates were washed three times with reconstitution buffer, then dissolved in 100 μl of 0.5 N NaOH. Protein was determined by the Lowry assay [15].

Reaction of Antibody With Ribosomal Particles

30S ribosomal subunits or subribosomal particles (16–32 pmoles) were "heat-activated" for 5 min at 40°C in Buffer I or TMA I. Particles were reacted with IgG in 25–100 μl of 0.10 M NH_4Cl, 0.01 M $MgCl_2$, 0.01 M Tris (pH 7.4) for 5 min at 40°C and 40 min at 0°C. The reaction mixtures were layered directly on 5 ml, 15–30% sucrose gradients in TMA I and centrifuged for 2.75 h at 50,000 rpm in a SW50.1 rotor at 4°C. Alternatively, the reaction mixtures were diluted with 1/2 volume of 20% sucrose in reaction buffer and layered between a 5-ml 15–30% sucrose gradient and 250 μl of reaction buffer overlay. Centrifugation was for 20 min (at speed) in a Beckman VTi65 rotor at 50,000 rpm and 4°C in an L5-50 centrifuge. The best gradient separations were obtained by accelerating the rotor at an acceleration rate setting of 1 up to 2,000 rpm, then at an acceleration rate of 10 up to speed. The rotor was braked down to 1,400 rpm, then allowed to coast to a stop. Absorbance at 260 nm was measured with a Gilford density gradient scanner by upward displacement of the gradients with sucrose.

RESULTS

In order to determine the degree of exposure of the antigenic determinants of E coli ribosomal protein S4 in situ, we have characterized the reactivity of S4-specific antibody (AS4) with intermediates of in vitro 30S subunit reconstitution and with the 30S subunit. Since we desired the widest variety of reactions, numerous bleedings were taken from eight rabbits during the course of immunization. Several bleedings from each animal containing high titers of AS4 were further studied. The spectrum of ribosomal protein-specific antibodies in each was determined by concentrating the serum (5–10)-fold and testing it for reaction with each of the 21 small-subunit proteins by double-immunodiffusion. The S4 used as immunogen was judged greater than 98% pure by polyacrylamide gel electrophoresis and the impurities were identified immunochemically as S3 and S7 (data not shown). Several antisera contained antibody to these impurities as well as to S4; others reacted only with S4 (Table I).

A nuclease-free IgG fraction was isolated from the antisera by ion-exchange chromatography or Protein A–Sepharose affinity chromatography. The IgG prepared by each method reacted with S4 in double-immunodiffusion experiments. S4-specific IgG in the IgG fraction was measured by quantitative immunoprecipitation analysis. The AS4 titer of the antibody preparations studied are listed in Table I. The AS4 titer varied from as low as 1.6% up to 16% of the total IgG. We generally saw a rise in the titer over the course of immunization.

To determine if S4 is accessible to antibody on subribosomal particles and to examine any changes in accessibility on completion of the 30S ribosomal subunit, intermediates of

TABLE I. Antibody Content of Rabbit AS4 IgG Preparations

Animal no.	Immunization period (weeks)	Method of IgG purification	Moles S4-specific IgG / 100 moles IgG	Contaminant r-protein antibodies
1	6	A	7	–
1	13	B	16	–
2	25	A	6	–
2	25	B	8	–
2	20–25	C	50–60	–
3	8	A	6	–
3	21	B	9	–
4	8	A	5	–
4	21	B	9	S7
5	24	A	ND	S3,S7
5	32	A	3	S3,S7
5	32	C	ND	–
6	6	A	5	S3,S7
6	6	C	ND	–
7	8	A	2	S3
7	8	C	ND	–
8	7	A	2	–
8	7	C	ND	–

For each rabbit individual IgG preparations are described by number of weeks between initial immunization and bleeding and method of IgG purification. IgG was purified by three methods (indicated in third column): A, DEAE-Sephadex ion-exchange chromatography; B, Protein A–Sepharose affinity chromatography; C, S4-Affigel affinity chromatography. S4-specific antibody in the IgG preparation was measured by quantitative immunoprecipitation (see Methods). Values indicated are percentage of total IgG added that was precipitated by antigen at equivalence (ND; Not Determined.) Contaminating r-protein-specific antibodies were identified by immunodiffusion of concentrated antisera against the 21 pure 30S subunit proteins.

in vitro 30S subunit reconstitution were prepared from purified components according to the following scheme:

$$16S\ RNA + S4,S7,S8,S15,S16,S17,S19 \rightarrow RI[4,7,8,15,16,17,19] \quad (1)$$

$$RI[4,7,8,15,16,17,19] + S5,S6,S9,S11,S12,S13,S18,S20 \rightarrow RI^* \quad (2)$$

$$RI^* + S1,S2,S3,S10,S14,S21 \rightarrow 30S\ \text{ribosomal subunit} \quad (3)$$

The first particle in this scheme is prepared by incubation of 16S RNA with the seven proteins in reconstitution buffer for 1 h at 40°C and purified by zone sedimentation in sucrose gradients (see Methods). The next intermediate in the scheme, the RI* particle, is a complex of 15 proteins and 16S RNA. Twelve of these proteins are required for RI* formation [9]; proteins S6, S13, and S20 were included to stabilize the particle during isolation. The RI* particle was prepared and purified in the same manner as the RI[4,7,8,15, 16,17,19] particle. In early experiments we isolated the particles directly by pelleting through 15% sucrose in reconstitution buffer. We found this method unsatisfactory since it resulted in reduced amounts of some proteins (eg, S5) as a consequence of the high salt in the reconstitution buffer through which they were pelleted.

The isolated RI[4,7,8,15,16,17,19] and RI* particles could be completed by the addition of the missing proteins to form 30S ribosomal subunits that were active in the poly-U translation assay (Table II). The isolated RI[4,7,8,15,16,17,19] particle was completed in two steps. It was first incubated for 1 h at 40°C in reconstitution buffer with the eight proteins needed to form the RI* particle. The remaining six proteins were then added and the incubation was continued for 10 min at 30°C. The isolated RI* particle was completed with this second step only. The results in Table II indicate that the RI[4,7,8,15,16,17,19] and RI* particles are inactive prior to completion. Both particles can be completed upon addition of the missing proteins to yield particles that have 80–100% of the control activity. The two-step reconstitution protocol was used to demonstrate that the RI[4,7,8,15,16,17,19] particle could be converted to the RI* particle and that the RI* particle had undergone the heat-dependent conversion step described by Held and Nomura [9].

Reconstituted 30S ribosomal subunits were prepared either with the 21 pure ribosomal proteins or total 30S protein (TP30). The protein and 16S RNA were incubated for 1 h at 40°C. The reconstituted particles were purified in the same manner as the RI[4,7,8,15,16,17,19] and RI* particles. We found that reconstituted 30S subunits purified in this manner were more active in the poly-U translation assay and had narrower sucrose gradient profiles than subunits isolated directly by pelleting through 15% sucrose in reconstitution buffer.

Analysis of the isolated particles in sucrose gradients indicated that the RI[4,7,8,15,16,17,19] and RI* particles sediment as narrow zones of about 22S and 26S, respectively. The reconstituted 30S subunit sedimented with native 30S ribosomal subunits.

The protein composition of the isolated particles was examined by two-dimensional gel electrophoresis and compared to native 30S subunits prepared from 70S ribosomes. Each of the isolated particles contained the expected proteins in amounts comparable to those found in the native 30S subunit (data not shown).

TABLE II. Completion of Isolated Subribosomal Particles

Particle	Proteins added in completion steps		Activity (pmoles [^{14}C] phenylalanine incorporated)
	Step 1	Step 2	
16S RNA	A + B + C	–	39
RI[4,7,8,15,16,17,19]	–	–	3
RI[4,7,8,15,16,17,19]	B	C	42
RI*	–	–	2
RI*	–	C	32
ΣSi 30S	–	–	40
Native 30S	–	–	80

30S subunits were reconstituted from 16S RNA or isolated subribosomal particles in two steps by addition of r-protein mixes A (S4,S7,S8,S15,S16,S17,S19), B (S5,S6,S9,S11,S12,S13,S18,S20), and C (S1,S2,S3,S10,S14,S21). 16S RNA or ribosomal particles were prepared in reconstitution buffer. Indicated protein mix(es) or buffer was added and the samples were incubated for 1 h at 40°C in step 1, then chilled on ice. In step 2 incubation was for 10 min at 30°C. As described in Held and Nomura [9], 16 pmoles of the completed particles was assayed directly for poly-U-directed [^{14}C] phenylalanine incorporation activity. ΣSi 30S are isolated 30S subunits reconstituted from 16S RNA and the 21 pure r-proteins. Native 30S subunits were prepared from 70S ribosomes without salt-washing. –; No addition of r-protein.

The AS4 IgG preparations were tested for AS4-dependent aggregation of the reconstitution intermediates and 30S subunits. Antibody was incubated with the ribosomal particles and the reaction mixtures were analyzed by zone sedimentation in sucrose gradients (Fig. 1). Incubation of AS4 IgG with RI[4,7,8,15,16,17,19] particles results in a reduction in the 22S monomer peak area and the formation of faster sedimenting aggregates. The major product was an IgG-linked dimer, although higher aggregates were always seen. Preabsorption of the antibody with S4 at equivalence precipitated the S4-specific IgG and eliminated any reaction of the antibody preparation with the RI[4,7,8,15,16,17,19] particle. The small amount of dimerized particles seen in the absorbed antibody control was also seen with nonimmune IgG and in the absence of IgG and thus represents nonspecific dimers. The production of aggregates larger than dimers indicates that multiple antigenic determinants are accessible on this particle. All of the AS4 IgG preparations reacted with this particle, but the reaction could not be shown to be AS4-specific for those preparations that contained antibody to proteins other than S4. Only a portion of the reactivity could be absorbed with S4 in those IgG preparations.

The IgG preparations also reacted with the RI* particle, but the extent of reaction was less than that seen under identical reaction conditions with the RI[4,7,8,15,16,17,19] particle (Fig. 2). The decrease in AS4 reactivity of the RI* particle may indicate a masking of S4 antigenic determinants on this assembly intermediate as compared to the RI[4,7,8,15,16,17,19]. Alternatively, the conformation of S4 may be altered such that

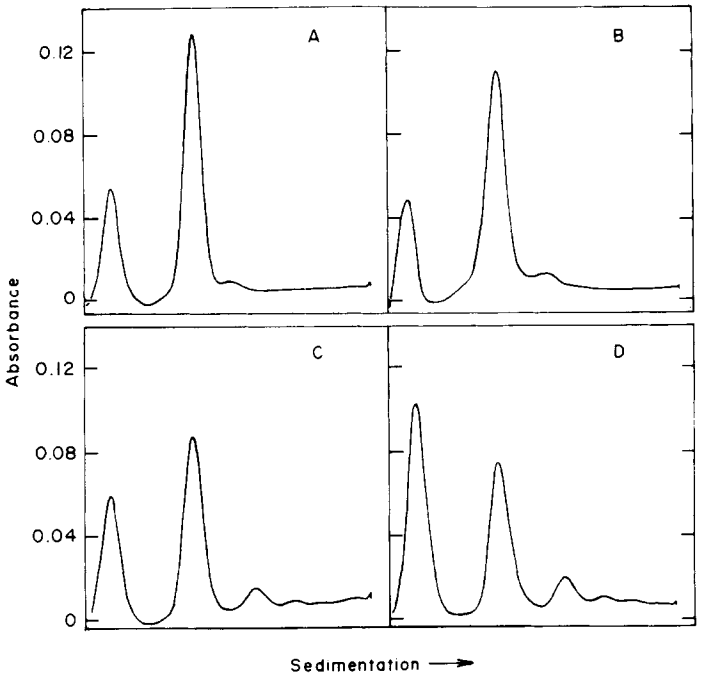

Fig. 1. Sucrose gradient analysis of the reaction of AS4 IgG (1-13B) with RI[4,7,8,15,16,17,19] particles. 24 pmoles of RI[4,7,8,15,16,17,19] was reacted with 140 μg of preimmune IgG (A), 140 μg of S4-absorbed AS4 IgG (B), 140 μg of AS4 IgG (48 pmoles AS4-specific IgG) (C), and 280 μg AS4 IgG (96 pmoles S4-specific IgG) (D). Reaction conditions were those described in Methods. Sedimentation was for 20 min at 50,000 rpm and 4°C in a VTi65 rotor through 15–30% sucrose gradients. Absorbance was monitored at 260 nm in a 2.5-mm pathlength flowcell.

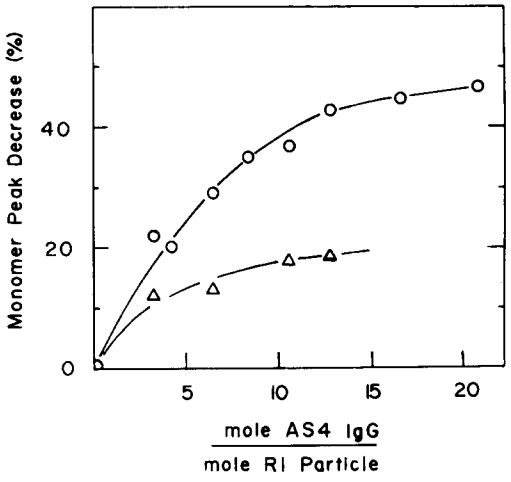

Fig. 2. Reaction of AS4 IgG (1-13B) with RI[4,7,8,15,16,17,19] (○) and RI* (△) particles as measured by antibody-dependent aggregation. The subribosomal particles were reacted with AS4 at various molar ratios and sedimented through sucrose gradient (see Methods and Fig. 1). Aggregation of particles was measured by decrease in monomer peak area from that observed when preimmune IgG was reacted with the same particles. Points shown are the average of 2–4 samples.

it is less strongly bound by antibody. Since the AS4 preparations did react specifically with the RI* particle S4 must be accessible to antibody on this intermediate.

In contrast to the results we observed with the reconstitution intermediates, AS4 IgG from four of the rabbits did not react with native or reconstituted 30S ribosomal subunits (Table III). These antibody preparations were tested over a wide range of antibody-antigen ratios. Reaction conditions such as reaction volume and incubation time were varied. Two methods of IgG purification were utilized and several bleedings over the course of immunization were tested. Glutaraldehyde fixation of the reaction products was attempted [16]. Despite all efforts to favor the reaction of antibody with the 30S ribosomal subunit and to stabilize the products, none of the antibody preparations from four of the eight rabbits reacted with the 30S subunit.

Several methods of 30S subunit preparation were also examined. Salt-washed 30S subunits, subunits isolated from salt-washed 70S ribosomes, reconstituted 30S subunits, low Mg^{2+}-inactivated 30S subunits [17], and 30S subunits from a different strain of E coli (MRE 600) were tested. None of the 30S subunit preparations reacted with these AS4 IgG preparations.

IgG preparations from four rabbits did react with the 30S ribosomal subunits (Table III). However, these preparations contained antibody to other r-proteins. The total IgG was therefore further fractionated by immunoabsorption chromatography on S4-Affigel (see Methods). S4-specific IgG, which bound to the immobilized S4 and was subsequently eluted, did not react with the 30S subunit. Figure 3 shows the results of the reaction of the S4-Affigel passthrough and bound IgG fractions with 30S subunits at various antibody-to-subunit ratios. All of the 30S subunit reactive antibody was found in the passthrough fraction. The bound, S4-specific fraction reacted with S4 and RI particles but not with the 30S ribosomal subunit. This result suggests that the antibody that reacted with the 30S subunit was directed against r-proteins other than S4.

TABLE III. Reactivity of AS4 IgG Preparations With 30S Ribosomal Subunits

IgG preparation[a]	30S subunit reactivity	Inhibition by S4 absorption	AS4 IgG:30S subunit molar ratios tested
1-6A	–		1–23
1-13B	–		1–15
2-25A	–		0.5–4
2-25B	–		0.5–27
2-(20–25)C	–		
3-8A	–		0.5–20
3-21B	–		0.5–19
4-8A	–		0.5–17
5-32A	+	No inhibition	0.2–1
5-32C	–		
6-6A	+	No inhibition	0.5–3
6-6C	–		
7-8A	+	No inhibition	0.5–8
8-7A	+	No inhibition	0.2–5

The 30S subunit reactivity of the IgG preparations was determined by antibody-dependent aggregation measured by zone sedimentation. A reaction is indicated by + and no reaction by –. In the cases where a reaction was detected, the IgG preparations were absorbed at equivalence with pure S4 and the supernatants were tested for reactivity with subunits. "No inhibition" indicates that absorption with S4 did not affect the reaction of the IgG preparation with 30S subunits.

[a]IgG preparations are identified as in Table I by rabbit number, period of immunization, and method of purification (A, DEAE-Sephadex; B, Protein A–Sepharose; C, S4-Affigel).

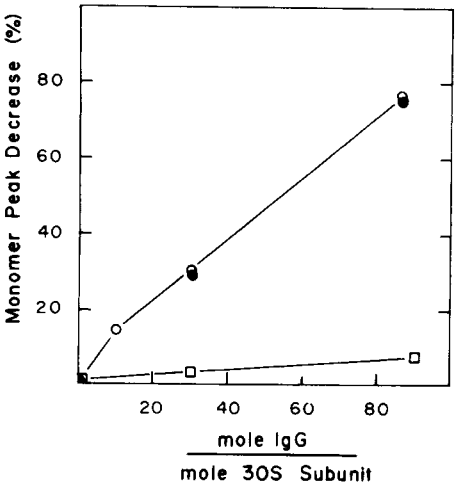

Fig. 3. Reaction of affinity-purified AS4 with 30S subunits. An IgG preparation (6-6A) that reacted with 30S subunits was further fractionated by immunoabsorption on S4-Affigel (as described in Methods). The unfractionated IgG (○), the nonbinding "passthrough" fraction (●), and the bound S4-specific fraction (□) were reacted with 30S subunits. Antibody-dependent aggregation of subunits was analyzed by sucrose gradient centrifugation (20 min at 50,000 rpm in a VTi65 rotor). Aggregation was measured by the decrease in the monomer peak area from that observed with nonimmune IgG. Carrier nonimmune IgG was added to the bound S4-specific fraction to bring the concentration to that of the original preparation.

If the reaction of the IgG preparations with 30S ribosomal subunits was S4-specific, preabsorption of the IgG with S4 should completely eliminate the reaction. However, if the reacting antibodies were directed against some other protein, absorption with S4 should not affect reactivity. Therefore, increasing amounts of S4 were incubated with the IgG preparations that reacted with the 30S subunit and immunoprecipitation was done as described in Methods. The quantitative immunoprecipitation curve for one of the IgG fractions is shown in Figure 4. The S4-absorbed supernatants were assayed for reaction with the 30S subunit and with S4. Despite the complete removal of the S4-specific antibody from the supernatants, there was no inhibition of the reaction of the IgG preparation with the 30S subunit. We concluded that the reaction of this IgG preparation with the 30S ribosomal subunit was due to a contaminant antibody reacting with a protein other than S4. Each of the antibody preparations that reacted with the 30S subunit was similarly tested. None reacted with the 30S subunit in a demonstrably S4-specific manner. Furthermore, in two cases the reaction was inhibited by preabsorption of the antibody with r-protein S7 (animals 5 and 6). These results are summarized in Table III.

Thus, none of the antibody preparations from eight rabbits reacted with the 30S ribosomal subunit in an AS4-specific manner. Since the antibody preparations do recognize S4 in situ on intermediates of 30S ribosome assembly, we have concluded that S4 is inaccessible to antibody on the surface of the 30S ribosomal subunit.

DISCUSSION

The accessibility of E coli ribosomal protein S4 to antibody on the 30S ribosomal subunit and ribosome reconstitution intermediates (RI particles) was studied with antibody preparations from eight rabbits. While the antibody preparations reacted with the RI[4,7,8, 15,16,17,19] and RI* particles in an S4-specific manner, no S4-specific reaction could be detected with complete 30S subunits.

The reaction of the antibody preparations with RI particles was demonstrated to be S4-specific by two methods. When the antibody preparations were passed over a column containing S4 covalently coupled to Bio-gel A, the fraction that bound to the column, the AS4-enriched fraction, reacted with the RI particles while the passthrough fraction showed greatly diminished reactivity. This result is consistent with an S4-specific reaction. However, the enrichment of antibody to contaminants in the S4 might also have occurred. Thus, it cannot be concluded that the antibody preparations react with S4 in reconstitution intermediates without additional supportive evidence.

Absorption of the antibody with S4 was also used to demonstrate the specificity of the reaction of the antibody preparations with RI particles. Immunoprecipitation of the antibody with an equivalent amount of S4 completely inhibited the reaction of some preparations and partially inhibited the reaction of other preparations which were known to be contaminated with antibody to other r-proteins. It is important to note that the immunoprecipitation control experiments used to demonstrate specificity were done at antigen-antibody equivalence. The addition of excesses of antigen may result in absorption of antibody directed against contaminants in the antigen, thus leading to an incorrect appearance of specificity.

We examined two intermediates of 30S subunit reconstitution and found that S4 was accessible on each of these intermediates. We did, however, see a decrease in the extent of reaction of the RI* particle when compared to the RI[4,7,8,15,16,17,19] particle (Fig. 2). The reactions of these two intermediates with several AS4 antibody preparations

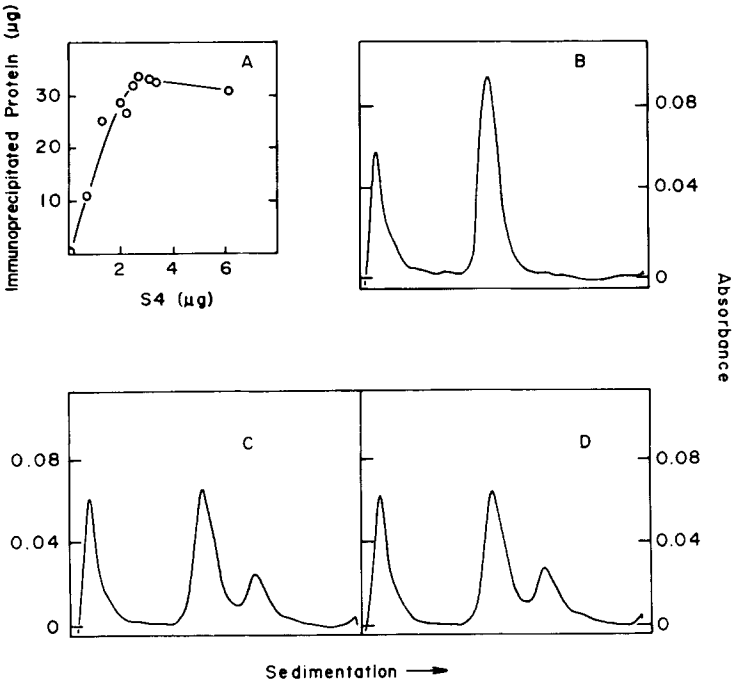

Fig. 4. A: Quantitative immunoprecipitation of IgG preparation 6-6A with S4. Aliquots of IgG (350 μg) were incubated with increasing amounts of pure S4 (0–6 μg) for 72 h in reconstitution buffer. Protein in the immunoprecipitate was measured as described in Methods. B–D: Sedimentation analysis of the reaction of the S4-absorbed IgG with 30S subunits. To measure antibody dependent aggregation of 30S subunits, 16 pmoles of 30S subunits were reacted with 100 μg of IgG and sedimented through sucrose gradients (2.75 h at 50,000 rpm and 4°C in a SW50.1 rotor). B: Preimmune IgG (control); C: "mock"-absorbed IgG (0 μg S4); and D: S4-absorbed IgG from the equivalence zone (3.4 μg S4).

were compared and the RI* was always found to be less reactive (data not shown). The decrease in reactivity may indicate a masking of S4 antigenic determinants by other ribosomal components or a conformational alteration in S4 that affects antibody binding. We have obtained preliminary evidence that S5 and S12 play major roles in the masking of S4 between the RI[4,7,8,15,16,17,19] and the RI* particles [18].

We have been unable to detect any reaction of S4-specific antibody with the 30S subunit. Antibody from eight rabbit antisera and several bleedings from each animal were examined to obtain a broad range of antibody specificities. These preparations have been shown to recognize antigenic determinants in peptide fragments covering residues 1–30, 31–102, 123–174, and 175–203 of S4 and thus recognize determinants widely distributed throughout the protein (data not shown). The antibody preparations do recognize the conformation of S4 in situ, as shown by their reaction with the RI[4,7,8,15,16,17,19] and RI* particles. Thus, we conclude that S4 is inaccessible to antibody on the 30S subunit. This conclusion directly contradicts several reports of S4 accessibility on the 30S subunit (2–5, 19–21). S4 was mapped on the surface of the 30S subunit by Lake et al [4, 19] using an AS4 preparation that was included in the experiments reported here (animal 5). We have now shown that the reaction of this antibody preparation with the 30S subunit was not S4-specific; thus, the reported location of S4 is incorrect.

Stöffler et al [1, 2] used several methods to investigate the accessibility of r-proteins, including S4, to antibody in the 30S subunit. Two of these methods, immunodiffusion and immunoprecipitation of intact 30S subunits, involved incubation of subunits with serum for long periods of time. Such conditions may result in substantial degradation of the subunits by serum nucleases and/or proteases and concomitant exposure of S4.

Sedimentation analysis and inhibition of poly-U translation were also used by Stöffler et al [2] to demonstrate a reaction of antibody with the 30S subunits. However, no test of specificity was reported. Therefore, these experiments did not eliminate the possibility that S4 is completely inaccessible to antibody and that the observed reaction was due to contaminating antibody directed against some other ribosomal protein.

This group has also mapped S4 on the surface of the 30S subunit using the immunoelectron microscopic approach [5, 20, 21]. The report of Tischendorf and Stöffler [5] contains the only data relevant to the specificity of the reaction of AS4 with the 30S subunit. Although it was stated that absorption experiments were done, the conditions and data were not reported. It is possible that contaminating antibodies were absorbed with a contaminant in the S4 used for absorption, particularly if an excess of antigen was used. The possibility of contaminating antibodies must be considered, since two of the antisera used gave multiple precipitin bands when reacted with 30S subunits in immunodiffusion experiments [2]. The explanation given, that these precipitin lines represent the reaction of AS4 antibodies with degraded or heterogeneous ribosomes, seems unlikely since such particles ought to have antigenic determinants of S4 in common and thus give a single precipitin band.

An additional argument for specificity given by Tischendorf and Stöffler [5] was the "enrichment" of the immunoglobulins from sera by affinity chromatography on S4-Sepharose. If the S4 antigen coupled to the column contained any contaminants, reactive antibody against that contaminant would also have been bound. Thus, antibody eluted from such an affinity column should not be assumed to be monospecific. These experiments did not eliminate the possibility that the observed subunit reactivity was due to antibody directed against another r-protein.

Although extensive regions of S4 are not accessible on the 30S subunit, small regions may still be exposed on the ribosome surface. Our findings are in good agreement with other approaches used to determine ribosomal protein accessibility. S4 in the 30S subunit is highly resistant to chemical modification by 2-methoxy-5-nitrotropone [22], trypsin digestion [23], glutaraldehyde modification [16], and reductive methylation with formaldehyde and $NaBH_4$ [24, 25].

Since S4 is inaccessible to antibody in the *intact* 30S subunit, direct immunochemical mapping of S4 determinants on the surface of the subunit will not be possible. However, S4 is exposed on the RI particles and these particles are similar in gross morphology to the intact 30S subunit [26, 27]. Thus, it may be possible to infer the location of S4 from mapping experiments carried out with subribosomal particles. The observation that S4 is accessible to antibody on ribosome assembly intermediates and masked on the 30S subunit also opens the possibility of probing the intermolecular interactions responsible for the masking of S4 antigenic determinants.

ACKNOWLEDGMENTS

We wish to thank M. Nomura, J. Lake, W. A. Strycharz, W. A. Held, and J. Schwarzbauer for helpful discussions, and Catherine Bloomer and Keiko Nomura for

valuable technical assistance. This work was supported by National Institute of Health grants GM22150 and GM07215.

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