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Localization of *Escherichia coli* Ribosomal Proteins S4 and S14 by Electron Microscopy of Antibody-Labeled Subunits

(*Bacillus stearothermophilus*/IgG antibody/ribosome topography/reconstitution)

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ABSTRACT **Binding sites for antibodies specific for proteins S4 and S14 of the small subunit of *E. coli* ribosomes have been mapped on the surface of the subunit by electron microscopy. Antibody binding to reconstituted subunits was shown to depend specifically on the presence of *E. coli* S4 and S14. Anti-S14 IgG was found to bind to a limited region of the ribosome surface. In contrast anti-S4 IgG was found to bind to three separated regions of the ribosome surface, suggesting S4 has an elongated conformation *in situ*.**

In order to understand the molecular basis of the functions performed by the small subunit of the *Escherichia coli* (*E.*) ribosome it is necessary to know the arrangement and function of the proteins and RNA in the ribosome. The availability of antibodies specific for individual proteins (1), the development of techniques for electron microscopic visualization of antibodies bound to subunits (refs. 2, 3, 4, and 5), and the interpretation of the two-dimensional images of small subunits seen in electron micrographs as views of a single three-dimensional structure, make possible the location of exposed ribosomal proteins in the three-dimensional structure of the ribosome.

In this paper we examine the distributions of binding sites for antibodies specific for two individual ribosomal proteins, S4 and S14, on the surface of the small subunit of E. ribosomes (preliminary report, ref. 5). Anti-S14 IgG (AS14) binding occurred only at one region of the small subunit, while anti-S4 IgG (AS4) binding occurred at three distinct regions of the subunit, indicating that S4 has an elongated structure *in situ*.

MATERIALS AND METHODS

E. (strain Q13) ribosomes were prepared according to Iwasaki *et al.* (6). Subunits were heated for 5 min at 40° in buffer I (10 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.8, 200 mM NH_4Cl), or for 2 min in buffer II (buffer I except 1 mM $MgCl_2$), then reacted with IgG antibodies for 2 min at 40° and then 20 min at 0°. Dimers and monomers of the small subunit were separated from each other and the unreacted antibodies by sedimentation on 15–30% sucrose gradients (3 hr at 234,000 × *g*, 4°, buffer I or buffer II). Sucrose was removed by gel filtration on Sephadex G-100. Alternately, 30S dimers and monomers were separated from unreacted antibodies by fractionation on a Sepharose 6B column (buffer I or buffer II). Both monomer and dimer fractions were examined by electron microscopy and the dimer fraction was occasionally augmented with part of the monomer peak to obtain optimum concentrations for negative staining.

Purification of *E. coli* and *Bacillus stearothermophilus* (*B.*) strain 799 ribosomal proteins, reconstitution of 30S subunits, and the assay of their activity were done as previously described (7, 8). The IgG fractions of specific rabbit antisera against purified ribosomal proteins (9) were purified by chromatography on DEAE-Sephadex A50 (10), ammonium sulfate precipitation, and gel filtration on Sephadex G-200. Electron micrographs were taken on a Phillips 301 microscope equipped with a liquid nitrogen cold finger at 80 kV. Samples were negatively contrasted by a double layer carbon modification of the method of Valentine (ref. 11, see also ref. 12) using 0.5%, 1.0%, or 2.0% uranyl acetate.

RESULTS

Small subunits were reacted with IgG antibodies against protein S14. Pairs of subunits joined by one or more antibodies were separated from monomers and free IgG, and the dimers were negatively stained and observed in the microscope (Fig. 1). The small subunit is characterized by a partition which divides its profile into an upper "one-third" and a lower "two-thirds" fraction. This feature is also observed in eukaryotic small subunits (13). Two views, corresponding to approximately orthogonal orientations of small subunits are shown schematically in Fig. 2. A quasi-symmetrical view characterized by an approximate line of mirror symmetry coinciding with the long axis of the subunit is diagrammed at the right, while an asymmetrical view which has both concave and convex profiles is shown at the left. In the asymmetrical view, the "one-third" region is tilted toward the concave side and a "hump" is present just below the partition on the convex side.

Fig. 1A shows pairs of subunits linked by IgG antibodies which form a "Y", with the Fab regions attached to the smaller third of the subunits. Usually a single AS14 antibody was attached to each subunit pair (e.g., the two central subunits); however, in about 30% of the pairs two antibodies joined them (Fig. 1A, pair at left). The simultaneous binding of more than two AS14 antibodies was never observed. Views of pairs in the symmetric and asymmetric views are shown in Fig. 1B and C, respectively. The exposed antigenic determinants of S14 are located on the concave side of the

Abbreviations: *E.*, *B.*, obtained from *Escherichia coli* and *Bacillus stearothermophilus*, respectively; AS14 and AS4, antibodies to proteins S14 and S4, respectively.

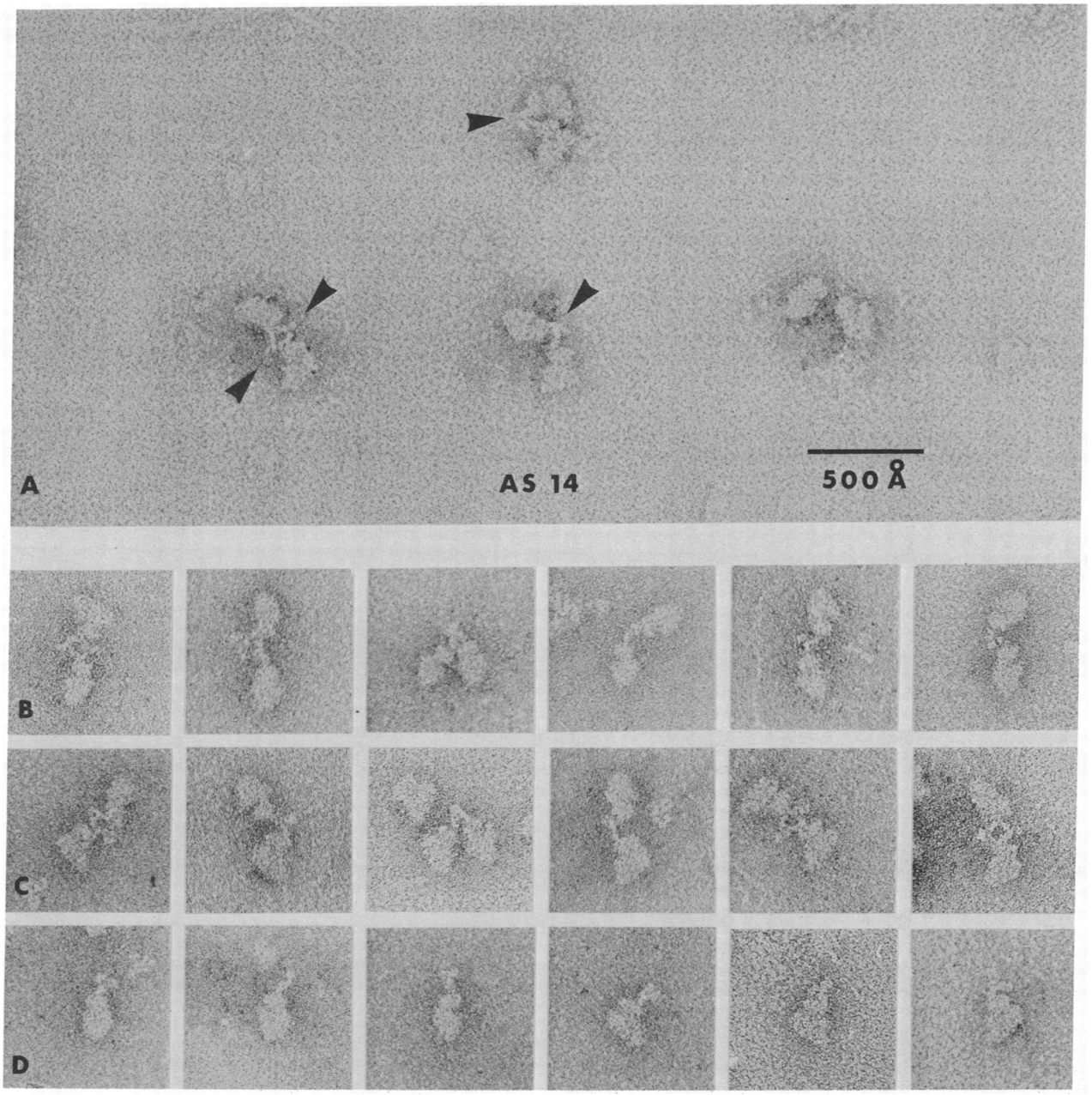

FIG. 1. Electron micrographs of small subunits reacted with AS14 antibodies. The antibodies attach only to a single region in the upper one-third of the subunit. The Fab regions and the Fc region of the antibodies are both visible. (A) A field showing pairs of subunits linked by one or two antibody molecules (indicated by arrows). (B) A gallery of dimers in which the symmetric profile is clearly identifiable (the lower subunit in each case). In the two frames at the left two antibody molecules are visible, bound to both subunits or to one subunit. (C) A gallery of dimers in which the asymmetric profile is visible (the lower subunit in each case). Two bound antibodies are visible in the frame at the left and the two frames at the right. (D) A gallery of monomers having a single bound AS14 antibody. The three frames at the left show monomers in the symmetric profile; the three frames at the right show monomers in the asymmetric profile.

smaller third in the asymmetric view and near the center line of the symmetrical view (Fig. 2).

AS14 binding was demonstrated to be dependent upon the presence of *E.* S14 by a technique that may in principle be applied to the binding of antibodies to all small subunit proteins. Each of the *E.* 30S ribosomal proteins (with the exception of S1) has a homologous counterpart in the *B.* 30S ribosome that may be substituted in the reconstituted *E.* ribosome without significant loss of functional activity (8). However, antisera to *E.* proteins are less reactive toward the corresponding *B.* protein (8, 14). Electron microscopic exami-

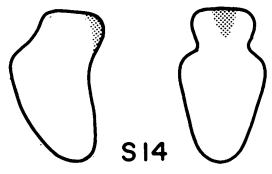

FIG. 2. A diagrammatic representation of the region of attachment of antibodies to protein S14. The site is represented by stippling in the symmetric view (right) and in the asymmetric view (left).

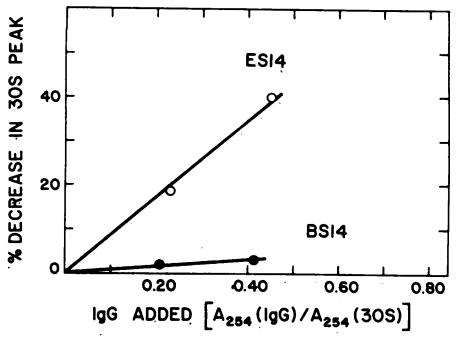

FIG. 3. *E.* S14 dependent aggregation of reconstituted subunits by AS14 antibody. Subunits were reacted with AS14 in buffer II and sedimented through sucrose gradients (buffer II, see *Materials and Methods*), and the amount of monomer 30S ribosomes was determined as the A_{254} of the monomer peak. The AS14-dependent aggregation of ribosomes containing *E.* S14 (O) and the corresponding *B.* protein (●) are shown expressed as percentage decrease from the monomer peak height obtained in the absence of IgG.

nation of subunits reconstituted from a mixture of purified *E.* proteins and 16S RNA, or a mixture of all *E.* proteins except S14, the *B.* homologue of *E.* S14, and *E.* 16S RNA showed no apparent differences in the electron microscopic profile of reconstituted subunits compared with those of isolated *E.* 30S subunits. Thus, it is possible to examine antibody binding to fully functional reconstituted subunits in which S14 has been replaced with a protein that has a reduced ability to bind antibody. Antibody binding to form dimers and higher aggregates was measured by the percentage reduction in the height of the 30S monomer peak in sucrose gradient sedimentation and is shown as a function of the amount of IgG added in Fig. 3. The hybrid subunits show a greatly reduced ability to form dimers (9% as many as nonhybrid subunits), indicating that antibodies bound to pairs of reconstituted *E.* subunits require the presence of *E.* S14. Additional control experiments demonstrating that the ability of AS14 to link subunits is similarly reduced by preabsorption with *E.* S14 showed that the attached antibodies are antibodies to S14.

Antibody labeling studies with AS4 show that antibodies to S4 attach at three distinct regions of the small subunit. These three sites are indicated by arrow heads in the field of subunits linked by AS4 antibodies in Fig. 4A. Two of the sites are in the head region and the third is located in the vicinity of the "hump" (see also Fig. 5). Galleries of AS4 antibodies attached to each of these three sites are shown in Fig. 4 in columns I, II, and III. The specificity of the observed antibody–ribosomal reaction is demonstrated by the binding of the identical combining sites of the antibody molecule with apparently the same location (Fig. 4A, B, and C) on two small subunits. Views of subunits joined by two antibodies were particularly valuable and facilitated the three-dimensional site determinations shown in Fig. 4. The off-center location of site III in the symmetrical view also establishes a relative hand for the structure.

Control experiments indicate that protein S4 is required for AS4 binding at all three sites. Fig. 6 shows the decrease in the amount of monomer as a function of added AS4 for reconstituted *E.* subunits and for reconstituted hybrid subunits with *E.* S4 replaced by the corresponding *B.* protein. The hybrid subunits are much less immunochemically reactive than reconstituted *E.* subunits, yet have the same functional activities as measured by poly(U)-dependent polyphenylalanine synthesis, and identical morphologies as determined by electron microscopy. An electron microscopic analysis of the frequency occurrence of antibodies bound to all three sites of both *E.* and hybrid subunits indicated that *distribution* of antibody binding to the sites of the hybrid subunit did not significantly differ from binding to reconstituted subunits containing *E.* S4 (Table 1). Since total AS4 binding to hybrid subunits is reduced by about 65% (Fig. 6), and the distribution of bound antibodies is not significantly altered, binding to each site is reduced and dependent on *E.* S4 (sites I, II, and III were reduced 72%, 59%, and 64%, respectively). The remaining reactivity might be due to some homology between the *E.* and the *B.* protein (14) [although this was not observed by Higo *et al.* (8)] or due to a small amount of *E.* S4 contaminant in one of the proteins used for reconstitution of hybrid subunits. Antibody binding to each of the three regions was also greatly reduced by preincubation of the AS 4 with a slight excess of *E.* S4, demonstrating that the attached antibodies are directed against S4.

DISCUSSION

In order to correctly interpret the results of experiments in which antibody molecules are reacted with complex antigens such as ribosomes (e.g., 2, 15, 16), it is necessary to determine the specificity of the interaction of the antibody with the ribosomal proteins *in situ*. The specificity of the interaction of antibodies with the purified ribosomal proteins in the precipitin reaction has been previously demonstrated (1, 9); however, these results indicate *only* that no pair of 30S ribosomal proteins share three or more common antigenic determinants (17, 18). While only limited sequence homologies have been found in the N-terminal regions of ribosomal proteins (19–21), it is possible that two proteins might have one or two common determinants which are accessible to antibody *in situ*, and that the reaction of antibody to a particular protein with homologous sites on some other protein might lead to incorrect interpretation of the results. In order to

TABLE 1. *Distribution of anti-S4 IgG bound to reconstituted 30S subunits*

Region	Binding to subunits containing *E.* S4, %	Binding to subunits containing *B.* "S4", %
Site I	29 ± 4	23 ± 7
Site II	27 ± 4	32 ± 9
Site III	44 ± 4	45 ± 11

Ribosomes were reconstituted from *E.* 16S RNA, and mixtures of either all of the *E.* 30S ribosomal proteins (ribosomes containing *E.* S4), or all of the *E.* 30S ribosomal proteins except S4 and the *B.* homologue of *E.* S4, *B.* "S4" [XII-1 in ref. (8)]. Reconstituted subunits were incubated with AS4 or IgG from nonimmune serum in buffer I at an A_{254} IgG/A_{254} ribosome ratio of 0.1 and analyzed by electron microscopy. IgG molecules bound to clearly identifiable sites were scored for binding to sites I, II, or III. The percentage of AS4 antibodies bound at each site to ribosomes is listed (225 antibodies bound to subunits containing *E.* S4 and 44 antibodies bound to subunits containing *B.* "S4" were scored) together with the standard deviation of each value.

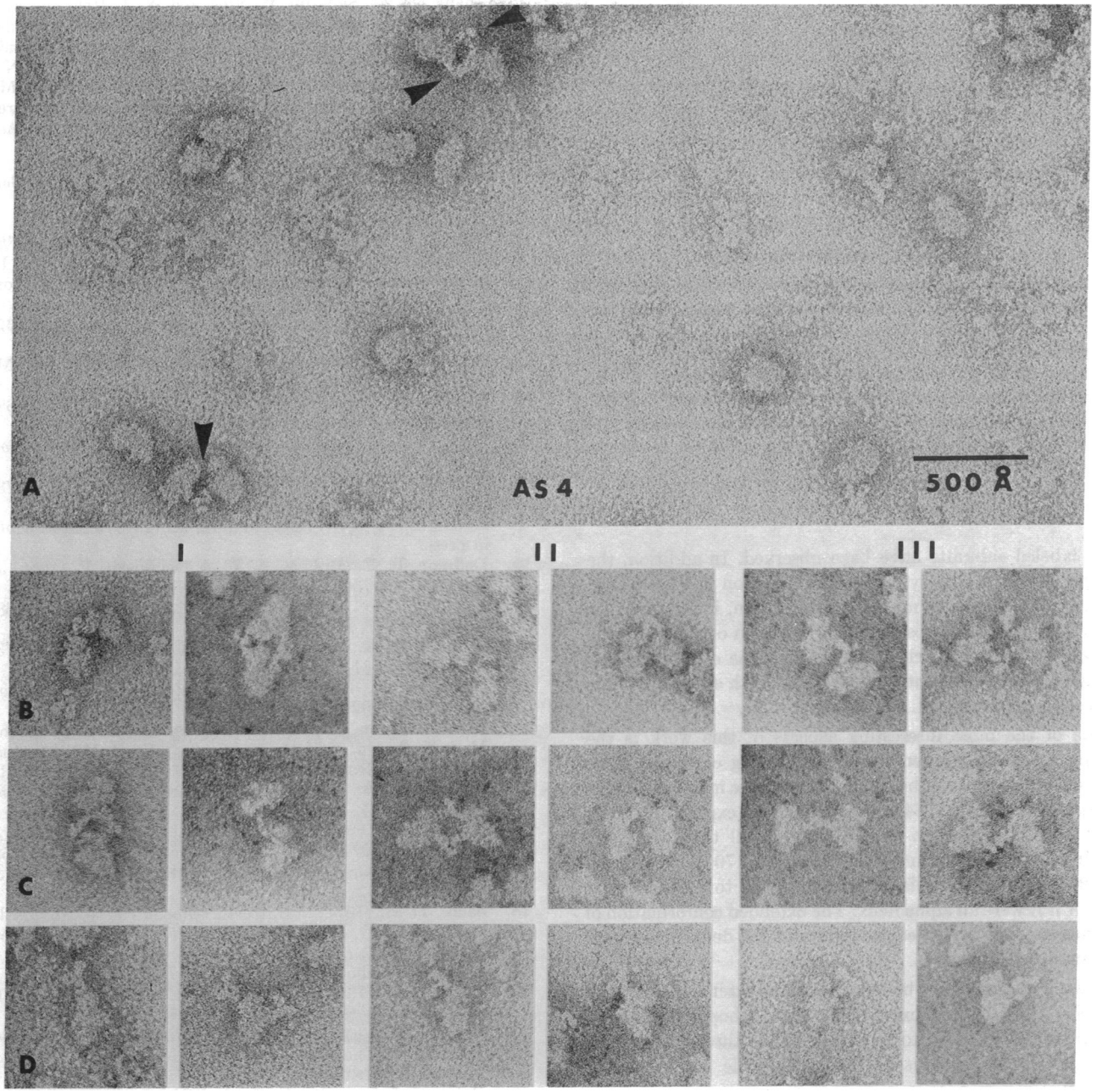

FIG. 4. Electron micrographs of small subunits reacted with AS4 antibodies. The antibodies attach to three regions of the subunit, shown schematically in Fig. 5. Sites I, II, and III are shown in the first, second, and third paired columns, respectively. Simultaneous bindings of antibodies to sites I and III, I and II, and II and III are shown in IB frame 2, IC frame 1 and IIC frame 1, respectively. (A) A field showing pairs of subunits with antibodies attached (arrows) at Sites III, I, and II from left to right. (B) Dimers in which the symmetrical profile is clearly shown (the lower subunit, or subunits, in each case). (C) Dimers in which the asymmetric profile is visible (the lower subunit in each case). (D) Monomers having single bound AS4 antibodies. The first frame of each site shows a symmetrical profile and the second frame shows an asymmetrical profile.

determine the specificity of the antibody-ribosomal protein reaction *in situ* we have replaced the *E.* ribosomal protein of interest with a corresponding *B.* protein (7) and thereby changed the antigenic composition of the ribosome without altering its functional activity and gross morphology, thus demonstrating that a particular *E.* protein is required for antibody binding.

We have considered the possibility of alterations in the structure of the subunit during the reaction with antibodies and during preparation of samples for electron microscopy. No major conformational differences between free and anti-

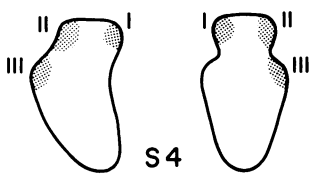

FIG. 5. A diagrammatic representation of the three regions of attachment of antibodies to protein S4. The regions are indicated by stippling in the symmetrical view (right) and in the asymmetrical view of the subunit (left) and are numbered.

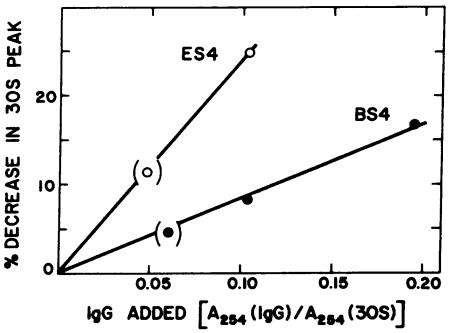

FIG. 6. *E.* S4 dependent aggregation of reconstituted subunits by AS4 antibody. Subunits were reacted with AS4 and the amount of 30S monomer ribosomes determined as described in the legend of Fig. 3. The AS4-dependent aggregation of subunits containing *E.* S4 (O) and the corresponding *B.* protein (●) are shown. The values in parentheses were obtained using buffer I, the other values using buffer II. Reaction with non-immune IgG produced no measurable aggregation with either of the reconstituted subunits.

body labeled subunits have been observed. In addition, the subunit profile in the symmetrical projection is consistently thinner than in the asymmetrical projection, indicating that there is probably no substantial compression occurring in the subunit in the symmetrical orientation. The electron microscopic profiles are not entirely consistent with either an oblate or prolate structure (ref. 22, see also ref. 3).

One of the most interesting aspects of this work is the finding that S4-dependent antibody binding occurs at three well-separated sites on the small subunit. The most reasonable interpretation is that S4 is elongated and exposed at these three sites. In order to extend through all three antibody binding sites, a minimum length of about 170 Å is required. S4 (203 residues) (23) is sufficiently large to extend about 30/5 Å if it were all alpha helix. The extended conformation of S4 thus suggests a new consideration in the determination of ribosome structure.

This approach can be succcessfully extended to other proteins (unpublished experiments). It is hoped that additional studies will lead to a solution of the three-dimensional arrangement of proteins in the prokaryotic 30S ribosomal subunit.

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