Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4031–4035, June 1985
Biochemistry

Secretory S complex of *Bacillus subtilis* forms a large, organized structure when released from ribosomes

(clathrin/ribosomal subunits/protein secretion/protein crosslinking)

MICHAEL P. CAULFIELD*†, DEIRDRE FURLONG*, PHANG C. TAI*‡§, AND BERNARD D. DAVIS*

*Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115; and ‡Department of Metabolic Regulation, Boston Biomedical Research Institute, Boston, MA 02114

Contributed by Bernard D. Davis, February 25, 1985

ABSTRACT **The S complex of *Bacillus subtilis*, a set of four proteins that appears to be involved in protein secretion, is shown to be attached to 70S ribosomes: antibody to its 64-kDa component can aggregate these ribosomes, and the complex can be chemically crosslinked to ribosomal proteins. Low Mg^{2+} or prolonged high-speed centrifugation in a sucrose gradient releases the S complex from the ribosomes, and it is recovered as an aggregate with an S value of 76. Electron microscopy shows that these aggregates have a regular structure, somewhat resembling clathrin cages, with a diameter of about 45 nm. If these aggregates are physiological, their function would differ significantly from that of the signal recognition particle of eukaryotes.**

A 64-kDa protein of *Bacillus subtilis*, present in the membrane fraction complexed with ribosomes (complexed membrane, CM), appears to be involved in protein secretion since the ribosomes protect it from interaction with protease or antibody (1); it is also present in the cytosol (2, 3). In addition, this protein is present in a complex of four proteins (the S complex), of 64, 60, 41, and 36 kDa, found in large amounts in the membrane-free monosomes (but not in the membrane–polysome complexes or in the cytosol) (3). These findings suggest that the S complex functions in the initiation step in protein secretion, like the signal recognition particle of eukaryote cells (4), but it differs significantly: it lacks an RNA molecule (at least as isolated), and it failed to cause translational arrest at the signal sequence of a secretory protein (3). The distribution of the 64-kDa protein further suggests that in the initiation cycle the other proteins of the S complex may be released after contact with the membrane, while the 64-kDa protein remains attached and then is released at a later stage.

This paper shows that the S complex separates from the ribosomes when they are dissociated into subunits by low Mg^{2+} or by high-speed centrifugation. However, the complex then sediments at about 76 S, rather than at the expected low values. Since the S complex in the monosome fraction can be chemically crosslinked to certain ribosomal proteins and anti-64-kDa IgG can aggregate the monosomes (as shown by electron microscopy), the 76S particle is evidently an aggregate of S complex, formed after release from attachment to the ribosomes. Moreover, after release, but not in the monosome fraction, the S complex is seen in the electron microscope as a specific, organized structure, larger than the ribosome. It is intriguing that this structure somewhat resembles the clathrin cages of eukaryotic cells, which have been implicated in membrane traffic (5).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Organism and Growth Conditions. *B. subtilis* ATCC 6051a was grown and labeled with [^{35}S]methionine as described (3).

Membrane and Ribosome Fractionation. Midlogarithmic phase cells were harvested, and the flagella were removed by shearing in an Omnimixer (Sorvall) at 9000 rpm for 30 sec before centrifugation in the high-salt wash (3). Cells were lysed by treatment with lysozyme and the French press, and the lysate was fractionated in a triphasic sucrose gradient as described (3). The CM-I fraction containing membrane, monosomes, and short polysomes banded at the 1.35/1.6 M interface.

Ribosomes lacking S complex were obtained as the pellet from a lysate centrifuged through a biphasic sucrose gradient, in the SW50.1 rotor, as described (6).

Preparation of S Complex Along with or Separated from Ribosomes. All sucrose solutions used were in TKM buffer [10 mM Tris·HCl, pH 7.6/50 mM KCl/10 mM $Mg(OAc)_2$], unless otherwise indicated.

To obtain a ribosome fraction containing S complex, CM-I was sedimented (45,000 rpm, 90 min, Spinco SW50.1 rotor) through a 10–30% linear sucrose gradient. The fractions containing 70S ribosomes (with the S complex, confirmed by $NaDodSO_4$ gel electrophoresis) were pooled, diluted in TKM buffer, and concentrated by centrifugation (50,000 rpm, 180 min, Spinco 60Ti rotor), and the pellet was dispersed in TKM buffer. To decrease contamination with 50S subunits and disomes the preparation was passed through a second 10–30% sucrose gradient and the 70S region was recovered.

In another method for preparing monosomes associated with S complex, the initial supernate from the lysate, after the bulk of the membrane/ribosomes had been removed by a 1-hr centrifugation (3), was centrifuged over a 1.35 M sucrose cushion (Spinco SW41, 37,000 rpm, 16 hr), which held back the membranes and allowed ribosomes to pellet. The pellet was dispersed in TKM buffer and the 70S fraction was recovered as above.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was conducted as described (2, 3).

Crosslinking of Ribosomal Proteins to the S Complex. Two A_{260} units of purified monosome/S complex, in 0.1 ml of 10 mM triethanolamine·HCl, pH 7.6/50 mM KCl/10 mM $Mg(OAc)_2$, was treated with 40 mM iodoacetamide (15 min, 37°C), followed by 0.4 mg of dithiobis(succinimidyl propionate) per ml as described (3) or by 12 mM 2-iminothiolane as described by Kenny and Traut (7). After boiling in 0.5% $NaDodSO_4$ and a 1:10 dilution with immunoprecipitation buffer (3) the material was treated with anti-64-kDa antibody

Abbreviation: CM, complexed membrane.

†Present address: Merck, Sharp and Dohme Research Laboratories, West Point, PA 19486.
§To whom correspondence and reprint requests should be addressed at: Boston Biomedical Research Institute, 20 Staniford Street, Boston, MA 02114.

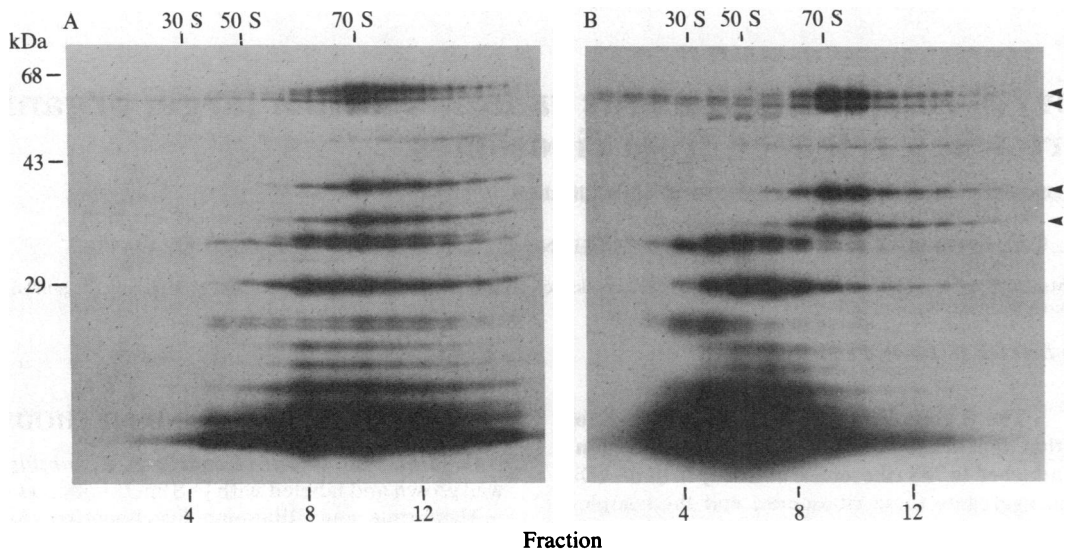

FIG. 1. Release of the S complex from ribosomes by low Mg^{2+}. (*A*) As a control, a sample of monosome/S complex, isolated from cells labeled with [^{35}S]methionine, was layered on a linear 10–30% sucrose gradient in TKM buffer. (*B*) To dissociate ribosomal subunits, a similar sample was diluted to lower the Mg^{2+} to 2 mM and was layered on a similar sucrose gradient with 0.1 mM $Mg(OAc)_2$. The gradients were centrifuged (Spinco SW50.1, 45,000 rpm, 90 min) and fractionated (eight drops). Fractions were analyzed for their protein composition by $NaDodSO_4$ gel electrophoresis. The positions of the 30S, 50S, and 70S ribosomes are marked, and arrowheads indicate the proteins (64, 60, 41, and 36 kDa) of the S complex; the other proteins are ribosomal.

bound to protein A-Sepharose (Pharmacia) as described (3). The immunoprecipitate was solubilized in $NaDodSO_4$ or 9 M urea, crosslinks were cleaved by mercaptoethanol, and the proteins were analyzed by one-dimensional $NaDodSO_4$ gel electrophoresis or by two-dimensional gel electrophoresis (8).

Preparation of Samples for the Electron Microscope. For examination under the electron microscope it was not necessary to concentrate the samples containing the S complex, described above. For staining a modification of the procedure described by Valentine and Green (9) was used. Samples were picked up between carbon and mica as described (10), the carbon was floated from the mica onto 0.5% uranyl acetate, and a copper grid was brought up quickly from the stain solution through an area of the carbon. The grid was then dried with a wick of filter paper.

To test for immune crosslinking, ribosomes (0.116 A_{260} unit/0.01 ml) or purified S complex (0.03 A_{280} unit/0.01 ml) was treated with rabbit antibody to the 64-kDa protein or with preimmune antibody (1 mg/ml of IgG) for 30 min on ice, diluted with TKM buffer to a final volume of 0.2 ml, and negatively stained. Control samples, without antibody treatment, were stained in a similar manner. Concentrations used were monosome/S complex, 0.116 A_{260} unit/0.2 ml and S complex alone, 0.01 A_{280} unit/0.2 ml.

A JEOL 100B electron microscope was used, operated at 80 kV with a 50-μm condenser and objective aperture.

Chemicals. All were reagent grade from commercial sources.

RESULTS

Separation of S Complex from Ribosomes. As a possible step toward isolating the S complex, we dissociated the ribosomes in the monosome fraction by lowering the Mg^{2+}. We expected the complex either to remain associated with one of the ribosomal subunits or to be released as a particle (of 200–400 kDa) that would sediment much more slowly than either subunit. However, although the S complex did separate from the dissociated 30S and 50S subunits, it sedimented around the 70S region (Fig. 1). A similar sedimentation of the S complex was observed when the ribosomes were dissociated and unfolded by excess EDTA (data not shown).

The S complex could also be separated from the ribosomes by centrifuging the 70S/S complex fraction to density equilibrium (Fig. 2). As would be expected, the separated protein complex was less dense than the ribosomes. When this complex was isolated and analyzed in a 10–30% sucrose gradient it was also found to sediment at about 70S, as in Fig. 1.

To determine the size of the separated complex more precisely it was fixed by glutaraldehyde, mixed with similarly fixed ribosomes lacking the complex, and centrifuged. As Fig. 3 shows, the S complex sedimented with an apparent S value of about 76.

Crosslinking of S Complex to Ribosomes. The similarity in S values of the ribosome and the S complex made it uncertain whether their cosedimentation in the monosome fraction is based on attachment or is only coincidental. In an attempt to answer this question the labeled, isolated monosome/S-

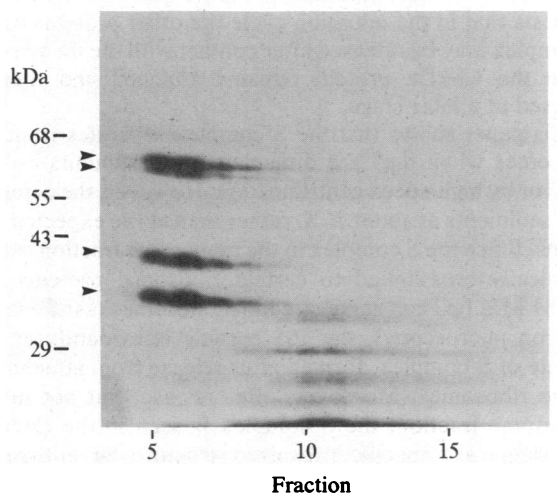

FIG. 2. Removal of the S complex from ribosomes by equilibrium density centrifugation. Monosome/S complex, isolated from cells labeled with [^{3}H]leucine, was centrifuged to equilibrium (SW50.1, 35,000 rpm, 60 hr) in a sucrose (1.6–2.4 M) gradient in TKM buffer. Fractions were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Arrowheads indicate the proteins of the S complex.

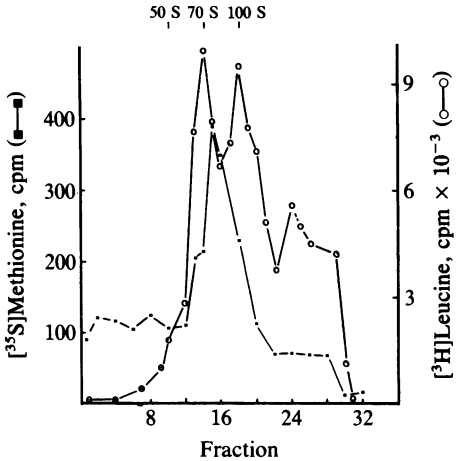

FIG. 3. Determination of the S value of the complex. S complex from cells labeled with [^{35}S]methionine was isolated by equilibrium density centrifugation (similar to fractions 5 and 6 of Fig. 2), crosslinked with 2% glutaraldehyde (2 min on ice), and passed through a G-25 Sephadex column to remove glutaraldehyde and sucrose. Ribosomes free of S complex, from cells labeled with [^{3}H]leucine, were treated similarly (fixation, Sephadex column). A mixture of the two preparations was centrifuged in a linear 10–30% sucrose gradient, and the radioactivity of each fraction (four drops) was counted. The positions of ^{3}H-labeled ribosomes are indicated by bars. The S-complex peak could be calculated to sediment at ≈76S.

complex peak was isolated and treated with dithiobis(succinimidyl propionate) or 2-iminothiolane, which might crosslink ribosomal proteins, directly or indirectly, to the 64-kDa protein. Samples were then solubilized with $NaDodSO_4$, diluted, and immunoprecipitated with the anti-64-kDa antibody. Dithiobis(succinimidyl propionate) was found to have crosslinked two ribosomal proteins to the S complex; 2-iminothiolane crosslinked a different ribosomal protein. These proteins coelectrophoresed with 50S ribosomal proteins (data not shown) in the two-dimensional electrophoresis system of Geyl *et al.* (8), but we have not yet identified them, since this system has not been correlated with the published numbers assigned to the *B. subtilis* ribosomal proteins. In a control, with the monosome/S-complex fraction treated with EDTA before treatment with dithiobis(succinimidyl propionate) or 2-aminothiolane, no crosslinking to ribosomal proteins was observed.

Electron Microscopic Demonstration of Attachment of the S Complex to Ribosomes. Though the above findings showed that some S complex could be crosslinked to ribosomes, they did not establish whether much or little of the S complex in the 70S preparation had that location. Therefore, we studied that fraction by electron microscopy. It revealed normal 70S ribosomes, occasionally appearing to have a small appendage (Fig. 4*A*). Only a rare aggregated S complex, with a distinctive structure (see below), was seen: fewer than one per 500–1000 ribosome particles, but more frequently after repeated freeze-thawing of the preparation. Thus, it appears that most of the S complex in this kind of preparation is attached to ribosomes, as a particle too small to be seen on most ribosomes.

To demonstrate the attachment more directly and to test for its frequency, we used immunoelectron microscopy, a technique in which dimerization of ribosomes by antibody to specific ribosomal proteins has been used to localize these proteins on the ribosome surface (11, 12). After incubation of the monosome/S-complex sample with antibody to the 64-kDa protein, the ribosomes appeared as dimers or as larger ag-

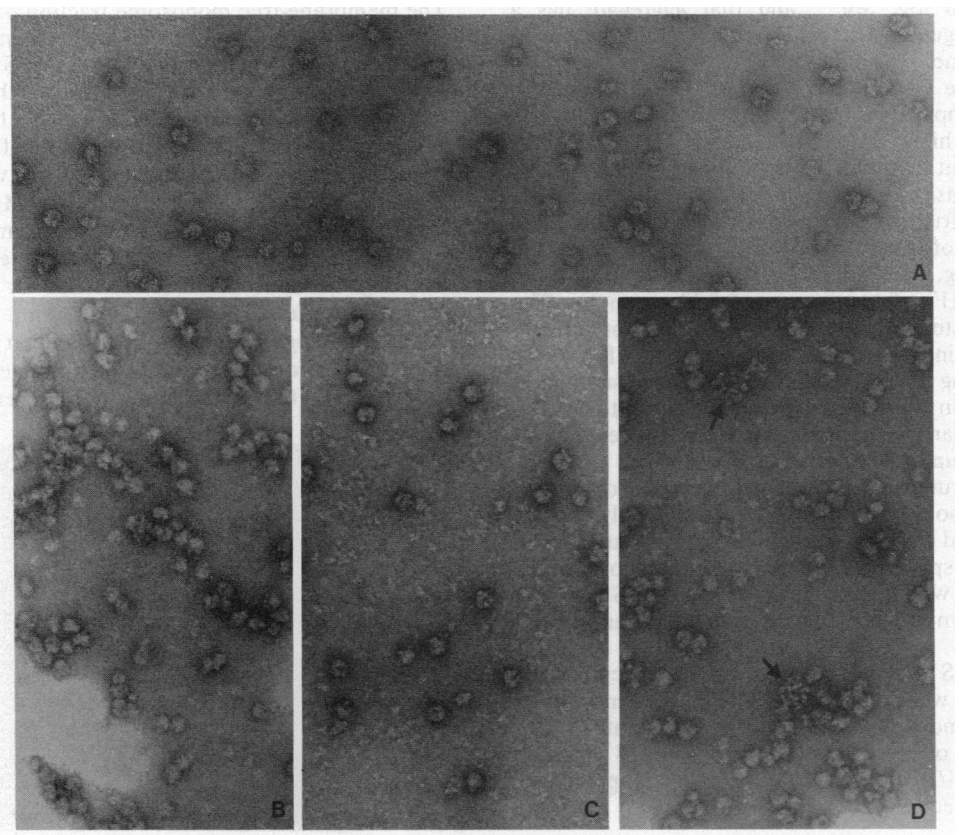

FIG. 4. Electron micrograph of monosome/S complex and its aggregation by antibody to the 64-kDa protein. (*A*) Ribosomes with S complex were applied to a grid and negatively stained with 0.5% uranyl acetate. (*B*) Monosome/S-complex sample treated with anti-64-kDa IgG. (*C*) Monosome/S complex treated with preimmune IgG. (*D*) Mixture of monosome/S complex and S complex treated with anti-64-kDa IgG. Organized structures (arrows) were often found to bind to ribosomes. (×105,000.)

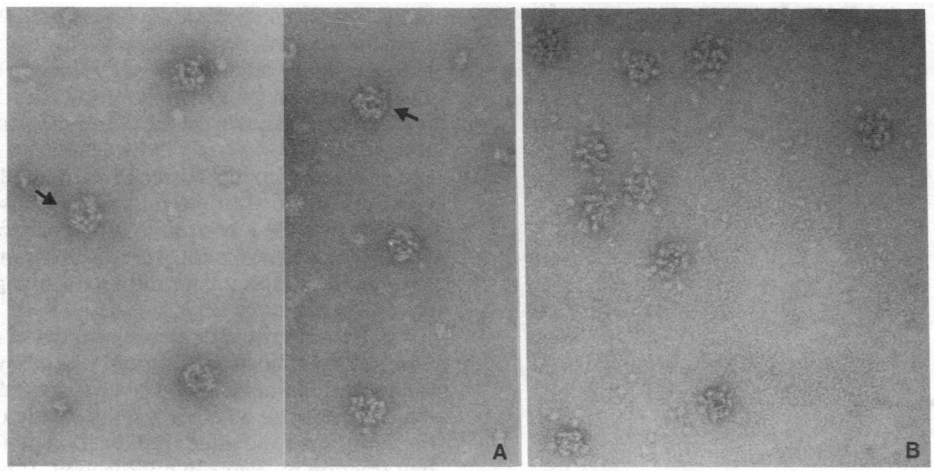

FIG. 5. Structure of the free S complex. (*A*) S complex was isolated by equilibrium density centrifugation (equivalent to fractions 5 and 6 of Fig. 2). Particles of *ca.* 45 nm in diameter, exhibiting 5-fold symmetry, were frequently seen (arrows). (*B*) S complex from *A* was centrifuged in a 10–30% sucrose gradient and the 76S region was isolated. (×105,000.)

gregates much more frequently (Fig. 4*B*) than in the untreated sample (Fig. 4*A*). In addition, small Y-shaped particles, presumably IgG molecules, are seen in the background, and a few appear to be attached to ribosomes. With preimmune serum (Fig. 4*C*) or with the anti-64-kDa antibody and 70S ribosomes lacking S complex (data not shown), no such attachment of IgG or aggregation of ribosomes was seen.

Thus, it is clear that the S complex is intimately associated with the 70S ribosome and that low Mg^{2+} or high centrifugal force reverses the association. Moreover, when the complex is released from ribosomes it forms a large aggregate with itself, resistant to low Mg^{2+}, and that aggregate has a remarkably homogeneous S value, coincidentally close to that of a monosome.

Structure of the S Complex Removed from Ribosomes. Electron microscopy also provided an explanation for the homogeneous and high S value of the released S complex. As Fig. 5*A* shows, that material, separated from the ribosomes by equilibrium density centrifugation, is not amorphous but has an organized structure highly suggestive of a hollow cage, with a diameter of 45–47 nm, which is larger than the ribosome (see Fig. 4*D*, arrows). These structures often exhibited a 5-fold (Fig. 5*A*, arrows) or 6-fold symmetry. The surface shows clusters of mass in distinctive pairs or in rings. In rotary shadowing the projection was round for every particle, supporting the inference that the dense staining in the center is within a hollow cage rather than within a flat structure. These particles evidently arose by aggregation of the released S complex in a specific manner.

Many smaller structures, of *ca.* 10 nm, were also observed, some in pairs and some in rings. These are evidently units of S complex released from the larger, organized structures in the preparation of specimens for electron microscopy, since a 76S preparation, which contained only the four S-complex proteins (not shown), also exhibited both 45-nm and 10-nm particles (Fig. 5*B*).

The presence of S complex both in the large structures and on the monosomes was confirmed by incubating a mixture of S complex and monosome/S complex with anti-64-kDa antibody. Linking of the cages and monosome/S complex can be seen (Fig. 4*D*). Furthermore, some of the presumed IgG Y-shaped molecules can be seen to attach to the large structure.

The aggregated S complex resembles in appearance published pictures of clathrin cages from eukaryotic cells, and so we compared the two structures in a mixture. As Fig. 6 shows, the bacterial structure, of *ca.* 45 nm, is somewhat smaller than the clathrin-coated vesicles, whose reported range is 65–125 nm (13). Small asymmetric structures of *ca.* 10–15 nm are also seen; they may be subunits (or pairs of subunits) released from either particle during specimen preparation.

In contrast to clathrin cages, which release triskelion subunits when the pH is raised from 6.5 to 7.5 (14), the S-complex cages did not dissociate into subunits at a pH as high as 8.5 or at pH 6.0 (data not shown).

DISCUSSION

The membrane-free monosome fraction of *B. subtilis* (probably including polysomes that were fragmented during cell lysis) contains a large amount of the S complex of four proteins (3). The present work shows that in equilibrium density centrifugation, which dissociates the ribosomes (15, 16), the S complex sedimented separately from the ribosomal particles, at a position of lower density as would be expected from its lack of RNA. Moreover, at low Mg^{2+} the S complex separated, in zonal sedimentation, from the dissociated ribosomal subunits. However, it sedimented at the unexpectedly high S value of 76 (established by comparison with ribosomes, after glutaraldehyde fixation).

These findings raised the possibility that in the original 70S fraction the S complex might be cosedimenting with, rather than attached to, the ribosomes. However, chemical cross-

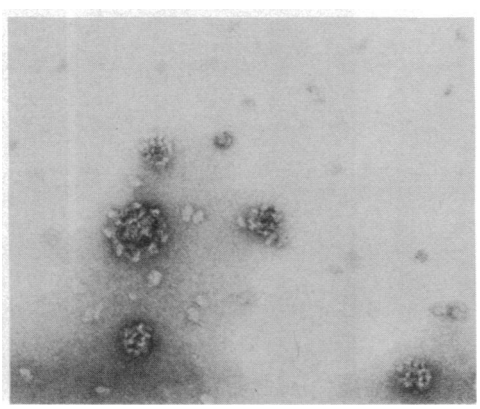

FIG. 6. Comparison of clathrin-coated vesicles and S-complex cages. Samples of bovine brain vesicles (40 μg/ml) and S complex (0.05 A_{280} unit/ml) were mixed and examined, as in Fig. 5. (×105,000.)

linking of that fraction demonstrated attachment of the S complex to several proteins of the 50S ribosomal unit, whereas at low Mg^{2+} this crosslinking was not observed. Moreover, antibody to the 64-kDa component of the complex caused ribosomes to dimerize and aggregate in the 70S/S-complex fraction but not in a ribosome preparation that lacked the S complex.

In the 70S/S-complex fraction evidently only one or very few S-complex units (of *ca.* 200 kDa) are attached per ribosome, for in the electron microscope the ribosomes did not differ in appearance from those in a preparation lacking the S complex: the images of a few suggested an additional small protuberance. The two kinds of preparations also did not differ significantly in density when examined in a sucrose gradient after fixation by glutaraldehyde (unpublished). On the other hand, after separation of the S complex from the ribosomes by low Mg^{2+} or by high centrifugal force, the resulting 76S particles were seen by electron microscopy to be a regular, organized structure, which appears to be icosahedral and hollow; it is clearly not an amorphous aggregate. This structure (diameter, *ca.* 45 nm) is larger than the ribosome, but its lower density evidently accounts for its sedimentation at a similar S value. The rarity of this structure in the monosome/S-complex fraction confirmed the conclusion that most of the S-complex units in that fraction are attached to ribosomes.

Curiously, the structure of the aggregated S complex somewhat resembles that of the clathrin cages seen in eukaryotic cells (17). However, there are major differences: the clathrin cages from various sources contain mainly three proteins, of molecular masses 180, 36, and 33 kDa; they have a larger diameter, of 65–125 nm (13); and the cages formed *in vitro* have an S value of about 300 (18). Also in contrast to clathrin cages (14), exposure to low pH (6.0) or high pH (8.5) did not dissociate the S-complex aggregates. Finally, clathrin is implicated in a late stage of secretion, the transfer of the proteins from the endoplasmic reticulum to the Golgi apparatus (19), whereas the S complex appears to be involved in initiating secretion. It is interesting that the dissociated clathrin triskelions can bind to membranes as well as form cages (20).

Since the S complex is present on ribosomes as a small particle, which is released by low Mg^{2+}, it may be viewed as a third ribosomal subunit, as has been suggested for the eukaryotic signal recognition particle (4). The large amount recovered in the 70S fraction suggests that a large fraction of the ribosomes in a growing bacterial cell carries the particle. Since the 64-kDa protein is also present, but without the S complex, in membrane–polysome complexes and in the cytosol, it is possible that the S complex breaks up following initiation of secretion; hence, the intact S complex may well not exist free in the cell. We have not yet studied its affinity for ribosomes in various stages of their cycle of translation.

The role of the organized aggregate of S complex is less clear. It may be an artifact of laboratory manipulation, resulting perhaps from hydrophobic forces that promote its interaction with the membrane in the initiation of protein secretion. On the other hand, its uniformity suggests that it has a function. As one possibility, once the S complex on a ribosome recognizes a signal sequence it may change its own conformation in such a way that other S-complex subunits will associate with it and form an organized structure around the emerging protein, thus keeping it isolated from the cytoplasm until contact with the membrane.

This proposed role for the S complex would be quite different from that of the signal recognition particle of eukaryotes, which arrests synthesis of secreted proteins on ribosomes in an *in vitro* system and then releases the arrest on interaction with added membrane (21). Using an *Escherichia coli in vitro* system, we have not been able to demonstrate translational arrest by the *B. subtilis* S complex (3). However, in the possible action proposed above for the complex there is no need to postulate such an arrest, as the formation of a protective structure would prevent release into the cytoplasm of a secreted protein whose contact with the membrane is delayed. Furthermore, in bacteria (as well as in eukaryotic cells) post-translational as well as cotranslational secretion have been demonstrated (22, 23), and the formation of a cage around the secreted protein could be particularly valuable for the former pathway.

Much more evidence would be needed to establish a function for the S-complex aggregates. However, if this model proves to be correct it would suggest that the clathrin system of intracellular protein transport in eukaryotes may have evolved from a system originally present in prokaryotes. It is also interesting to speculate that bacteria may have evolved such a system because their mRNA, unlike that of eukaryotes, is translated as it is being transcribed from the DNA; hence, if bacteria employed a system involving a signal recognition particle their DNA would have many points of indirect contact, via RNA and via secreting ribosomes, with the membrane. In contrast, a protein transport system involving a sequestering organized structure would simplify the network of contacts.

We thank Daniel Branton and Steve Moseley for providing clathrin-coated vesicles; Donna Bielinski, Evelyn Schweig, and Peter Tang for technical assistance; and Dotty Syrigos for typing the manuscript. This work was supported by grants from the National Institutes of Health and the American Cancer Society.

1. Horiuchi, S., Tai, P. C. & Davis, B. D. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3287–3291.
2. Horiuchi, S., Marty-Mazars, D., Tai, P. C. & Davis, B. D. (1983) *J. Bacteriol.* **154,** 1215–1221.
3. Caulfield, M. P., Horiuchi, S., Tai, P. C. & Davis, B. D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7772–7776.
4. Walter, P. & Blobel, G. (1982) *Nature (London)* **299,** 691–698.
5. Salisbury, J. L., Condeelis, J. S. & Satir, P. (1983) *Int. Rev. Exp. Pathol.* **24,** 2–63.
6. Marty-Mazars, D., Horiuchi, S., Tai, P. C. & Davis, B. D. (1983) *J. Bacteriol.* **154,** 1381–1388.
7. Kenny, J. W. & Traut, R. R. (1979) *J. Mol. Biol.* **127,** 243–263.
8. Geyl, D., Bock, A. & Isono, K. (1981) *Mol. Gen. Genet.* **181,** 309–312.
9. Valentine, R. C. & Green, M. (1967) *J. Mol. Biol.* **27,** 615–617.
10. Lake, J. A. (1979) *Methods Enzymol.* **91,** 250–257.
11. Wabl, M. R. (1974) *J. Mol. Biol.* **84,** 241–247.
12. LaVie, J. A., Pendergast, M., Kahan, L. & Nomura, M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4688–4692.
13. Kirchhausen, T. & Harrison, S. C. (1981) *Cell* **23,** 755–761.
14. Woodward, M. P. & Rothe, T. F. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4394–4398.
15. Infante, A. A. & Baierlein, R. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1780–1785.
16. Spirin, A. S. (1971) *FEBS Lett.* **14,** 349–353.
17. Pearse, B. M. F. (1975) *J. Mol. Biol.* **97,** 93–98.
18. Van Jaarsveld, P. P., Nandi, P. K., Lippoldt, R. E., Saroff, H. & Edelhock, H. (1981) *Biochemistry* **20,** 4129–4135.
19. Rothman, J. E. & Fine, R. E. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 780–784.
20. Schmid, S. L., Matsumoto, A. K. & Rothman, J. E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 91–95.
21. Walter, P. & Blobel, G. (1981) *J. Cell Biol.* **91,** 551–556.
22. Chen, L. L., Rhoads, D. B. & Tai, P. C. (1985) *J. Bacteriol.,* **161,** 973–980.
23. Muller, M. & Blobel, G. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7421–7425.