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Structural Properties of Fibrillar Proteins Isolated from the Cell Surface and Cytoplasm of *Streptococcus salivarius* (K^+) Cells and Nonadhesive Mutants

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Most *Streptococcus salivarius* (K^+) cells contain two protein antigens with different adhesive functions. The subcellular distribution and some structural properties of purified proteins were studied. Antigen B (AgB), a protein involved in interbacterial coaggregation with gram-negative bacteria, was present in the cell wall fraction only of the wild-type strain and was absent from the cells of a nonadhesive mutant. Antigen C (AgC), a glycoprotein involved in host-associated adhesive functions, was predominantly associated with the cell wall of the wild-type strain (AgC_w), but accumulated in high amounts in the cytoplasmic fraction (AgC_{in}) of mutants lacking the wall-associated form. AgB, AgC_w, and AgC_{in} had molecular weights of 380,000, 250,000 to 320,000, and 488,000, respectively, upon gel electrophoresis under nondenaturing conditions. In the presence of sodium dodecyl sulfate and β-mercaptoethanol the molecular weights were only slightly lower, suggesting that the free, isolated molecules exist as monomers under native conditions. AgC_{in} readily stained with periodate-Schiff reagent, indicating a significant content of carbohydrate, similar to AgC_w. Circular dichroism spectra showed that about 45% of the amino acids of AgC_w were involved in α-helical coiled structures. AgB had a significantly lower proportion of ordered coiled structure. Electron microscopic observations of low-angle-shadowed preparations of purified antigens showed that they were flexible, thin rods with thickened or globular ends. Measurements corrected for shadow thickness showed lengths of 184 nm (AgB), 112 nm (AgC_{in}), and 87 nm (AgC_w). Treatment of AgC_w with protease destroyed the fibrillar core, but seemed not to affect the globular ends. Comparison of the results with the localization of the antigens in wild-type and specific mutant strains suggested that each antigen molecule may represent a single, characteristic surface fibril with a specific adhesive capacity.

A generalized model of bacterial adhesion to host surfaces involves adhesins on the bacterial surface which interact by a lectinlike mechanism with components of the host surface (2, 10, 17). The bacterial adhesins often appear associated with surface appendages of the cells, such as pili, fibrils, or fimbriae. These models have mainly been derived from studies on piliated gram-negative bacteria. Although a similar mechanism may apply to gram-positive bacteria, the direct demonstration of the presence of adhesins, the identification and characterization of these compounds, and their subcellular localization have only been provided in a limited number of cases (3, 9, 15).

A variety of adhesive functions have been attributed to different strains of streptococci, e.g., binding of salivary proteins (6), human cell surface components (23), serum proteins (12), and bacterial cell surface carbohydrate (11, 30). Multiple functions may be expressed by the same cell (11, 29). However, with the exception of the M protein of group A streptococci, very little is known about the localization, the chemistry and structure, and the mechanisms of synthesis, translocation, and regulation of expression of adhesins and other cell wall-associated proteins of the streptococci.

Streptococcus salivarius Lancefield group K, a successful and early colonizer of the human oral cavity, is an interesting organism in this respect. These bacteria possess a complex surface morphology (8) and surface biochemistry (28) and a variety of adhesive capabilities (29). At least three protein antigens were observed at the cell surface of *S. salivarius* HB, two of which were identified as adhesins with different specificities (28). These protein antigens were previously isolated, purified, and biochemically characterized (28). Antigen B (AgB) mediates the interbacterial coaggregation with particular gram-negative oral bacteria (26, 31), whereas antigen C (AgC) is mainly involved in the adhesion of the bacteria to human oral surfaces (28). In an accompanying paper (27) we have shown that the adhesins are associated with specific classes of surface fibril.

In this paper we report on the fibrillar morphology and biochemical characteristics of the purified adhesins and survey their subcellular localization. The results indicate that synthesis and assembly of both adhesins are independent and that intact AgC is accumulated intracellularly in a mutant lacking the molecule at the cell surface.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. salivarius* HB and mutant strains HB-7 and HB-V5 have been described previously (27–29). Mutant strain HB-V51 was prepared from HB-V5 by nitrosoguanidine mutagenesis according to previously described procedures (29) and was unable to attach to buccal epithelial cells or to aggregate with saliva; in addition it was unable to coaggregate with veillonellae.

Unless otherwise stated, the strains were grown in batch culture in Todd-Hewitt broth for 16 h at 37°C in air with 5% CO_2.

Purification of protein antigens. Wall-associated protein antigens were prepared from mutanolysin-solubilized cell

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walls as described previously (28), with the following modifications: after chromatography on DEAE-Sephadex A25 and Sepharose CL-2B columns, the fractions containing peak antigen activity were subjected to preparative isoelectric focusing in a granular gel bed (LKB, Bromma, Sweden) using a pH gradient of 2.5 to 4.6. AgB was thus purified to homogeneity as judged from polyacrylamide gel electrophoresis and crossed immunoelectrophoresis. Preparations of AgC were found to be contaminated by a minor antigen which was successfully removed by affinity chromatography on a column of Concanavalin A-Sepharose (Pharmacia Fine Chemicals).

Cytoplasmic AgC was purified by immunoaffinity chromatography. After cells were broken by shaking with glass beads in a Braun cell disintegrator (31), the preparation was centrifuged for 15 min at 18,000 × *g* to remove unbroken cells and cell wall debris. Subsequently the supernatant was centrifuged for 45 min at 40,000 × *g*, which yielded a membrane-enriched fraction. This supernatant was dialyzed for 24 h at 4°C against a 50 mM potassium phosphate buffer (pH 7.3) and subsequently applied to a column containing immunopurified antibodies against AgC immobilized on Affigel-10 (Biorad Laboratories; 0.2 nmol of immunoglobulin G per 50 nmol of active ester in MES [morpholineethanesulfonic acid] buffer [pH 6.5]; column dimensions, 6 by 1.0 cm). The column was preequilibrated with 0.1 M phosphate buffer (pH 7.3) containing 0.5 M NaCl and eluted with the same buffer until no more UV-adsorbing material (220 nm) was detected in the eluate. Bound antigens were released by washing the column with 0.1 M glycine hydrochloride buffer (pH 2.5). The pH of the eluate was quickly neutralized by the addition of solid Tris, and the eluate was dialyzed against distilled water and freeze dried.

Immunological procedures. Specific polyvalent antisera against AgB and AgC were prepared by immunoaffinity chromatography as described previously (28). The antisera were stored at -20°C in the presence of 1% bovine serum albumin.

Crossed immunoelectrophoresis and rocket immunoelectrophoresis (RIEP) in 1% agarose gels using a Tris-Veronal buffer system (pH 8.6) were described previously (28). Bromophenol blue was used as a marker for electrophoretic mobility in the first dimension.

PAGE. Polyacrylamide gel electrophoresis (PAGE) in slab gels in the presence of sodium dodecyl sulfate (SDS) was done in 5 to 9% gradient gels as previously described (28). The samples were solubilized by boiling for 5 min in sample buffer containing 2% SDS and 2.5% β-mercaptoethanol. Nondenaturing PAGE was carried out with commercially available 4 to 30% slab gels (Pharmacia), using the borate buffer system recommended by the manufacturer, for 16 h at 150 V. The samples were solubilized in electrophoresis buffer at room temperature. Proteins were stained with Coomassie blue, and carbohydrates were stained with the periodate-Schiff reagent.

Rotary shadowing. Low-angle (6°) rotary shadowing of purified antigen preparations was carried out by the procedures described by Shotton et al. (22). Briefly, very small drops of the antigen solution were applied to a freshly cleaved mica surface. The antigen solutions contained 0.2 to 1 μg of protein per ml of 0.3 M ammonium acetate (pH 6.8) containing 70% (vol/vol) glycerol. The droplets were evaporated in vacuo in a Balzers freeze-etch apparatus, and the mica surfaces were coated with a 10-nm-thick supporting film of carbon. A platinum/carbon mixture (95% Pt) was then deposited at a 6° shadow angle to a thickness of 1.1 nm, using a sample rotation speed of 120 rpm. The replicas so formed were examined in a Philips 300 electron microscope at 60 kV.

Dimensions of the molecules were estimated by measuring about 100 well-resolved molecules and correcting for shadow thickness using the true width of concomitantly shadowed tropomyosin molecules (2 nm), as described by Phillips et al. (20).

Circular dichroism spectra. Circular dichroism spectra of AgB and AgC were recorded over the range 190 to 260 nm with a Roussel-Jouan Dichrograph II recording spectropolarimeter. A quartz cuvette with a 1.0-mm light path length was used at room temperature. The test substances were dissolved in double-distilled water at a concentration of about 0.2 mg (dry weight) per ml. Protein concentrations of the samples were measured by the method of Peterson (19) with bovine serum albumin as standard.

RESULTS

Subcellular distribution of protein antigens. RIEP of fractions obtained after differential centrifugation of preparations of broken cells was used to establish the subcellular localization of the antigens (Table 1). In the wild-type strain HB, both AgB and AgC were predominantly associated with the cell wall fraction. Only traces of the antigens were detected in both the cytoplasmic and membrane fractions of this strain, which is in accordance with immunoelectron microscopic data (27). AgB could not be detected in significant amounts in any subcellular fraction of mutants HB-V5 and HB-V51, but was normally expressed in the cell walls of mutant HB-7. AgC was virtually absent from the cell wall fractions of mutants HB-7 and HB-V51. However, high intracellular concentrations of AgC (AgC_{in}) were detected in both these mutants. Mutant HB-V5 had very low AgC_{in} levels, comparable to the wild-type strain HB. In all mutants the amount of antigen associated with the membrane-enriched fractions was very low when tested in RIEP. The low activity in these preparations was confirmed by Western immunoblot analysis after SDS-PAGE (not shown here).

Structure of the protein antigens. Preparations of AgB, AgC, and AgC_{in} showed apparent homogeneity both in nondenaturing PAGE (Fig.1) and crossed immunoelectrophoresis (Fig. 2).

R_f values relative to bromophenol blue indicated that AgC_{in} has a lower electrophoretic mobility than AgC derived from mutanolysin-solubilized cell walls (AgC_w) (Fig. 2). Comparison of the molecular weights of these preparations also showed differences (Table 2). AgC_w gave rise to two

TABLE 1. Subcellular distribution of protein antigens in *S. salivarius* HB and mutants defective in adhesive properties, measured by RIEP[a]

Strain	AgB (rocket area, mm²) from:		AgC (rocket area, mm²) from:	
	Solubilized cell walls	Intracellular fraction	Solubilized cell walls	Intracellular fraction
HB	36	3	44	trace
HB-7	33	trace	0	60
HB-V5	0	0	50	trace
HB-V51	0	0	0	85

[a] Cells were fractionated as described in the text. The equivalent of about 100 μg (dry weight) of walls and 40 μg (dry weight) of intracellular material were applied. Cytoplasmic membrane fractions were also tested, but did not contain detectable amounts of soluble antigens under the conditions applied.

major protein bands with molecular weight (mol wt) between 250,000 and 320,000 under native conditions, which is similar to those shown previously (33). AgC_{in}, however, showed a single band with a mol wt of 488,000. All AgC bands also stained with periodate-Schiff reagent, confirming the glycoprotein nature of AgC (28), and reacted with AgC-specific antiserum in Western immunoblots (not shown). In the presence of SDS and β-mercaptoethanol, AgC_w had only a slightly lower mol wt (28, 33). AgB had a native mol wt of 380,000, which is slightly higher than the value of 320,000 obtained in SDS-PAGE (28). The relatively small differences obtained under native and denaturing conditions are likely to result from conformational changes induced by boiling in the presence of SDS. This suggests that both AgB and AgC exist essentially as monomers under native conditions.

Circular dichroism spectra of wall-associated AgB and AgC_w were prepared to obtain additional information on the structure of these compounds. Both spectra showed negative ellipticities, with AgC_w having minima at 208 nm and 222 nm characteristic for α-helical structures (33). AgC_w showed an ellipticity of 15,300 degrees cm^{-2} per dmol of protein at 222 nm, which indicates that approximately 45% of the amino acid residues are involved in α-helix structures. This relatively high α-helix content suggests that at least part of the molecule is fibrillar. AgB showed less distinct features of α-helical structures with a broad and shallow minimum at about 217 nm. An ellipticity at 222 nm of 3,900 degrees cm^{-2} per dmol would indicate that about 19% of amino acids are involved in ordered coiled structures. The results therefore show that AgB has a significantly higher proportion of random coiled structure than AgC_w. It should be noted, however, that the values obtained may be underestimates since the antigens were prepared in the presence of Triton X-100, which still may be partly bound to the proteins. Also the carbohydrate moiety of AgC_w may affect the results, so that the values presented have to be taken as approximations.

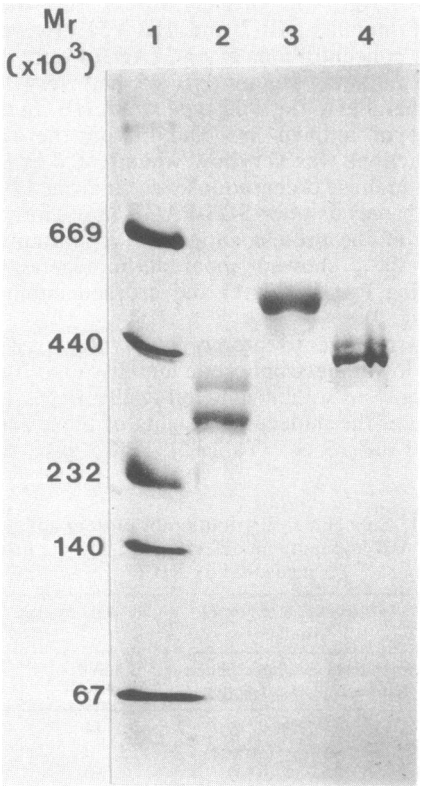

Fig. 1. Nondenaturing PAGE of purified protein antigens. The samples were dissolved at room temperature and applied to a 4 to 30% gradient gel. Proteins were stained with Coomassie brilliant blue. Lane 1, Molecular weight markers; lane 2, AgC_w; lane 3, AgC_{in}; lane 4, AgB.

Electron microscopy of antigens. Low-angle rotary shadowing was used to visualize the isolated, free antigens in an electron microscope (Fig. 3). AgC_w, AgC_{in}, and AgB showed a typical flexible, rodlike structure with slightly or conspiciously swollen ends. These globular ends were most prominently seen in preparations of AgB. In each case the core structure seemed to be a very thin fibril, to which various amounts of material could be appositioned, sometimes giving rise to an irregular outline. No particular tendency to aggregate into larger complexes was noted, although occasionally possible dimers of polarly connected fibrils were seen. Length measurements of the free, isolated fibrillar structures are presented in Table 2. Comparison of AgC_{in} and AgC_w shows that the latter is significantly shorter, which is commensurate with its lower mol wt. AgB was the longest of the fibrillar preparations tested. It was not possible to obtain reliable estimates of the true width of the fibrils by this method, although the fibril core appeared not significantly different from the width of tropomyosin molecules (2 nm).

To confirm the proteinaceous nature of the fibrils, purified AgC_w was subjected to treatment with pronase under conditions known to remove fibrils from the surface of whole cells (8). PAGE showed that under such conditions a variety of lower-mol-wt breakdown products were present, whereas the immunological reactivity in RIEP was almost completely lost (not shown). Protease treatment resulted in the disappearance of the fibrillar core, but apparently did not affect the structural integrity of the globular fibril ends (Fig. 3D). This suggests that the fibril core represents the protein moiety of the glycoprotein.

DISCUSSION

Cell walls of *S. salivarius* HB and most other group K strains of *S. salivarius* contain three major proteinaceous components (28). Two of these, AgB and AgC, are associated with different adhesive functions of the cell (28, 31). These adhesins were subsequently located in the fibrillar layer peripheral to the solid cell wall (8, 27, 32). The electron microscopic observations made in the present study on the isolated and purified adhesins indicate that under conditions which do not break covalent bonds, the smallest antigen molecules available have a fibrillar structure. Consequently, it seems likely that each adhesin represents a single cell surface fibril, belonging to one of the fibril classes observed on whole cells (27). Each fibril class of *S. salivarius* would thus carry a specific function. However, recent observations of cell surface properties of mutants of an *S. sanguis* strain could be interpreted in favor of different functions carried on the same fibril (7). Based on their presence and localization on the cell surface of particular mutant strains, AgC would be identical with the 72-nm fibrils and AgB would be identical with the 91-nm fibrils seen in negatively stained preparations of whole cells (27). The length measurements made of free, isolated AgC are comparable with those made on negatively stained whole cells, considering the widely different techniques applied. AgC_{in}, isolated from the cytoplasm of strain HB-7, was larger, both in length and mol wt,

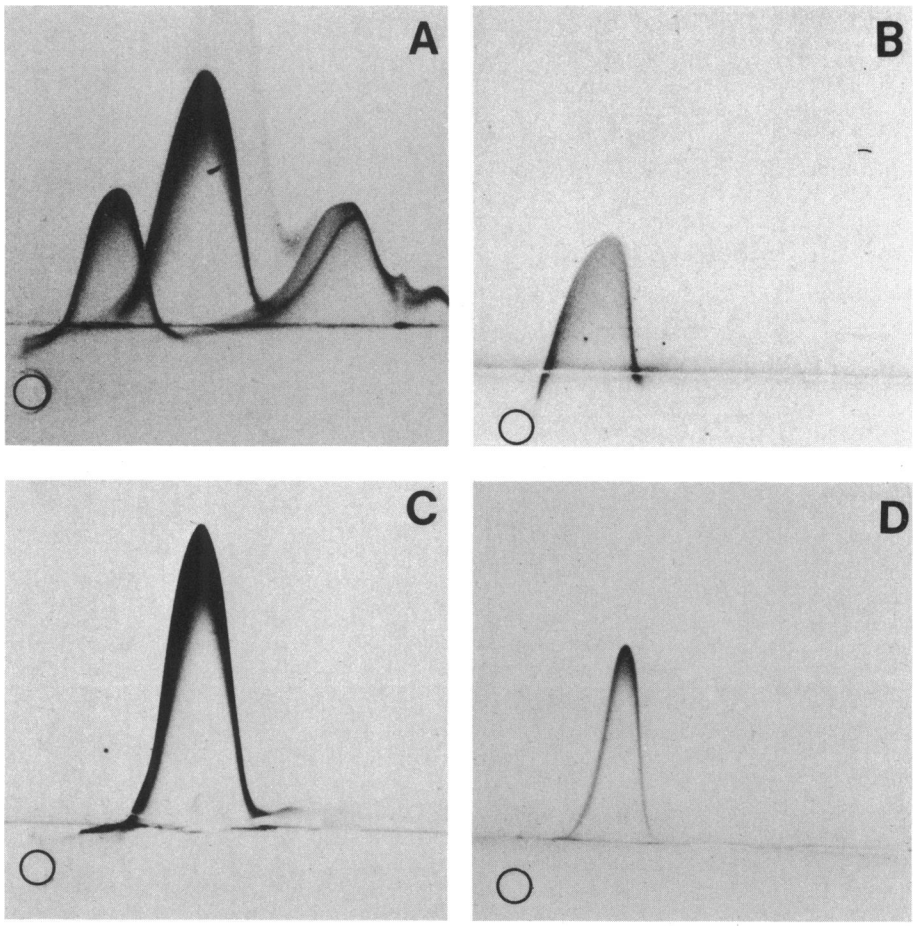

Fig. 2. Crossed immunoelectrophoresis of purified protein antigens against whole antiserum developed against *S. salivarius* HB cell walls. Samples (10 μl) were applied to the wells and electrophoresed for 90 min at 10 V/cm for 90 min. Bromophenol blue was used as a marker for electrophoretic mobility in the first dimension. The relative mobilities were AgB, 0.14; AgC_w, 0.30; and AgC_{in}, 0.21. Electrophoresis in the second dimension was done for 16 h at 5 V/cm in agarose containing 1% (vol/vol) of whole anti-HB serum. (A) Mutanolysin digest of strain HB containing the equivalent of about 80 μg (dry weight) of walls; (B) AgB, 5 μg (dry weight); (C) AgC_w, 5 μg; (D) AgC_{in}, 5 μg. The gels were stained with Coomassie brilliant blue.

than AgC_w. One explanation for this may be that during the mutanolysin treatment of the walls part of the molecule is lost. In this respect it may be important that an extracellular form of AgC which is excreted into the culture medium during growth also has a higher mol wt, although not as high as AgC_{in} (28). Alternatively, it may be that AgC_{in} is associated with components which are not present in the surface-associated antigen. The length of free, isolated AgB appears to be twice the length of the corresponding fibril on the surface of the cells (27). This apparent discrepancy cannot be readily explained at present. The similarity in mol wt of AgB under both native and denaturing conditions argues against the possibility that the isolated antigen represents a dimer of the molecule.

Considerable evidence indicates that the protein moiety of AgC is at least partly responsible for the fibrillar configuration of this compound. It was suggested from circular dichroism data that a high proportion of amino acid residues was involved in α-helical structures and that treatment with protease destroyed the fibrillar core, leaving globules resembling the ends of the fibrils. AgB, which contains little carbohydrate, showed a higher content of randomly coiled structure. The basic configuration of this fibril is therefore unclear. The structural properties of AgB and AgC show similarities with bacterial surface proteins of group A streptococci (M protein) and *Staphylococcus aureus* (protein A), which may also form projections on the cell surface (5, 20). Whether one or both fibrillar antigens are arranged according to the basal α-helical plans suggested by Cohen and Phillips (4) and based on the M protein and protein A structures, respectively, is not clear and will require a more detailed structural study. A further similarity between the *S.*

TABLE 2. Mol wts and dimensions of purified fibrillar protein antigens of *S. salivarius* HB

Antigen	Source of prepn	Molecular wt[a]		Fibril length (nm ± SD)[b]
		Native	SDS	
AgC_w	Solubilized cell wall	250,000–320,000	220,000–280,000	87 ± 3.5
AgC_{in}	Cytoplasm	488,000	NT	112 ± 5.0
AgB	Solubilized cell wall	380,000	320,000	184 ± 9.0

[a] Determined from PAGE in the absence and presence of SDS and β-mercaptoethanol; data partly from reference 28. NT, Not tested.

[b] Determined by low-angle rotary shadowing. About 100 fibrils of each type were measured.

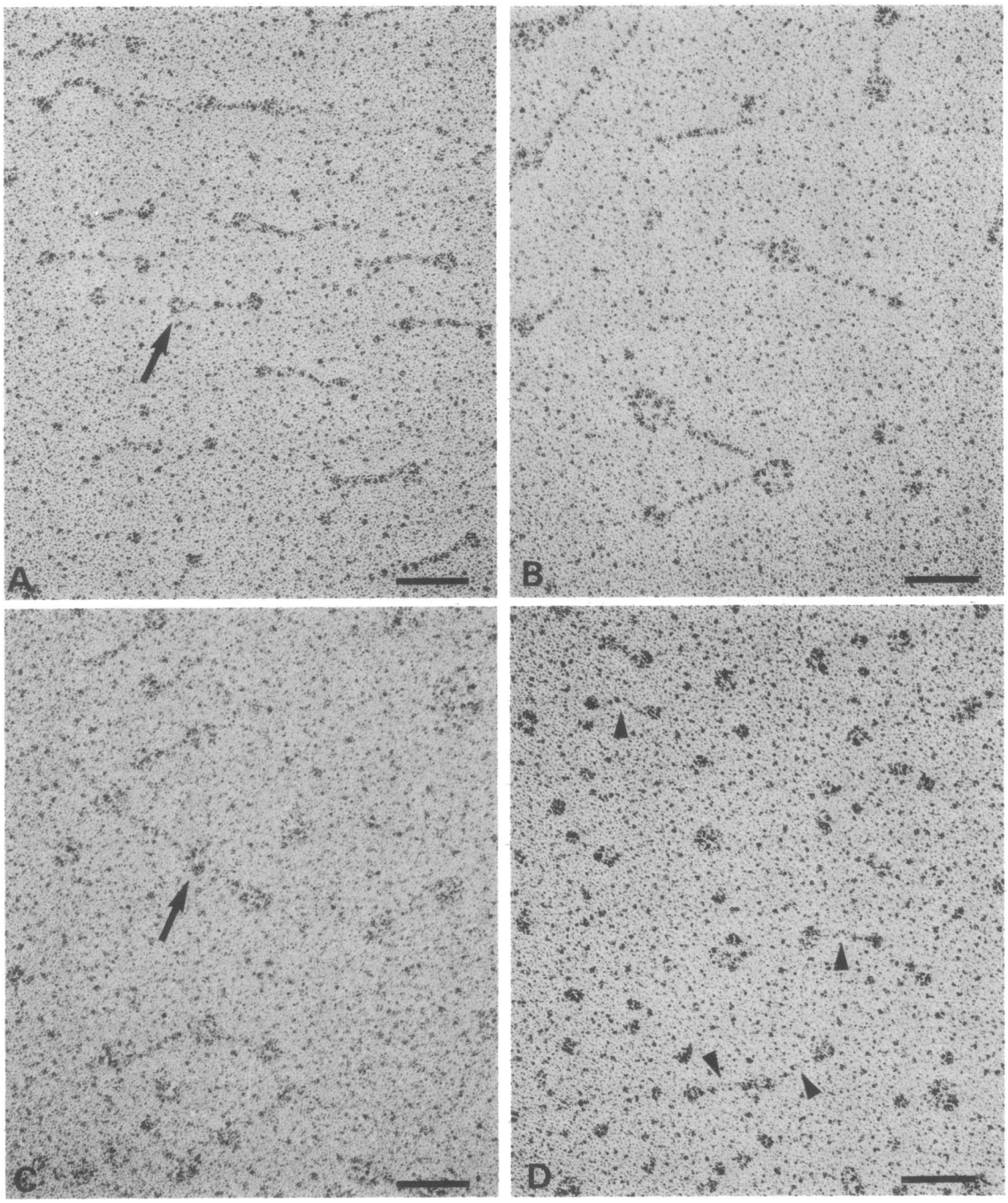

Fig. 3. Electron micrographs of low-angle rotary-shadowed individual molecules of purified protein antigens. (A) AgC_w prepared from cell walls of strain HB; (B) AgB prepared from cell walls of strain HB; (C) AgC_{in} prepared from the cytoplasm of strain HB-7; (D) AgC_w treated with pronase before electron microscopy. Arrows indicate possible dimers. Arrowheads in D indicate incompletely hydrolyzed AgC molecules. Bar markers, 70 nm.

salivarius fibrillar proteins (32), M protein and protein A (25), and surface fibrils of other streptococcal strains (7) is that these compounds may confer a high surface hydrophobicity to the cells.

Surface morphologies similar to those observed for *S. salivarius* are also found on a variety of other streptococci (13, 16, 20), and surface-associated proteins are common constituents of different streptococci (1, 21). Although in most of these cases a direct proof of an association with adhesive functions has not been provided, this association would be strongly suggested from our results with *S. salivarius*. Other gram-positive bacteria, such as oral actinomycetes and *Corynebacterium renale* (9, 34), contain adhesive fibrils with biochemical properties very similar to those of the *S. salivarius* adhesins.

It was remarkable to note that AgC was accumulated in the cytoplasm of mutant strains HB-7 and HB-V51 in the form of structurally intact fibrils, whereas intracellular accu-

mulation of AgB was not observed. This indicates that these adhesins are expressed independently and at different biochemical levels. The former conclusion is supported by the independent expression of these antigens which was observed under various growth conditions in continuous cultures (32). Interestingly, our observations also differ from those made with *Streptococcus mutans* isolates, which lose cell wall-associated proteins upon repeated subculture, apparently through the failure to incorporate the translocated proteins into the cell wall (14). *S. salivarius* wild-type strain HB excretes both AgB and AgC in the culture supernatant (28), but the mutants which lack the wall-associated antigens do not excrete the missing compound (unpublished data). The mutation leading to the absence of AgB is therefore most likely expressed at the level of protein synthesis. In case of AgC, however, the translocation of the fibril, or a fibrillar precursor, over the cytoplasmic membrane seems blocked in a specific way. An analogous intracellular accumulation of an extracellular protein was observed for alkaline phosphatase in a nonsecreting mutant of *Bacillus licheniformis* (24). This was explained by the absence of an as yet putative membrane receptor-mediated secretory apparatus. Alternatively, the accumulation of AgC_{in} may result from the accidental assembly of the fibrils inside the cell, thus preventing the translocation of precursors over the membrane. Since little is known about the synthesis, translocation, and incorporation of cell surface appendages in gram-positive bacteria, our observations cannot be completely explained. These mechanisms are clearly different in certain respects from those in gram-negative bacteria (18). Further studies on the translocation and incorporation of AgB and AgC into the cell wall are now in progress in our laboratory and should help to elucidate these mechanisms.

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