

## Oligomerization of the Bacteriophage Lambda S Protein in the Inner Membrane of *Escherichia coli*

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**Western blot (immunoblot) analysis of cell extracts from induced bacteriophage lambda lysogens probed with S-protein-specific antibody (raised against an S-β-galactosidase fusion protein) demonstrated that the bacteriophage lambda S protein begins to appear 10 min after phage induction and is localized to the inner membrane at all times during the lytic cycle. Between 100 and 1,000 molecules of S protein per cell were present at the time of phage-induced lysis. Western blots of chemically cross-linked membranes from induced lysogens showed a ladder of bands at 18, 24, 32, and 42 kilodaltons (the S-protein monomer ran at 8 kilodaltons) that reacted with anti-S-protein antibody. Thus, the S protein appears to reside in the inner membrane as a multimer, and the molecular weights of the cross-linked species are consistent with those of S-protein homopolymers. Sodium dodecyl sulfate-resistant dimers were also detected when S protein was purified by immunoprecipitation.**

The lytic cycle of bacteriophage lambda concludes with the synchronous lysis of the host *Escherichia coli* cells and the efficient release of approximately 100 newly synthesized phage particles per cell. Two phage genes are sufficient for this synchronous lysis, as demonstrated by the cloning of the lambda lysis region into a plasmid under the transcriptional control of the *lac* operon (14, 15). The lambda *R* gene encodes a soluble 17.5-kilodalton (kDa) transglycosylase that is synthesized in an active form in the cytoplasm and is capable of degrading the periplasmic peptidoglycan layer (4-7). The lambda *S* gene product increases the permeability of the inner membrane at 40 to 50 min postinfection and thereby initiates cell lysis (15, 35, 36, 50). A third phage gene product, the Rz protein, is required for efficient lysis when the cells are grown in the presence of high concentrations of magnesium and is thought to encode an endopeptidase activity which cleaves peptide bonds that are present in the peptidoglycan layer (53). The simplest model for the mechanism of *S* gene-induced lysis is the formation or activation of a large nonspecific pore(s) through the inner membrane that allows the cytoplasmic *R* (and possibly Rz) gene product access to the periplasm at the time of lysis (50). This hypothesis accounts for the increased membrane permeability that was observed in the presence of the S protein and incorporates a reversible element (the opening and closing of the pore), which is compatible with experimental results that have been obtained by using the temperature-sensitive *S* allele (50).

Initial reports identified both a 15-kDa acidic protein in the inner membrane fraction of lambda-infected cells and a 5.5-kDa protein found in phage particles as the products of the *S* gene (50, 51). In contrast, when maxicells and UV-irradiated cells were used to suppress host gene expression, Altman et al. (1, 2) identified the *S* gene product as an 8.5-kDa protein that was localized primarily to the inner membrane. Recently, genetic analysis of a battery of mutations in the *S* gene has revealed an unexpected complexity in *S* gene expression (33, 34). Evidence has been presented

which suggests that there are two translational start sites controlled by an upstream stem-loop structure, resulting in the production of two proteins, S<sub>107</sub> and S<sub>105</sub>, which differ by two N-terminal residues (8a). It has been proposed that the regulation of the timing of lysis might involve the rate of synthesis or degradation of both S-protein species.

Despite its central role in phage-induced lysis, the *S* gene product remains almost entirely uncharacterized. S protein is present in the cell in very low quantities, even at the time of lysis, and its lethal function makes it impossible to overproduce it from a multicopy plasmid. Moreover, without an enzymatic activity assay or specific labeling technique, the detection of S protein has presented a formidable barrier to its identification, localization, and purification. The approach taken in this study was to construct an inducible *S-lacZ* fusion gene that overexpressed an S-β-galactosidase fusion protein. Antibody raised against this S-β-galactosidase fusion protein was then used to determine the time course of S-protein synthesis, its localization during the phage lytic cycle, and the state of oligomerization of S protein in the cell membrane.

### MATERIALS AND METHODS

**Bacterial strains, bacteriophages, plasmids, and growth conditions.** The bacterial strains, bacteriophages, and plasmids used in this study are described in Table 1. When required, antibiotics were added to the growth media at the following concentrations: 50 μg of ampicillin per ml, 30 μg of chloramphenicol per ml, 50 μg of kanamycin per ml, or 15 μg of tetracycline per ml.

Bacteriophage lambda strains were maintained as thermally inducible lysogens of CSH7. To induce a lambda prophage, the lysogenic culture was grown at 32°C in LB medium (24) to an optical density at 600 nm (OD<sub>600</sub>) of between 0.3 and 0.4, incubated at 45°C for 8 min, and then placed at 37°C for 60 min. A *Salmonella typhimurium* culture to be infected with phage P22 was grown at 30°C in LB medium supplemented with 0.2% maltose and 10 mM MgCl<sub>2</sub> to an OD<sub>600</sub> of between 0.3 and 0.4, infected with phage at a multiplicity of infection of 5, and incubated for 60 min. All other general phage procedures were performed as described by Arber et al. (3).

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TABLE 1. Bacterial strains, bacteriophage strains, and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
<i>E. coli</i> K-12		
CSH7	F <sup>-</sup> <i>lacY</i> <i>strA</i> <i>thi</i>	26
JM101	$\Delta$ ( <i>lac-proAB</i> ) <i>thi</i> <i>supE</i> (F' <i>traD36 proAB lacI</i> <sup>s</sup> $\Delta$ M15)	52
<i>S. typhimurium</i> LT2 DB7000	<i>leuA414</i> (Am)	A. R. Poteete
<i>E. coli</i> K-12 phage		
$\lambda$ S <sup>+</sup> Ram	$\lambda$ cI857 Ram60	J. W. Roberts
$\lambda$ Sam Ram	$\lambda$ cI857 Sam7 Ram60	J. W. Roberts
<i>S. typhimurium</i> LT2 phage		
P22 13 <sup>+</sup> 19am	P22 cI-7 19amH447	A. R. Poteete
P22 13 am19 <sup>+</sup>	P22 cI-7 13amH101	A. R. Poteete
Plasmids		
pJG1	Amp <sup>r</sup> <i>lacPO</i> S <sup>+</sup> Ram54am60 Rz <sup>+</sup>	15
pJG3	Amp <sup>r</sup> <i>lacPO</i> Sam7 Ram54am60 Rz <sup>+</sup>	15
pJG200	Amp <sup>r</sup> $\lambda$ p <sub>R</sub> $\Phi$ ('collagen'- <i>lacZ</i> )	16
pLys	Chl <sup>r</sup> T7 gene 3.5	F. W. Studier
pMC1871	Tet <sup>r</sup> ' <i>lacZ</i>	12
pSZ5	Amp <sup>r</sup> <i>lacPO</i> $\Phi$ (S' <sub>1-104</sub> '- <i>lacZ</i> ) <sup>a</sup>	This study
pSCZ5	Amp <sup>r</sup> <i>lacPO</i> $\Phi$ (S' <sub>1-104</sub> '-collagen'- <i>lacZ</i> )	This study

<sup>a</sup> Subscripts indicate S codons.

**Enzymes and chemicals.** Restriction enzymes, T4 DNA ligase and kinase, the Klenow fragment of *E. coli* polymerase I, and *Bal* 31 exonuclease were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), or P-L Biochemicals, Inc. (Milwaukee, Wis.), and were used according to the recommendations of the suppliers. Unphosphorylated *Bam*HI linkers (12-mer) were obtained from New England BioLabs and were treated with T4 kinase prior to use. The detergents Triton X-100 and octyl- $\beta$ -D-glucoside were purchased from Pierce Chemical Co. (Rockford, Ill.) and Calbiochem-Behring (La Jolla, Calif.), respectively. Unless otherwise noted, all other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.), including isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside, *o*-nitrophenyl- $\beta$ -D-galactopyranoside, purified  $\beta$ -galactosidase, and *p*-aminobenzyl 1-thio- $\beta$ -D-galactopyranoside-agarose.

**Assays and gel electrophoresis.** Protein concentrations were determined by the method of Bradford (9), with bovine serum albumin used as a standard. The trichloroacetic acid-Lowry procedure (23, 32) was used when dithiothreitol (DTT) or high levels of detergents were present.  $\beta$ -Galactosidase activity was assayed as described by Miller (26), using CHCl<sub>3</sub>-sodium dodecyl sulfate (SDS) treatment to permeabilize whole cells. One unit of  $\beta$ -galactosidase activity was defined as the amount that hydrolyzed 1  $\mu$ mol of *o*-nitrophenyl- $\beta$ -D-galactopyranoside per min at 28°C in Z buffer.

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was based on the protocol of Laemmli (21), except that the Tris hydrochloride concentration was doubled in both the running buffer and the running gel (but not the stacking gel) to produce narrower, better-resolved protein bands (13). Prestained molecular weight markers were purchased from Bethesda Research Laboratories. Following electrophoresis, SDS-polyacrylamide gels were set up for Western blot (immunoblot) transfer, cut into slices for electroelution, or stained with Coomassie blue or silver (30).

**S-*lacZ* and S-collagen-*lacZ* fusion gene construction.** All

standard DNA cloning procedures were performed as described by Maniatis et al. (24). Plasmid pJG1 carries the lambda lysis region of  $\lambda$  S<sup>+</sup> Ram under control of *lacPO* (15). Digestion of pJG1 with *EcoRV*, followed by treatment with *Bal* 31 exonuclease, resulted in a pool of plasmid fragments that was estimated to be deleted of the 3' end of the S gene. After the addition of *Bam*HI linkers, these fragments were ligated with T4 DNA ligase and used to transform competent WFK cells, resulting in approximately 1,000 transformants. Plasmid DNA was isolated from the transformation plates, purified on a CsCl gradient, and digested with *Bam*HI and *Sal*I. The 4.5-kilobase-pair *Bam*HI-*Sal*I fragment was ligated with a 3.1-kilobase-pair *Sal*I-*Bam*HI fragment (containing *lacZ* sequences from codons 8 to 1021) isolated from pMC1871 (12). Competent JM101 cells were transformed with the ligation mix and plated onto 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside-ampicillin plates (26); 19 of 1,100 transformants showed  $\beta$ -galactosidase activity in this screening procedure. Plasmid pSZ5 (Fig. 1A) was chosen for further study because it generated the highest levels of  $\beta$ -galactosidase activity after induction with 1 mM IPTG and contained almost the entire S gene sequence, as estimated by restriction mapping. The DNA sequence at the S-*lacZ* fusion junction of pSZ5 was determined by dideoxy sequencing (39, 40) using a single-stranded template derived from the sequencing vector M13mp10 (25) with the *Eco*RI-*Bam*HI fragment of pSZ5 subcloned into the polylinker region.

To create a tribrid fusion gene that encoded a protein with N-terminal S-protein sequences fused to collagen- $\beta$ -galactosidase sequences, the 5.8-kilobase-pair fragment isolated from a *Pst*I-*Bam*HI double digestion of pJG200 (16) was ligated with the 1.5-kilobase-pair fragment (containing the *lacPO* and S' sequences) isolated from a *Pst*I-*Bam*HI digestion of pSZ5 to generate the plasmid pSCZ5 (Fig. 1B). Like the S- $\beta$ -galactosidase tribrid protein encoded by pSZ5, the expression of the S-collagen- $\beta$ -galactosidase tribrid protein SCZ5 is under the transcriptional control of *lacPO* sequences.

**Characterization and purification of the S- $\beta$ -galactosidase fusion protein SZ5.** By using anti- $\beta$ -galactosidase antibody,

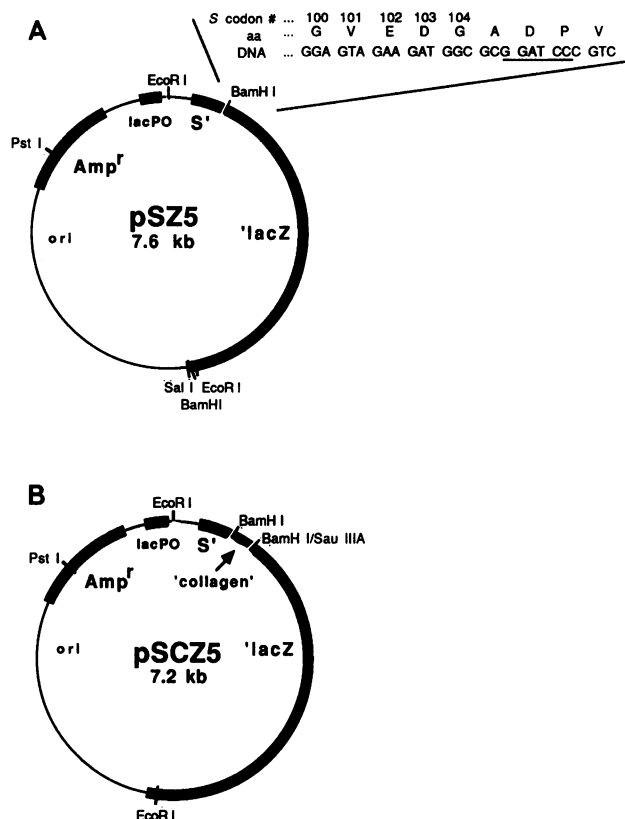


FIG. 1. Schematic representation of the pSZ5 and pSCZ5 plasmids. (A) Derived from pJG1 and pMC1871, plasmid pSZ5 carries an *S-lacZ* fusion gene, which contains the first 104 codons of the *S* gene fused to codon 8 of the *lacZ* gene, under the control of *lacPO*. The nucleotide sequence at the *S-lacZ* fusion junctions was determined by dideoxy sequencing (underlined sequences are from the *Bam*HI linker); protein sequences were deduced from the nucleotide sequences. aa, Amino acid. (B) Derived from pSZ5 and pJG200, plasmid pSCZ5 contains approximately 63 codons of collagen inserted between and in frame with the *S* gene and *lacZ* sequences. Transcription of each fusion gene was induced by IPTG; translation was directed by the wild-type lambda signals associated with the *S* gene.

Western blots of total cell extracts from induced and uninduced JM101(pSZ5) cultures detected an IPTG-dependent 125,000-kDa protein, which was in good agreement with the DNA sequence prediction. By using two different criteria, neither the SZ5 fusion protein nor the SCZ5 tribrid protein retained the *S* function. First, their expression was not lethal even after large quantities had accumulated intracellularly; electron microscopy of induced cultures revealed no abnormal morphology or inclusion bodies (data not shown). Second, high levels of the fusion proteins did not allow access of cytoplasmic lysozyme activity to the peptidoglycan layer. When JM101(pJG1) (*S*<sup>+</sup> Ram) cells were transformed with the compatible pACYC184-based plasmid pLys, which constitutively produces T7 lysozyme (19, 27), the cells lysed approximately 40 min after induction. Equivalent experiments with JM101(pJG3) (*S*am Ram) showed no lysis (Fig. 2). Thus, T7 lysozyme can effectively complement a deficiency in lambda transglycosylase. Because the T7 lysozyme enzymatically degrades the cell wall once it passes through the inner membrane, it provides a very sensitive test of *S*-protein function; even a small amount of membrane dam-

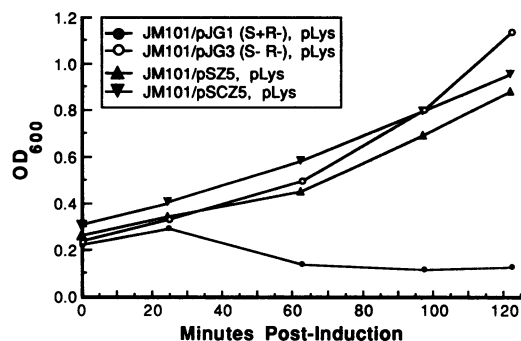


FIG. 2. Growth curve after IPTG induction of JM101(pJG1, pLys), JM101(pJG3, pLys), JM101(pSZ5, pLys), and JM101(pSCZ5, pLys). Plasmid pLys constitutively expresses the T7 3.5 gene, which encoded a 17-kDa *N*-acetylmuramyl-L-alanine amidase (19, 27). Cultures of each pLys-containing strain were grown at 37°C in M9 medium (24) supplemented with Casamino Acids (Difco), thiamine, glycerol, and ampicillin until they reached an OD<sub>600</sub> of 0.2; induced at time zero with 1 mM IPTG; and sampled over a 2-h time period.

age results in cell lysis. However, after IPTG induction of JM101(pSZ5) cells or JM101(pSCZ5) cells that were transformed with pLys, no detectable cell lysis occurred (Fig. 2).

The lack of lethal *S*-protein activity displayed by the SZ5 protein greatly facilitated the isolation and purification of large amounts of hybrid protein for subsequent rabbit immunization. After growth overnight in the minimal medium of Tanaka et al. (47) supplemented with Casamino Acids (Difco Laboratories, Detroit, Mich.), thiamine, glycerol, ampicillin, and 1 mM IPTG, a 330-ml culture of JM101(pSZ5) was assayed for  $\beta$ -galactosidase activity and harvested by centrifugation at 3,000  $\times$  *g* for 20 min. The cell pellet, containing 1,060 U of activity, was suspended in 15 ml of lysis buffer (0.1 M sodium phosphate [pH 7], 0.01 M KCl, 6 mM MgSO<sub>4</sub>, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), disrupted at 20,000 lb/in<sup>2</sup> by using a French pressure cell (Aminco; American Instrument Co.), and centrifuged at 100,000  $\times$  *g* for 1 h at 4°C.  $\beta$ -Galactosidase assays of the ultracentrifugation supernatant and pellet revealed that over 94% of the total activity was localized to the pellet fraction. To solubilize the SZ5 fusion protein, the ultracentrifugation pellet was suspended in 8 ml of 100 mM MOPS (morpholinepropanesulfonic acid; pH 7.5)–1% Triton X-100–35 mM MgCl<sub>2</sub>; incubated at room temperature for 1 h; and recentrifuged at 100,000  $\times$  *g* for 1 h at 4°C. The supernatant from this Triton X-100 extraction (containing 860 U) was further purified by affinity chromatography. A 10-ml column of *p*-aminobenzyl 1-thio- $\beta$ -D-galactopyranoside-agarose (similar to that described by Steers et al. [45]) was packed in column 1 buffer (lysis buffer containing 1% Triton X-100). Solubilized SZ5 fusion protein (550 U) was then loaded, and the column was washed with 375 ml (over 30 column volumes) of column 1 buffer to remove any proteins that were nonspecifically bound to the matrix. This extensive washing was required to deplete the column of a particularly abundant 50-kDa protein that interacted strongly with *p*-aminobenzyl 1-thio- $\beta$ -D-galactopyranoside-agarose. To elute the SZ5 fusion protein, one column volume each of three IPTG concentrations (0.1, 0.25, and 0.5 M in column 1 buffer) was used. The purity of the fraction with the highest level of  $\beta$ -galactosidase activity from each elution was analyzed by SDS-polyacrylamide gel electrophoresis. Each of the IPTG concentrations eluted approximately equivalent

amounts of SZ5 protein (100 U total); higher IPTG concentrations eluted slightly more  $\Delta$ M15  $\beta$ -galactosidase, but no other proteins were detectable. These fractions were pooled and used for rabbit immunization.

The solubilization of the S- $\beta$ -galactosidase fusion protein by Triton X-100 suggests that the SZ5 protein resides primarily in the inner membrane (42), the proposed location of the wild-type S protein (2), and distinguishes it from other highly expressed proteins which form insoluble inclusion bodies (43). Furthermore, the localization of the SZ5 protein is consistent with the current model for the arrangement of S protein in the inner membrane (34); the SZ5 protein includes the three proposed membrane-spanning regions and all but three residues of the hydrophilic carboxy terminus of the S protein, which is predicted to be exposed to the cytoplasm.

**Purification and collagenase digestion of the S-collagen- $\beta$ -galactosidase tribrid protein SCZ5.** After induction with IPTG, a culture of JM101(pSCZ5) produced high levels of the tribrid protein SCZ5 with a rate of induction and specific activity identical to those of the SZ5 fusion protein. Furthermore, the SCZ5 tribrid displayed similar membrane localization and nonlethality and could be purified by techniques similar to those used for the SZ5 hybrid protein. During the purification, 1.25% octyl- $\beta$ -D-glucopyranoside was used in place of 1% Triton X-100 because the SCZ5 tribrid fusion was not as efficiently solubilized by Triton X-100.

The SCZ5 tribrid protein was digested with *Achromobacter iophagus* collagenase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by using the conditions suggested by Germino and Bastia (16). The purified SCZ5 tribrid protein was precipitated with 90% acetone and redissolved in collagenase buffer (0.25 M NaCl, 10 mM CaCl<sub>2</sub>, 0.2 mM DTT, 10 mM Tris hydrochloride [pH 7.4]) supplemented with 1.25% octyl- $\beta$ -D-glucopyranoside to achieve a final protein concentration of approximately 0.1 mg/ml. The SCZ5 tribrid was then digested with 0.05 or 0.5 U of collagenase per ml at 28°C for 0, 10, or 60 min, followed by precipitation with 10% trichloroacetic acid. The digestion products were fractionated on a 10 to 20% gradient SDS-polyacrylamide gel and visualized by the Western blot procedure.

**Antibody preparation.** Antibody directed against the SZ5 fusion protein was prepared in two female New Zealand White rabbits. Each rabbit was initially injected with 100  $\mu$ g of purified SZ5 fusion protein and then boosted every 2 to 4 weeks (four times total) with 10  $\mu$ g of SZ5 protein. To test for the presence of anti- $\beta$ -galactosidase antibody in the serum, double immunodiffusion was performed by using Ouchterlony plates (31). Preimmune serum, which was collected just before the first injections, showed no anti- $\beta$ -galactosidase antibody; all subsequent serum collections contained anti- $\beta$ -galactosidase antibody, although the titer varied with the rabbit and the date of collection.

**Western blot procedure.** The Western blot procedure of Towbin and colleagues (48, 49) was performed with modifications to improve the detection of small basic membrane proteins. The transfer buffer for preequilibration and electrophoretic transfer contained 25 mM ethanolamine, which was adjusted to pH 9.5 with glycine, and 20% methanol (46). Nitrocellulose (pore size, 0.1  $\mu$ m; Schleicher & Schuell, Inc., Keene, N.H.) was used, but neither Triton X-100 nor Tween 20 was included in any incubations or washes (22).

Prior to use on Western blots, the anti-SZ5 serum was preabsorbed with a large excess of CSH7 extract overnight at 4°C. The preabsorbed anti-SZ5 serum was used at a 1:2,000 dilution; anti- $\beta$ -galactosidase antibody (Organon Teknika-Cappel, Durham, N.C.) was used at a 1:1,000

dilution. A second antibody solution contained goat anti-rabbit immunoglobulin G (IgG) antibody (Bio-Rad Laboratories, Richmond, Calif.) conjugated with either horseradish peroxidase or alkaline phosphatase and the enzymatic color reactions were performed according to the directions of the manufacturer.

**Immunoprecipitation.** By using the S-protein-specific antibody, S protein was immunoprecipitated by a procedure based on that of Muller and Muller (28). The crude membrane fraction from an induced CSH7 ( $\lambda$  S<sup>+</sup> Ram) culture was extracted with 10 mM MOPS (pH 7.5) containing 1% Triton X-100 and 35 mM MgCl<sub>2</sub> for 1 h at room temperature and recentrifuged. To 100  $\mu$ l of supernatant containing Triton X-100-solubilized proteins at approximately 1 mg/ml, 10  $\mu$ l of anti-SZ5 serum (preabsorbed with the membrane fraction from the nonlysogenic parent strain) was added. After the anti-SZ5 serum had incubated with the Triton X-100-solubilized membrane proteins for 15 to 24 h at 4°C, antibody-antigen complexes were precipitated with 100  $\mu$ l of a 20% (vol/vol) solution of protein A-agarose beads (Calbiochem-Behring) that was prewashed three times in 100 mM imidazole hydrochloride (pH 7.0) containing 154 mM NaCl, 1 mM EDTA, and 1% Triton X-100 (IP buffer). After incubation at 4°C for 3 h on a shaker, the beads were washed at least three times with IP buffer and three times with 50 mM Tris hydrochloride (pH 6.8). After the final wash, antibody and antigen were eluted from the beads by vortexing and boiling for 5 minutes in SDS-PAGE loading buffer.

**Protein localization.** The localization of S protein was determined by the method of Schnaitman (41, 42), which was based on the preferential extraction of inner membrane proteins by 1% Triton X-100. An induced CSH7 ( $\lambda$  S<sup>+</sup> Ram) culture, which was suspended in 1/10 volume of 10 mM MOPS (pH 7.5), was disrupted in a French pressure cell and centrifuged at 100,000  $\times$  g for 1 h at 4°C in the presence of 150 mM KCl. After the ultracentrifugation pellet was resuspended in 10 mM MOPS (pH 7.5), this crude membrane fraction was extracted for 1 h at room temperature with 1% Triton X-100, followed by another centrifugation step at 100,000  $\times$  g in the presence of 35 mM MgCl<sub>2</sub> (to minimize the solubility of outer membrane proteins). The presence of S protein in each cell fraction was determined on Western blots by using anti-S-protein antibody.

**Chemical cross-linking of membrane proteins.** The homobifunctional cleavable reagent dithiobis(succinimidylpropionate) (DSP; Pierce Chemical Co.) was used to cross-link primary amines of proteins in close proximity with each other in the cell membrane (11, 20). The crude cell membrane fraction from an induced lysogen or infected cell culture, which was produced by ultracentrifugation of a French-pressed culture at 100,000  $\times$  g for 1 h, was suspended in 10 mM MOPS (pH 7.5) to a final concentration of 5 to 15 mg of protein per ml. The conditions for cross-linking were as follows: 2.5 mg of membrane protein per ml; 0.1 M MOPS (pH 7.5); 20 mM NaCl; and 0, 0.3, or 0.6 mM DSP in dimethyl sulfoxide. After 30 min of shaking at room temperature, the reaction was stopped by the addition of 1/10 volume of 1 M glycine, extracted with 1% Triton X-100 in the presence of 35 mM MgCl<sub>2</sub> for 1 h at room temperature, and recentrifuged at 100,000  $\times$  g for 1 h. The supernatant was then analyzed by SDS-PAGE both in the presence and in the absence of DTT, followed by Western blotting with anti-S-protein antibody.

## RESULTS

**Specificity of anti-SZ5 serum.** The low amount of S protein produced in a lambda-infected cell has hindered previous attempts to detect its presence throughout the lambda lytic cycle. To facilitate the production of enough S protein to demonstrate the specificity of the anti-SZ5 serum, a second fusion gene was constructed that encodes a tribrid protein, SCZ5, with a stretch of collagen (containing six collagenase cleavage sites) inserted between the S-protein and  $\beta$ -galactosidase sequences. Digestion of the purified SCZ5 tribrid with collagenase was expected to yield a large  $\beta$ -galactosidase fragment and a small S-protein fragment, as neither wild-type  $\beta$ -galactosidase nor S protein contains the consensus site for collagen cleavage. The collagenase cleavage products of the SCZ5 tribrid, which were visualized on a silver-stained SDS-polyacrylamide gel, included a large fragment about the size of wild-type  $\beta$ -galactosidase and a set of three low-molecular-weight fragments (consistent with cleavage at the different collagenase sites in the collagen segment). With increasing levels of collagenase or increasing time of digestion, these small fragments reduced to a single 8-kDa band that was not degraded further. The control cleavage experiment with the SZ5 hybrid protein showed no low-molecular-weight bands, even at the highest levels of collagenase used for the SCZ5 tribrid, indicating that these small fragments were not derived from  $\beta$ -galactosidase sequences or collagenase self-digestion but, instead, contained S-protein sequences. To provide evidence of anti-S-protein antibodies in the anti-SZ5 serum, two Western blots of collagenase digestion products of the SCZ5 tribrid were performed; one used anti- $\beta$ -galactosidase antibody, while the second used anti-SZ5 serum. The low-molecular-weight peptides were labeled only with the anti-SZ5 serum, showing that they did not contain  $\beta$ -galactosidase sequences and that the anti-SZ5 serum contained S-protein-specific antibody (Fig. 3, lanes 1 through 6). As further evidence that collagenase did not digest the SZ5 protein, anti-SZ5 serum and anti- $\beta$ -galactosidase serum labeled the same two bands in the control collagenase reaction with the SZ5 protein, and the mobilities of the labeled bands corresponded to the sizes of the uncleaved SZ5 fusion protein and  $\Delta$ M15  $\beta$ -galactosidase (Fig. 3, lanes 7 and 8). In equivalent Western blots incubated with preimmune serum (collected prior to immunization with the SZ5 protein) as the first antibody, neither the fusion proteins nor the S-protein fragment derived from collagenase cleavage of the SCZ5 protein was labeled.

**Identification and localization of S protein.** By using anti-SZ5 serum as anti-S-protein antibody, the amount of S protein present in CSH7 ( $\lambda$  S<sup>+</sup> Ram) cells after phage induction was visualized on Western blots. S protein, with a mobility corresponding to 8 kDa, was slightly visible in total cell extracts 30 min after induction and was seen clearly in cell extracts 90 min after induction. By densitometry scanning of a Western blot containing samples from a time course study, the time of appearance and accumulation of S protein throughout the lytic cycle were determined (Fig. 4). Extrapolation of the time course of accumulation to zero S protein suggested that S protein begins to be synthesized approximately 10 min postinduction, concurrent with the onset of late protein synthesis. Like R protein (18), S protein continued to accumulate in the cell until the time of lysis.

When infected cells from any time point during the lytic cycle were disrupted in a French pressure cell and centrifuged at  $100,000 \times g$  for 1 h, S protein partitioned into the pellet, suggesting that the S protein resides in the cell

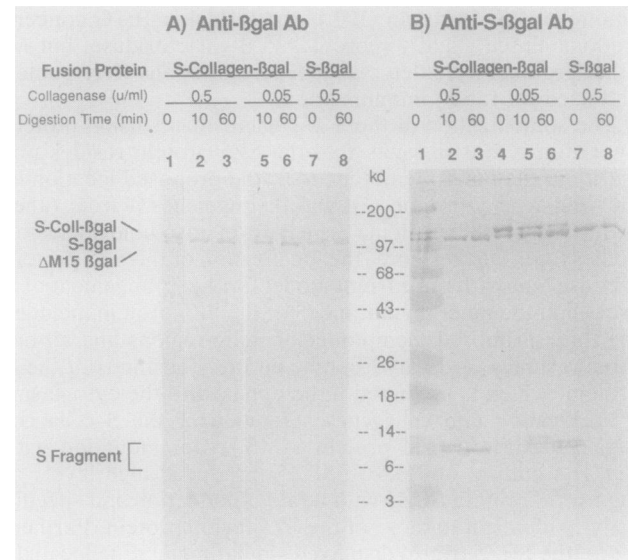


FIG. 3. Western blot of the collagenase cleavage products of the SCZ5 tribrid and SZ5 fusion proteins: evidence of anti-S-protein antibody in the anti-SZ5 serum. Collagenase digestion products of the two fusion proteins, SCZ5 (S-Coll-βgal) and SZ5 (S-βgal), were loaded onto a 10 to 20% gradient SDS-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose paper. The nitrocellulose was then incubated with either anti- $\beta$ -galactosidase antibody (Anti-βgal Ab) (A) or anti-S- $\beta$ -galactosidase (Anti-S-βgal Ab) serum prepared against the SZ5 protein (B). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the second antibody for both Western blots. Lanes: 1 through 6, purified SCZ5 protein was subjected to collagenase digestion at two different concentrations (0.05 or 0.5 U/ml) for 0, 10, and 60 min; 7 and 8, purified SZ5 protein was digested with 0.5 U of collagenase per ml for 60 min. Only the collagenase digestion of the SCZ5 protein produced low-molecular-weight peptides that reacted with the anti-S- $\beta$ -galactosidase serum. Prestained molecular weight markers were added to the collagenase reaction in lane 1 of panel B just before gel loading.

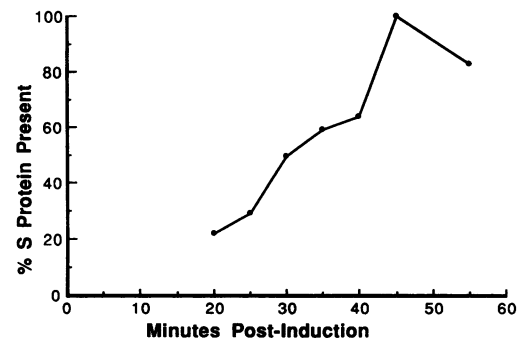


FIG. 4. Time course study of S-protein accumulation. Cultures of CSH7 ( $\lambda$  Sam Ram) or CSH7 ( $\lambda$  S<sup>+</sup> Ram) were grown at 30°C in LB-glucose until they reached an OD<sub>600</sub> of 0.3, induced at time zero by incubation at 45°C for 8 min, and then transferred to 37°C. The cultures were sampled at 20, 25, 30, 35, 40, 45, and 55 min postinduction; French pressed; spotted onto nitrocellulose; and incubated with anti-SZ5 serum and <sup>125</sup>I-labeled protein A. The S-protein signal in the autoradiogram of the nitrocellulose blot was quantitated by densitometry. An S-protein level of 100% was defined as the amount of S protein present at the normal time of lysis.

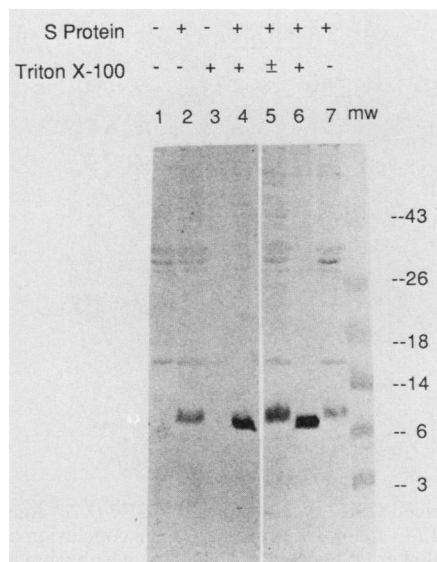


FIG. 5. Western blot of Triton X-100 extraction of S protein. At 60 min postinduction, cultures of CSH7 ( $\lambda$  Sam Ram) or CSH7 ( $\lambda$  S<sup>+</sup> Ram) were harvested, French pressed, and centrifuged at  $100,000 \times g$  for 1 h at 4°C. The resultant membrane pellet was then extracted with 1% Triton X-100 and recentrifuged at  $100,000 \times g$  for 1 h at 4°C in the presence of 35 mM MgCl<sub>2</sub>. Both the membrane pellet and the Triton X-100-solubilized membrane fraction were electrophoresed on an SDS-15% polyacrylamide gel, transferred to nitrocellulose, and incubated with anti-SZ5 serum and an alkaline phosphatase-conjugated second antibody by the Western blot procedure. Lanes: 1 and 2, total membrane pellet from induced CSH7 ( $\lambda$  Sam Ram) and CSH7 ( $\lambda$  S<sup>+</sup> Ram) lysogens, respectively; 3 and 4, Triton X-100-solubilized membrane fraction from induced CSH7 ( $\lambda$  Sam Ram) and CSH7 ( $\lambda$  S<sup>+</sup> Ram) lysogens, respectively; 5, Triton X-100-solubilized membrane fraction from CSH7 ( $\lambda$  S<sup>+</sup> Ram) mixed with the total membrane pellet from CSH7 ( $\lambda$  S<sup>+</sup> Ram); 6, Triton X-100-solubilized membrane fraction from CSH7 ( $\lambda$  S<sup>+</sup> Ram); 7, the total membrane pellet from CSH7 ( $\lambda$  S<sup>+</sup> Ram). Each lane contains the protein derived from a membrane pellet which contained approximately 200  $\mu$ g of protein, except lane 5, which contained protein derived from twice as much membrane. Molecular weights (mw) are indicated to the right of the gel (in thousands).

envelope both before and after the time of lysis. Triton X-100 solubilization of the ultracentrifugation pellet, followed by another centrifugation step at  $100,000 \times g$  in the presence of MgCl<sub>2</sub>, extracted S protein into the supernatant, providing evidence that the S protein is localized to the inner membrane. Surprisingly, this solubilized S protein displayed an enhanced antibody signal and a slightly increased mobility on Western blots compared with the membrane-bound form (Fig. 5). When a Triton X-100-extracted sample was mixed with an unextracted sample, the S-protein signal resembled that of the unextracted sample alone, as characterized by a decreased mobility and reduced labeling with antibody (Fig. 5, lane 5). These results indicate that a component in the unextracted sample interferes with the mobility of S protein in an SDS-polyacrylamide gel and either the efficiency with which S protein can transfer to the nitrocellulose or the reaction of S protein with anti-S-protein antibody during the Western blot procedure. When a lipoprotein deletion mutant (*E. coli* SB221; kindly provided by M. Inouye) was used as the lysogenic strain in a lambda induction experiment, the membrane fraction did not show an enhanced signal for S protein prior to Triton X-100 extraction (data not shown); thus, lipoprotein was not the cause of the interference.

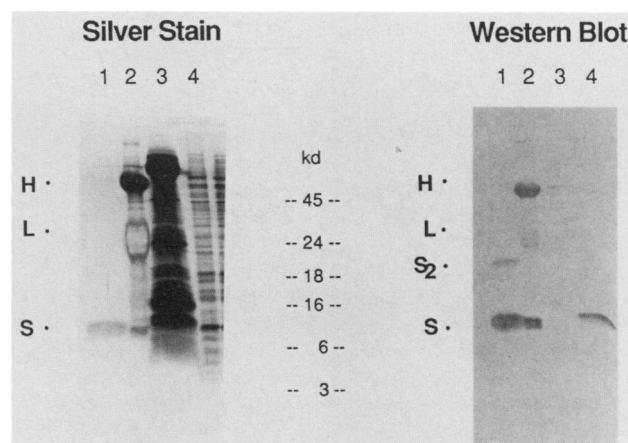


FIG. 6. Purification of S protein. Aliquots removed during the purification procedure of S protein were run on an SDS-17% polyacrylamide gel and visualized both with silver staining and by Western blot with anti-SZ5 serum and an alkaline phosphatase-conjugated second antibody. Silver-stained lanes contained three times more protein than Western blot lanes. Lanes: 1, electroeluted S protein; 2, immunoprecipitated S protein; 3, protein in the Triton X-100-solubilized membrane that was not immunoprecipitated; 4, Triton X-100-solubilized membrane fraction from an induced CSH7 ( $\lambda$  S<sup>+</sup> Ram) lysogen. H, Heavy chain; L, light chain; kd, kilodaltons.

Experiments to demonstrate whether lipopolysaccharide might be responsible for decreasing the Western blot signal of S protein were not conducted.

**Estimation of the number of molecules of S protein per cell.** The number of S-protein molecules per cell at the time of phage-induced lysis was estimated by comparing the Western blot signal of S protein in Triton X-100 extracts of cell membranes collected 90 min after induction of a CSH7 ( $\lambda$  S<sup>+</sup> Ram) culture with the signal of the S-protein fragment from the collagenase cleavage of a known amount of the SCZ5 tribrid. Assuming that the Triton X-100 extraction results in the efficient solubilization of membrane-bound S protein, roughly 100 to 1,000 molecules of S protein mediated the lysis of the host *E. coli* cell at the conclusion of the lytic cycle.

**Purification of S protein.** Immunoprecipitation with anti-S-protein antibody efficiently pulled the S protein out of a mixture of Triton X-100-solubilized membrane proteins, leaving no detectable S protein in solution (Fig. 6, lanes 2 and 3). After a large-scale immunoprecipitation of Triton X-100-solubilized membranes from approximately 200 ml of an induced CSH7 ( $\lambda$  S<sup>+</sup> Ram) culture, the precipitated proteins (including S protein and heavy and light chains of the antibody) were loaded onto an SDS-17.5% polyacrylamide gel (45 cm long to improve resolution). The protein band corresponding to the S protein, which was identified by comparison with a silver-stained gel strip, was electroeluted into ammonium carbonate buffer containing 0.05% SDS. Approximately 20 pmol (0.2  $\mu$ g) of S protein was isolated by this procedure. Equivalent samples of the purified S protein were boiled in SDS-PAGE loading buffer and run on each half of an SDS-17.5% polyacrylamide gel; one half of the gel was used for a Western blot with anti-SZ5 serum, and the other half was silver stained for total protein (Fig. 6). Comparison of the signals from these two labeling methods suggests that the only protein eluted was S protein. Interestingly, the Western blot showed a faint band running with the mobility of an S-protein dimer.

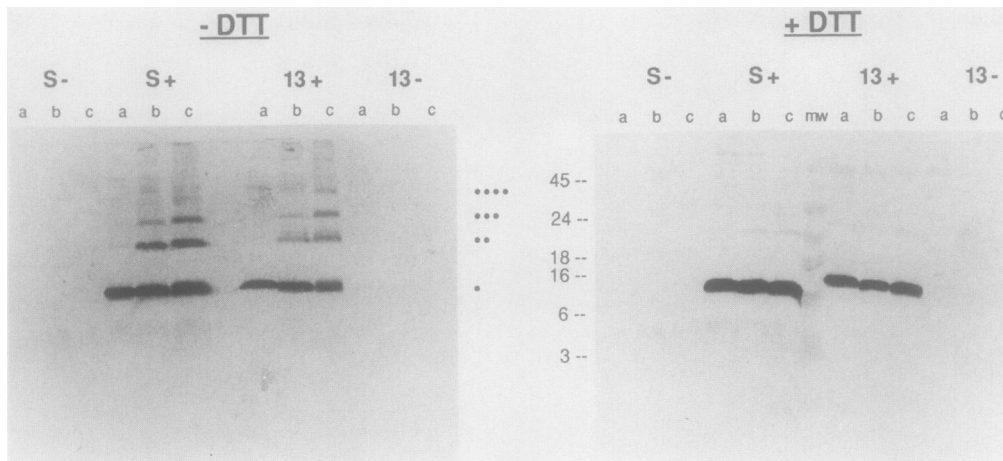


FIG. 7. Western blot of DSP cross-linking of membrane proteins. Cultures of induced CSH7 ( $\lambda$  Sam Ram) or CSH7 ( $\lambda$   $S^+$  Ram) lysogens and cultures of *S. typhimurium* DB700 which were infected with P22  $13$  am $19^+$  or P22  $13^+$   $19$ am were collected at the conclusion of the phage life cycle and cross-linked with the amine-reactive homobifunctional cleavable cross-linker DSP. Membrane pellets, which were prepared by ultracentrifugation of French-pressed cultures at  $100,000 \times g$  for 1 h at  $4^\circ\text{C}$ , were reacted with dimethyl sulfoxide alone, 0.3 mM DSP in dimethyl sulfoxide, or 0.6 mM DSP in dimethyl sulfoxide for 30 min; stopped by the addition of glycine; extracted with Triton X-100; and electrophoresed on an SDS-17.5% polyacrylamide gels in the presence or absence of DTT in the SDS-PAGE loading buffer. Western blots of these gels were incubated with anti-SZ5 serum and an alkaline phosphatase-conjugated second antibody. Lanes: a, dimethyl sulfoxide control; b, 0.3 mM DSP in dimethyl sulfoxide; c, 0.6 mM DSP in dimethyl sulfoxide. The dots between the Western blots mark the approximate location of monomers ( $\cdot$ ), dimers ( $\cdot\cdot$ ), trimers ( $\cdot\cdot\cdot$ ), and tetramers ( $\cdot\cdot\cdot\cdot$ ). mw, Molecular weight (indicated between the panels, in thousands).

**Oligomerization of S protein in the inner membrane.** When the membrane fractions from induced CSH7 ( $\lambda$   $S^+$  Ram) and CSH7 ( $\lambda$  Sam Ram) cultures (collected 60 min after induction) were subjected to cross-linking with the cleavable cross-linking reagent DSP, extracted with Triton X-100, and visualized on Western blots with anti-S-protein antibody, a DTT-sensitive ladder of bands that contained S protein was observed. Bands corresponding to molecular weights of 8, 18, 24, and 32 kDa (and often 42 kDa) were labeled with the anti-S-protein antibody in the  $S^+$  lane, and all migrated at the 8-kDa position when exposed to DTT (Fig. 7). In membrane fractions collected at intermediate times during the phage life cycle, the 18- and 24-kDa species were detected (data not shown). The low level of S protein at these intermediate time points hindered the detection of other cross-linked species. Previous experiments demonstrated that anti-S-protein antibody also recognizes the bacteriophage P22  $13$  gene product; this was not surprising since it differs from S protein by only 10 amino acid residues (37). DSP cross-linking of cell membranes from P22-infected cultures of *S. typhimurium* DB700 showed a similar DTT-sensitive, gene  $13$ -dependent ladder of bands (Fig. 7). The molecular weights of the cross-linked species visualized on the Western blot were consistent with the sizes predicted for dimers, trimers, tetramers, and pentamers of the lambda S protein or the P22  $13$  gene product.

## DISCUSSION

**Identification and localization of S protein.** By using antibody raised against an *S-lacZ* fusion gene product, a Western blot of induced CSH7 ( $\lambda$   $S^+$  Ram) and CSH7 ( $\lambda$  Sam Ram) cell extracts produced a labeled band that correlated with the presence of the wild-type *S* allele, and was thus identified as the *S* gene product. The mobility of the band was identical at all time points sampled during the lytic cycle and corresponded to an 8-kDa protein that first appeared

approximately 10 min postinduction (Fig. 4). Within the limits of resolution of the SDS-17.5% polyacrylamide gel, this result suggests that the size of S protein is not posttranslationally modified to effect its activation at the time of lysis. The immunological identification of S protein with S-protein-specific antibodies is consistent with previous reports of the detection of S protein by using UV-irradiated cells (1). In those studies, however, intermediate times during the phage lytic cycle could not be distinguished from late times because the cells did not display the normal time course of phage infection. In some cases, high doses of UV light have been observed to significantly alter the regulation of synthesis and degradation of viral proteins in irradiated cells (44).

The molecular weight estimate of 8,000 for the S protein is significantly less than its predicted size of 11,600 and most likely reflects aberrant SDS binding as a consequence of its very hydrophobic nature. While the molecular weight assignment derived from this Western blot experiment agrees well with that seen for S protein synthesized in both maxicells and UV-irradiated cells (1), the report of an acidic 15-kDa protein whose appearance correlated with S-protein-dependent changes in membrane permeability (50) could not be confirmed. Often, an 18-kDa species was detected with anti-S-protein antibody on Western blots of SDS-polyacrylamide gels, although its signal was always much less than that of the 8-kDa species. The cross-linking results suggest that this species corresponds to an S-protein dimer, which would be just as basic as an S-protein monomer (predicted pI,  $>9$ ), unless it was posttranslationally modified. Indeed, Western blots of equilibrium isoelectric focusing gels showed no acidic proteins labeled by the anti-S-protein antibody (data not shown). Thus, the acidic 15-kDa protein most likely does not contain S-protein sequences and probably arose from S-protein-dependent changes in the membrane composition, as there are many differences in the protein profiles of two-dimensional gels containing mem-

branes from either  $\lambda$  S<sup>+</sup> Ram- or  $\lambda$  Sam Ram-infected cells (50).

The report of a 5.5-kDa S gene product incorporated into lambda phage particles (51) was not reproduced with anti-S-protein antibody. A Western blot of purified phage (10<sup>11</sup> phage per lane) failed to show any protein species recognized by anti-SZ5 serum (data not shown), in agreement with the results reported by Altman et al. (1). The sensitivity limits for alkaline phosphatase-conjugated antibody are such that this procedure would have detected one molecule of S protein per phage.

Two experiments confirmed that S protein resides in the inner membrane at the conclusion of the phage lytic cycle: (i) fractionation based on the differential solubility of membrane vesicles versus soluble proteins, followed by the preferential extraction of inner membrane proteins with Triton X-100 in the presence of Mg<sup>2+</sup> (Fig. 5), and (ii) equilibrium density centrifugation of membrane vesicles in sucrose, as observed using UV-irradiated cells (2) and reproduced using unirradiated cells and anti-S-protein antibody (M. T. Zagotta, unpublished data). Furthermore, immunological detection of the S protein was sensitive enough to allow us to observe the inner membrane localization of S protein at intermediate times during the lytic cycle, after late transcription but before lysis. While this result suggests that inactive S protein present at intermediate times also resides in the inner membrane, it must be interpreted with some caution since any cell-harvesting procedure might trigger S protein into its active state merely by dissipating the membrane potential, analogous to the triggering seen in premature lysis (10, 36). However, our conclusion that S protein is localized to the inner membrane throughout the lytic cycle is consistent with the very hydrophobic nature of the S protein. There is no evidence to implicate membrane localization as the triggering event for lysis at the conclusion of the phage lytic cycle.

**Quantitation and oligomerization of S protein in the inner membrane.** By comparing the Western blot signal of S protein extracted from a known amount of cell membrane by Triton X-100 with the signal generated by the S-protein fragment produced by collagenase cleavage of a known amount of S-collagen- $\beta$ -galactosidase tribrid protein, the number of S-protein molecules present at the conclusion of the phage lytic cycle was estimated to be between 100 and 1,000 per cell. This conclusion roughly agrees with the results from the purification of S protein by immunoprecipitation, in which 20 pmol of S protein was recovered from 200 ml of cells (corresponding to approximately 100 molecules per cell). The actual number of inner membrane pores, however, is expected to be much lower since S protein appears to reside in the inner membrane as an oligomer and would need multiple subunits to form a channel large enough to allow a protein the size of phage lysozyme to pass through it. Minimum estimates of the oligomerization, based on the dimensions of the lambda lysozyme and the diameter of the  $\alpha$ -helical membrane-spanning segments, range from 15 to 20  $\alpha$  helices per channel or at least five molecules of S protein per channel, assuming that no host proteins participate in the structure of the channel multimer. Given that the nonhomologous P22 lysozyme (a 16-kDa N-acetylmuramidase [37]) and T7 lysozyme (a 17-kDa N-acetylmuramyl-L-alanine amidase [19]) are also allowed access to the periplasm by S protein (37) (Fig. 2), the S-protein channel appears to function nonspecifically. For such a nonspecific channel, the diameter of the pore could be much larger than these

minimum estimates, requiring higher states of oligomerization.

DSP-cross-linked membranes from induced lambda lysogens of *E. coli* and from P22-infected *S. typhimurium* cells produced a ladder of bands that reacted with the anti-S-protein antibody (Fig. 7). Estimates of the molecular weights corresponding to these cross-linked bands were consistent with the formation of dimers, trimers, tetramers, and pentamers of S protein (or the P22 13 gene product). Oligomers containing more than five molecules would not have been detected for technical reasons. Two other thiol-cleavable chemical cross-linkers were used which, like DSP, react with primary amines through a 1.2-nm spacer arm. Both the membrane-permeable dimethyl-3,3'-dithiobispropionimide (Pierce Chemical Co.) and the membrane-impermeable 3,3'-dithiobis(sulfosuccinimidylpropionate) (Pierce Chemical Co.) cross-linkers produced band patterns on Western blots identical to those seen with DSP (data not shown), demonstrating that the cross-linked species were not dependent on a specific chemical.

Recently, Bläsi et al. (8a) have presented evidence for the utilization of two translational initiation sites on the S gene, resulting in the expression of two protein species, S<sub>107</sub> and S<sub>105</sub>. The amine-reactive chemical cross-linker DSP has the potential to react with any of the six lysine residues of the S<sub>105</sub> protein (or seven lysine residues of the S<sub>107</sub> protein) or the amino terminus. While the anti-S-protein antibody recognizes both forms of the S protein (29), the resolution of the polyacrylamide gels used to fractionate the cross-linked species was not great enough to distinguish between S<sub>105</sub> and S<sub>107</sub>. The current model for the S protein hypothesizes that one lysine residue (or two lysine residues for S<sub>107</sub>) and the amino terminus are exposed to the periplasm, two lysine residues are buried in the first two membrane-spanning regions, and the three remaining lysine residues are at the carboxy-terminal end exposed to the cytoplasm (34). Since the pore-forming function of S protein allows the cross-linker potential access to all regions of the protein, any of these amino groups are possible candidates for cross-linking. Experiments with French-pressed membrane vesicles (containing primarily inverted vesicles [38]) derived from cells infected with lambda carrying the temperature-sensitive S allele might distinguish which lysine residues are involved in the cross-linking if a membrane-impermeable chemical cross-linker is reacted with the vesicles at the nonpermissive temperature at which the S-protein channel is not functional.

Although the low molecular weight of S protein suggests that it must form a multimeric structure to construct a pore with a diameter large enough to allow a 17.5-kDa protein to pass through nonspecifically, this is the first biochemical evidence for its oligomerization. The genetic analysis of the partially or codominant missense mutations in the S gene is consistent with an oligomeric mechanism in which mutant S protein interferes with wild-type S protein (33). Furthermore, when purified S protein was visualized on an SDS-polyacrylamide gel, two protein species were labeled with anti-S-protein antibody, the expected 8-kDa protein and an 18-kDa protein (Fig. 6). The size estimation of this latter protein correlates well with that of the DSP-cross-linked dimer, suggesting that S protein has the ability to form very stable dimers. Oligomer formation by small lysis proteins may prove to be a common theme in the lysis mechanisms of bacteriophages. SDS-resistant oligomers of the E gene product of bacteriophage  $\phi$ X174 have recently been observed (8), and the lysis proteins of RNA phages have been proposed to



form alamethicinlike oligomeric structures in the presence of a membrane potential (17).

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