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Angelo Scorpio
University of Rhode Island

Paul Johnson
University of Rhode Island

Ann Laquerre
University of Rhode Island

David R. Nelson
University of Rhode Island, dnelson@uri.edu

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Subcellular localization and chaperone activities of Borrelia burgdorferi Hsp60 and Hsp70

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Subcellular Localization and Chaperone Activities of *Borrelia burgdorferi* Hsp60 and Hsp70

ANGELO SCORPIO,[1,2] PAUL JOHNSON,[1] ANN LAQUERRE,[1] AND DAVID R. NELSON[1,2]*

*Department of Biochemistry, Microbiology, and Molecular Genetics[1] and Center for
Vector-Borne Disease,[2] University of Rhode Island, Kingston, Rhode Island 02881*

Subcellular locations and chaperone functions of Hsp60 and Hsp70 with flagellin were investigated in *Borrelia burgdorferi*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis of fractionated cells showed Hsp60 to be present in the soluble fractions and the Triton X-100 detergent-soluble membrane fraction at growth temperatures ranging from 20 to 37°C. The relative amount of Hsp60 associated with the membrane increased with growth temperature. Hsp70 was found in soluble fractions at growth temperatures between 28 and 37°C, but at 20°C it was also present in the Triton X-100-insoluble membrane fraction. Immunoelectron microscopy revealed that the majority of Hsp60 was localized in the cytoplasm but a detectable fraction (~30%) was associated with the cell envelope. The chaperone functions of Hsp60 and Hsp70 were analyzed by immunoprecipitation of [^{35}S]methionine-labeled cell lysates under nondenaturing conditions in the presence or absence of ATP. Hsp70 was found to bind flagellin at all temperatures tested between 33 and 41°C. This association could be decreased with ATP when cells had been incubated at 41°C during radioactive labeling but not at lower temperatures. Both flagellin and Hsp70 were found to associate with Hsp60, forming a complex of the three proteins. Hsp70 association with this complex could be decreased with ATP, but flagellin binding to Hsp60 was ATP independent at all temperatures studied. Both Hsp70 and flagellin were inaccessible to monoclonal antibodies against them when bound to Hsp60. These studies suggest that in *B. burgdorferi*, a major function of Hsp60 and Hsp70 is in the molecular processing of flagellin.

Lyme disease, a tick-borne bacterial disease transmitted by ticks of the genus *Ixodes*, is caused by the spirochete *Borrelia burgdorferi* (13). Initially, the disease affects the skin, with lesions (erythema migrans) developing at the site of the tick bite. Early-stage disease is also manifested by fever, chronic fatigue, dizziness, and general malaise. Late-stage symptoms include various neuropathies and the development of arthritis in large joints (18).

Heat shock proteins (Hsps) are a stress response class of proteins whose synthesis is increased when cells are exposed to elevated temperatures (30). We have previously characterized the heat shock response of *B. burgdorferi* (14). Among the Hsps identified were Hsp60 and Hsp70. Hsp60 and Hsp70 are homologous to GroEL and DnaK of *Escherichia coli*, respectively. While GroEL and DnaK are found predominantly in the cytoplasm (12, 44), several recent reports indicate that these proteins are found in several subcellular locations. It has been reported that the GroEL homologs in *Mycobacterium leprae* and *Coxiella burnetii* sediment with the insoluble pellet following cell lysis (22, 47). Additionally, in *Neisseria gonorrhoeae*, Hsp60 can be detected in outer membrane preparations following exposure to environmental stress (37). Further, the DnaK homolog of *Vibrio* sp. strain S14 has been reported to be located in both the periplasmic space and the cytoplasm (27). We were interested in determining the subcellular location of Hsp60 and Hsp70 in *B. burgdorferi* and whether the location was related to the functions of these Hsps.

In other organisms studied, Hsp60 and Hsp70 bind to nascent or misfolded proteins and help maintain their secondary structure during stress conditions (5, 20, 30, 39). They also

function as chaperones in the cell; that is, they aid in transporting proteins in the cytoplasm and across the cell membrane and/or aid in the proper posttranslational folding of peptides (15, 17, 20, 45, 48). Hsps may also play a major role in disease pathogenicity. Major antigenic components observed in a variety of bacterial and parasitic infections and involved in the pathogenesis of disease have been identified as members of stress protein families (21, 24, 36, 40). In Lyme disease, Hsp60 is an immunodominant antigen, and the immunological recognition of human Hsp60 may be involved in the development of Lyme arthritis (23).

In Lyme disease, *B. burgdorferi* flagellin (~41 kDa) is an immunodominant antigen and is generally the first protein recognized serologically during the course of infection in humans (43). The assembly process of the flagellum in most bacteria involves the polymerization of flagellin monomers onto the distal end of the flagellum to form a filament outside the cell (28). In spirochetes, the flagella are located in the periplasmic space, wrapped around the cell. The synthesis of flagellin in *E. coli* has been shown to be dependent on the Hsps DnaK, DnaJ, and GrpE (42). However, the role of the Hsps in the biosynthesis of the flagellum was not defined.

In this report, we show that a major function of both Hsp60 and Hsp70 is involvement in the molecular processing of flagellin, that both can be observed in multiple subcellular compartments of *B. burgdorferi*, and that the subcellular distribution of these two proteins is temperature dependent.

MATERIALS AND METHODS

Bacterial strains. DnaK was purified from *E. coli* MC4100(PBB1) (35). GroEL was purified from *E. coli* F-18 (16). *B. burgdorferi* B-31 was used as the source of Hsp60 and

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* Corresponding author. Phone: (401) 792-5902. Fax: (401) 792-7148. Electronic mail address: dnelson@uriacc.uri.edu.

Hsp70 and for fractionation experiments. *B. burgdorferi* T15, a tick salivary isolate (19), was used to study chaperone activity.

Media and growth conditions. *E. coli* strains were grown at 37°C in L broth. *B. burgdorferi* strains B-31 and T15 were grown at 33°C in tubes of BSKII broth (1).

DnaK and Hsp70 purification. DnaK was purified by the method of McCarty and Walker (35). For *B. burgdorferi* Hsp70 purification, strain B-31 was grown in 1 liter of BSKII and heat shocked 1 h at 40°C. The cells were pelleted by centrifugation (8,000 × g, 15 min) and resuspended in 10 mM Tris-HCl (pH 7.5). The suspension was sonicated with four 30-s pulses and centrifuged at 10,000 × g for 20 min, and the supernatant was saved. The supernatant was brought to 45% ammonium sulfate and pelleted by centrifugation at 30,000 × g for 20 min. The pellet was washed in buffer A (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 10% glycerol, 0.1 mM NaCl, 10 mM β-mercaptoethanol [pH 8.0]) and dialyzed against buffer A overnight at 4°C, and the dialysate was loaded onto a DEAE-Sephadex anion-exchange column equilibrated with buffer A. A 50 to 500 mM step gradient of NaCl in buffer A was used to elute the column, and the collected fractions were checked for the 70-kDa band by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (29). Fractions containing Hsp70 were pooled and dialyzed against buffer B (25 mM HEPES, 20 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 5 mM MgCl$_2$ [pH 8.0]) overnight. The supernatant was applied to an ATP-agarose column equilibrated with buffer B. Five volumes of buffer B plus 500 mM NaCl was passed through the column, followed by 2 volumes of buffer B plus 50 mM NaCl. ATP-binding proteins were eluted with 5 mM Na-ATP in buffer B.

GroEL and Hsp60 purification. *E. coli* GroEL was purified as described by Hendrix (25). For *B. burgdorferi* Hsp60 purification, the 500 mM NaCl eluent from the ATP-agarose column obtained from the purification of Hsp70 was dialyzed in 10 mM Tris-HCl (pH 7.5), concentrated by lyophilization, and resuspended in 10 mM Tris (pH 7.5). The dialysate was then centrifuged on a 10 to 30% glycerol gradient (160,000 × g, 1 h). Aliquots (0.6 ml) were collected and analyzed by SDS-PAGE.

Antibody preparation. Protein was further purified by electrophoresis in an 11% SDS-polyacrylamide gel and lightly stained with Coomassie blue in water. The bands were cut out and emulsified in Freund's incomplete adjuvant (8). This mixture was then injected intramuscularly and subcutaneously into rabbits. One booster shot was given at 3 weeks, and serum was collected at 6 weeks.

Subcellular fractionation of *B. burgdorferi*. Cultures of *B. burgdorferi* B-31 (36 ml) were grown at five different temperatures: 20, 28, 33, 35, and 37°C. Another culture was grown at 33°C and heat shocked for 1 h at 41°C. Cells from all cultures were pelleted by centrifugation (8,000 × g, 15 min), washed twice, and resuspended in 1 ml of 10 mM HEPES (pH 7.5). The cell suspensions were kept on ice for 10 min with occasional shaking, centrifuged (17,000 × g, 2 min), and resuspended in 1 ml 20% sucrose–10 mM HEPES (pH 7.5). After further incubation on ice (15 min with shaking); the cells were centrifuged (17,000 × g, 4 min), and the pellet was resuspended in 1 ml of 10 mM HEPES (pH 7.5) plus 10 mM EDTA and kept on ice for 10 min. The final cell suspension was centrifuged (17,000 × g, 4 min), and the supernatant was saved as the osmotic shock or periplasmic fraction. The remaining pellet was resuspended in 10 mM HEPES (pH 7.5) and sonicated with three 15-s pulses, and the resulting suspension was examined microscopically to be sure the cells were completely broken. The suspension was centrifuged (17,000 × g, 2 min), and the supernatant was transferred to another tube

and centrifuged at 160,000 × g for 60 min to yield a membrane pellet and a cytoplasmic supernatant. The membrane pellet was resuspended in 1 ml of 10 mM HEPES (pH 7.5) plus 1 mM EDTA and centrifuged along with the cytoplasm (160,000 × g) to wash each fraction. The pellet was then rinsed three times with 10 mM HEPES (pH 7.5). The soluble fraction was saved, and the membrane pellet was resuspended in 0.5 ml of 10 mM HEPES with 2% Triton X-100. This suspension was kept at 25°C for 15 min with occasional shaking and then centrifuged (160,000 × g for 60 min) to give Triton-soluble and -insoluble membrane fractions. Pyruvate dehydrogenase activity of subcellular fractions was measured to ensure that there was no mixing of cytoplasmic with periplasmic contents during fractionation. The specific activity of pyruvate dehydrogenase in the periplasmic extracts never exceeded 3.5% (0.001 U/mg of protein) of the cytoplasmic extracts (0.029 U/mg of protein). No pyruvate dehydrogenase activity was detected in the membrane extracts. Activity was measured as previously described (6). Protein concentrations in the periplasmic extracts were about 10% of the concentrations in cytoplasmic and membrane fractions. Periplasmic proteins were concentrated by freeze-drying.

Western blot (immunoblot) analysis. Fractionated proteins were separated in SDS–11% polyacrylamide gels. The proteins were then transferred electrophoretically to nitrocellulose sheets (46). The sheets were blocked 10 min in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl [pH 7.2]) with 2% Tween 20 (Fisher), washed in TBS, and immersed in either rabbit anti-DnaK or anti-GroEL antiserum diluted 1:1,000 in TBS containing 0.05% Tween 20. This mixture was incubated with shaking at room temperature for 1 h. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Sigma) diluted 1:1,000 in TBS containing 0.05% Tween 20 was used as the secondary antibody. Hydrogen peroxide was used as the substrate, and *N*′,*N*′,*N*′-tetramethylbenzidine dissolved in 95% ethanol was used for color development. Hsp60 and Hsp70 in each fraction were quantified by image analysis (Java image analysis system; Jandel Scientific, Corte Madera, Calif.).

Radioactive labeling. Cultures (30 ml) of *B. burgdorferi* were grown to late log phase at 33°C, harvested by centrifugation (8,000 × g, 15 min), and washed in RPMI (GIBCO, Gaithersburg, Md.). The pellets were resuspended in 0.5 ml of RPMI, and 10 μCi of [^{35}S]methionine was added to the mixture (14). The cells were then labeled at either 33, 38, or 41°C for 1 h. Radioactive labeling was stopped by adding excess unlabeled methionine (1.5 mg/ml). Cells were broken by sonication, the lysates were centrifuged (17,000 × g, 2 min) to remove cellular debris, and the supernatants were saved for use in immunoprecipitation experiments.

Immunoprecipitation of flagellin, Hsp60, and Hsp70. *B. burgdorferi* (30 ml) was grown at 33°C and labeled with [^{35}S]methionine in 0.5 ml of RPMI as described above. Labeling was for 1 h at either 33, 38, or 41°C. Labeled mixtures were divided into two aliquots. To one half, ATP (2.5 mM) was added. Apyrase (10 U/ml) was added to the second aliquot. At the end of the labeling period, the cells were immediately broken by sonication and the lysates were immunoprecipitated by adding 10 μl of a monoclonal antibody (MAb) to either Hsp60 (LAλ8), Hsp70 (LA3) (generous gifts from Michael Kramer, Institut für Immunologie, Universität Heidelberg), or flagellin (H9724) (generous gift from Alan Barbour, University of Texas Health Science Center) for 1 h at 4°C. Antibody complexes were precipitated by incubation with protein A-bearing *Staphylococcus aureus* cells (10 min, 4°C), collected by centrifugation (17,000 × g, 2 min), and washed three times in

RPMI. The pellets were resuspended in 50 µl of solubilization buffer and boiled for 5 min. Equal counts of protein for each mixture were separated on SDS–12% polyacrylamide gels and analyzed by fluorography. It should be noted that equal amounts of the labeled cell lysates were immunoprecipitated. In most experiments, nearly equal amounts of labeled material were immunoprecipitated by each treatment, and only minor adjustments were made to load equal counts of each mixture for separation and analysis on 12% polyacrylamide gels and fluorography. For pulse-chase experiments, cells were radioactively labeled as described above. [^{35}S]methionine was chased either by adding 50 µl of nonradioactive methionine (10 mg/ml) or by washing and resuspending the cells in BSKII. Cells resuspended in BSKII were then incubated for an additional 30 min at 33°C, washed twice in RPMI, and broken by sonication, and the lysates were immunoprecipitated with MAb LAλ8 or MAb LA3 as described above. In pulse-chase and continuous-labeling experiments, equal volumes of immunoprecipitated material were loaded onto gels for analysis.

Electron microscopy. Cultures of cells grown to late log phase were harvested and washed two times with phosphate-buffered saline–5 mM MgCl$_2$ (PBS-Mg) (4). The cells were then fixed for 30 min in either 0.5% formaldehyde or 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The samples were centrifuged at 10,000 × g for 30 min, the supernatant was discarded, and pieces of the pellet were enrobed in molten 1.5% purified agar in 0.1 M sodium cacodylate. The agar was then trimmed around the small bits of pellet, and the agar cubes containing cells were placed in 3-ml vials containing cacodylate buffer. Following three washes in cacodylate buffer, half of each sample was postfixed with osmium tetroxide in 0.1 M cacodylate buffer, while the other half was not treated. The samples were then dehydrated in solutions of 10, 25, 50, and 70% ethanol for 20 min each step at room temperature and then in a 1:1 mix of 70% ethanol and LR White resin. Each sample was infiltrated overnight in LR White resin, resuspended in the same, and incubated for an additional 1 hour. The LR White was again removed, and fresh resin was added. The samples were placed in gelatin capsules and cured at 50°C for 24 h or until the resin hardened. Thin sections were prepared from each block with a diamond knife on a Sorvall MT2-B ultramicrotome and placed on folding nickel grids. The grids were incubated in PBS-Mg containing 1% bovine serum albumin for 30 min at 30°C. They were then washed three times in PBS-Mg (10 min each wash) and incubated for 1 h at 30°C with a MAb to Hsp60 (LAλ8 diluted 1:10). Following three more washes in PBS-Mg, the grids were incubated with anti-mouse IgG–gold diluted 1:50 for 1 h (30°C). After three washes, the samples were stained with uranyl acetate and lead citrate and examined by scanning electron microscopy in a JEOL 1200EX scanning/transmission electron microscope operating in the transmission mode at 80 kV.

RESULTS

Hsp60 localization. *B. burgdorferi* grown at various temperatures between 20 and 37°C was fractionated. The proteins in each fraction were separated by SDS-PAGE and analyzed for Hsp60 by Western blotting (Fig. 1). At all growth temperatures, Hsp60 was found in the soluble (cytoplasmic and periplasmic) and Triton X-100-soluble membrane fractions. Of the Hsp60 in the soluble fractions, most was detected in the cytoplasm (data not shown). Little Hsp60 was observed in the Triton X-100-insoluble fraction. Image analysis of Western blots demonstrated that the amount of Hsp60 in the Triton
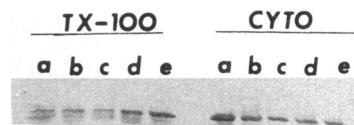

FIG. 1. Western blot comparing relative amounts of Hsp60 in the cytoplasm (CYTO) and Triton X-100 (TX-100)-soluble membrane fractions in cells grown at 20°C (lanes a), 28°C (lanes b), 33°C (lanes c), 35°C (lanes d), or 37°C (lanes e). Equal amounts of protein (10 µg) were loaded onto each lane and separated by SDS-PAGE (17). Blots were prepared and probed for Hsp60 as described in Materials and Methods.

X-100-soluble fraction was approximately threefold greater in cells grown at 37°C than in cells grown at 20°C (Fig. 1). No increase was observed in the soluble fractions.

Localization of Hsp60 by immunoelectron microscopy. The subcellular location of Hsp60 in *B. burgdorferi* was also examined by immunoelectron microscopy. Thin sections from fixed whole cells (grown at 33°C) were probed with a MAb against *B. burgdorferi* Hsp60 (LAλ8) followed by gold-labeled goat anti-mouse IgG. The samples fixed initially in formaldehyde showed more gold particles than samples fixed in glutaraldehyde. Postfixing with osmium tetroxide did not alter antibody binding and provided greater definition of subcellular structures than in samples that were not postfixed. The majority of the gold was found attached in the cytoplasmic space. Gold labeling in each subcellular compartment was counted in 10 fields at ×20,000 magnification and compared with the total number counted. Gold particles were designated as associated with the membrane if no distinguishable space was observed between the gold and the membrane. Gold found in tangential sections was also counted as membrane bound. Particles localized to the periplasmic side of the cytoplasmic membrane but inside the outer membrane were counted as periplasmic. Of the particles counted (648), 31% (σ = 1.5%) were associated with the cell envelope (18% were associated with the cytoplasmic membrane, and 13% were in the periplasm or on the periplasmic side of the cytoplasmic membrane) and 69% (σ = 4.5%) were found in the cytoplasmic space. Less than 1% were associated with the outer membrane. An example of the appearance of probed cells is shown in Fig. 2.

Hsp70 localization. The subcellular localization of Hsp70 was determined by Western blot analysis. Cells grown at temperatures ranging from 20 to 37°C were fractionated, and the proteins in each fraction separated by SDS-PAGE and then analyzed for the presence of Hsp70. At growth temperatures of 33 and 37°C, Hsp70 was found to be almost exclusively located in the soluble fractions of the cell (Fig. 3). However, when the cells were grown at 20°C, Hsp70 was found in the Triton X-100-insoluble membrane fraction in addition to the soluble fractions.

Protein binding activity of Hsp70. Previous reports of protein binding activity by Hsp60 and Hsp70 homologs suggested to us that Hsp60 and Hsp70 may have chaperone functions in *B. burgdorferi*. To determine whether this was the case and the identity of any proteins bound by Hsp60 or Hsp70, the following experiment was carried out. Briefly, whole-cell lysates of *B. burgdorferi* grown at 33°C and labeled with [^{35}S]methionine at 33, 38, or 41°C were immunoprecipitated under nondenaturing conditions in the presence or absence of ATP, using a MAb against *B. burgdorferi* Hsp70 (LA3) or Hsp60 (LAλ8) followed by treatment with protein A-bearing *S. aureus* cells. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Immunoprecipitation with LA3 resulted in
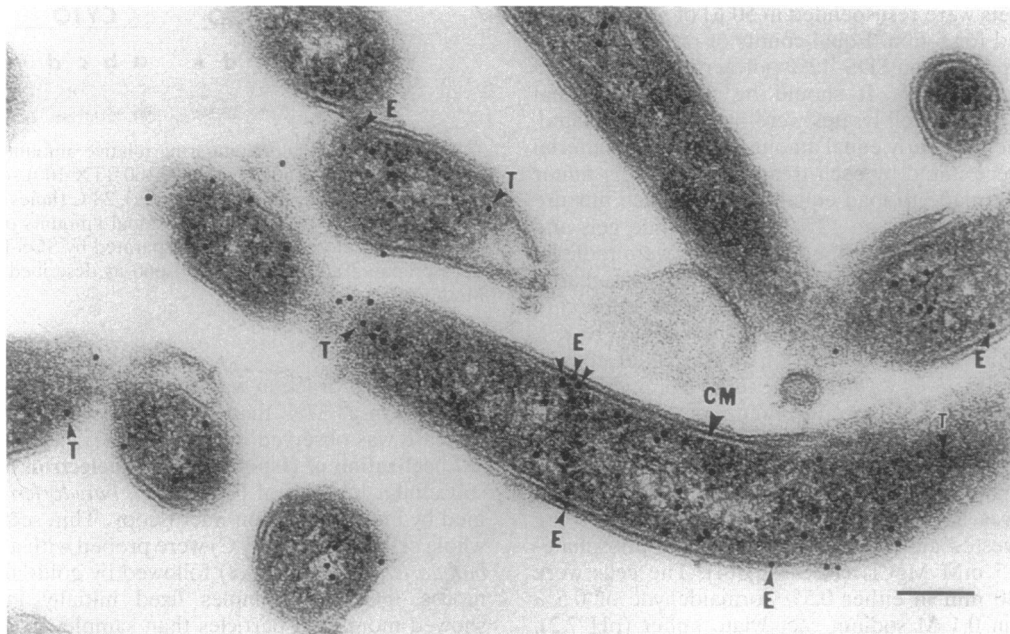
FIG. 2. Electron micrographs showing immunogold detection of Hsp60 in ultrathin sections of *B. burgdorferi* B-31 cells grown at 33°C. Thin sections were probed with an anti-Hsp60 MAb (LAλ8) followed by a 10-nm gold–anti-mouse IgG conjugate. Arrows indicate examples of Hsp60 associated with the cell envelope (E) and with tangential sections through the envelope (T). The cytoplasmic membrane is also indicated (CM). Bar = 100 nm.

the coprecipitation of flagellin with Hsp70 (Fig. 4). Addition of ATP to lysates of cells labeled at 38°C had no effect on the amount of Hsp70 precipitated. However, in cells labeled at 41°C, the addition of ATP to the immunoprecipitation mixture resulted in smaller amounts of Hsp70 precipitated compared with mixtures to which apyrase was added (Fig. 4B). No discernible effect on the coprecipitation of flagellin was observed regardless of the labeling temperature (Fig. 4). To determine whether Hsp70 binding to flagellin occurred at growth temperature (33°C), whole-cell lysates labeled with [^{35}S]methionine at 33°C were immunoprecipitated with LA3. This resulted in flagellin being the only protein detected in the fluorogram (Fig. 4C). No ^{35}S-labeled Hsp70 was detected. We have previously shown that Hsp70 is not highly expressed at 33°C (not visible on an autoradiogram) (14). To investigate the possibility of cross-reactivity between either LAλ8 or LA3 and flagellin under nondenaturing conditions, Hsp60 and Hsp70 were precipitated in the presence of 1% Triton X-100 and 0.5 M NaCl (to disrupt chaperone complexes). Under these conditions, no cross-reactivity with flagellin was observed (Fig. 5). These data strongly suggest that previously synthesized Hsp70 can bind flagellin and that coprecipitation of flagellin with either LAλ8 or LA3 is due to chaperone binding and not cross-reactivity.

Whole-cell lysates labeled with [^{35}S]methionine were also immunoprecipitated with an anti-*B. burgdorferi* flagellin MAb (H9724) in the presence or absence of ATP. Immunoprecipitates of cells labeled at 38°C contained both flagellin and Hsp70 (Fig. 4A). Addition of ATP had a minor effect on the coprecipitation of Hsp70 with slightly smaller amounts of Hsp70 coprecipitating in the presence of ATP. However, in cells labeled at 41°C, the amount of Hsp70 coprecipitating with flagellin was greatly reduced in the presence of ATP (Fig. 4B).

Protein binding activity of Hsp60. Immunoprecipitation experiments performed under nondenaturing conditions as described above were also carried out with anti-*B. burgdorferi* Hsp60 (LAλ8). Immunoprecipitates from cells labeled with [^{35}S]methionine at 38 or 41°C contained Hsp60, Hsp70, and flagellin (Fig. 4A and B). Further, if labeled whole-cell lysates were immunoprecipitated first with LA3 (anti-Hsp70) and then with LAλ8, the amount of Hsp70 coprecipitated with LAλ8 was nearly unaffected (data not shown). When ATP was added to the precipitation mixtures, the amount of Hsp70 that was coprecipitated by LAλ8 was significantly reduced at both labeling temperatures. The coprecipitation of flagellin, however, was unaffected by ATP. Immunoprecipitates from cells labeled at 33°C contained only Hsp60 and flagellin, consistent with the results obtained for LA3 at this temperature (Fig. 4C). Thus, flagellin was bound by both chaperones at all temperatures studied. When immunoprecipitation with LAλ8 was performed after the [^{35}S]methionine was chased by incubating the cells in BSKII for 30 min, the amount of Hsp70 and flagellin coprecipitated was decreased (Fig. 6). In contrast, immunoprecipitation with LA3 following the BSKII chase did not affect the coprecipitation of flagellin with Hsp70.

FIG. 3. Western blot analysis of temperature-dependent subcellular localization of Hsp70 in *B. burgdorferi*. Cells grown at 20, 33, or 37°C or at 33°C and heat shocked for 1 h at 41°C were fractionated to yield cytoplasmic (C), periplasmic (P), Triton X-100-soluble membrane (Ts), and Triton X-100-insoluble membrane (Ti) fractions. Equal amounts of protein (10 μg) were loaded onto all lanes, and the proteins were separated by SDS-PAGE. Blots were prepared and Hsp70 was detected with rabbit anti-*E. coli* DnaK as described in Materials and Methods.
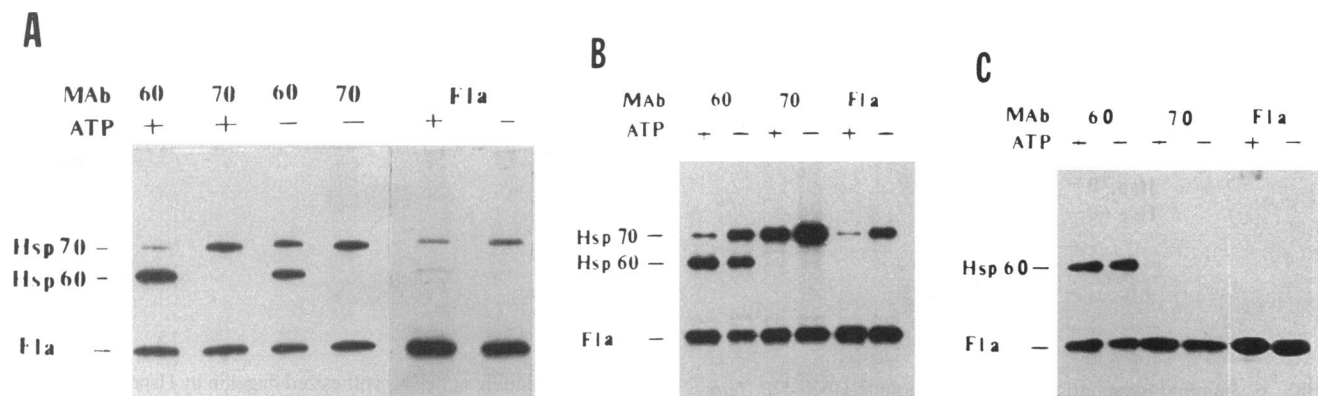
FIG. 4. Immunoprecipitation of *B. burgdorferi* whole-cell lysates under nondenaturing conditions with anti-Hsp60 (LAλ8; 60), anti-Hsp70 (LA-3; 70), or anti-flagellin (H9724; Fla) in the presence or absence of ATP. Cells were grown at 33°C and labeled with [^{35}S]methionine (10 μCi) at either 38°C (A), 41°C (B), or 33°C (C). Lysates were treated with ATP (+) or apyrase (−) and immunoprecipitated with LAλ8 (MAb 60), LA-3 (MAb 70), or H9724 (MAb Fla), and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography as described in Materials and Methods.

To determine the sequence of binding of the two Hsps to flagellin, *B. burgdorferi* cells grown at 33°C were labeled with [^{35}S]methionine at 38°C, and samples were removed after various times (1, 5, 13, and 40 min). Each sample was divided in half, and each aliquot was immunoprecipitated with either anti-Hsp70 (LA3) or anti-Hsp60 (LAλ8). The fluorogram presented in Fig. 7 indicates that by 5 min, Hsp60 and flagellin were synthesized in detectable amounts. Immunoprecipitation with either LA3 or LAλ8 resulted in the coprecipitation of flagellin after a 5-min pulse of [^{35}S]methionine. Newly synthesized Hsp70 was present by 13 min but was not coprecipitated by LAλ8 until 40 min. These data suggest that Hsp60 and Hsp70 are able to bind flagellin independently and that a complex of Hsp60, Hsp70, and flagellin is formed during the processing of flagellin.

DISCUSSION

In this report, we show that Hsp60 and Hsp70 can be observed in multiple cellular compartments in *B. burgdorferi* and that a major function of each is to chaperone flagellin. The subcellular location of the chaperones, Hsp60 in particular, has been unclear. While Hsp60 is generally considered a cytoplasmic protein in most prokaryotes (44), there have been reports suggesting that Hsp60 is membrane associated in some organisms (22, 37, 47). However, it is possible that chaperone proteins may associate with the membrane artifactually because of their chaperone functions (12). It has previously been reported that a 66-kDa protein in *B. burgdorferi* appears to be located in the outer membrane and has amino-terminal sequence similarity to Hsp60 (2, 33, 34). Indirect immunofluorescence performed by us on whole *B. burgdorferi* cells with a MAb against Hsp60 (LAλ8) was negative (data not shown), consistent with studies previously reported (24). Immunogold staining of whole cells was also negative (data not shown). Any membrane association of Hsp60, then, is probably with the inner membrane. Immunoelectron microscopy of thin sections suggested that Hsp60 binds to the cytoplasmic membrane of *B. burgdorferi* and can be detected in the periplasm (Fig. 2). However, the large size of Hsp60-antibody-gold complexes (~20 nm) makes it difficult to determine whether gold particles that appear in the periplasm are periplasmic or cytoplasmic membrane associated. Taking this into account, the amount of membrane-associated Hsp60 may actually be as high as 30%.

Further, under all growth conditions, we found that Hsp60 sediments with the membrane pellet and is solubilized by Triton X-100, a nonionic detergent which is known to solubilize the inner membrane proteins of *E. coli* (41). Furthermore, Hsp60 retains its high degree of mobility in density gradient ultracentrifugation after exposure to the detergent, suggesting that it is still in its oligomeric form during extraction (data not shown). Taken together, these data suggest that (i) Hsp60 associates with the cytoplasmic membrane and (ii) Hsp60 sediments with the membrane fraction because of this association.

It has been shown that *B. burgdorferi* contains fragile membranes (3) and that repeated washings with buffer can result in membrane disruption. Thin-section electron microscopy of washed cells shows blebbing and scattering of outer membranes with release of flagellin. Freeze fracture techniques used to analyze the ultrastructure of *B. burgdorferi* inner and outer membranes show a much greater density of particles in the cytoplasmic membrane than in the outer membrane (39a), which probably results in lower structural integrity of the outer membrane. The protoplasmic (or inner membrane) cylinder, however, seems to stay intact with up to three washings (11).
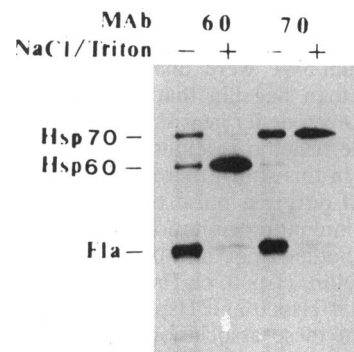

FIG. 5. Immunoprecipitation of Hsp60 and Hsp70 in the presence of 1% Triton X-100 and 0.5 M NaCl. Cells were grown at 33°C and labeled with [^{35}S]methionine at 38°C. The lysates were adjusted to 1% Triton X-100 and 0.5 M NaCl and immunoprecipitated with either an anti-Hsp60 (MAb 60) or anti-Hsp70 (MAb 70) MAb. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Fla, flagellin.
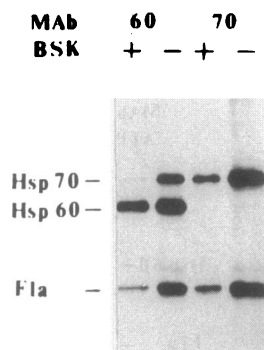
FIG. 6. Immunoprecipitation of [^{35}S]methionine-labeled *B. burgdorferi* whole-cell lysates by either an anti-Hsp60 (MAb 60) or anti-Hsp70 (MAb 70) MAb under nondenaturing conditions before (BSK −) and after (BSK +) a 30-min chase. Cells grown at 33°C were labeled with 10 μCi of [^{35}S]methionine at 38°C for 1 h. One half of the samples were immunoprecipitated at the end of the labeling period; the remainder were allowed to incubate in BSKII for 30 min and then immunoprecipitated. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Fla, flagellin.

FIG. 7. Binding of newly synthesized flagellin by Hsp60 and Hsp70. *B. burgdorferi* cells were pulsed with [^{35}S]methionine (10 μCi) for the indicated times (1, 5, 13, and 40 min). Lysates from each time point were immunoprecipitated with LAλ8 (MAb 60) or LA3 (MAb 70) and analyzed by SDS-PAGE and autoradiography. Fla, flagellin.

We confirmed this with ultrathin-section electron microscopy of spirochetes washed up to three times in 10 mM HEPES buffer (pH 7.6). We also did not observe significant pyruvate dehydrogenase activity in our periplasmic fractions compared with the cytoplasm. It is, therefore, probable that we lose some of the periplasmic and outer membrane contents during the washing steps but do not appear to mix the cytoplasmic or inner membrane contents with them. However, we cannot rule out the possibility that the observed periplasmic location of some Hsp60 and Hsp70 is a result of a small amount of cell disruption during the extraction steps.

In *E. coli*, DnaJ, GrpE, and GroES are involved in peptide processing by DnaK and GroEL (30). We observed other bands that coprecipitated with Hsp60 and Hsp70 and had molecular masses similar to those of DnaJ and GroES but were not present consistently and generally were very faint. Antibodies against GrpE and DnaJ were not reactive to a *B. burgdorferi* cell lysate.

The classical function of the chaperones generally involves their binding to unfolded or misfolded proteins, with an ATP-dependent release of bound polypeptides from either Hsp70 or Hsp60 (5, 15, 17, 45, 48). Immunoprecipitation of *B. burgdorferi* whole-cell lysates under nondenaturing conditions with anti-Hsp60 or anti-Hsp70 results in the specific coprecipitation of flagellin. We were unable to reproducibly detect proteins other than flagellin that coimmunoprecipitate with the chaperones even under heat shock conditions. It should be noted that while flagellin is abundantly expressed at all temperatures used in this study, there are other highly expressed proteins labeled during a pulse of [^{35}S]methionine (14, 19). Additionally, immunoprecipitation experiments performed with other antibodies (e.g., anti-OspA) failed to coimmunoprecipitate flagellin, Hsp60, or Hsp70 (data not shown). Our studies show that Hsp60 and Hsp70 both have high affinities for flagellin, but these associations are not ATP dependent under normal conditions. Rather, ATP-dependent flagellin dissociation from Hsp70 occurs only when *B. burgdorferi* cells are heat shocked at 41°C prior to immunoprecipitation and not at lower temperatures (33 and 38°C). The data presented in Fig. 4 suggest at least two possibilities. First, there may be multiple Hsp70-binding sites on the flagellin molecule. The sites exposed at 41°C are sensitive to ATP-induced release of
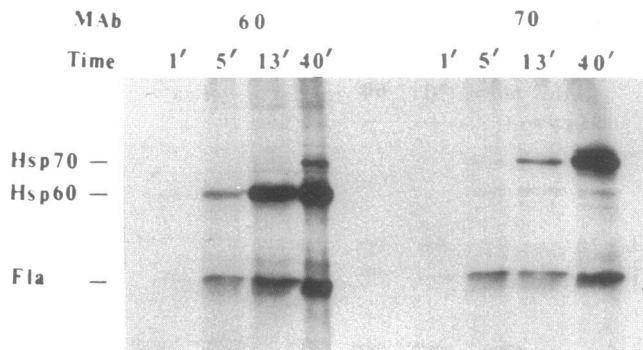
Hsp70, while those exposed at lower temperatures are insensitive to ATP. Alternatively, it is possible that the ATPase activity or the ATP-binding activity of Hsp70 is enhanced at 41°C but is weak or inactive at lower temperatures. Some combination of these two possibilities may also occur. We fail to observe any ATP-dependent relationship between flagellin and Hsp60.

In *E. coli*, DnaK is believed to bind nascent polypeptides in extended conformations (38), while GroEL may associate with intermediates containing secondary structure (26). Furthermore, it has been shown that DnaK interacts with newly synthesized polypeptides to prevent misfolding, after which GroEL aids in proper folding. GroES then acts to release the folded protein from GroEL by using ATP hydrolysis (30). Such an ordered sequence of events is not evident from our data. We observed that flagellin is bound by either Hsp60 or Hsp70 immediately after its synthesis. Subsequently, a complex containing Hsp60, Hsp70, and flagellin is formed. In vitro, only Hsp70 is released from this complex upon addition of ATP. The factors involved in activating the release of flagellin from Hsp60 in *B. burgdorferi* are still to be elucidated. We were unable to observe release of flagellin unless cells were transferred to BSKII following labeling. Even after 30 min in BSKII, some flagellin was still found associated with Hsp60. Thus, the rate-limiting step in the processing of flagellin may be release of folded flagellin from Hsp60. Incubating labeled cells in RPMI (a non-growth medium) with unlabeled methionine did not result in significant loss of Hsp60-bound flagellin, even after 3 h (data not shown). Perhaps growth and flagellum assembly are necessary for the processing of flagellin. To our knowledge, the events between synthesis and export of flagellin subunits through the inner membrane are still to be determined. It would be interesting if Hsp60 association with the membrane is related to this process in *B. burgdorferi*. We also note that the complex formed between Hsp70 and flagellin is not transient in vivo when labeling is carried out at heat shock temperatures (Fig. 6). Under these conditions, the formation of Hsp70-flagellin complexes may represent a dead end. Beckmann et al. (5) noted similar nontransient complexes between Hsp70 and newly synthesized proteins in stressed HeLa cells.

Finally, it was interesting that Hsp60 could not be coprecipitated with a MAb to either Hsp70 or flagellin, since it appears that the three form a complex. Recent reports indicate that polypeptides are bound in the central core of Hsp60 (10, 31). At this location, Hsp70 and flagellin may be inaccessible to

antibodies. Thus, the Hsp60-Hsp70-flagellin complex can be observed only when immunoprecipitating with an antibody to Hsp60. We speculate that the formation of this complex may be related to the export of flagellin across the cytoplasmic membrane to the distal end of the periplasmic flagella in *B. burgdorferi*. Perhaps flagellin is kept in an extended conformation by Hsp70 and is then partially folded into an export-competent shape by an ATP-dependent association between Hsp60 and Hsp70. Since it is proposed that flagellin is exported through the central core of the flagellum (28), it may be that Hsp60 and Hsp70 hold flagellin in an export-competent state during this process.

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