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Ca^{2+}-Stabilized Oligomeric Protein Complexes Are Major Components of the Cell Envelope of "*Thermus thermophilus*" HB8

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The major cell envelope proteins of the gram-negative thermophilic eubacterium "*Thermus thermophilus*" gave an electrophoretical pattern characterized by two well-defined groups of bands. One of them showed up as four regularly spaced proteins (HMrPs) with M_rs higher than 310,000, a value corresponding to the smaller HMrP. The second one was formed by two proteins with M_rs of 100,000 (P100) and 84,000 (P84). HMrPs P100 and P84 were apparently located in the outer layer of the cell envelope, as indicated by their accessibility, in intact cells, to external lactoperoxidase and by their association, in fractionation experiments, with a high-density membrane fraction devoided of NADH-oxidase activity. Removal of Ca^{2+} unstabilized the HMrPs, which dissociated into P100 when heated at 80 to 85°C in 10% (wt/vol) sodium dodecyl sulfate, indicating that HMrPs were oligomeric complexes of P100. In the presence of Ca^{2+}, HMrPs were extremely stable, withstanding prolonged incubation in boiling 10% (wt/vol) sodium dodecyl sulfate–2% (vol/vol) β-mercaptoethanol. Solubilization of P100 and HMrPs by detergents was severely constrained by interactions with the peptidoglycan layer of the cell envelope.

"*Thermus thermophilus*" is a thermophilic gram-negative eubacterium able to grow at temperatures ranging from 60 to 85°C. It was originally isolated from a hot spring (11). The taxonomic position of "*T. thermophilus*," in particular its rank as a species, is still under discussion, being at present considered a particular isolate (HB8) of *T. aquaticus* (5), although studies of numerical taxonomy do not support a close taxonomic relationship between *T. aquaticus* and "*T. thermophilus*" (7).

The cellular components of members of the genus *Thermus* thus far studied are intrinsically more heat stable and, in general, more resistant to damaging agents than their counterparts in mesophilic bacteria (1, 6).

The hypothesis, first proposed by Brock (3), that the integrity of the cell membrane could be the limiting factor for thermal death of bacteria stimulated research on the properties of the cell envelope of thermophilic bacteria, among them the genus *Thermus* (12).

Under the electron microscope, the cell envelope of "*T. thermophilus*" consists of a well-defined outer layer and a thin murein sacculus covering the cytoplasmic membrane (4). However, its chemical composition seems to be rather peculiar compared with that of mesophilic gram-negative bacteria. Peptidoglycan contains ornithine as a diamino acid instead of *meso*-diaminopimelic acid, and neither heptose sugars nor 3-desoxy-octulonic acid has been identified in the outer layer. The fatty acid composition of phospholipids is closer to that of gram-positive bacteria, and unique phospholipids and neutral lipids are major constituents of the membranes (4). Furthermore, many of its components are still poorly characterized, hampering studies on the molecular interactions relevant to the thermal stability of the cell envelope as a whole. These considerations prompted us to investigate the properties of the putative major proteins of the cell envelope of "*T. thermophilus*." The rationale behind this was the assumption that envelope proteins of high relative abundance could act as structural stabilizers of the cell envelope by means of protein-protein or protein-lipid interactions, in addition to a potential involvement in other functions.

Our results indicate that the major proteins of the cell envelope of "*T. thermophilus*" are in fact a group of Ca^{2+}-stabilized oligomeric proteins, accessible to enzymatic proteins in the external medium and associated with a high-density membrane fraction. The oligomeric complexes exhibited exceptionally high resistance against thermal denaturation in the presence of detergents.

MATERIALS AND METHODS

Strains and conditions of growth. "*T. thermophilus*" HB8 (11, 12) was obtained from the American Type Culture Collection (ATCC 27634). The organism was routinely grown at 75°C under strong aeration in a rich medium containing 4 g of yeast extract, 8 g of Trypticase, 3 g of NaCl, and 1 g of glucose per liter. Growth was monitored by turbidimetry.

Preparation of cell envelopes. Mid-log-phase cultures were harvested by centrifugation (10,000 × *g*, 5 min). Cells were suspended to the initial volume in 10 mM Tris hydrochloride (pH 7.8) buffer, centrifuged as above, and resuspended in 1/50th the original volume in the same buffer. Cells were broken by sonication (one 30-s pulse) on a sonicator (MSE Scientific Instruments, Sussex, U.K.) set at an amplitude of 18 μm. Unbroken cells were removed by low-speed centrifugation (5,000 × *g*, 5 min), and cell envelopes were recovered by centrifuging the supernatant at high speed (30,000 × *g*, 30 min). Cell envelopes were washed once by suspension in an appropriate volume of the same buffer and centrifuged (30,000 × *g*, 30 min) before being resuspended in the same buffer at a concentration of 30 mg of protein per ml, as measured by the method of Lowry et al. (9). All manipulations were performed in the cold (4 to 6°C). Cell envelope preparations were either used immediately or kept frozen at −70°C.

Solubilization of cell envelope proteins by detergents. Purified cell envelopes (200 μl, 30 mg of protein per ml) were

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incubated for 30 min at 60°C in 1% (wt/vol) solutions of sodium dodecyl sulfate (SDS), Triton X-100, cetyl-trimethylammonium bromide, and sodium dodecyl sarkosinate. After separation of solubilized and insoluble material by centrifugation (10,000 × *g*, 20 min), the pellets were resuspended in an equal volume of 10% (wt/vol) SDS, incubated for 30 min at 100°C to extract material insoluble in the detergent previously tested, and centrifuged as before. Proteins in the supernatant and pellet from each sample were identified by SDS-polyacrylamide gel electrophoresis (PAGE) as described below.

Electrophoretic separation of cell envelope proteins. Separation of cell envelope proteins was routinely performed by SDS-PAGE essentially as described by Laemmli and Favre (8), with discontinuous slab gels in which the concentration of acrylamide was 11% (wt/vol) in the lower half and 6% (wt/vol) in the upper half. The total length of the gels was 18 cm. The concentrations of SDS and β-mercaptoethanol in the sample buffer were increased to 4% (wt/vol) and 2% (vol/vol), respectively.

For the determination of the M_r of very high M_r proteins, we used 3.3% acrylamide slab gels prepared by the method of Weber et al. (15) with protein standard mixture III (catalog no. 15126; E. Merck, Darmstadt, Federal Republic of Germany) as standards.

Bidimensional slab gel electrophoresis of cell envelope proteins. The electrophoresis system was based on the fact that in the presence of EDTA, high-M_r proteins (HMrPs) were dissociated at elevated temperatures in SDS.

A sample of 15 mg of cell envelopes was solubilized by boiling in 2 ml of 10% (wt/vol) SDS, and insoluble material was removed by centrifugation (15,000 × *g*, 15 min). The sample was layered on top of a 12-cm-wide, 2-mm-thick slab gel and electrophoresed in the first dimension as described for SDS-PAGE. The gel was then cut into 1.5-cm-wide strips that were boiled for 5 min in 10 mM EDTA–2% (wt/vol) SDS–125 mM Tris hydrochloride, pH 6.8. Afterwards, one strip was put on top of a second SDS-PAGE slab gel (8% polyacrylamide) containing 1 mM EDTA and electrophoresed as above. The running buffer for the second dimension was supplemented with 1 mM EDTA.

Fractionation of membranes by centrifugation in sucrose density gradients. Cells were treated as described by Osborn et al. (10) and Schnaitman (13) for the fractionation of cell envelopes in sucrose density gradients.

When cell envelopes were fractionated by the method developed by Osborn et al., a sample of 0.5 g (wet weight) of cells was incubated for 5 min at 37°C in 4 ml of a solution of lysozyme (100 μg/ml) in 10 mM Tris hydrochloride–1 mM EDTA–0.25 M sucrose, pH 7.8. Cells were broken by sonication (45 s at an amplitude of 18 μm, 4°C), the suspension was centrifuged (30,000 × *g*, 30 min, 4°C), the pellet was suspended in 0.5 ml of 10 mM Tris hydrochloride–1 mM EDTA, pH 7.8, and layered on top of a discontinuous sucrose gradient, prepared in a Beckman SW40 rotor tube, consisting of the following layers of sucrose (from the bottom to the top): 1 ml of 65% (wt/wt); 2 ml of 55%; 2 ml of 50%; 4 ml of 40%; 3 ml of 30%. Samples were centrifuged at 4°C in an SW40Ti rotor (Beckman Instruments Inc., Palo Alto, Calif.) for 16 h at 30,000 × *g*.

When the method developed by Schnaitman was used, cells (0.5 g wet weight) were suspended in 4 ml of 10 mM Tris hydrochloride–1 mM EDTA, pH 7.8, containing DNase I and RNase A at 40 μg/ml each and subjected to sonication as above. $MgCl_2$ was added immediately afterwards to the sample, and cell envelopes were recovered by centrifugation (30,000 × *g*, 30 min, 4°C). The pellet was suspended in 0.8 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate) buffer, pH 7.5, and processed as above.

Gradients were fractionated into a number of equal fractions with a gradient fractionator (ISCO, Lincoln, Neb.). Density of the fractions was estimated by weighing fixed volumes of each on a precision balance (Mettler Instrumente, Greifensee-Zurich, Switzerland). To identify the proteins on each fraction, samples of 50 μl were diluted 1:3 in water, centrifuged (13,000 × *g*, 15 min), suspended in 10 mM Tris hydrochloride, pH 7.8, and subjected to SDS-PAGE as described above.

Assay of NADH-oxidase activity in membrane fractions. The activity was measured spectrophotometrically following the initial rate of decline in the absorbance at 340 nm produced by adding 30-μl samples of the fraction to be tested to a cuvette, held at 75°C, containing 1 ml of 50 mM Tris hydrochloride, 0.2 mM dithiothreitol, and 12 mM NADH, pH 7.8 (11). The protein content of each fraction was measured by the method of Lowry et al. (9).

Radioiodination of cell surface proteins by lactoperoxidase. Cells harvested by centrifugation (10 min, 10,000 × *g*) of a mid-log-phase culture were suspended in an equal volume of cold (4°C) 50 mM phosphate buffer–120 mM NaCl, pH 7.2, centrifuged as above, and suspended to 1/20th the original volume in the same buffer supplemented with 20 mM glucose. Radioiodination was performed by sequentially adding 10 mU of lactoperoxidase (EC 1.11.17; Sigma Chemical Co.), 10 μg of lactalbumin, 4 mU of glucose-oxidase (EC 1.1.3.4; type V; Sigma), and 100 μCi of ^{125}INa (2.22 Ci/μmol; The Radiochemical Center, Amersham, U.K.) per ml. Samples were incubated for 15 min at 4°C. To stop the reaction, cells were diluted to 1:20th the original buffer and washed twice by centrifugation and resuspension in an equal volume of the same buffer. To identify the labeled membrane proteins, cell envelopes were fractionated by centrifugation in sucrose density gradients by the method of Osborn et al., modified as indicated above. Samples of each fraction of interest to us were subjected to electrophoresis as described, and after drying the gels, radioactive material was detected by autoradiography on Kodak X-Omat X-ray film.

RESULTS

Solubilization of cell envelope proteins of "*T. thermophilus*" by detergents. Figure 1 (lane C) shows the picture of a Coomassie blue-stained gel obtained by SDS-PAGE of the material solubilized after incubation of purified cell envelopes in 10% (wt/vol) SDS and 2% (vol/vol) β-mercaptoethanol at 100°C for 30 min, conditions generally assumed to solubilize cell envelope constituents except the murein layer and proteins covalently bound to it (2). The most remarkable features were a group of four regularly spaced bands at positions corresponding to M_rs higher than 300,000, and two prominent bands at apparent M_rs of 100,000 and 84,000. Henceforth the group of proteins with apparent M_rs higher than 300,000 are referred to as HMrPs a to d and the proteins with M_rs of 100,000 and 84,000 as P100 and P84, respectively. Densitometric quantification gave the following figures for the relative abundance of each protein in the cell envelope: P100, 5%; P84, 20%; HMrP a, 20%; HMrP b, 5%; HMrP c, 1%; and HMrP d, 0.5%. Therefore, these six proteins account for more than 50% of total cell envelope protein by themselves.

Solubilization of HMrPs P100 and P84 by detergents of different nature was examined. While P84 was solubilized by

all detergents tested except cetyl-trimethylammonium bromide, the HMrPs and P100 were virtually unextractable by any of them except partially by 1% SDS under the conditions adopted.

Fractionation of cell envelopes by centrifugation in sucrose density gradients. The distribution of the major envelope protein among membrane fractions of different densities was studied by the methods described by Osborn et al. (10) and Schnaitman (13), both in common use in topological studies of membrane proteins in gram-negative bacteria.

NADH-oxidase was used as a specific marker for the identification of fractions enriched in cytoplasmic membrane (10). Unfortunately, no appropriate marker for the outer layer of this organism has been described so far.

Fractionation of cell envelopes by both methods gave two well-defined bands in the gradients at densities of 1.15 ± 0.005 and 1.19 ± 0.005 g/ml (Fig. 2). Estimation of the amount of protein in each band indicated that the denser band was about 10-fold richer in protein than the lighter one; however, this could be partially due to preferential loss of cytoplasmic membrane vesicles during sample preparation. NADH-oxidase activity was mainly associated with the lighter band irrespective of the method used for fractionation of the envelopes. The percentage of cytoplasmic membrane protein contaminating the denser fractions after separation by the methods of Osborn et al. (10) and Schaitman (13) was estimated as 12 and 32%, respectively, on the basis of the activity of NADH-oxidase in the dense fraction. SDS-PAGE analysis of the fractions indicated that the patterns of proteins in the dense fractions were strongly dependent on the fractionation method (Fig. 2). When membranes were fractionated by the Schnaitman method, the HMrPs, P100, and P84 were quantitatively recovered in the dense band. However, when the method of Osborn et al. was used, the HMrPs could not be visualized in the band of corresponding density or in any other fraction of the gradient. However, the relative abundance of P100 and P84 was considerably increased compared with the unfractionated control sample.

Hypothetically, these results could be explained by assuming that HMrPs were oligomeric proteins whose stability was drastically reduced during fractionation by the method of Osborn et al., dissociating into monomeric subunits.

Modifications in the pattern of major envelope proteins induced by Ca^{2+} chelators and lysozyme; reversibility by divalent cations. As the main difference between the methods of Osborn et al. and Schnaitman is the inclusion in the former of an EDTA-lysozyme treatment to form spheroplasts, experiments were run to study the individual effects of EDTA and lysozyme on the SDS-PAGE pattern of the major envelope proteins of "*T. thermophilus*."

Figure 3A shows the results obtained when samples of cell envelopes were incubated for 5 min with increasing concentrations of EDTA before SDS-PAGE. A drastic change in the pattern of proteins, characterized by a reduction in the amount of HMrPs and a concomitant increase in P100 and P84, was clearly induced by EDTA at a low concentration (0.1 mM). The same effect was observed when the more specific Ca^{2+} chelator EGTA [ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetracetate] was used instead of EDTA (Fig. 3B).

The effect of lysozyme was also investigated. As shown in Fig. 3C, lysozyme at the concentration used in the spheroplasting treatments (100 μg/ml) had the same effect as EDTA or EGTA on the relative proportions of HMrPs, P100, and P84 upon fractionation of treated cell envelopes by SDS-PAGE. Interestingly, a thermally denatured enzyme had no effect on the major envelope proteins (Fig. 3C, lane 14), indicating that the action of lysozyme was dependent on its native structure or its enzymatic activity.

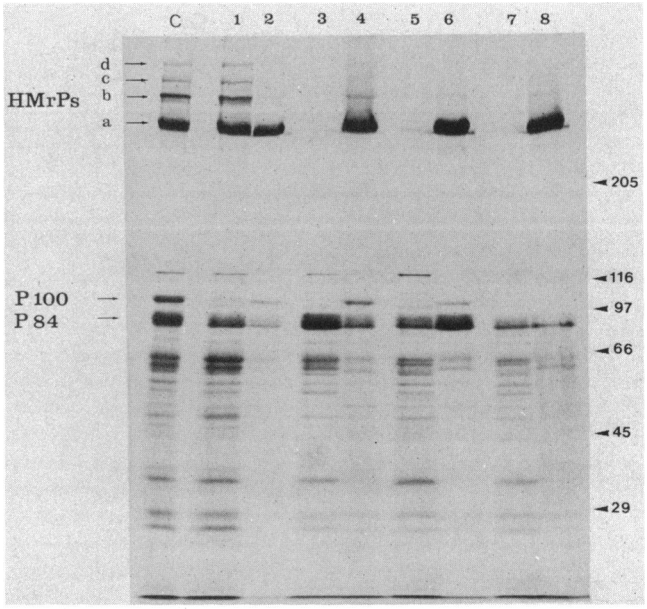

FIG. 1. Solubilization of cell envelope proteins of "*T. thermophilus*" by detergents. Samples of purified cell envelopes were treated at 60°C with 1% (wt/vol) solutions of SDS, Triton X-100, cetyl-trimethylammonium bromide, and sodium dodecyl sarkosinate and fractionated into soluble and insoluble fractions as indicated in the text. Sample C (control) was incubated at 100°C in 10% (wt/vol) SDS–2% (vol/vol) β-mercaptoethanol, and the solubilized material was directly applied onto the gel. Lanes: 1, SDS soluble; 2, SDS insoluble; 3, Triton X-100 soluble; 4, Triton X-100 insoluble; 5, cetyl-trimethylammonium bromide soluble; 6, cetyl-trimethylammonium bromide insoluble; 7, sodium dodecyl sarkosinate soluble; 8, sodium dodecyl sarkosinate insoluble. The positions of the following marker proteins are indicated (in kilodaltons); myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorilase B, 97.4 kDa; bovine serum albumin, 66 kDa; albumin from egg, 45 kDa; and carbonic anhydrase, 29 kDa.

To study the reversibility of the effects of EDTA and lysozyme, cell envelopes pretreated with either of them were washed and resuspended in EDTA- or lysozyme-free buffer, treated with Ca^{2+} or Mg^{2+} at increasing concentrations, and analyzed by SDS-PAGE (Fig. 4). Addition of Ca^{2+} at low concentrations (0.5 mM) readily reversed the effects of both EDTA and lysozyme on cell envelopes, preventing dissociation of the HMrPs into monomeric subunits by boiling SDS. In contrast, Mg^{2+} proved to be a quite inefficient antagonist of EDTA in this particular case.

No preference in the stabilization of any given species of HMrP could be detected in these experiments. All of them increased in parallel depending on the concentration of Ca^{2+}.

Incubation of cell envelopes with either EDTA (10 mM) or lysozyme (100 μg/ml) did not by itself promote solubilization of P100 or P84. However, preincubation with lysozyme (30 min, 100 μg/ml) but not with EDTA made P100 and HMrPs extractable by nonionic detergents under relatively mild conditions (1% Triton X-100, 60°C).

Subunit composition and stoichiometry of HMrPs. Taking advantage of the change in the stability of HMrPs induced by EDTA, we designed a bidimensional electrophoresis system for identification of the monomeric subunits of the HMrPs

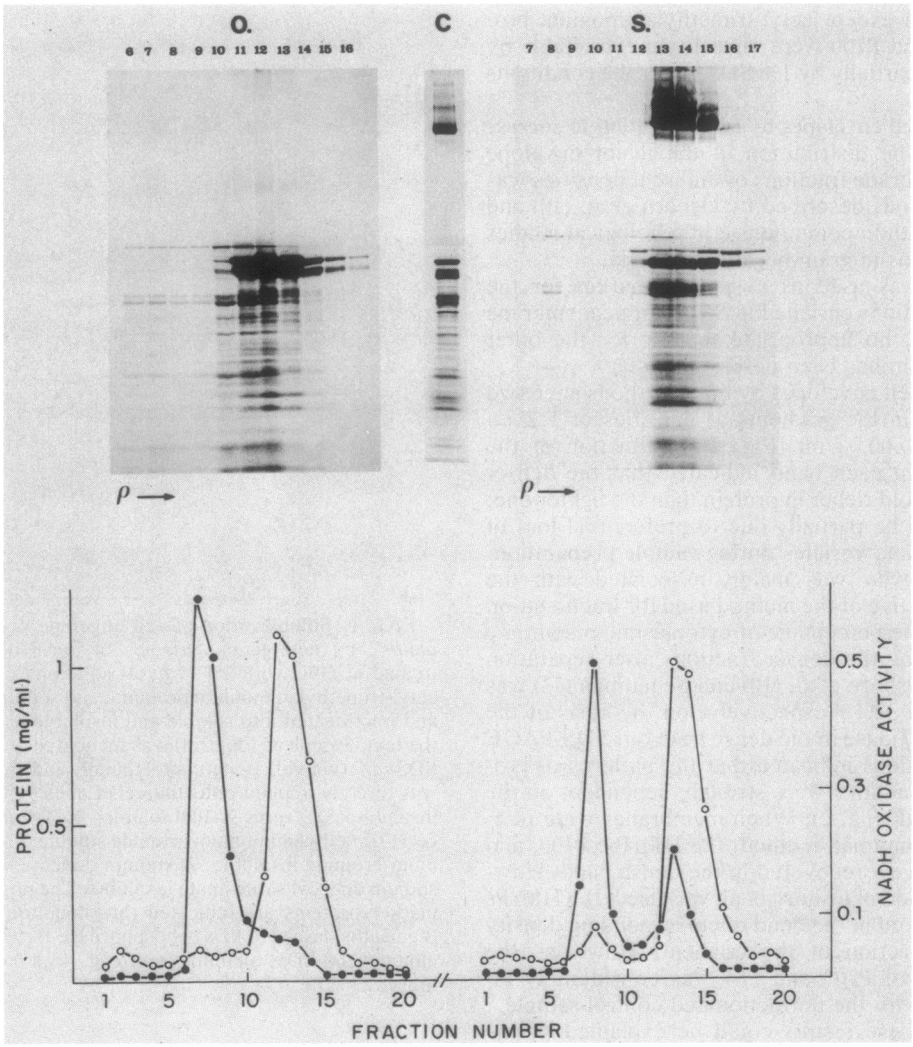

FIG. 2. Fractionation of cell envelopes by centrifugation in sucrose density gradients. Cell envelopes were fractionated in sucrose gradients as described in the text. (O) Fractionation by the method of Osborn et al. (S) Fractionation by the method of Schnaitman. The upper part shows Coomasie blue-stained gels obtained by SDS-PAGE of the gradient fractions. Numbers above the lanes are fraction numbers. The lower part shows graphic representations of protein concentration (○) and NADH-oxidase activity (●) along the gradients. (C) Coomasie blue-stained gel obtained by SDS-PAGE of a sample of unfractionated cell envelopes.

(see above). In short, cell envelope proteins were first fractionated by standard SDS-PAGE. Afterwards, a strip was cut along the upper part of the slab gel containing the HMrPs and boiled in an EDTA-containing buffer to unstabilize and dissociate the HMrPs. The strip was then put on top of a second SDS-polyacrylamide slab gel containing EDTA and subjected to electrophoresis (Fig. 5). Apparently, the HMrPs are oligomeric proteins essentially built up from a single kind of subunit, P100. The arrows in Fig. 5 indicate possible minor components of HMrPs.

If the HMrPs were homotypic proteins, their stoichiometry could be roughly estimated from their apparent M_rs. Using the electrophoretical method of Weber et al. (15), we obtained an apparent M_r of 310,000 for HMrP a, suggesting that the smaller and more abundant HMrP is a trimer of P100. Due to lack of appropriate M_r markers, the M_rs of the other HMrPs could not be determined accurately.

The outcome of this experiment raised a puzzling question: if P84 is not a subunit of the HMrPs, what is its fate when Ca^{2+} is added to cell envelopes before SDS-PAGE?

The possibility that the reduction in the amount of P84 observed in Ca^{2+}-treated samples was due to formation of SDS-insoluble complexes seems unlikely. In fact, when samples of the P84-rich dense fraction of the cell envelope, isolated by the method of Osborn et al., were treated with Ca^{2+}, solubilized in SDS, and directly analyzed by SDS-PAGE, no protein precipitates could be detected in the upper part of the gels (data not shown).

Effect of EDTA and Ca^{2+} on the stability of solubilized HMrPs. The influence of Ca^{2+} on the stability and resistance to thermal denaturation of solubilized HMrPs was also investigated. The results of an experiment in which the effects of EDTA and Ca^{2+} on the stability of SDS-solubilized and particulate HMrPs were compared indicated that solubilization did not alter the stability of the oligomeric complexes (data not shown). This observation, together with the fact that the state of aggregation of the SDS-solubilized HMrPs could be tested by subjecting the samples directly to SDS-PAGE, facilitated the determination of the temperature causing dissociation of HMrPs in both the presence and

absence of EDTA. For this purpose, SDS-solubilized HMrPs were incubated (15 min) at different temperatures in the presence or absence of 1 mM EDTA and analyzed by SDS-PAGE. The results indicated that in the presence of EDTA, HMrPs were readily dissociated at 80 to 85°C (>80% of total protein migrating in the gels as P100), whereas in the absence of the chelator they could withstand at least 100°C. Furthermore, once HMrPs had been dissociated in the presence of EDTA, they failed to reassociate upon addition of excess Ca^{2+} (data not shown).

^{125}I-radiolabeling of cell envelope proteins with lactoperoxidase. Additional information about the localization of the major proteins of the cell envelope of "*T. thermophilus*" was obtained by studying their accessibility to external lactoperoxidase in intact cells.

Figure 6 displays the results of an iodination experiment in which cell envelopes were fractionated by the method of Osborn et al. upon completion of labeling. HMrPs as well as P100 were efficiently labeled by lactoperoxidase. Furthermore, the fact that the bulk of labeled proteins appeared in the denser fraction supports the idea that this fraction is enriched in fragments in direct contact with the external medium, as happens in *Escherichia coli* and *Salmonella typhimurium* (10, 13).

DISCUSSION

Evidence presented in this report indicates that the major cell envelope proteins of "*T. thermophilus*" are actually a family of oligomeric proteins, the HMrPs, apparently built up from a single kind of monomeric subunit, protein P100. Nevertheless, the possible presence of additional proteins as minor components of the HMrPs could not be ruled out at this stage of the investigation.

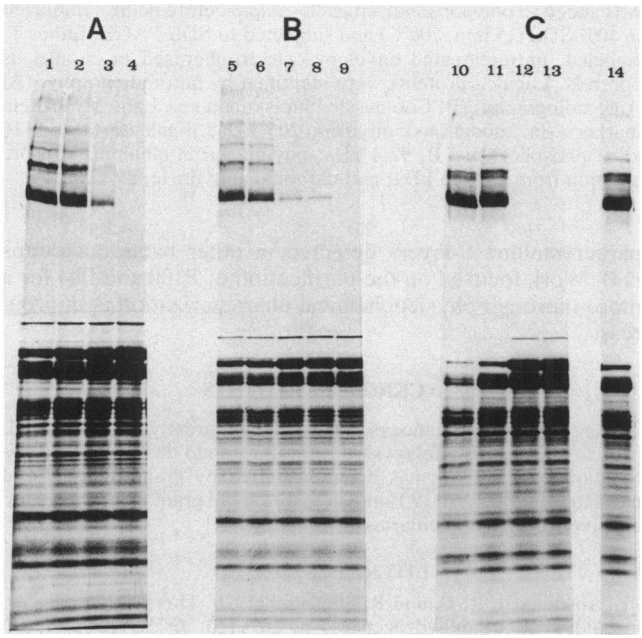

FIG. 3. Effect of Ca^{2+} chelators and lysozyme on the electrophoretic pattern of cell envelope proteins. Purified cell envelopes were incubated for 30 min at 37°C in the presence of EDTA, EGTA, or lysozyme at the concentrations indicated below and analyzed by SDS-PAGE. (A) Samples treated with EDTA at 0 mM (lane 1), 0.01 mM (lane 2), 0.1 mM (lane 3), and 1 mM (lane 4). (B) Samples treated with EGTA at 0 mM (lane 5), 0.01 mM (lane 6), 0.1 mM (lane 7), 0.5 mM (lane 8), and 1 mM (lane 9). (C) Samples treated with lysozyme at 0 μg/ml (lane 10), 10 μg/ml (lane 11), 100 μg/ml (lane 12), and 200 μg/ml (lane 13). The sample in lane 14 was treated like the sample in lane 13 but with enzyme inactivated by heating at 100°C for 15 min before use.

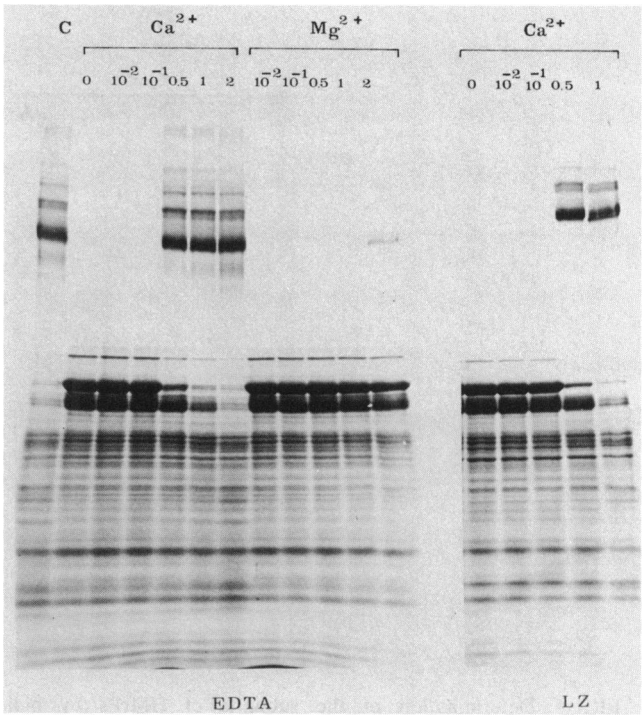

FIG. 4. Reversion of the effects of EDTA and lysozyme by divalent cations. Purified cell envelopes were treated with 1 mM EDTA (gel labeled EDTA) or lysozyme (200 μg/ml; gel labeled LZ) for 30 min at 37°C. EDTA or lysozyme was washed out by repeated centrifugation and resuspension in the original buffer. Samples were incubated (5 min, 37°C) with $CaCl_2$ or $MgCl_2$ at the concentrations (millimolar) indicated and subjected to SDS-PAGE. Lane C shows the pattern of proteins for untreated cell envelopes.

The drastic effect of Ca^{2+} on the stability of the HMrPs and its freely reversible nature strongly argue against covalent bonding of the monomeric subunits. Furthermore, the fact that HMrPs were not dissociated by strongly denaturing agents suggests that aggregation of P100 to form an HMrP might involve extensive conformational alterations of the protein, stabilized by its interaction with Ca^{2+}.

In opposition to P84, which was easily extracted by detergents, solubilization of HMrPs and P100 required rather harsh treatments, suggesting a strong association with other cell envelope constituents, very likely with the peptidoglycan layer, whose degradation by lysozyme made these proteins soluble under milder conditions.

Information about the exact location of HMrPs, P100, and P84 is still far from definitive. However, our observations suggest that all of them are associated with the outer layer of the cell envelope.

Apparently, dissociation into P100 was efficiently prevented by concentrations of Ca^{2+} (0.1 to 0.5 mM) which can be considered "ecological" in the sense that such concentrations might be found in the natural habitat of "*T. thermophilus*" (11). Therefore, it seems logical to think that in nature P100 is in the form of thermostable protein complexes.

Interestingly, no differential effects of Ca^{2+} or EDTA on the individual HMrPs were detected, suggesting that the

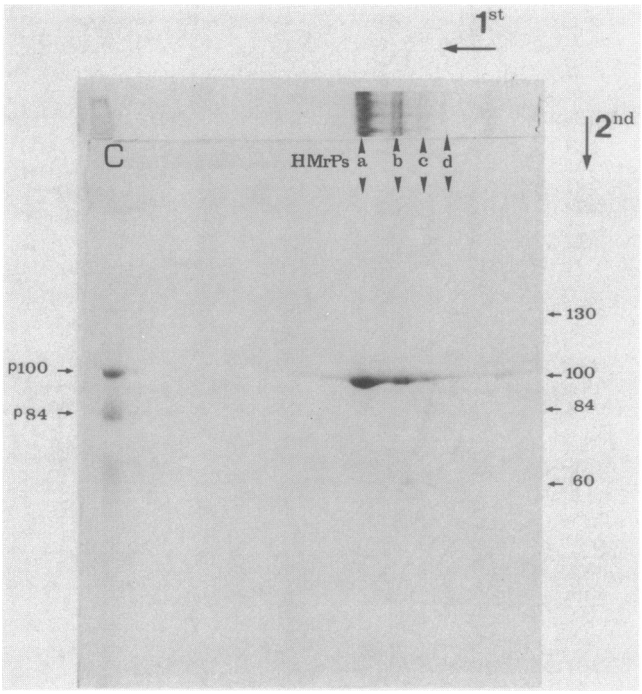

FIG. 5. Determination of the subunits of HMrPs by bidimensional slab gel electrophoresis. Purified cell envelopes solubilized in 10% (wt/vol) SDS were subjected to two-dimensional gel electrophoresis as described in the text. To facilitate identification of the subunits of the HMrPs, a sample of EDTA-treated cell envelopes was electrophoresed in the left side of the same second-dimension gel (lane C). In the picture, a Coomassie blue-stained strip of the first-dimension gel has been put on top of the second-dimension slab to mark the positions of the different HMrPs. The positions of P100 and P84 in lane C and of the spots detected in the second-dimension slab are indicated on the left and right (in kilodaltons), respectively.

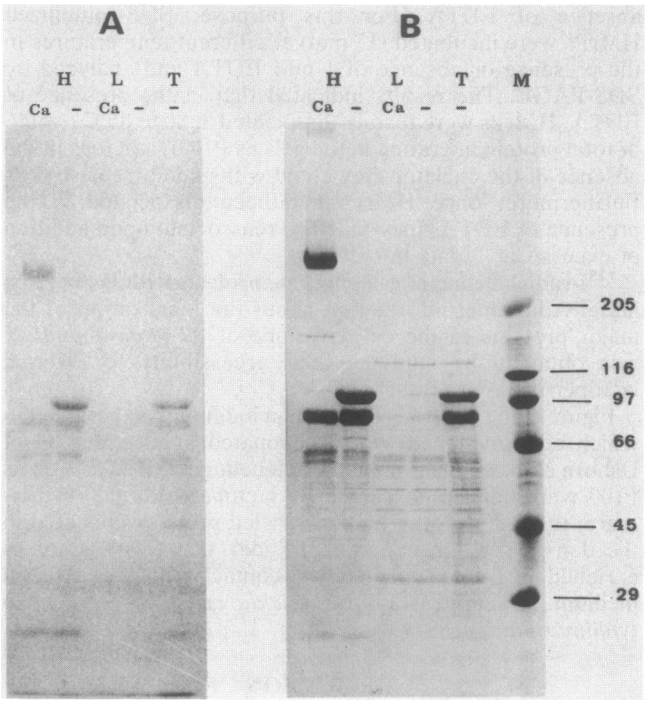

FIG. 6. Radioiodination of cell envelope proteins in intact cells by lactoperoxidase. A sample of exponentially growing cells was labeled with ^{125}INa and lactoperoxidase and fractionated as described in the text. Samples of the heavy (lanes H) and light (lanes L) membrane fractions were divided into two portions. Ca^{2+} (5 mM) was added to one portion from each sample before being solubilized in 10% SDS (15 min, 100°C) and subjected to SDS-PAGE. Lanes T, Labeled, unfractionated envelopes electrophoresed in parallel as controls. Labeled proteins were identified by autoradiography. (A) Autoradiography. (B) Coomassie blue-stained gel. Lane M, Protein markers (in kilodaltons): myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 66 kDa; albumin from egg, 45 kDa; and carbonic anhydrase, 29 kDa.

stoichiometry of the complexes would most likely not be determined in vivo by the concentration of Ca^{2+}.

Two particularly conflicting aspects of our results were the fate of P84 and the discovery that lysozyme alone had the ability to facilitate dissociation of HMrPs in purified cell envelopes. With respect to the former point, our initial supposition that P84 would be a subunit of HMrPs was neatly eliminated by the fact that dissociation of HMrPs did not render P84 at detectable amounts. Based on some casual observations that are still difficult to evaluate, we are presently investigating the possibility that P84 would itself be an oligomeric protein whose association-dissociation equilibrium responds to changes in the concentration of Ca^{2+} in a way opposite to that of P100.

As to whether lysozyme is able to unstabilize HMrPs, the straightforward interpretation of a stabilizing action of the peptidoglycan layer cannot be maintained considering that the effect of the enzyme can be reversed by addition of Ca^{2+} and that solubilized HMrPs were as stable as particulate HMrPs in the presence of Ca^{2+}.

Alternatively, the effect of lysozyme could be due to its casual interaction with a critical domain of P100, for instance, regions involved in monomer-monomer or Ca^{2+}-HMrP interactions; however, this interpretation is purely speculative at present.

An interesting possibility suggested by the features of the HMrP-P100 system is that HMrPs might be intermediate oligomeric subunits of a higher-order structure, such as the paracrystalline S-layers detected in other bacterial groups (14). Work focused on the purification of P100 and P84 for a more thorough physicochemical characterization is in progress.

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