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Identification and Characterization of *Vibrio cholerae* Surface Proteins by Radioiodination

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Whole cells and isolated outer membrane from *Vibrio cholerae* (Classical, Inaba) were radiolabeled with Iodogen or Iodo-beads as catalyst. Radiolabeling of whole cells was shown to be surface specific by sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis of whole cells and cell fractions. Surface-labeled whole cells regularly showed 16 distinguishable protein species, of which nine were found in radiolabeled outer membrane preparations obtained by a lithium chloride-lithium acetate procedure. Eight of these proteins were found in outer membranes prepared by sucrose density gradient centrifugation and Triton X-100 extraction of radiolabeled whole cells. The mobility of several proteins was shown to be affected by temperature, and the major protein species exposed on the cell surface was shown to consist of at least two different peptides.

Little is known about the surface or outer membrane structures of *Vibrio cholerae*. *V. cholerae* cells have two types of appendage: a single, polar flagellum enclosed in a sheath which appears contiguous with the outer membrane (5), and pili (1, 36). The lipopolysaccharide of *V. cholerae* is chemically different from that of the family *Enterobacteriaceae* (13). *V. cholerae* cytoplasmic membrane and outer membrane are not differentially solubilized by Triton X-100 extraction (18), as are the membranes of *Escherichia coli* and *Pseudomonas aeruginosa*. Furthermore, the electrophoretic profile of *V. cholerae* outer membrane proteins in polyacrylamide gels is distinct from those of other gram-negative organisms. These studies and others (16, 17, 34) indicate that the outer membrane of *V. cholerae* is a complex structure containing many proteins.

Proteins exposed on the bacterial surface are likely to contribute to colonization of the host, and improved knowledge of the surface proteins should further our understanding of their role in colonization. The surface proteins of several bacterial species have been identified by iodination with membrane-impermeable enzymes (15, 20, 29, 31) or the water-insoluble reagent, 1,3,4,6-tetrachloro-3,6-diphenylglycouril (Iodogen) (33). We used Iodogen-coated vessels and a covalently immobilized catalyst (Iodo-beads) to radioiodinate whole cells and isolated outer membrane of *V. cholerae*. The major surface proteins on *V. cholerae* accessible to radioiodination were identified and shown to correspond, with a single exception, to the proteins in radiolabeled outer membrane obtained by lithium chloride-lithium acetate extraction (14). Outer membrane obtained by sucrose density centrifugation and Triton X-100 extraction (18) of labeled whole cells was shown to closely resemble the lithium-extracted outer membrane.

MATERIALS AND METHODS

Bacterial strains and media. *V. cholerae* CA401, originally obtained from C. Lankford (7) and characterized in earlier studies (2, 3, 9, 18), was stored at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 15% glycerol. Bacteria were grown in semisynthetic broth medium (4).

Materials. Lysozyme, pepsin, trypsinogen, bovine serum albumin (for iodination), Tris, lithium acetate, lithium chloride, and Coomassie brilliant blue R were obtained from Sigma Chemical Co., St. Louis, Mo.; Iodogen and covalently immobilized catalyzst (Iodo-beads) were obtained from Pierce Chemical Co., Rockford, Ill.; bovine serum albumin for precipitations was obtained from United States Biochemical Corp., Cleveland, Ohio; carrier-free sodium [^{125}I]iodide, [^{35}S]methionine, and [^{3}H]glucose were obtained from New England Nuclear Corp., Boston, Mass.; and all electrophoresis reagents, including molecular weight markers, were obtained from Bio-Rad Laboratories, Richmond, Calif. All other chemicals were reagent grade or the purest grade available.

Lithium chloride-lithium acetate outer membrane preparation. Outer membrane was prepared by a modification of the lithium chloride-lithium acetate extraction method of Johnston et al. (14). Bacteria were grown to late log phase in broth at 35°C with shaking at 250 rpm. The cells were washed once with fresh medium and then suspended in 200 mM lithium chloride–100 mM lithium acetate (adjusted to pH 6.0 with acetic acid) to give 1 g (wet weight) of cells per 20 ml of buffer. Membrane vesicles were generated by shaking the cell suspension at 250 rpm at 45°C for 2 h (Lab-Line Orbit Incubator Shaker; Lab-Line Instruments, Inc., Melrose Park, Ill.) in a flask containing a layer of 3-mm-diameter glass beads. After differential centrifugation at 100,000 × *g* for 1 h in Tris buffer (10 mM Tris, 100 mM NaCl [pH 8.0]), the final pellet was resuspended in Tris buffer and stored at −70°C. The protein concentration was determined by the method of Lowry et al. (23).

Triton X-100 cell fractionation. Cells were radiolabeled with ^{125}I, and membrane was isolated by the method of Kelley and Parker (18). Briefly, freeze-thawed cells were incubated with DNase and then sonicated. Whole cells were removed by centrifugation at 5,000 × *g* for 20 min, and a total envelope fraction was obtained after ultracentrifugation on a 15 and 65% sucrose step gradient. Crude outer and cytoplasmic membrane fractions were obtained after ultracentrifugation on a 45 to 60% linear sucrose gradient. Purified outer membrane was obtained by extraction of crude outer membrane with Triton X-100.

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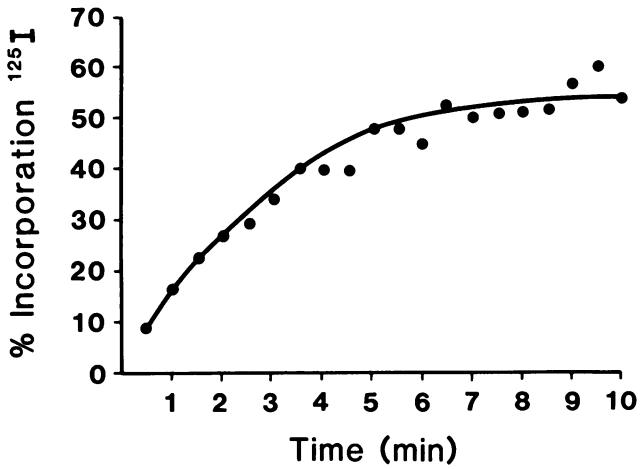

FIG. 1. Incorporation of ^{125}I into whole cells. Whole cells were labeled by the Iodogen method. At 30-s intervals, portions were removed, and radioactivity was determined for washed cells and the total reaction mixture to calculate the percent incorporation of ^{125}I.

Radioiodination. Bacteria grown to late log phase were washed twice with 0.01 M sodium phosphate-buffered normal saline (pH 7.2) (PBS) and resuspended to give 0.1 g (wet weight) of cells per ml of PBS. Cell suspension (1 ml) was reacted with 500 μCi of ^{125}I in an Iodogen-coated 20-ml borosilicate scintillation vial at 25°C with shaking, by the method of Markwell and Fox. (25). Radioiodination was terminated by transferring the samples to a microcentrifuge tube containing 1.5 mg of NaI in 0.2 ml of PBS. For a determination of the optimum reaction time, 50-μl portions were removed at 30-s intervals for 10 min, and the percent incorporation of ^{125}I into the washed cells was determined (Fig. 1). The optimal reaction time chosen was 5 min. Isolated outer membrane was radioiodinated by reacting 50 μg of protein in 0.1 ml of PBS with 50 μCi of ^{125}I in an Iodogen-coated 2-dram (1.5-ml) glass vial at 25°C for 10 min. The reaction was terminated by transferring the sample to an ultracentrifuge tube containing 5 mg of NaI in 0.5 ml of PBS. Iodination of isolated outer membrane and whole cells catalyzed by Iodo-beads instead of Iodogen-coated vials was performed similarly. A single Iodo-bead was used to radiolabel outer membrane, and five Iodo-beads were used to radiolabel whole cells or lysed cells. Lysed cells were obtained by freeze-thawing 1 ml of whole cells (0.1 g [wet weight] per ml of PBS) in a dry-ice–ethanol bath and a 56°C water bath seven times. The lysate was centrifuged at 8,000 × *g* for 10 min to remove whole cells, and the supernatant was centrifuged for 1 h at 100,000 × *g* to remove cytosol. The pellet (total envelope) was resuspended to 1 ml with PBS and labeled as for whole cells.

To remove unreacted ^{125}I from the reaction mixtures of outer membrane or lysed cell preparations, the samples were placed in ultracentrifuge tubes, the volume was brought to 5 ml with PBS containing 1 mg of NaI per ml, and the samples were centrifuged at 100,000 × *g* for 1 h. The membrane pellets were resuspended in PBS. Labeled whole cells were washed three times with PBS containing 1 mg of NaI per ml to remove unreacted ^{125}I. To determine the efficiency of labeling for all samples, portions of the original supernatant and resuspended pellets were counted. Samples were stored at −20°C and used within 2 weeks of labeling.

To determine whether the catalyst became soluble during the iodination reaction, intact cell suspensions were incubated in Iodogen-coated vials or with Iodo-beads but without ^{125}I for 10 min. The cell suspensions were then placed in new vessels without catalyst, ^{125}I was added, and the sample was treated as in a normal labeling experiment.

To determine whether these procedures iodinated cytoplasmic proteins, whole cells which had been labeled in vivo with [^{35}S]methionine and [^{3}H]glucose were radioiodinated. In vivo labeled cells were obtained by growing cells in minimal A salts medium (26) containing 0.2% glucose and 1.0 μM methionine for 4 h at 35°C; the cells were centrifuged at 8,000 × *g* for 10 min, suspended in fresh medium containing 0.02% glucose, 1.0 μM methionine, and 100 μCi of [^{35}S]methionine (1,059 Ci/mmol), and incubated at 35°C with shaking; 60 min later, 100 μCi of [^{3}H]glucose (33 Ci/mmol) was added, and the incubation was continued for 90 min. The cells were divided into two equal portions, and one portion was iodinated. The non-iodinated half of the culture was treated as for iodination except that no ^{125}I was added, and ^{35}S and ^{3}H determinations were performed on these portions.

Radioiodinated molecular weight markers were obtained by reacting 200 μg of each protein in 0.2 ml of PBS with 100 μCi of ^{125}I in an Iodogen-coated vial for 15 min at 25°C and then transferring the solution to a microcentrifuge tube. To remove unreacted ^{125}I, each protein was precipitated by adding 5 μl of the sample to 1 ml of cold PBS containing 1 mg of bovine serum albumin, to which 1 ml of 20% ice-cold trichloroacetic acid was then added. The sample was mixed, and incubated for 30 min at 4°C, and centrifuged for 5 min in a microcentrifuge (Eppendorf, Brinkmann Instruments Inc., Westbury, N.Y.), and the supernatant was removed. The pellet was washed three times with ice-cold 10% trichloroacetic acid and suspended in 0.2 ml of PBS. Protein was dissolved by the addition of 5 N NaOH, and portions were counted. For polyacrylamide gel electrophoresis, protein solutions were mixed to give ca. 10,000 cpm of each protein in the sample applied to a single well.

Radioactivity measurements. Kodak X-Omat XAR-2 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen (Cronex Lightning-plus ZB; Du Pont Co., Wilmington, Del.) was exposed to dried gels at room temperature for 18 h (unless otherwise specified). Wet gels were exposed at −70°C. Radioiodinated samples in solution were counted in a Beckman 8000 gamma counter. ^{35}S and ^{3}H were measured in Aquasol (New England Nuclear) with a Beckman 6000 scintillation counter.

Heat and mercaptan treatment. Samples containing about 50,000 cpm were mixed with an equal volume of denaturing buffer (12.5% glycerol, 1.25% sodium dodecyl sulfate, 0.005% bromphenol blue in water) with or without 1.25% 2-mercaptoethanol. The samples were incubated at various temperatures for 5 min and then electrophoresed.

To identify the origin of protein bands observed after treatment at various temperatures, labeled outer membrane (about 4 × 10^6 cpm in denaturing buffer) heated at 25°C was electrophoresed on a polyacrylamide slab gel. After electrophoresis, the gel was exposed to X-ray film for 96 h at −70°C. The developed X-ray film exposure pattern was used as a template for cutting individual bands from the gel with a razor blade. These gel slices were placed in 1 ml of half-strength denaturing buffer, disrupted with a spatula, and incubated for 1 h at 25°C to extract labeled protein(s). The extracted protein(s) from each band was divided into four equal portions (counts ranged from 2 × 10^3 to 1 × 10^4 cpm per portion for the various bands). Samples were incubated at 25 (control), 55, 85, or 100°C for 5 min and electrophor-

esed on a second polyacrylamide gel, and the dried gel was exposed to X-ray film for 48 h.

Lysozyme and trypsin treatment. Radioiodinated cells (0.5 ml, 0.1 g [wet weight] per ml in PBS) were incubated with 0.5 mg of lysozyme at 25°C. At 5, 10, 20, 40, and 60 min, a portion of the reaction mixture was added to an equal volume of denaturing buffer and boiled for 5 min. Samples of radioiodinated outer membrane (50 μg of protein) in 0.2 ml of PBS containing 5 μg of trypsin or 5 μg of lysozyme were treated similarly.

SDS-urea PAGE. A 10 to 20% polyacrylamide gradient gel was used. The gel system of Laemmli (19) was used, with the following modifications: (i) both the stacking and resolving gel contained 0.5 M urea, and (ii) the resolving gel stock contained 0.54% bisacrylamide. The gel was 0.75 mm thick. The gels were fixed with 50% methanol–10% acetic acid for 30 min and dried onto Whatman 3MM chromatography paper under vacuum with heat.

RESULTS

Radioiodination of intact cells of *V. cholerae*. We examined the kinetics of ^{125}I incorporation when the iodination of whole cells was catalyzed by Iodogen or Iodo-beads. Figure 1 shows the results for iodination of whole cells with Iodogen as the catalyst. Similar results were obtained for Iodo-bead-catalyzed iodination, except that the percent incorporation was greater. In both cases, maximum incorporation had occurred by 5 min.

We also investigated the optimum number of Iodo-beads needed to label whole cells. Whole cells iodinated for 5 min incorporated 40, 52, 67, 75, and 75% of the radionuclide when 1, 2, 3, 4, and 5 beads were used, respectively.

A comparison of the efficiency of radiolabeling of various preparations by the two techniques is shown in Table 1. Whole cells incorporated approximately twofold more ^{125}I when Iodo-beads rather than Iodogen were used as catalyst. Incorporation of ^{125}I in the absence of catalyst was 0.1%, a level too low for detection on polyacrylamide gels. However, incubation of whole cells with Iodogen followed by incubation with ^{125}I without catalyst resulted in 3.5% incorporation. A similar experiment with Iodo-beads showed that incorporation of ^{125}I was not significantly greater (0.9%) than the control of cells exposed to ^{125}I only.

To ascertain whether radioiodination occurred only at the bacterial surface, we examined the cytoplasm for the presence of ^{125}I after iodination of intact cells. Cells were labeled in vivo with [^{3}H]glucose and [^{35}S]methionine to allow quantitation of the various fractions. In early experiments we had observed that the supernatant wash fluid from labeled whole cells contained ^{125}I even after 12 to 15 washes. However, the amount of ^{125}I associated with the supernatant decreased and became constant, usually after eight washes. The ratio of ^{125}I to ^{35}S or ^{3}H was constant for all the wash fluids. Only 10% of whole-cell-associated ^{125}I was released after cell lysis. The ratio of ^{125}I to ^{35}S and ^{3}H, respectively, was 2.20 and 1.17 before lysis, whereas released cytoplasm had ratios of 0.054 and 0.045, respectively. The differences in ratios of radioactivity of the cells and the cytosol suggested that iodination was envelope specific.

Initially, we radioiodinated lysed cell preparations containing lysed cells, released cytoplasm, and intact cells. We expected that the autoradiogram of the SDS-urea gel would resemble the profile observed when similar gels were stained for protein. However, we saw a profile similar to the pattern from radiolabeled whole cells. The amount of ^{125}I associated with the total envelope portion of the mixture was low (ca. 7% incorporation), and the gel profile showed considerable background. Differential centrifugation of lysed cells allowed us to remove the remaining unbroken cells and the cytoplasmic proteins and gave a total envelope preparation with higher ^{125}I incorporation (ca. 28%) into the particulate fraction. This total envelope fraction gave an SDS-urea PAGE profile which showed many more proteins than observed for labeled whole cells (Fig. 2, lane F).

TABLE 1. Incorporation of ^{125}I by *V. cholerae* cell fractions[a]

Iodination method	% Incorporation of ^{125}I in[b]:		
	Whole cells	Total envelope	Lithium-extracted outer membrane
Iodogen	31 (8)	29 (2)	33 (8)
Control[c]	3.5	ND[d]	ND
Iodo-bead	60 (4)	26 (2)	13 (3)
Control[c]	0.90 (2)	ND	ND
No catalyst	0.10 (2)	ND	0.45 (2)

[a] Samples were iodinated as described in the text.
[b] Values are from a single experiment or reflect the average value from multiple experiments (number of experiments is given in parentheses).
[c] Samples were incubated with the catalyst and then added to ^{125}I in the absence of catalyst.
[d] ND, Not determined.

Radioiodination of *V. cholerae* outer membrane proteins. A *V. cholerae* outer membrane fraction obtained by lithium chloride-lithium acetate extraction was radioiodinated and analyzed by SDS-urea PAGE. Radiolabeling with one or two Iodo-beads resulted in 6% incorporation of ^{125}I, whereas incubation with three Iodo-beads resulted in 8% incorporation. The kinetics of ^{125}I incorporation into outer membrane with Iodogen or Iodo-beads as catalyst were similar to the kinetics observed for whole cells. One distinct difference from labeling of cell preparations was the decreased efficiency of ^{125}I incorporation when Iodo-beads were used as the catalyst (Table 1). There was almost threefold less incorporation of ^{125}I into isolated outer membrane when Iodo-beads rather than Iodogen were used as the catalyst. Inclusion of SDS (0.5%) in the Iodo-bead catalyzed reaction did not increase the incorporation of ^{125}I into outer membrane, nor was a significant amount of labeled precipitable protein released from Iodo-beads boiled in 0.1% SDS after labeling of outer membrane.

Radioiodinated proteins observed by SDS-urea PAGE. Sixteen protein bands were regularly observed from surface-labeled whole cells (Fig. 2, lanes D and E), regardless of the catalyst used. The 16 bands corresponded to those labeled 1 through 9 and a through g in Fig. 2. However, as many as 21 bands were sometimes seen, depending on the radiolabeled preparation, gel, and length of exposure to X-ray film. The bands labeled 1 through 9 were consistently observed from radioiodinated outer membrane preparations (Fig. 2, lanes B and C) and corresponded to 9 of the 16 bands observed in iodinated whole cells (lanes D and E). The seven bands observed in radioiodinated whole cells but not in radiolabeled outer membrane were given letter designations. Band 9 of the outer membranes was sometimes seen as a doublet. Both surface-labeled whole cells and iodinated outer membranes exhibited a prominent band (band 4) migrating as a species of molecular weight 43,000 (a 43K species). Band 4 corresponded to the major protein band from outer membrane preparations prepared with the lithium technique as well as to outer membranes obtained by sucrose density gradient separation and observed on polyacrylamide gels stained with Coomassie blue (18). To com-

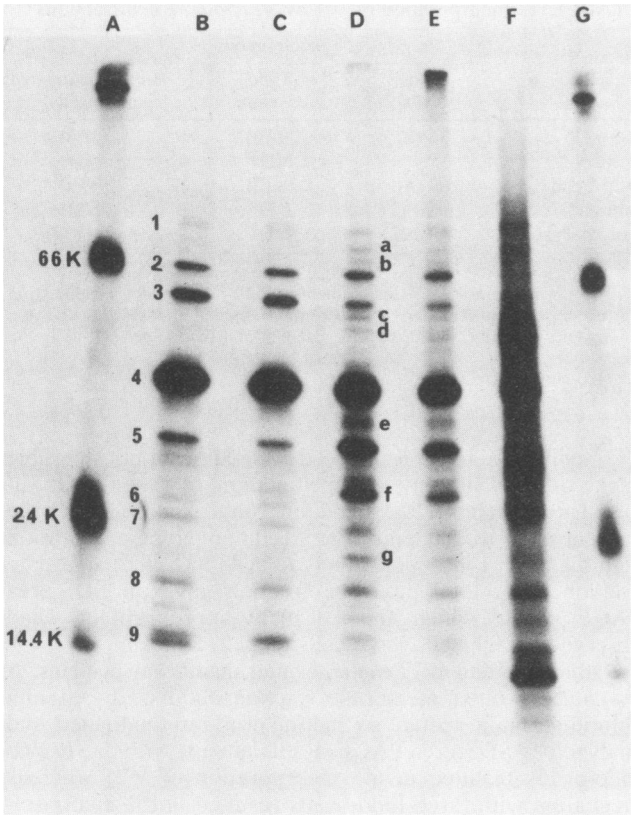

FIG. 2. SDS-urea PAGE analysis of iodinated outer membrane, intact cells, and lysed cells of *V. cholerae*. Samples (50,000 cpm) were boiled for 5 min in denaturing buffer containing 2-mercaptoethanol and electrophoresed, and the gel was exposed to X-ray film. Lanes A and G, Molecular weight markers (bovine serum albumin, 66K; ovalbumin, 45K; trypsinogen, 24K; and lysozyme, 14.4K) labeled with Iodogen as catalyst. Lanes B and C, Lithium chloride-extracted outer membrane labeled, respectively, with Iodogen and Iodo-beads as catalyst. Lanes D and E, Intact cells labeled, respectively, with Iodogen and Iodo-beads as catalyst. Lane F, Total envelope labeled with Iodogen as catalyst. Numbers have been assigned to iodinated proteins observed in labeled whole cells and outer membrane. Bands unique to labeled whole cells are given a letter designation. Bands not labeled are those which are not always seen.

pare the outer membrane preparations obtained by the sucrose gradient-Triton extraction with those obtained by the lithium extraction technique, we used SDS-urea PAGE analysis of outer membranes prepared from surface-labeled whole cells (Fig. 3). Whole cells, total envelope, and gradient-separated outer membrane (Fig. 3, lanes B, C, and D, respectively) showed similar radioiodinated species. After extraction with Triton X-100, the 60K to 62K species corresponding to band 3 disappeared (Fig. 3, lane E). Lithium-extracted outer membrane preparations contained band 3 but lacked band f (Fig. 2, lanes B and C). The inner membrane fraction from the linear sucrose gradient (Fig. 3, lane F) contained labeled proteins including a radiolabeled 43K species, indicating some contamination with outer membrane, in agreement with the earlier study (18).

One band (Fig. 4, band 0) unique to iodinated outer membranes, migrated as a 100K species.

Effect of heat treatment on radioiodinated proteins of surface-labeled cells and lithium-extracted outer membrane. Protein bands which shifted position in SDS-urea PAGE after incubation at higher temperatures were essentially the same for radiolabeled whole cells and outer membrane (Fig. 4). The single clear exception was band 0, observed only in radioiodinated isolated outer membrane (Fig. 4, bottom panel). The relative amount of band 4 increased as the samples were heated. The opposite effect was seen for band III, which gradually decreased in intensity as samples were incubated at higher temperatures. Bands 0, 1, 5, 7, and 8 were seen in samples boiled for 5 min (lanes J and K) but were not present in samples incubated at room temperature (lanes B and C). Bands 1 and 8 appeared after incubation at 55°C (lanes F and G), the temperature at which bands I, II, and III disappeared from radiolabeled outer membrane. Bands 0 and 7 were observed after incubation at 85°C (lanes H and I), the temperature at which band IV of the outer membrane disappeared. Band 0 is a heat-modifiable protein, in that it was seen at higher apparent molecular weight after treatment at 100°C than any other band observed at any other temperature.

To determine the origin of bands 0, 1, 5, 7, and 8, radioiodinated outer membrane incubated at room temperature was electrophoresed, protein(s) extracted from gel slices of bands I, III, and IV and portions of the extracts re-electrophoresed after incubation at various temperatures (Fig. 5). (Band II could not be reproducibly extracted by this technique.) Band I shifted from one band with an apparent molecular weight of 100K to three bands after treatment at

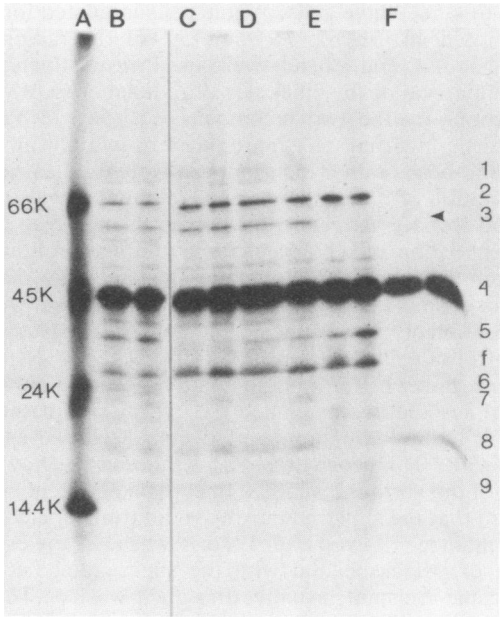

FIG. 3. SDS-urea PAGE analysis of radiolabeled whole cells fractionated by sucrose density centrifugation and Triton X-100 extraction. Samples (50,000 cpm) were boiled for 5 min in denaturing buffer containing 2-mercaptoethanol and electrophoresed, and the gel was exposed to X-ray film. Lane A, Molecular weight markers (bovine serum albumin, 66K; ovalbumin, 45K; trypsinogen, 24K; and lysozyme, 14.4K) labeled with Iodogen as catalyst. Lane B, Radiolabeled whole cells. Lane C, Radiolabeled total envelope (fraction from the step gradient). Lane D, Crude outer membrane from the linear sucrose gradient. Lane E, Purified outer membrane (Triton X-100 extracted). Lane F, Inner membrane fraction from the linear sucrose gradient. Whole cells were labeled with Iodo-beads as catalyst. All lanes without a designation are irrelevant. The arrow to the right of lane F indicates the surface-labeled protein lost from outer membrane extracted with Triton X-100.

85°C (Fig. 5, I, lane C). Two of the new bands corresponded to bands 0 and 1 with apparent molecular weights of 110K and 80K, respectively. The third band migrated as a 39K protein at the leading edge of band 4. Band III, seen as a smear migrating from 34K to 40K at temperatures below 55°C (Fig. 4, lanes B through E) migrated as band 4 (43K), regardless of heat treatment. Band IV, which originally migrated at the leading edge of band III (Fig. 4, lanes B and C), migrated as a smear with two bands at 34K and 43K after incubation at 55°C (Fig. 5, IV, lanes A and B). After treatment at 85°C, all of band IV migrated as band 4 (43K; Fig. 5, IV, lane C). Thus there were at least two proteins, one from band III and one from band IV, which migrated as 43K species, after treatment at 100°C.

Effect of chemical treatment on radioiodinated proteins of surface-labeled cells and lithium-extracted outer membranes. Figure 2, band 4 represents one or more of the major proteins observed in *V. cholerae* outer membranes. The pattern of this band in Coomassie-stained polyacrylamide gels and the effect of heat on its migration behavior suggested that protein in this band might be associated with peptidoglycan. Lysozyme or trypsin treatment of radioiodinated outer membrane and whole cells had no effect on the major protein band (data not shown). Trypsin did, however, alter the migration of minor radiolabeled proteins. Lysozyme had no effect on minor bands.

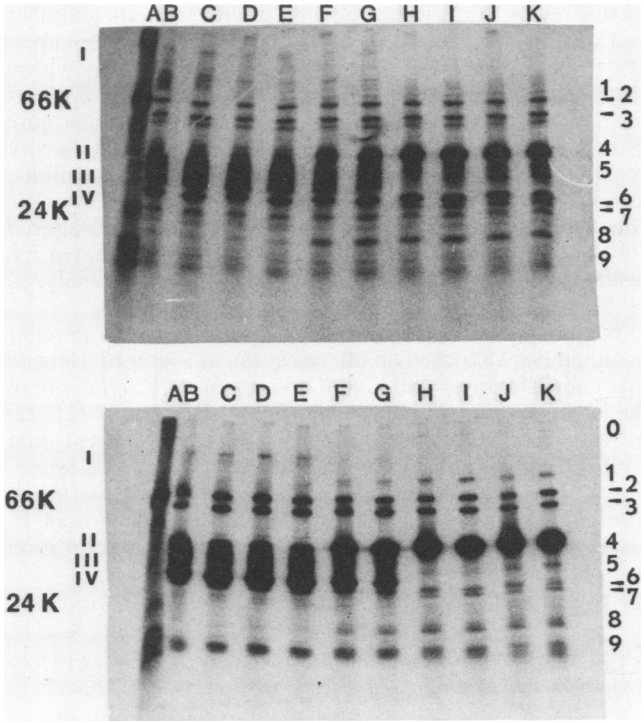

FIG. 4. Effect of heat and 2-mercaptoethanol on ^{125}I-labeled *V. cholerae* outer membrane proteins. X-ray film exposure of polyacrylamide gels. Whole cells (top panel) and outer membrane (bottom panel) were radioiodinated with Iodogen as catalyst. Samples in lanes B, D, F, H, and J were incubated in denaturing buffer without 2-mercaptoethanol at 25, 37, 55, 85, and 100°C, respectively, for 5 min before electrophoresis on 10 to 20% polyacrylamide gradient SDS-urea gels. Samples in lanes C, E, G, I, and K were incubated at the same respective temperatures but with 2-mercaptoethanol. Molecular weight marker proteins (lanes A) were prepared as described in the text. Numbers 1 through 9 (right) correspond to similarly numbered bands in Fig. 2. Band 0 corresponds to the irregularly seen 110K species found in outer membranes. Bands which disappeared as the samples were heated were designated with roman numerals (left).

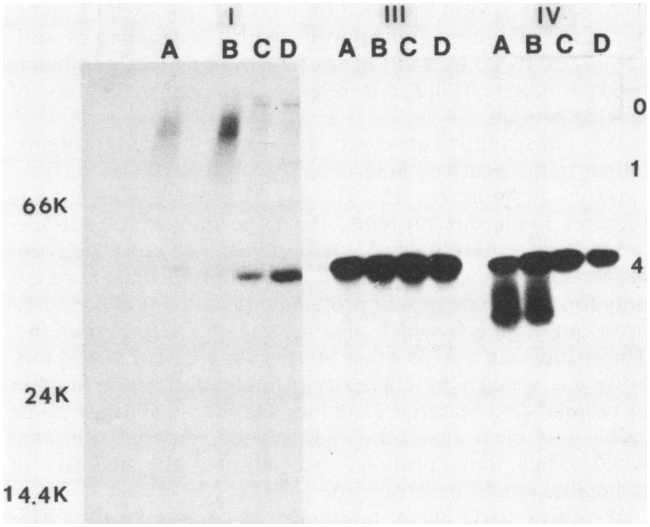

FIG. 5. Determination of the origin of iodinated protein bands whose migration is affected by heat. Iodinated outer membrane was incubated at 25°C and examined by SDS-urea PAGE. X-ray film was exposed to the gel at −70°C for 96 h, and the film was developed and used as a template to cut out bands which were observed to shift in previous experiments as the samples were heated (groups I, III, and IV). Iodinated protein(s) were extracted from the band, and portions were incubated at various temperatures. They were then re-electrophoresed, and the X-ray film was exposed to the gel. Lanes: A, Control samples incubated at 25°C; B, samples incubated at 55°C; C, samples incubated at 85°C; D, samples incubated at 100°C for 5 min.

DISCUSSION

We identified several proteins from *V. cholerae* which are exposed on the cell surface. The conclusion that radiolabeling was surface restricted relies on the following evidence. First, sequential exposure of cells to the covalently immobilized catalyst (Iodo-beads) and ^{125}I did not result in significant labeling of cells, indicating that the catalyst was not solubilized. Second, examination of the cytoplasm from cells fractionated after radioiodination showed that the amount of ^{125}I present was only 10% of the total counts. Third, additional proteins (not detected when whole cells were iodinated) were observed when lysed cells (total envelope) were iodinated. Examination of radioiodinated whole cell cytoplasmic, inner membrane, and outer membrane fractions (Fig. 3) indicated that the label was primarily restricted to the outer membrane. Finally, no significant incorporation of ^{125}I occurred in the absence of catalyst. Caution must be used in identifying minor bands, or those not regularly observed, as surface proteins, since they could represent transient species secreted by the organism or proteins exposed by any lysed cells in the culture. Furthermore, the incorporation of label reflects not only the availability of a particular protein but also the availability of tyrosine residues within the protein for activation and reaction.

Radiolabeling of intact whole cells with Iodo-beads as catalyst consistently resulted in greater incorporation of ^{125}I than when Iodogen was used as catalyst. However, the opposite was observed for ^{125}I incorporation into isolated outer membrane. We have no explanation for these observations. We could not demonstrate adsorption of isolated outer membrane to polystyrene Iodo-beads, nor did the

inclusion of SDS in the reaction mixture increase ^{125}I incorporation. SDS does not interfere with ^{125}I labeling of antiserum catalyzed by Iodo-beads (24). Boiling the Iodo-beads used as catalyst did not release significant amounts of labeled protein.

We consistently observed 16 surface-labeled proteins. Other gram-negative bacteria appear to have fewer surface-exposed proteins (8, 15, 31, 33, 35). Since *V. cholerae* secretes numerous proteins, the large number of surface-labeled proteins obtained could reflect secretory activity. However, Lambert and Booth (20) reported that there are only four surface exposed proteins on *Pseudomonas aeruginosa*, an organism which also secretes numerous proteins. Their study, as well as other studies (8, 15, 31, 33), did not, however, specify the efficiency of iodination or the amount of radiolabeled material examined by SDS-PAGE. In some cases it is clear that little radiolabeled material was analyzed. Thus, only proteins incorporating the majority of radiolabel would be observed.

We were particularly interested in comparing the SDS-urea PAGE profile of radioiodinated whole cells with that of labeled outer membrane obtained by lithium chloride-lithium acetate extraction, since this technique was developed by Johnston et al. (14) to analyze a particular outer membrane complex of *Neisseria gonorrhoeae* rather than as a method for outer membrane isolation. Comparison of the radiolabeled protein gel profiles of *V. cholerae* whole cells and isolated outer membrane has allowed us to examine the degree to which this outer membrane preparation reflects the exposed proteins on intact cells. Of the prominent iodinated proteins from whole cells, the radioiodinated outer membrane isolated by lithium chloride-lithium acetate extraction contained all but one (Fig. 2, band f). Also, the relative intensity of the radioiodinated proteins from outer membrane parallels well that observed for whole cells, the single exception being band 5, which had increased intensity. In addition, comparison of an outer membrane obtained from radiolabeled whole cells by a well characterized technique showed that one of the major surface-exposed proteins (band 3) is lost during Triton X-100 extraction. The lithium chloride-lithium acetate-extracted outer membrane contained this protein. Outer membrane obtained by lithium chloride-lithium acetate extraction therefore appears to contain most of the major surface-exposed proteins (prominent iodinated proteins) of *V. cholerae*.

We, as well as others (16, 34), observed an effect of heat on the migration pattern of *V. cholerae* outer membrane proteins in polyacrylamide gels. Such an effect has been observed for other gram-negative organisms (10, 30, 32). Schnaitman (32) first observed that migration of certain outer membrane proteins of *E. coli* was affected when the membrane preparation was heated in the presence of SDS. Changes of outer membrane migration patterns in SDS-polyacrylamide gels as a result of heating at various temperatures in the presence of SDS reflects the dissociation of oligomers or conformational changes of a single polypeptide (27, 28, 32). Our data showed that dissociation of oligomeric complexes and disruption of intramolecular associations occurred when iodinated whole cells or isolated outer membrane were treated with heat. Band I (100K) represents an oligomer containing three different proteins (proteins 0, 1, and one at the leading edge of band 4; 110K, 80K, and 39K, respectively). Bands III and IV contained heat-modifiable proteins. A β-configuration for band III may be responsible for its migration as a smear. The abrupt disappearance of the smear is characteristic of changes in migration due to disruption of β-structure (28). Whether these associations reflect the in vivo arrangement of proteins on the bacterial surface will require further studies.

Disulfide linkages do not appear to play a significant role in the secondary structure of *V. cholerae* outer membrane proteins. The presence of 2-mercaptoethanol in denaturing buffer did not alter the migration of any iodinated surface protein.

The gradual disappearance of band III and concomitant increase in band 4 in the initial studies of the effect of heat, as well as the unusual banding pattern in this region of Coomassie blue-stained polyacrylamide gels, led us to investigate whether the proteins in band IV might be complexed with peptidoglycan. Noncovalent protein-peptidoglycan association has been observed in *E. coli* (37) and *P. aeruginosa* (11). Hebeler et al. (12) have investigated peptidoglycan-associated protein(s) of *N. gonorrhoeae* which appear to be covalently linked, since the complex is not dissociated by boiling in 4% SDS but is dissociated by lytic enzymes such as lysozyme and trypsin. Our lysozyme treatment of iodinated whole cells and outer membranes suggests that no lysozyme-sensitive association of proteins in band IV with peptidoglycan occurred. Kelley and Parker (18) also failed to detect any effect of lysozyme on outer membrane proteins.

It is likely that the anomalous banding pattern in the 39K to 50K region of the gel is due, at least in part, to the number and quantity of protein species which migrate in this area. The experiments identifying the iodinated proteins whose migration is effected by heat showed that three proteins migrate in this region. Additionally, studies of flagellar core proteins (submitted for publication) have identified two core proteins in lithium-extracted outer membrane preparations, as well as whole cells, which migrate in this region. We showed that the migration of the flagellin proteins is altered substantially when a mixture of purified radioiodinated flagellin proteins and unlabeled outer membranes are analyzed by SDS-urea PAGE.

We have shown that several proteins in *V. cholerae* outer membrane are exposed on the cell surface. Freter (6) showed that copro-antibody will inhibit absorption of *V. cholerae* to the intestinal lumen. Recent studies by Levine et al. (21, 22) in volunteers challenged with *V. cholerae* suggest that antibacterial rather than, or in addition to, antitoxic immunity is important for protection against cholera. It is therefore likely that further study of the surface proteins of *V. cholerae* will contribute to an understanding of colonization and perhaps identify potential protective antigens.

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