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Biochemical Characterization of $HgCl_2$-Inducible Polypeptides Encoded by the *mer* Operon of Plasmid R100

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Minicells carrying the subcloned *mer* operon from plasmid R100 were pulse-labeled with [^{35}S]methionine, and the labeled polypeptides were analyzed at various subsequent times by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Hg(II) reductase monomer encoded by plasmid R100 occurred as two proteins of 69 and 66 kilodaltons (kd). The minor 66-kd protein is a modified form of the 69-kd protein. This modification occurs in vivo. Both of these *mer* proteins are found in the soluble fraction of the cell; however, the 66-kd protein appears to have a slight affinity for the cellular envelope. Both the 69- and 66-kd *mer* proteins have pI values greater (pI = 5.8) than that reported (pI = 5.3) for the analogous monomer encoded by plasmid R831. The 15.1- and 14-kd *mer* proteins are localized in the inner membrane and are probably elements of the *mer*-determined Hg(II) uptake system. These two *mer* membrane proteins, which are antigenically unrelated to the Hg(II) reductase monomer, are quite basic (pI values greater than 7.8). The 12-kd *mer* protein is also a basic polypeptide that is present in the soluble fraction of the cell. Unlike the two membrane-bound *mer* proteins, the 12-kd *mer* protein is processed from a 13-kd precursor.

Resistance to mercuric chloride is one of the most widely disseminated plasmid-determined phenotypes found in gram-negative and gram-positive bacteria (10, 21, 24, 30). The basis for $HgCl_2$ resistance is the reduction of Hg(II) to the volatile, inert Hg(0) form (30–32). This reaction is catalyzed by the intracellular Hg(II) reductase (HR). This enzyme is a flavin adenine dinucleotide-containing protein that utilizes NADPH as an electron donor (25, 30). Recent studies (9a) with the HR encoded by the mercury resistance transposon, Tn*501* (27), demonstrate that this enzyme possesses an active-site cysteine thiolate that acts as an electron donor. This is a characteristic property of other flavin adenine dinucleotide-containing disulfide oxidoreductases (35) and may indicate an evolutionary relationship between such enzymes and the HR.

Both the HR and the Hg(II) uptake system are inducible by subtoxic levels of $HgCl_2$ (9, 20, 31). Previous genetic studies have shown that the reductase and the transport function are elements of an operon, *mer*, which has the gene order promoter-transport-reductase (9, 31). Additional evidence indicates that the regulatory function influences the expression of *mer* in a positive fashion and is located on the opposite side of the promoter region from the transport and reductase genes (9, 28).

Recently, we have shown that exposure to $HgCl_2$ induces the synthesis of four common polypeptides from *Escherichia coli* minicells carrying different $HgCl_2$ resistance-conferring (HgR) plasmids (12). Examination of cloned subfragments of the *mer* operon from the plasmid NR1 (=R100) (17) in the minicell system indicates that the "consensus" *mer* proteins of 15.1, 14, and 12 kilodaltons (kd) participate in the transport of Hg(II) or in the regulation of the operon or both (12). This same study indicated that the largest consensus *mer* protein (69 kd) determined by plasmid R100 is the HR. This conclusion was based on the molecular weight and the cross-reactivity of the 69-kd protein with rabbit antiserum to the HR. However, these properties alone are not sufficient to assign the HR enzymatic activity to this 69-kd *mer* protein for two reasons. First, a faintly labeled 66-kd protein appears to be induced along with the 69-kd protein in some HgR minicells, including those carrying R100 and Tn*501*. This 66-kd polypeptide also reacts with anti-Hg(II) reductase sera (12). Second, the purified Tn*501*-determined HR used to generate our antiserum has a mass of 66 rather than 69 kd. We have hypothesized that the 66-kd *mer* protein is a proteolytically modified form of the more abundant 69-kd *mer* protein and that the purified HR we used as antigen had been modified entirely to the 66-kd form (12).

In this paper, we report that the 66-kd *mer* protein is indeed derived from the 69-kd *mer*

protein and that this modification takes place in intact minicells. We also demonstrate that both the 15.1- and 14-kd *mer* proteins determined by plasmid R100 are inner-membrane peptides, whereas the 12-kd *mer* protein, like the 69- and 66-kd *mer* peptides, is found in the soluble cell fraction. The 12-kd *mer* protein is processed from a 13-kd precursor. We have found no evidence of processing of the 14- and 15.1-kd peptides.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used were derivatives of the *E. coli* K-12 minicell producer DS410 (*minA minB strA*) (7), which we received from J. Reeve. The plasmid pRR130 is a HgR recombinant derivative of RSF2124 (ColE1::Tn*3*) (26) that carries the subcloned *mer* operon from the IncFII plasmid NR1 (=R100) (17). The plasmid pSC101, which confers inducible tetracycline resistance, has been described previously (3, 4). Plasmid-containing derivatives of DS410 were constructed by the transformation procedure of Lederberg and Cohen (13), using plasmid DNA isolated by the cleared-lysate dye-buoyant density technique (2).

Minicell purification and labeling. Cells were grown with aeration in 150 ml of M9 minimal medium (18) supplemented with 0.5% Casamino Acids for 10 to 12 h at 37°C into late exponential or early stationary phase. Minicells were purified as previously described (12), using two discontinuous sucrose gradients. Purified minicells were then suspended at an absorbance at 620 nm of 0.200 ± 0.02 in M9 minimal medium containing 50 μg of all common amino acids, except methionine, per ml. Unless otherwise stated, minicells were labeled, after a 15-min preincubation period at 37°C, with [^{35}S]methionine at a concentration of 50 μCi/ml (>1,200 Ci/mmol, Amersham Corp.) and induced with $HgCl_2$ as previously described (12). To induce the expression of the tetracycline resistance proteins from minicells carrying the plasmid pSC101, tetracycline (5 μg/ml) was added to the labeling suspension. Labeled minicells were washed with an equal volume of cold 50 mM Tris-hydrochloride (pH 8.0) and lysed by suspending the pellet in 50 μl of cold 50 mM Tris-hydrochloride (pH 8.0) containing 500 μg of lysozyme per ml. EDTA and phenylmethylsulfonyl fluoride (to prevent protease activity) were added to final concentrations of 25 mM and 5 mM, respectively, unless the preparation was to be used for electrofocusing. Preparation for one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as previously described (12, 34).

Labeling in the presence of ethanol. Minicells were labeled with 50 μCi of [^{35}S]methionine per ml as described above, except that ethanol was present at a final concentration of 9.5% (19). Approximately 25,000 trichloroacetic acid (TCA)-precipitable counts of each sample were electrophoresed on an 18% SDS-polyacrylamide gel and autoradiographed as previously described.

Minicell pulse-chase labeling. A 3.0-ml volume of purified pRR130 minicells suspended in M9 labeling solution was prewarmed for 15 min at 37°C before [^{35}S]methionine was added to a final concentration of 50 μCi/ml. Unlike the labeling procedure above, nonradioactive carrier methionine was not added to this mixture. Minicells were then induced by adding $HgCl_2$ and glutathione to final concentrations of 1 and 3 μm, respectively. Immediately thereafter ("0-min" sample) and at various subsequent times, a 250-μl sample of the labeling mixture was removed into 3.0 ml of "holding mix" (cold 50 mM Tris-hydrochloride [pH 7.5] containing 250 μg of chloramphenicol per ml). Samples were kept on ice until the assay was complete. After 10 min of incubation with label (the pulse period), incorporation of [^{35}S]methionine into minicell polypeptides was inhibited by adding chloramphenicol (final concentration, 250 μg/ml) and nonradioactive methionine (final concentration, 30 μM). Incubation and sampling were continued for an additional 95 min (the chase period). Upon the completion of the chase, all samples were washed, lysed by an EDTA-lysozyme treatment, and prepared for one-dimensional SDS-polyacrylamide gel electrophoresis as described previously (12). After electrophoresis and autoradiography, each lane on the autoradiogram was traced with a Joyce-Loebl MKIII microdensitometer, using a slit width of 100 nm and a tracing-to-scanning ratio of 2:1. The area under each protein peak and the total area under all peaks in a lane were determined by using a Tektronics microcomputer and digitizing pad. The amount of [^{35}S]methionine in each protein peak was normalized to the total labeled protein present in the lane.

Separation of minicell membranes. Separation of minicell inner and outer membranes by sucrose density centrifugation was performed as described by Achtman et al. (1), except that phenylmethylsulfonyl fluoride (final concentration, 5 mM) was added before the minicells were lysed in a French pressure cell. The density of each fraction was determined with an Abbe refractometer (Bausch & Lomb, Inc.). TCA-precipitable radioactivity in each fraction was measured by using prewashed 0.45-μm membrane filters (Metricel GN-6) (12).

Selective solubilization of inner-membrane proteins with sodium lauryl sarcosinate (Sarkosyl) was as described by Levy (14). The envelopes recovered from 10 ml of labeled minicells were extracted with 175 μl of 50 mM Tris-hydrochloride (pH 7.5) containing 0.5% Sarkosyl for 45 min at 4°C. Sarkosyl-soluble and -insoluble materials were separated by centrifugation at 50,000 × *g* for 1 h at 25°C in a Beckman Airfuge (Beckman Instruments, Inc.). The top, middle, and bottom portions of the supernatant (Sarkosyl-soluble fraction) were removed, and a sample from each was analyzed by SDS-polyacrylamide gel electrophoresis. The Sarkosyl-insoluble material (the pellet) was also analyzed on SDS-polyacrylamide gels.

Gel electrophoresis. One-dimensional SDS-polyacrylamide gel electrophoresis was performed by the method of Thomas and Kornberg (34). Molecular weight standard proteins were obtained from Sigma Chemical Co. The standards and their respective subunit masses (kd) are: phosphorylase *a*, 94; catalase, 60; ovalbumin, 43; α-chymotrypsinogen, 25; lysozyme, 14.4; and cytochrome *c*, 12. Sample preparation, equilibrium, and non-equilibrium isoelectric focusing for two-dimensional polyacrylamide gel electrophoresis were performed by the methods of O'Farrell (22). Isoelectric focusing equilibrium was considered to have occurred by 5,600 volt-hours (V-h) of electropho-

resis, whereas non-equilibrium focusing proceeded for only 1,600 V-h.

RESULTS

Identifying modified forms of the *mer* polypeptides. (i) Pulse-labeling $HgCl_2$-induced minicells. To demonstrate that the apparently $HgCl_2$-inducible 66-kd protein is derived from the larger 69-kd *mer* protein, we pulse-labeled $HgCl_2$-induced minicells carrying pRR130 with [^{35}S]methionine for 10 min. Incorporation of label after 10 min was prevented by adding chloramphenicol and excess nonradioactive methionine. During the 10-min pulse period, the 69-kd *mer*-encoded protein was heavily labeled (Fig. 1, lanes B to E). However, the 66-kd *mer* protein was not observed until 5 min after the addition of chloramphenicol (Fig. 1, lane F). The appearance of label in the 66-kd protein under conditions which inhibit protein synthesis indicated that this *mer* protein was derived from a labeled precursor protein. The increase in intensity of the signal from the 66-kd *mer* protein during the chase period was accompanied by a concomitant decrease in the signal from the 69-kd protein (Fig. 1, lanes H to M).

We determined the relative abundance of each peptide present in the autoradiogram (Fig. 1) by densitometry (data not shown). As expected of a precursor-product relationship, the increase in abundance of the 66-kd protein was accompanied by a simultaneous decrease in the 69-kd protein. During the chase period, the sum of the 69- and 66-kd *mer* proteins was a constant fraction of the total labeled minicell proteins. The loss of the 69-kd peptide was therefore directly related to the production of the 66-kd protein and was not the result of nonspecific proteolysis. Together with our previous evidence showing that these two *mer* proteins are immunologically cross-reactive (12), these data prove that the 66-kd *mer* peptide is a modified (presumably by proteolysis) form of the 69-kd *mer* protein.

Like the 69-kd *mer* polypeptide, the 15.1-, 14-, and 12-kd *mer* proteins were synthesized immediately after the minicells were exposed to $HgCl_2$ (Fig. 1, lane C). These three *mer* proteins

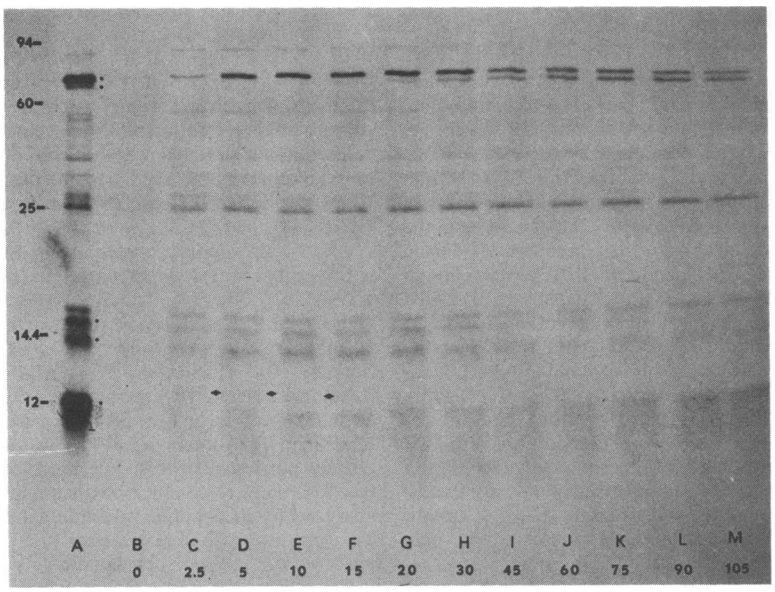

FIG. 1. Pulse-chase of $HgCl_2$-inducible polypeptides synthesized from minicells containing pRR130. $HgCl_2$-induced minicells were labeled with [^{35}S]methionine for 10 min, and the label was chased for 95 min by adding chloramphenicol and excess nonradioactive methionine (see the text). The minicell proteins present at various times were analyzed on an 18% SDS-polyacrylamide gel, followed by autoradiography for 14 days at −70°C. Each lane, except A and B, was loaded with approximately 10,000 TCA-precipitable counts. Lane A was loaded with roughly 25,000 counts, and lane B (due to the low incorporation of label at the initial sampling time) was loaded with approximately 2,000 counts. The numbers on the left margin designate molecular-mass standards. The $HgCl_2$-inducible proteins are marked with dots in lane A. The diamonds in lanes C, D, and E designate the 13-kd protein referred to in the text. Lanes: (A) labeling and induction control; minicells were labeled for 60 min without a chase period; (B) 0 min; (C) 2.5 min; (D) 5 min; (E) 10 min; after this sample, the label was chased; (F) 15 min; (G) 20 min; (H) 30 min; (I) 45 min; (J) 60 min; (K) 75 min; (L) 90 min; (M) 105 min.

remained relatively intact throughout the chase period. The 30-kd precursor of the β-lactamase (*bla*) protein encoded on RSF2124 (26) and the 28-kd processed β-lactamase were both produced early in the pulse period (Fig. 1, lane C). However, within 5 min, most of the 30-kd *bla* protein was converted into the 28-kd active form. Thus, the processing of the β-lactamase precursor occurred much more rapidly than the modification of the 69-kd *mer* protein. When pRR130 minicells had been labeled for 60 min under standard conditions (see above) (Fig. 1, lane A), only 14% of the total HR synthesized was present in the modified form, whereas 72% of the total β-lactamase had been processed (data not shown).

A previously undetected 13-kd protein was also synthesized early in the pulse period. This novel 13-kd protein rapidly disappeared in the chase (Fig. 1, lane F), indicating that it is extremely labile to degradation or modification. The loss of the 13-kd protein may have been associated with a slight increase in the abundance of the 12-kd *mer* peptide. However, this is difficult to argue since the low-molecular-mass proteins (including the 12-kd *mer* protein) are not resolved very well and thus are difficult to quantitate precisely.

(ii) Labeling $HgCl_2$-induced minicells in the presence of ethanol. Mooi et al. (19) have shown that periplasmic and outer-membrane protein processing in *E. coli* minicells is inhibited in the presence of 9.5% ethanol. To confirm that the 13-kd protein observed during pulse-labeling is the precursor of the 12-kd *mer* protein, we labeled pRR130 minicells in the presence of ethanol. Under these conditions, only the 30-kd *bla* precursor, and not the 28-kd processed form of β-lactamase, was produced by minicells carrying pRR130 (Fig. 2, lanes C and D). The 69-, 15.1-, and 14-kd *mer* proteins were not affected by the presence of ethanol (Fig. 2, lane D). However, both the 66- and 12-kd *mer* proteins disappeared. In the presence of ethanol, two new polypeptides with molecular masses of 17.5 and 13 kd appeared (Fig. 2, lane D). The 17.5-kd protein, but not the 13-kd protein, was also detected in minicells containing RSF2124 (the vehicle used to construct pRR130) when processing was inhibited by ethanol (data not shown). Therefore, only the 13-kd protein is *mer* specific and is probably the precursor of the 12-kd *mer* protein.

Location of *mer* polypeptide in the inner membranes of minicells. Since the *mer* operon of plasmid R100 determines an inducible mercuric ion transport system (9, 20, 31), we asked whether any of the $HgCl_2$-inducible proteins were membrane associated. Labeled minicells were lysed with a French pressure cell and separated into envelope and soluble fractions by centrifugation (1). A portion of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis. As expected (25, 32), both forms of the Hg(II) reductase monomer (the 69- and 66-kd *mer* proteins) were found in the soluble fraction of minicells (Fig. 3, lane C). The soluble fraction also contained the 12-kd *mer* peptide and most of the 30- and 28-kd *bla* proteins. The membrane fraction contained the 15.1- and 14-kd *mer* proteins as well as the 16-kd mobilization (*mob*) protein encoded by the ColE1 component (16) of pRR130 (Fig. 3, lane D).

Minicell envelopes were further separated into inner- and outer-membrane fractions by sucrose density centrifugation (1). A sample from the peak of each fraction (Fig. 4A) was examined by SDS-gel electrophoresis. (Achtman [1] has reported that earlier methods [11, 14] for separating the inner and outer membranes of minicells yield less than satisfactory results for DS410 minicells. However, an acceptable level of membrane separation can be

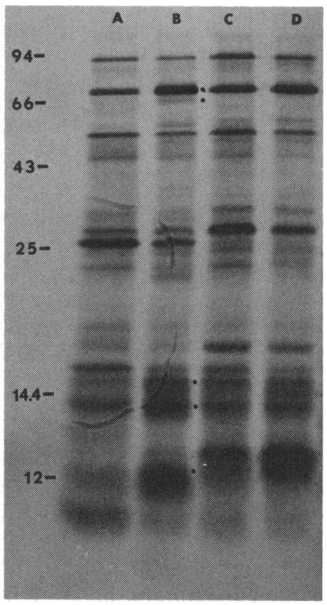

FIG. 2. Effect of ethanol on pRR130-directed proteins synthesized in minicells. Minicells were labeled with 50 μCi of [^{35}S]methionine per ml as described above, except that ethanol was present at a final concentration of 9.5%. Approximately 25,000 TCA-precipitable counts of each sample were loaded onto a 18% SDS-polyacrylamide gel and autoradiographed as previously described. Lanes: (A) total uninduced minicell proteins; (B) total $HgCl_2$-induced minicell proteins; (C) total uninduced minicell proteins labeled in the presence of 9.5% ethanol; (D) total $HgCl_2$-induced minicell proteins labeled in the presence of 9.5% ethanol. Dots designate $HgCl_2$-inducible proteins.

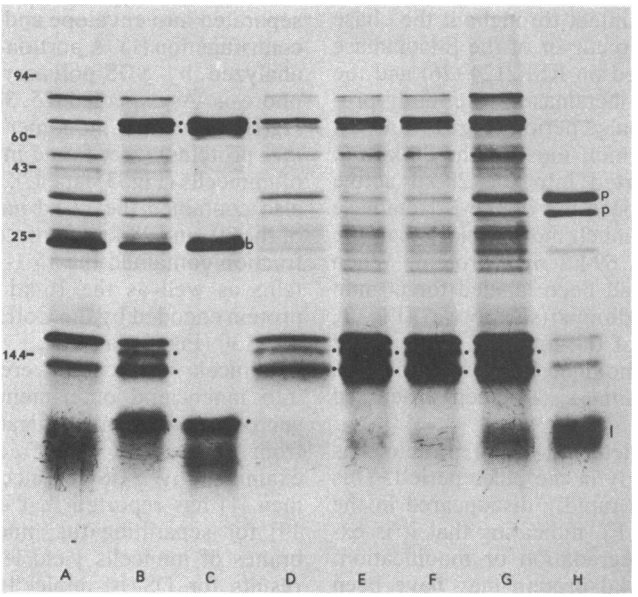

FIG. 3. Detergent fractionation of soluble and membrane-bound *mer* minicell proteins. Minicells harboring pRR130 were labeled and lysed, and the soluble and envelope components were isolated as described in the text. Envelopes were further extracted with Sarkosyl to solubilize the inner membrane. Treated membranes were centrifuged to separate soluble and insoluble fractions. Samples were run on an 18% SDS gel containing approximately 75,000 TCA-precipitable counts per lane. Symbols are as described in Fig. 1. Abbreviations: b, β-lactamase proteins; p, porin proteins; l, lipoprotein. Lanes: (A) total uninduced minicell proteins; (B) total induced minicell proteins; (C) induced soluble proteins; (D) induced envelope proteins; (E) upper portion of the Sarkosyl-soluble material; (F) middle portion of the Sarkosyl-soluble material; (G) lower portion of the Sarkosyl-soluble material; (H) Sarkosyl-insoluble material.

obtained with these minicells by using slight modifications of the earlier techniques [1].) The 16-, 15.1-, and 14-kd proteins were found predominantly in fractions having light (ρ = 1.164 g/cm^3) and intermediate (ρ = 1.183 g/cm^3) densities (Fig. 4B, lanes C and D). These are the same fractions where most of the 36-kd tetracycline-inducible inner-membrane protein (33) was found when minicells harboring pSC101 were similarly examined (data not shown). The outer-membrane porin proteins of 36 and 34 kd were present in fractions with greater densities (ρ = 1.210 and 1.235 g/cm^3, respectively) (Fig. 4B, lane A). The presence of the porin proteins, as well as the outer-membrane lipoprotein, in the higher-density fractions was more evident in protein staining than in autoradiography (data not shown). We also observed that a small but consistent amount of the 66-kd *mer* protein sedimented with the envelope fraction. Like the other membrane-bound *mer* proteins, most of this 66-kd protein was found in light- and intermediate-density fractions (Fig. 4B, lanes C and D). We believe that this phenomenon is not explainable simply in terms of soluble protein contamination of the envelopes, since neither the 69-kd nor the 12-kd *mer* proteins sedimented along with the membranes. The 66-kd *mer* protein may therefore associate weakly with the inner membrane.

To confirm the inner-membrane location of the 15.1- and 14-kd *mer* proteins, labeled minicell envelopes were also extracted with Sarkosyl, which specifically solubilizes the inner membrane of *E. coli* (8). Both *mer* membrane proteins and the 16-kd *mob* peptide were solubilized with Sarkosyl (Fig. 3, lanes E to G). A small amount of intracellular protein contamination was indicated by the presence of the 69-kd peptide in all three of the Sarkosyl-soluble fractions. The 36-kd inner-membrane tetracycline-inducible protein was also solubilized when pSC101 minicell envelopes were extracted with Sarkosyl (data not shown). The Sarkosyl-insoluble material contained the majority of the outer-membrane porins and lipoprotein (Fig. 3, lane H). These data and those described above indicate that the 15.1- and 14-kd *mer* proteins are components of the inner membrane.

Two-dimensional gel electrophoresis of *mer* polypeptides. The pI values for the *mer* proteins encoded by pRR130 were determined by the method described by O'Farrell (22). Preliminary results showed that in the two-dimensional

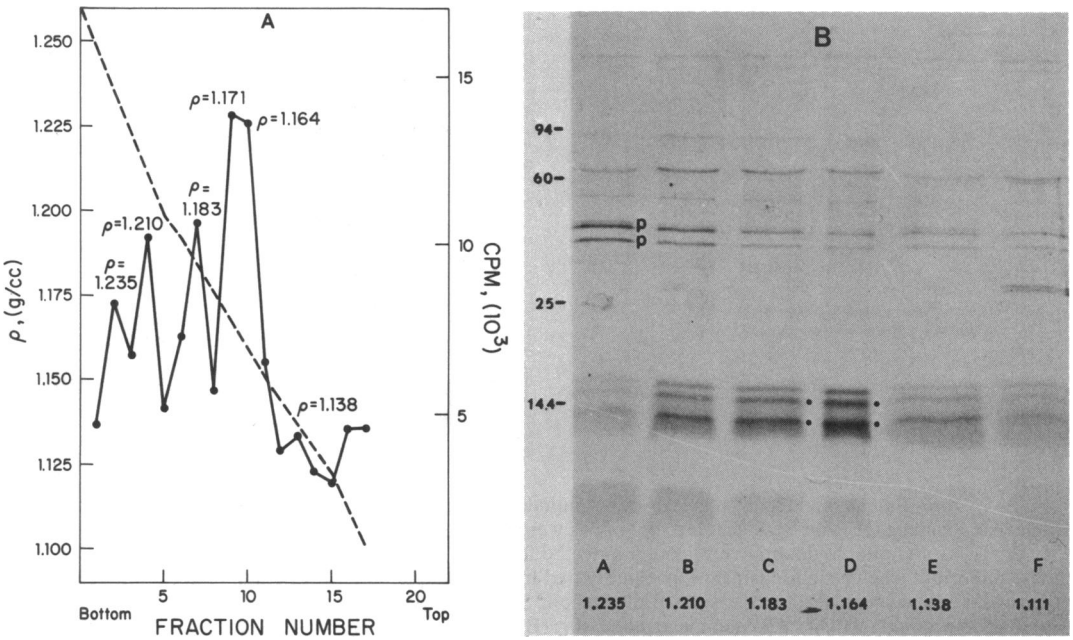

FIG. 4. (A) Sucrose gradient fractionation of the inner and outer membranes of minicells containing pRR130. (---). Buoyant density. The densities of each peak fraction are indicated above the TCA-precipitable counts (——). (B) Autoradiogram of the labeled minicell proteins present in the indicated sucrose gradient fractions. An 18% SDS-polyacrylamide gel was run with approximately 15,000 TCA-precipitable counts from the indicated fractions and autoradiographed for 7 days at −70°C. Symbols are as described in Fig. 1 and 2. The density (g/cm^3) of each fraction analyzed is denoted below each lane.

system, the 15.1-, 14-, and 12-kd *mer* proteins were less prominent on autoradiograms than the larger *mer* proteins. The diminution in the signal of the low-molecular-mass proteins was due to their preferential loss from the isoelectric focusing gel during its equilibration in SDS-sample buffer before electrophoresis in the second dimension (data not shown). This effect can be partially overcome by decreasing the equilibration time in SDS-sample buffer, loading an excess of labeled protein, and increasing the exposure time of the autoradiogram. Unfortunately, these remedies also enhance the background with many faintly labeled contaminating whole-cell proteins. However, the $HgCl_2$ inducibility of the smaller *mer* proteins is sufficient to differentiate them from the whole-cell contamination.

At equilibrium, the major form of the HR monomer (69 kd) migrated as a single spot with a pI of 5.8 (data not shown). This HR monomer is considerably more basic than the HR monomer encoded by plasmid R831, which has a pI of 5.3 (25). This result is consistent with our earlier study (12) which indicated that the HR monomers encoded by R100 and R831 are biochemically distinct. The 66-kd processed HR monomer migrated as a single species, with an isoelectric point slightly greater (pI = 5.9) than the unmodified major HR form (data not shown).

Two-dimensional gel analysis suggested that a sixth $HgCl_2$-inducible protein was determined by pRR130 (Fig. 5A and B, spot D). This abundant protein has a molecular mass of approximately 54 kd and migrated as a single species with an isoelectric point nearly equal to that (pI = 5.5, data not shown) of the 30-kd TEM-type β-lactamase precursor protein encoded by RSF2124 (26). (The 28-kd processed β-lactamase has a pI of 5.2; data not shown.) We suspect that this 54-kd protein is a degradation product of the 69-kd *mer* protein which is produced by proteolysis when samples are analyzed by the two-dimensional method. (Since the protease inhibitor, phenylmethylsulfonyl fluoride, is dissolved in a polar solvent [ethanol] which might interfere with ampholyte focusing, it was not used when samples were prepared for isoelectric focusing.) This conclusion is consistent with our observation that the 54-kd protein is only occasionally seen on two-dimensional autoradiograms and has never been observed when the *mer* proteins are examined on one-dimensional SDS gels (unpublished data). Similarly, Dempsey et al. (5, 6) have reported that a faintly labeled 52-kd protein is occasionally induced by

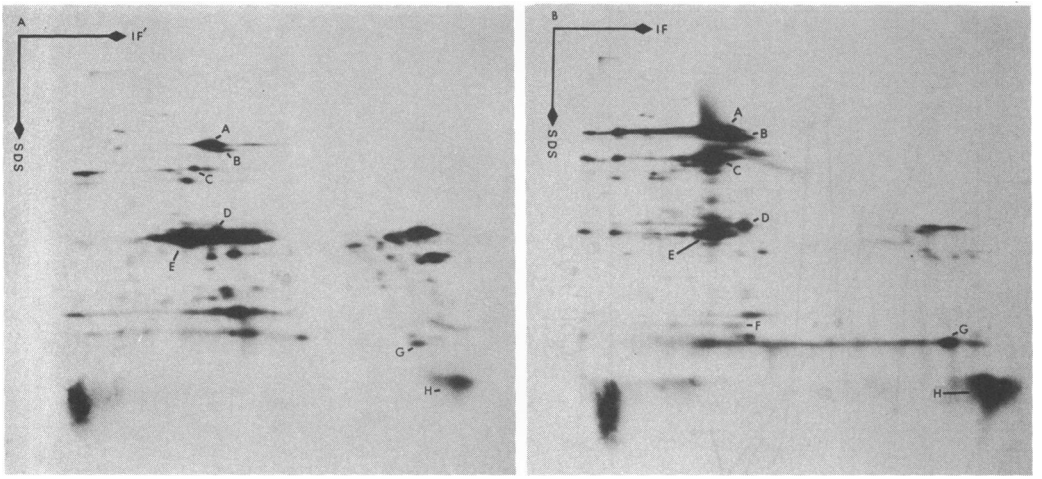

FIG. 5. Two-dimensional electrophoresis of (A) uninduced and (B) $HgCl_2$-induced pRR130 minicell proteins. Isoelectric focusing (IF) and SDS-gel electrophoresis were performed in the directions indicated. Approximately 250,000 TCA-precipitable counts were loaded onto the focusing gel which, after electrophoresis for 1,600 V-h (non-equilibrium isoelectric focusing), was equilibrated in SDS-sample buffer for 30 min at 25°C. An 18% SDS-polyacrylamide gel was used in the second dimension. Symbols: (A) 69-kd HR; (B) 66-kd HR; (C) 54-kd HR degradation product; (D) 30-kd β-lactamase precursor; (E) 28-kd β-lactamase; (F) 15.1-kd *mer* protein; (G) 14-kd *mer* protein; (H) 12-kd *mer* protein.

$HgCl_2$ when UV-treated cells are infected with λ *mer* phages derived from the IncFII plasmid R100.

The 15.1-kd *mer* protein migrated under equilibrium conditions as a single species, with a pI of 8.2 (data not shown). The 14- and 12-kd peptides have isoelectric points so basic that they migrate through the ampholyte into the anode buffer (data not shown). To visualize the 14- and 12-kd *mer* proteins, labeled minicell proteins were electrophoresed in the first dimension for only a short time before the proteins were separated on the second dimension (i.e., non-equilibrium polyacrylamide gel electrophoresis) (22). This procedure allowed the basic peptide to enter the isoelectric focusing gel but not to migrate through it. The 14- and 12-kd *mer* proteins each migrated as a single species and had almost reached the end of the focusing gel after 1,600 V-h of electrophoresis (Fig. 5B, spots E and F). It is interesting to note that there are many proteins in the 20- to 30-kd range that are very basic.

DISCUSSION

We have shown that the HR monomer determined by the *mer* operon of plasmid R100 exists in minicells as two distinct polypeptides of 69 and 66 kd. The minor 66-kd form arises from an in vivo modification of the 69-kd *mer* protein. Preliminary data suggest that the 69-kd protein loses a 3-kd segment from its amino terminus when it is converted into the smaller form (W. J. Jackson and A. O. Summers, manuscript in preparation).

Susceptibility to this limited, but apparently specific, in vivo modification appears to be a characteristic feature of the Hg(II) reductase monomers determined by Tn*501* and the IncFII HgR plasmids such as R100 and R538-1. Previous studies have shown that both a 69-kd *mer* protein and a minor 66-kd *mer* protein are present in minicells harboring these HgR plasmids (12). However, only one high-molecular-mass (>25 kd) *mer* protein is produced in minicells carrying either the IncP plasmid, R702, or the IncH plasmid, R831 (12). Thus, it appears that the HR encoded by Tn*501*, R100, and R538-1 are more closely related to each other than to those encoded by R702 and R831. J. Miller and R. H. Rownd (personal communication) have suggested, on the basis of hybridization data, that the *mer* operon encoded by Tn*501* is the prototype of the *mer* operons found on all of the IncFII HgR plasmids. Our findings support such a relationship.

At present, there are no available data on whether the conversion of the 69-kd *mer* protein into the 66-kd form affects the HR activity or serves some other biological purpose. Fox and Walsh (9a) have shown that SDS-gel electrophoresis resolves a partially purified Tn*501* HR activity into two proteins of 62 and 56 kd. (These correspond to the 69- and 66-kd *mer* proteins seen in our gel system.) The proportion of each form varied considerably in different prepara-

tions of the enzyme without affecting the HR activity [as measured by Hg(II)-dependent NADPH-oxidizing activity]. Even though these preparations were not tested for their ability to reduce Hg(II), this result suggests that the modification of the 69-kd protein may have little effect on the enzymatic activity of the HR. The 66-kd *mer* protein, like the 69-kd *mer* protein, is present predominantly in the soluble fraction of the cell. However, the 66-kd modified form may have a slight affinity for the envelope fraction, since a small amount sedimented with the inner-membrane component (ρ = 1.164 g/cm^3) in a sucrose gradient.

Indirect support for an association between the HR protein and the cell envelope also comes from our findings that ethanol prevents the in vivo modification of the 69-kd *mer* protein. Ethanol has been shown to inhibit the processing of both periplasmic (Fig. 2) and membrane-bound proteins (19). Palva et al. (23) have suggested that ethanol at low concentrations inhibits processing, like carbonyl cyanide-*m*-chlorophenyl hydrazone and 2,4-dinitrophenol, by dissipating the electrochemical membrane potential. It is interesting that a compound which affects membrane potential and membrane-associated processing also affects the modification of the HR monomer. The association of the HR with the membrane might serve a biological purpose and, in any case, would permit more effective reduction of Hg(II) as it enters the cell (29).

The 15.1- and 14-kd *mer* proteins are components of the inner membrane. Since many membrane (36) and periplasmic proteins (such as β-lactamase) are processed from larger precursors, we hypothesized that the 15.1- and 14-kd *mer* proteins might also be derived from larger polypeptides. However, neither pulse-labeling $HgCl_2$-induced minicells nor labeling in the presence of 9.5% ethanol revealed any precursor forms for these *mer* proteins. These experiments did show that the 12-kd *mer* protein is the processed form of a 13-kd *mer*-specific polypeptide. Like the 30- and 28-kd *bla* proteins encoded by pRR130, the 12-kd *mer* protein was also found predominantly in the soluble fraction of French-pressed minicells. These data are consistent with the hypothesis that the 12-kd *mer* protein is in the periplasmic space along with the processed 28-kd *bla* protein rather than the cytosol.

From previous studies (9, 20), we know that the Hg(II) transport system and the *mer* regulatory function are determined by the *Eco*RI-H fragment of plasmid R100. When subcloned and examined in the minicell system, this restriction fragment determined the 15.1-, 14-, and 12-kd *mer* proteins as well as 40 kd of the amino terminus of the Hg(II) reductase (12). Since both the 15.1- and 14-kd *mer* proteins are localized in the inner membrane, they very likely participate in the uptake of Hg(II) into the cell. The 12-kd *mer* protein may function as a periplasmic Hg(II)-binding protein. We have preliminary evidence that the 12-kd protein can be found in the periplasmic fraction (unpublished data). Experiments to test our hypotheses about the roles of these proteins and the energetic basis of Hg(II) transport are currently in progress.

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