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Outer Membrane Proteins of *Escherichia coli*

V. Evidence that Protein 1 and Bacteriophage-Directed Protein 2 Are Different Polypeptides

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Protein 1 from the outer membrane of *Escherichia coli* K-12 and protein 2 from a phage PA-2 lysogen of the same strain were isolated by differential sodium dodecyl sulfate extraction and purified by ion-exchange and gel filtration chromatography. Rabbit antisera were prepared against these proteins and showed no cross-reaction between proteins 1 and 2. The proteins have the same N-terminal amino acid but show small yet significant differences in amino acid composition. The proteins were cleaved with cyanogenbromide in solvents containing both formic acid and trifluoroacetic acid. By comparing the cleavage in these solvents, it was established that protein 1 yielded 5 cyanogen bromide peptides, and the sum of the molecular weights of these was equivalent to the molecular weight of the uncleaved protein. Protein 2 yielded 4 cyanogen bromide peptides, none of which was identical to those of protein 1, and the sum of these peptides was also equivalent to the apparent molecular weight of the uncleaved protein. Significant differences were also observed when tryptic peptides from the two proteins were compared. These results indicate that protein 1 and the phage-directed protein 2 are distinct, different, and apparently homogeneous proteins.

Two major protein bands are observed when the outer membrane protein of *Escherichia coli* K-12 grown on minimal medium is examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (1, 5, 15). We designate these protein bands as protein 1 and protein 3a, and these correspond to the bands designated as proteins I and II* by Henning et al. (5, 6). On phosphate-buffered SDS-gels run at pH 7.2, these two proteins migrate as a single band, whereas on alkaline SDS-gels prepared by the formulation of Bragg and Hou (2) or on SDS-gels utilizing the discontinuous Laemmli buffer system (1, 11), protein 3a migrates faster than protein 1. Protein 1 from *E. coli* K-12 appears heterogeneous in that it migrates as two closely spaced bands in high-resolution gels in the Laemmli buffer system (1, 11). We term these bands protein 1a and 1b (1), corresponding to proteins Ia and Ib of Schmitges and Henning (11). Protein 1 of *E. coli* B yields only a single band identical to band 1a when it is examined with the Laemmli gel system (1, 11). Protein 1 from *E. coli* B has been purified and extensively characterized by Rosenbusch (10), who has termed this protein the "matrix protein." He found this protein to have a molecular mass of 36,500 daltons.

Our initial investigation of *E. coli* outer membrane proteins was carried out with strain J-5, a derivative of *E. coli* $O111_{B4}$. This strain produced only a small amount of protein 1, instead producing a protein, which we designated as protein 2, that exhibited a more rapid migration on alkaline Bragg-Hou SDS-gels (13). More recently, we have found that this protein is found in many wild strains of *E. coli*. Protein 2 is the consequence of an unusual lysogenic conversion by a lambdoid phage, which we have called PA-2 (16). We have established that protein 1b is a component of the receptor for phage PA-2 (1), and thus the consequence of the replacement of protein 1 by protein 2 in PA-2 lysogens is the surface exclusion of superinfecting phage.

This novel form of lysogenic conversion raises interesting questions about the interrelationship between the genes of phage PA-2 and its host. In other examples of lysogenic conversion that have been studied in detail, the products of prophage genes have been relatively minor components of the cell, such as toxins or enzymes that provide altered carbohydrate biosynthesis. However, in the case of PA-2, pro-

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tein 2 may comprise as much as 30 to 40% of the total outer membrane protein or as much as 3 to 5% of the total protein of the cell. The amount of protein 2 on the cell surface is regulated, both by catabolite repression (15, 16) and by the same type of physiological response observed for other major outer membrane proteins (1). The question brought to mind is whether the structural gene for protein 2 resides in the host or the phage genome, and how this gene is regulated in the lysogenic state.

A necessary prerequisite for studies on the genetics and regulation of protein 2 production by lysogens is to demonstrate unequivocally that protein 2 is a unique polypeptide and not simply a modified form of protein 1. Our previous studies (14) on the cyanogen bromide (CNBr) peptides derived from proteins 1 and 2 suggested that these were different polypeptides, but the data were not convincing because it was not possible to reconcile the apparent molecular weights of the peptides with the apparent molecular weight of the uncleaved protein. This report deals with a more detailed examination of the chemical properties of proteins 1 and 2, and we conclude that these proteins are different polypeptides.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* K-12 strain W1485F$^-$ was used for the preparation of protein 1, and protein 2 was isolated from a ParI mutant derived from the above strain (strain PB102), which was lysogenic for hy7, a derivative of PA-2. Cultures were grown with shaking at 37°C to mid-exponential phase (ca. 5×10^8 to 7×10^8 cells/ml) on minimal salts medium (19). Unless otherwise noted, the sole carbon source was 0.5% lactic acid. This was prepared as a 5% stock solution, adjusted to pH 7.0 with KOH, and autoclaved separately. The medium was supplemented with 1 μg of thiamine, 20 μg of carrier L-leucine, and 0.1 μCi of [4,5-^{3}H]leucine per ml unless otherwise noted.

Cell breakage and fractionation. Cultures were chilled and centrifuged, and the cells were suspended in 0.1 of the culture volume in 0.01 M HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), pH 7.4, at 0°C. The cells were centrifuged and suspended in the above buffer at 0.01 of the culture volume, and a small amount of ribonuclease and deoxyribonuclease was added. The cells were broken by passage through a French pressure cell, and $MgCl_2$ was added to give a final concentration of 1 mM. After centrifugation for 5 min at 5,000 × *g* to remove unbroken cells, the envelope was sedimented by centrifugation at 200,000 × *g* for 45 min. Cytoplasmic membrane was removed by suspending the pellet at the same volume used for breakage in HEPES buffer, as above, containing 2% Triton X-100. After standing for 10 min at 23°C, this suspension was centrifuged at 200,000 × *g* for 45 min to sediment the outer membrane-peptidoglycan fraction. This was washed once by suspension in the same HEPES buffer without detergent and centrifugation as above.

Differential extraction and purification of proteins 1 and 2. Since proteins 1 and 2 exhibit similar resistance to solubilization by SDS in the presence of Mg^{2+}, a modification of the selective extraction procedure of Rosenbusch (10) was used to isolate both proteins. The washed outer membrane-peptidoglycan fraction was suspended in either distilled water or in 10 mM HEPES buffer as above to which 0.2 mM $MgCl_2$ had been added. This suspension contained 10 mg of protein per ml. To this suspension was added an equal volume of solution containing 4% SDS (Bio-Rad, electrophoresis grade), 20% glycerol, 0.2% 2-mercaptoethanol, and 0.1 mM $MgCl_2$ in 10 mM HEPES buffer, pH 7.4. The final suspension was allowed to stand for 30 min at 37°C and was then centrifuged at 200,000 × *g* at 25°C for 45 min to sediment the peptidoglycan-protein 1 (or protein 2) complex. The pellet was washed twice by suspension in distilled water containing 0.1 mM $MgCl_2$ followed by centrifugation as above to remove excess SDS.

The washed fragments of the SDS-insoluble peptidoglycan-protein complex were suspended at a concentration of 5 mg of protein per ml in 10 mM HEPES buffer, pH 7.4, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 2% Triton X-100 and allowed to stand for 20 min at 23°C. This suspension was then centrifuged at 200,000 × *g* for 45 min at 25°C. After this centrifugation, about 60% of the protein 1 or 2 was present in the supernatant fraction. The supernatant fraction was chilled, and the protein was precipitated by adding 2 volumes of ice-cold 95% ethanol and allowing the mixture to stand overnight at −20°C to insure complete precipitation. The precipitate was collected by centrifugation, drained carefully of supernatnatant fluid, and dissolved by gentle homogenization at a concentration of 5 mg of protein per ml in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.2, containing 5 mM EDTA and 2% Triton X-100 (Tris-Triton buffer). This and all subsequent steps were performed at 23°C.

The protein was applied to a diethylaminoethyl (DEAE)-cellulose column (Whatman DE-52) equilibrated with Tris-Triton buffer. The original suspension was opalescent, but this did not affect the binding to the column or subsequent elution. The sharpest elution profiles were obtained when the column had a bed volume of 2 ml for each milligram of protein applied. After sample application, the column was washed with 2 column volumes of Tris-Triton buffer, followed by a linear gradient of from 0 to 0.2 M NaCl in Tris-Triton buffer extending over 2 column volumes.

Peak fractions containing protein 1 or 2 were pooled, and the protein was precipitated with ethanol as above. The precipitated protein was dissolved in 0.1 M sodium phosphate buffer containing 2% SDS, 0.1% 2-mercaptoethanol, and 5 mM EDTA at a concentration of 10 mg of protein per ml. This was applied to a Sephadex G-200 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.2,

containing 0.1% SDS and 0.5 mM EDTA and eluted with the same buffer. The "A" peak (12), eluting just after the void volume, containing aggregated protein 1 or 2 was concentrated to 10 mg of protein per ml with an Amicon PM-10 filter and an ultrafiltration apparatus, and SDS was added to make the final concentration 4%. The sample was heated in a boiling bath for 10 min and was then applied to a second Sephadex G-200 column exactly as described above. The "B" peak of dissociated protein, eluted from this column (12), was concentrated by pressure ultrafiltration as above and lyophilized. When necessary, SDS was removed from the lyophilized protein by washing the protein by low-speed centrifugation at least four times with a solution consisting of six parts acetone and one part water. No detectable SDS remained with the protein after this procedure. The SDS-free protein was generally suspended in water and lyophilized again.

CNBr cleavage. Samples of SDS-free protein were cleaved with CNBr after solubilization of the protein in 70% formic acid (14), or by the method of Schroeder (17) after solubilization of the protein in 70% trifluoroacetic acid. Cleavage in formic acid was carried out in the dark for 24 h at 23°C with a 1,000-fold molar excess of CNBr over methionine in the protein. When trifluoroacetic acid was the solvent, the cleavage time was reduced to 12 h for protein 1 and 3 h for protein 2 to minimize artifactual acid hydrolysis by the solvent. The proteins were cleaved at concentrations ranging from 0.5 to 5.0 mg of protein per ml. After cleavage, the samples were diluted 10- to 15-fold with water and lyophilized, taking care to avoid thawing the sample during drying. When proteins were cleaved at a concentration of greater than 1 mg/ml, the dried protein was suspended in water and lyophilized again to insure complete removal of solvent.

Polyacrylamide gel electrophoresis. Uncleaved protein samples were boiled in SDS-urea solution and analyzed on 7.5% acrylamide gels by either the alkaline phosphate-buffered system of Bragg and Hou or the neutral-pH Maizel buffer system as previously described (15). Lyophilized CNBr peptides were dissolved in the Tris-SDS solubilizing solution described by Swank and Munkres (18), heated in a boiling bath for 5 min, and analyzed on 15% acrylamide gels by the Maizel (pH 7.2, phosphate buffer) system as above. In all cases the gels contained 0.5 M urea and were prepared in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS. The acrylamide-to-bisacrylamide ratio was constant at 37.5:1 for all gels. Electrophoresis was carried out at 5 mA/gel, and gels were either stained with Coomassie blue (18) or frozen and sliced into 1.2-mm slices. The slices were incubated for 2 h at 50°C in 0.5 ml of a mixture of NCS solubilizer (Amersham/Searle) and water at a ratio of 9:1 for the 7.5% acrylamide gels and 7.5:1 for the 15% acrylamide gels. After cooling, 10 ml of toluene-based counting fluid was added, and the vials were allowed to stand for a few hours prior to counting.

Determination of C-terminal peptides. Cultures were grown with lactate as carbon source in minimal medium supplemented with a mixture of vitamins and all of the standard amino acids (9) with the exception of leucine and methionine, which were reduced to 20 μg of each per ml. The medium contained 0.2 μCi of [4,5-^{3}H]leucine and 1.0 μCi of [1-^{14}C]methionine per ml. Proteins were isolated and cleaved with CNBr, as above, and analyzed on 15% acrylamide gels to determine which peptide was not labeled with methionine.

Amino acid analysis. Samples of SDS-free proteins 1 and 2 were reduced and carboxymethylated (14) and hydrolyzed in constantly boiling HCl for 18 h at 105°C in vacuo. Hydrolysis was also carried out for 72 h to assess any increase in leucine and isoleucine. Analysis was performed on a Beckman model 121 automated amino acid analyzer.

N-terminal amino acid determination. The N-terminal amino acids of proteins 1 and 2 were determined as the fluorescent dansyl derivatives by two-dimensional thin-layer chromatography on Baker polyamide sheets by the method of Gray (4). Dansyl amino acid standards were from Sigma Chemical Co.

Analysis of tryptic peptides. Protein 1 from the ParI mutant strain PB102 and protein 2 from an hy7 lysogen of the same strain (1) labeled, respectively, with [^{14}C]leucine and [^{3}H]leucine were isolated by a simplified procedure in which the protein of the SDS-Mg^{2+}-insoluble peptidoglycan-protein complex was solublized by extraction with 0.1 M sodium phosphate buffer, pH 7.2, containing 2% SDS, 0.1% 2-mercaptoethanol, and 5 mM EDTA for 20 min at 37°C. The peptidoglycan was removed by centrifugation at 200,000 $\times$ g for 45 min at 25°C, and the solublized protein was purified by chromatography on Sephadex G-200 before and after boiling in SDS solution as described above. SDS-free protein 1 (0.7 mg) mixed with SDS-free protein 2 (0.3 mg) was suspended in 1.0 ml of 0.1 M ammonium bicarbonate buffer, pH 8.5, containing 1 mM $MgCl_2$. Tolysulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (7) was added (200 μg), and the reaction mixture was incubated with stirring at 37°C for 6 h. An additional 200 μg of TPCK-trypsin was added, and incubation was continued for an additional 12 h. The resulting suspension was lyophilized, and the peptides were dissolved in water. After adjustment of the pH to 1.5 with HCl, the peptide sample was loaded onto an Aminex A-6 column (Bio-Rad) and developed with pyridine-acetate buffer (8). Column fractions were collected into counting vials, dried overnight in an oven, and counted after the addition of NCS solublizer and counting fluid.

Molecular weight of CNBr peptides. The molecular weight of the CNBr peptides was estimated either by determining the migration on 15% polyacrylamide gels compared with the migration of bovine serum albumin, ovalbumin, pepsin, α-chymotrypsinogen, myoglobin, and insulin, or (see Results) by estimating the amount of radioactivity in each peptide peak derived from a uniformly ^{14}C-labeled culture. In the latter case, the culture was grown in a stoppered flask on minimal medium with 0.1% glucose as the sole carbon source and with 0.2 μCi of [U-^{14}C]glucose added per ml. Protein 1 was isolated from this culture as described above. This

latter method could not be used with protein 2 due to problems of catabolite repression by glucose (15).

Immunological procedures. Rabbits were immunized with partially purified protein 1 or 2. Each animal received a primary inoculation of 1 mg of protein. This inoculation consisted of protein 1 or 2 purified as described above by selective SDS extraction, DEAE-cellulose chromatography, and the first step of chromatography on G-200. Instead of boiling this protein, as in the complete purification procedure, the eluent from the first G-200 column in 0.1% SDS was concentrated to 3 mg of protein per ml, and this was mixed with 2 volumes of complete Freund adjuvant (Difco) and dispersed by ultrasonic treatment. Portions (0.25 ml) of this material were injected into each of the four major footpads of each animal. Each animal received two subsequent booster injections at 1-month intervals of the same protein sample prepared in the same way, except that incomplete Freund adjuvant was used and these booster injections (1 mg of protein per animal) were administered intradermally at multiple sites on the back. The animals were then rested for 2 months, and a final booster injection of protein 1 or 2 purified only through the DEAE-cellulose chromatography step of purification was given. The pooled protein peak eluted from the DEAE-cellulose column was precipitated with ethanol, and the precipitate was dissolved in 0.1 M NaCl containing 1% Triton X-100 at a protein concentration of 3 mg/ml. This was dispersed as above in Freund incomplete adjuvant, and 1 mg of protein was administered to each animal intradermally at multiple sites. One week after the final injection, the animals were sacrificed and serum was collected.

Immune precipitation of proteins 1 and 2 was carried out in 0.05 M Tris-chloride buffer, pH 7.2, containing 0.05 M NaCl and 0.5% Triton X-100 (Triton-salt buffer). The samples of protein 1 and 2 used in immune precipitation and immunodiffusion experiments consisted of protein purified through the DEAE-cellulose chromatography step, as above, dissolved at 1 mg of protein per ml in Triton-salt buffer. These protein samples were labeled with [^{3}H]leucine. Quantitative immune precipitation was carried out by mixing 0.1 mg of protein 1 or 2 with various dilutions of the immune or preimmune sera in Triton-salt buffer, adjusting the volume to 1 ml with Triton-salt and incubating for 1 h at 37°C and then overnight at 4°C. The immune precipitate was sedimented by centrifugation at 5,000 × *g* for 10 min, washed by suspension in 2 ml of cold Triton-salt buffer and centrifugation as above, dissolved in NCS solublizer, and counted. Precipitin reactions were also examined by double diffusion on slides of 1.5% agar in Triton-salt buffer.

RESULTS

Purification of proteins 1 and 2. Protein purification was facilitated by a modification of the Rosenbusch (10) procedure for selective SDS extraction. When the Triton-insoluble outer membrane fraction from a nonlysogenic culture of W1485F$^-$ was extracted with SDS in the presence of a small amount of Mg^{2+}, almost all of the protein 3 and the majority of the other minor proteins were solublized. Protein 1, a trace of protein 3, and some of the murein lipoprotein remained associated with the insoluble peptidoglycan-protein fraction. In the case of W1485F$^-$ lysogenic for PA-2, protein 2 was also associated with the insoluble peptidoglycan-protein fraction.

Proteins 1 and 2 could then be extracted completely from the peptidoglycan by a second, mild (37°C, 30 min) extraction with SDS solution containing EDTA. Alternatively, we found that about 65% of the protein 1 (and/or protein 2) could be extracted from the peptidoglycan-protein fraction with 2% Triton X-100 in Tris-chloride buffer containing 5 mM EDTA. Both of these procedures offer an advantage over the hot SDS extraction of Rosenbusch (10) in that the protein may be extracted in a more native form. In the case of Triton-EDTA extraction, the protein may then be subject to further purification on DEAE-cellulose.

When protein 1 solublized from the peptidoglycan-protein fraction with Triton-EDTA was chromatographed on DEAE-cellulose, it eluted at slightly less than 0.1 M NaCl. Protein 2 eluted from such a column at lower salt, about 0.075 M NaCl. Thus it was possible to separate the proteins effectively at this step. It was also possible to achieve separation of proteins 1 and 2 by chromatography on Sephadex G-200 after dissolution of the protein in SDS solution without heating. The oligomeric "A" peak of protein 2 elutes from such a column just after the void volume, whereas the "A" peak of protein 1 was slightly included and eluted several fractions later.

Thus by a combination of the selective Rosenbusch extraction technique followed by solublization with Triton-EDTA, chromatography in Triton X-100 on DEAE-cellulose, and chromatography of the unheated protein in SDS on Sephadex G-200, it was possible to obtain protein that was highly purified and still retained antigenic activity. The final preparations of proteins 1 and 2 obtained by this procedure yielded single bands on alkaline Bragg-Hou tube gels (data not shown). When analyzed on a high-resolution slab-gel system in the Laemmli buffer system (1), protein 2 yielded a single band while protein 1 yielded roughly equal amounts of the 1a and 1b components (1). The phosphate content of protein 1 purified as above with the inclusion of a second Sephadex G-200 chromatography step after boiling in SDS was analyzed. The protein contained less than 0.3 mol of phosphate per mol of protein (assumed molecular mass of 36,500 daltons) and thus ap-

peared to be to be substantially free of contaminating lipopolysaccharide or phospholipid.

Immunological comparison of proteins 1 and 2. Purified protein 1 yielded a single precipitin line when tested by double immunodiffusion against anti-protein 1 serum, and no precipitin line against preimmune serum or anti-protein 2 serum. Likewise, protein 2 gave a single line against anti-protein 2 serum and none against pre-immune serum or anti-protein 1 serum. The equivalence point of protein 1 and the anti-protein 1 serum was determined by measuring the amount of ^{3}H-labeled protein 1 precipitated when a constant amount of protein 1 was incubated with various dilutions of anti-protein 1 serum. At the optimum ratio, more than 90% of the labeled protein 1 was precipitated by the antiserum, and, at this ratio of protein and antiserum, no reduction in precipitation of protein 1 was observed when a 10-fold excess of unlabeled protein 2 was included in the incubation mixture. These data indicate that the anti-protein 1 serum must react with both the 1a and 1b components of protein 1, and that there is little or no cross-reactivity between proteins 1 and 2. The final titers of both sets of antisera were similar, and, at equivalence as defined above, approximately 1.1 ml of antiserum was sufficient to precipitate 1 mg of protein.

Total and N-terminal amino acid analysis. Alanine was the N-terminal amino acid of both proteins 1 and 2, and no "minor" N-terminal amino acids were detected. The amino acid composition of proteins 1 and 2 is given in Table 1. Although the composition is similar for both proteins, significant differences in lysine, histidine, aspartic acid, and glycine were observed. No cysteine was detected in either of the proteins.

CNBr peptides of proteins 1 and 2. Earlier (14) we were unable to reconcile the apparent molecular weights of the CNBr peptides of proteins 1 and 2 with the apparent molecular weight of the uncleaved proteins. Garten et al. (3) reported a more complete study of CNBr peptides from protein 1 isolated from *E. coli* B and demonstrated that incomplete cleavage occurs in formic acid. They were able to obtain more complete cleavage by the use of trifluoroacetic acid as the solvent and, by comparison of cleavage in both solvents, to identify the partial cleavage products.

CNBr peptides of proteins 1 and 2 of *E. coli* K-12 cleaved in formic acid and trifluoroacetic acid, respectively (Fig. 1 and 2). For this experiment, the cultures were labeled with [^{3}H]leucine and with methionine labeled with ^{14}C on carbon 1 to demonstrate two things: first, this allowed identification of the C-terminal peptide, since this peptide would not contain labeled methionine. Second, by measuring the amount of methionine radioactivity in each peak we can identify partial cleavage products and also demonstrate whether the peaks represent single peptides.

The peptides from protein 1 have the same letter designations of Garten et al. (3; Fig. 1 and 2). However, the relative order of peptide peaks D1, D2, and D3 is reversed in the neutral-pH phosphate-buffered gel system we use with respect to that observed by Garten et al. (3), who used a discontinuous Tris-buffered system. To avoid confusion, we identified peptides from protein 2 by Roman numerals.

The results shown in Fig. 1 and 2 for protein 1 confirm those of Garten et al. (3). Peptide D1 contains few ^{14}C counts and thus is the C-terminal peptide. Peaks A and C are reduced when the protein is cleaved in trifluoroacetic acid, indicating that these represent partial cleavage products. The sum of the ^{14}C counts in peaks A and B in the formic-cleaved protein (Fig. 1) is the same as the counts in peak B in the trifluoroacetic acid-cleaved protein (Fig. 2), indicating that the partial cleavage fragment in peak A is composed of peptides B and D1. The sum of the

TABLE 1. *Amino acid composition of proteins 1 and 2*[a]

Amino Acid	Protein 1		Protein 2	
	mol/mol	mol%	mol/mol	mol%
Lys	11	3.31	20	6.04
His	1	0.30	4	1.21
Arg	13	3.92	14	3.93
Half Cys	0	0	0	0
Asp	49	14.76	39	11.78
Thr	20	6.02	23	6.95
Ser	17	5.12	18	5.44
Glu	27	8.13	29	8.76
Pro	5	1.51	4	1.21
Gly	49	14.76	40	12.09
Ala	30	9.04	32	9.67
Val	33	9.94	32	9.67
Met	4	1.21	4	1.21
Ile	10	3.01	7	2.12
Leu	22	6.63	22	6.65
Tyr	23	6.93	22	6.65
Phe	18	5.42	22	6.65

[a] The number of moles of amino acid per mole of protein is rounded off to the nearest integer. A molecular weight of 36,500 was assumed for each protein. The data represent the average of two separate determinations. Cystine was determined as *S*-carboxymethylcysteine and cysteic acid. Tryptophan was not determined.

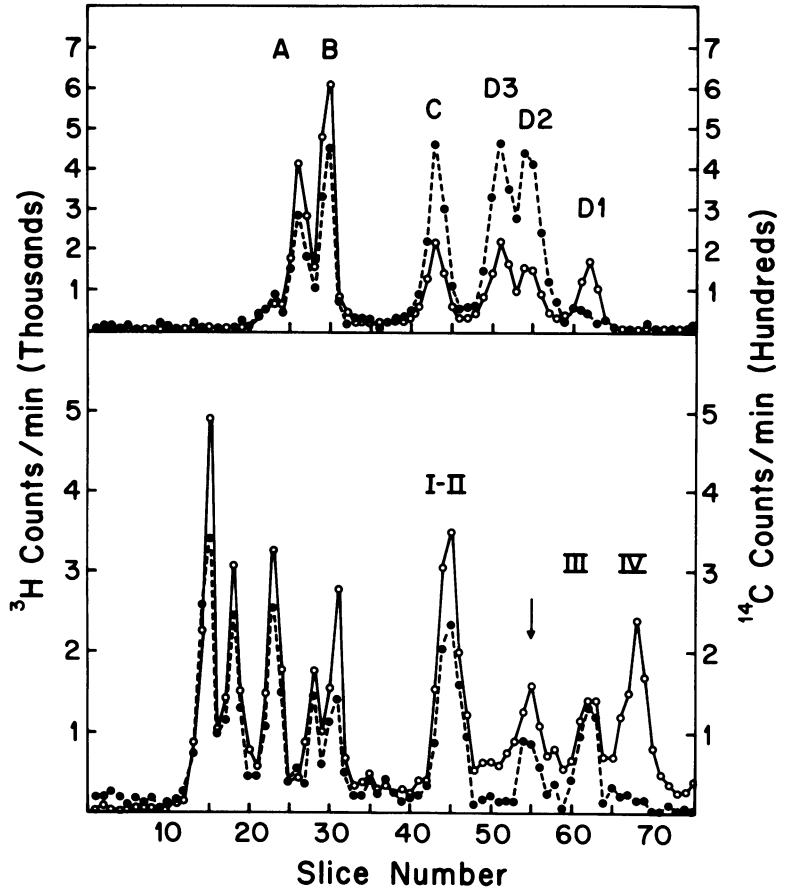

FIG. 1. *Polyacrylamide (15%) gels of CNBr peptides of protein 1 (top panel) and protein 2 (bottom panel) after cleavage in formic acid. Cultures, from which the proteins were obtained, were labeled with [^{3}H]leucine (solid line) and [^{14}C]methionine (dashed line). Peptides from protein 1 are designated by the letters A through D as described by Garten et al. (3), and peptides from protein 2 are designated by roman numerals. The top of the gel is to the left.*

^{14}C counts in peaks C and D2 is the same in protein cleaved in either solvent, and cleavage in trifluoroacetic acid reduces peak C and correspondingly increases peak D2. Peak D2 from the sample cleaved in trifluoroacetic acid (Fig. 2) contained nearly twice the ^{14}C counts of the other peptides. Taken together, these data indicate that peak D2 contains two peptides (D2a and D2b) that are not resolved, and that peak C is a partial cleavage fragment consisting of peptides D2a and D2b.

The difference in CNBr cleavage in the two solvents is more striking in the case of protein 2. Cleavage of protein 2 in formic acid resulted in a significant amount of uncleaved protein (slowest migrating peak, Fig. 1) and a number of high-molecular-weight partial cleavage fragments. Four peaks are observed when protein 2 is cleaved in trifluoroacetic acid (peptides I and II appear as a doublet on stained gels, but cannot be resolved by slicing). Peak IV contains few ^{14}C counts and is the C-terminal peptide. The presence of a partial cleavage product migrating between peaks II and III (arrow, Fig. 1) indicates that peptides III and IV are contiguous.

Although amino acid analysis (Table 1) indicated that protein 2 contained four Met residues, we have been able to detect only four CNBr peptides. We attribute this discrepancy to error in the amino acid analysis. When methionine-labeled CNBr peptides from a sample of protein 2 cleaved in trifluoroacetic acid were applied to a variety of gel filtration columns, we were unable to detect any low-molecular-weight-labeled material, indicating that there is no small fifth peptide which was not detected on the gels. As seen in Fig. 2, the ^{14}C counts are

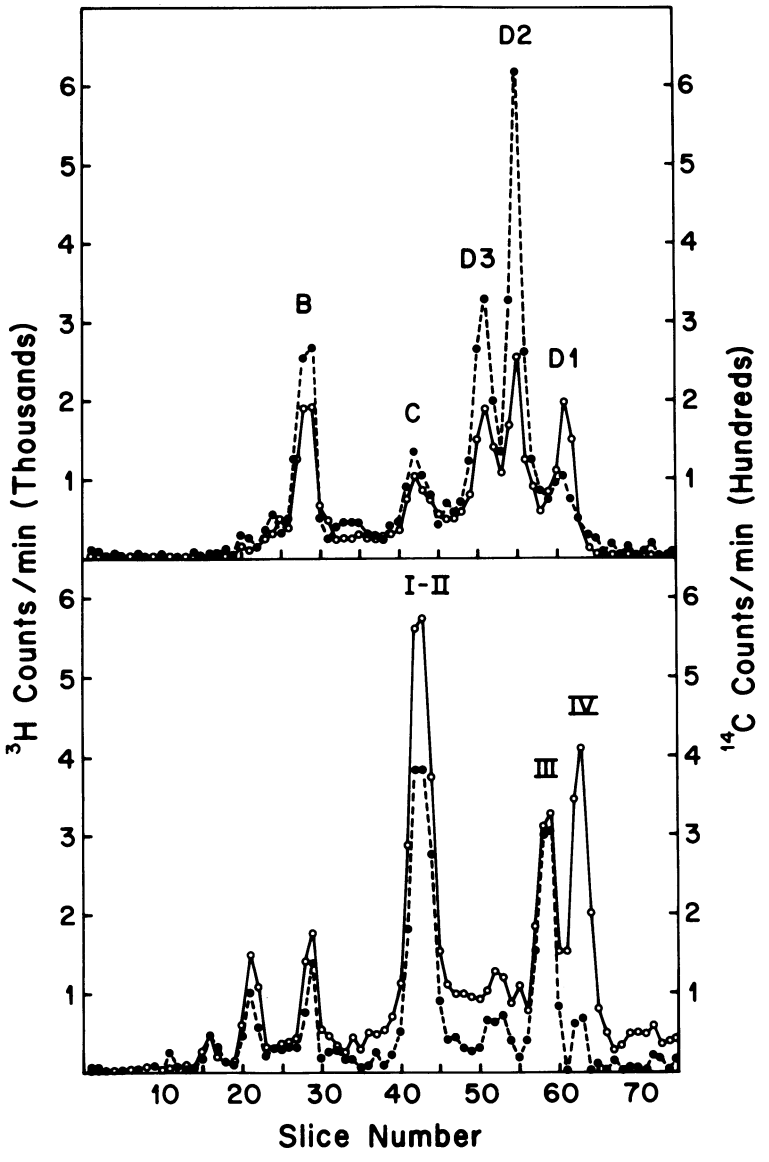

FIG. 2. *CNBr peptides of protein 1 (top panel) and protein 2 (bottom panel) obtained after cleavage in trifluoroacetic acid. Labeling is the same as in Fig. 1.*

roughly equal in peaks I, II, and III. Thus, none of these peptides contain an uncleaved -Met-Met- sequence.

To further confirm our analysis of the peptides derived from protein 1 and to validate our measurements of the apparent molecular weights of the peptides on gels, we grew a nonlysogenic culture on [*U*-^{14}C]glucose as the sole carbon source. Protein 1 was isolated from this culture and cleaved with CNBr in both solvents. Since the peptides are uniformly labeled, we could estimate the relative amount of each partial and final cleavage product produced in each solvent quite accurately. The data in Table 2 provide additional chemical evidence for the relationship between partial and final cleavage products.

The molecular weight of each of the peptides was also calculated from the data on the relative mass of each peptide given in Table 2. The molecular weights are as follows: peptide B, 15,800; peptide D1, 2,800; peptide D2, 5,500; and peptide D3, 6,200. The scheme in Fig. 3 summarizes the CNBr peptides of proteins 1 and 2 and

their arrangement, as deduced from partial cleavage products. The molecular weights given in Fig. 3 were calculated from the relative mobility on neutral-pH 15% polyacrylamide gels, and those for protein 1 agree well with the values given above.

Absence of cysteine in proteins 1 and 2. Rosenbusch (10) has reported the presence of one cysteine residue in protein 1 isolated from *E. coli* B. However, we were unable to detect reasonable amounts of *S*-carboxymethylcysteine by analyzing reduced, carboxymethylated protein (Table 1). We also attempted to label protein 1 with [^{14}C]iodoacetate, and we obtained less than 1% of the predicted incorporation based on 1 mol of cysteine per mol of protein. Since both of these techniques would yield negative results if the −SH group of cysteine was oxidized or blocked, we tested this more directly by following the fate of ^{35}S-labeled protein after cleavage with CNBr in both solvents (Table 3). For both proteins, the amount of labeled sulfur remaining after cleavage was far less than would be predicted on the basis of one cysteine residue per polypeptide. In addition, these data indicate that the conversion of methionine to homoserine is essentially complete in both proteins after cleavage in trifluoroacetic acid.

Tryptic peptides of proteins 1 and 2. To minimize contamination of protein 2 by protein 1, we routinely isolated protein 2 from a λ-PA-2 hybrid phage (hy7) lysogen of a ParI *E. coli* mutant that is partially defective in protein 1 production but produces normal protein 2 (1). To make a valid comparison of tryptic peptides between proteins 1 and 2, we isolated protein 1 (in this case only the 1a component) from the same ParI mutant strain from which we isolate protein 2. The mixture of the two proteins, labeled respectively with ^{14}C and ^{3}H, was digested with trypsin, and the resulting peptides were separated by Dowex-50 column chromatography (Fig. 4). Both proteins yielded 18 peptide peaks, which were resolved on the column, and of these, 7 showed identical elution of the two radioactive labels. The remainder were clearly different, as is seen particularly in fractions 40 through 90.

TABLE 2. *Relative distribution of ^{14}C label in peptides of uniformly labeled protein 1 after cleavage in formic acid and trifluoroacetic acid*[a]

CNBr peptide	Relative amount of label (% of total counts on gel)	
	Cleaved in formic acid	Cleaved in trifluoroacetic acid
A	18.6	0
B	29.7	43.7
C	11.9	3.9
D3	18.2	18.5
D2	14.5	22.6
D1	7.1	11.3

[a] The protein 1 used in this experiment was from a culture grown with [*U*-^{14}C]glucose as the sole carbon source.

DISCUSSION

Protein 1 and protein 2 are similar in many properties, including apparent molecular weight on neutral-pH SDS-gels, formation of aggregates that can be dissociated only by heating or other harsh treatment, lack of cysteine, and the unique property of resistance to solubilization by SDS in the presence of divalent cations. However, the results presented clearly show that these are different polypeptides. These data may be summarized as follows: (i)

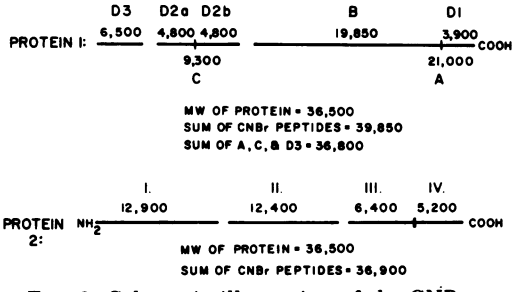

FIG. 3. *Schematic illustration of the CNBr peptides from proteins 1 and 2, with molecular weights as calculated by migration in neutral-pH phosphate-buffered 15% polyacrylamide gels.*

TABLE 3. *Absence of cysteine in proteins 1 and 2 as determined by ^{35}S labeling*

Sample	Specific radioactivity (cpm/mg of protein)			
	Uncleaved protein	Predicted, after CNBr cleavage[a]	Observed, after CNBr cleavage in formic acid	Observed, after CNBr cleavage in trifluoroacetic acid
Protein 1	34,625	6,925	356	295
Protein 2	41,666	10,417	4,045	389

[a] Calculated on the basis of complete conversion of methionine to homoserine, and on the assumption that protein 1 contained four methionine residues and one cysteine and that protein 2 contained three methionine residues and one cysteine.

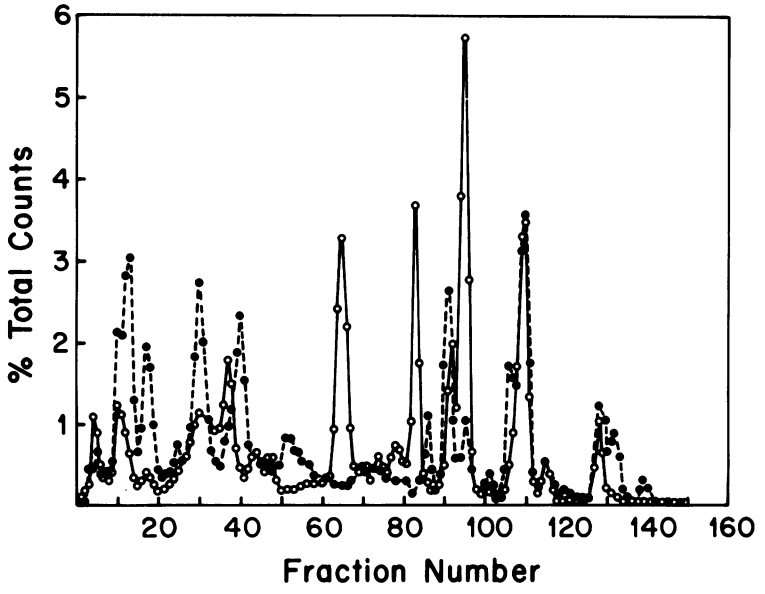

FIG. 4. *Tryptic peptides of protein 1 (dashed line, ^{14}C label) from strain PB102, and of protein 2 (solid line, ^{3}H label) from an hy7 lysogen of PB102 cleaved simultaneously and analyzed on an Aminex A-6 column.*

proteins 1 and 2 exhibit different elutions from DEAE-cellulose columns run in the presence of Triton X-100; (ii) no immunological cross-reactivity exists between the two proteins; (iii) there are distinct and significant differences in the amino acid composition of the two proteins; (iv) cleavage of the proteins with CNBr in trifluoroacetic acid has shown that each protein yields distinct CNBr peptides that can be reconciled with the molecular weight of the uncleaved proteins; and (v) proteins 1 and 2 yield different profiles of tryptic peptides upon resolution by column chromatography.

Neither the structural gene for protein 1 nor the structural gene for protein 2 has been identified unambiguously. The observation that protein 2 is a distinct polypeptide and not a modified form of protein 1 is consistent with the hypothesis that the structural gene for this protein is carried on the PA-2 phage genome.

Protein 2 migrates as a single, sharp band on neutral and alkaline phosphate-buffered SDS-gels as well as on a higher-resolution gel system using the discontinuous Laemmli buffer system (1). Since this protein yields a single N-terminal amino acid, a single C-terminal CNBr peptide, a display of CNBr peptides that can be reconciled with the methionine content and the molecular weight of the uncleaved protein, there is no evidence indicating that this protein is heterogeneous and not a single polypeptide. The situation is less clear with respect to protein 1, since this protein, while meeting almost all of the criteria described above, does yield two bands on Laemmli-buffer-system gels (1, 11). Genetic loci (*par*, *tolF*) which affect only one or the other of these two bands are known (1). The data presented here, while supporting the hypothesis that protein 1 is a single polypeptide which may exist in different forms, do not distinguish between this hypothesis and an alternate one stating that subcomponents 1a and 1b are two nearly identical polypeptides coded for by distinct structural genes. This question can only be resolved by additional biochemical and genetic investigation.

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