Properties of a Major Protein Released from *Escherichia coli* by Osmotic Shock†

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ABSTRACT: A large fraction of a constitutively synthesized polypeptide, comprising 5% of the total *Escherichia coli* protein, is released when plasmolysed cells are subjected to osmotic shock into ice-cold water. Since the protein is not liberated by the conversion of cells to spheroplasts, it is not a typical periplasmic protein. A complex pattern of association with the cell envelope indicates that it is bound to this structure in vivo. Its susceptibility to trypsin and its interaction with specific antibodies vary with the type of preparations used. Based on these observations, we postulate a peripheral location at the inner surface of the plasma membrane. The protein has been purified to homogeneity from osmotic shock fluid. It has a mass of 44 000 daltons. Some of its physical and chemical properties have been investigated. Most remarkable are its strongly aggregating and adhesive characteristics and its precipitation by vinblastine and calcium ions. These unusual properties, its presumed location, and the observation that it is present in large amounts (approximately 70 000 molecules per cell) suggest a structural role for this protein.

In a recent study, we have observed that a major protein from *Escherichia coli* with an apparent polypeptide chain molecular weight of 44 000 is associated with the envelope fraction under defined conditions (Takacs and Rosenbusch, 1975). Subsequently, we found that it is released from cells during osmotic shock treatment, but is retained by conversion of cells to spheroplasts. This unusual characteristic led us to investigate the interaction of this protein with the cell envelope further. In this report, we present the results of these observations and describe a purification procedure for this protein as well as some of its properties. The possibility of a structural function of this protein is considered.

Experimental Procedures

Bacteria and Culture Conditions. The strains used were the same as those employed previously (Rosenbusch, 1974) and include ten derivatives of *E. coli* B, K12, C, W, and 15. In addition, the *E. coli* K12 derivative BHB 960 (*su* III$^+$, ton B) and ASH 102 *cet* B3 were kindly provided by Drs. B. Hohn and I. B. Holland. Culture conditions have been described (Takacs and Rosenbusch, 1975). As alternatives to the M9 medium containing 0.4% glucose, we also used maltose (0.4%), succinate (1%), or glycerol (0.4%) as carbon sources, or a phosphate-depleted medium (Levinthal et al., 1962). Cells grown on 0.4% glycerol were induced for β-galactosidase by addition of 0.1 mM 2-propyl β-D-thiogalactoside at a cell density of 10^8 cells/ml. Radioactive labeling of bacterial cultures with ^{14}C-labeled amino acids has been described (Takacs and Rosenbusch, 1975).

Cell Disruption Procedures. Cells were disintegrated using a French pressure cell (20 000 psi), by spheroplast formation followed by osmotic lysis (Osborn et al., 1972) or by agitation with glass beads (Salton and Horne, 1951) as described previously (Takacs and Rosenbusch, 1975). Osmotic shock was performed and viability estimated according to the procedure of Nossal and Heppel (1966). Minor modifications are specified below. Preparation of envelopes from disrupted cells and their extraction with detergents and guanidinium chloride have been described in detail (Takacs and Rosenbusch, 1975).

Purification of the Major Protein Released by Osmotic Shock. All strains of *E. coli* tested released the polypeptide to be described during osmotic shock with ice-cold water. The most reproducible results and highest yields were obtained with a derivative of *E. coli* K12 which was used for its purification. Cultures (5 l.) were grown to cell densities of 6×10^8/ml in M9 medium. They were harvested by centrifugation at 15 000*g* for 10 min. The pellet was suspended in 100 ml of 33 mM Tris-HCl, pH 7.1, containing 20% sucrose and 0.5 mM EDTA (Nossal and Heppel, 1966). Plasmolysis at room temperature was followed by centrifugation at 15 000*g* for 10 min. The pellet was taken up in 5 ml of the same solution and rapidly diluted with 200 ml of ice-cold deionized water. After centrifugation, 5 ml of 1 M Tris-HCl, pH 8, 1 ml of 0.1 M DTT,[1] and 94.4 g of $(NH_4)_2SO_4$ were added to the supernatant. Subsequent steps were performed at 4 °C unless otherwise indicated. The solution was stirred for 1 h and centrifuged at 22 000*g* for 15 min. Precipitated protein was redissolved in 5 ml of 25 mM Tris-HCl, pH 8.0, containing 1 mM DTT and 0.1 mM EDTA (buffer A), dialyzed against this buffer (three changes), and applied to a DEAE-cellulose column (1.8×20 cm), equilibrated with buffer A. After 50 ml each of buffer A without and with 0.1 M NaCl, the protein sample was eluted with a linear gradient (total volume 200 ml) of 0.1–0.3 M NaCl in buffer A and 1.5 ml fractions were collected. The major protein peak eluted between 0.21 and 0.24 M NaCl. According to gel electrophoresis in sodium dodecyl sulfate, fractions corresponding to the trailing half of the peak were found to contain most of the major protein released by osmotic shock. They were pooled and concentrated to 4 ml by pressure

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[1] The abbreviations used are: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, DEAE, diethylaminoethyl; P_i, inorganic phosphate.

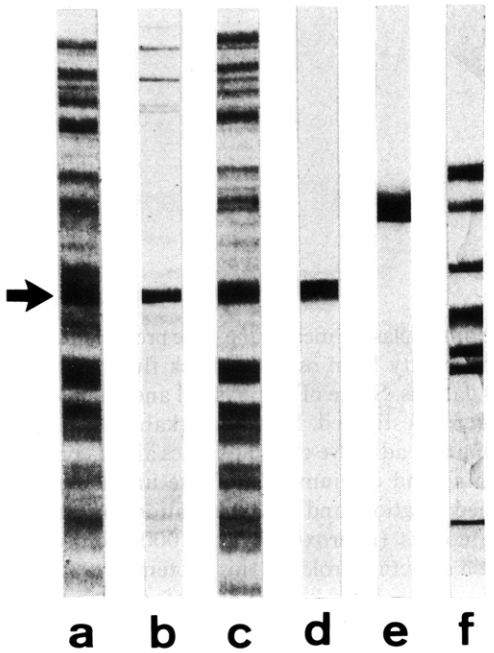

FIGURE 1: Electrophoresis on polyacrylamide slab gels (10%) in sodium dodecyl sulfate. A culture of *E. coli* B was grown in tryptone broth. a, Unfractionated cells; b, supernatant fraction after osmotic shock in water; c, pellet (30 000*g*) after breakage of cells by agitation with glass beads; d, purified major protein released by osmotic shock; e, flagellin prepared from a motile strain (*E. coli* K12, grown in minimal medium (M9) with 1% glycerol as the carbon source); f, standards (from top to bottom): bovine serum albumin (68 000), catalase (58 000), fumarase (49 000), aldolase (40 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), aspartate transcarbamylase, catalytic chain (33 000), and regulatory chain (17 000). The position of the protein with a mass of 44 000 daltons is indicated by an arrow.

dialysis, and the solution was then brought to room temperature. Isoelectric precipitation was performed by adding solid NaH_2PO_4 with stirring until the pH of the solution was 5.3, causing formation of a precipitate. Stirring was continued for 10 min, the suspension was centrifuged, and the pellet was redissolved in buffer A. At this point, most preparations were homogeneous, as judged by gel electrophoresis in sodium dodecyl sulfate. Occasionally, preparations were contaminated with higher molecular weight proteins (90 000–100 000). These were removed by layering 0.5 ml of the preparation onto a 12-ml 5–20% sucrose gradient in buffer A, centrifugation at 200 000*g* (average) for 20 h, and collection of the gradient in 200-μl fractions from the bottom of the tube. The final yield, as estimated from the band intensities in stained sodium dodecyl sulfate gels of unfractionated shock fluids and of pure preparations, was approximately 5%. Recoveries in all steps were <50%.

Enzyme and Ligand Binding Assays. Alkaline phosphatase and β-galactosidase were assayed by the procedures of Levinthal et al. (1962) and Malamy and Horecker (1961), respectively. Binding of maltose was assessed by equilibrium dialysis at 4 °C in multicavity dialysis cells. To estimate binding of inorganic phosphate, a small Sephadex G-25 column (0.5 × 7 cm), preequilibrated with radioactive ligand (Hummel and Dreyer, 1962), was used.

Immunological Techniques. Antibodies to the major protein released by osmotic shock were obtained from rabbits by three intramuscular injections in 18-day intervals. Purified protein (0.5 mg) was emulsified with complete Freund's adjuvant. Ten days following the last injection, blood was collected and antibodies purified by precipitation with ammonium sulfate (final saturation 50%) and by chromatography on Sephadex G-200 in 0.15 M sodium phosphate buffer, pH 7.0.

Immunodiffusion was carried out on glass microscope slides overlayered with 2 ml of a solution of 2% agarose in 0.05 M sodium barbital buffer, pH 8.2. Antigen and antibody (5 μl) were placed in wells in the agarose layer and were allowed to diffuse for 24 h at room temperature. The slides were washed for 24 h with 0.15 M NaCl, dried, stained with 0.25% Coomassie blue in methanol:acetic acid:H_2O (5:1:5), and destained in the same solution without the dye. Immunoprecipitation was performed in small tubes. The solutions to be tested and purified antibodies were mixed, incubated at 37 °C for 15 min, and then kept at 4 °C overnight. Precipitates were collected, and the pellets were washed once with 0.15 M sodium phosphate, pH 7.0, and directly analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Analytical Procedures. Polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate, densitometric scanning of electropherograms and autoradiograms, quantitation of radioactivity eluted from gels, and electron microscopy were performed as previously described (Takacs and Rosenbusch, 1975). Standard procedures for sedimentation equilibrium experiments, recording of ultraviolet spectra, amino acid analysis, and performic acid oxidation have been quoted and the conditions for isoelectric focusing in polyacrylamide gels reported previously (Rosenbusch, 1974). Amino-terminal sequence determinations were performed according to the procedure of Weiner et al. (1972).

Materials. ^{14}C-Labeled maltose, *N*-acetylglucosamine, sodium acetate, glycerol, palmitic acid, and amino acid mixtures were products of Amersham (England). Guanidinium chloride (ultrapure) was obtained from Schwarz/Mann and vinblastine sulfate from Sigma. Sources of protein standards have been reported previously (Takacs and Rosenbusch, 1975). Lysozyme (grade A, three times recrystallized) was purchased from Calbiochem and *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin was a product of Serva. All other chemicals used were analytical grade.

Results

Occurrence and Quantitation of the Protein. When electropherograms in sodium dodecyl sulfate of unfractionated *E. coli* B cells are examined, two bands are prominent (Figure 1a). The one with a mobility corresponding to a mass of 44 000 daltons (indicated by an arrow in Figure 1) accounts for approximately 5% of the total cell protein, as judged by densitometric scanning of stained gels or by quantitation of eluted radioactivity. The band is second in intensity only to the major envelope protein which has a mass of 36 500 daltons (Rosenbusch, 1974; Garten et al., 1975). We have observed similar intensities of the 44 000-dalton band in all strains of *E. coli* used and under all growth conditions we have tested (cf. Experimental Procedures).

Release from the Cell. The protein corresponding to this band has very unusual characteristics of release when cells are subjected to various treatments. Osmotic shock of cells in mid-exponential growth phase into ice-cold water released from 60 to 90% of this polypeptide in the absence of Mg^{2+} (Figure 1b and Table I). The viability of shocked cells was low (10–20%), as expected with the conditions used (Neu et al., 1966). Release was much less effective if 0.5 mM MgCl was present in the diluting solution or if cells grown to late log or stationary phase were used. Conversion of cells to spheroplasts by lysozyme–EDTA treatment released only 10–20% of this

TABLE I: Comparison of the Release into the Soluble Fraction of the 44 000-Dalton Polypeptide with that of Periplasmic, Cytoplasmic, and Cell Envelope Marker Proteins after Various Treatments.[a]

Treatment	Percentage of Proteins Released[b]			
	44 000-Dalton Polypeptide[c]	Alkaline Phosphatase	β-Galactosidase	Matrix Protein[d]
Osmotic Shock				
With H_2O	60–90	80–90	10–15	nd
With 0.5 mM $MgCl_2$	20–40	80–90	5–10	nd
Spheroplast Formation	10–20	80–90	10–20	nd
Osmotic Lysis of Spheroplasts	>90	>90[e]	>90	nd
Agitation with Glass Beads	10–20	80–90	80–90	nd
French Pressure Cell	>90[f]	>90	>90	nd

[a] The data are expressed as the range of values obtained in at least three experiments for each treatment. [b] The sums of the values obtained for the soluble and particulate fractions were in all cases equal to those of whole cells within experimental error. [c] Determined by scanning densitometry of stained gels. When the intensity of a particular band was estimated, the reproducibility of the values obtained was within 10%. [d] The matrix protein is the major envelope protein from *E. coli* on a mass basis (Rosenbusch, 1974). It corresponds to protein I of Garten et al. (1975). nd, none detected. [e] The figure given refers to the enzymatic activity which remained associated with spheroplasts after lysozyme-EDTA treatment. [f] Similar results were observed with the Manton-Gaulin mill and with extensive sonification. Mild sonification yielded intermediate values.

TABLE II: Amino Acid Composition of the Major Protein Released by Osmotic Shock.[a]

Amino Acid	Residues/44 000 Daltons
Lys	24.3
His	10.5
Arg	22.3
Asp	34.2
Thr	25.8
Ser	17.9
Glu	49.9
Pro	18.1
Gly	42.8
Ala	31.7
Cys	3.5
Val	32.5
Met	10.4
Ile	25.8
Leu	32.2
Tyr	10.3
Phe	14.9
Trp	Not determined

[a] Values for Ser and Thr were calculated by extrapolation to 0 time from 24-, 48-, and 72-h hydrolysates. Values for Leu and Ile are from 72-h hydrolysates. Values for the rest are averages of the three hydrolysis periods. Cysteine is expressed as cysteic acid after performic acid oxidation.

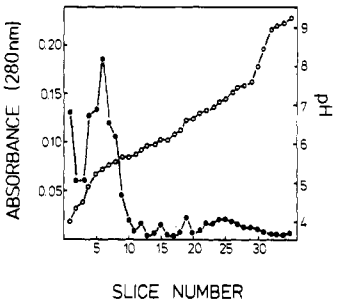

FIGURE 2: Isoelectric focusing of the purified protein (0.5 mg) in a polyacrylamide tube gel (7 cm) in the presence of 7.6 M urea. The procedure used has been described (Rosenbusch, 1974). The gel was cut into 35 slices which were eluted with 6 M urea. The pH (O–O) and absorbance at 280 nm (●–●) of the eluates were determined for each fraction.

protein, but osmotic lysis of spheroplasts liberated it quantitatively.

When cells were disrupted mechanically, the partition of this protein between soluble and particulate fractions depended on the forces applied. Table I shows that, upon agitation of cells with glass beads, this polypeptide remained largely with the envelope fraction (Figure 1c), whereas cell disintegration with the French pressure cell released it nearly quantitatively into the soluble fraction. If these results are compared with the distribution of the cytoplasmic marker β-galactosidase, the periplasmic enzyme alkaline phosphatase and the major envelope protein from *E. coli*, a pattern of release for this protein is revealed which is inconsistent with those typical of cytoplasmic, periplasmic, or of cell envelope proteins.

Physical and Chemical Characterization of the Pure Protein. Analysis of a purified preparation yielded a single band in sodium dodecyl sulfate gel electrophoresis (Figure 1d) with a mobility identical with that of the major band in osmotic shock supernatants. Its apparent mass is 44 000 daltons. The following observations indicate that the purified protein was homogeneous. Sedimentation equilibrium of the carboxymethylated protein in 6 M guanidinium chloride, 0.2 M Tris-HCl, pH 8.2, gave a polypeptide molecular weight of 41 000, ± 2000 and a linear plot of the log (concentration) vs. R^2. Sucrose gradient centrifugation yielded a single peak with a sedimentation coefficient of 3.5 S. Isoelectric focusing in polyacrylamide gels in the presence of 7.6 M urea also gave a unique peak with an isoelectric point of 5.3 (Figure 2).

The amino acid composition of the purified protein is presented in Table II. The percentage of nonpolar amino acid residues was calculated to be 42% (Heller, 1968). Attempts to determine the amino-terminal sequence of the protein showed no detectable aminoacyl derivatives after reaction of the polypeptide with 1-dimethylaminonaphthaline-5-sulfonyl chloride either before or after each of three consecutive cycles of Edman degradations. Therefore, the amino terminus of the protein is blocked.

Covalent binding of carbohydrates and lipids to this protein appears unlikely. This conclusion is based on the following experiments. Staining of sodium dodecyl sulfate-polyacrylamide gels of whole *E. coli* cells with periodic acid-Schiff reagent (Zacharius et al., 1969) and autoradiography of electrophoretic analyses obtained with cells labeled either with *N*-acetyl[^{14}C]glucosamine, with $^{32}P_i$, or with ^{14}C-labeled acetate, glycerol, or palmitate showed no detectable amounts of lipids or sugars to migrate with the 44 000-dalton band. Further support for this conclusion also comes from the excellent agreement between the polypeptide chain molecular

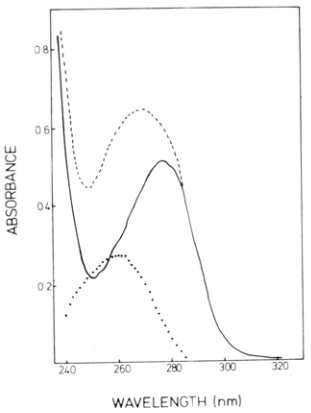

FIGURE 3: Ultraviolet absorption spectra of the purified protein recorded on a Cary 17 spectrophotometer. (- - -) Spectrum of the protein (0.5 mg/ml) purified through the isoelectric precipitation step; (—) spectrum of the same sample dialysed exhaustively against 33 mM Tris-HCl, 1 mM DTT, pH 8; (• • •) calculated difference spectrum.

weight, determined in guanidinium chloride or dodecyl sulfate, with the mass obtained from the sedimentation coefficient, if a monomeric, compact structure of the protein is assumed. However, the noncovalent binding of mono- or oligonucleotides is suggested by the ultraviolet difference spectrum (Figure 3).

Homogeneity of the Major Band in Osmotic Shock Fluids. When unfractionated osmotic shock supernatants were subjected to sucrose gradient centrifugation or isoelectric focusing, a major peak with characteristics similar to those described for the purified protein was observed in each case. Analyses by sodium dodecyl sulfate–gel electrophoresis confirmed that these peaks consisted uniquely of the 44 000-dalton polypeptide. To obtain quantitative estimates, we used antibodies specific to the purified protein.

Ouchterlony double diffusion tests indicated identity of the purified protein with a constituent of the unfractionated osmotic shock supernatant. This allowed us to quantitate the 44 000 dalton protein in unfractionated shock fluids by incubation of radioactively labeled osmotic shock fluid with the antibody. Precipitates were analyzed by sodium dodecyl sulfate–gel electrophoresis. Densitometric scanning of the resulting autoradiograms (Figure 4) demonstrated that over 90% of the band in the shock fluid consisted of the protein we have purified.

Corresponding experiments were also performed to examine the composition of the 44 000 dalton band observed in gels from whole cells. The immunological experiment described above showed that the specific antibody removed over 90% of the constituent of this band also from extracts of whole cells after extensive sonification. When the material of this band was eluted from gels, the amino acid analysis yielded a composition identical within experimental error with that shown in Table II. The amino-terminal sequence determination again failed to show any derivatized aminoacyl residue.

Sensitivity to Trypsin and Interaction of the Protein with Antibodies in Situ. From experiments reported previously (Rosenbusch, 1974), we knew that the 44 000-dalton protein was sensitive to trypsin treatment after cell disintegration by agitation with glass beads. Since this polypeptide is not released by treatment of cells with lysozyme and EDTA (see above), we subjected spheroplasts to tryptic hydrolysis in order to determine whether the protein was located on the surface of spheroplasts. Figure 5 shows that trypsin does not degrade the polypeptide in intact spheroplasts, but that upon their osmotic lysis, it was rapidly degraded. If the conditions used (3% trypsin

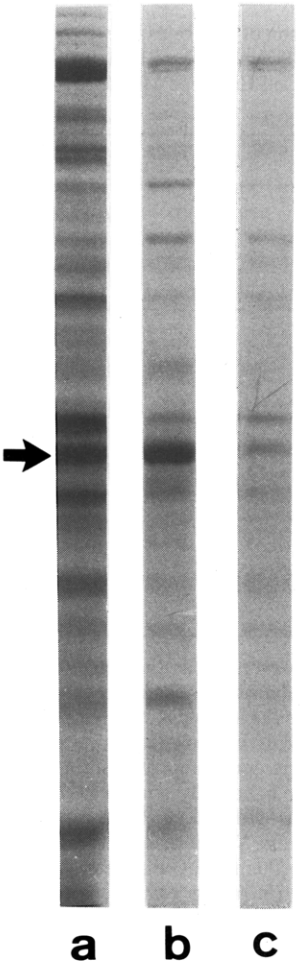

FIGURE 4: Autoradiograms of immunoprecipitates and controls, analyzed by slab gel electrophoresis in sodium dodecyl sulfate. A culture of *E. coli* BE was grown in minimal medium (M9) and labeled with [^{14}C]amino acids. a, 10 μg of osmotic shock supernatant before incubation with antibodies; b, immunoprecipitate after incubation of 10 μg of the same preparation with 1 mg of purified antibodies specific for the homogeneous protein; c, control: immunoprecipitate as in b, but with antibodies (1 mg) purified from preimmune serum. The arrow indicates the position of the 44 000-dalton protein. It will be noted that the electropherogram shown in a differs from that presented in Figure 1b. The pattern of polypeptides released by osmotic shock varies with the growth conditions used.

for 15 min at 25 °C) were applied to the pure protein (Figure 5e), quantitative conversion to an intermediate degradation product (Figure 5d) was observed. This intermediate can also be seen in the preparations of osmotically lysed spheroplasts after trypsin treatment (Figure 5c).

The results of the tryptic hydrolysis experiment indicated that no trypsin-sensitive bond of this protein was exposed on the surface of spheroplasts. To determine whether other methods could detect parts of the polypeptide on the surface, we reacted spheroplasts with antibodies specific to the pure protein at approximate equivalence. Macroscopically, no aggregation was observed. Under the light microscope, we could not detect any difference between this preparation and a control treated with a preimmune serum. Since preliminary experiments, using pyridoxal [^{32}P]phosphate and subsequent reduction of the resulting Schiff bases, also failed to label this protein,[2] we conclude that it is unlikely to be exposed at the surface of these structures.

[2] A. Gould, G. R. J., and J. P. R., unpublished results.

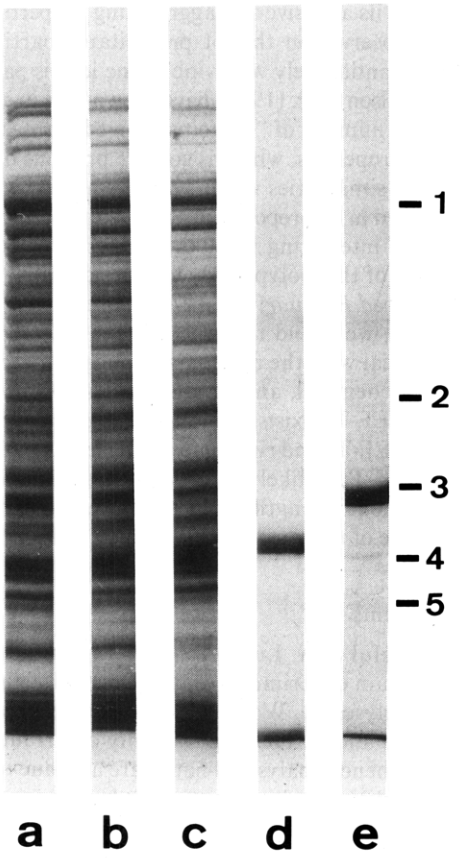

FIGURE 5: Tryptic degradation of the 44 000-dalton protein in various states as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. a, untreated spheroplasts; b–d, spheroplasts, osmotically lysed spheroplasts and purified protein after treatment with 20 μg of trypsin/ml for 15 min at 25 °C; e, untreated purified protein. The molecular weight standards indicated are: 1, phosphorylase a, (95 000); 2, catalase (58 000); 3, ovalbumin (45 000); 4, matrix protein of *E. coli* (36 000); 5, aspartate transcarbamylase, catalytic chain (33 000).

Aggregating Properties of the Protein. The polypeptide, either dissociated from cells by osmotic shock or in the purified state, proved to be unstable. It precipitated quantitatively within days upon storage at 4 °C, and within 2 min at 60 °C. When subjected to Sephadex G-100 chromatography, a large but variable fraction eluted close to the exclusion volume, indicating that aggregation had occurred. Furthermore, the protein showed strong adhesion to surfaces such as dialysis membranes. Incubations of osmotic shock supernatants or of the pure protein with 10 mM vinblastine sulfate in 0.1 M sodium phosphate, pH 7.0, at 24 °C caused quantitative precipitation within 10 min. Incubation under the same conditions with $CaCl_2$ (10–50 mM) precipitated approximately 50%.

Lack of Functional Identity with Other Proteins of Similar Molecular Weight. The retention of this protein in spheroplasts, its heat lability, and its constitutive synthesis made an identity with most periplasmic proteins unlikely a priori. Nevertheless, we tested a possible identity with the major periplasmic proteins of similar molecular weight (Heppel, 1971). No alkaline phosphatase activity could be detected in the purified protein. The absence of detectable phosphate binding activity in the range of 1–20 μM P_i and the amino acid composition of the polypeptide (Table II) preclude an identity with the phosphate-binding protein (Gerdes and Rosenberg, 1974). We also did not detect maltose-binding activity of the purified protein over a range of 2.5–50 μM of this sugar, ruling out an identity with this binding protein (Kellermann and Szmelcman, 1974) as well.

Recently, two envelope-associated proteins have been described in *E. coli*. One of them is the receptor of colicin E_2 whose mass has been reported to be 44 000 daltons (Holland and Darby, 1973). The other one, protein X, was shown to be partitioned between the envelope and the soluble fraction after sonification, and is related to DNA synthesis (Gudas and Pardee, 1975). We have tested the colicin E_2 tolerant strain (ASH 102 cet B), and *E. coli* BE under the conditions in which protein X with an apparent molecular weight of approximately 40 000 is produced. Both colicin E_2 receptor and protein X migrated faster (3% and 4%, respectively) in sodium dodecyl sulfate electropherograms and clearly separated from the protein described here.

Finally, since motile strains produce flagellin in large amounts and its mass remained unclear in *E. coli*, we prepared flagella according to De Pamphilis and Adler (1971). The preparation whose purity we confirmed in the electron microscope yielded a single band in sodium dodecyl sulfate–gel electrophoresis (Figure 1e). The apparent molecular weight of *E. coli* flagellin was determined to be 57 000, similar in mass to phase 2 flagellin of *S. typhimurium* (Ames, G.F.-L., 1974), but different from the protein we have described (Figure 1d).

Discussion

Treatment of plasmolysed *E. coli* cells with ice-cold water releases a number of proteins. When the shock fluid was analyzed by gel electrophoresis in sodium dodecyl sulfate, a band with a mobility corresponding to a mass of 44 000 daltons was clearly prominent. In the electrophoretic pattern of whole cells, a band migrating in the same position accounted for approximately 5% of the total cellular protein. Quantitation of the protein recovered in the shock fluid, and the comparison of the gel patterns of cells before and after osmotic shock, indicated that this treatment released 60–90% of the total cellular complement of this protein. The quantitative precipitation of the constituent(s) of this band, either by elevated temperature or by addition of calcium or vinblastine ions, suggested that it may consist of a single polypeptide component.

When we purified this protein from the osmotic shock supernatant, the final recovery was 5% of the amount initially present. We therefore examined whether the low yield did not reflect the presence of more than one protein species rather than losses of a unique protein. Using physical, chemical, and immunological techniques, we have shown that the purified protein is homogeneous, and that the major band in the gel pattern of osmotic shock fluid or the corresponding band in patterns from whole cells consists of over 90% of the polypeptide we have purified. The low yield is thus likely to be due to losses arising from the aggregating and adhesive properties of the protein.

The low viability of the cells after the osmotic shock treatment we used indicated that this procedure was rather harsh. It was therefore necessary to determine whether this protein was not a cytoplasmic constituent of the cell and its release simply concomitant with cell lysis. Since conversion of cells to spheroplasts did not release the protein, a careful examination of this possibility was particularly important. However, the fraction of the protein released was always approximately four times larger than the fraction of lysed cells, clearly ruling out such an explanation. Since it is known (Neu, et al., 1966) that under the conditions we have used a rearrangement of the membrane occurs during osmotic shock, we also considered the possibility that the 44 000-dalton protein (with a Stokes

radius of 2.8 nm, estimated from its sedimentation coefficient) could escape on the basis of its size, while the cytoplasmic marker protein used (β-galactosidase, Stokes' radius 7 nm) could not. However, since only a few selected proteins were liberated under the conditions we used, release on the basis of size is unlikely unless it were assumed that all of the retained polypeptides with masses below 44 000 daltons are present in cells in complexed form.

From these considerations it appears unlikely that this protein is a cytoplasmic constituent or a typical periplasmic protein. Thus, an envelope association of this protein appeared most probable. Evidence supporting such an assignment came from our observation that most of it is found in the particulate fraction when cells were disrupted by agitation with glass beads. It seems unlikely that such an association is artifactual, since it was not observed with envelope fragments obtained by other cell disruption procedures, such as passage through a French pressure cell. In our opinion, it is more plausible that high shear cell disruption causes the release of a protein originally associated with the envelope than that a protein associates preferentially, but artifactually, with envelope fragments obtained by a milder procedure.

If the assignment to the envelope were correct, one may wonder where in that structure the protein would be found. Clearly, it does not belong to one of the major components of the cell wall (Garten et al., 1975; Rosenbusch, 1974), as these proteins are not released from spheroplasts, but are recovered in those fractions in isopycnic sucrose gradient centrifugation which contain the cell wall constituents (Osborn et al., 1972). Indeed, the observation that the 44 000-dalton protein is released from spheroplasts upon their osmotic lysis (the step preceding isopycnic centrifugation) argues against its being deeply embedded in any part of the envelope. Also, if this protein were an integral envelope constituent, it would be difficult to understand why vesicles which do not contain it (Takacs and Rosenbusch, 1975) should still be semipermeable and capable, at least in part, of performing active transport (Kaback, 1972).

A peripheral location on one of the surfaces of spheroplast envelopes thus appears most likely. We subjected intact spheroplasts to trypsin, to antibodies specific to the purified protein, or to chemical labeling procedures in order to determine whether this protein was exposed at the outer surface of spheroplasts. Since the results of these experiments were negative, we considered a location at the inner surface a valid working hypothesis. Such a location also appears to best explain its unusual pattern of release. Thus, liberation during osmotic shock could be due to its proximity to "pores" arising during the rearrangement of the membrane. A similar hypothesis has recently been suggested to account for the partial eversion of a number of enzymes known to be located at the inner surface of the membrane (Altendorf and Staehelin, 1974). Although not uncontested as an explanation of that phenomenon (Hare et al., 1974), the proposed mechanism could adequately explain our findings. We therefore tentatively conclude that this polypeptide is a peripheral protein at the inner surface of the plasma membrane. Attempts to test this hypothesis by visualization of the protein with ferritin-labeled antibodies are currently in progress in this laboratory.

From the amount present in bacteria, it can be calculated that of the order of 70 000 molecules of this protein exist per genome equivalent. What then is the function of this major component in the cell? Three of its main characteristics suggest that it may play a structural role in the cell: its presumed localization at the inner surface of the plasma membrane, its abundance, and its adhesive and aggregating properties. In this context, the observation that it precipitates partially with calcium and quantitatively with vinblastine ions is particularly remarkable. Wilson et al. (1970) have shown that in eucaryotic cells a sizeable number of "proteins derived from structure" showed these properties, whereas soluble proteins did not. In view of certain similarities which we have noted between the protein described in this report and actin-like proteins in higher cells, it will be interesting to investigate the structural and functional role of this polypeptide in more detail.

Note (*Received February 17, 1976*). After this manuscript was completed, we found that the protein described in this report is identical with the ribosomal elongation factor Tu in its functional, chemical, and immunological properties. We also found that EF-Tu exists at a fourfold higher concentration in the cell than EF-Ts and ribosomes. Our results indicate that the "excess" EF-Tu is likely to be located at the membrane. These observations strengthen our assignment of this protein to the inner face of the plasma membrane, as suggested in the present report.

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The pH Dependence of the Conformation of Angiotensin Peptides by Nuclear Magnetic Resonance: Cis–Trans Isomerism of Proline 7†

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ABSTRACT: The pH dependence of the proton NMR spectrum of [Asn^1,Val^5]angiotensin II in aqueous solution shows the existence of one major and one minor conformation above pH 6.5, the minor conformation representing 12 ± 2% of the total peptide. A similar observation has been made for [Asn^1, Val^5]angiotensin I and Val-Tyr-Val-His-Pro-Phe. This effect is not due to the presence of angiotensin-like impurities in the peptide samples. We have shown two expected impurities, [β-Asp^1,Val^5]angiotensin II and [Asn^1,3-Bzl-Tyr^4,Val^5]-angiotensin II, to be absent, and a third impurity, [Asn^1,Val^5,D-His^6]angiotensin II, to be present at less than or equal to 2.1 mol %, too little to account for the observed amount (12 ± 2%) of minor conformation. The carbon-13 spectrum of the hexapeptide at high pH shows that the major conformation has Pro^7 in the trans form and the minor conformation has Pro^7 in the cis form.

Several models have been proposed for the solution conformation of angiotensin II (Smeby et al., 1962; Weinkam and Jorgensen, 1971; Printz et al., 1972a; Fermandjian et al., 1972). The one common feature of all of these models is a compact, ordered conformation, which was also found by Deslauriers et al. (1975) by ^{13}C NMR. However, Marshall et al. (1973) have shown that the peptide NH-C$^\alpha$H coupling constants observed for angiotensin II are inconsistent with all of the proposed models. Nevertheless, a compact conformation and the two intramolecular hydrogen bonds proposed by Printz et al. (1972b) and confirmed by Bleich et al. (1973) seem to be necessary components of the solution structure of angiotensin II.

A major difficulty in determining the solution conformation of angiotensin at high resolution is the availability of highly purified material. Interpretation of physical data which suggest different populations of conformations relies on a large supply of synthetic peptide of high, quantitatively known purity. There are several angiotensin-like contaminants known to be formed during synthesis of angiotensin by both the solution and solid phase methods. Solution synthesis of [Asp^1,Val^5]- and [Asn^1,Val^5]angiotensin II has led to the acid-catalyzed rearrangement product [β-Asp^1,Val^5]angiotensin II (Riniker, 1964). Solid phase synthesis has yielded as much as 40% [D-His^6]angiotensin (Windridge and Jorgensen, 1971; Khosla et al., 1972). In addition, acid-catalyzed rearrangement of *O*-benzyltyrosine to 3-benzyltyrosine can occur during cleavage from the resin in solid phase synthesis (Erickson and Merrifield, 1973). We intend to show here that our synthetic [Asn^1,Val^5]angiotensin II, [Asn^1,Val^5]angiotensin I, and C-terminal hexapeptide of angiotensin II are free of β-Asp^1 peptides and of 3-benzyltyrosine peptides within the limits of our analytical methods, and that these peptides contain less than 2.1 mol % of D-His^6 peptides. Therefore, the observation of the minor conformation for angiotensin II above pH 6.5, representing 12 ± 2% of the total peptide, cannot be due to the presence of any of the above angiotensin-like impurities, and is the result of trans to cis isomerization of Pro^7.

The aim of our earlier NMR study (Bleich et al., 1973) was the identification of those amide protons that had been previously observed by the tritium technique to exchange rather slowly (Printz et al., 1972b) near the pH of minimum exchange rate (pH 3–4). The assignments of all amide resonances reported in that study were subsequently confirmed by Glickson et al. (1974). The NMR study of angiotensin and analogues at and above physiological pH is beset by two problems: the base-catalyzed exchange rate of all amide protons is so large that the resulting line broadening prevents observation of the respective coupling constants, and limited solubility in this pH range requires long data accumulation time (Glickson et al., 1973). The present study by proton magnetic resonance has, therefore, been performed in deuterium oxide as solvent, and the use of Fourier transform NMR has circumvented the problem of peptide solubility.

Materials and Methods

Synthesis of Angiotensin Peptides. The tetrapeptide fragment of [Val^5]angiotensin II was obtained from material used

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