PURIFICATION AND CHARACTERIZATION OF A HEAT-MODIFIABLE PROTEIN FROM THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

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1. Introduction

The outer membrane of Gram-negative bacteria is unique in that it contains lipopolysaccharide (LPS) and is tightly associated with peptidoglycan [1]. Phospholipid is not required for the structural integrity of this membrane [2], indicating that the fluid mosaic model of Singer and Nicolson [3] may not adequately describe its structure. While the inner, cytoplasmic membrane of *E. coli* is composed of a large number of protein components, the outer membrane contains less than a dozen major proteins [1]. The unique properties and the simple protein composition of the outer membrane make it an interesting system for study.

The properties of only a few outer membrane proteins from *E. coli* have been described. The amino acid sequence of the lipoprotein which attaches the outer membrane to the peptidoglycan has been determined [4]. The protein receptors for colicin E3 [5], and for both phage T5 and colicin M [6] have recently been purified. Phospholipase A_1 activity has been localized to the outer membrane [7].

In this communication, we report the purification and preliminary characterization of a major protein component of the outer membrane of *E. coli*. This protein may be involved in maintaining the shape of the bacterium [8]. Protein B [9] with an apparent molecular weight of 28 500 is quantitatively converted to a form B* with a molecular weight of 33 400 upon heating in sodium dodecyl sulfate (SDS)-containing solutions at temperatures higher than 50°C. Cyanogen bromide cleavage and N-terminal analysis of B and B* showed that they were the same protein. The conversion allowed the isolation of protein B* in a homogeneous form.

2. Experimental

2.1. *Purification of membrane proteins*

The cell wall layer, consisting of outer membrane and peptidoglycan was prepared from *E. coli* NRC 482 by solubilization of the inner membrane with Triton X-114 as previously described [10]. Proteins were sequentially extracted from this fraction by treatment with 0.5% SDS at 37°C for 1 hr, followed by extraction with 2.5% SDS at 100°C for 15 min. The membranes were centrifuged at 100 000 *g* for 30 min after each extraction step to produce two extracts, 1 and 2, and a residue of peptidoglycan. The extracts were dialyzed against distilled water at room temperature for 24 hr and then at 4°C for 5 days, to remove SDS. 10 g (wet weight) of frozen cells produced an average yield of 20 and 35 mg of protein in extracts 1 and 2, respectively.

In a typical experiment, 40 mg of extract 1 was dissolved in 10 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 0.1% β-mercaptoethanol (ME), and incubated at 37°C for 1 hr. The clear solution was applied to a column of Sephadex G100 (2.5 × 40 cm) connected in series to a column of Sepharose 6B of the same dimensions. The proteins were eluted at room temperature with sodium phosphate buffer, pH 7.2, containing 1% SDS. Fractions (10 ml) were collected and the

absorbance measured at 280 nm. Fractions were assayed for protein and lipopolysaccharide (as 2-keto-3-deoxyoctonic acid (KDO)) according to Lowry [11] and Osborn [12], respectively. Column samples (50 and 100 μl) were incubated with a few crystals of urea and ME (final concentration, 0.1%) for 15 min at 37°C and run on 10% SDS-polyacrylamide gels as described before [9].

The fractions containing protein B were pooled, dialyzed against distilled water, and lyophilized. One half of this fraction was saved for characterization. The other half was dissolved in 0.1 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 0.1% ME, heated at 100°C for 15 min and reapplied to the double column gel filtration system. The fractions from the column were examined as before and those containing protein B* were pooled, dialyzed and lyophilized.

2.2. *Protein characterization*

Amino acid analyses were carried out with a Beckman model 120C amino acid analyzer. Cyanogen bromide cleavage was done as described by Schnaitman [13]. N-terminal analyses were performed according to Weiner et al. [14]. SDS-disc gel electrophoresis was done as previously described [9]. Gels were stained with Coomassie Blue and scanned at 600 nm using a Gilford spectrophotometer.

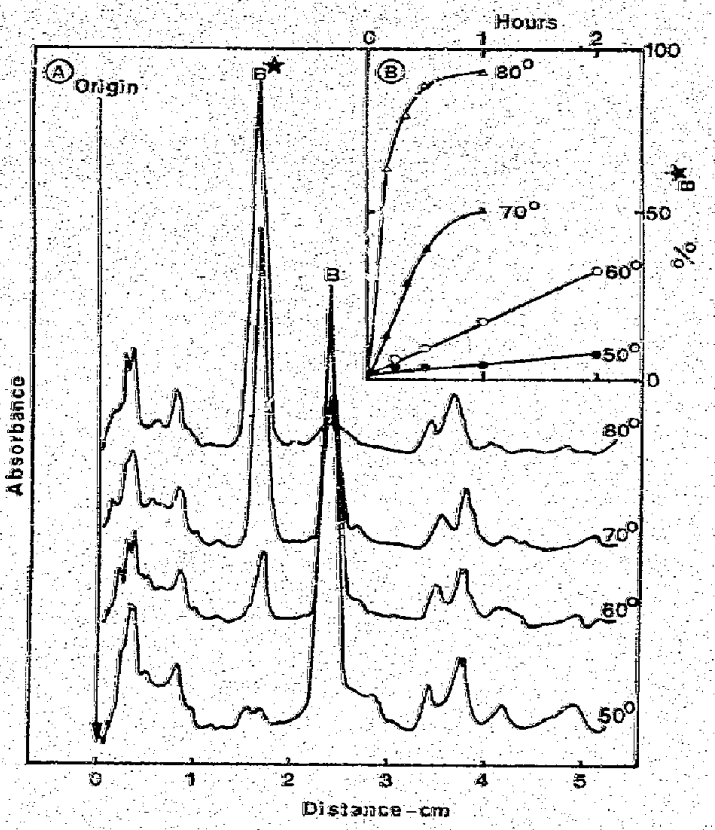

Fig. 1. Effect of heating on migration of protein B in SDS polyacrylamide gels. A: Densitometer scanning traces of gels. Samples were heated at the indicated temperatures for 20 min. B: Time course of conversion of protein B to B* at various temperatures.

3. Results and discussion

Sixty percent of the protein in extract 1 is protein B, with an apparent molecular weight of 28 500, as determined by SDS-gel electrophoresis. Upon heating, protein B was converted to a higher molecular weight form B*, molecular weight 33 400, while all other protein bands were unaffected (fig. 1A). Measurement of peak areas of the gel scans showed a quantitative conversion of protein B to B*, proceeding more rapidly at higher temperatures (fig. 1B). No change was detected after a 2 hr incubation of the sample at 40°C, while heating at 100°C resulted in a rapid conversion. The activation energy for this process is 36.5 kcal per mole. Protein B* did not revert back to form B on cooling or after prolonged storage. This temperature dependent conversion accounts for the three protein bands seen in the region of protein B on SDS-polyacrylamide gels by a number of authors [15–17]. These workers had heated membrane extracts at 70°C for 20 min. As seen in fig. 1B, only 25% of protein B was converted to B* under these conditions. Peak 7 of Inouye and Yee [16], peak C of Schnaitman [15], and band D of Koplow and Goldfine [17] correspond to unconverted protein B, while peak 6 of Inouye and Yee, peak B of Schnaitman, and band C of Koplow and Goldfine correspond to protein B*. Protein band II* of Henning et al. [2] corresponds to protein B*.

Extract 1 was resolved into well-separated protein peaks by gel filtration in the presence of SDS (fig. 2a). The largest peak absorbing at 280 nm contained Triton. Protein B could be separated from contaminating Triton, most of the LPS and the other proteins

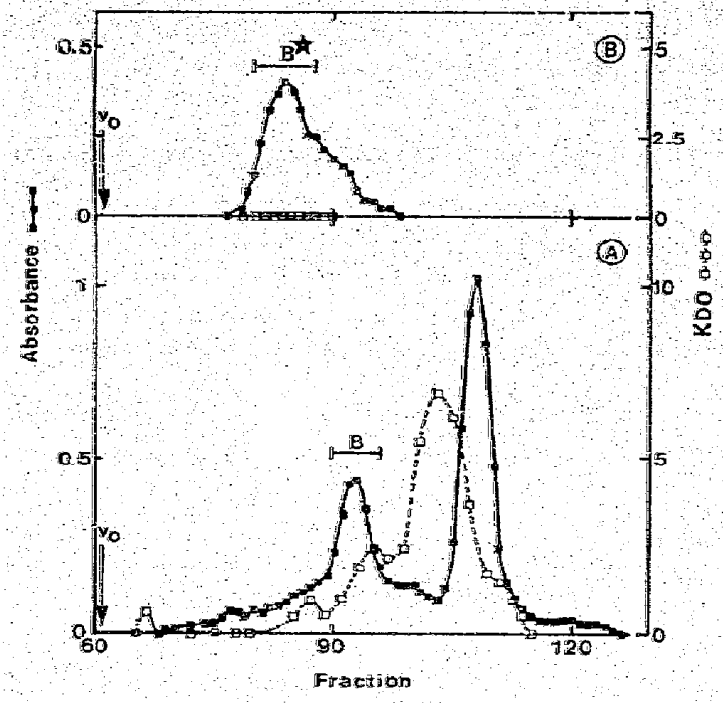

Fig. 2. Separation of proteins of extract 1 by gel filtration in the presence of 1% SDS. A: gel filtration of extract 1 without prior heat-treatment of sample. The fractions under the bar contained only protein B. B: gel filtration of half of protein B from separation A following heating at 100°C for 15 min. The fractions under the bar contained only B*. The concentration of 2-keto-3-deoxyoctonic acid (KDO) is expressed as nmoles/0.2 ml sample. The absorbance of the fractions was measured at 280 nm. Fraction volume, 1.0 ml. V_o, void volume of double column system.

of the outer membrane by this method. Protein B contained in fractions 90 to 96 migrated as a single band of molecular weight 28 500 on SDS-polyacrylamide gels. When protein B was heated at 100°C for 15 min in SDS and rerun in the double column system, it eluted in fractions 80 to 88 (fig. 2B). The protein in these fractions gave a single band of molecular weight 33 400 on SDS-polyacrylamide gels. The heating procedure enabled resolution of pure protein B* from small amounts of contaminating proteins and LPS which co-eluted with protein B.

The amino acid analysis of extracts 1 and 2, and of protein B are given in table 1. More vigorous conditions were required to obtain extract 2 than extract 1, suggesting that the proteins of extract 2

Table 1
Amino acid composition of extracts and protein B

Amino acid	Extract 1		Extract 2		Protein B	
Lys	5.35		5.60		6.17	
His	1.50		0.90		1.84	
Arg	4.90		4.30		4.97	
		11.7		10.8		13.0
Asp	11.80		16.70		12.08	
Glu	9.40		10.70		9.35	
		21.2		27.4		21.4
Val	6.35		6.30		7.64	
Met	1.90		1.70		0.31	
Ile	4.15		3.00		4.99	
Leu	7.30		6.20		7.80	
Tyr	4.30		5.20		4.67	
Phe	6.30		3.50		2.72	
		30.0		25.9		28.1
Thr	5.05		6.30		5.58	
Ser	5.25		6.60		4.44	
Pro	4.75		1.50		5.75	
Gly	10.80		9.30		11.66	
Ala	10.20		12.30		10.50	
½Cys	0.0		0.0		0.0	
		37.1		36.0		37.9

Values are expressed as moles percent. The data is grouped and summed as basic, acidic, hydrophobic and neutral residues. Duplicate analyses were performed after hydrolysis in 6 N HCl at 105°C for 25 hr. Tryptophan was not determined.

were more tightly associated in the membrane. This difference was not reflected in the amino acid compositions which were similar. Protein B had a high content of proline and acidic amino acids. No cysteine was detected and only a single methionine was present. These features agree with the compositions determined for a number of membrane protein preparations [18], namely, the lack of cysteine, the high content of acidic amino acids and the percentage of hydrophobic residues.

Treatment of either protein B or B* with cyanogen bromide produced the same two fragments which had molecular weights of about 18 000 and 15 000 on SDS-polyacrylamide gels (fig. 3). This cleavage confirms the presence of a single methionine residue. The position of migration of either of the fragments on polyacrylamide gels was not modified by heating. The simple cleavage pattern obtained, contrasts with the complex peptide pattern found by

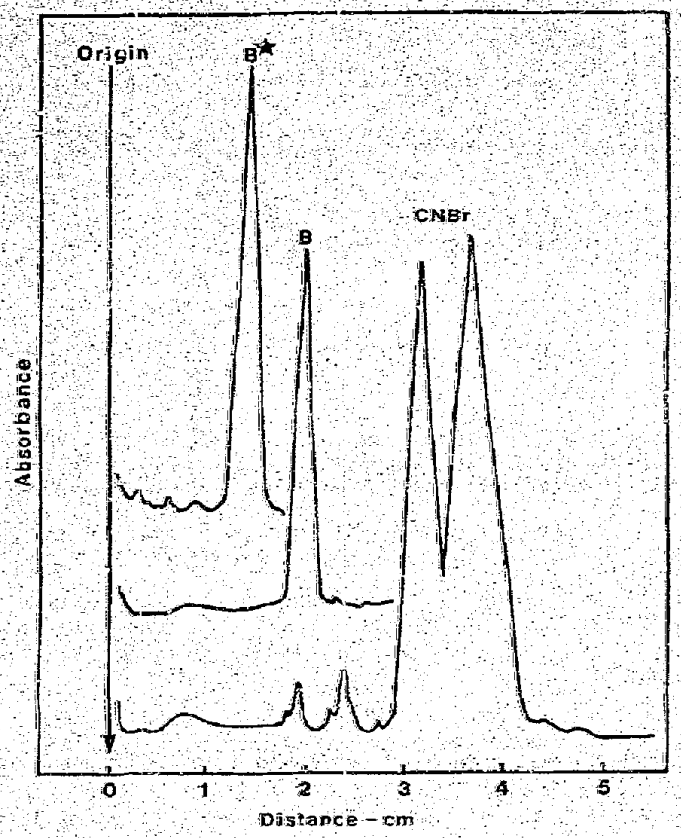

Fig. 3. Cyanogen bromide (CNBr) cleavage of protein B. CNBr cleavage was carried out as described in Experimental. The lower gel scan shows the separation of the cleavage products. Gel scans of proteins B and B* are given for reference.

Schnaitman [13], indicating significant contamination of his preparation with other proteins.

N-terminal analysis of proteins B and B* failed to produce an N-terminal dansyl derivative suggesting that the N-terminal residue was blocked. The blocking group is unlikely to be carbohydrate since there was no periodic acid–Schiff base staining of either proteins B or B* on polyacrylamide gels.

These results suggest that proteins B and B* are the same. It can be calculated from the molecular weights of the cyanogen bromide fragments that the true molecular weight of the protein is 33 400. This value was obtained by SDS-gel electrophoresis after heating in SDS solutions. Presumably the non-heated protein migrates atypically on electrophoresis and gel filtration in the presence of SDS. The reason for this behaviour is under investigation.

Acknowledgements

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