PORE PROTEIN e OF THE OUTER MEMBRANE OF *ESCHERICHIA COLI* K12

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1. Introduction

Several outer membrane proteins of Gram-negative bacteria function in the formation of pores through which nutrients and other solutes pass the outer membrane. General pores facilitate the permeation of a large number of structurally unrelated solutes through the outer membrane whereas other pores are used by one or only a few structurally related solutes. An extensive description of the properties of various pores can be found in [1–12]. The peptidoglycan-associated proteins b and c (nomenclature of [13]) are involved in the functioning of general pores [7,8,10,11] in wild type strains of *Escherichia coli* K12. The purification and partial characterization of these proteins have been described extensively [14–19]. Recently we described that another peptidoglycan-associated protein, protein e, which was detected in pseudo-revertants of mutants lacking proteins b and c (b^-c^- mutants), is also involved in the functioning of pores [10]. The isolation and properties of e^+ mutants and a comparison of the purified protein e with proteins b and c is described here. Moreover we will show that protein e and the proteins Ic [16] and E [20] are identical.

2. Experimental

2.1. *Strains and growth conditions*

Strain PC0479 (*thr, leu, thi, pyrF, thyA, argG, ilvA, his, codA, lacY, tonA, tsx, rpsL, deoC, supE, uvrB*) is the parental strain of most mutants. Mutant strain CE1107 was isolated as a phage Mel-resistant *ompB* (b^-c^-) mutant [10,19]. It does not form colonies on media supplemented with 3% sodium dodecyl sulphate (SDS). Strain CE1108 ($e^+b^-c^-$) was isolated as a SDS-resistant pseudo-revertant of strain CE1107. Strains CE1129 (b^-), CE1062 (c^-) and CE1131 (d^-) are phage-resistant derivatives of PC0479 [10,19]. Mutant strains CE1041 and CE1034, derivatives of strain AB1859 [21], were used for the isolation of proteins b and c, respectively [15,19]. Heptose-deficient mutants were isolated as in [22]. Strains W620 ($Ic^+b^-c^-$) [16] and JF694 [20] were obtained from U. Henning and J. Foulds, respectively. Unless otherwise indicated cells were grown in yeast broth [23] at 37°C under vigorous aeration. In order to study the influences of the growth medium on the level of protein e, glucose minimal medium [23], brain–heart infusion [23] and yeast broth supplemented with 0.3 M NaCl [24] were used. For studying the growth rate of colonies on minimal medium plates, the medium was solidified with 1.4% agar. Phage TC45 was kindly donated by J. Foulds [25].

2.2. *Uptake of amino acids*

Cells grown exponentially in yeast broth were washed twice with glucose minimal medium and resuspended in this medium to $A_{660\,nm} = 0.3$. After 10 min preincubation of 0.9 ml of this suspension at 37°C, uptake was started by the addition of 0.1 ml 5.2 μM L-[5-^{3}H]proline or L-[G-^{3}H]glutamine (both 1 Ci/mmol) (Radiochemical Centre, Amersham). Samples of 0.1 ml were removed at various times, immediately filtered and washed with minimal medium salt solution [23] of 37°C. After drying, the radioactivity of the filter was determined.

2.3. Isolation and characterization of membrane fractions

Cell envelopes were isolated as in [13,19]. Protein—peptidoglycan complexes were isolated after incubation of cell envelopes in 2% SDS at 60°C (unless otherwise indicated), followed by sedimentation and washing of the complexes [14,15,19]. The protein(s) were dissociated from the peptidoglycan by incubation in 2% SDS at 98°C [14], further purified by column chromatography using Biogel P150, precipitated with 90% acetone, washed several times with acetone and water, and lyophilized [19,26]. Proteins III and d were isolated as in [27]. Methods used for the determination of the amino acid composition, determination of N-terminal amino acids, fragmentation of purified proteins with cyanogen bromide and partial degradation of purified proteins in SDS solution with trypsin and chymotrypsin [28] will be described [19].

2.4. Analytical methods

Methods used for the determination of protein, phospholipid and lipopolysaccharide (LPS) have been described [29,30]. The gel system in [14] with 11% acrylamide was used for the analysis of the proteins of the membrane fractions. Convex exponential gradient gels (10–13.5% acrylamide) [19] were used for the analysis of CNBr fragments. Both systems were used for the analysis of proteolytic fragments.

3. Results

3.1. Isolation and partial characterization of $e^+b^-c^-$ mutants

We observed that b^-c^- mutants tend to revert to strains which, in contrast to the original b^-c^- mutants, are resistant to 3% SDS and have regained the ability to take up several nutrients with a wild-type rate. Examination of cell envelope protein patterns by SDS–gel electrophoresis showed that some of the revertants had greatly increased amounts of protein with the electrophoretic mobility of protein a. In order to study this phenomenon we selected spontaneous SDS-resistant mutants from strain CE1107, our most stable b^-c^- mutant. Cell envelopes of all 5 revertants tested had a very heavy protein band in the electrophoretic position of band a. Strain CE1108 was further studied as an example of such a strain

(fig.1). The increased amounts of protein with the electrophoretic mobility of protein a is not due to increased amounts of protein a but is caused by the presence of another protein which, in contrast to protein a [23], is peptidoglycan-associated (fig.1). Moreover, in cells grown at 30°C the new peptidoglycan-associated protein is synthesized in large amounts whereas protein a is hardly synthesized under those conditions [23]. We have designated the new peptidoglycan-associated protein as protein e [10,11].

Protein e differs from proteins b and c in that the amount of protein e is not significantly different after growth in glucose minimal medium, yeast broth, brain–heart infusion or yeast broth supplemented with 0.3 M NaCl. The amounts of proteins b and c, which are extremely dependent on the growth medium [23,24], obviously are under control of another regulation mechanism than that of protein e. Protein e has in common with protein b, but not with protein c [21,22] that its amount is strongly decreased in mutants which lack heptose in their LPS (unpublished results).

A preliminary genetic localization of the mutation which results in the appearance of protein e showed that it is located between min 80 and min 84 on the E. coli K12 linkage map [31]. Then J. Foulds informed us that the gene responsible for the appearance of protein E, designated as nmpA, is located at min 82.7 (J. Foulds, personal communication). Subsequently we have shown cotransduction between ilvA and the mutation leading to the e^+ phenotype (unpublished data). From gel electropherograms of cell envelope proteins from b^+c^+ strains in which the gene responsible for the e^+ phenotype was transduced, it could be concluded that proteins e, b and c can coexist in one strain (gel not shown).

a
b
c
d

A B C D E

Fig.1A–E. SDS–polyacrylamide gel electrophoresis of cell envelopes (A–C) and protein–peptidoglycan complexes (D,E) of the wild-type parental strain PC0479 (A,D), the b^-c^- strain CE1107 (B) and the $e^+b^-c^-$ strain CE1108 (C,E). 11% gel. Only the relevant part of the gel is shown.

3.2. Pore properties of protein e

We have already shown that proteins e and b, but not protein c, are involved in the formation of pores for nucleoside monophosphates and bis-paranitrophenyl phosphate [10], whereas proteins b and c, but not protein e, facilitate the permeation of cephaloridine and ampicillin [11]. Evidence for a role of protein e in the uptake of various small nutrients, which was expected to be more evident at low nutrient concentrations [10], comes from experiments in which the growth of visible colonies was followed after spreading ~1000 exponentially growing cells on minimal medium plates in which one of the required nutrients was present in a very low concentration (sugars, 0.05%; auxotrophic requirements, 5 μg/ml; ions, 5-fold lower than usual, see [23]). Whereas the colonies of the parent strain PC0479 and its $e^+b^-c^-$ derivative CE1108 (and also those of its b^-, c^- and d^- derivatives) grew about equally fast, those of the b^-c^- strain CE1107 grew considerably slower at the decreased concentration of all tested nutrients: the sugars, glucose and fructose; the amino acids, arginine, isoleucine, histidine, threonine and leucine; and the ions, magnesium, sulphate and phosphate. As strain CE1107 grew equally fast as the other strains at the usual concentrations of these nutrients, these results indicate that the presence of at least one of the proteins b, c and e enables the cell to grow fast at low nutrient concentrations. Presumably the growth rate of a strain which lacks all these proteins is decreased by the low rate of uptake of the nutrient. Comparison of the rates of uptake of radioactive amino acids in low concentrations (0.5 μM) confirms this assumption (table 1). Assuming that the permeation through the outer membrane is the rate-limiting step, as was shown for 5'-adenosine monophosphate in b^-c^- cells [10], the present results indicate that protein e, like proteins b and c, is involved in the formation of general pores in the outer membrane. In contrast to results in [32] we did not detect a significant decrease in the rate of uptake of proline and glutamine in protein d-deficient strains of *E. coli* K12, see f.i. strain CE1131 (table 1).

3.3. Comparison of purified protein e with other outer membrane proteins

Whereas proteins b and c dissociate from the peptidoglycan at 70–80°C [15], protein e–peptidoglycan complexes dissociate at 60–70°C. Protein e, purified as described, was free of protein contaminants (fig.2) and phospholipid, whereas it contained < 0.3% LPS (w/w). A comparison of the overall amino acid composition of protein e with those of proteins b and c (table 2) shows many similarities but protein e contains relatively more asparagine and methionine and less arginine, valine and tyrosine than the two other proteins. The amino terminus is H_2N–Ala–Glu as was also observed for proteins b and c [16,18,19].

The pattern of the cyanogen bromide fragments of protein e depends on the solvent used as described for proteins b and c [19,33]. In both formic acid and trifluoroacetic acid the pattern differs strongly from that of the other peptidoglycan-associated proteins b and c (fig.3). In contrast to the fragment patterns of the former 3 proteins, those of proteins d and III are not dependent on the solvent used. Figure 4 shows that the CNBr fragments of all 5 proteins are completely different.

Cleavage of proteins e, b and c in a solution of SDS

Table 1
Rate of uptake of proline and glutamine by mutants lacking various major outer membrane proteins

Strain	Outer membrane protein abnormalities	Rate of uptake (pmol/min/mg dry wt cells)	
		[³H]Proline	[³H]Glutamine
PC0479	None	185	173
CE1107	b^-c^-	63	36
CE1108	$b^-c^-e^+$	209	224
CE1129	b^-	191	204
CE1062	c^-	189	198
CE1131	d^-	178	169

Table 2
Amino acid composition of proteins (mol %)[a]

Amino acid	Protein e (strain CE1108)	Protein b[b] (strain CE1041)	Protein c[b] (strain CE1034)
Lys	7.16	6.46	5.12
His	0.40	0.44	0.81
Arg	4.14	4.88	5.78
Half Cys	<0.10	0.21	0.16
Asp	17.76	12.79	13.28
Thr	5.61	5.97	6.08
Ser	4.66	5.10	5.34
Glu	9.59	7.61	9.17
Pro	1.31	0.95	0.55
Gly	11.50	12.67	11.50
Ala	8.53	9.09	8.02
Val	3.63	5.90	6.16
Met	1.94	0.94	1.14
Ile	3.99	3.36	3.22
Leu	6.61	6.67	7.65
Tyr	6.66	10.35	9.37
Phe	6.51	6.61	6.65

[a] The data are averages of at least two determinations after hydrolysis for 24 h. Tryptophan was not determined. The values for serine and threonine have been corrected for losses of 10% and 5%, respectively, during hydrolysis

[b] Data from [19]

A B C D

Fig.2A–D. SDS– polyacrylamide gel electrophoresis of cell envelopes of strain PC0479 (A) and purified proteins b (B), c (C) and e (isolated from strain CE1108) (D). 11% gel.

A B C D E F
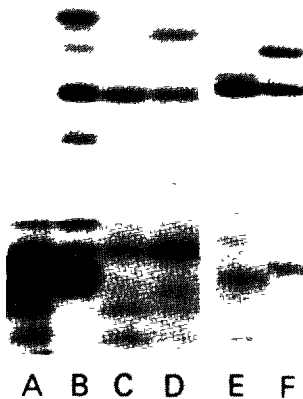
Fig.3A–F. Comparison of cyanogen bromide fragments of protein e (A,B) with those of protein b (C,D) and protein c (E,F). Left, trifluoroacetic acid; right, formic acid. Gradient gel.
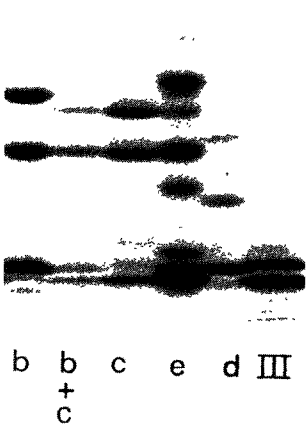

b   b + c   c   e   d   III

Fig.4. Comparison of cyanogen bromide fragments obtained after cleavage in formic acid of various outer membrane proteins. Gradient gel.

Fig.5. Tryptic fragments of proteins e, b and c. Gradient gel. The position of undegraded protein is indicated by an arrow.

with trypsin or chymotrypsin, followed by analysis of the fragments on SDS—polyacrylamide gels leads to time-dependent band patterns. The patterns obtained for the 3 proteins after degradation with trypsin (fig.5) and chymotrypsin (fig.6) differ in

almost all fragments. This result was observed for all stages of degradation. Control incubations showed that none of the bands was due to fragments of the added enzyme.

3.4. *Comparison of protein e with proteins Ic and E*

Recently two other peptidoglycan-associated proteins were described which appear, like protein e, in b^-c^- strains [16,20]. Protein Ic has the same electrophoretic mobility as protein b in the Laemmli gel system [16] whereas protein E has about the same mobility as our e protein in the system described by us and seems to have pore properties for antibiotics [20]. The published CNBr fragment pattern of protein Ic [16] looks very much like that of protein e (fig.3). Comparison of cell envelope protein patterns of strains W620 ($Ic^+b^-c^-$), JF694 ($E^+b^-c^-$) and CE1108 ($e^+b^-c^-$) showed that all 3 proteins had the same electrophoretic mobility as protein b in the Laemmli system and the same electrophoretic mobility as protein a in our system (not shown). Cyanogen bromide fragment patterns of the purified proteins Ic, E and e were indistinguishable (fig.7). The recently
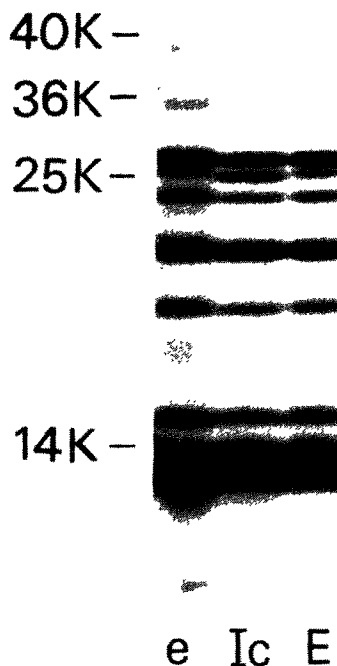

Fig.6. Chymotryptic fragments of proteins e, b and c. Gradient gel. The position of undegraded protein is indicated by an arrow.

Fig.7. Cyanogen bromide fragments of proteins e, Ic and E. Gradient gel. Cleavage was in formic acid. The positions of molecular weight standard proteins are indicated.

isolated protein E-specific bacteriophage TC45 [25] formed plaques on strains possessing proteins E, Ic and e but not on their parent strains. Phage TC45-resistant mutants of strain CE1108 lack protein e. These data clearly show that proteins Ic, E and e are identical.

4. Discussion

The new proteins e, Ic [16] and E [20] all appeared in a $b^- c^-$ background. As no differences could be detected between the 3 proteins with respect to the electrophoretic mobility of the purified proteins and of the CNBr fragments (fig.7) and the phage specificity of the mutants we conclude that the 3 proteins are identical.

Protein e has several properties in common with proteins b and c. All 3 proteins are involved in the formation of intramembranous protein—LPS particles [34] and in the formation of aqueous pores. They are peptidoglycan-associated and serve as the protein part of phage receptors, indicating a transmembrane arrangement which is convenient for a pore protein. Their amino termini are strikingly similar [16]. Differences are observed between protein e and the other 2 proteins with respect to their overall amino acid composition (table 1), to the amounts present in wild-type cells under laboratory conditions and to the mechanism of regulation of the amounts. Moreover, we observed striking differences between the three proteins after fragmentation with CNBr (fig.3) and partial degradation with trypsin (fig.5) and chymotrypsin (fig.6). It became clear recently that proteins b and c are coded for by different structural genes [18,19,30] which probably are derived from one structural gene during the evolution. The differences between the fragments of the proteins can then be explained by assuming differences in posttranslational modification [16,18,19]. Two possibilities can be given to explain the relation between proteins b, c and e.

(i) The structural gene for protein e is also derived from the same original structural gene as those of proteins b and c whereas differences in posttranslational modification could explain the rather drastic differences in the patterns of the fragments. The experiments on the regulation of the amounts of the three proteins and the amino acid composition (table 2) suggest that proteins b and c are more related with each other than with protein e.

(ii) Alternatively, protein e can be completely unrelated to proteins b and c and the strong similarity between their amino termini [16] might be related to one or more properties which these 3 proteins share like their function as general aqueous pores or their interaction with LPS.

Our present results together with those in [10,11] clearly show that protein e, like proteins b and c, is involved in the formation of general aqueous pores. The fact that protein e is not synthesized in wild-type cells under laboratory conditions but can be found as a real major protein in mutants, suggests that its synthesis can be induced by certain growth conditions or by a certain (so far unknown) nutrient, analogous to the synthesis of another pore protein, the receptor of phage lambda, which is induced by maltose [35]. The existence of phages which are specific for protein e [25] indicates that the protein is indeed expressed under certain growth conditions.

The frequent spontaneous appearance of protein e^+ pseudorevertants in $b^- c^-$ cultures indicates that the presence of protein e in a $b^- c^-$ background increases the survival chances of the cell, presumably by a partial restoration of the pore function (table 1) and of the architecture of the outer membrane. Evidence for a restoration of the outer membrane's architecture is the observation that most $e^+ b^- c^-$ cells, like wild-type cells but in contrast with $e^- b^- c^-$ cells, are resistant to 3% SDS. This resistance is probably caused by the presence of more protein and consequently less phospholipid in the outer membrane [29].

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