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PhoE PROTEIN PORES IN THE OUTER MEMBRANE OF *ESCHERICHIA COLI* K-12 NOT ONLY HAVE A PREFERENCE FOR P_i AND P_i-CONTAINING SOLUTES BUT ARE GENERAL ANION-PREFERRING CHANNELS

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Previous studies on the question of whether the PhoE protein pore has a preference for P_i and P_i-containing solutes only or whether it constitutes a general anion-preferring channel, have not given an unequivocal answer either because the presence of the phosphate binding protein was not ascertained or because only arsenate was tested as a non P_i-containing control solute. Permeability properties of PhoE, OmpF and OmpC protein pores for negatively charged solutes were measured in vivo in the presence of phosphate-binding protein. It appeared that the PhoE protein pore is the most efficient channel for the three tested solutes phosphate, succinate and sulphate. Conditions were established to measure the frequency of ethyl methane sulphonate induced mutations as a function of the presence of pore proteins. These results indicate that PhoE protein also forms the most efficient channel for ethyl methane sulphonate. We conclude that the preference of the PhoE protein pore is not restricted to P_i and P_i-containing solutes but also concerns several other negatively charged solutes.

Introduction

Most hydrophilic nutrients and antibiotics pass the outer membrane of Gram-negative bacteria through water-filled channels formed by proteins. Wild type cells of *Escherichia coli* K-12 produce two types of pore protein, OmpF and OmpC proteins. The osmolarity of the growth medium is a major factor in determining the relative levels of expression of these proteins [1]. The synthesis of another pore protein, PhoE protein, is derepressed upon phosphate starvation [2]. The latter condition also results in the synthesis of a number of other proteins, all of which are more or less involved in transport of phosphate and phosphate-containing nutrients across the cell envelope. (For a review, see Ref. 3).

With respect to their structure and function the three pore proteins share many properties. (i) The primary amino acid sequences are very similar [4]. (ii) The proteins can be isolated complexed to peptidoglycan [5–7] and function as part of the receptor for phages [8–10]. (iii) The channels formed by the proteins all display general pore properties [7,11–15] depending on the hydrophobicity, charge and size of the solute diffusing through the pore [16,17]. (iv) The effective diameters of the pores have been calculated in the range between 1.0 and 1.4 nm [17,18], that of OmpC

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protein being smaller than that of OmpF protein and that of PhoE protein having a similar or lower value than that of OmpF protein.

The observation that the expression of the pore proteins is regulated by growth conditions suggests that OmpF, OmpC and PhoE pores have different channel properties. Although permeation through OmpC and OmpF proteins seems to be determined mainly by the effective diameters of the protein channels [17], the PhoE pore has besides general pore properties additional characteristics which speed up the rate of permeation of certain solutes.

A preference for negatively charged solutes has been reported in several studies [16–19], a phenomenon which was explained by the presence of a recognition site for anionic solutes on the PhoE pore [19]. As the experiments were carried out either with in vitro systems [17,18] or with intact cells constitutively producing PhoE protein due to a *phoS* or a *phoT* mutation [16,19], the phosphate-binding protein, the product of the *phoS* gene, was not present in these systems. Only in the case of a *phoT* mutation its absence cannot be claimed with certainty. However, as *phoT* maps very close to *phoS* [20], it is conceivable that many *phoT* mutations cause the absence of the phosphate binding protein. As the phosphate binding protein, analogous to the maltose binding protein in case of the LamB protein pore [21,22] may contribute to the solute-specificity of the PhoE pore, the possibility exists that results obtained in the absence of the phosphate binding protein underestimate the specificity of the PhoE protein pore.

In our previous study [23] on the pore properties of PhoE protein we used a mutant containing a *phoR* regulatory mutation, a mutation which induces both the PhoE protein and the PhoS protein. The results showed that PhoE protein forms a more efficient channel for P_i and P_i-containing solutes than OmpF and OmpC protein and that arsenate, which was used as a negatively charged, non P_i-containing solute, permeates about equally well through all three pores [23]. Thus, whereas our results indicate a preference for phosphate moieties only, the former set of experiments indicate that PhoE pores have a more general preference, namely for negatively charged solutes.

As these seemingly contradictory conclusions about the pore properties of PhoE protein could either be due to the role of the phosphate-binding protein or to the possibility that arsenate was an unfortunately chosen control, we extended our experiments with the *phoR* mutant by the measuring of pore-determined rates of uptake of succinate and sulphate as examples of other non P_i-containing, negatively charged solutes. In addition, a method is introduced to estimate the uptake of the mutagenic agent ethyl methane sulphonate by intact cells.

For all three solutes, succinate, sulphate and ethyl methane sulphonate it appeared that the PhoE pore is the most efficient of the three channels. We conclude that arsenate behaved as an anomalous solute and that PhoE pores are relatively efficient channels not only for P_i and P_i-containing solutes but also for several non P_i-containing compounds.

Materials and Methods

Bacterial strains and growth conditions. All strains used in this study are isogenic derivatives of *Escherichia coli* K-12 which either produce only one type of pore protein or no pore protein at all. Their construction and relevant characteristics have been described in a previous paper [23]. Strains CE1230, CE1233 and CE1234 produce PhoE protein, OmpF protein and OmpC protein as the only pore protein, respectively. Strain CE1231 was used as the pore-deficient control strain. Unless otherwise indicated cells were grown in L-broth at 37°C under aeration.

Rates of permeation of phosphate, succinate and sulphate through outer membrane pores. The rate of uptake of radiolabelled nutrients by intact cells was measured under conditions in which the rate of permeation through the outer membrane pores is the rate-limiting factor. Details were as described previously [23] except that the growth medium was adapted such that it allowed the measurement of uptake of phosphate, succinate and sulphate. Thus, cells were grown to the exponential phase in a Hepes-based succinate-minimal salts medium [24], supplemented with the appropriate growth requirements. Succinate (85 mM) was chosen as the carbon source in order to induce dicarboxylate transport [25]. After growth

the cells were harvested, washed twice with minimal salts solution lacking phosphate, sulphate and succinate, and adjusted to a cell density of 10^7, $5 \cdot 10^7$ and $5 \cdot 10^8$ cells/ml in cases the rate of uptake of phosphate, succinate and sulphate, respectively, were to be measured. Part of the cell suspensions was used for the isolation of cell envelopes and subsequent analysis of the protein pattern by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. To remove the last traces of phosphate, sulphate and succinate from the uptake medium the cells were starved by incubation for about one hour at 37°C. Just before starting the uptake experiment by the addition of radiolabelled solute, glucose was added in a final concentration of 0.2%. The specific activities of the solutes were in the range between 0.1 and 1.0 Ci/mmol.

Uptake of ethyl methane sulphonate. Cells were grown to the exponential phase in L-broth, harvested and washed twice with the described minimal salts solution supplemented with glucose (0.2%). The suspension was adjusted to a cell density of 10^9 cells/ml and uptake was started by the additon of 200 μl ethyl methane sulphonate (concentrations were in the range of 0.05 M up to 1.0 M) to 1.8 ml of prewarmed cell suspension. At time intervals of about 2.5 min samples of 100 μl were filtered (Schleicher and Schüll, type BA85, pore diameter 0.45 μm) and the cells on the filter were washed twice with 2.0 ml of a solution of thiosulphate (5%). They were subsequently resuspended in L-broth, grown overnight at 37°C and appropriate dilutions were plated on L-broth agar and L-broth agar containing 100 μg/ml rifampicin (Sigma Chemical Co). The increase in the number of rifampicin-resistant mutants/10^9 cells was used as an indicator for the amount of ethyl methane sulphonate taken up by intact cells.

Isolation and characterization of cell envelopes. Cell envelopes were prepared by differential centrifugation after desintegration of the cells by ultrasonic treatment [26]. Protein patterns were analyzed by SDS-polyacrylamide gel electrophoresis [26]. The relative amounts of pore protein were determined by scanning of the gel [23].

Materials. [^{32}P]Phosphate, [2,3-^{14}C]succinate and [^{35}S]sulphate were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Results

Rates of permeation of succinate, sulphate and phosphate through OmpC, OmpF and PhoE protein pores

In a previous paper we have shown that strains CE1230, CE1233 and CE1234 grown in L-broth produce comparable amounts of PhoE, OmpF and OmpC protein, respectively [23]. However, after growth in minimal salts medium containing succinate as the carbon source, the medium used for the experiments described in the present paper, the level of expression of OmpF protein is decreased and that of OmpC protein is increased in comparison with that of PhoE protein. As the amount of OmpA protein per cell for strains CE1230, CE1233 and CE1234 was found to be constant [23], the relative amounts of pore protein per cell were estimated relative to OmpA protein by gel scanning. Values of 0.61 (PhoE), 0.30 (OmpF) and 1.16 μg (OmpC) pore protein/μg OmpA protein were calculated.

At sufficiently low solute concentrations, the rate of permeation through outer membrane pores is the rate-limiting step in the translocation of nutrients across the cell envelope [15,27,28]. In order to ascertain that pore-limited uptake of phosphate, succinate and sulphate was measured, solute concentrations were chosen below the K_m values of overall transport for these substrates [23,25,29]. Consistent with this idea were the following observations. (i) Experiments with various strains among which one containing increased amounts of PhoE protein due to the presence of a multicopy plasmid carrying the *phoE* gene have shown that at low solute concentrations (1.0–2.0 μM) the rates of uptake are directly proportional to the amounts of PhoE protein produced per cell (not shown). (ii) By decreasing the solute concentrations 2-fold, the rates of uptake decreased directly proportionally (Table I).

The permeability properties of the various pores for phosphate, succinate and sulphate are summarized in Table I. Relative pore activities were calculated from data corrected for differences in the levels of expression between PhoE, OmpF and OmpC protein. The major conclusions are the following. (i) As the rates of uptake measured for the pore-deficient strain are much lower than those

TABLE I

RATE OF PERMEATION OF VARIOUS NEGATIVELY CHARGED SOLUTES ACROSS THE OUTER MEMBRANE OF ISOGENIC STRAINS PRODUCING ONLY ONE GENERAL DIFFUSION PORE PROTEIN [a]

Experiments were carried out with strains CE1230, CE1233, CE1234 and CE1231 containing PhoE, OmpF and OmpC protein and no pore protein at all, respectively [23].

Solute	Concn. [a] (μM)	Rate (nmol substrate per min per mg dry wt.) [b,c] Pore used for uptake			
		PhoE protein	OmpF protein	OmpC protein	'None'
Phosphate	1.0	12.8 (100%)	1.4 (13%)	6.6 (23%)	0.6
	2.0	28.4 (100%)	3.0 (14%)	12.4 (20%)	1.0
Succinate	1.0	3.5 (100%)	0.9 (51%)	1.0 (14%)	< 0.01
	2.0	7.1 (100%)	1.7 (56%)	2.2 (15%)	< 0.01
Sulphate	1.0	0.18 (100%)	0.06 (25%)	0.05 (17%)	0.02
	2.0	0.32 (100%)	0.13 (32%)	0.08 (14%)	0.04

[a] Concentrations used are below the apparent K_m values for the substrates under study.

[b] Data represent initial rates and are the averages of three experiments in the case of phosphate and succinate (maximal standard deviation: 0.1). Uptake data calculated for sulphate are the average of five experiments (maximal standard deviation: 0.01).

[c] Relative rates were calculated as follows. Uptake data found for the pore-deficient strain were subtracted from the data found for the pore-producing strains. Subsequently the resulting pore activity for PhoE protein was set at 100% and the corresponding pore activities for OmpF protein and OmpC protein were expressed as percentages relative to the pore activity of PhoE protein. Finally, the percentages were corrected for differences in the relative amounts of pore protein produced.

measured for the pore-containing strains, by far most solute molecules are taken up through pores. It should be noted that the pore-deficient cells allow significant permeation of phosphate and sulphate but not of succinate. Apparently, a small fraction of the phosphate and sulphate molecules diffuses through other channels in the outer membrane than the three studied pores. (ii) In agreement with our previous observation (23), inorganic phosphate prefers the PhoE channel despite the fact that its diameter is not larger or even smaller than that of the other two channels [17,18]. (iii) A key observation was that, in addition to phosphate, also succinate and sulphate diffuse significantly faster through PhoE channels than through OmpF and OmpC channels. Thus, in this respect these two non-Pi-containing negatively charged solutes differ from arsenate [23]. (iv) Consistent with its larger diameter [17,18] is the observation that the OmpF protein channel forms a more efficient pore for succinate and sulphate than the OmpC protein channel. (v) Despite the calculated smaller diameter for the OmpC pore we confirmed our previous observation [23] that phosphate permeates faster through OmpC pores than through OmpF pores. Obviously, other factors than only channel diameter are involved in determining differences in the characteristics of OmpF and OmpC pores.

Permeation of ethyl methane sulphonate through pores

The accumulation of ethyl methane sulphonate by intact cells was estimated by measuring the increase in the frequency of rifampicin-resistant mutants due to the presence of ethyl methane sulphonate at concentrations between 10 and 100 mM. Fig. 1 shows that the number of rifampicin-resistant mutants per 10^9 PhoE producing cells increased by a factor 100 within 10 min after the addition of 100 mM ethyl methane sulphonate. The same increase in the frequency of rifampicin-resistant mutants was found for cells containing OmpF and OmpC protein (not shown). As the frequency increased only 5-fold for the pore-deficient strain, these results indicate that the molecules pass the outer membrane mainly through PhoE, OmpF and OmpC protein pores. Differences in the number of ethyl methane sulphonate-induced mutants for strains CE1230, CE1233

TABLE II

FREQUENCY OF RIFAMPICIN-RESISTANT MUTANTS INDUCED BY ETHYL METHANE SULPHONATE IN CELLS CONTAINING ONLY ONE PORE PROTEIN OR NO PORE PROTEIN AT ALL

Strain	Pore protein	Number of rifampicin-resistant mutants per 10^9 cells [a]		
		Zero time [b] (A)	After incubation [c] (B)	EMS induced ($B-A$)
CE1230	PhoE protein	6 ± 2	108 ± 5	102 ± 5
CE1233	OmpF protein	8 ± 2	46 ± 2	38 ± 3
CE1234	OmpC protein	11 ± 2	54 ± 3	43 ± 4
CE1231	None	5 ± 1	9 ± 2	4 ± 2

[a] The frequency of rifampicin-resistant mutants/10^9 cells was determined as described in Materials and Methods.

[b] Represents spontaneous rifampicin-resistant mutants.

[c] Total number of spontaneous ethyl methane sulphonate (EMS) induced, rifampicin-resistant mutants after 10 min of incubation with ethyl methane sulphonate at a final concentration of 10 mM. The data were calculated from plots of log (number of rifampicin-resistant mutants per 10^9 cells) versus time.

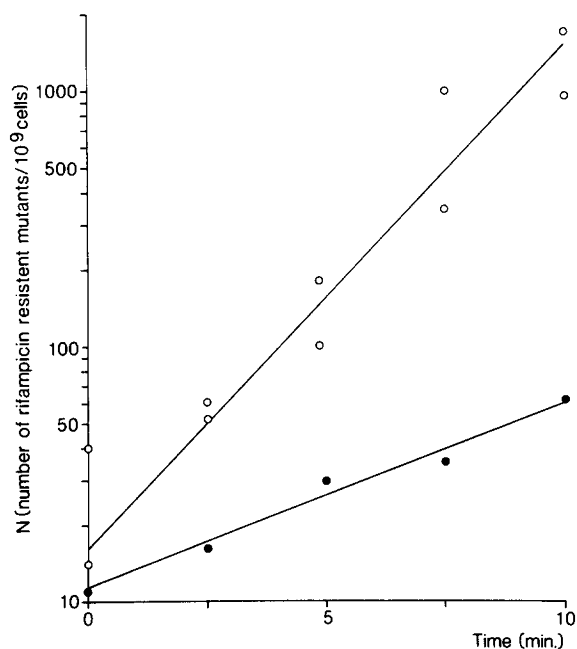

Fig. 1. Accumulation of ethyl methane sulphonate as measured by the increase in the number of rifampicin-resistant mutants per 10^9 cells. Cell suspensions of strain CE1230 containing PhoE protein (○) and of strain CE1231 lacking pore protein (●) were incubated with ethyl methane sulphonate at a final concentration of 100 mM for the indicated periods of time. The zero-values represent controls without ethyl methane sulphonate.

and CE1234 became apparent at concentrations below 100 mM. Obviously, at concentrations equal to or above 100 mM permeation of ethyl methane sulphonate through the pores is not the rate-limiting factor for the generation of mutations. No significant increase in the frequency of ethyl methane sulphonate-induced mutants was observed for strains CE1230, CE1233 and CE1234 when ethyl methane sulphonate concentrations of 5 mM were used, not even after incubation for one hour.

Table II shows the data obtained with cell suspensions incubated with ethyl methane sulphonate at a final concentration of 10 mM, the concentration for which the largest differences were found. Although the increase in the frequency of mutations will not be directly proportional with the pore activities, it is clear that the presence of PhoE protein pores results in the accumulation of a larger amount of ethyl methane sulphonate in the cell than the presence of equal amounts of OmpC or OmpF protein pores. We conclude that similar to the negatively charged solutes phosphate, succinate and sulphate, also ethyl methane sulphonate permeates more efficiently into cells producing PhoE protein pores.

Discussion

The main purpose of the experiments described in this paper was to answer the question of whether PhoE pore protein channels in wild type cells have a preference for P_i and P_i-containing solutes only or whether there is a preference for negatively charged solutes in general. As mentioned in the Introduction section all in vitro and most in vivo results are hampered by the fact or the probability that the phosphate binding protein, which might increase the specificity of the PhoE pore, was lacking. Only in the experiments described in one of our previous papers [23] the presence of the phosphate-binding protein was ascertained. These experiments were also the only ones in which another negatively charged molecule than P_i or P_i-containing ones, namely arsenate, did not permeate faster through PhoE pores than through OmpF and OmpC pores [23]. In the present paper we have extended the number of non P_i-containing solutes from which the rates of permeation through pores can be measured or estimated. The experiments were carried out in cells containing high levels of phosphate-binding protein. It was observed that, like phosphate the solutes succinate, sulphate (Table I) and ethyl methane sulphonate (Table II) were taken up faster by cells containing PhoE protein than by those containing OmpC or OmpF protein. Therefore the results show that arsenate must be considered as an exception and indicate that the PhoE protein pore, in addition to preferring P_i and P_i-containing solutes, it also is a more efficient pore for several other negatively charged solutes.

This notion agrees with another observation from our laboratory, namely that the recognition site at the PhoE protein pore is not only sensitive to P_i and P_i-containing solutes [19] but also to other negatively charged solutes, although to a lower extent.

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