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Membrane-derived oligosaccharides (MDO's) promote closing of an *E. coli* porin channel

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The outer membrane of *Escherichia coli* is a diffusion barrier for macromolecules, but allows the passage of small hydrophilic solutes through non-specific channels, the porins. Some electrophysiological studies find reconstituted porins in a mostly open state, while those done with the patch-clamp technique performed on live cells suggest that the vast majority of the native channels are closed. We present here current measurements through porins from reconstituted outer membrane, which demonstrate that bacterial metabolites, the MDO's, which bathe the periplasmic side of the outer membrane, induce the channels to close. These findings illustrate that the degree of openess of porins can be regulated by compounds naturally found in bacteria.

Porin; Membrane-derived oligosaccharide; Ion channel; Outer membrane; Patch-clamp; *Escherichia coli*

1. INTRODUCTION

Membrane-derived oligosaccharides (MDO's) from *Escherichia coli* are heterogeneous polymers of glucose which differ in the size of the sugar backbone, as well as in the amount of substitution with phosphoethanolamine, phosphoglycerol and succinyl ester residues, the latter two substituents conferring to the molecule a net negative charge [1]. Biosynthesis of MDO is initiated at the cytoplasmic side of the inner membrane and completed at its external side, resulting in the release of soluble polymers into the periplasmic space between the outer and inner membranes. The regulation of MDO synthesis by the medium osmolarity is documented [1–3], although its mechanism is still unknown. High periplasmic concentrations of MDO's are found in cells growing in low osmolarity medium, and may play a significant role in osmotic adaptation by reducing the turgor pressure exerted against the inner membrane. Low periplasmic concentrations of MDO reflect high osmolarity medium. Because of their large size, the MDO molecules are essentially trapped in the periplasm, unable to diffuse outside through the porins. As fixed negative charges, they are the major contributors to the Donnan potential established across the outer membrane [2,4]. Since their concentration varies with osmolarity, a range of potentials is generated across the outer membrane depending on the external conditions [3].

We have performed electrophysiological studies of a wild-type strain of *E. coli* and have described a channel, which is absent in mutants lacking the major porins OmpC and OmpF. This channel is similar to OmpC in cation-selectivity, high probability of being open, cooperative opening and closing, voltage-dependent closing frequency [5,6]. This channel is observed in outer membrane fractions reconstituted into liposomes and, less frequently, in spheroplasts and giant cells [7]. For these reasons, we believe that it represents a protein of the porin family, possibly the heterotrimers of OmpC and OmpF previously described [8]. The work presented here demonstrates that a preparation of MDO from *E. coli* acts as a blocker and a regulator of this porin channel activity, at concentrations which can be found in the periplasmic space under conditions of low external osmolarity. This finding points to a new mechanism of modulation of outer membrane permeability, which might be of importance for cell survival in a stressful environment.

2. MATERIALS AND METHODS

Outer membrane of *E. coli* wild-type AW740 [9] was purified by sucrose gradient centrifugation and reconstituted into liposomes as described [10]: bacterial membrane fractions were mixed with exogenous lipids (azolectin (Sigma)) at a protein-to-lipid ratio of at most 1:600 and giant artificial liposomes were made by submitting these combined fractions to a dehydration–rehydration cycle overnight. Previous studies [5,7,10] have shown that bacterial channels, including porins, reconstituted with this dehydration–rehydration method have conserved their native properties. Measurement of porin activity was

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performed with the electrophysiological technique of patch-clamp [11]. This technique allows the measurement of the current flowing through individual channel-proteins. Each gating event (opening followed by closing, or vice versa) is represented on a recorded trace as a squared-shape deflection in the measured current. Our recordings were made from inside-out patches formed by pulling away the pipette from the liposomes and excising a small area of membrane such that the periplasmic side of the outer membrane is facing the bath solution. Because the native asymmetric voltage dependence of the channels is preserved in the liposomes [5,7], we know the orientation of the channels and can assign pipette side as outside. The membrane potentials are expressed here as cellular potentials, i.e. their signs are given with respect to the side of the membrane normally facing the outside of the cell (which in this case is facing the pipette interior). Hence, positive voltages are referred to as depolarizing, and negative voltages are hyperpolarizing.

To study the modulation of the channels we have perfused the periplasmic side of the patches with a solution of unfractionated MDO's, which we call the crude MDO fraction. This fraction was prepared from *E. coli* by extraction of the cells with 50% ethanol and fractionation of the concentrated water-soluble extract on Biogel P6 [12]. The crude MDO fraction is collected from this chromatography as a peak of intermediate molecular weight. The concentrations used were calculated on the basis of an average molecular weight of 2,300.

3. RESULTS

The typical activity pattern of the porin channel in control condition is shown in the top trace of Fig. 1. Many channels are open at the same time. Frequent brief closures of 1 channel, which at this time resolution appear as a thick baseline, are interspersed by cooperative closures of many channels. Because we cannot ascertain whether these closing events are produced by individual channels or substates of one (or a few) large channel(s), we prefer to refer to each event as the closure of N units of conductance [5]. The number N of units of conductance which close and re-open cooperatively to give rise to a given observed event is calculated as the ratio of the current amplitude of the observed event to the current amplitude of the smallest event taken as unity (single-channel current) [5]. The subsequent traces of Fig. 1 show that the addition of increasing concentrations of the crude MDO fraction to the periplasmic side of the channel increases the frequency of closing of all units. The traces, displayed at a low time resolution, most dramatically emphasize the increased frequency of closures of multiple units of conductance. When the concentration of MDO is high enough, for example at 5 and 13 mM, prolonged closures of 3 or 4 units are observed, which can have a major impact on reducing the total amount of charges carried through the outer membrane. The overall effect of the MDO on the channel gating properties is rapid in its onset and reversibility, and has been observed in 10 separate patches.

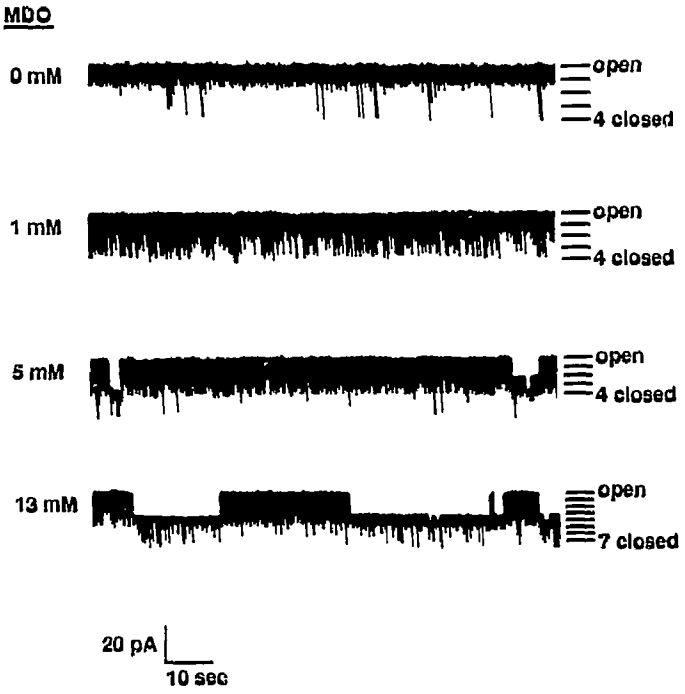

Fig. 1. Current traces at low time resolution of porin activity in the absence of MDO (top trace) and at the indicated MDO concentrations applied to the bath. The membrane potential was +80 mV. Both bath and pipette solutions contain 150 mM KCl, 0.1 mM EDTA, 0.01 mM $CaCl_2$ and 5 mM HEPES, pH 7.2. The level where all the channels are open is labelled 'open'; the other levels, indicated by tick marks, correspond to the closure of 1 or more units of conductance simultaneously.

The traces of Fig. 2 are displayed on an expanded time scale to illustrate the detail of the increased closing activity. At +80 mV, the number of closures of 1 unit of conductance is dramatically increased but their average duration decreases from 1.2 ms in the control to 0.25 ms at 20 mM MDO. At high concentrations of MDO, most 1-unit events are spikes lasting for less than 0.4 ms, but larger events, especially those corresponding to the cooperative closure of 4 units, are lengthened, as well as more frequent. At +80 mV, the proportion of 4-unit closures lasting for more than 1 s goes from 33% at 0 mM MDO to 95% at 9 mM MDO. This effect is more pronounced at depolarizing voltages than hyperpolarizing ones.

Similarly, the amount of current passing through a single channel at a defined voltage decreases with in-

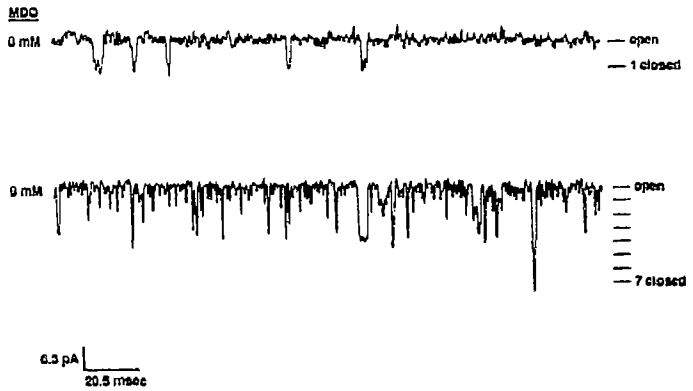

Fig. 2. Current traces at high time resolution of porin activity from the same patch as in Fig. 1 at 0 and 9 mM MDO. The membrane potential was again +80 mV. The bath and pipette solutions are the same as in Fig. 1.

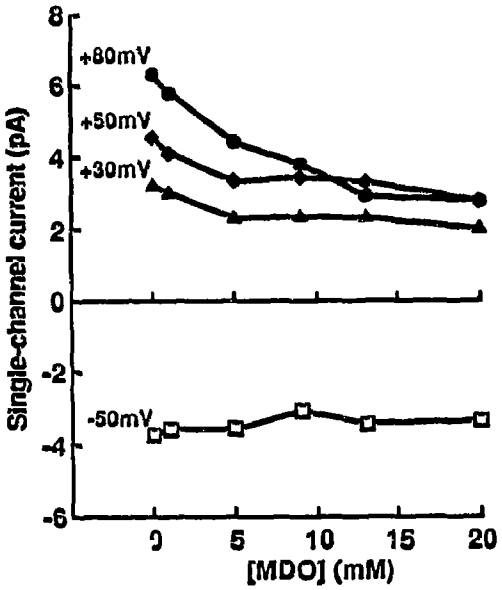

Fig. 3. MDO concentration-dependence of single channel current at the indicated voltages. By convention, the current (positive charges) flowing out of the cell has a positive sign, and the current flowing into the cell has a negative sign.

creasing MDO concentrations (Fig. 3). The single-channel current was measured from events lasting longer than 0.3 ms, which is the resolution time of our equipment. Any event lasting less than 0.3 ms is considered as unresolved and not taken into account. The steepness of the concentration dependence is increased at more depolarized voltages, but the effect is negligible at hyperpolarizing ones.

The modulation of porin gating is represented by plotting the frequency of closures vs. each concentration of MDO tested. Fig. 4 depicts the results of 1 out of 5 experiments with similar concentration dependencies. Each bar of the histograms represents the frequency of closures at concentrations of MDO increasing from left to right. It is clear that closing is promoted in a voltage- and concentration-dependent fashion. The effect is negligible at −50 mV, or at any other hyperpolarizing voltages (data not shown). Note that very large events, simultaneous closures of more than 4 units, are observed only at high MDO concentrations and voltages of at least +50 mV.

In order to find out more about the nature of the regulation of the gating activity, we performed the following experiments (data not shown). (i) We tested a 'cocktail' of negatively-charged low-molecular weight compounds which mimic the ionic content of the crude MDO fraction at 10 mM. A mixture of glycero-

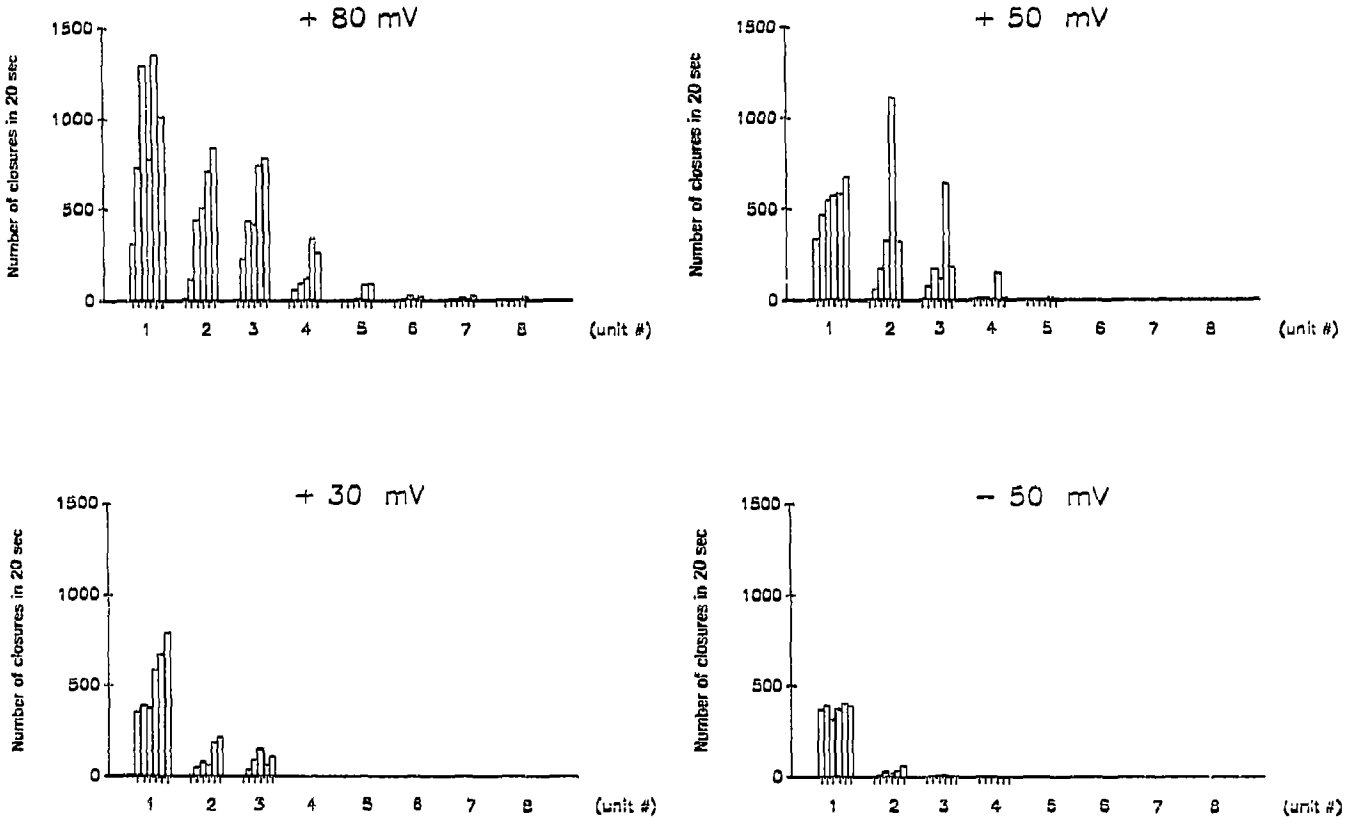

Fig. 4. Concentration-dependence of the closure-promoting activity of MDO on the channel. Each bar of the histograms represent the number of closures per 20 s. The MDO concentrations, indicated by six tick marks below the X axis, are, from left to right: 0, 1, 5, 9, 13 and 20 mM. The 'unit #' refers to the number of units which closed cooperatively. The absence of a bar means that no event of that size was detected.

phosphate and sodium succinate was without effect, while 1 mM of the crude MDO fraction had a reversible activation of the closing activity in the same patch. (ii) An MDO fraction enriched in MDO molecules carrying mostly anionic substituents (fraction C) [13] as well as other anionic polymers such as heparin (MW 3,000) and dextran sulfate (MW 5,000 and 8,000) were also ineffective. (iii) However, 5 mM phosphoethanolamine increased the frequency of closing of single and multiple units of conductance, in a fashion qualitatively similar to that of the crude MDO fraction (C. Cui, personal communication). This suggests that it may be the MDO molecules carrying phosphoethanolamine residues, perhaps as a zwitterionic form at the low pH presumed to exist in the periplasm, which are the active principle of the crude MDO fraction.

4. DISCUSSION

This study provides evidence that the porin channels of the *E. coli* outer membrane can be down-modulated by cellular metabolites. This inhibition is seen at concentrations of MDO which are physiologically relevant. At low medium osmolarity, concentrations of up to 20 mM MDO are not uncommon [3]. Taken all together, the results suggest that the effect of MDO's on the porin channel might be complex. It seems to involve a partial and voltage-dependent block of the ionic flow which results in a decrease in the mean closed time of 1-unit events (fast flickering of the current in the open-channel state) and a MDO-concentration dependent decrease in single-channel conductance. Similar effects of blocking agents on various types of eukaryotic channels have been reported [14]. That MDO reduces the conductance suggests that the interaction is fast and intimate, either by physical occlusion or preventing access, and would involve binding of MDO to some site(s) at or within the mouth of the channel. The finding that the cooperativity of gating is enhanced in the presence of MDO's points to possible allosteric effects as well, following binding of the MDO molecules to the channel or the surrounding lipids. These observations point to a specific effect of MDO on the porin channels, although they do not completely rule out the possibility that other periplasmic components which might be present in the crude MDO fraction contribute to the regulation of the channel as well. Further studies of the effect of various MDO and non-MDO fractions issued from the crude MDO fraction will be useful at clarifying this point.

The effect is seen at transmembrane voltages which are opposite to the Donnan potential established across the outer membrane in vivo as a result of the presence of MDO's (negative inside). This depolarization (positive inside) of the outer membrane could arise – perhaps transiently – when cells, which have been adapted to a low osmolarity medium and which have accumulated MDO's in their periplasm, are suddenly facing a high-salt medium. The resulting rush of positive charges into the periplasm could be quenched by the voltage- and MDO-dependent closure of the porins. This decrease in outer membrane permeability would serve as a rapid defense mechanism while the cell initiates the appropriate and slower regulation mechanisms for adaptation to high osmolarity.

Purified porins reconstituted in planar lipid bilayers are either permanently open [15] or mostly open with voltage-dependent closing at membrane potentials which might be non-physiological [16–20]. Along with results from permeability measurements of cells to antibiotics and other solutes [21–23], those electrophysiological observations led to the view that the outer membrane is a static sieve full of permanently open pores. We have challenged this view by the simple observation that we can form high-resistance seals with pipettes on the outer membrane of live cells and, therefore, concluded that most of the porins are closed [7]. We do not believe that the inhibition of porins by MDO is responsible for the low conductivity of the outer membrane observed in spheroplasts [7], because (i) the cells are grown in rich medium and most likely have very low concentrations of MDO and (ii) the porins remain closed when a membrane patch is excised from the spheroplast, giving a chance for potential soluble inhibitory factors (such as MDO) to be washed away. However, the work presented here confirms our proposal that the outer membrane is a dynamic structure whose permeability can be controlled. We have identified one type of channel regulated by one type of compound (MDO); it is not unlikely that other channels of the outer membrane might also be modulated by MDO's or other periplasmic or external factors.

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REFERENCES

[1] Kennedy, E.P. (1987) in: *Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology (F.C. Neidhardt Ed.) American Society for Microbiology.
[2] Kennedy, E.P. (1982) Proc. Natl. Acad. Sci. USA 79, 1092–1095.
[3] Sen, K., Hellman, J. and Nikaido, H. (1988) J. Biol. Chem. 263, 1182–1187.
[4] Stock, J.B., Rauch, B. and Roseman, S. (1977) J. Biol. Chem. 252, 7850–7861.
[5] Delcour, A.H., Martinac, B., Adler, J. and Kung, C. (1989) J. Memb. Biol. 112, 267–275.
[6] Delcour, A.H., Adler, J. and Kung, C. (1991) J. Memb. Biol. 119, 267–275.
[7] Buechner, M., Delcour, A.H., Martinac, B., Adler, J. and Kung, C. (1990) Biochim. Biophys. Acta 1024, 111–121.
[8] Gehring, K.B. and Nikaido, H. (1989) J. Biol. Chem. 264, 2810–2815.
[9] Ingham, C., Buechner, M. and Adler, J. (1990) J. Bacteriol. 172, 3577–3583.

[10] Delcour, A.H., Martinac, B., Adler, J. and Kung, C. (1989) Biophys. J. 56, 631–636.
[11] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
[12] Kennedy, E.P., Rumley, M.K., Schulman, H. and van Golde, L.M.G. (1976) J. Biol. Chem. 251, 4208–4213.
[13] Van Golde, L.M.G., Schulman, H. and Kennedy, E.P. (1973) Proc. Natl. Acad. Sci. USA 70, 1368–1372.
[14] Hille, B. (1984) Ionic Channels of Excitable Membranes. Sinauer Associates Inc., Sunderland, MA.
[15] Benz, R. (1988) Annu. Rev. Microbiol. 42, 359–393.
[16] Dargent, B., Hofmann, W., Pattus, F. and Rosenbusch, J.P. (1986) EMBO J. 5, 773–778.
[17] Mauro, A., Blake, M. and Labarca, P. (1988) Proc. Natl. Acad. Sci. USA 85, 1071–1075.
[18] Schindler, H. and Rosenbusch, J.P. (1978) Proc. Natl. Acad. Sci. USA 75, 3751–3755.
[19] Schindler, H. and Rosenbusch, J.P. (1981) Proc. Natl. Acad. Sci. USA 78, 2302–2306.
[20] Xu, G., Shi, B., McGroarty, E.J. and Tien, H.T. (1986) Biochim. Biophys. Acta 862, 57–64.
[21] Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 19, 1–32.
[22] Nikaido, H. (1986) Methods Enzymol. 125, 265–278.
[23] Zimmermann, W. and Rosselet, A. (1977) Antimicrob. Agents Chemother. 12, 368–372.