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Biochimica et Biophysica Acta 1463 (2000) 55–64

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Origin of antibacterial stasis by polymyxin B in *Escherichia coli*

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Received 6 July 1999; received in revised form 9 September 1999; accepted 14 September 1999

Abstract

We show that blockage of hyperosmotic shock induced plasmolysis by polymyxin B (PxB) is related to its selective antimicrobial action against Gram-negative organisms. The rapid wrinkling of the cytoplasmic membrane induced by the hyperosmotic shrinkage of cytoplasmic volume due to the water efflux is monitored as an increase in the 90° light scattering. The rapid scattering response is complete within 1 min after the addition of hyperosmolar NaCl. PxB decreases the amplitude of the rapid increase in the light scattering due to the shrinkage of the cytoplasmic volume by hyperosmotic shock. The amplitude is highest with cells in the early log phase of growth. The effect of PxB is induced rapidly and the maximum effect is seen within 1 min preincubation of cells. The effect of PxB is concentration dependent, and about 50% decrease in the amplitude is seen in the range of the growth inhibitory concentrations of PxB. The effect of PxB is not seen if added after the onset of the up-shock. As a heuristic model we suggest that PxB forms contacts between the two phospholipid interfaces that enclose the periplasmic space. The plasmolytic response results with osmY$^-$ mutant suggest that, like PxB, the osmY gene product in the periplasmic space prevents the shrinkage of the cytoplasmic compartment. Since PxB induces *osmY* transcription, we propose that, as a possible locus for the origin of the PxB induced stress, a contact between the phospholipid interfaces surrounding the periplasmic space triggers the metabolic changes leading to bacterial stasis.

Keywords: Plasmolysis; Periplasmic contact; Antibacterial stress; Membrane wrinkling

1. Introduction

Polymyxin B (PxB) and related antibacterial peptides induce selective and independent transcription of *osmY* and *micF* genes in the early growth phase of *Escherichia coli* [1–3]. The same response is induced in an hyperosmolar medium, but not under the hypoosmolar conditions [2–4]. Although detailed function of the *osmY* gene product is not established, earlier studies have shown that *osmY* is expressed near the stationary phase under the normal growth condition of *E. coli* [4–7]. Since the transcription and growth responses are typically measured on the time scale of the doubling time of 30 to 60 min, it is of interest to characterize the coupling between the early PxB-induced and hyperosmolarity-induced events. The underlying primary locus of action could control the systemic metabolic response that leads to stasis.

Our working hypothesis is that the hyperosmolarity and PxB induced transcription events are some-

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; LPS, lipopolysaccharide; MIC, minimum growth inhibitory concentration in 60 min; NP, polymyxin B (2–10)-nonapeptide; OM, outer membrane of Gram-negative *Escherichia coli*

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0005-2736/00/$ – see front matter
PII: S0005-2736(99)00178-9

how proximally related to the rapid response of the cytoplasmic membrane to such stresses. Results in this paper show that the rapid shrinkage of the cytoplasmic volume in response to hyperosmotic stress is blocked by PxB. The hyperosmotic response from viable *E. coli* cells in growth medium is measured as a change in the 90° light scattering due to water efflux and consequent shrinkage of the cytoplasmic volume leading to the wrinkling of the plasma membrane [8–10]. Results show that PxB blocks the scattering change in response to hyperosmotic stress on osmY$^+$ and osmY$^-$ strains in the early growth phase. Surprisingly, during the late growth phase, the hyperosmotic shrinkage is observed only with the *osmY*$^-$ strain but not with the wild-type *osmY*$^+$ strain. During the late growth phase of both strains, the effect of PxB is considerably less pronounced. The effects of PxB are mainly on the cell's early growth phase, yet the *osmY* gene product acts during the late growth phase. These results suggest that like PxB, the *osmY* gene product also counteracts the membrane wrinkling. We suggest that the primary locus for the metabolic changes leading to stasis by PxB or hyperosmotic shock, have their origin in the changes in the relationship between the phospholipid interfaces surrounding the periplasmic space.

2. Materials and methods

2.1. Reagents

Sources of the materials were essentially as described [1–3]. Typically cultures of *E. coli* strain (the wild-type RFM443 or the *osmY*$^-$ strain) in LB medium were grown at 24°C in LB medium in 100-ml batches in 250-ml Erlenmeyer flasks. All measurements were carried out at 24°C in the growth medium. The osmY$^-$ strain of *E. coli* was kindly provided by Prof. Hengge-Aronis who has described its construction and characterization [7].

2.2. Protocols for plasmolysis

The change in the 90° light scattering induced by the hyperosmotic stress was monitored in cultures of *E. coli* strain RFM443 in LB medium at 24°C. Typically, plasmolysis was initiated by adding an aliquot of 4 M NaCl to a magnetically stirred 2.0-ml aliquot of the culture in the early log phase with OD_{600} of 0.15 to 0.3. The change in the 90° light scattering at 600 nm was monitored on a SLM AB2 spectrophotometer equipped with a computer data acquisition system. The change in the scattered light intensity is expressed as the normalized change, I/I_o-1, where I_o is the initial stable intensity, and I is the time-dependent intensity after the perturbation. Note that hyperosmotic shock induced by sucrose cannot be measured by this technique because a change in the refractive index adversely affects the scattering signal [9]. The error bars are not shown; however, replicate assays were routinely carried out. Independent runs under comparable and somewhat different conditions were also carried out to optimize the hyperosmotic response with the growth conditions. Many of such controls are described in Section 3. Typically the scatter in repeat results was less than 10%; however, the scattering changes are dramatically affected with the growth phase.

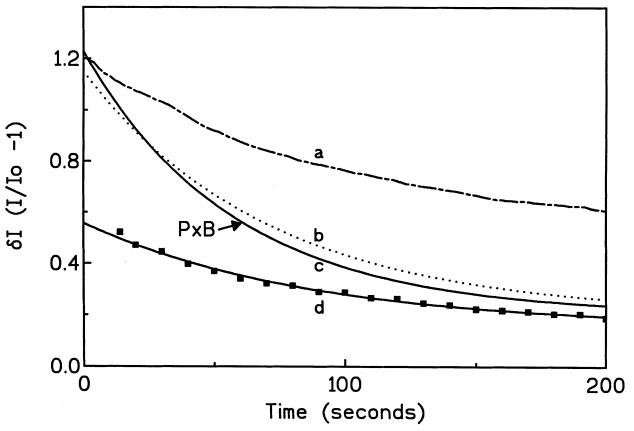

Fig. 1. The time course of the change in the 90° scattered light intensity at 600 nm of 1.6 ml *E. coli* culture in LB medium in response to 0.3 M NaCl added at time zero: (dot, curve b) in the absence of any additive; (curve d with points) with cells preincubated with 0.5 μM PxB for 1 min; (full, curve c) PxB added at 50-s arrow; (dot-dash, curve a) with cells preincubated with 19 μM CCCP for 1 min. Elsewhere we have reported the effects of CCCP and cecropin on the plasmolytic response [3]; however, in the caption to Fig. 6 of this report the label for the effect of CCCP was interchanged with that for the control curve in the absence of any additive. In accord with the present study (curve a), the presence of CCCP in the incubation medium increases the recovery period but not the amplitude of the rapid plasmolytic response.

3. Results

The minimum inhibitory concentration of PxB for the osmY$^+$ and osmY$^-$ *E. coli* strain was 0.25 μM, which also correlates well with the ability of PxB to induce transcription of *osmY* and *micF* [1–3]. Results described below show that in the same concentration range PxB also blocks the rapid phase response of *E. coli* to the hyperosmotic shock. For example, the scattered light response of *E. coli* to hyperosmotic shock is biphasic. In response to 0.3 M NaCl, added to the medium at time = 0 s in Fig. 1 (curve b), the 90° scattered light intensity increases rapidly. The rising phase is essentially complete in less than 10 s, and the increase is followed by a slow recovery phase that is complete in about 5 min. In the presence of 0.5 μM PxB, added 1 min before the addition of NaCl, the amplitude of the rapid increase in the intensity is considerably lower (Fig. 1, curve d). However, the effect on the recovery phase is modest at best. As also shown in Fig. 1 (curve a), the recovery phase response is considerably slower in the presence of CCCP, a proton ionophore. While the plasmolytic shrinkage is a physical process related to the water efflux from the cytoplasm [8–10], the recovery phase is attributed to biological accommodation that involves proton dependent influx of compatible solutes from the concentrated cytoplasm to recover the volume change [11–14].

In this paper we have analyzed the amplitude of the rapid rise in the 90° scattered light which is essentially complete in less than 10 s under most of the conditions. Results in Fig. 1 clearly show that preincubation of *E. coli* with PxB prevents the rapid cytoplasmic shrinkage caused by hyperosmotic stress. However, as also shown in Fig. 1 (curve c), addition of PxB after the onset of the recovery phase did not have any noticeable effect on the time course of the recovery. During the rapid phase the cytoplasm shrinks due to water efflux in response to the higher osmolarity in the external medium. As the turgor pressure on the cytoplasmic membrane is reduced or eliminated, and the shape and size of cytoplasmic compartment changes to accommodate the decrease in the cytoplasmic volume, the cytoplasmic membrane wrinkles [8,9]. Such changes are readily tracked as changes in the intensity of the transmitted [8–10,15] or the 90° scattered light [16]. Although we did not investigate in detail, virtually identical hyperosmotic response profiles were observed with several nonmetabolizable pentoses which do not penetrate the cytoplasmic membrane [8]. For example, the magnitude of the scattering change was virtually identical with 0.6 M xylose as with 0.3 M NaCl, and the hyperosmotic change is also inhibited to the same extent by 0.5 μM PxB. On the other hand, such scattering changes could not be monitored with non-utilizable hexoses and sucrose, presumably because they change the refractive index of the medium [8]. These results rule out the possibility that electrostatic effects of NaCl form the basis for any of the PxB effects that we have characterized.

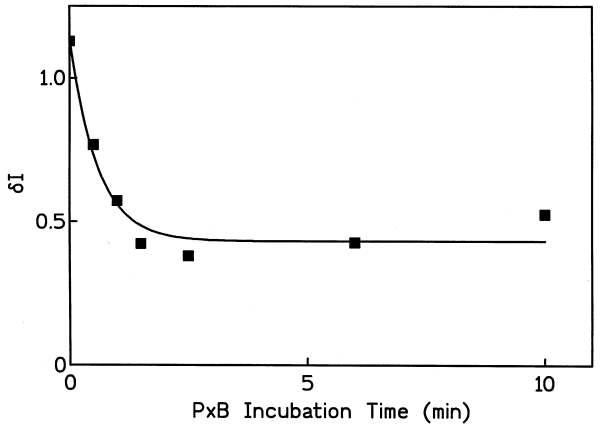

Fig. 2. The dependence of the amplitude of the rapid increase in the scattered light intensity of *E. coli* culture preincubated for the indicated time period with 0.5 μM PxB before the addition of 0.3 M NaCl.

The primary effect of adding PxB before the onset of the osmotic shock is to decrease the peak intensity of the scattered light. This effect on the change in the peak intensity is induced in a very short period. For example, the effect of the time of pre-incubation of 0.5 μM PxB on the amplitude of the light-scattering change in response to 0.3 M NaCl shock (Fig. 2), shows that the maximum effect of PxB is established in about 1 min after the addition of PxB. Independent controls, not shown here, demonstrated that the time to establish the maximum effect does not noticeably change with the PxB concentration in the preincubation medium, and the time course of the onset of the PxB effect does not depend on its concentration. These results reaffirm our earlier conclusion

that the effect of antibacterial peptides is not due to proton leakage or disruption of the cytoplasmic membrane [2]. Similar results were observed with cecropin [3]; however, in Fig. 6 of that report the label on the curve for the effect of CCCP was inadvertently interchanged with that for the control curve for the plasmolytic response in the absence of any additive. In short, preincubation with cecropin or PxB lowers the amplitude of the plasmolytic response. The maximum effect of cecropin is seen after 10 min of preincubation. Although the MIC for cecropin is somewhat lower than that for PxB, the onset of the effect of PxB is essentially complete in about 1 min. We attribute the difference between the onset of the same effect by the two peptides to their ability to breach the outer membrane and to enter the periplasmic space.

The peak intensity of the scattered light depends on the magnitude of the hyperosmotic stress, which is proportional to the concentration of NaCl added to the medium. As shown in Fig. 3, the amplitude of the 90° scattered light intensity increases with the salt concentration, and at all the three NaCl concentrations measured the peak intensity is lower in the presence of 0.5 μM PxB. Results at higher NaCl showed non-linearity of the response [2]. Results in Fig. 3 show that PxB counteracts the changes leading to the shrinkage of the cytoplasmic compartment. The rapid effect of PxB on the scattering change is consistent with the hypothesis that PxB in the periplasm establishes contacts between the outer monolayer of the cytoplasmic membrane and the inner monolayer of the outer membrane, which implies that such contacts prevent wrinkling of the cytoplasmic membrane as the cytoplasmic volume shrinks.

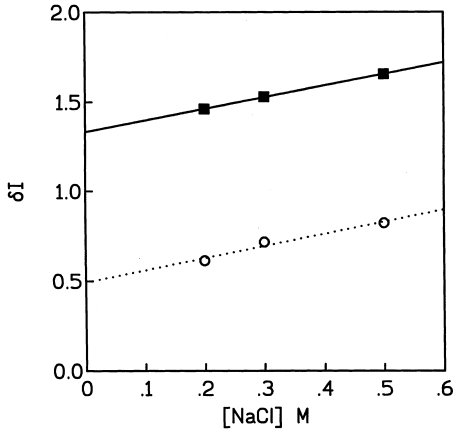

Fig. 3. The effect of the NaCl concentration on the scattered light intensity in the absence (squares) or presence (circles) of 0.5 μM PxB. Other conditions as in Fig. 1 for curves b and d.

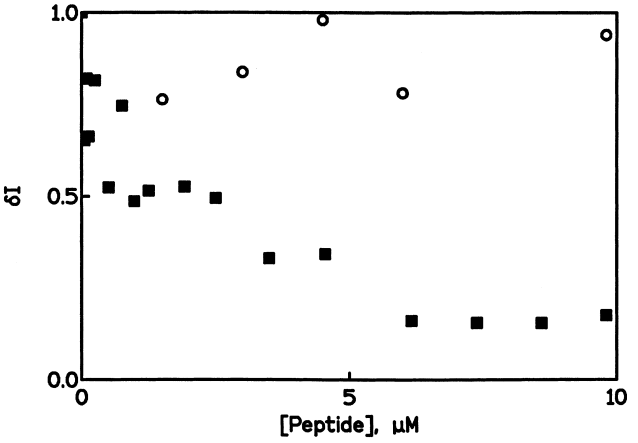

Fig. 4. The effect of the peptide concentration on the scattered light amplitude in response to 0.3 M NaCl added at time zero to *E. coli* culture preincubated for 1 min with indicated concentrations of PxB (squares) or NP (circles).

The effect of PxB concentration, at which the cells are preincubated for 1 min before the addition of 0.3 M NaCl, on the amplitude of the rapid scattering increase is shown in Fig. 4. The change in the scattering intensity decreases monotonically with the PxB concentration. The initial region of the curve is noisy; however, a significant decrease is observed above 0.2 μM PxB. These results show that the effect of PxB on the scattering changes from hyperosmotic shock occur in the same concentration range as the minimum concentrations for inhibition of growth, that is 0.2 μM [1,2]. As also shown in Fig. 4 the decrease in the scattering amplitude is not seen on incubation of cells with NP, the truncated (2–10)-polymyxin nonapeptide. This is a particularly informative control. Recall that NP has little effect on cell growth or on the transcription of *osmY* or *micF* [1–3]. This analog binds to LPS and disrupts the outer membrane of Gram-negative organisms at about 1 μM concentration, and makes the cytoplasmic membrane accessible to solutes, which do not otherwise cross the outer membrane [17]. Together, we conclude that disruption of the outer membrane is not a sufficient condition for the inhibitory effect of PxB on growth, transcription, or the hyperosmotic response. Also, these results show that the short

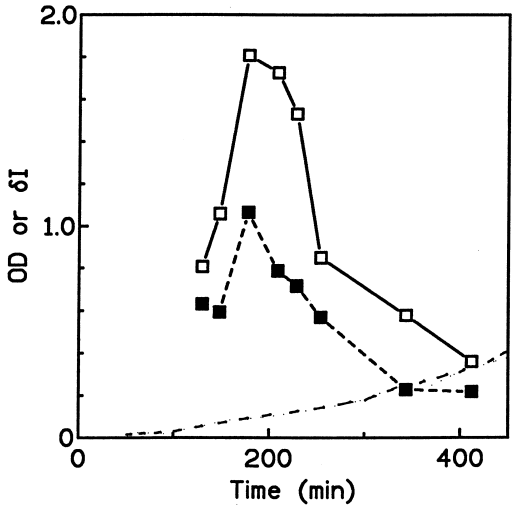

Fig. 5. The amplitude of the scattered light intensity in response to 0.3 M NaCl added to *E. coli* culture without (open symbols) or after preincubation with 0.5 μM (filled symbols) PxB for 1 min. OD_{600} change associated with the growth is shown as the dashed line.

and long term responses of PxB are both qualitatively and quantitatively correlated.

The amplitude of the rapid scattering response changes with the growth phase of the culture. As summarized in Fig. 5, the amplitude of the plasmolytic response to 0.3 M NaCl is biphasic, both in the absence or presence of 0.5 μM PxB. The amplitude of the plasmolytic response reaches a maximum in the early log phase. Note that the amplitude decreases in the presence of 0.5 μM PxB at all points along the growth curve. In the stationary phase of growth the plasmolysis amplitude is low. Independent controls with several batches of the cells in the late growth phase and stationary phase show less than 30% change in the scattering in response to the hyperosmotic shock. At these low levels of the scattering change it is difficult to ascertain whether or not PxB has a significant effect on the scattering change. Controls with diluted cultures showed that the reduction in the amplitude is not due to inner filter effects.

While PxB influences the scattering response to the hyperosmotic stress, both of these stresses independently stimulate the *osmY* transcription [1–3], which is also induced by stresses related to late growth phase and starvation [4–7,18]. *osmY* is a representative of a large group of σ^S-dependent genes in *E. coli*; however, the function of its product, a periplasmic outer membrane protein, is not known. Therefore, to characterize the functional role of *osmY* induction in stationary-phase and its osmotic regulation, we tested the effect of hyperosmotic shock on the cytoplasmic shrinkage of the *osmY*$^-$ mutant. This strain produces a *lacZ* fusion protein with only one quarter of the amino terminal sequence of the wild-type *osmY* protein [7]. The fusion protein has the glycosidase activity which remains confined to the cytoplasm.

The effect of the hyperosmotic shock and PxB on the *osmY*$^-$ strain is shown in Fig. 6. The hyperosmotic shrinkage is seen during the entire cell growth period of the *osmY*$^-$ strain. The amplitude of the shrinkage is apparently higher during the late growth phase, and remains so even in the stationary phase (data not shown). Surprisingly, the effect of PxB is quite pronounced with the *osmY*$^-$ strain. During the early growth phase, 0.5 μM PxB virtually completely abolishes the rapid shrinkage of the cytoplasmic volume in response to hyperosmotic stress. On the other hand, in the late growth phase the shrinkage is not sensitive to 0.5 μM, or even to 5 μM PxB (data not shown). A comparison of results in Figs. 5 and 6 clearly show that the effects of PxB on the hyperosmotic shrinkage is dominant in the early growth phase, yet a PxB-like effect on the shrinkage in both strains is induced by the *osmY* gene product during the late growth phase. These results imply that sim-

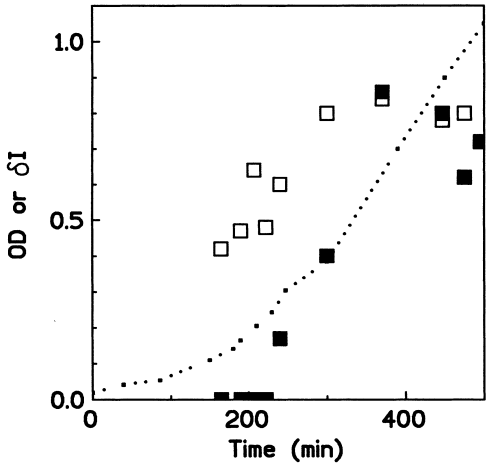

Fig. 6. The amplitude of the scattered light intensity in response to 0.3 M NaCl added to the culture of *osmY*$^-$ strain of *E. coli* without (open symbols) or after preincubation with 0.5 μM (filled symbols) PxB for 1 min. OD_{600} change associated with the growth is shown as the dashed line.

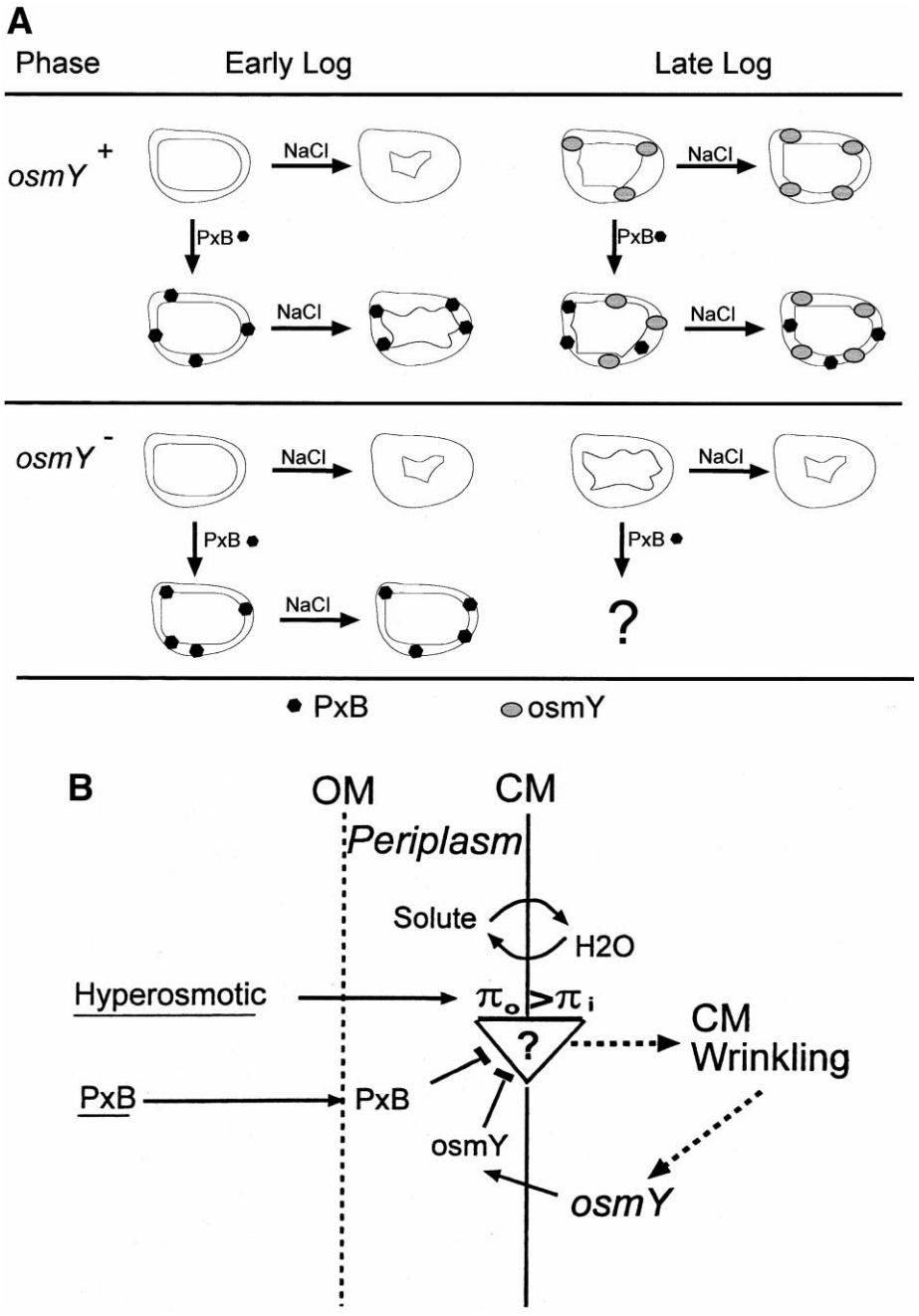

Fig. 7. (A) An operational model to account for the observed changes in the NaCl-induced light scattering from WT and *osmY*⁻ *E. coli*. The physical contacts of PxB (early growth phase) and the *osmY* gene product (late growth phase) in the periplasmic space between the outer and cytoplasmic membranes counteract the effect of hyperosmotic shock by preventing the wrinkling of the cytoplasmic membrane due to shrinkage of the cytoplasmic compartment. (B) The chain of rapid events that in response to hyperosmotic shock lead to wrinkling of the cytoplasmic membrane followed by the influx of compatible solutes by pumps. Entry of PxB in the periplasmic space interferes with the plasmolytic wrinkling of the membrane. Possibly, a similar effect is mediated by osmY protein in the periplasmic space. We suggest that the changes in the state of the cytoplasmic membrane is the primary signal for the *osmY* transcription which ultimately induces stasis.

ilar to PxB, the *osmY* gene product counteracts the effect of hyperosmotic shock by preventing the shrinkage of the cytoplasmic compartment; however, other explanations cannot be ruled out yet.

4. Discussion

PxB and related antimicrobial peptides promote the expression of *osmY* and *micF* [1–3], as also induced by the hyperosmotic stress [2–7,18]. The antimicrobial response is a cumulative metabolic end effect as most conveniently monitored over the cell division time in the culture. Results of the present study are particularly instructive in constructing a plausible chain of initial events that ultimately trigger the metabolic and transcriptional responses associated with a late growth phase like stasis induced by PxB. We propose that the rapid effects of PxB, below or at the minimum growth inhibitory concentrations, on the rapid response phase of hyperosmotic up-shock is the early, if not the primary, locus of PxB and hyperosmotic stresses. A model relating the underlying chain of events is outlined in Fig. 7.

4.1. Antibacterial peptides block the rapid phase of the up-shock

Although PxB is a prototype for the present study, several other antibacterial peptides that induce the *osmY* transcription associated with the growth also blocks the rapid scattering response to the hyperosmotic up-shock [1–3]. The present study was in fact motivated by the hypothesis that the primary locus of action of PxB is the contact formation between the phospholipid interfaces surrounding the periplasmic space [22]. Plasmolysis, the shrinkage of cytoplasmic compartment in response to hyperosmotic up-shock, is a rapid event. The light scattering changes provide us a rapid and sensitive measure of the shrinkage of the cytoplasmic volume. Even though the scattering response does not provide an insight into the underlying molecular events, analysis of the time course of the light-scattering changes does place a limit on the nature and origin of the underlying events. The rising phase of the scattering change is rapid with half-time of about 0.1 s [15]. The major effect of PxB is on the peak amplitude, rather than on the rise-time itself (data not shown), which is consistent with the suggestion that the number of PxB contacts, rather than the rate of formation of the contacts, is responsible for the decrease in the amplitude. The half-time for the recovering phase, i.e., the falling phase in Fig. 1, is 1000-fold slower with half-time of about 100 s. The effect of PxB on the rise phase is dramatic, and the recovery phase is only modestly affected by the presence of PxB. Therefore, we conclude that the events of the recovery phase have little if any effect on the amplitude of the scattering change that was measured within 10 s after the up-shock. A lack of the effect of CCCP on the peak amplitude also rules out a role for the proton gradient in the events of the up-shock. Only the recovery time increases in the presence of CCCP (Fig. 1, curve a). This observation supports the view that as an osmoregulatory event, the solute influx in the recovery phase is driven by the proton gradient [12,13,19]. In *E. coli*, such a proton-coupled solute efflux occurs through the ProP transporter [14].

Note that a rapid effect of PxB on the rapid hyperosmotic shrinkage correlates with the effects of PxB seen on the growth or *osmY* transcription. The effect of PxB is seen mostly on the amplitude of the scattering change in the rapid phase. The onset of the scattering increase is rapid, and the time course does not seem to change significantly with the concentration of the antibacterial peptide (Fig. 4). Similarly, the effect of PxB on the plasmolytic changes in cells in the early log phase is fully developed in a matter of a minute or so. These results show that the rapid onset of the effect of PxB near the minimum growth inhibitory concentrations on the hyperosmotic shrinkage is associated with a biophysical change rather than a metabolic change. With the assumption that the rapid phase of the scattering response is dominated only by the efflux of cytoplasmic water [8,9], the protocols and criteria developed in this paper establish that the antimicrobial peptides block the rapid hyperosmotic stress in growing cells.

4.2. Role of osmY gene product

OsmY is a 20 kDa outer membrane or periplasmic

protein. Along with several other proteins it is expressed during the entry of culture into stationary phase. These proteins confer resistance to a range of stresses including higher temperature, heat-shock, wider pH-range, and higher concentrations of hydrogen peroxide, NaCl, alkylating agents, ethanol, acetone, toluene and deoxycholate [6,7,18]. The hyperosmolar environment and the cationic peptide antimicrobials promote *osmY* transcription in the early growth phase, and the effect of PxB is minimum during the late growth phase where the hyperosmotic scattering change is also marginal (Fig. 5). This would be expected if the osmY protein made PxB-like contacts in the periplasmic space. This interpretation is also consistent with results in Fig. 6, where under the hyperosmotic condition we observed the cytoplasmic shrinkage during the entire cell growth period with an *osmY*$^{-}$ strain. In this strain the amplitude of the hyperosmotic scattering is high in the late growth phase, yet surprisingly it does not respond to PxB.

In terms of the working hypothesis in Fig. 7A,B, we propose that during the initial chain of events the *osmY* gene product counteracts the effect of hyperosmotic shock by forming or stabilizing the periplasmic contact of the cytoplasmic membrane with the outer membrane. Note that the effects of PxB are seen mainly during the cell's early growth phase, yet the effect attributed to the *osmY* gene product are seen after its expression in the late growth phase. Thus, the hyperosmotic shrinkage is not seen when the *osmY* gene product is expressed. The effects of PxB are measurable in the early growth phase because the osmY contacts are not established yet. Whether or not such contacts are formed during the stationary phase remains to be established. The fact that the effect of PxB on the osmY^{-} strain is seen with the early but not in the late growth phase implies that the two surfaces surrounding the periplasmic space are either well separated to form the PxB-contacts in the late growth phase, or that PxB does not reach the periplasmic space in the late growth phase. Additional studies are required to resolve these and other plausible explanations for the role of the osmY protein.

4.3. Basis for the stress signal

Possible origin of the signal triggered by the various stresses and consequent osmoregulatory processes can only be speculated. A teleological rationale can be constructed on the basis of the physiological observation that the osmY gene product prevents wrinkling of the cytoplasmic membrane in the late growth phase of the wild-type strain. Apparently this control is lost in the *osmY*$^{-}$ strain, which also grows to much higher culture densities. It is unlikely that the hyperosmotic efflux of water is prevented under these conditions, and it also appears unlikely that the osmoregulatory solute influx is very rapid in the stationary phase. To find a common basis we focus on the fact that the membrane wrinkling is triggered by hyperosmotic response in the log phase, where initial water efflux triggers influx of compatible solutes. If during the steady state the pumps develop a high turgor pressure from the cytoplasmic side, the cytoplasmic membrane may come close to the outer membrane. Such an apposition could be a possible trigger for the metabolic events. Irrespective of the detailed mechanism, the situation in the periplasmic space could be operationally similar to that after the formation of stable PxB-contacts.

It is unlikely that the outer membrane (OM) and the peptidoglycan layer (PGL) of periplasm directly responds to osmotic shock because these membranes are permeable to small molecular mass solutes. If OM or PGL respond at all to the shrinkage of the cytoplasmic volume, it is not seen after 45 s of sample preparation for electron microscopy, or after 15 s of sample preparation for phase contrast microscopy [9,21]. Shrinkage of the cytoplasmic space would create a decrease in the turgor pressure. Since the cytoplasmic membrane cannot shrink laterally, the cytoplasmic membrane responds by wrinkling. In EM studies the cytoplasmic membrane is found to be detached within the 45 s required for preparing the sample [10]. Since the turbidity and scattering changes associated with up-shock occurs with half time of a fraction of a second, we postulate that the wrinkling of CM in the growth phase of WT is a rapid event. It is not clear whether CM detaches as

rapidly from the outer membrane structures in the presence of the obligatory attachments sites putatively formed during the cell division [20].

4.4. Control of cell growth through periplasmic contacts

Cell growth occurs under constant turgor pressure maintained by linking wall growth to the increase in cytoplasm. In addition, other control mechanisms maintain the turgor pressure in response to environmental challenges encountered during the cell division. If PxB or the osmY protein form stable contacts between outer and inner membranes, such growth processes will be interrupted or altered. We do not have direct evidence for the formation of such contacts in the periplasmic space; however, putative properties of such contacts provide for a potential function for the osmY protein.

Blockage of the scattering change by PxB suggests that somehow the wrinkling of the cytoplasmic membrane is prevented by PxB. A role for contacts, formed by antimicrobial peptides between the two phospholipid monolayers surrounding the periplasmic space, could account for the blockage of the up-shock response by PxB as assumed in the model in Fig. 7A. As shown in the preceding paper, efficacy of several peptides in the phospholipid exchange assay correlates with their efficacy for growth inhibition or the induction of *osmY* transcription. Here we are guided by the observation that PxB forms a stable contact between anionic phospholipid vesicles and promote intervesicle exchange of phospholipids. The heuristic guide for our efforts to identify the primary locus of action of PxB [22] is based on the observation that PxB promotes a rapid and specific exchange of monoanionic phospholipid by forming a stable contact between the outer membrane of anionic vesicles [23,24]. In terms of the model shown in Fig. 7A, a plausible interpretation of the PxB-induced loss of the shrinkage of the cytoplasmic space would be that PxB-mediated stable contacts are formed between the cytoplasmic membrane and some other periplasmic component. in our opinion the most plausible partner for the formation of the peptide-mediated contact of the cytoplasmic membrane is the phospholipid containing anionic inner monolayer of the outer membrane; however, we cannot yet rule out a role for the peptidoglycan layer or for the LPS that may enter the periplasmic space under the influence of PxB.

Antimicrobial activity is a growth phase response. Like the up-shock response, the effect of PxB is also most prominent in the early log phase of growth (Fig. 5). In the context of the PxB selectivity against Gram-negative organisms, during their growth the inner and outer membranes come in contact with each other to transfer the components necessary for cell division. The intervening peptidoglycan layer putatively hinders spontaneous formation of such contacts. The zones of adhesion, where the inner and outer membranes are in close apposition, have been observed in normal cells during growth [20]. Formation and stabilization of contacts with the inner anionic phospholipid layer of the outer membranes would prevent hyperosmotic shrinkage of the cytoplasmic membrane is consistent with a correlation with the phospholipid exchange results developed in the preceding paper [1].

To recapitulate, parallel biophysical and metabolic consequences of the hyperosmotic stress and the specific effects of the antimicrobial peptides are intriguing. We believe that the peptide does not enter the cytoplasm and that antibacterial mechanism is based on interaction of the peptide with phospholipid interfaces. Both the hyperosmotic shock and the antibacterial peptide trigger a fundamental physiological function, i.e., the onset of stasis. This novel antibacterial strategy is suggested by the fact that PxB is able to trigger the stasis response in the early growth phase. With the evolutionary success of PxB type antimicrobials, nature seems to have taken a biophysical course of action to induce a rapid stress response leading to stasis.

Acknowledgements

We thank Prof. Hengge-Aronis for providing the *osmY*− mutant, and Arthur Koch for useful insights into bacterial plasmolysis. This work was supported by the NIH (GM29703 to M.K.J.).

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