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HOW DOES LYSOZYME PENETRATE THROUGH THE BACTERIAL OUTER MEMBRANE?

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SUMMARY

Lysozyme fails to penetrate through the outer membrane of stationary phase cells of *Escherichia coli* when it is simply added to suspensions of plasmolyzed cells. Lysozyme penetrates the outer membrane only when these cells are exposed to a mild osmotic shock in the presence of EDTA and lysozyme.

In the presence of Mg^{2+}, the outer membrane is stabilized sufficiently so that there is no lysozyme penetration during osmotic shock. If Mg^{2+} is added after an osmotic shock has been used to cause lysozyme to penetrate a destabilized outer membrane, the outer membrane is stabilized once again. In this case however, cells are converted to spheroplasts by the lysozyme which has gained access to the murein layer prior to the addition of Mg^{2+}. Mg^{2+} stabilizes the outer membranes of these spheroplasts sufficiently so that they remain immune to lysis even in the absence of osmotic stabilizers such as sucrose.

These results are discussed in terms of current information on the structure of the murein layer and the outer membrane.

INTRODUCTION

Gram-negative cells are surrounded by an envelope which consists of an inner cytoplasmic membrane, a murein layer, and an outer membrane [1]. Such cells can be converted to spheroplasts by removal of the intermediate murein layer; this is usually accomplished by enzymatic degradation with lysozyme [2–12].

The success of lysozyme in converting gram-negative cells to spheroplasts raises the interesting question of how this enzyme penetrates through the outer membrane, since recent evidence indicates that this membrane, which shields the murein layer from the external environment, is permeable only to small molecules with molecular weights of up to 900–1000 [13]. This problem is partially eliminated by the fact that lysozyme treatments are carried out in plasmolyzing buffers [2–6, 8, 12] which

Abbreviations: Tris/EDTA/sucrose buffer, 200 mM Tris · HCl/0.5 mM EDTA/0.5 M sucrose (pH 8.0); Tris/EDTA/sucrose lysozyme treatment, lysozyme treatment of cells suspended in Tris/EDTA/sucrose buffer.

contain Tris and/or EDTA to destabilize the outer membrane [2–11]. Nevertheless, in most of these treatments large amounts of lysozyme* are used to degrade the cell murein, indicating that the extent of lysozyme access to murein remains unsatisfactory. Moreover, while these procedures work with exponentially growing cells, they fail with stationary phase cells, the outer membrane of which is more resistant to destabilizers such as Tris and EDTA [12, 15].

We have recently become interested in the membranes of stationary phase *Escherichia coli* [16]. To isolate and purify these membranes it was necessary to develop a new procedure to prepare spheroplasts from stationary phase cells; the resulting procedure is very effective in rendering such cells susceptible to small amounts of lysozyme [15]. In the course of developing this procedure we became interested in the mechanism which caused the effective penetration of lysozyme through the outer membrane of stationary phase cells.

In this paper we show that it is not sufficient to merely plasmolyze the cells and destabilize the outer membrane. Instead, it is necessary to drive lysozyme through the outer membrane by means of a transient influx of water, which is engendered by a mild osmotic shock. The effectiveness of the osmotic shock depends on the state of the outer membrane; it is successful when the outer membrane is destabilized with EDTA, and unsuccessful when the outer membrane is stabilized with Mg^{2+}**.

MATERIALS AND METHODS

Cell growth

Stationary phase cultures of *E. coli* JC411 (*leu*$^-$ *his*$^-$ *arg*$^-$ *met*$^-$ *lac*$^-$ *mal*$^-$ *xyl*$^-$ *mtl*$^-$ *str*^{r}) and *E. coli* W3110 were grown in minimal media as described previously [15, 16]. The harvested cells, centrifuged 5000 × *g* for 10 min at 0–4 °C, were suspended in 1 mM $MgCl_2$ and 200 mM Tris · HCl (pH 8.0) to a density of 25 mg/ml, stored on ice, and used for the production of spheroplasts over a period of 8 h. $MgCl_2$ prevented autolysis. Cell densities (dry mass) were determined as described previously [17].

Lysozyme treatment

The lysozyme treatment described below is an analytical variant of the procedure described previously [15]. To 0.2 ml suspended cells was added 0.3 ml 200 mM Tris · HCl (pH 8.0). This time was defined as zero minutes. At 1 min, 5 µl 100 mM EDTA (pH 7.6) was added. At 2 min, 0.5 ml 1 M sucrose in 200 mM Tris · HCl (pH 8.0) (Tris/sucrose) was added. Thus, given the excess of EDTA over Mg^{2+}, these cells were in fact suspended in a Tris/EDTA/sucrose buffer (200 mM Tris · HCl/0.5 mM EDTA/0.5 M sucrose (pH 8.0). At 3.5 min, 20 µl lysozyme (EC 3.2.1.17, C.F. Boehringer und Soehne GmbH, Mannheim, G.F.R., 0.55 mg/ml in 200 mM Tris · HCl (pH 8.0) was added to the Tris/EDTA/sucrose suspended

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* The amount of lysozyme used is often 10–50 % of the cell dry mass [4, 5, 7–10]; given that murein accounts for only 1.2 % of the cell dry mass [14], many lysozyme treatments employ an 8–40-fold (w/w) excess of enzyme over substrate.

** A preliminary report of this work was presented at the 1974 Symposium on The Bacterial Cell Envelope held in Lunteren, The Netherlands.

cells. At 4 min, the cells were exposed to a mild osmotic shock by the addition of 1 ml water.

In the experiments of Figs. 1 and 2 there was no mild osmotic shock. These experiments were started by adding 0.4 ml 200 mM Tris · HCl (pH 8.0) to 0.1 ml suspended cells at zero minutes. EDTA and Tris/sucrose were added as above, and 10 μl lysozyme solution was added at 3.5 min, so that the final cell and lysozyme concentrations were identical in all experiments.

The effect of these procedures was determined by two criteria. The conversion of rod shaped cells to spheres was followed by phase contrast microscopy. Osmotic sensitivity was measured turbidimetrically; the time course of lysis was followed by recording A_{450} on a Cary 15 spectrophotometer immediately (within 10 s) after a portion of a treated cell suspension had been diluted 11-fold in either 10 mM EDTA (pH 7.6), water, or 20 mM $MgCl_2$.

Fig. 1 shows how these experiments were set up; dilutions were made at various times after the onset of the Tris/EDTA/sucrose lysozyme treatment, and the corresponding lysis curves (A_{450} vs. time) were obtained. Figs 2–4 are compressed versions of Fig. 1; the time at which each dilution was made is indicated for the corresponding lysis curve. It always took from 8 to 10 s before a given dilution had been completed and inserted in the spectrophotometer, and the lysis curves of Figs. 2–4 therefore did not start at zero time. In some experiments there was a significant amount of cell lysis during the initial nonrecorded 8–10 s; therefore the recorded portions of the different lysis curves obtained in such experiments did not necessarily start at the same absorbance (Figs. 3a, b, and 4a).

RESULTS

When does lysozyme attack the murein layer of stationary phase cells?

A suspension of stationary phase *E. coli* W3110 was diluted into Tris/EDTA/sucrose buffer by sequential addition of the buffer components at 0, 1, and 2 min as described in Materials and Methods and as indicated in Fig. 1. Lysozyme was added at 3.5 min to a concentration of 5.5 μg/ml; there was no mild osmotic shock. A portion of the resulting lysozyme treated Tris/EDTA/sucrose suspension was immediately diluted 11-fold with 10 mM EDTA. Curve 1 shows that the cells lysed steadily after dilution, indicating lysozyme activity in the cuvette. It might have been expected that lysozyme should be active in the Tris/EDTA/sucrose suspension as well, because the cells were plasmolyzed [2, 6, 12], and because the outer membrane was destabilized by EDTA [9]; the fraction of osmotically sensitive cells in the lysozyme treated Tris/EDTA/sucrose suspension should therefore have increased with time. Such osmotically sensitive cells should lyse immediately upon 11-fold dilution into 10 mM EDTA; subsequent cell lysis curves could therefore have been expected to start at progressively lower absorbances as the lysozyme incubation proceeded. Fig. 1 shows, however, that lysis curves obtained at 8, 12, and 20 min were essentially identical to the first lysis curve, which was obtained only 10 s after the addition of lysozyme. Thus, there was no detectable lysozyme activity in the lysozyme treated Tris/EDTA/sucrose suspension, at least as judged by absorbance measurements.

Further evidence that lysozyme was inactive prior to dilution is shown in Fig. 2b. An experiment similar to that of Fig. 1 was carried out, except that lysozyme was

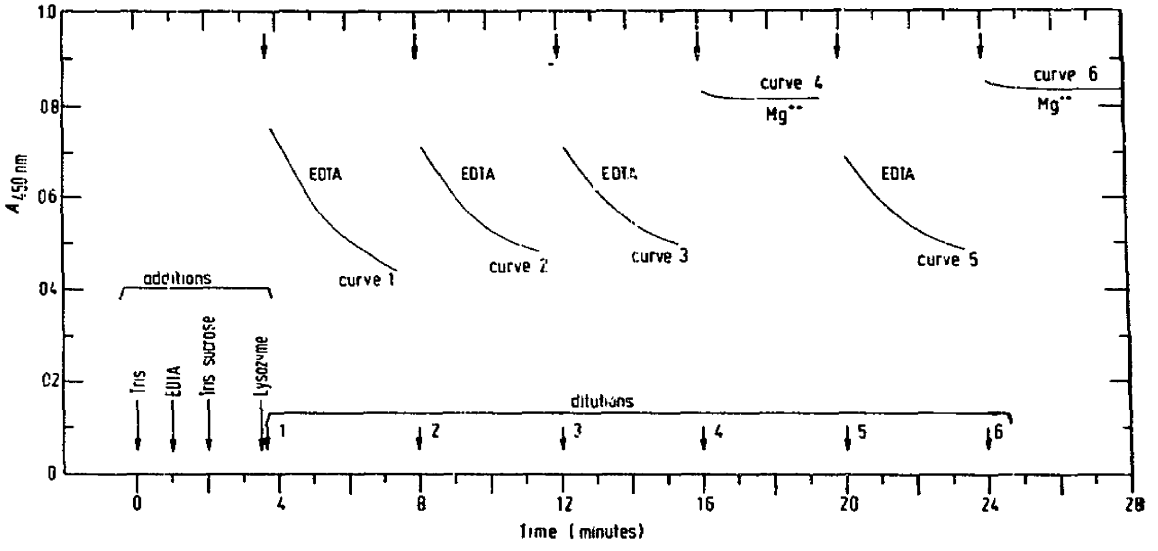

Fig. 1. Effect of lysozyme on the osmotic sensitivity of stationary phase *E. coli* W3110. Tris, EDTA and Tris/sucrose were added to a cell suspension to final concentrations of 200 mM Tris · HCl (pH 8.0), 0.5 mM EDTA, and 0.5 M sucrose. Lysozyme was added at 3.5 min to a concentration of 5.5 μg/ml. The osmotic sensitivity of the resulting suspension was determined at various times after the addition of lysozyme by diluting portions of the suspension 11-fold and following the absorbance for about 4 min. Dilutions were carried out in 10 mM EDTA (curves 1, 2, 3, and 5) and in 20 mM $MgCl_2$ (curves 4 and 6).

not added to the Tris/EDTA/sucrose suspended cells at 3.5 min. Instead, lysozyme was present in the diluents in which the 11-fold dilutions were carried out at a final concentration of 0.5 μg/ml; thus, the final lysozyme concentration after dilution was identical in both experiments. The lysis curves of Fig. 1 have been redrawn in Fig. 2a to facilitate comparison between these curves and those of Fig. 2b. Whether lysozyme was added before (Fig. 2a) or was present only during and after (Fig. 2b) 11-fold dilution into 10 mM EDTA, the resulting lysis curves were identical.

The activation of lysozyme after dilution into 10 mM EDTA was not related to the EDTA as such; similar results were obtained when water was used as a diluent. In addition, stationary phase cells of *E. coli* W3110 remained resistant to lysozyme prior to dilution when the EDTA concentration in the Tris/EDTA/sucrose buffer was varied from 0.5 to 45 mM.

The presence of Mg^{2+} in the diluent prevented cell lysis, as can be seen in Fig. 2. Whether lysozyme was present before (Fig. 2a), not at all (Fig. 2b) or during (Fig. 2b) dilution into 20 mM $MgCl_2$, cells simply failed to lyse in this diluent. When lysozyme was omitted altogether, and Tris/EDTA/sucrose suspended cells were diluted into 10 mM EDTA, there was no lysis (Fig. 2b). The experiments of Fig. 2 have also been carried out with stationary phase *E. coli* JC411, with identical results.

Passage of lysozyme through the outer membrane during osmotic shock

The triggering of lysozyme activity by 11-fold dilution in 10 mM EDTA or water was probably related to the drastic osmotic shock which resulted from such

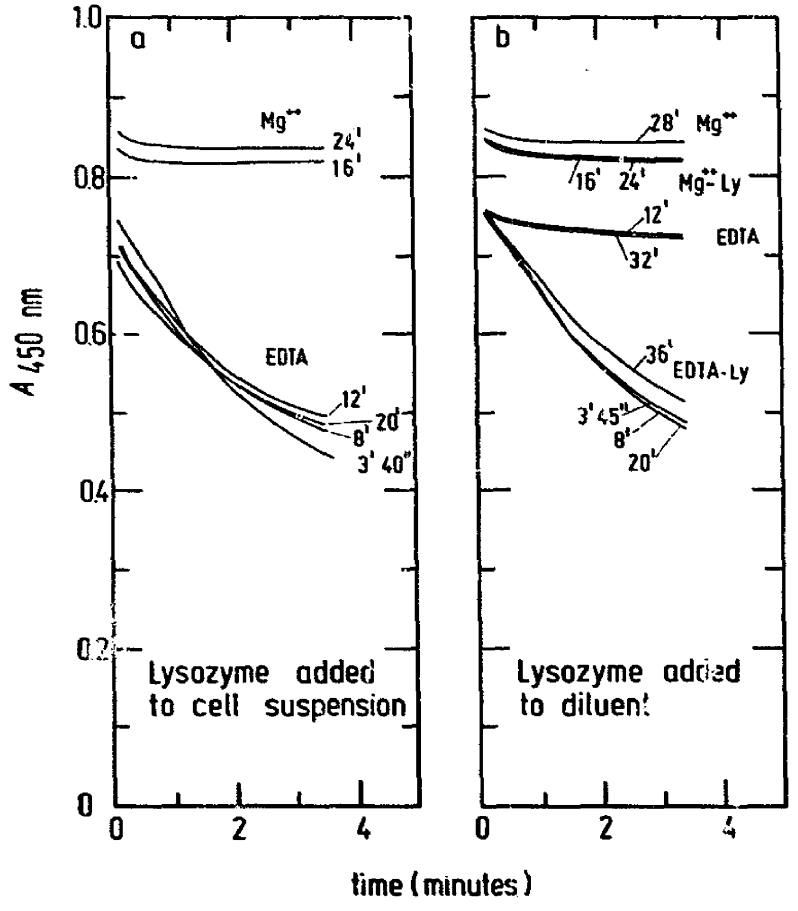

Fig. 2. Effect of lysozyme on stationary phase *E. coli* W3110, when added before and after 11-fold dilution of cells suspended in Tris/EDTA/sucrose buffer. The conditions of this experiment were similar to those of Fig. 1, except as noted. (a) Lysozyme was added to a concentration of 5.5 μg/ml at 3.5 min, after which portions of this suspension were diluted 11-fold into 10 mM EDTA or 20 mM $MgCl_2$, at the times indicated. These data are identical to those of Fig. 1, but replotted to facilitate comparison of the different curves. (b) Cells suspended in Tris/EDTA/sucrose buffer were diluted 11-fold into 10 mM EDTA, 20 mM $MgCl_2$, or the same diluents containing 0.5 μg/ml lysozyme.

dilutions. The effect of a milder osmotic shock was therefore examined. Fig. 3a shows that when lysozyme treated cells were diluted 2-fold in water at 4 min (30 s after the addition of lysozyme, Fig. 1) and subsequently diluted 11-fold into 10 mM EDTA at different times, the resulting lysis curves were no longer superimposable as was the case in Fig. 2. Instead, the 11-fold dilutions caused successively more lysis as the time between the mild osmotic shock (2-fold dilution in water) and the 11-fold dilution in EDTA increased, indicating that lysozyme degraded the murein layer after mild osmotic shock. When lysozyme was added 30 s after, instead of before the mild osmotic shock, the activity of lysozyme on the shocked cells was considerably less, as shown in Fig. 3b. This effect was even more dramatic with *E. coli* JC411, where the

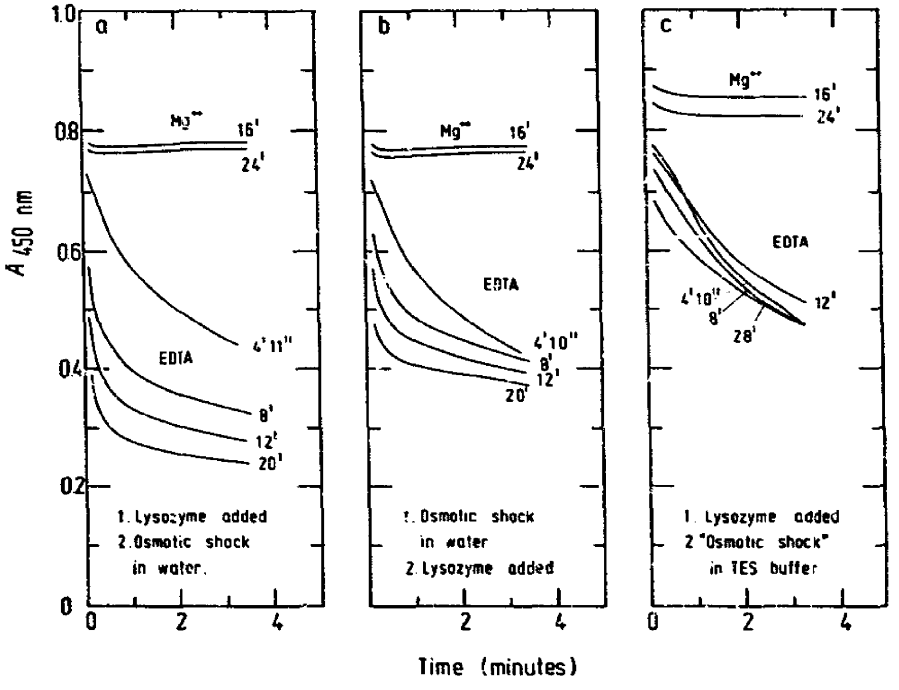

Fig. 3. Effect of mild osmotic shock on the accessibility of *E. coli* W3110 murein to lysozyme. Tris/EDTA/sucrose suspensions (1 ml) were prepared containing twice the cell density used in Fig. 2. (a) Lysozyme was added to 11 μg/ml at 3.5 min, followed by 1 ml water at 4 min, thus reducing both cell and lysozyme concentrations to those used in the experiment of Fig. 2a. Portions of this suspension were diluted into 10 mM EDTA or 10 mM $MgCl_2$ at the times shown. (b) Identical to a, except that 1 ml water was added at 3.5 min, followed by lysozyme after 4 min, to a final concentration of 5.5 μg/ml. (c) Identical to a, except that 1 ml Tris/EDTA/sucrose buffer was added instead of water at 4 min.

addition of lysozyme after mild osmotic shock allowed no lysozyme penetration even when lysozyme was added as fast as possible after mild osmotic shock. Thus, the mild osmotic shock caused by 2-fold dilution appeared to cause a transient increase in the permeability of the outer membrane to lysozyme. Optimal penetration occurred only when lysozyme was present during mild osmotic shock. When the Tris/EDTA/sucrose suspended, lysozyme treated cells were diluted into Tris/EDTA/sucrose buffer instead of water there was no osmotic shock. Fig. 3c shows that under these conditions the results resembled those of Fig. 2a, implying that there was essentially no murein degradation prior to 11-fold dilution into 10 mM EDTA.

Effect of Mg^{2+} on the accessibility of the murein layer to lysozyme

Fig. 4a shows that Mg^{2+} did not inhibit the action of lysozyme on murein. When lysozyme was added to Tris/EDTA/sucrose suspended cells at 3.5 minutes, followed by mild osmotic shock at 4 min, and $MgCl_2$ was added to a final concentration of 25 mM at 4.5 min, lysozyme acted on these cells prior to their subsequent dilution in 10 mM EDTA (compare Figs. 4a and 3a). When Mg^{2+} was added prior to

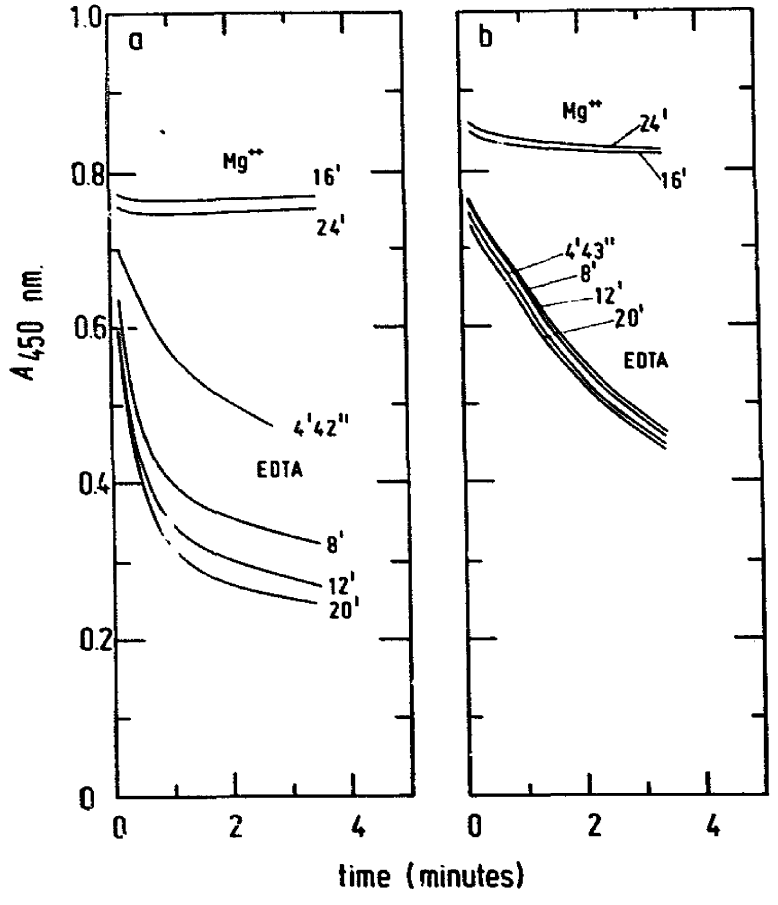

Fig. 4. Effect of Mg^{2+} on the hydrolysis of murein by lysozyme. (a) Cells were treated as described for Fig. 3a, except that Mg^{2+} was added to 25 mM after the mild osmotic shock; 50 μl 1 M $MgCl_2$ was added at 4.5 min. Portions of this suspension were diluted into 10 mM EDTA and 20 mM $MgCl_2$ at the times shown. (b) Identical to a, except that Mg^{2+} was added prior to osmotic shock; 50 μl 1 M $MgCl_2$ was added at 4 min, followed by 1 ml water at 4.5 min.

the mild osmotic shock however (but following the addition of lysozyme to the cells), mild osmotic shock had no effect in rendering murein accessible to lysozyme, as shown in Fig. 4b, which is essentially identical to Fig. 2a. Thus, Mg^{2+} stabilized the outer membrane of stationary phase cells to such an extent that mild osmotic shock failed to cause lysozyme penetration.

Effect of Mg^{2+} on the stability of stationary phase spheroplasts

The Tris/EDTA/sucrose lysozyme treatment rendered stationary phase cells spherical as well as osmotically sensitive [15, 16]. Nevertheless, while osmotically sensitive cells were always spherical, spherical cells were not necessarily always osmotically sensitive. Specifically, spherical cells (after lysozyme treatment) were resistant to 11-fold dilution in 20 mM $MgCl_2$. This was true even when lysozyme had obviously acted on the murein layer as judged by the fact that the cells were pro-

gressively more susceptible to lysis in 10 mM EDTA (Figs 3a and 4a); even after prolonged lysozyme treatment (up to 4 h) the spheroplasts were as stable to dilution into 20 mM $MgCl_2$ as to dilution into osmotic stabilizers. About two-thirds of these spheroplasts lysed within 15 s when diluted 11-fold into 10 mM EDTA (data not shown).

DISCUSSION

Lysozyme activity before and after osmotic shock

The addition of lysozyme to a suspension of plasmolyzed stationary phase cells, the outer membrane of which was destabilized with EDTA, resulted in little if any murein hydrolysis. Lysozyme became active only after the cells were shocked osmotically. It was possible to reduce the extent of the osmotic shock sufficiently so that there was no cell lysis, even though lysozyme still gained access to the murein layer, as shown in Fig. 3. The presence of EDTA facilitated, while Mg^{2+} prevented the action of lysozyme during the osmotic shock, as shown in Fig. 4.

These results are in clear contrast with the situation for exponentially growing cells, where the action of lysozyme is generally taken to be straightforward; lysozyme is added to a suspension of plasmolyzed cells, it gains access to the murein layer, and it proceeds to hydrolyze this layer [2–12, 18].

Penetration of lysozyme through the outer membrane and murein layer of stationary phase E. coli

The murein of *E. coli* consists of parallel polysaccharide chains which are crosslinked by peptide bridges [14, 19, 20], as indicated in Fig. 5. About 10 % of the murein peptides are bound to the outer membrane lipoprotein [21–23], and the peptide side of the murein layer must therefore face the outer membrane. Since the murein peptides do not fit in the lysozyme groove [24], lysozyme can bind to a polysaccharide chain only from the inside surface of the murein layer [22]. Thus, lysozyme must penetrate both the outer membrane and the murein layer before it can hydrolyze the murein polysaccharide chains.

The results of this paper show that this penetration is greatly enhanced by osmotic shock in the presence of EDTA. The penetration of lysozyme could be nonspecific: fissures may be created in the outer membrane, which permit the entry of lysozyme, just as they allow the release of periplasmic proteins [25]. Nevertheless, the outer membrane is anchored to the murein layer via the lipoprotein [21–23, 26] at about 250 000 sites per cell, or approx. every 13 nm^2 on average [14]. Given a thickness of 7.5 nm [26], it is difficult to see how such small and relatively thick membrane segments might be ruptured sufficiently to allow the penetration of lysozyme, which has a cross-sectional surface of about 8 nm^2 [24, 27]. In fact, no rupturing of the outer membrane of stationary phase spheroplasts has been detected by electron microscopy (ref. 15 and unpublished results). In addition, the penetrability caused by mild osmotic shock is transient, indicating that there can be no gross damage to the outer membrane.

An alternative to a nonspecific effect of osmotic shock is that lysozyme might be driven through the outer membrane channels postulated by Inouye [26]; lysozyme, which resembles a sphere with a diameter of 3.2 nm [24, 27], could penetrate through

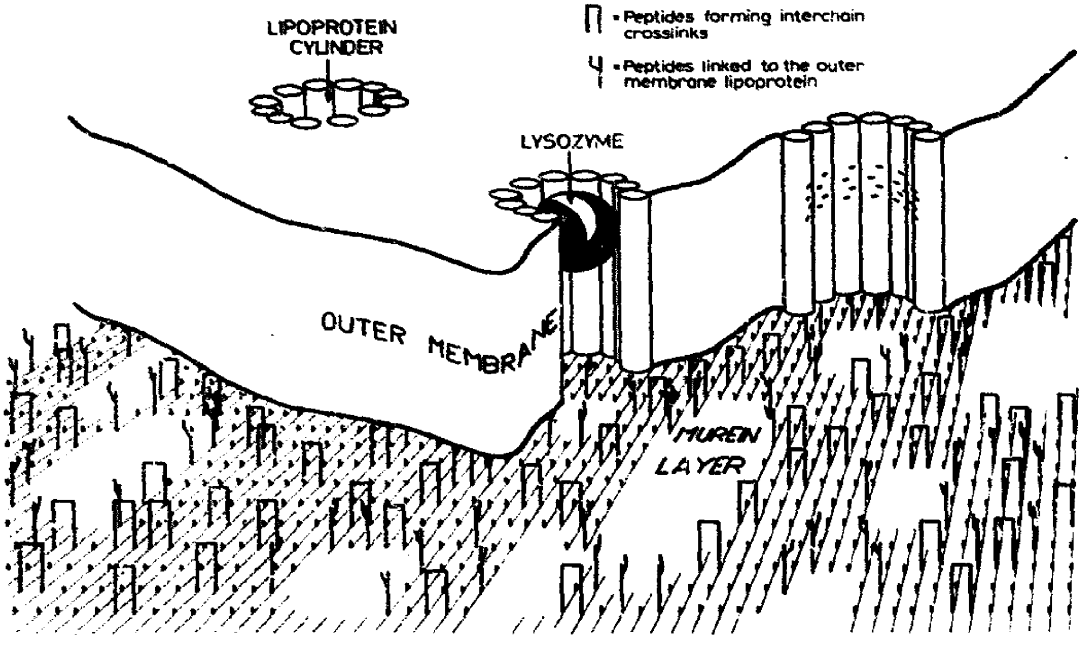

Fig. 5. Possible model for the penetration of lysozyme through the outer membrane and murein layer. The murein polysaccharide chains are indicated by parallel lines. The murein peptides originate at the short vertical marks. About 18 %* of them form interchain crosslinks [19], about 10 % of them are linked to the outer membrane lipoprotein [21–23], while the others form interchain crosslinks or remain free (not shown). The empty spaces indicate gaps in the murein layer [14, 20]. Cylindrical assemblies of lipoprotein form channels in the outer membrane [26] through which lysozyme might penetrate, as shown for the middle channel (see text for details).

an outer membrane channel formed by 12 lipoprotein molecules [26], as illustrated in Fig. 5. Such presumed channels are lined internally with negative charges [26], and the inrush of water which results from an osmotic shock might be necessary to drive the positively charged lysozyme molecules [24] past these negative charges.

Outer membrane channels, if they exist, should be closed to molecules with molecular weights in excess of 1000 [13]. Mg^{2+} might normally block these channels by forming ionic bridges between the negative charges within the channels, thereby preventing the entry of lysozyme upon mild osmotic shock. The addition of Mg^{2+} after lysozyme has been shocked through a channel might reblock that channel, but should have no effect on the activity of the lysozyme which has already penetrated underneath the murein layer (Fig. 4a).

Even after penetrating through the outer membrane and the murein layer, lysozyme cannot bind to polysaccharide chains that are stacked as closely as predicted by Formanek et al. [20]. Before a hexasaccharide segment can fit into the lysozyme cleft, the distance between the substrate chain and the two neighbouring polysaccharide chains must increase from the 0.44 nm predicted by Formanek et al. [20] to at

* The values of Schwarz and Leutgeb [19] concerned only peptides which were free or crosslinked to each other. Since 10 % of the peptides are linked to outer membrane lipoprotein [23] the data of Schwarz and Leutgeb [19] were used to calculate the distribution of the 90 % remaining peptides.

least 1.2 to 1.5 nm [27]. Braun et al. [14] have determined for several strains of *E. coli* that on the average there is one murein unit per 1.29 nm^2 of cell surface. Since each murein unit occupies 0.45 nm^2 in the structure determined by Formanek et al. [20], the murein of *E. coli* is likely to consist of a monolayer interrupted by gaps over about 65 % of its surface, as shown in Fig. 5, providing room for distension of adjacent polysaccharide chains near such gaps. The sudden water influx, due to osmotic shock, may increase the distance between adjacent polysaccharide chains near these gaps in the murein layer; this distance can potentially increase from 0.44 nm [20] to about 4 nm when the peptide crosslinks between adjacent polysaccharide chains are fully extended [14]. Thus, it is possible that the mild osmotic shock acts to loosen the murein structure in addition to allowing lysozyme penetration through the outer membrane.

Stabilization of stationary phase spheroplasts by Mg^{2+}

The Tris/EDTA/sucrose lysozyme treatment converted stationary phase rod shaped cells to spheroplasts very effectively. Nevertheless, there was no simple correlation between sphere formation and osmotic sensitivity; while spheroplasts lysed upon 11-fold dilution in water or 10 mM EDTA, they withstood the same dilution in 20 mM Mg^{2+} (Figs 3a and 4a).

Thus, the outer membrane plays a role in protecting stationary phase cells from dilution. Osmotic sensitivity arises only if lysozyme has degraded the murein layer and EDTA has destabilized the outer membrane. Although EDTA weakens the interactions between outer membrane components (lipopolysaccharides, proteins, phospholipids [28–30]), it probably causes no large scale damage; spheroplasts from stationary phase cells were stabilized quantitatively when Mg^{2+} was added within a few minutes after lysozyme treatment, indicating that the outer membrane still covered these spheroplasts completely. This was also seen in thin sections of Mg^{2+} stabilized spheroplasts (unpublished results).

These results are in contrast to those found for spheroplasts from exponential phase cells. EDTA treatment of such spheroplasts ruptures the outer membrane [2], while 5 mM $MgCl_2$ has been used to lyse spheroplasts of exponential phase cells [4, 8, 31]. Thus, the outer membrane of stationary phase cells appears to be significantly more stable than that of exponential phase cells.

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