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Ca^{2+}-Induced Permeabilization of the *Escherichia coli* Outer Membrane: Comparison of Transformation and Reconstitution of Binding-Protein-Dependent Transport

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Ca^{2+} treatment renders the outer membrane of *Escherichia coli* reversibly permeable for macromolecules. We investigated whether Ca^{2+}-induced uptake of exogenous protein into the periplasm occurs by mechanisms similar to Ca^{2+}-induced uptake of DNA into the cytoplasm during transformation. Protein import through the outer membrane was monitored by measuring reconstitution of maltose transport after the addition of shock fluid containing maltose-binding protein. DNA import through the outer and inner membrane was measured by determining the efficiency of transformation with plasmid DNA. Both processes were stimulated by increasing Ca^{2+} concentrations up to 400 mM. Plasmolysis was essential for a high efficiency; reconstitution and transformation could be stimulated 5- and 40-fold, respectively, by a high concentration of sucrose (400 mM) in cells incubated with a suboptimal Ca^{2+} concentration (50 mM). The same divalent cations that promote import of DNA (Ca^{2+}, Ba^{2+}, Sr^{2+}, Mg^{2+}, and Ni^{2+}) also induced import of protein. Ca^{2+} alone was found to be inefficient in promoting reconstitution; successive treatment with phosphate and Ca^{2+} ions was essential. Transformation also was observed in the absence of phosphate, but could be stimulated by pretreatment with phosphate. The optimal phosphate concentrations were 100 mM and 1 to 10 mM for reconstitution and transformation, respectively. Heat shock, in which the cells are rapidly transferred from 0 to 42°C, affected the two processes differently. Incubation of cells at 0°C in Ca^{2+} alone allows rapid entry of protein, but not of DNA. Transformation was observed only when exogenous DNA was still present during the heat shock. Shock fluid containing maltose-binding protein inhibited transformation (with 6 μg of DNA per ml, half-maximal inhibition occurred at around 300 μg of shock fluid per ml). DNA inhibited reconstitution (with 5 mg of shock fluid per ml, half-maximal inhibition occurred at around 3 mg of DNA per ml).

The *Escherichia coli* cell envelope consists of an outer and an inner membrane separated by the periplasmic space (21). The periplasm contains peptidoglycan, presumably in the form of a gel (16). The outer membrane contains pore-forming proteins in high copy number and acts as a molecular filter allowing passive entry of hydrophilic molecules with a molecular weight of up to 600. The inner membrane is the osmotic barrier of the cell. Active transport of substrates through this membrane is accomplished by specific transport systems. The periplasmic space harbors some enzymes and a variety of soluble binding proteins needed for transport of sugars, amino acids, and ions (27). The binding-protein-dependent maltose transport system (15) was used in this study to monitor permeability changes of the outer membrane for proteins.

Macromolecules like DNA and proteins cannot pass through either membrane (14). Competence for uptake of DNA through both membranes can, however, be accomplished in *E. coli* by Ca^{2+} treatment at 0°C followed by a short temperature shift up to 42°C (23, 32). We have recently shown that Ca^{2+} treatment of *E. coli* and *Salmonella typhimurium* at a low temperature induces a reversible permeabilization of the outer membrane of these organisms. This phenomenon allows efficient reconstitution of transport and chemotaxis in binding-protein-deficient mutants after import of exogenous binding protein into the periplasm (4–6). Under optimal conditions, about 20% of the cells were fully competent for uptake of proteins into the periplasm. This was seen by microscopic observation of single cells reconstituted in maltose chemotaxis (6). The inner membrane is apparently much less susceptible to Ca^{2+}-induced permeabilization (5). Under optimal conditions, with strains with a high efficiency of transformation, uptake of plasmid DNA was seen only in about 0.1 to 1% of the cells. The same individual cells preferentially took up a second plasmid (1, 13). The amount of DNA introduced into the periplasm was much higher than that found in the cytoplasm (29).

In the present study we compared conditions affecting import of plasmid DNA during transformation with those affecting reconstitution of maltose transport by maltose-binding protein (MBP). Most parameters, such as the Ca^{2+} concentration optimum, the effect of other divalent cations, and stimulation by plasmolysis and phosphate ions, were similar for both processes. We found that transformation is inhibited by the addition of protein, and reconstitution is inhibited by the addition of DNA. We suggest that DNA and protein might gain access to the periplasm via the same route. An important difference, however, was that permeation of protein through the outer membrane was achieved by Ca^{2+} treatment at 0°C alone, whereas permeation of DNA through the cell envelope needed an additional heat shock at 42°C.

MATERIALS AND METHODS

Growth of the cells. Overnight cultures were grown at 37°C in minimal medium A (24) plus 0.4% glycerol.

Preparation of shock fluid. Shock fluid of maltose-induced cells of strain pop1080 [HfrG6 *lamB102*(Am) *metA trpE*(Am) *galE galY*] (18), containing between 30 and 50% of total protein as MBP, was prepared by osmotic shock by the

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method of Neu and Heppel (25). The shock fluid was dialyzed and lyophilized as described previously (5).

Preparation of DNA. Plasmid DNA for transformation was isolated by the method of Birnboim and Doly (3) from a derivative of MC4100 (8), strain DL291 [*gyrA* Δ(*glpT-glpA*)*593 zei-724*::Tn*10 glpR*] (20), harboring plasmid pACYC184 (Tet^r Cam^r) (9). The DNA was further purified by $CsCl_2$ gradient centrifugation followed by phenol extraction as described by Schumann and Bade (30). The DNA was dissolved in TE buffer (10 mM Tris-hydrochloride [pH 7.8] and 1 mM EDTA [pH 8]) to a final concentration of 60 μg/ml.

DNA for the inhibition experiments (see Fig. 7) was calf thymus DNA (type I) from Sigma Chemical Co. DNA was sheared by sonification. The resulting DNA fragments were precipitated with ethanol (final concentration, 50%) and suspended in TE buffer to a final concentration of 13 mg/ml. The size of the fragments was 300 to 500 base pairs as estimated by agarose gel electrophoresis.

Standard procedure for reconstitution of maltose transport. If not otherwise indicated, reconstitution experiments were done with slight modifications of the procedure described previously (5). Cultures of strain HS3018 (MC4100 Δ*malE444 malT^c-1*) (31) were grown in minimal medium A containing 0.2% glycerol at 37°C. In this strain the remaining genes of the maltose regulon are expressed constitutively due to the *malT^c-1* allele. Cells were harvested in the exponential phase.

(i) Pretreatment. Samples of 2×10^9 cells were spun down and washed at room temperature with 5 ml of 50 mM Tris-hydrochloride (pH 7.2), followed by 1 ml of 100 mM potassium phosphate buffer (pH 7.2). The supernatant was completely removed, and the cells were washed with 1 ml of 50 mM Tris-hydrochloride (pH 7.2) containing 300 mM $CaCl_2$ (0°C).

(ii) Introduction of MBP. The cells were suspended at 0°C in 50 μl of the above Tris-Ca^{2+} buffer containing 1 mg of lyophilized shock fluid (final MBP concentration of about 10 mg/ml, corresponding to about one-fourth of the periplasmic MBP concentration of wild-type cells [11]) and vigorously shaken at 0°C for 30 min. Shock fluid was used, since purified MBP and crude shock fluid had virtually the same activity in reconstitution (5).

(iii) Transport assay. After centrifugation, the cells were washed with 1 ml of 0.9% NaCl at room temperature, suspended at room temperature in 1 ml of minimal medium containing 10 mM glycerol, and assayed for initial rates of maltose uptake as described previously (5).

Standard procedure for transformation. If not otherwise indicated, the regimen for transformation of strain HS3018 was very similar to that used for reconstitution. The efficiency of transformation was the same as that obtained with a commonly used transformation procedure (10). The efficiency in strain HS3018, an MC4100 derivative, was, as expected, lower than that of strains selected for high transformability (8). Cultures of strain HS3018 were grown and harvested as for reconstitution.

(i) Pretreatment. Samples of 2×10^9 cells were washed in 5 ml of 50 mM Tris-hydrochloride (pH 7.2) at room temperature and then washed once with 1 ml of 50 mM Tris-hydrochloride (pH 7.2) containing 300 mM $CaCl_2$ at 0°C; in one experiment (see Fig. 5), cells were pretreated with 50 mM Tris-hydrochloride (pH 7.2) containing 1 to 100 mM potassium phosphate buffer (pH 7.2).

(ii) Introduction of DNA. The cells were suspended at 0°C in 50 μl of the above Tris-Ca^{2+} buffer and immediately mixed with 5 μl of pACYC184 DNA dissolved in TE buffer. The DNA concentration in the transformation mixture (6 μg/ml) was kept limiting. The suspension was kept at 0°C for 30 min and then transferred to 42°C for 2 min (heat shock). A 1-ml sample Luria broth medium (24) (1 ml) was added, and the cells were incubated at 37°C for 40 min to allow phenotypic expression of resistance genes. Samples (200 μl) of the transformation mixture were plated on Luria broth plates containing 5 μg of tetracycline per ml and incubated overnight at 37°C. The viability of the recipient cells was determined by plating appropriate dilutions of the transformation mixture onto Luria broth plates. The efficiency of transformation is given as the number of tetracycline-resistant transformants per viable cell.

RESULTS

Ca^{2+} dependence of transformation and reconstitution. The efficiency of transformation increased with increasing Ca^{2+} concentration up to 400 mM (Fig. 1A). A very similar Ca^{2+} dependence, with an identical Ca^{2+} optimum at 400 mM, was found for reconstitution of maltose transport (Fig. 1B). At this Ca^{2+} concentration the initial rate of reconstituted

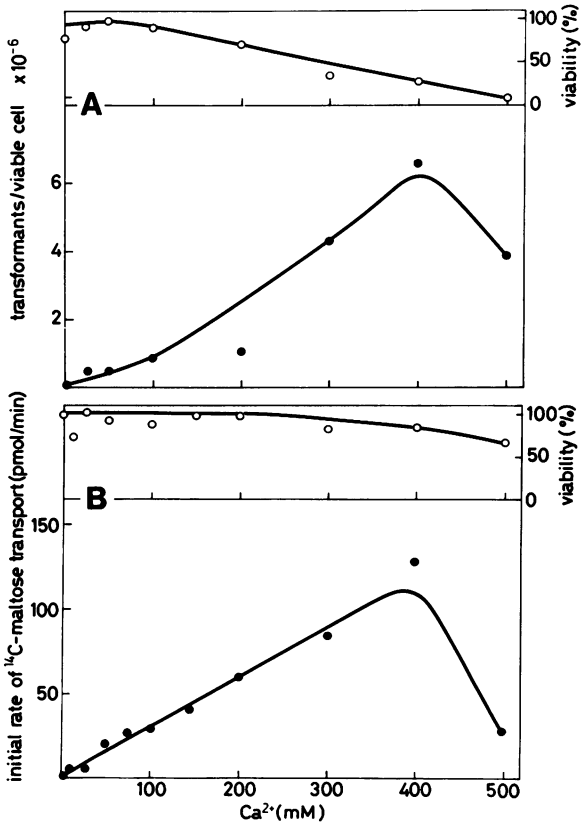

FIG. 1. Calcium dependence of transformation and reconstitution in strain HS3018 (Δ*malE444 malT^c-1*). Samples of 2×0^9 cells were subjected to the standard procedures of transformation and reconstitution, except that the Ca^{2+} concentration was varied from 0 to 500 mM in the wash medium and in the transformation and reconstitution mixture. The efficiency of transformation is expressed as Tet^r transformants per viable cell. The efficiency of reconstitution is expressed as picomoles of [^{14}C]maltose taken up per minute by 3×10^7 cells. Symbols: (○) viability, (●) efficiency of transformation (A) or reconstitution (B).

maltose transport reaches 40 to 50% of the wild-type rate (see Fig. 7). In contrast to the high viability of cells subjected to the reconstitution procedure (80% at 400 mM $CaCl_2$), the viability of cells subjected to the transformation procedure strongly decreased with increasing Ca^{2+} concentration (20% at 400 mM $CaCl_2$), presumably due to the heat shock (Fig. 1A).

Plasmolysis of the cells is essential for efficient transformation and reconstitution. The high Ca^{2+} concentration needed for optimal transformation and reconstitution caused plasmolysis of the cells. This could be concluded from the observed increase of the optical density at 578 nm of the cell suspension from 0.95 to 1.55 after the addition of 300 mM Ca^{2+}. A similar effect has been described for $CaCl_2$ and other nonpermeable, plasmolyzing solutes (22). To test whether plasmolysis contributed to the efficiency of transformation and reconstitution, cells of strain HS3018 were treated with 50 mM Tris-hydrochloride (pH 7.2) containing 50 mM Ca^{2+} and increasing concentrations of sucrose. The addition of sucrose stimulated transformation and reconstitution of maltose transport by factors of 40 and 5, respectively. The sucrose concentration optimum was 400 mM for both processes (Fig. 2). The viability of the cells at the optimal sucrose concentration of 400 mM decreased to about 60 and 80%, respectively, in transformation and maltose transport reconstitution. A treatment of cells in buffer containing 400 mM sucrose and shock fluid (MBP concentration, 10 mg/ml) in the absence of $CaCl_2$ did not allow reconstitution (data not shown).

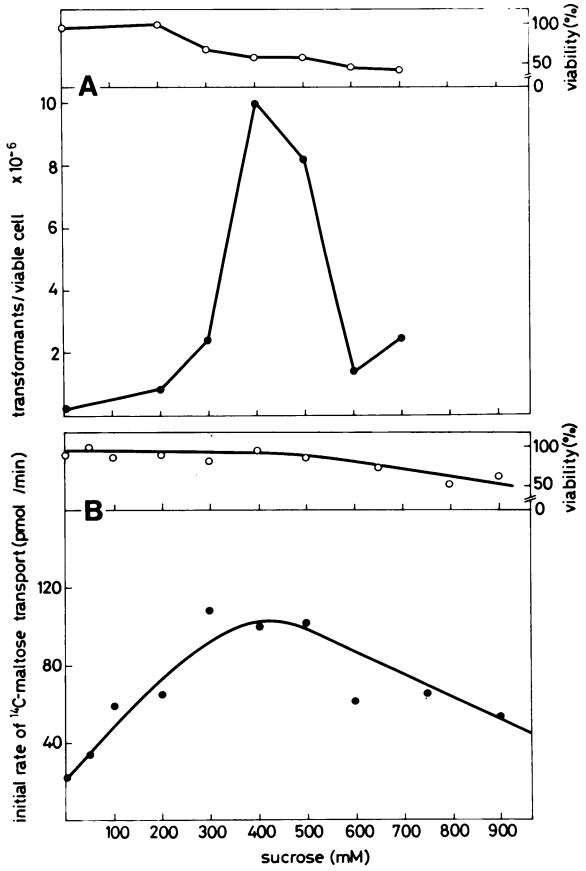

FIG. 2. Stimulation of transformation and reconstitution by plasmolysis. Cells of strain HS3018 were subjected to the standard procedures of transformation and reconstitution, except that the cells were treated with 50 mM $CaCl_2$ and increasing concentrations of sucrose (0 to 900 mM) in the prewash and the transformation and reconstitution mixture. Symbols: (○) viability, (●) efficiency of transformation (mean values of four independent experiments are given) (A) and reconstitution (B) as defined in the legend to Fig. 1.

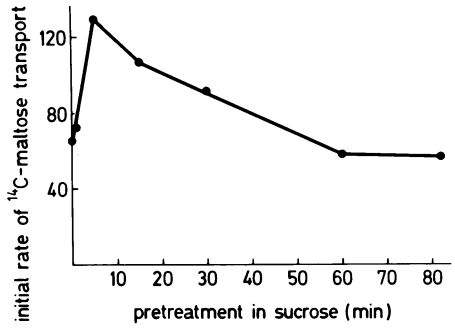

FIG. 3. Temporal course of stimulation of reconstitution by plasmolysis. A sample of 1.4×10^{10} cells of strain HS3018 was subjected to the standard procedure of reconstitution, except that the cells were pretreated at 0°C with 2.1 ml of 50 mM Tris-hydrochloride (pH 7.2), containing 50 mM $CaCl_2$ and 400 mM sucrose. Samples of this mixture were centrifuged at 4°C immediately or at the times indicated, suspended in the same buffer containing MBP, and further incubated for 30 min at 0°C. The efficiency of reconstitution is defined as in the legend to Fig. 1. Transport rates are given in picomoles per minute.

We studied the time dependence of the stimulatory effect of plasmolysis on reconstitution by adding MBP to cells pretreated for various times with 50 mM Tris-hydrochloride (pH 7.2) containing 400 mM sucrose and 50 mM $CaCl_2$ (Fig. 3). Reconstitution performed in the same buffer was optimal after 5 min of pretreatment. The plasmolysis effect declined slowly. Even after 80 min of pretreatment in sucrose, the efficiency of reconstitution was still 3 times higher (Fig. 3) than in the absence of sucrose (Fig. 2).

Ion specificity of reconstitution. The permeabilization of the outer membrane can also be induced by divalent cations other than Ca^{2+}. Ba^{2+}, Sr^{2+}, Mg^{2+}, and Ni^{2+} can promote reconstitution of maltose transport with the efficiency de-

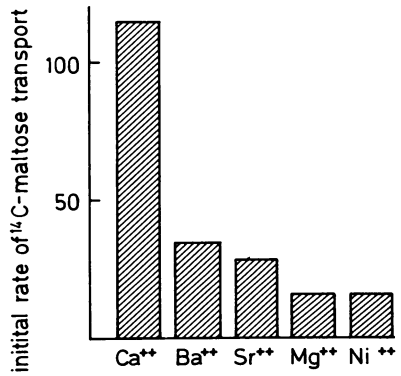

FIG. 4. Ion specificity of reconstitution. Cells of strain HS3018 were subjected to the standard procedure of reconstitution, except that other divalent cations were substituted for Ca^{2+}. All ions were tested at a concentration of 150 mM. Transport rates (picomoles per minute) are defined as in the legend to Fig. 1.

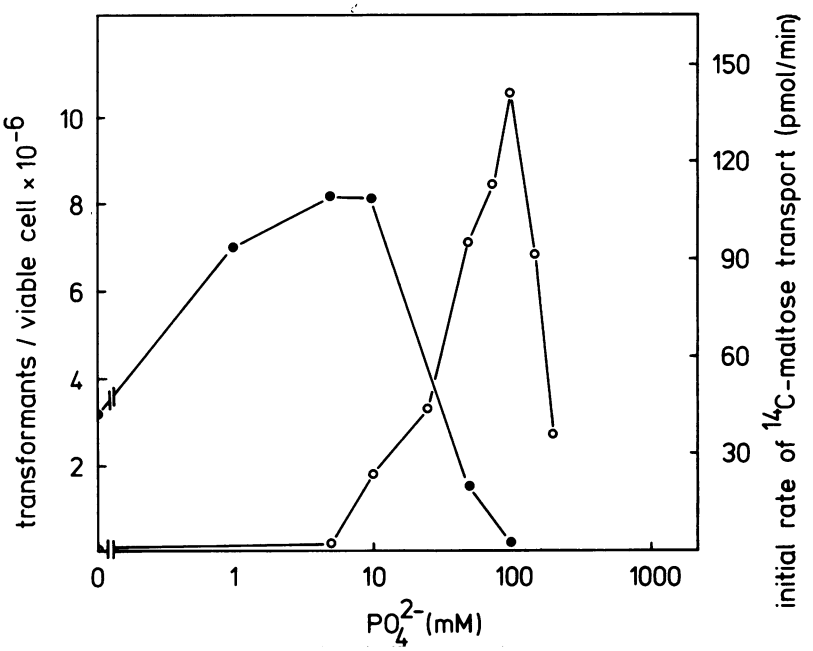

FIG. 5. Combined action of phosphate and calcium ions in reconstitution and transformation. Cells were washed at room temperature with 50 mM Tris-hydrochloride (pH 7.2) containing between 0 and 200 mM of potassium phosphate buffer (pH 7.2). After centrifugation the supernatant was completely removed, and the cell pellet was suspended in 50 μl of Tris-Ca^{2+} buffer containing DNA or MBP and subjected to the standard procedure for transformation and reconstitution. The efficiency of transformation (●) is the mean value of three or four determinations. The efficiency of reconstitution (○) is defined as in the legend to Fig. 1.

creasing in that order (Fig. 4). All monovalent cations tested (NH_4^+, Li^+, Rb^+) were ineffective.

Combined action of Ca^{2+} and phosphate is essential for reconstitution and stimulates transformation. Washing HS3018 cells with Ca^{2+}-free 100 mM Tris-hydrochloride (pH 7.2) or NaCl (0.9%) before the addition of Ca^{2+} resulted in a complete loss of competence for reconstitution (5). Pretreatment of the cells with 50 mM Tris-hydrochloride (pH 7.2) containing increasing concentrations of potassium-phosphate buffer (pH 7.2), however, fully restored competence. Optimal restoration was observed with 100 mM phosphate (Fig. 5). The efficiency of reconstitution of cells subjected to this pretreatment was even higher than that of cells treated with Ca^{2+} immediately after harvesting from the phosphate-containing growth medium.

A treatment with phosphate buffer alone at 0°C did not permeabilize the outer membrane (Table 1). When the order of washing steps was reversed and the cells were pretreated first with Ca^{2+} and then with phosphate, the efficiency of reconstitution was reduced by 80% (Table 1). Phosphate was almost totally ineffective when added to the Ca^{2+}-containing permeabilization mixture before or after the addition of the cells (Table 1).

To test whether phosphate pretreatment also stimulates transformation of *E. coli*, Tris-washed cells of strain HS3018 were subjected to a second wash with various concentrations of phosphate before the addition of DNA in the presence of Ca^{2+}. Low phosphate concentrations (1 to 10 mM) enhanced transformation by a factor of 2 to 3. Higher concentrations resulted in a strong decrease in transformation efficiency (Fig. 5). Phosphate was inhibitory at all concentrations when added to the transformation mixture itself (cells, Ca^{2+}, and DNA) (data not shown).

Simultaneous addition of shock fluid and DNA inhibits transformation and reconstitution. To determine whether protein inhibits DNA uptake and vice versa, transformation and reconstitution were performed in the presence of increasing concentrations of the potentially inhibitory macromolecule. Because of the different phosphate optima for DNA and protein uptake, suboptimal, compromise concentrations of phosphate had to be used. Thus, transformation

TABLE 1. Phosphate dependence of reconstitution[a]

Pretreatment conditions in wash steps:			Permeabilization conditions (30 min, 0°C)	Reconstitution efficiency ([^{14}C]maltose transport [pmol/min])
1	2	3		
Tris			Ca^{2+}-Tris-MBP	0.4
Tris	Phosphate		Phosphate-MBP	0
Tris	Ca^{2+}-Tris	Phosphate	Ca^{2+}-Tris-MBP	35
Tris	Phosphate	Ca^{2+}-Tris	Ca^{2+}-Tris-MBP	164
Tris			Ca^{2+}-Tris-phosphate-MBP	1.8
Tris			Ca^{2+}-Tris-MBP added to cells, phosphate added 1 min later to cell suspension	5

[a] Cells were subjected to the standard procedure of reconstitution, except that they were washed, in the order indicated, with 50 mM Tris-hydrochloride (Tris [pH 7.2]) at room temperature, the same buffer containing 300 mM $CaCl_2$ (Ca^{2+}-Tris) at 0°C, and 100 mM potassium phosphate buffer (phosphate [pH 7.2]) at 0°C. Lyophilized shock fluid containing MBP was dissolved in 50 μl of Ca^{2+}-Tris, phosphate, or Ca^{2+}-Tris premixed with 5 μl of 700 mM phosphate and incubated with cells for 30 min at 0°C. In one sample, 5 μl of 700 mM phosphate was added to the Ca^{2+}-Tris-MBP mixture (50 μl) 1 min after addition to the cells. The efficiency of reconstitution was determined as described for the standard procedure for reconstitution.

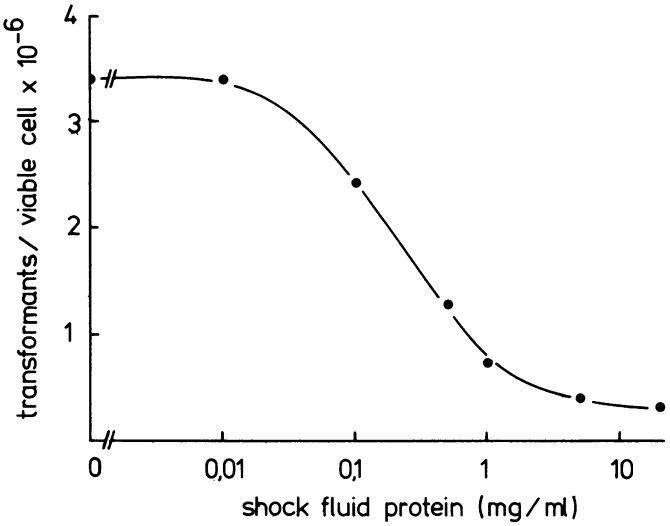

FIG. 6. Inhibition of transformation by the addition of MBP-containing shock fluid. Samples of 10^9 cells of strain HS3018 were subjected to the standard procedure for transformation, except that the cells were pretreated with 20 mM phosphate buffer and that different concentrations of MBP-containing shock fluid were added simultaneously with DNA to the transformation mixture. The efficiency of transformation is defined as in the legend to Fig. 1.

and reconstitution were measured with cells pretreated with 20 and 100 mM phosphate, respectively.

Inhibition of transformation with 6 μg of DNA per ml was half maximal at 300 μg of shock fluid per ml, but was not complete even at much higher concentrations (Fig. 6). This inhibition was not due to DNase activity in the shock fluid, since pACYC184 DNA incubated under the conditions of the experiment did not show any signs of degradation when checked by agarose gel electrophoresis (data not shown).

Inhibition of reconstitution with shock fluid containing 2.5 mg of MBP per ml was half maximal at 3 mg of DNA per ml and appeared to be complete at 7 mg of DNA per ml. Wild-type cells subjected to an identical treatment showed no inhibition of maltose transport (Fig. 7).

Inhibition of reconstitution was not due to Ca^{2+}-induced precipitation of DNA-MBP complexes. This could be concluded from the finding that samples taken from the supernatant of the reconstitution mixture after pelleting the cells showed virtually the same maltose-binding activity at all DNA concentrations (data not shown).

Role of the heat pulse in transformation and reconstitution. The essential role of a heat pulse in the presence of Ca^{2+} for entry of DNA during transformation has been established (2, 23). We observed transformation only after a shift of the cells for a short period (2 min) to 42°C. In contrast, reconstitution of maltose transport was not stimulated by a heat pulse (Fig. 8). This held for optimal (300 mM) and suboptimal (25 mM) Ca^{2+} concentrations.

To examine the possibility that DNA might at least enter the periplasm at 0°C, cells were incubated on ice for 30 min in Ca^{2+}-containing buffer in the presence of DNA. One sample of these cells was heat pulsed immediately; a second sample was washed twice with NaCl, suspended in Ca^{2+}-containing buffer at 0°C, and then heat pulsed. A 1,000-fold higher efficiency of transformation (5.3×10^{-6} transformants per viable cell) was seen when exogenous DNA was still present during the heat pulse than when it was removed by washing (5.6×10^{-9} transformants per viable cell). The viability was similar in both samples. This indicates that the heat pulse might be necessary for the uptake of DNA

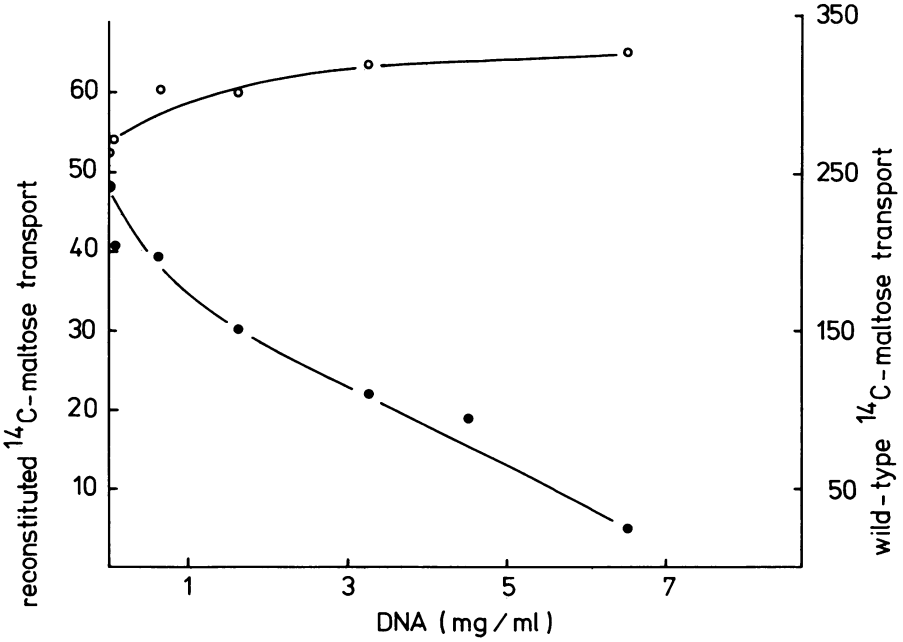

FIG. 7. Inhibition of reconstitution by the addition of DNA. Samples of 10^9 cells of strains HS3018 (MC4100, Δ*malE444 malT*^{c}-*1*) and JB3018-2 (MC4100, *malE*$^+$ *malT*^{c}-*1*) were subjected to the standard procedure for reconstitution, except that the MBP concentration was about 2.5 mg/ml (fourfold below our standard concentration) and that different concentrations of sheared calf thymus DNA were added simultaneously with shock fluid to the reconstitution mixture. The efficiency of reconstitution (●, picomoles per minute; mean values of two independent experiments) is defined as in the legend to Fig. 1. Wild-type maltose transport in strain JB3018-2 (○, picomoles per minute) is given as a control.

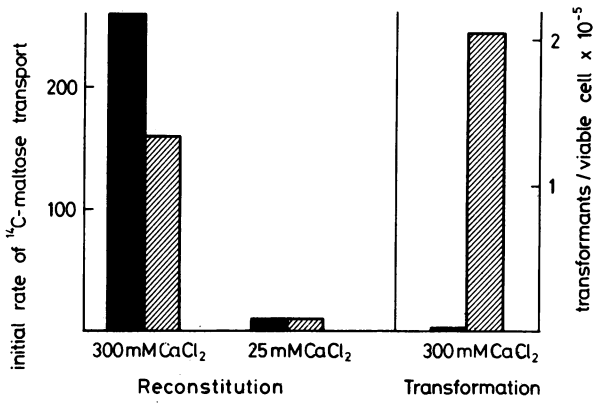

FIG. 8. Effect of heat shock of Ca^{2+}-treated cells on transformation and reconstitution. Cells for reconstitution were subjected to the standard procedure, except that they were treated with 300 or 25 mM $CaCl_2$ and that the cells suspended in the reconstitution mixture were subjected to a heat shock for 2 min at 42°C (□) or kept at 0°C (■). After washing in 0.9% NaCl, cells were assayed for maltose transport. Cells for transformation were subjected to the standard procedure at 300 mM $CaCl_2$, except that the cells suspended in the transformation mixture were subjected to a heat shock for 2 min at 42°C (□) or kept at 0°C (■). Transport rates are given in picomoles of [^{14}C]maltose taken up per minute by 3×10^7 cells.

through the outer membrane. The assumptions under which this conclusion holds are discussed below.

DISCUSSION

In the present paper we investigated whether the Ca^{2+}-induced uptake of protein into the periplasm (4, 5) is mechanistically related to the Ca^{2+}-induced uptake of DNA during transformation (23). Using an *E. coli* strain containing a nonpolar deletion in *malE*, we compared the conditions required for transformation by plasmid DNA with the conditions necessary for reconstitution of maltose transport by exogenous MBP (4, 5).

Plasmolysis of the Ca^{2+}-treated cells turned out to be an important prerequisite for optimal efficiency of transformation and reconstitution of transport. Sucrose strongly stimulated the efficiency of both processes at a suboptimal Ca^{2+} concentration (Fig. 2). At 400 mM sucrose a Ca^{2+} concentration optimum of about 150 mM was found (data not shown). These results show that the high Ca^{2+} optimum (400 mM) found in the absence of sucrose (Fig. 1) partially reflects a requirement for plasmolysis. Plasmolyzed cells take up increased amounts of MBP, and possibly also DNA, into the enlarged periplasm. As monitored by sucrose-stimulated reconstitution, the cells adapted only slowly to plasmolysis (Fig. 3), indicating that entry of DNA can probably also be stimulated by plasmolysis even after longer periods of incubation (e.g., during heat shock). The stimulatory effect of monovalent cations at concentrations of around 100 mM reported for transformation procedures employing much lower Ca^{2+} concentrations (13) may also partially be due to plasmolysis.

The much stronger effect of plasmolysis on transformation as compared with reconstitution (Fig. 2) suggests that plasmolysis also might permeabilize the inner membrane. The alternative explanation, that imported MBP is not limiting for reconstituted maltose transport, is less likely, since MBP was added at a concentration fourfold below that needed for half-maximal reconstitution (5).

Ca^{2+} ions alone appeared to be ineffective in permeabilization of the outer membrane for entry of proteins. Treatment of Tris-washed cells with phosphate before the addition of Ca^{2+} was essential for competence for reconstitution (Table 1). Phosphate at lower concentrations also stimulated transformation (Fig. 5). Stimulation by phosphate was seen only when phosphate was added to the cells before the addition of Ca^{2+}. Conditions that do not allow the precipitation of calcium phosphate complexes on the membrane surface, such as acidic pH or the addition of preformed calcium-phosphate crystals, resulted in a very low efficiency of reconstitution (5) (Table 1). Our interpretation of the dual requirement for Ca^{2+} and phosphate is that calcium-phosphate complexes are formed in close contact with the cell envelope and interact with the highly negatively charged lipopolysaccharide molecules to permeabilize the outer membrane.

Phosphate also strongly stimulates the Ca^{2+}-induced permeabilization and fusion of phospholipid vesicles (12) and of erythrocyte ghosts (17). This stimulation also requires treatment with phosphate before the addition of Ca^{2+}. Calcium-phosphate complexes are believed to lead to a local destabilization of membrane bilayers by dehydration, transition from the gel to the liquid-crystalline phase, and phase separation of the phospholipids. Multivalent calcium-phosphate complexes are more effective than free calcium in this respect, presumably because they cause extensive lipid cross-linking (7, 12, 26).

The reason that different phosphate concentration optima were found for stimulation of reconstitution (100 mM) and transformation (1 to 10 mM) (Fig. 5) might be that binding of DNA to outer and inner membranes (28, 36) is competitively inhibited by phosphate. Alternatively, high phosphate concentrations (100 mM) might lead to firm attachment of Ca^{2+}-phosphate-DNA complexes to the outer surface of the cells, blocking subsequent transport of DNA through the cell envelope.

The fact that transformation (in contrast to reconstitution) also occurred in the absence of P_i may indicate that DNA, complexed at its own phosphate groups with Ca^{2+}, catalyzes its own entry. Binding of Ca^{2+} to DNA also might induce an extensive condensation of the DNA molecule, which may be important for efficient permeation through the cell envelope.

The findings that the addition of shock fluid to the transformation mixture inhibited transformation (Fig. 6) and that the addition of DNA to the reconstitution mixture inhibited reconstitution (Fig. 7) support the hypothesis that transformation and reconstitution are mechanistically related. DNA and protein might compete for a common binding site on the cell surface. Binding might be a prerequisite for permeation through Ca^{2+}-induced disruptions of the outer membrane. DNA or protein tightly bound to the cell envelope in the form of a dense matrix also may block the free entry of protein or DNA through Ca^{2+}-induced disruptions. Alternatively, inhibition could occur if binding of DNA and protein interfered with the process of outer membrane permeabilization itself, for instance, by inhibition of phase separation of lipopolysaccharide molecules.

A striking difference between transformation and reconstitution became apparent when the role of the heat shock was examined. Proteins can pass through the outer membrane during a Ca^{2+} treatment at 0°C, whereas DNA can pass through the cell envelope only during or after a temperature shift of Ca^{2+}-treated cells from 0 to 42°C (Fig. 8) (2, 23, 35, 36).

We examined the possibility that DNA might enter the periplasm during the Ca^{2+} treatment at 0°C. The finding that

a high efficiency of transformation was seen only when exogenous DNA was still present during the heat pulse does not support this possibility. Rather, entry of DNA through the outer membrane may occur only during or after a heat pulse. Our experiment did not unequivocally prove this interpretation, but it could be valid if the following two assumptions hold. (i) DNA is not removed from the periplasm by washing in NaCl (MBP introduced into the periplasm could not be washed out under identical conditions; data not shown). (ii) Washing does not abolish the competence of the cells for uptake of DNA through the cytoplasmic membrane.

The conclusion that DNA might pass through the outer membrane during the heat step has also been arrived at by others, in part because imported DNA molecules became resistant to DNase only after heat shock (2, 36). We think, however, that results obtained with DNase have to be interpreted with care, because we have previously shown that proteins of low and high molecular weight easily enter the periplasm of Ca^{2+}-treated cells at 0°C (5). The drastic effect of heat shock on the outer membrane, and perhaps also the inner membrane, is indicated by the strongly increased release of periplasmic proteins from heat-shocked cells (35) and by their reduced viability compared with that of cells kept at 0°C (Fig. 1). A stronger perturbation of the membranes is obviously essential for uptake of DNA molecules (e.g., pACYC184; molecular weight about 3×10^6), whereas much smaller protein molecules (e.g., MBP; molecular weight about 4×10^4) can enter the periplasm during a Ca^{2+} treatment at 0°C.

In summary, most conditions affecting reconstitution and transformation were similar: the Ca^{2+} concentration and pH optima (Fig. 1) (5, 34), the effect of other divalent cations (Fig. 4) (19), stimulation by plasmolysis (Fig. 2) and phosphate (Fig. 5), the kinetics of entry of DNA and MBP (5, 33), and the requirement for exponential-phase cells (5, 34). In addition, a mutual exclusion of DNA and MBP was observed (Fig. 6 and 7). Optimal conditions for reconstitution specifically reflect optimal permeabilization of the outer membrane. We conclude from the similar requirements for transformation that most aspects of the transformation protocol mainly influence the permeation of DNA through the outer membrane. Both macromolecules seem to gain access to the periplasm via the same route.

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