***Bacillus subtilis* levansucrase: the efficiency of the second stage of secretion is modulated by external effectors assisting folding**

Régis Chambert, El Arbi Haddaoui and Marie-Françoise Petit-Glatron

Author for correspondence: Régis Chambert. Tel: +33 1 44 27 47 19. Fax: +33 1 44 27 59 94.

Institut Jacques Monod,
CNRS/Université Denis
Diderot (Paris 7),
Laboratoire Génétique
et Membranes, Tour 43,
2 place Jussieu, 75251 Paris
Cedex 05, France

We investigated whether the concentration of H^+ or metal ions such as Ca^{2+} , or both, on the external side of the cytoplasmic membrane is involved in coupling of folding and secretion of *Bacillus subtilis* levansucrase by studying the modulation of each isolated event. *In vitro* at 30 °C, in the absence of Ca^{2+} , the equilibrium between the unfolded and the folded states of levansucrase was rapidly and totally displaced toward the folded state by a small pH shift from 7.4 to 6.0. Ca^{2+} (> 5 mM) acted as a catalyst of folding at $pH \geq 7$. *In vivo* pulse-chase experiments at 30 °C showed that, in the absence of Ca^{2+} , the rate and the yield of the second step of levansucrase secretion were strongly dependent on the external pH. In acidic growth medium (pH 5.8), secretion was efficient. In contrast, at $pH \geq 7$, the presence of Ca^{2+} was essential for secretion. In bacteria grown at high temperature (48 °C), both external acidic pH and Ca^{2+} were required for efficient secretion. Moreover, a levansucrase variant altered in its calcium affinity was efficiently secreted only under acidic growth conditions. Depending on the culture conditions, the differences in H^+ or Ca^{2+} concentrations which are maintained between the opposite sides of the energized cytoplasmic membrane could be adequate to catalyse conformational transition which could play a critical role in the second step of the protein release. These environmental parameters could also affect the yield of secretion of some other secretory proteins, leading to the hypothesis that several different secretion mechanisms could coexist in *B. subtilis*.

Keywords: levansucrase, *Bacillus subtilis*, protein folding, protein secretion, folding catalysts

INTRODUCTION

Levansucrase is an extracellular enzyme synthesized by *Bacillus subtilis* during the exponential phase of growth. It is secreted by a two-step mechanism (Petit-Glatron *et al.*, 1987). The first step of this mechanism consists of the proteolytic cleavage of the N-terminal signal sequence to give a mature membrane-associated form. The second step, which is rate limiting, is the release and concomitant conformational change of the protein. We have suggested that the energy required for the vectorial diffusion of the protein from the membrane phase to the external aqueous phase (driven reaction) is provided by the energy change accompanying the folding process (driving reaction). The efficiency of such a hypothetical coupling could strongly depend on the rate of the driving reaction, as we previously proposed from an irreversible thermodynamic point of view (Chambert *et al.*, 1990). This model predicts

that changes in the external concentration of any effector that modulates protein folding will also affect the efficiency of the second step. We have recently shown that in addition to Fe^{3+} (Chambert *et al.*, 1990), Ca^{2+} ions could also play such a role; these two metal ions catalyse *in vitro* the spontaneous folding of levansucrase at pH 7 and can be concentrated within the cell wall in the micro-environment of the external side of the cytoplasmic membrane (Beveridge & Murray, 1976, 1980; Petit-Glatron *et al.*, 1993).

In this work we focused our attention on the H^+ ion as a potential effector of folding and secretion, for several reasons. Preliminary studies have shown that the unfolding–folding transition of levansucrase is dependent on the ionization state of the protein within the physiological pH range (Chambert & Petit-Glatron, 1990). The involvement of H^+ ions can be studied because their

concentration on the external side of the membrane can be easily manipulated. At culture pH ranging from 5.8 to 7.8, *B. subtilis* grows normally and maintains its cytoplasmic pH unchanged near 7.4 (Khan & Macnab, 1980; Shioi *et al.*, 1980). This means that under acidic conditions of the culture medium, the H^+ concentration can be 200 times greater on the external side of the membrane than on the internal side.

Finally, effects of the transmembrane ΔpH on the *B. subtilis* protein secretion process have been observed (Hemilä *et al.*, 1992) but never clearly understood. Kemper *et al.* (1993) proposed that the cell wall may serve as a proton sink, causing the wall to have a relatively low pH in the immediate vicinity of the membrane. This may regulate cell-wall-associated enzymes in *B. subtilis*. From a thermodynamic point of view, it has been proposed that ΔpH is involved in secretion via its participation in the proton-motive force which has been postulated to supply the energy for protein translocation in prokaryotes (Randall *et al.*, 1987).

The question arises as to whether the proton concentration, *per se*, on the external side of the membrane promotes a spontaneous molecular event helping the protein vectorial translocation across the membrane. We here investigated the effect of pH on the *in vitro* unfolding–folding transition and on the *in vivo* efficiency of levansucrase secretion. Moreover, since we have demonstrated that Ca^{2+} ions can modulate both events under the usual pH growth conditions, i.e. pH 7 (Petit-Glatron *et al.*, 1993), we assessed the relative involvement of this metal ion and interactions, competition or co-operation, between these two cations in levansucrase secretion under various growth conditions.

METHODS

Enzymes, substrates and reagents. Subtilisin and Protein-A–Sepharose CL4B were from Sigma; *p*-nitrophenyl-maltoheptaoside was purchased from bio-Merieux; [^{35}S]methionine, $^{45}CaCl_2$ and ^{125}I -labelled protein A were from Amersham.

Purification of extracellular levansucrases. Levansucrase and its variant were purified from culture supernatants of induced strains QB112 and QB250 according to the procedure described by Dedonder (1966).

Purification of extracellular α -amylase. α -Amylase was purified from *B. subtilis* culture supernatant according to the method of Mäntsälä & Zalkin (1979).

Levansucrase assay. The sucrase activity of levansucrase was assayed as described by Dedonder (1966). Glucose and fructose release from sucrose hydrolysis were measured by the method of Somogyi (1945).

α -Amylase assay. α -Amylase activity was assayed using *p*-nitrophenyl-maltoheptaoside as a substrate under the conditions recommended by the supplier.

Pulse-chase experiments. *B. subtilis* cells in the exponential phase of growth were pulse-chased with [^{35}S]methionine as described previously (Chambert & Petit-Glatron, 1988).

Characterization of labelled transient membrane forms.

Bacteria in the exponential phase of growth and induced for levansucrase synthesis (50 mM sucrose final concentration) were labelled with [^{35}S]methionine [0.25 mCi (9.25 MBq) ml^{-1} , 1000 Ci (37 TBq) $mmol^{-1}$]. Samples (0.4 ml) were withdrawn at intervals. All reactions were stopped by diluting the samples with 5 vols ice-cold stopping buffer (0.1 M potassium phosphate, pH 7, containing 2 M KCl, 200 μg chloramphenicol ml^{-1} , 5 mM EDTA and 0.2 mM PMSF). The high-ionic-strength treatment releases any levansucrase remaining associated with the cell wall (Chambert & Petit-Glatron, 1984). After centrifugation, the pellets were washed with 0.5 ml 0.1 M sodium phosphate, pH 7, containing the protease inhibitors. The pellets from this step were resuspended in 0.3 ml 50 mM Tris/HCl, pH 7.4, containing 5 mM EDTA, 150 mM NaCl and 2% (w/v) SDS. The cells were then disrupted by sonication, the suspensions were incubated for 5 min at 95 °C and diluted in 1.7 ml TNET (50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 5 mM EDTA and 1%, w/v, Triton X-100). Purified anti-levansucrase antibodies (8 μl , 4 mg ml^{-1}) and 80 μl 10% Protein-A–Sepharose in TNET were then added and incubated overnight at 4 °C. The immunoprecipitates were recovered by centrifugation. The pellets were washed three times with 1 ml TNET, and finally resuspended in the electrophoresis sample buffer. After 5 min at 95 °C, the samples were analysed by SDS-PAGE.

Culture and strains. Strains QB112 (*sacA*₃₂₁ *sanU*₃₂) and QB250 [*sacA*₃₂₁ *sacU*₃₂ *sacB*(250)] were grown in minimal medium supplemented with 1% (w/v) glucose or 1% (w/v) glycerol as carbon source (Petit-Glatron *et al.*, 1993).

Gel electrophoresis and immunoblotting. Proteins were analysed on 10% SDS-polyacrylamide gels and the membrane-associated forms of levansucrase were analysed by immunoblotting under the conditions described previously (Petit-Glatron *et al.*, 1987).

Folding experiments. The unfolding–folding transitions of levansucrase were monitored by following changes in intrinsic fluorescence measured with a F2000 Hitachi thermoregulated spectrofluorimeter (Chambert & Petit-Glatron, 1990).

Protein– Ca^{2+} binding assays. Ca^{2+} binding to levansucrase and α -amylase was assayed by quantitative dot-blot (Maruyama *et al.*, 1984) using $^{45}CaCl_2$ [specific activity 10 mCi (370 MBq) mg^{-1}] on nitrocellulose.

RESULTS

In vitro unfolding–folding transition of levansucrase

Modulation of the refolding kinetics by pH and calcium concentration. The kinetics of the unfolding–folding transition of levansucrase were followed by measuring the changes in the intrinsic fluorescence of the protein as previously described (Chambert & Petit-Glatron, 1990). In a first set of experiments, the protein was unfolded in 8 M urea at 30 °C. Refolding was promoted by dilution of the denaturing agent in 0.1 M potassium phosphate buffer at a series of pH values from 5.8 to 7.8, which is in the range of physiological pH allowing normal *B. subtilis* growth. At each pH value, the kinetics of the transition were monitored in the absence or presence of 0.5 mM Ca^{2+} (Fig. 1). The protein displayed two different behaviours depending on the pH. From pH 5.8 to 6.8, the rate of

Fig. 1. Kinetics of levansucrase refolding with respect to the pH of the renaturation mixture at 30 °C. A sample of 1 μ l stock solution of purified levansucrase (5 mg ml⁻¹) was preincubated for 4 min in 20 μ l 8 M urea at pH 7. Refolding was initiated by adding 1 ml 0.1 M potassium phosphate at the pH indicated, containing (a) 1 mM EDTA, or (b) 0.5 mM calcium. Fluorescence intensity was recorded after urea dilution. The 'dead' time was estimated to be 5 s.

refolding decreased slightly and was not affected by Ca²⁺. At pH $\geq$ 7, the rate and the yield of refolding decreased strongly and became more calcium dependent as the pH value increased.

Effects of a small shift of pH or a jump in Ca²⁺ concentration on the transition. For these experiments, the amplitudes of the variations were chosen to correspond roughly to the differences in H⁺ or Ca²⁺ concentrations between the two sides of the cytoplasmic membrane. Levansucrase was unfolded at pH 7.4 in the absence of Ca²⁺, which are the conditions usually prevailing in the cytosol (Khan & Macnab, 1980). Refolding was promoted either by a small downward shift in pH or by the injection of Ca²⁺ in the renaturing mixture to give conditions simulating the environment of the external side of the membrane. When the pH was shifted from 7.4 to 6, the refolding reaction monitored by variation of the intrinsic fluorescence of the protein was fast ($t_{1/2}$ = 10 s) (Fig. 2a). Similar refolding kinetics were obtained when measuring refolding by the resistance to proteolytic degradation by subtilisin (Fig. 2b). The transition occurred in the absence of Ca²⁺. With the pH maintained at 7.4, complete refolding was obtained at 5 mM Ca²⁺, but the rate of the unfolding-refolding transition increased as a function of the increase in Ca²⁺ concentration (Fig. 3).

Thus, we can conclude that small variations in H⁺ or Ca²⁺ concentrations, compatible with physiological transmembrane pH or Ca²⁺ gradients, can significantly and rapidly displace the equilibrium between the unfolded and folded states of levansucrase.

Fig. 2. Unfolding-refolding transition of levansucrase following a pH shift from 7.4 to 6.0 at 30 °C in the presence of 1 mM EDTA. Levansucrase was unfolded by mixing (arrow 1) 5 μ l levansucrase stock solution (5 mg ml⁻¹) with 1 ml 0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA. After 2 min of unfolding, the pH was shifted to 6.0 by the addition of 10 μ l 5 M phosphoric acid (arrow 2). The kinetics of levansucrase unfolding and refolding were monitored by (a) the fluorescence intensity changes and (b) the resistance to subtilisin proteolytic degradation. Aliquots of 20 μ l were withdrawn at intervals and mixed with 10 μ l subtilisin solution (1 mg ml⁻¹). After 2 min incubation, 400 μ l 0.1 M sucrose solution containing 0.2 mM PMSF was added and sucrose activity was assayed. Subtilisin rapidly degrades the unfolded form of levansucrase but not the folded enzyme (Chambert et al., 1990).

***In vivo* efficiency of the second step of levansucrase secretion is modulated by the pH and Ca²⁺ concentration of the growth medium**

The rate of *B. subtilis* growth is similar at all pH values within the range 5.8 to 7.8. Since the *in vitro* folding properties of levansucrase differ greatly according to the external pH value, we have postulated that the folding properties of the transient processed membrane form of levansucrase might similarly vary. Thus, we evaluated the yield of levansucrase production, the kinetics of its release into the supernatant and its stability by pulse-chase experiments. We previously demonstrated that this type of experiment characterizes the kinetics of the second step of the secretion process (Chambert & Petit-Glatron, 1988).

Fig. 3. Kinetics of levansucrase refolding at pH 7.4 and 30 °C at various Ca^{2+} concentrations. A sample of 1 μl purified levansucrase stock solution (5 mg ml^{-1}) was preincubated for 4 min in 20 μl 8 M urea at pH 7.4. Refolding was initiated by adding 1 ml 0.1 M potassium acetate, pH 7.4, containing various concentrations of calcium chloride. The ionic strength was held constant in each experiment by the addition of potassium chloride. The unfolding-refolding transition was measured by following intrinsic fluorescence intensity changes. Calcium concentrations (mM) were 50, 20, 5, 1, 0.2, as indicated on the traces.

At 30 °C, levansucrase production was ten times higher at pH 5.8 than at pH 7.8 (Fig. 4a, b). The results demonstrated that it was the yield of the protein release which was affected at pH 7.8 and not the stability of the protein in the culture medium. As suggested by *in vitro* folding experiments, the spontaneous folding of levansucrase on the external side of the membrane may be too low at pH 7.8 and thereby prevent efficient release. In contrast, folding and secretion appear to be optimally coupled at pH 5.8. However, these results may also have been due to an effect of pH on the synthesis and processing of the transient membrane forms of levansucrase. We therefore examined the steady-state concentration of these

Fig. 4. Production of extracellular levansucrase by *B. subtilis* cultivated at pH 5.8 or pH 7.8. (a) SDS-PAGE of labelled extracellular levansucrase from a pulse-chase experiment as described in Methods. A, Culture at pH 5.8; B, culture at pH 7.8. Approximately the same amount of [^{35}S]methionine was incorporated in the bacteria at each pH during a pulse period of 1 min. (b) Curves obtained by quantification of the radioactive bands from the SDS gels. ●, Gel A; ○, Gel B.

Fig. 5. Steady-state production of transient levansucrase membrane forms by *B. subtilis* grown at pH 5.8 or pH 7.8 and 30 °C. (a) Pulse experiment. Bacteria fully induced by sucrose were labelled with [^{35}S]methionine at OD_{600} 2.0. Samples removed at the times indicated were treated and analysed as described in Methods. Immunoprecipitates of labelled levansucrase were obtained from bacteria grown at pH 5.8 (A) and pH 7.8 (B). Uptake of labelled methionine by bacteria was constant throughout the pulse period. (b) Immunoblotting. Bacteria grown at pH 5.8 (A) or pH 7.8 (B) and 30 °C were fully induced by sucrose (50 mM final concn). After two generation times (OD_{600} 1.5) 5 mM EDTA and 5 mM PMSF were added. Samples (5 ml) were removed at the times indicated. Cell extracts were prepared for immunoblotting analysis as previously described (Petit-Glatron *et al.*, 1987). (c) Quantification of immunoblotting analysis. ●, Gel A; ○, gel B.

Fig. 6. Effects of Ca^{2+} on the production of extracellular levansucrase by *B. subtilis* grown at pH 7.4. (a) SDS-PAGE of labelled extracellular levansucrase from a pulse-chase experiment as described in Methods. A, Culture at pH 7.4 in the presence of 0.2 mM Ca^{2+} ; B, culture at pH 7.4 with no added Ca^{2+} . EDTA (0.5 mM) was added to the culture 5 min before the pulse-chase. (b) Curves obtained by quantification of the radioactive bands from the SDS gels. ●, Gel A; ○, gel B.

intermediates by pulse experiments in bacteria grown at the two pH values (Fig. 5a). There were no significant differences either in the amount of labelled 50 kDa membrane form or in the processing of prelevansucrase during the continuous uptake of [^{35}S]methionine. The fact that the cell-associated form was not accumulated at pH 7.8 suggested that this unfolded form is proteolysed on the external side of the membrane rather than being folded and released. This hypothesis was substantiated by the strong accumulation of the 50 kDa membrane form only observed at pH 7.8 when two protease inhibitors, EDTA and PMSF, were added to the culture medium (Fig. 5b). The steady-state level of the 50 kDa membrane levansucrase form was increased by a factor of three as evaluated by quantitative immunoblotting analysis (Fig. 5c).

To confirm the correlation between the efficiency of protein release and the ability to fold rapidly, we tested whether Ca^{2+} ions are involved in the coupling between these two events at defined pH values for which Ca^{2+}

Fig. 7. Production of extracellular levansucrase by the mutant QB250 grown at pH 5.8 and pH 7 and 30 °C. (a) SDS-PAGE of labelled extracellular levansucrase from a pulse-chase experiment as described in Fig. 4. Production of extracellular wild-type levansucrase by strain QB112 grown at pH 7.0 and 30 °C (A) was compared with production of extracellular levansucrase by the mutant QB250 at pH 7 (B) and pH 5.8 (C) and 30 °C. (b) Curves obtained by quantification of the radioactive bands from the SDS gels. ■, Gel A; ○, gel B; ●, gel C.

catalyses folding *in vitro*. The pulse-chase experiments were conducted with bacteria cultivated at pH 7.4. The culture medium was complemented with either Ca^{2+} (0.2 mM) or the calcium chelator EDTA added at 5 mM, 5 min before the pulse. The yield of protein released into the extracellular medium was strongly dependent on the presence of free Ca^{2+} (Fig. 6a, b). A similar pulse-chase experiment carried out at pH 5.8 (result not shown) led us to conclude that Ca^{2+} is not required at this pH for efficient levansucrase secretion.

A levansucrase variant with altered calcium-binding affinity is secreted normally at pH 5.8 and poorly at pH 7.0

The amino acid at position 236 on mature levansucrase has been shown to play a crucial role in the unfolding–folding transition, since residue 236 is part of a calcium-binding site. The amino acid substitution Thr → Ile at this position alters both the folding transition and the efficiency of secretion of levansucrase under standard conditions of *B. subtilis* growth (pH 7). Increasing the Ca^{2+} concentration in the culture medium improves the yield of secretion of this variant (Petit-Glatron *et al.*, 1993).

Fig. 8. Levansucrase calcium binding as a function of pH. Samples of purified levansucrase (2 µg) were blotted onto nitrocellulose. Each piece of membrane was equilibrated for 15 min at room temperature in buffer at pH varying from 5.4 to 7.5, and then incubated for 30 min in 1 ml of the same buffer containing 20 µCi $^{45}\text{CaCl}_2$. Each membrane was washed for 20 min in a large volume of the buffer and air-dried. The dried membranes were first exposed to Kodak X-Omat film then excised and their radioactivity measured. (a) Autoradiography of the nitrocellulose pieces treated at the following pH values 1, 5.4; 2, 5.6; 3, 5.8; 4, 6.0; 5, 6.2; 6, 6.4; 7, 6.6; 8, 6.8; 9, 7.0; 10, 7.2; 11, 7.5. (b) Graphic representation of the counts (means of three individual experiments) of the excised spots versus the pH of the incubation and washing buffers. The inflexion point at pH 6.5 is arrowed.

The data presented in this work suggest that the secretion efficiency of the levansucrase variant (Thr₂₃₆ → Ile₂₃₆) might be affected at pH ≥ 7 only and not at lower pH values because at lower pH the protein would not need the presence of Ca²⁺ ions for folding. As expected, variant 236 was secreted at pH 5.8 with the same efficiency as the wild-type protein (Fig. 7a, gels A and C), whereas at pH 7 its secretion efficiency was very low compared to wild-type levansucrase (Fig. 7a, gel B). These findings support the hypothesis that the efficiency of the second step of secretion is coupled to the folding event.

H⁺ and Ca²⁺ may cooperate to couple the folding and secretion events when bacteria are grown at high temperature

We have shown above that Ca²⁺ is not required at 30 °C for efficient protein secretion when the external pH of the growth medium is acidic enough. Thus, it appears that Ca²⁺ does not function as a folding catalyst at low pH values. We therefore tested the calcium binding affinity throughout the pH range 5.8 to 7.8 by the method of Maruyama (1984). Surprisingly, the protein displayed a

Fig. 9. Production of extracellular levansucrase by *B. subtilis* grown at 48 °C at pH 5.8, in the absence or presence of Ca²⁺. (a) SDS-PAGE of labelled extracellular levansucrase from a pulse-chase experiment as described for Fig. 5. A, Culture in the presence of 0.2 mM Ca²⁺; B, culture with no Ca²⁺ added. EDTA (0.5 mM) was added to the culture 5 min before the pulse-chase. (b) Curves obtained by quantification of the radioactive bands from the SDS gels. ●, Gel A; ○, gel B.

higher affinity for Ca²⁺ at acidic pH (Fig. 8a). The calcium titration curve showed an inflexion point at pH 6.5 (Fig. 8b). This unexpected result suggests that Ca²⁺ and H⁺ concentrations could cooperate to promote an efficient coupling between folding and secretion under particular environmental conditions. We tested this possibility by pulse-chase experiments at high temperature since, in such conditions, levansucrase folding is very sensitive to Ca²⁺ and H⁺ concentrations (Chambert & Petit-Glatron, 1990). The efficiency of the final step of secretion in bacteria grown at 48 °C and pH 5.8 was significantly different in the presence and in the absence of Ca²⁺ (Fig. 9). The efficiency of the protein release and not its subsequent stability in the growth medium was modified by the presence of Ca²⁺.

Is it possible to extend the effects of pH and Ca²⁺ on the secretion efficiency of other secretory proteins?

Several difficulties arise in addressing the above question. Firstly, the secretion level of extracellular proteins is

Fig. 10. Pattern of labelled exoproteins produced by *B. subtilis* 168 Marburg during the exponential phase of growth at various pH values. Bacteria grown at pH 5.8, pH 7.8 and pH 7.0 were labelled for 5 min at 37 °C with [³⁵S]methionine (0.6 mCi ml⁻¹; 1000 Ci mmol⁻¹) at OD₆₀₀ 1.5. A portion of bacteria grown at pH 7.0 was labelled in the presence of 5 mM EDTA. After centrifugation, aliquots of supernatants were directly analysed by SDS-PAGE. Molecular masses of the major labelled proteins are indicated on the left.

negligible in the *sacU*₃₂ strain used in this work during the exponential phase of growth (Chambert & Petit-Glatron, 1984). Secondly, few data have hitherto been available concerning the folding properties of the *B. subtilis* secretory proteins. Given these limitations, we adopted two preliminary approaches to this question.

The first approach was based on the evaluation of the effects of medium parameters on the total production of extracellular proteins by a wild-type strain of *B. subtilis* (168 Marburg). The pattern of labelled exoproteins released by this strain was analysed by pulse experiments (Fig. 10) under the environmental conditions which modulate levansucrase production in strain QB112. Since in this experiment attention was given to other secretory proteins, bacteria were not induced for levansucrase synthesis. The modification of pH, or the presence of a metal chelator, or both, modified the production of several exoproteins. One of these, a 55 kDa protein, was dramatically modulated by these medium parameters, but in a different way from levansucrase in strain QB112.

For the second approach, we focused on the properties of extracellular α -amylase, even though this protein is mainly produced during the stationary phase of growth. Folding properties of levansucrase and α -amylase produced by the same *B. subtilis* strain were compared. The folding-unfolding transition of α -amylase occurred at such a high

Fig. 11. The graph shows a comparison of the unfolding-folding transition of α -amylase (curve A) and levansucrase (curve B). Thermal unfolding was followed by the intrinsic fluorescence intensity changes at 336 nm. The excitation wavelength was 285 nm. The protein concentrations were 1.5 μ g ml⁻¹ for α -amylase and 4 μ g ml⁻¹ for levansucrase in 0.1 M sodium phosphate, pH 7. The temperature of the protein solutions in the thermoregulated spectrofluorimeter cell was gradually increased from 25 °C to 85 °C, at a constant rate of 2 °C per 90 s. Temperatures of the mid-point are indicated by arrow 1 for levansucrase and arrow 2 for α -amylase. The inset shows calcium-binding properties of α -amylase. Pure proteins (1 μ g) were blotted onto nitrocellulose. The radioactive dots corresponded to levansucrase (1), α -amylase of *B. subtilis* (2), α -amylase of *B. licheniformis* (3). The non-calcium-binding proteins BSA (4) and ovalbumin (5) were used as controls.

temperature ($T_m = 62$ °C) (Fig. 11a) that it was not reasonable to postulate that this protein has a tertiary structure flexibility similar to that exhibited by levansucrase under the conditions of pH and temperature of *B. subtilis* growth (Chambert & Petit-Glatron, 1990). Moreover, the two proteins have very different affinities for calcium since no calcium binding was observed for α -amylase (Fig. 11b) in contrast to levansucrase and *Bacillus licheniformis* α -amylase. Furthermore, the secretion level of α -amylase assayed in the culture supernatant during the exponential phase of *B. subtilis* growth at pH 5.8 or 7.8 was not substantially different. These results seem to indicate that several different secretion mechanisms coexist in *B. subtilis*.

DISCUSSION

Bioenergetic aspects of the translocation of proteins across bacterial membranes have been the subject of many investigations (Palmen *et al.*, 1994). There is also considerable evidence in both prokaryotic and eukaryotic systems that the dynamics and the free energy of protein folding play a role in the efficiency of secretion (London, 1992; Eilers & Schatz, 1988). Several folding effectors have been identified in the cytosol which maintain the protein in a state competent for export (Kumamoto, 1991; Stuart *et al.*, 1994; Baker & Craig, 1994). Other effectors on the external side of the membrane assist the protein folding step which results in the irreversibility of translocation (Langer & Neupert, 1991; Kontinen *et al.*, 1991; Petit-Glatron *et al.*, 1993). Thus, the chemical asymmetries between the *cis* and the *trans* sides of the membrane could be the driving forces for translocation,

allowing the coupling of a scalar event (folding) with a vectorial event (translocation) (Simon *et al.*, 1992).

Levansucrase of *B. subtilis* does not possess a prosequence, unlike the serine protease subtilisin (Otha *et al.*, 1991) or α -amylase (Sasamoto *et al.*, 1989). These prosequences may play a role in the coupling of secretion and folding (Fujishige *et al.*, 1992). There is no evidence that levansucrase undergoes a late chemical modification. Apparently it is the high conformational flexibility of levansucrase under the conditions of its synthesis in *B. subtilis* which allows it to be efficiently secreted.

Fe^{3+} or Ca^{2+} ions catalyse levansucrase folding at pH 7. These ions are concentrated only on the external side of the membrane due to the metal-binding properties of the cell wall (Beveridge & Murray, 1976, 1980; Petit-Glatron *et al.*, 1993). The transmembrane gradient of these metal ions may be the force which drives the release of levansucrase from the membrane due to coupling with the folding process. However, this model can only apply to particular environmental conditions. Our studies suggest that the H^+ concentration on the external side of the membrane could play the same role as Ca^{2+} or Fe^{3+} ions.

However, this raises the issue of whether the pH on the external side of the membrane is the same as that in the bulk growth medium. Several theoretical and experimental analyses have shown that there is a very steep pH gradient between a very acidic interface layer which generates a surface electrostatic potential and the neutral bulk aqueous phase (Teissié *et al.*, 1993; Koch, 1986). In the case of phosphatidylethanolamine bilayers, it was calculated that in a low ionic strength buffer (1 mM) the drop was more than 2 pH units across a distance of less than 1.5 nm (Prats *et al.*, 1986). This phenomenon seemingly complicates the interpretation we propose. However the findings with model membranes may not apply to natural membranes. The *B. subtilis* cytoplasmic membrane contains a large amount of protein (about 60% dry weight), and relatively high proportions of cationic phospholipids (about 15% lysylphosphatidylglycerol) (Archibald, 1993) and neutral glycolipids (Minnikin & Abdeolrahimzadeh, 1974). This structural heterogeneity may greatly reduce the surface charge density which generates the electrostatic potential. Moreover, the magnitude of the potential is strongly dependent on the ionic strength (McLaughlin, 1977). In the ionic conditions of bacterial growth (0.1 M potassium phosphate), this potential will be very low according to theoretical calculations; consequently, the H^+ concentrations on the bacterial membrane outer surface and in the bulk solution may not be very different.

On the other hand, our findings and interpretations have focused on the effects of pH and ions only on the proteins 'en route' to secretion, and not on the secretion machinery components. Obviously, this alternative must be considered as a reasonable working hypothesis even though experimental approaches are not easy. One possible heuristic way could be the comparison of *in vitro* and *in vivo* effects of these environmental parameters on PrsA

activity, since this surface-exposed lipoprotein has been proposed to function as a molecular chaperone catalysing the folding of translocated secretory proteins such as α -amylase (Kontinen *et al.*, 1991) in *B. subtilis*.

ACKNOWLEDGEMENTS

We are grateful to EBSG (European *Bacillus* secretion group) for helpful discussions. We thank A. Edelman for help with the English text. This work was supported in part by a grant from the EEC (contract BIO2-CT93-0254).

REFERENCES

- Archibald, A. R. (1993). The *Bacillus* cell envelope. In *Bacillus*, pp. 217–254. Edited by C. R. Harwood. New York: Plenum Press.
- Baker, J. & Craig, E. A. (1994). Heat shock proteins as molecular chaperones. *Eur J Biochem* **219**, 11–23.
- Beveridge, T. J. & Murray, R. G. E. (1976). Uptake and retention of metals by cell walls of *Bacillus subtilis*. *J Bacteriol* **127**, 1502–1518.
- Beveridge, T. J. & Murray, R. G. E. (1980). Sites of metal deposition in the cell wall of *Bacillus subtilis*. *J Bacteriol* **141**, 1876–1887.
- Chambert, R. & Petit-Glatron, M. F. (1984). Hyperproduction of exocellular levansucrase by *Bacillus subtilis*: examination of the phenotype of a *sacU^h* strain. *J Gen Microbiol* **130**, 3143–3152.
- Chambert, R. & Petit-Glatron, M. F. (1988). Secretion mechanism of *Bacillus subtilis* levansucrase: characterization of the second step. *J Gen Microbiol* **134**, 1205–1214.
- Chambert, R. & Petit-Glatron, M. F. (1990). Reversible thermal unfolding of *Bacillus subtilis* levansucrase is modulated by Fe^{3+} and Ca^{2+} . *FEBS Lett* **275**, 61–64.
- Chambert, R., Benyahia, F. & Petit-Glatron, M. F. (1990). Secretion of *Bacillus subtilis* levansucrase. Fe(III) could act as cofactor in an efficient coupling of the folding and translocation processes. *Biochem J* **265**, 375–382.
- Dedonder, R. (1966). Levansucrase from *Bacillus subtilis*. *Methods Enzymol* **8**, 500–506.
- Eilers, M. & Schatz, G. (1988). Protein unfolding and the energetics of protein translocation across biological membranes. *Cell* **52**, 481–483.
- Fujishige, A., Smith, K. R., Silen, J. L. & Agard, D. A. (1992). Correct folding of α -lytic protease is required for its extracellular secretion from *Escherichia coli*. *J Cell Biol* **118**, 33–42.
- Hemilä, H., Pokkinen, M. & Palva, I. (1992). Improving the production of *E. coli* β -lactamase in *Bacillus subtilis*: the effect of glucose, pH and temperature on the production level. *J Biotechnol* **26**, 245–256.
- Kemper, M. A., Urrutia, T. J., Beveridge, A. L. & Doyle, R. J. (1993). Proton motive force may regulate cell wall associated enzymes of *Bacillus subtilis*. *J Bacteriol* **175**, 5690–5696.
- Khan, S. & Macnab, R. M. (1980). Proton chemical potential, proton electrical potential and bacterial motility. *J Mol Biol* **138**, 599–614.
- Koch, A. L. (1986). The pH in the neighborhood of membranes generating a protonmotive force. *J Theor Biol* **120**, 73–84.
- Kontinen, V. P., Saris, P. & Sarvas, M. (1991). A gene (*prsA*) of *Bacillus subtilis* involved in a novel late stage of protein export. *Mol Microbiol* **5**, 1273–1283.
- Kumamoto, C. A. (1991). Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. *Mol Microbiol* **5**, 19–22.

- Langer, T. & Neupert, W. (1991).** Heat shock proteins hsp60 and hsp70: their roles in folding, assembly and membrane translocation of proteins. *Curr Top Microbiol Immunol* **167**, 3–30.
- London, E. (1992).** How bacterial toxins enter cells: the role of partial unfolding in membrane translocation. *Mol Microbiol* **6**, 3277–3282.
- Mäntsälä, P. & Zalkin, H. (1979).** Membrane-bound and soluble extracellular α -amylase from *Bacillus subtilis*. *J Biol Chem* **254**, 8540–8547.
- Maruyama, K., Mikawa, T. & Ebashi, S. (1984).** Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membranes after sodium dodecyl sulfate gel electrophoresis. *J Biochem* **95**, 511–519.
- McLaughlin, S. (1977).** Electrostatic potentials at membrane–solution interfaces. *Curr Top Membr Transp* **9**, 71–144.
- Minnikin, D. E. & Abdeolrahimzadeh, H. (1974).** Effect of pH on the proportions of polar lipids in chemostat cultures of *Bacillus subtilis*. *J Bacteriol* **120**, 999–1003.
- Otha, Y., Hojo, H., Aimoto, S., Kobayashi, T., Zhu, X., Jordan, F. & Inouye, M. (1991).** Pro-peptide as an intramolecular chaperone: renaturation of denatured subtilisin E with a synthetic pro-peptide. *Mol Microbiol* **5**, 1507–1510.
- Palmen, R., Driessen, A. J. & Hellingwerf, K. J. (1994).** Bioenergetic aspects of the translocation of macromolecules across bacterial membranes. *Biochim Biophys Acta* **1183**, 417–451.
- Petit-Glatron, M. F., Benyahia, F. & Chambert, R. (1987).** Secretion of *Bacillus subtilis* levansucrase: a possible two step mechanism. *Eur J Biochem* **163**, 379–387.
- Petit-Glatron, M. F., Grajcar, L., Munz, A. & Chambert, R. (1993).** The contribution of the cell wall to a transmembrane calcium gradient could play a key role in *Bacillus subtilis* protein secretion. *Mol Microbiol* **9**, 1097–1106.
- Prats, M., Teissié, J. & Tocanne, J. F. (1986).** Lateral proton conduction at lipid–water interfaces and its implications for the chemiosmotic-coupling hypothesis. *Nature* **322**, 756–758.
- Randall, L. L., Hardy, S. J. S. & Tom, J. R. (1987).** Export of protein: a biochemical view. *Annu Rev Microbiol* **41**, 507–541.
- Sasamoto, H., Nakasawa, K., Tsutsumi, K., Takase, K. & Yamane, K. (1989).** Signal peptide of *Bacillus subtilis* α -amylase. *J. Biochem* **106**, 376–382.
- Shioi, J. I., Matsuura, S. & Imae, Y. (1980).** Quantitative measurements of proton motive force and motility in *Bacillus subtilis*. *J Bacteriol* **144**, 891–897.
- Simon, S. M., Peskin, C. S. & Oster, G. F. (1992).** What drives the translocation of proteins? *Proc Natl Acad Sci USA* **89**, 3770–3774.
- Somogyi, M. (1945).** A new reagent for the determination of sugars. *J Biol Chem* **160**, 61–73.
- Stuart, R. A., Cyr, D. M., Craig, E. A. & Neupert, W. (1994).** Mitochondrial molecular chaperones; their role in protein translocation. *Trends Biochem Sci* **19**, 87–92.
- Teissié, J., Gabriel, B. & Prats, M. (1993).** Lateral communication by fast proton conduction: a model membrane study. *Trends Biochem Sci* **18**, 243–246.

Received 30 August 1994; revised 28 December 1994; accepted 10 January 1995.