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Secretion of *Bacillus subtilis* levansucrase

Fe(III) could act as a cofactor in an efficient coupling of the folding and translocation processes

Régis CHAMBERT,* Fadila BENYAHIA and Marie-Françoise PETIT-GLATRON
Institut Jacques Monod, Laboratoire Génétique et Membranes, Centre National de la Recherche Scientifique; Université Paris 7, 2 place Jussieu, 75251 Paris Cedex 05, France

The refolding of levansucrase denatured by urea was studied as a possible model for the second step of the secretion pathway of this protein. The folding–unfolding transition was monitored by measuring intrinsic fluorescence and resistance to proteolysis. Both methods provided the same estimation for the unfolding free energy of levansucrase, ΔG_D, which was 30.1 ± 1.7 kJ·mol^{-1} (7.2 ± 0.4 kcal·mol^{-1}) at pH 7 in 0.1 M-potassium phosphate buffer. The rate of refolding was greatly enhanced by Fe^{3+}, whereas the Fe^{3+} chelator EDTA prevented correct refolding. Fe^{3+} allowed the protein to reach its folded form in medium in which the dielectric constant had been lowered by ethanol. The efficiency *in vivo* of the export of levansucrase bearing an amino acid modification which blocks the second step of the translocation pathway was greatly increased by high concentrations of Fe^{3+} in the culture medium. Assuming that the protein folding governs the second step of the secretion process of levansucrase, we discuss from an irreversible thermodynamic point of view the possible role of Fe^{3+} in the efficient coupling of the two events.

INTRODUCTION

Biochemical [1] and, more recently, genetic [2] techniques have been used to identify the components of the putative apparatus for protein secretion in *Bacillus subtilis*. Levansucrase (EC 2.4.1.10) may be used as a model to study the working of the secretion machinery in the exponentially growing bacteria, as this enzyme is the only one secreted in large quantities (about 8% of total proteins) by the *sacU*^{h} *strain* [3].

We have previously characterized two steps in the secretion process of *B. subtilis* levansucrase [4]. The first step involves the proteolytic processing of a membrane precursor, and the second, rate-limiting, step is the secretion of the resulting mature membrane form. This second step appears to be coupled to the continuous uptake of Fe^{3+} by the bacteria [5], and the partially purified transient membrane forms are enzymically active only in the presence of Fe^{3+} [4]. This suggests that the mature levansucrase undergoes a conformational change, during export, in which Fe^{3+} plays an important role.

The question arises: can the folding rate of a transient membrane form of an exoprotein modulate its unidirectional movement? This possibility exists for several prokaryotic systems in which folding is involved in protein transport [6,7]. It has also been suggested that the free energy drop accompanying folding could be the driving force of the secretion event [8]. Under these circumstances, the efficiency of such a coupling between a chemical reaction and a vectorial process should, in terms of non-equilibrium thermodynamics [9], depend on the rate of this energy change. It is therefore very likely that the efficiency of such coupling, within the membrane, is ensured by one or more specific factors.

Fe^{3+} could be one of these factors for levansucrase secretion. This study examines the effect of Fe^{3+} on the kinetics of refolding of purified exocellular levansucrase which has been reversibly denatured by urea. The refolding is used as a model for the second step of secretion. The study also analyses the ability of *B. subtilis* to secrete efficiently a levansucrase molecule bearing, in its mature form, an amino acid modification which blocks the second step of secretion [10]. Such an approach should provide information on the nature and function of Fe^{3+} in the secretion process of levansucrase. Fe^{3+} is not associated with the purified protein [11], whose activity is unaffected by the presence of a ferric chelator [5]. Fe^{3+} only increases the enzyme's thermostability [12].

Levansucrase is a 50000-M_r monomeric protein which contains no disulphide bonds, has five tryptophan residues [13] and is resistant to proteolysis in its native state [14]. The unfolding–folding transition may therefore be monitored by measuring changes in either intrinsic fluorescence or in the resistance to proteolytic digestion.

MATERIALS AND METHODS

Purification of exocellular levansucrase

Levansucrase was prepared from the culture supernatant of the induced QB112 *B. subtilis* strain according to the published procedure [15].

Levansucrase activity assay

Levansucrase activity was estimated by measuring the initial rate of the exchange reaction catalysed by this enzyme [16]. One unit of enzyme activity is defined as the amount of enzyme exchanging 1 μmol of glucose·min^{-1} under the following conditions; 22 °C, 0.05 M-potassium phosphate (pH 6.0)/0.1 M-sucrose/0.2 M-[^{14}C]glucose.

Unfolding and refolding experiments

Unfolding and refolding experiments were carried out in buffer A (0.1 M-sodium phosphate, pH 7) or in buffer

Abbreviations used: CAF, ammonium ferric citrate.
* To whom correspondence should be sent.

B (0.1 M-potassium phosphate, pH 7), with the temperature maintained at 22 °C for all experiments. The stock solution of levansucrase (0.3 mM) was diluted in urea solution. The unfolding reaction was allowed to proceed for the time specified in the text for each experiment. For refolding experiments, the urea/levansucrase solutions were diluted with buffer A or buffer B to produce a final urea concentration below 1 M.

Fluorescence was measured at 340 nm with a Jobin et Yvon spectrofluorimeter (excitation at 285 nm).

Unfolded levansucrase was digested with subtilisin (50 $\mu g \cdot ml^{-1}$) for 15 min at 22 °C, and the reaction was stopped by adding SDS/polyacrylamide-gel electrophoresis sample buffer [17] containing phenylmethanesulphonyl fluoride (0.2 mM). After incubation at 90 °C for 5 min, samples were analysed by SDS/polyacrylamide gel electrophoresis (10 % gels). Levansucrase activity was assayed in aliquots removed before the addition of sample buffer.

Immunoblotting

Immunoblotting on nitrocellulose sheets (0.45 μm pore size), in 25 mM-Tris/HCl (pH 8.3)/192 mM-glycine/20 % (v/v) methanol/0.02 % SDS, was carried out using a constant current of 400 mA for 2 h. Incubation with purified rabbit anti-levansucrase antibodies and radioiodinated Protein A was adapted from the method of Burnette [18] as described previously [4].

The amounts of levansucrase in the samples were determined from a standard curve established from the immunoblotting of purified levansucrase, within the range of 5–50 ng.

Preparation and purification of antibodies

Levansucrase was covalently coupled to glutaraldehyde-activated Ultrogel (Act Ultrogel AcA 22, I.B.F.) and antibodies were purified by affinity chromatography [19].

Fe^{3+} assay

The concentrations of Fe^{3+} were estimated by the method of Tangeras *et al.* [20]. The Fe^{3+} concentration of a 1 $mg \cdot ml^{-1}$ ferric-ammonium citrate (CAF) solution was estimated to be 3 mM.

Culture and strains

The BF 211-D strain was constructed and characterized in our laboratory [10].

Products and enzymes

Trypsin, papain and subtilisin were purchased from Sigma. Urea was from Merck, CAF was from Prolabo and EDTA–Fe^{3+} was from Fluka.

RESULTS

Unfolding–folding transition of levansucrase measured by proteinase sensitivity

The proteinase resistance of native levansucrase [14] indicates that the change from a folded to an unfolded structure may be monitored by measuring the sensitivity of the protein to proteolysis. This technique has been used successfully to study the refolding of many proteins [21]. Urea was used as the unfolding agent.

Levansucrase was first incubated in 8 M-urea, and the urea concentration was then reduced to 0.8 M. Both the kinetics of unfolding (i.e. the disappearance of a subtilisin-resistant form versus the time of incubation in 8 M-urea) and the kinetics of refolding (the appearance of a subtilisin-resistant form after urea dilution) were analysed by immunoblotting and enzyme activity (Fig. 1). Unfolding is completely reversible in such conditions, and the half-times of unfolding and refolding were 20–22 s and 60–65 s respectively.

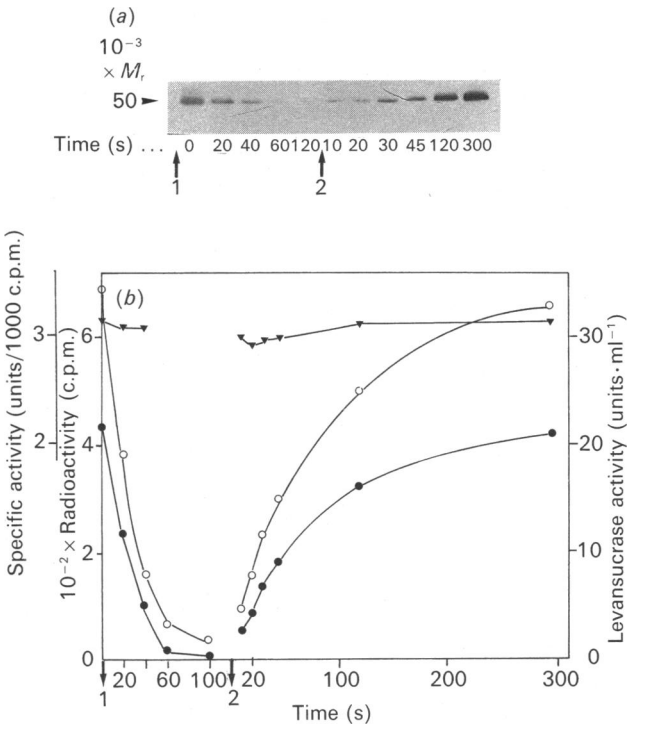

Fig. 1. Kinetics of levansucrase unfolding and refolding measured by resistance to degradation by subtilisin

Unfolding was promoted (arrow 1) by mixing levansucrase (final concentration 15 μM) with urea (final concentration 8 M) at 22 °C in buffer A. Samples (20 μl) were withdrawn at the times indicated and quickly mixed with 200 μl of a solution of 50 μg of subtilisin $\cdot ml^{-1}$ in the same buffer, and incubated for 15 min at 22 °C. Refolding was initiated (arrow 2), after 2 min of unfolding, by mixing 400 μl of the unfolding mixture with 3.6 ml of buffer A (final urea concentration 0.8 M). Samples (200 μl) were withdrawn at the times indicated, quickly mixed with 20 μl of a 500 $\mu g \cdot ml^{-1}$ subtilisin solution and incubated for 15 min at 22 °C. All samples were then treated as described in the Materials and methods section before being submitted to SDS/polyacrylamide-gel electrophoresis and immunoblotting, or were assayed for levansucrase activity. (*a*) Immunoblot; (*b*) radioactivity of the immunoblot spots (○); levansucrase activity ($units \cdot ml^{-1}$) (●) and specific activity of levansucrase (▼).

The changes in levansucrase activity during unfolding and refolding had the same time-dependence as did the amounts of subtilisin-resistant protein. Thus the specific activity of the enzyme remained constant during both events, indicating that the enzymically active structure and the subtilisin-resistant form are undissociable. The kinetics of refolding may therefore be established from direct measurements of enzyme activity recovered after the concentration of urea is decreased.

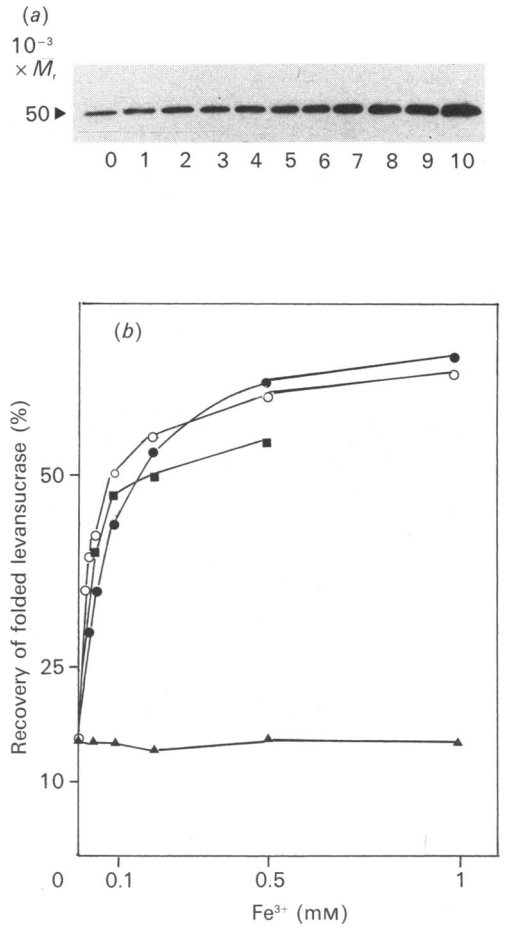

Fig. 2. Refolding as a function of Fe^{3+} concentration in the refolding medium

Unfolding and refolding were carried out as described for Fig. 1. Denaturation was allowed to proceed for 3 min at 22 °C. The refolding medium, buffer B, contained CAF, $FeCl_3$ or EDTA–Fe^{3+} at the concentrations indicated. After 15 s of refolding, the samples were mixed with subtilisin (final concentration 50 $\mu g \cdot ml^{-1}$). Aliquots (20 μl) of each sample were treated as described in the Materials and methods section for subsequent immunoblot analysis. (*a*) Immunoblot analysis of refolding performed in the presence of various concentrations of CAF. Concentrations of Fe^{3+} were: lane 1, 1 μM; 2, 5 μM; 3, 10 μM; 4, 20 μM; 5, 50 μM; 6, 0.1 mM; 7, 0.2 mM; 8, 0.5 mM; 9, 1.0 mM; lane 10, control. Lane 0, in this sample the concentration of Fe^{3+} in the buffer, assayed by the method of Tangeras *et al.* [20], was about 1 μM. (*b*) Yield of refolding obtained from quantitative analysis of the immunoblot presented in (*a*) (○) and from measurements of enzyme activity displayed in the samples refolded in the presence of CAF (●), $FeCl_3$ (■) and EDTA–Fe^{3+} (▲). [An insoluble precipitate of $Fe_2(PO_4)_3$ formed at $FeCl_3$ concentrations above 0.5 mM].

Effect of Fe^{3+} on levansucrase refolding

Two soluble forms of Fe^{3+}, CAF and $FeCl_3$, were used to study the effect of Fe^{3+} on the refolding reaction *in vitro*.

Increasing concentrations of CAF or $FeCl_3$ in the refolding medium strongly enhanced the yield of refolded protein recovered 15 s after the reduction in urea concentration (Fig. 2). However, when complexed with EDTA, Fe^{3+} did not modify the rate of refolding. EDTA is known to be a more potent Fe^{3+} chelator than citrate; their affinity constants (K_A) are respectively 1×10^{25} and 1×10^{12}. Neither free nor complexed Fe^{3+} had any effect on subtilisin activity.

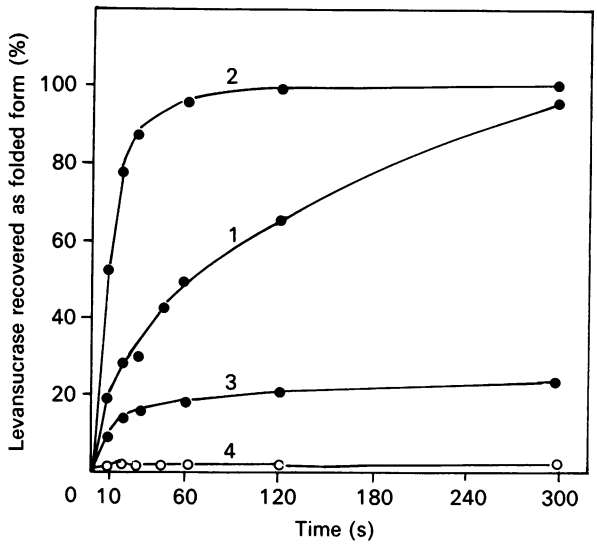

Fig. 3. Effects of Fe^{3+} on the kinetics of levansucrase refolding

Unfolding was carried out by mixing 100 μl of 8.8 M-urea in buffer B with 10 μl of 0.3 mM-levansucrase at 22 °C. After 3 min, refolding was promoted by adding 1 ml of buffer B, containing (1) no CAF, (2) CAF, 1 $mg \cdot ml^{-1}$ (3 mM-Fe^{3+}), (3) EDTA, 1 mM. Aliquots (100 μl) were withdrawn at the times indicated and quickly mixed with 0.9 ml of subtilisin (50 $\mu g \cdot ml^{-1}$) in the same buffer containing CAF (100 $\mu g \cdot ml^{-1}$ (1 and 3) or no CAF (2). The control (4) was obtained by promoting refolding by adding 1 ml of subtilisin (50 $\mu g \cdot ml^{-1}$) in buffer B containing CAF (1 $mg \cdot ml^{-1}$) to the unfolding mixture. Aliquots of each sample were treated as described in the Materials and methods section for subsequent immunoblot analysis or enzymic assay.

The refolding kinetics in the presence and absence of 1 mg of CAF $\cdot ml^{-1}$ (3 mM-Fe^{3+}) are shown in Fig. 3. The equation for the appearance of refolding levansucrase, assuming that the rate of refolding is proportional to the concentration of the protein remaining under an unfolded form, is:

$$\frac{dE}{dt} = k \cdot (E_T - E); (E_T - E) = E_T \cdot e^{-kt}$$

where E_T is the total amount of protein which can be refolded when time $t = \infty$, and E is the amount of the form refolded at any time after start ($t = 0$) of refolding.

The regression adjustment, using the least-squares method, gave correlation coefficients for the first-order kinetics of refolding of $r^2 = 0.99$ in the absence of CAF and $r^2 = 0.96$ in the presence of CAF. The resulting kinetic parameters were $k = 1.16 \times 10^{-2}\ s^{-1}$ and $t_{\frac{1}{2}} = 60$ s in the absence of CAF, and $k = 5.7 \times 10^{-2}\ s^{-1}$ and $t_{\frac{1}{2}} = 12$ s in the presence of CAF. Thus the folding process was more than four times faster in the presence of CAF than in the absence of added Fe^{3+}.

Control experiments on the effect of Fe^{3+} on subtilisin activity showed that: (1) there was no refolding of levansucrase in the presence or absence of Fe^{3+} when

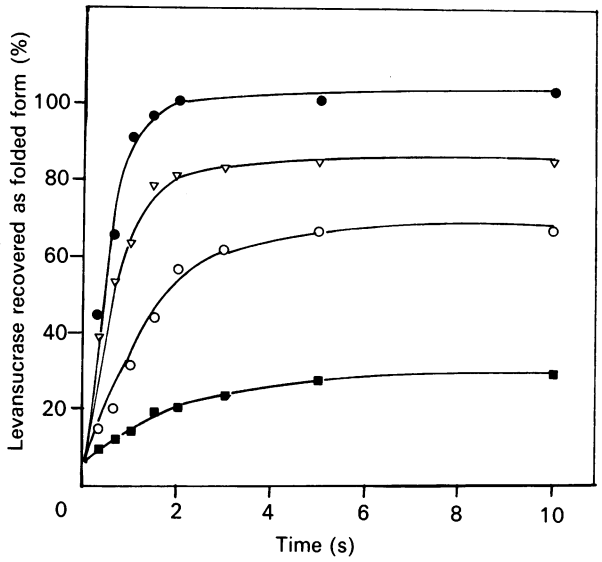

Fig. 4. Effect of Fe^{3+} on the kinetics of levansucrase refolding in the presence of ethanol

Unfolding was carried out as described in Fig. 3. Refolding was promoted by mixing 100 μl of the unfolding mixture with 1 ml of buffer B with or without CAF (1 mg·ml^{-1}) and containing ethanol at the concentrations indicated. Samples were withdrawn at the times indicated and quickly mixed with 0.9 ml of subtilisin (50 μg·ml^{-1}) in the same buffer containing 0.1 mg of CAF. ●, 20% ethanol, with CAF; ○, 20% ethanol, without CAF; ▽, 25% ethanol, with CAF; ■, 25% ethanol, without CAF.

subtilisin was added at the beginning of the refolding; and (2) the same effect of Fe^{3+} was obtained when trypsin or papain were used instead of subtilisin (results not shown).

The yield of correctly refolded levansucrase was less than 30% when the refolding experiment was performed in phosphate buffer containing 1 mM-EDTA. However, 70% of the initial amount of active enzyme was recovered when 2 mg of CAF·ml^{-1} (6 mM-Fe^{3+}) was added to the refolding medium after 10 min of EDTA action. Therefore correct refolding is blocked in the presence of EDTA, but the protein partly retains its potential to regain its folded form. This result and those presented in Fig. 2, showing the lack of catalytic effect of the EDTA-Fe^{3+} complex on folding, indicate that EDTA complexed the free Fe^{3+} present in the refolding buffer (estimated at 1 μM by the Tangeras method) in a form unavailable for levansucrase refolding.

Finally, the catalytic effect of Fe^{3+} on the refolding of levansucrase in a medium having a dielectric constant lowered by increasing concentrations of ethanol was examined. There was a differential effect of ethanol at concentrations above 15% (Fig. 4). These results show that the catalytic effect of Fe^{3+} on the rate of the refolding process remains effective in a medium of lower dielectric constant, which mimics the membrane environment. Moreover, the yield of correct refolding is much higher in the presence of Fe^{3+}.

Unfolding–folding transition of levansucrase measured by intrinsic fluorescence

The fluorescence emission spectra of levansucrase in its native state and in the presence of 8 M-urea show that fluorescence is quenched in the presence of high concentrations of denaturant, suggesting that the environments of the tryptophan residues in the folded and unfolded proteins are very different. The wavelength of 340 nm was selected for measurement, as the change in fluorescence intensity between native and denatured forms of levansucrase is greater than 2-fold.

Fig. 5(*a*) shows the change in fluorescence intensity of

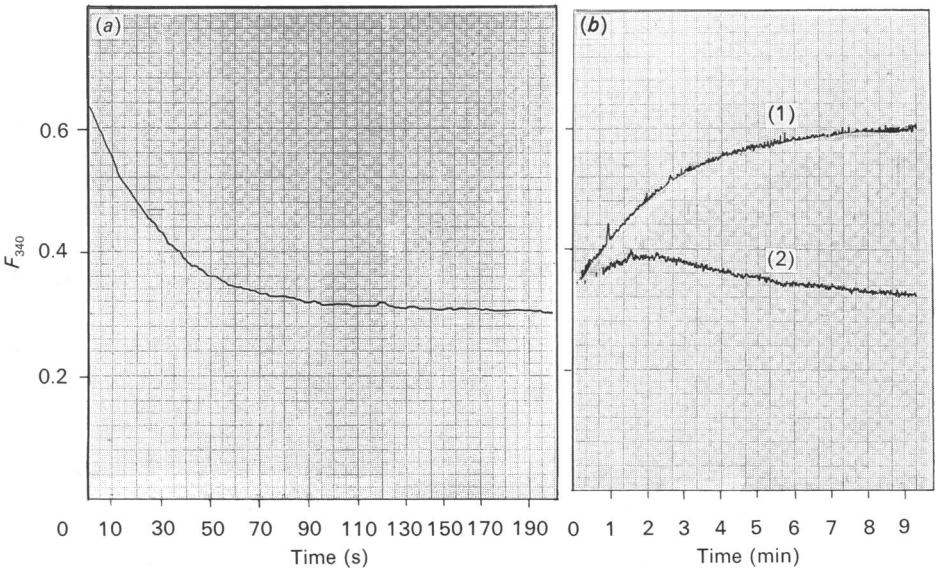

Fig. 5. Kinetics of levansucrase unfolding and refolding measured by fluorescence intensity changes

Traces are given for fluorescence intensity changes for unfolding (*a*) and refolding (*b*). (*a*) Fluorescence intensity of the solutions of enzyme (0.4 μM final concentration) and urea (8 M final concentration in buffer B) manually mixed were recorded; the dead time was 5 s. Excitation wavelength was 285 nm. (*b*) The solution of levansucrase (0.4 μM) was preincubated for 3 min in 8 M-urea at 22 °C. Fluorescence intensity was recorded after a decrease in urea concentration from 8 M to 0.8 M in the absence (1) or in the presence (2) of 1 mM-EDTA.

the protein after dilution in 8 M-urea. The maximum decrease in fluorescence intensity is reached with a half-time of about 22 s. Fig. 5(*b*) (curve 1) shows the change in fluorescence intensity of levansucrase preincubated for 5 min in 8 M-urea, followed by a decrease in the denaturant concn. from 8 M to 0.8 M. The protein fully recovered the fluorescence properties of its native form after 10 min, showing that unfolding caused by urea is completely reversible. Both unfolding and refolding were monophasic, and from the semi-logarithmic representation of fluorescence intensity changes during the unfolding and refolding processes, we estimated the apparent first-order rate constants. These were $k = 3.1 \times 10^{-2} \cdot s^{-1}$ and $t_{\frac{1}{2}} = 22$ s for unfolding in 8 M-urea and $k = 7.3 \times 10^{-3} \cdot s^{-1}$ and $t_{\frac{1}{2}} = 95$ s for refolding in 0.8 M-urea. These values for the kinetic parameters are of the same order of magnitude as those obtained from the measurement of proteinase resistance. The fluorescence spectroscopic approach was, unfortunately, unsuitable for studying the effect of Fe^{3+} on the refolding process, as the intrinsic fluorescence emission of the protein was strongly quenched in the presence of the low concentrations of Fe^{3+} used. This behaviour is not specific to levansucrase, as the fluorescence emission at 340 nm of a tryptophan solution is 90% quenched by 0.1 mM-Fe^{3+}, as either CAF or $FeCl_3$. The effect is probably due to the absorbance of these compounds at wavelengths between 320 and 360 nm. However, the kinetics of refolding were monitored by fluorescence changes in the presence of EDTA. Fig. 5(*b*) (curve 2) shows that unfolding was apparently not reversible in the presence of EDTA. These results agree with those obtained from proteinase sensitivity measurements.

Conformational stability of levansucrase and determination of the free energy of unfolding (ΔG_D)

The curve of levansucrase denaturation by urea was established by fluorescence measurements, proteinase sensitivity and estimation of residual enzyme activity. Levansucrase was exposed to various concentrations of urea in 0.1 M-potassium phosphate buffer, pH 7, at 22 °C. The fluorescence intensity changes and the proteinase sensitivity of the protein were measured as a function of the incubation time. There were no significant modifications of these parameters after 6 h of incubation. The results obtained after this incubation time are shown in Fig. 6. The profiles of urea denaturation of levansucrase, given by the change in the fluorescence intensity at 340 nm, the proteinase sensitivity and the residual enzyme activity, were represented as a function of urea concentration (Fig. 7). The observed single step denaturation allowed us to analyse the urea-induced unfolding process using the simple two-state equilibrium model proposed by Pace [22]. The free energy for the unfolding of levansucrase in the transient region varied linearly with the denaturant concentration as shown in Fig. 8.

These data were fitted to the equation proposed by Pace [22] by least-squares analysis:

$$\Delta G_D = \Delta G_D^{H_2O} - m \cdot [\text{urea}]$$

The values for $\Delta G_D^{H_2O}$, m (a constant, expressed in $kJ \cdot mol^{-1}$) and $D_{\frac{1}{2}}$ (the mid-point of the denaturation curves) determined by each method (Table 1) are in good agreement.

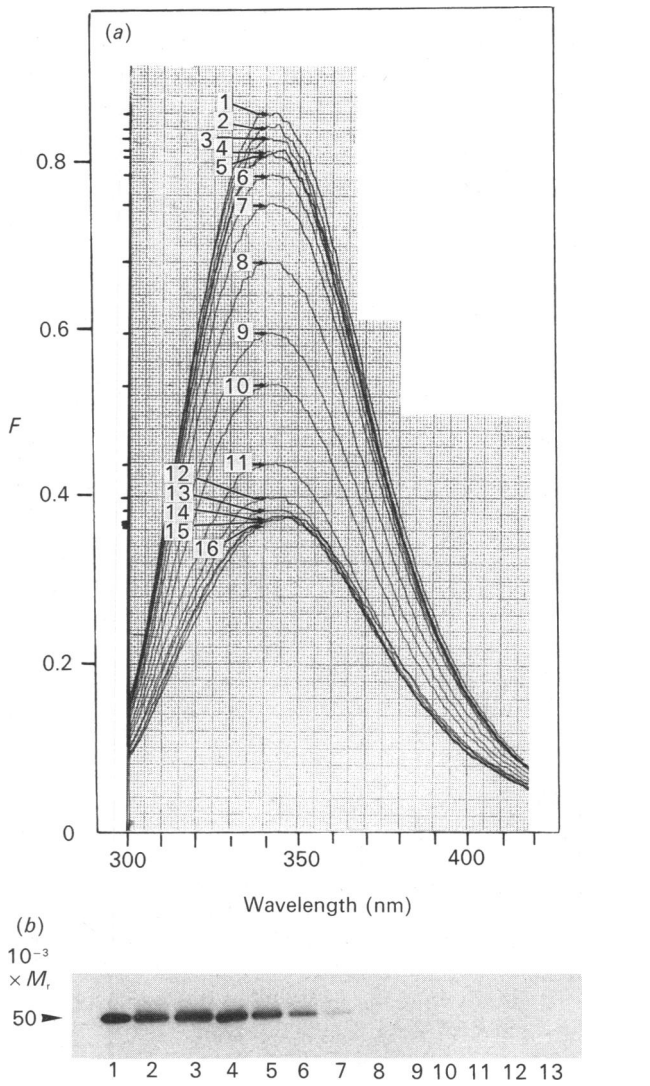

Fig. 6. Fluorescence intensity changes and proteinase sensitivity of levansucrase in the presence of various concentration of urea.

Samples of levansucrase (0.5 μM final concentration) were incubated for 6 h at 22 °C with the urea concentrations indicated in buffer B. An aliquot of each sample was then treated with subtilisin as described in the Materials and methods section before being submitted to immunoblot analysis and enzymic assay. (*a*) Traces of fluorescence emission spectra of each sample (excitation wavelength = 285 nm). Urea concentrations (M): trace 1, 0.5; 2, 0; 3, 1.0; 4, 1.1; 5, 1.2; 6, 1.3; 7, 1.4; 8, 1.5; 9, 1.6; 10, 1.7; 11, 1.8; 12, 1.9; 13, 2.0; 14, 2.2; 15, 2.5; 16, 3.0. (*b*) Immunoblot. Urea concentrations (M): lane 1, 0.8; 2, 1.0; 3, 1.2; 4, 1.4; 5, 1.6; 6, 1.8; 7, 2.0; 8, 2.2; 9, 2.4; 10, 2.6; 11, 2.8; 12, 3.0; 13, 3.2.

Effect of Fe^{3+} on the production of a modified levansucrase which is blocked at the second step of its secretion pathway

The *B. subtilis* mutant BF 211-D produces a levansucrase which is modified in its mature part [10] by substitution of aspartate for the glycine residue at position 366. The differential rate of levansucrase synthesis by this strain is 1.5% of the wild-type value, but the protein produced remains stable in the culture super-

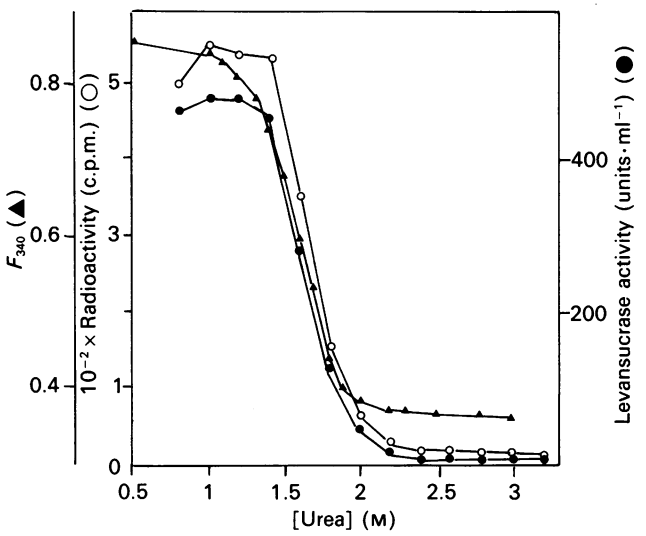

Fig. 7. Denaturation of levansucrase, measured by fluorescence intensity changes, subtilisin sensitivity and residual enzyme activity, as a function of urea concentration

▲, Fluorescence intensity at 340 nm measured at each urea concentration; ○, radioactivity of the immunoblot spots; ●, assay of residual enzyme activity (see Fig. 6).

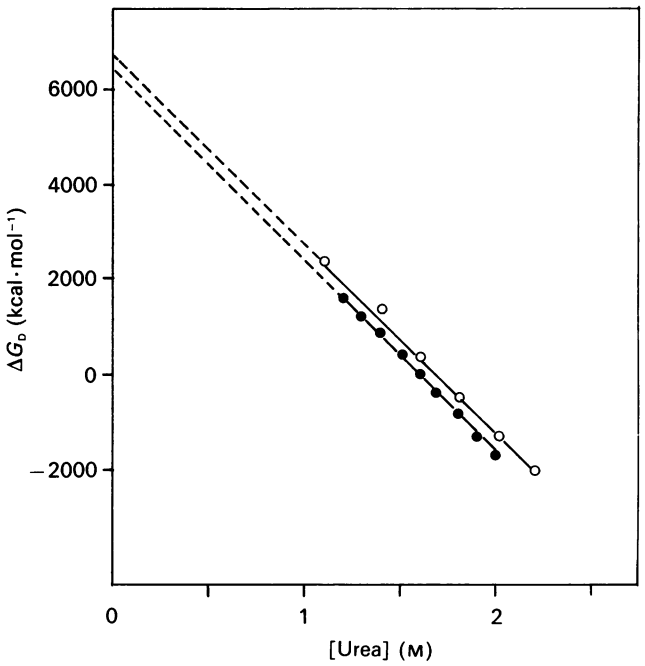

Fig. 8. Levansucrase free energy (ΔG_D) of unfolding as a function of urea concentration

ΔG_D from fluorescence data (●) was calculated using the equation: $\Delta G_D = -RT \cdot \ln (f_N/f_D)$ (f_N is the fraction of native protein and f_D the fraction of denatured protein). ΔG_D for proteinase sensitivity data (○) was defined as $\Delta G_D = -RT \cdot \ln (P_r/P_s)$ (P_r is the fraction of proteinase-resistant enzyme and P_s the fraction of proteinase-sensitive enzyme). 1 kcal = 4.184 kJ.

Table 1. Parameters of levansucrase urea denaturation

$D_{\frac{1}{2}}$ is the mid-point of the denaturation curve ($\Delta G_D = 0$). The data in Fig. 8 were fitted to the equation: $\Delta G_D = \Delta G_D^{H_2O} - m \cdot$[urea] by least-squares analysis. The results are means ± S.D. ($n = 6$ or 9)

Method of monitoring refolding	$\Delta G_D^{H_2O}$ (kJ·mol^{-1})	$D_{\frac{1}{2}}$ (M)	m (kJ·mol^{-1})
Fluorescence	30.1 ± 1.3	1.6	18.8
Sensitivity to degradation by subtilisin	30.5 ± 1.3	1.65	18.8

natant and is subtilisin-resistant. Pulse-labelling experiments demonstrated that the modification affects the efficiency of the second step in the levansucrase secretion pathway. The ability of Fe^{3+} to enhance levansucrase production in this mutant was tested by increasing the concentration of Fe^{3+} in the culture medium. The production of the modified exocellular levansucrase increased greatly to reach 12–15% of the wild-type value in the presence of CAF (0.02–5 mg·ml^{-1}) (Fig. 9). The generation time remained unchanged. These results strongly suggest that high concentrations of Fe^{3+} ions increase the rate of folding of the M_r-50000 intermediate membrane form, and thus increase the yield of protein secretion.

DISCUSSION

This study shows that Fe^{3+} modulates the rate of levansucrase refolding *in vitro*. The catalytic event occurs in the same conditions of pH and ionic strength as does levansucrase secretion by *B. subtilis*. These results must be considered in the context of the considerable amount of data accumulated over the past 20 years on the interference of Fe^{3+} with levansucrase production by this bacterium. (1) The presence of Fe^{3+} in the culture medium has been shown to be necessary for synthesis and secretion of levansucrase [12]. (2) Partially purified transient membrane forms of the enzyme become active only in the presence of this metal [4]. (3) The second step of the secretion process, which was characterized as the release of the processed precursor into the culture medium, appears to be coupled to the continuous uptake of Fe^{3+} by the bacteria [5]. Considering all these data, it is tempting to assume that Fe^{3+} is a catalytic effector of the folding of the membrane-processed protein form, and that this catalysed conformational change plays an important role in the export process.

It is now accepted that folding is important for the export of proteins in several prokaryotic systems [8,23–26]. It is generally agreed that a protein is competent for membrane translocation only when it is devoid of its final stable tertiary structure. However, there is considerable debate as to whether this unfolded structure is required for recognition of a membrane component of the secretion machinery governing the secretion event, or whether it plays a direct dynamic role via the folding event in transmembrane diffusion as postulated in the membrane trigger hypothesis [27].

Since at present there is little experimental evidence for the existence of an apparatus for protein secretion in *B. subtilis*, we focus our attention on the latter hypothesis, which raises an interesting question: is the rate of protein folding critical for its translocation across the membrane,

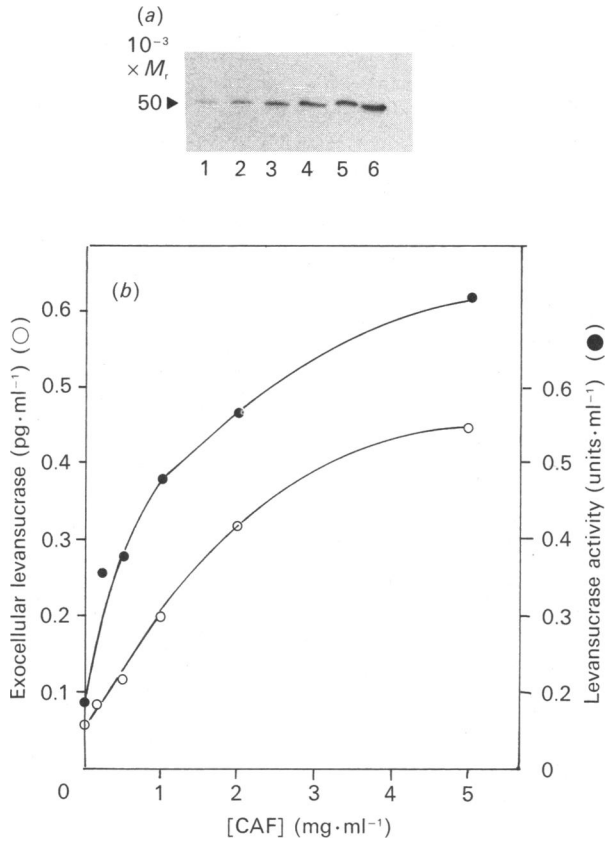

Fig. 9. Production of exocellular levansucrase by *B. subtilis* BF211-D as a function of CAF concentration in the culture medium

A cell suspension (A_{600} = 0.8 units) was distributed equally into flasks containing the indicated CAF concentrations. Levansucrase synthesis was induced by adding 50 mM-sucrose. After 2 h, cell suspensions, which had all reached A_{600} = 3.2 units, were centrifuged. (*a*) A 0.05 ml portion of each supernatant was analysed by SDS/polyacrylamide-gel electrophoresis and immunoblotting. CAF concentrations (mg·ml^{-1}): lane 1, 0; 2, 0.2; 3, 0.5; 4, 1.0; 5, 2.0; 6, 5.0. (*b*) Levansucrase production/ml of cell supernatant (immunoblot) (○), and levansucrase activity/ml of cell supernatant (●) are shown.

or for its release from the cell surface? We propose that a possible answer takes into account the source of energy for transfer. If we postulate, as others have done [8,27], that the energy used for the vectorial diffusion of the protein through the membrane is provided by the energy change accompanying folding, we must include the rate of energy change in this thermodynamic approach.

The so-called dissipation function ϕ [9], which is the rate of free energy dissipation of the system, is related to the events taking place during energy conversion. In the case of protein secretion, the first event is the folding of the protein, occurring with a free energy difference, ΔG ($= -\Delta G_D$), at a rate v, and the second is the diffusion rate, J, of the protein through the membrane space in which its electrochemical potential is $\bar{\mu}_{mb}$, to the exocellular space ($\bar{\mu}_{exo}$). The dissipation function is the sum of the products of conjugate 'flows' and 'forces' as follows:

$$\phi = v \cdot \Delta G_D + J \cdot (\bar{\mu}_{exo} - \bar{\mu}_{mb})$$

In such conditions, the putative non-spontaneous diffusion of the protein could be coupled to the spontaneous process of folding as long as ϕ remains positive. Such a relationship shows that the efficiency of levansucrase release from the membrane to the exocellular space could depend strongly on the rate of its folding.

An alternative model is that the energy of folding is used only to release the protein from the cell surface [28]. The efficiency of such an event should depend again on the rate of this energy change. Moreover, Fe^{3+} via its catalytic effect on folding, should rapidly protect levansucrase against the membrane-bound proteinases which are known to be present in *B. subtilis* [29]. To choose one or the other hypothesis, we have to establish the location of the processed transient membrane form of levansucrase. Several lines of evidence allow us to assume that it should be strongly embedded within the membrane. (1) The solubilization of this form was only obtained on total disintegration of the membrane by a high concentration of ionic detergent, and all methods failed to obtain differential solubilization of unprocessed and processed transient membrane forms [4]. (2) It has been shown [30] that addition of high concentration of subtilisin (1 mg·ml^{-1}) to the culture medium slightly increases levansucrase production by *B. subtilis*. This means that the precursor forms of the enzyme are inaccessible to the exocellular proteinase. (3) Finally, pulse–chase experiments [5] demonstrated unambiguously that all the effectors which prevent Fe^{3+} uptake by the bacteria lead to the inhibition of the second step of levansucrase secretion.

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