Peptide carrier potentiality of *Bacillus subtilis* levansucrase

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(Received 30 October 1991; revised 18 January 1992; accepted 11 February 1992)

A synthetic oligodeoxynucleotide encoding the vasopressin peptide was ligated to the 3' terminal codon of *sacB*, the structural gene of levansucrase. This gene fusion was integrated into the chromosome of a *Bacillus subtilis* strain able to overproduce levansucrase. The extracellular production of the hybrid protein, consisting of the whole levansucrase primary sequence plus the nine amino acids of the vasopressin peptide added at the C-terminal end, represented 50–55% of that found for the wild-type levansucrase (20 mg l⁻¹). The purified hybrid protein displayed the same conformational stability, protease insensitivity and enzymic properties as the wild-type levansucrase. However, the rate and the yield of the unfolding–folding transition at the pH and temperature used for bacterial growth were lower in the case of the hybrid protein; the latter also required a higher iron concentration to be completely folded.

Introduction

Levansucrase is one of the few proteins secreted by *B. subtilis* during the exponential growth phase (Dedonder, 1966). The expression of its structural gene, *sacB*, requires induction by sucrose and is regulated by a number of genes (Lepesant *et al.*, 1976). Among them, *sacU* is of particular interest since *sacU*^h strains secrete levansucrase in large quantities, about 8% of total protein and the only one released into the culture medium during the exponential phase of growth (Chambert & Petit-Glatron, 1984). These properties make the expression/secretion signals of *sacB* particularly attractive for the construction of secretion vectors (Wong, 1989). However, attempts to translocate normally intracellular prokaryotic proteins or extracellular eukaryotic proteins by fusing their genes to the *sacB* signal sequence have so far failed (Dion *et al.*, 1989), the secretion block occurring at the post-translational level (Sarvas, 1986).

Such results were not in our view surprising since we concluded (Chambert & Petit-Glatron, 1990), from an extensive investigation of its secretion mechanism, that the tertiary structure flexibility of mature levansucrase plays a key role in its secretion process. We proposed that the newly synthesized protein remains in an unfolded state which is essential for insertion into the membrane. The acquisition of the folded state of the protein, catalysed by metal ions such as Fe³⁺ or Ca²⁺ whose

intracellular levels are low (Norris *et al.*, 1991), occurs on the external side of the membrane and is tightly coupled with its release into the external medium. It would therefore appear difficult to engineer a foreign protein for secretion through the same pathway as levansucrase. If, on the other hand, levansucrase secretion is strongly dependent upon its intrinsic folding properties, this protein could be of value as a carrier for foreign peptides which have little effect on such properties.

The approach of peptide secretion via the expression of gene fusions has been recently developed with different envelope proteins in *Escherichia coli* (Charbit, 1991), but is poorly developed in *B. subtilis*. One of the reasons for the latter is the proteolytic degradation of hybrid proteins expressed during the later phases of growth (Ulmanen *et al.*, 1985). However, since proteases are only weakly expressed during exponential growth (Kunst *et al.*, 1974), levansucrase–peptide hybrids should provide model systems with which to gain further insight into the mechanism of the translocation process of levansucrase. We have chosen the levansucrase–vasopressin fusion as a model system to investigate. Vasopressin is a nonapeptide that contributes very little to the net charge and hydrophobicity of levansucrase. It contains an intramolecular disulphide bond which may be expected to direct its self-governing folding independently of the protein-carrier. We have focused our attention both on the secretion efficiency of this hybrid and on the folding properties of its purified extracellular form.

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Methods

Purification of extracellular levansucrases. Wild-type and hybrid levansucrases were prepared from culture supernatants of the induced *B. subtilis* strains GM9150 and GM9151, respectively, according to the published procedure (Dedonder, 1966).

Levansucrase assay. Levansucrase activities were estimated by measuring the initial rate of the fructosyl exchange reaction and of fructosyl polymerase activity (Chambert & Gonzy-Tréboul, 1976; Chambert & Petit-Glatron, 1989).

Pulse-labelling experiments. Cells of *B. subtilis* were pulse-labelled with [³⁵S]methionine as described previously (Chambert & Petit-Glatron, 1988).

Gel electrophoresis and immunoblotting. Proteins were analysed on 10% (w/v) SDS polyacrylamide gels. Electrophoresis was performed using a slab-gel apparatus with the discontinuous system described by Laemmli (1970). Immunoblotting on nitrocellulose sheets (0.45 µm pore size), in 25 mM-Tris/HCl pH 8.3, 192 mM-glycine, 20% (v/v) methanol and 0.02% SDS, was carried out using a constant current of 400 mA for 2 h. Incubation with purified rabbit anti-levansucrase antibodies and radio-iodinated Protein A was as described previously (Petit-Glatron *et al.*, 1987).

Preparation and purification of antibodies. Antibodies against pure extracellular levansucrase were raised in rabbits. Levansucrase was covalently coupled to glutaraldehyde-activated ultrogel (Act Ultrogel AcA, Pharmacia) and the antibodies purified by affinity chromatography (Avrameas & Ternynck, 1969).

Unfolding and refolding experiments. The unfolding–refolding transitions of levansucrase were monitored using changes either in intrinsic fluorescence measured with a F2000 Hitachi thermoregulated spectrofluorimeter or in resistance to proteolytic degradation (Chambert *et al.*, 1990).

Culture and strains. Strains were grown at 37 °C in the following minimal medium: 0.1 M-potassium phosphate pH 7, 25 mM-(NH₄)₂SO₄, 0.5 mM-MgSO₄, 0.01 mM-MnSO₄, 22 µg ferric ammonium citrate ml⁻¹ (60 µM Fe³⁺), 0.1 M-glucose. *B. subtilis* QB112 (*sacA321*, *sacU32*; Kunst *et al.*, 1974) was used as a host strain for the integration into the chromosome of DNA fragments containing the gene fusion or *sacB* wild-type gene.

B. subtilis QB112 was transformed by the procedure of Anagnostopoulos & Spizizen (1961). Transformants were selected on LB plates supplemented with kanamycin (10 µg ml⁻¹) or chloramphenicol (5 µg ml⁻¹). *B. subtilis* strains GM9150 and GM9151 were constructed for this work.

E. coli XL1-B was used as a host for plasmid propagation as described previously (Chambert & Petit-Glatron, 1991).

Plasmids. The amplified DNA fragment was cloned into the *Bam*HI and *Xho*I sites of pBluescript plasmid. The *Xho*I end of the DNA fragment was repaired to flush end with the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates before ligation into the *Eco*RV site of pGMK50. Plasmid pGMK50 was obtained by insertion of the kanamycin-resistance gene cassette purified from pBEST502 (Itaya *et al.*, 1989) into the *Pst*I site of plasmid pLS50 (Steinmetz *et al.*, 1985), indicated by an asterisk in Fig. 1(a). After digestion of pLS50 with *Pst*I and ligation with the kanamycin-resistance gene cassette, transformants were selected on plates containing ampicillin and kanamycin.

In vitro construction of the gene fusion. The nucleotide sequence of vasopressin was fused in-frame to the C-terminal codon of the *sacB* gene using a synthetic oligonucleotide including this sequence as a

primer for the polymerase chain reaction (PCR). The codons used for the nucleotide sequence of the hormonal peptide were chosen from the 'optimal' *B. subtilis* codons as defined by Sharp *et al.* (1990).

The first primer, 5'-dCGCGGATCCGGCCCGTAGTCTGCAATCCT-3', corresponds to nucleotides 169–190 of the published sequence of the *sacB* region. The second primer, 5'-dCCGCTCGAGTTAACAGTAGAATTGGTTACACGGGCGGCCTTTGTTAACTGTTAATTGTCC-3', corresponds to nucleotides 1862–1882 of *sacB* plus the vasopressin sequence. *Bam*HI or *Xho*I sites (underlined) were added to the 5' end of each oligonucleotide, respectively.

Plasmid pLS50 was used as a template for the amplification of the wild-type *sacB* gene by PCR under conditions already described (Chambert & Petit-Glatron, 1991).

Results

Construction of a *B. subtilis* strain containing the gene fusion as a single copy in the chromosome

The fusion gene coding for the levansucrase–vasopressin hybrid (*sacBV*) was ligated into the *Bam*HI and *Eco*RV sites of pGMK50 after the *Xho*I end had been repaired to form a blunt end. The resulting plasmid, pGMK51, was used to transform strain QB112. Transformants in which the DNA fragments were integrated into the chromosome by double crossing-over events between two homologous sequences, as indicated in Fig. 1(b), were selected for their kanamycin (Km) resistance and chloramphenicol (Cm) sensitivity. About 10% of the Km^R transformants were found to be Cm^S. A similar construction was carried out with pGMK50 to provide a reference strain for wild-type levansucrase production. The resulting *B. subtilis* strains were called GM9151 and GM9150, respectively.

Production of levansucrase–vasopressin hybrid

Extracellular production of the hybrid protein by strain GM9151 was compared with that of the wild-type levansucrase by strain GM9150 (Fig. 2a). Quantitative analysis of immunoblots showed that the proteins exhibited the expected size difference (approximately 1 kDa) and that the major part of both proteins (>80%) was released in a homogeneous form (Fig. 2b). Production of the hybrid protein, at 1.9 µg ml⁻¹ per OD₆₅₀ unit, was about half that of the wild-type levansucrase at 3.6 µg ml⁻¹ per OD₆₅₀ unit.

Kinetics of levansucrase secretion in both strains were studied by pulse-chase experiments (not shown). The total amount of hybrid protein produced by strain GM9151 represented approximately 55% of the labelled levansucrase released by strain GM9150 and both proteins displayed the same stability in the culture supernatant. In both cases, they were the only proteins secreted into the culture medium.

Fig. 1. (a) Plasmid pGMK50 results from the insertion of a kanamycin-resistance gene cassette into the *Pst*I site of pLS50, indicated by an asterisk, as described in Methods. Plasmid pGMK51 results from the insertion of the structural gene *sacBV*, encoding the levansucrase-vasopressin hybrid, into the *Bam*HI-*Eco*RV sites of pGMK50. Heavy black lines correspond to the original plasmid pBR322. Stippled boxes represent *B. subtilis* sequences flanking the *sacB* gene (dense stippling indicates the sequenced fragment). (b) Schematic representation of integration of *sacB* or *sacBV* genes from pGMK50 or pGMK51, respectively, into *B. subtilis* QB112 by double crossing-over.

Purification and enzymic properties of the levansucrase-vasopressin hybrid

Purification of the hybrid protein was achieved by chromatography on hydroxyapatite. This protein displayed the same chromatographic behaviour as the wild-type protein and eluted at 1.1 M-potassium phosphate pH 6. Stock solutions of 1 mg ml⁻¹ of the purified hybrid protein in 0.1 M-potassium phosphate pH 6 were prepared for further characterization and could be stored at -20 °C for several weeks without apparent loss of enzyme activity.

The kinetic parameters of the reaction of levan synthesis from sucrose were evaluated at 25 °C for both proteins (Chambert & Petit-Glatron, 1989). Both enzymes had the same k_{cat} ($2.5 \pm 0.3 \times 10^4$ min⁻¹);

however, the values of K_m for sucrose were slightly different - $3.6 \pm 0.6 \times 10^{-2}$ M for wild-type levansucrase and $7 \pm 1.6 \times 10^{-2}$ M for the hybrid protein. The fact that the hybrid protein displayed similar chromatographic and enzymic properties to those of wild-type levansucrase suggested that the elongation at the carboxyl terminus had a rather small effect on the final tertiary conformation of hybrid levansucrase-vasopressin.

Conformational stability of the hybrid protein under bacterial growth conditions

In the pulse-chase experiments described above we showed that wild-type levansucrase and the hybrid protein remained stable in the culture supernatant during the experiments. A more precise estimation of the

Fig. 2. Comparison of production of levansucrase and levansucrase-vasopressin hybrid by *B. subtilis* strains GM9150 and GM9151. (a) Extracellular release of proteins was measured by quantitative immunoblotting. The arrow indicates the beginning of the induction promoted by the addition of 50 mM-sucrose. ○, GM9150; ●, GM9151. (b) Electrophoresis on 10% (w/v) SDS polyacrylamide gel and immunoblotting analysis of the extracellular medium and the bacterial pellet of induced strains GM9150 and GM9151 grown to an OD₆₅₀ of 4. Lanes 2 and 5: 10 µl of cell supernatant of GM9151 and GM9150, respectively. Lanes 3 and 6: 60 µl of cell supernatant of GM9151 and GM9150, respectively. Lanes 4 and 7: 60 µl of cell pellet of GM9151 and GM9150, respectively, resuspended in the initial volume and sonicated before analysis. Lane 1 contains 50 ng of purified levansucrase as a control.

respective stabilities under such conditions can be obtained from the determination of the free energy of unfolding (ΔG_D) for each protein. The curves of denaturation by urea were therefore established by exposing each protein to various concentrations of this denaturant at the same pH (7.0), temperature (37 °C) and salt composition as that used for bacterial growth. The change in proteinase sensitivity occurring under these conditions allowed us to analyse the urea-induced unfolding process (Chambert *et al.*, 1990). The free energies of unfolding for each protein are represented as a function of urea concentration in Fig. 3, according to

Fig. 3. Free energy (ΔG_D) of unfolding for wild-type levansucrase (○) and the hybrid protein (●) as a function of urea concentration at 37 °C, pH 7. ΔG_D values estimated from proteinase sensitivity data were defined as $\Delta G_D = -RT \cdot \ln(P_r/P_s)$; P_r is the amount of subtilisin-resistant protein and P_s the amount of subtilisin-sensitive protein evaluated for each urea concentration after 5 h incubation at 37 °C [1 cal = 4.184 J]. No significant modifications of this parameter were observed after this incubation time.

Pace's plot (Pace, 1986). The values of $\Delta G_D^{H_2O}$ obtained by extrapolation to zero urea concentration were estimated as $\Delta G_D^{H_2O} = 2300 \pm 200$ cal mol⁻¹ and $\Delta G_D^{H_2O} = 1700 \pm 200$ cal mol⁻¹ for the wild-type levansucrase and the hybrid protein, respectively [1 cal = 4.184 J]. This means that the protein elongation slightly decreases the overall stability of levansucrase, the native extracellular hybrid protein being protease-resistant under the pH and temperature conditions of bacterial growth.

Kinetics of the unfolding-refolding transition of the levansucrase-vasopressin hybrid mediated at pH 7 and 37 °C

The kinetics of unfolding-refolding transition were compared for both proteins under two different conditions (Chambert *et al.*, 1990).

First, denaturation of both proteins was performed in 0.1 M-potassium acetate buffer, pH 7, in the presence of EDTA. The refolding was then promoted by the addition of Ca²⁺. Under such conditions, changes in intrinsic fluorescence intensity provided a suitable method for monitoring the unfolding-refolding transition. Traces of fluorescence intensity changes (Fig. 4) showed that both proteins were rapidly denatured in the presence of 0.1 mM-EDTA. After addition of 50 mM-Ca²⁺ to the solution of the denatured form, 90% and 50% of the fluorescence properties of the native forms were

Fig. 4. Unfolding-refolding transition of wild-type levansucrase (A) and levansucrase-vasopressin hybrid (B) mediated by Ca^{2+} at 37°C , pH 7, measured by intrinsic fluorescence intensity changes. Traces represent fluorescence intensity as a function of time (excitation wavelength 285 nm, emission wavelength 336 nm). Aliquots ($7.5\ \mu\text{l}$) of the stock solutions of the purified proteins ($1\ \text{mg}\ \text{ml}^{-1}$) were mixed (arrow 1) with $1\ \text{ml}\ 0.1\ \text{M}$ -potassium acetate pH 7 containing $0.1\ \text{mM}$ -EDTA and preincubated at 37°C . After 1 min of unfolding, refolding was initiated by the addition of $50\ \mu\text{l}\ 1\ \text{M}$ - CaCl_2 in $0.1\ \text{M}$ -potassium acetate pH 7 (arrow 2).

recovered in the case of wild-type levansucrase and the hybrid protein, respectively. Assuming a simple two-state model for the unfolding-refolding transition, the relaxation time, τ , for each event and for each protein could be calculated by using the equation $(I_0 - I_\infty) = (I - I_\infty)e^{t/\tau}$, where I , I_0 , I_∞ are the values of fluorescence intensity at any time, zero time and infinite time, respectively, of the unfolding or the refolding process. These estimations gave the same value for unfolding, $\tau_{\text{un}} = 29 \pm 1\ \text{s}$, but different ones for refolding, $\tau_{\text{re}} = 104 \pm 2\ \text{s}$ and $179 \pm 6\ \text{s}$ for wild-type levansucrase and the hybrid protein, respectively.

Secondly, both proteins were denatured by $8\ \text{M}$ -urea and then their refolding initiated by diluting this denaturing agent with buffer containing the same salt concentrations as the growth medium. The unfolding-refolding transitions were followed by monitoring the appearance of resistance to proteolytic degradation. As shown in Fig. 5, the recovery of protease resistance was 70% for the wild-type levansucrase and only 30% for the hybrid protein. Moreover, the initial rate of refolding was lower for this latter protein. In the presence of a tenfold concentration of ferric ions in the renaturation buffer, we observed a greater increase of the yield of refolding for the hybrid protein than for the wild-type levansucrase.

Fig. 5. Unfolding-refolding transition of wild-type levansucrase and levansucrase-vasopressin hybrid at 37°C , pH 7, measured by changes in proteolytic resistance. Unfolding (arrow 1) was promoted by mixing $10\ \mu\text{l}$ of the solution of the purified proteins ($1\ \text{mg}\ \text{ml}^{-1}$) with $100\ \mu\text{l}\ 8\ \text{M}$ -urea, $0.1\ \text{M}$ -potassium phosphate pH 7. Refolding (arrow 2) was initiated by the addition of $5\ \text{ml}$ of the same buffer containing the same salt concentrations as the growth medium (filled symbols) or a tenfold concentration of ferric ions (open symbols), preincubated at 37°C . Aliquots were withdrawn at the times indicated, mixed with subtilisin ($50\ \mu\text{g}\ \text{ml}^{-1}$ final concentration) and assayed for levansucrase activity. ■, □, Levansucrase; ●, ○, hybrid protein.

The results obtained from both approaches allowed us to conclude that the extension of the levansucrase polypeptide chain alters both the rate and the yield of correct refolding under the conditions of temperature and pH used for its production from *B. subtilis*.

Endoproteinase treatment of the hybrid protein

When this work was initiated, it was postulated that vasopressin could direct its self-governing folding independently of the carrier levansucrase. It might therefore have been expected that the peptide bond between the two parts of the fusion protein would display a particular sensitivity to hydrolysis by proteinases. Endoproteinase Lys-C was therefore used to attempt to digest the hybrid protein, since the peptide bond involves the carbonyl group of the terminal lysine of levansucrase. As shown in Fig. 6, the hydrolysis of the hybrid protein did not release the carrier levansucrase. It was therefore concluded that our first hypothesis was not correct and the insertion of a recognition sequence for a restriction protease between levansucrase and the piloted peptide would be necessary to exploit levansucrase as a potential peptide carrier.

Fig. 6. Hydrolysis of the hybrid protein by endoproteinase Lys-C. A 24 µg sample of the hybrid protein was submitted to hydrolysis with 1 µg endoproteinase Lys-C (30 units mg⁻¹, from Sigma) in 120 µl 50 mM-ammonium bicarbonate buffer pH 8, at 25 °C. Aliquots (40 µl) of the reaction mixture were withdrawn at intervals, mixed with 100 µl of electrophoresis sample buffer (containing 2% SDS) and analysed by 10% PAGE. Proteins were stained with Coomassie blue as described by Laemmli (1970). Lanes: A, levansucrase (8 µg); B, hybrid protein (8 µg); C, a mixture of levansucrase and hybrid protein (8 µg each); D, E, and F, kinetics of hybrid protein digestion by proteinase, 1, 5 and 60 min, respectively.

Discussion

The model we have proposed (Chambert & Petit-Glatron, 1990) for levansucrase release in *B. subtilis* emphasized the key role of the polypeptide chain flexibility and the modulation of the folding rate by metal ions such as Ca²⁺ and Fe³⁺. It has been recently postulated that α-amylase, another enzyme of *B. subtilis*, is also subject to conformational changes during its secretion process (Kontinen *et al.*, 1991).

The results obtained here with the levansucrase-vasopressin hybrid protein strengthen the hypothesis of a tight coupling between the efficiency of secretion and subsequently correct folding catalysed by metal ions such as Fe³⁺ and Ca²⁺. We have postulated (Chambert *et al.*, 1990), as others have done (Eilers & Schatz, 1988), that the energy used for the vectorial diffusion of the protein through the membrane is provided by the energy change accompanying folding. However, we proposed from an irreversible thermodynamic point of view that the rate of folding could be important for an efficient coupling of

these two processes. This hypothesis is supported by this work, since the decrease of the secretion yield of the hybrid may be correlated with a lower rate of unfolding–folding transition and a lower yield of correct refolding compared with wild-type levansucrase.

It has been pointed out repeatedly that proteolytic degradation of heterologous or hybrid proteins is one of the major obstacles to the use of *B. subtilis* as producer of secreted proteins (Henner, 1990). It appears from this work that in the case of levansucrase such an obstacle may be overestimated since our hybrid protein remains protease-insensitive in the extracellular medium. However, it was shown that the rate of correct protein folding is fundamental for a high yield of secreted protein recovery. Identification in the mature levansucrase of sites playing a critical role in the folding event, particularly potential metal-binding domains, would be essential to a further understanding of the process. From previous genetic analyses (Lepesant *et al.*, 1976) various mutants of *B. subtilis* producing levansucrase altered both in its secretion and in its conformational stability have been isolated. Studies of these *sacB* alleles are presently in progress in our laboratory. Such an approach could give us a rational way to study the peptide carrier potentialities of levansucrase.

We thank M. Steinmetz for the generous gift of plasmid pBEST502. We are indebted to Dr A.G. Smith (University of Cambridge, UK) for reading the manuscript.

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